

Acta Medica Okayama

Volume 48, Issue 1

1994

Article 9

FEBRUARY 1994

Synergistic Effect of Tumor Necrosis Factor- α and Interferon- α on the Induction of Apoptosis Detected by BM-I/JIMRO: A New Marker of Apoptosis

Wei Zhang*

Yoshio Naomoto[†]

Noriaki Tanaka[‡]

Akio Hizuta**

Kunzo Orita^{††}

*Okayama University,

[†]Okayama University,

[‡]Okayama University,

**Okayama University,

^{††}Okayama University,

Synergistic Effect of Tumor Necrosis Factor- α and Interferon- α on the Induction of Apoptosis Detected by BM-1/JIMRO: A New Marker of Apoptosis*

Wei Zhang, Yoshio Naomoto, Noriaki Tanaka, Akio Hizuta, and Kunzo Orita

Abstract

The effects of the combination of natural human tumor necrosis factor- α (nHuTNF- α) and natural human interferon- α (nHuIFN- α) on the induction of apoptosis were investigated by immunohistochemical analysis with BM-1/JIMRO monoclonal antibody in RPMI 4788 tumor cells. Few tumor cells in the control culture could spontaneously undergo apoptosis. The number of positive cells increased at 2 and 4 h after treatment with nHuTNF- α (1×10^5 U/ml) and nHuIFN- α (1×10^5 IU/ml). This effect was clearly maintained from 8 h up to 72 h of culture. The number of apoptotic cells also greatly increased with doses, suggesting that the apoptosis induced by nHuTNF- α and nHuIFN- α in combination was dose-dependent. nHuTNF- α or nHuIFN- α alone could induce apoptosis, but the induction increased significantly when the two cytokines were combined. These findings indicate that by combining nHuTNF- α and nHuIFN- α apoptosis can be synergistically induced in RPMI 4788 tumor cells, and may have specific therapeutic implications for clinical treatments using these two cytokines.

KEYWORDS: apoptosis, tumor necrosis factor-?, interferon-?

*PMID: 8191917 [PubMed - indexed for MEDLINE]

Copyright (C) OKAYAMA UNIVERSITY MEDICAL SCHOOL

Synergistic Effect of Tumor Necrosis Factor- α and Interferon- α on the Induction of Apoptosis Detected by BM-1/JIMRO: A New Marker of Apoptosis

WEI ZHANG, YOSHIO NAOMOTO*, NORIAKI TANAKA, AKIO HIZUTA AND KUNZO ORITA

First Department of Surgery, Okayama University Medical School, Okayama 700, Japan

The effects of the combination of natural human tumor necrosis factor- α (nHuTNF- α) and natural human interferon- α (nHuIFN- α) on the induction of apoptosis were investigated by immunohistochemical analysis with BM-1/JIMRO monoclonal antibody in RPMI 4788 tumor cells. Few tumor cells in the control culture could spontaneously undergo apoptosis. The number of positive cells increased at 2 and 4 h after treatment with nHuTNF- α (1×10^5 U/ml) and nHuIFN- α (1×10^5 IU/ml). This effect was clearly maintained from 8 h up to 72 h of culture. The number of apoptotic cells also greatly increased with doses, suggesting that the apoptosis induced by nHuTNF- α and nHuIFN- α in combination was dose-dependent. nHuTNF- α or nHuIFN- α alone could induce apoptosis, but the induction increased significantly when the two cytokines were combined. These findings indicate that by combining nHuTNF- α and nHuIFN- α apoptosis can be synergistically induced in RPMI 4788 tumor cells, and may have specific therapeutic implications for clinical treatments using these two cytokines.

Key words: apoptosis, tumor necrosis factor- α , interferon- α

Tumor necrosis factor (TNF) is a cytokine secreted by activated macrophages, and was first identified by Carswell *et al.* in sera of mice infected with *bacillus Calmette-Guérin* and subsequently treated with endotoxin (1). It has been well known that TNF is cytotoxic and cytostatic to some tumor cells *in vivo* and *in vitro*, but to date the precise mechanism by which the tumoricidal effect of TNF occurs is still not well known (2). Although TNF

receptors can be demonstrated on the surface of the cells, the sensitivity to TNF cytotoxicity does not seem to correlate with number of TNF receptors or affinity to TNF (3). Another cytokine, interferon (IFN), which was originally recognized for its antiviral effect and later found to be directly cytolytic to some tumor cell lines (4), can markedly enhance the susceptibility of tumor cells to cytotoxicity of TNF (5-9). The synergistic effect of these cytokines on target cells may involve the increase of TNF receptors (10, 11), damage of membrane structures (7, 8), changes in cell cycle progression (12) or regulation of immune response (13).

Two distinct forms of cell death have been described in many branches of cell biology, necrosis and apoptosis (14, 15). The morphological event in necrosis involves swelling of cytoplasm with karyorrhexis and karyolysis, and eventual rupture of the entire cell (15). Apoptosis, in contrast, is mainly characterized by cytoplasmic condensation and nuclear DNA fragmentation by endonuclease into multiples of approximately 180 base pairs. It is a basic biological process that plays an important role in the regulation of cell populations, the cell turnover in normal tissues and the active cellular self-destruction in tumor tissues. Recent studies have revealed that TNF-induced cytotoxicity is associated with apoptosis, and that apoptosis induced by TNF accounts for target cell killing in some tumor cell lines (16-21). Furthermore, The changes of cellular glycosylation pattern are known to be correlated with apoptosis and Le^y expression is a phenotypic marker predictive of apoptosis (22, 23). In the present immunohistochemical analysis, BM-1/JIMRO anti-Le^y mouse IgM monoclonal antibody (BM-1 Mab) was used to study apoptosis induced by a combination of natural human tumor necrosis factor- α (nHuTNF- α) and natural human interferon- α (nHuIFN- α) in

* To whom correspondence should be addressed.

RPMI 4788 human colon cancer cells.

Materials and Methods

Cell line and reagents. The human colon cancer, RPMI 4788 cells were provided by Roswell Park Memorial Institute, Buffalo, New York, USA and were grown in RPMI 1640 medium (Nissui, Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum. nHuTNF- α and nHuIFN- α , produced from HVJ (Hemagglutinating Virus of Japan)-stimulated BALL-1 cells and purified to homogeneity as estimated by sodium dodecylsulfate (SDS)-polyacrylamide gel, were obtained from Hayashibara Biochemical Laboratories Inc., Okayama, Japan. The specific activity of nHuTNF- α was 10^9 U/mg protein. Our standard was titrated against a Japan Reference (J-PS 5K01, National Institute of Health, Tokyo, Japan) and 350U equaled 1 JRU. BM-1 Mab was purchased from Otsuka Pharmaceuticals, Tokushima, Japan.

Preparation of cell suspensions. RPMI 4788 tumor cells were cultured in RPMI 1640 medium at 37°C in a 5% CO₂ atmosphere with nHuTNF- α and/or nHuIFN- α at various concentrations for 2-72 h. Additional cultures without any cytokine treatment were used as controls. The cells were collected, washed with phosphate buffered saline (PBS) and then prepared for immunohistochemical analysis.

Immunohistochemical analysis. Cell suspensions were smeared by cytospin (1,000 rpm for 5 min) on glass slides pre-treated with neoprene toluen solution. The slides were fixed in cold acetone for 10 min and then air dried. The immunohistochemical analysis with BM-1 Mab was performed by strept-avidin-biotin (SAB) method as described by Hiraishi *et al.* (23). Briefly, smears were rinsed in PBS, immersed in 10% normal rabbit serum for 10 min, and then incubated with BM-1 Mab at room temperature for 60 min. Smears were washed three times in PBS for about 10 min, followed by biotinylated anti-mouse IgG + IgA + IgM (Nichirei Co., Tokyo, Japan) for 30 min and washed with PBS. Then the smears were treated with peroxidase-conjugated strept-avidin (Nichirei Co., Tokyo, Japan) for 20 min, washed with PBS, and stained with diaminobenzidine-H₂O₂ solution for 5-20 min at room temperature. Finally, the smears were sequentially counterstained with hematoxylin, washed, dehydrated, penetrated and enclosed. In each experiment, additional smears were also stained with

haematoxylin and eosin (HE) to compare with the immunohistochemical analysis.

Statistical analysis. Data were expressed as mean values plus or minus standard deviations of the number of BM-1 Mab-positive cells of at least two separate experiments, and three times for each experiment. Analysis of the differences between cultures treated and untreated with cytokines was performed by Student's *t*-test, and statistical significance was considered for a *p* value of less than 0.05.

Results

Induction of apoptosis with nHuTNF- α and nHuIFN- α . Positive cells undergoing apoptosis were occasionally observed in the tumor cells without any treatment (Fig. 1a). Two hours after exposure to nHuTNF- α (1×10^5 U/ml) and nHuIFN- α (1×10^5 IU/ml), the positive cells had increased in number. Under higher magnification, the nuclei of some of these positive cells had broken into a number of discrete fragments, which is the typical nuclear feature of apoptosis. However, for an unexplained reason, a portion of the positive cells stained with BM-1 Mab had not obviously reduced size. In the presence of nHuTNF- α and nHuIFN- α , typical necrotic tumor cells characterized by swelling, eosinophilic and vacuolar cytoplasm with nuclear pyknosis and karyorrhexis were also observed.

Effects of nHuTNF- α and nHuIFN- α on induction of apoptosis. Time courses of apoptosis induced by nHuTNF- α and nHuIFN- α are shown in Table 1. A few of untreated RPMI 4788 tumor

Table 1 Time course of apoptotic cells induced by nHuTNF- α (1×10^5 U/ml) and nHuIFN- α (1×10^5 IU/ml) in RPMI 4788 cells

Time (h)	Apoptotic cells*		P Value
	Control	Cytokines	
2	2.00 ± 0.84	2.81 ± 1.12	< 0.05
4	2.67 ± 1.28	3.67 ± 1.11	< 0.05
8	4.33 ± 1.59	10.90 ± 1.48	< 0.001
12	5.43 ± 1.63	11.76 ± 2.17	< 0.001
24	7.76 ± 2.05	34.00 ± 3.21	< 0.001
48	11.00 ± 2.53	34.52 ± 4.90	< 0.001
72	17.19 ± 2.25	58.38 ± 7.46	< 0.001

*Figures are mean of seven randomly selected microscopic fields (average of 350 cells each).

February 1994

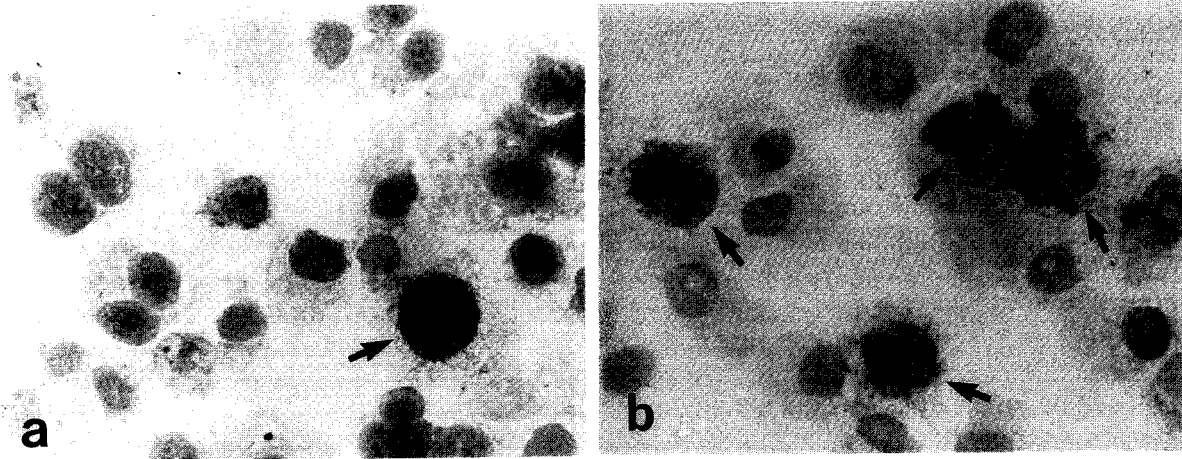
Apoptosis Induced by nHuTNF- α and nHuIFN- α 

Fig. 1 Apoptosis induced by nHuTNF- α and nHuIFN- α in RPMI 4788 cells. (a) Control. A positive cell is occasionally observed (arrow). (b) Positive cells 24 h after treatment with nHuTNF- α (1×10^5 U/ml) and nHuIFN- α (1×10^5 IU/ml) (arrows). Staining with BM-1 Mab. $\times 200$.

Table 2 Dose-response at 24 h for apoptotic cells induced by nHuTNF- α (U/ml) and nHuIFN- α (IU/ml)

Cytokines	Apoptotic cells*	P Value
0	7.86 ± 1.56	
1×10^3	17.95 ± 2.89	< 0.001
1×10^4	25.52 ± 2.71	< 0.001
1×10^5	36.14 ± 4.02	< 0.001

*Figures are mean of seven randomly selected microscopic fields (average of 350 cells each).

Table 3 Synergistic induction of apoptosis at 24 h after treatment with nHuTNF- α (1×10^5 U/ml) and/or nHuIFN- α (1×10^5 IU/ml)

Cytokines	Apoptotic cells*	P Value
0	7.76 ± 2.05	
nHuIFN- α	9.29 ± 2.57	< 0.001
nHuTNF- α	10.62 ± 2.91	< 0.001
nHuTNF- α + nHuIFN- α	34.00 ± 3.21	< 0.001

*Figures are mean of seven randomly selected microscopic fields (average of 350 cells each).

cells also underwent apoptosis and increased in number with time. In the presence of both nHuTNF- α and nHuIFN- α at predetermined concentrations of 1×10^5 U/ml and 1×10^5 IU/ml, respectively, the number of positive cells increased at 2 h and 4 h of culture ($p < 0.05$). Marked increases in number of positive cells were observed when RPMI 4788 tumor cells were exposed to nHuTNF- α and nHuIFN- α for 8 h to 72 h (Fig. 1b). Analysis after 72 h of culture was discontinued because many of the tumor cells had died in culture.

The response of tumor cells treated with various concentrations of nHuTNF- α and nHuIFN- α at 24 h as compared to untreated control revealed that the combination of nHuTNF- α and nHuIFN- α at doses of 1×10^3 U/ml and 1×10^3 IU/ml, respectively, caused a marked increase of positive cells which greatly increased with dose ($p < 0.001$, Table 2).

Synergistic effects on apoptosis induced by nHuTNF- α and nHuIFN- α . As demonstrated in Table 3, treatment with nHuIFN- α alone induced apoptosis in RPMI 4788 tumor cells at a concentration of 1×10^5 IU/ml after 24 h of culture ($p < 0.001$). Similarly, an increased number of positive cells was also observed 24 h after treatment with nHuTNF- α alone at a concentration of 1×10^5 U/ml ($p < 0.001$). No statistically significant difference in number of positive cells was found between nHuIFN- α and nHuTNF- α treated cells. The maximal induction of apoptosis was seen in RPMI 4788 tumor cells treated with both nHuTNF- α and nHuIFN- α . The number of positive cells induced by this combination was greater than that induced by the two cytokines separately ($p < 0.001$), thus suggesting a synergistic effect of nHuTNF- α combined with nHuIFN- α on the induction of apoptosis.

Discussion

In this experiment, the combination of nHuTNF- α and nHuIFN- α induced apoptosis in RPMI 4788 cells as observed by immunohistochemical analysis with BM-1 Mab. Kerr *et al.* described the morphological changes of the cell undergoing apoptosis. The cells shrink in volume and the plasma membrane becomes ruffled and blebbed. Finally the cells break apart into a number of apoptotic bodies (14). However, recent studies have suggested that the concept and mechanism of apoptosis are diverse and that the structural feature of apoptosis are also numerous (24). Apoptosis is activated under the appropriate physiological conditions and triggered by signaling through cytoplasmic receptors or cell surface membrane. Recently, it has been reported that Le^y expression is a predisposing marker for apoptosis and increased Le^y expression in apoptotic cells is confirmed in the early stage of the process (23).

Several reports have demonstrated that TNF-induced DNA fragmentations contributed to TNF-mediated cytotoxicity in some sensitive target cells (16–21). This DNA fragmentation is thought to be caused by DNA endonuclease, although the nature of this enzyme has not been demonstrated. The topoisomerase II inhibitor, which might enhance DNA endonuclease activity, accelerated significantly the rate of DNA fragmentation (19) and in contrast, the percentage of DNA fragmentation was greatly reduced by Zn²⁺ which was considered to be an inhibitor of the endogenous endonuclease (16, 21). The fact that nHuTNF- α and nHuIFN- α induce apoptosis in RPMI 4788 tumor cells may suggest that nHuTNF- α and nHuIFN- α can stimulate DNA endonuclease activity, that results in DNA fragmentation, although we were not able to prove it.

In addition to apoptosis, TNF also induced necrosis of tumor cells, depending on different cell lines (17, 25). In the present study, the typical necrotic cells were also detected after exposure to nHuTNF- α and nHuIFN- α , suggesting that these cytokines in combination could activate both apoptotic and necrotic forms of cell death. Only a few RPMI 4788 cells spontaneously underwent apoptosis without any stimulus as shown in our controls.

The findings that the number of apoptotic cells significantly increased after 8 h and progressed until 72 h in RPMI 4788 cells following treatment with nHuTNF- α and nHuIFN- α in combination suggested that the

apoptosis induced by these cytokines was a time-dependent process. Wright and co-workers (16) demonstrated that the percentage of apoptotic cells in U937 cells peaked at 2 h after exposure to TNF and then decreased thereafter. Other studies showed that the specific DNA fragmentation in MCF-7 cells started 12 h after treatment with rHuTNF (21). These differences could be due to the selected tumor cell lines which had different sensitivity to TNF. Our results also demonstrated that the apoptotic cells induced by nHuTNF- α and nHuIFN- α in combination increased in a dose-dependent manner, and indicated that RPMI 4788 tumor cells were very sensitive to the dose of these cytokines.

Previous studies have demonstrated that IFN- γ alone failed to induce specific DNA fragmentation in tumor cells (21, 25), but significantly enhanced TNF-mediated DNA fragmentation in MCF-7 breast cancer cells after pretreatment with rHuIFN- γ 1 h prior to rHuTNF treatment (21). Recently, the typical DNA fragmentation of apoptosis has also been observed in pre-B cells exposed for 3 days to IFN- γ alone (26). These studies, however, evaluated the ability of IFN- γ to fragment DNA, and in this study, we have observed nHuIFN- α . The present findings showed that nHuIFN- α alone also induced apoptosis in RPMI 4788 cells and caused a synergistic effect with nHuTNF- α . We have proved that the combination of nHuTNF- α and nHuIFN- α causes a marked tumor cell accumulation in S phase of the cell cycle progression using two-parameter flow cytometry *in vitro* and an immunohistochemical analysis *in vivo* (12). Recent reports have shown that the activation of apoptosis is also related to the cell cycle-mediated event (27). The combined evidence supports the notion that the synergistic effect of these two cytokines on induction of apoptosis may be concerned with the prevention of cell cycle progression. Because the process of apoptosis is thought to be caused by DNA endonuclease, probably the enhanced activity of TNF on apoptosis by IFN is a result of the synergistic activation of DNA endonuclease (21).

The possibility of synergistic induction of apoptosis by nHuTNF- α and nHuIFN- α in combination may have specific therapeutic implications for patients with cancer. nHuTNF- α and nHuIFN- α can induce both apoptosis and necrosis of tumor cells and for this reason can cause the death of tumor cells and reduction in tumor size. Furthermore, the synergistic effect of these two cytokines in combination allows the clinical use of lower doses to reduce their side effects. In fact, nHuTNF- α and

nHuIFN- α in combination have been applied to clinical treatment of the patients with cancer, and the results have been satisfactory (28).

Acknowledgments. The authors would like to thank Mrs. K. Nasu for her excellent technical help.

References

- Carswell EA, Old LJ, Kassel RL, Green S, Fiore N and Williamson B: An endotoxin induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* (1975) **72**, 3666-3670.
- Larrick JW and Wright SC: Cytotoxic mechanism of tumor necrosis factor- α . *FASEB (Fed Am Soc Exp Biol) J* (1990) **4**, 3215-3223.
- Tsujimoto M, Yip YK and Vilcek J: Tumor necrosis factor: Specific binding and internalization in sensitive and resistant cells. *Proc Natl Acad Sci USA* (1985) **82**, 7626-7630.
- Le J, Yip YK and Vilcek J: Cytolytic activity of interferon-gamma and its synergism with 5-Fluorouracil. *Int J Cancer* (1984) **34**, 495-500.
- Orita K, Ando S and Kurimoto M: Synergism between human tumor necrosis factor and human interferon- α : Effects on cells in culture. *Acta Med Okayama* (1987) **41**, 155-160.
- Naomoto Y, Tanaka N and Orita K: Antitumor effect of natural human tumor necrosis factor- α and natural human interferon- α in combination against human cancer transplanted into nude mice. *Acta Med Okayama* (1989) **43**, 211-221.
- Yoshimura T and Sone S: Different and synergistic actions of human tumor necrosis factor and interferon- γ in damage of liposome membranes. *J Biol Chem* (1987) **262**, 4597-4601.
- Williams TW and Bellanti JA: *In vitro* synergism between interferons and human lymphotoxin: Enhancement of lymphotoxin-induced target cell killing. *J Immunol* (1983) **130**, 518-520.
- McIntosh JK, Mulé JJ, Krosnick JA and Rosenberg SA: Combination cytokine immunotherapy with tumor necrosis factor α , interleukin 2, and α -interferon and its synergistic antitumor effects in mice. *Cancer Res* (1989) **49**, 1408-1414.
- Tsujimoto M, Yip YK and Vilcek J: Interferon- γ enhances expression of cellular receptors for tumor necrosis factor. *J Immunol* (1986) **136**, 2441-2444.
- Ruggiero V, Tavernier J, Fiers W and Baglioni C: Induction of the synthesis of tumor necrosis factor receptors by interferon- γ . *J Immunol* (1986) **136**, 2445-2450.
- Muro M, Naomoto Y and Orita K: Mechanism of the combined antitumor effect of natural human tumor necrosis factor- α and natural human interferon- α on cell cycle progression. *Jpn J Cancer Res* (1991) **82**, 118-126.
- Williamson BD, Carswell EA, Rubin BY, Prendergast JS and Old LJ: Human tumor necrosis factor produced by human B-cell lines: Synergistic cytotoxic interaction with human interferon. *Proc Natl Acad Sci USA* (1983) **80**, 5397-5401.
- Kerr JFR, Wyllie AH and Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* (1972) **26**, 239-257.
- Wyllie AH, Kerr JFR and Currie AR: Cell death: The significance of apoptosis. *Int Rev Cytol* (1980) **68**, 251-306.
- Wright SC, Kumar P, Tam AW, Shen N, Varma M and Larrick JW: Apoptosis and DNA fragmentation precede TNF-induced cytolysis in U937 cells. *J Cell Biochem* (1992) **48**, 344-355.
- Laster SM, Wood JG and Gooding LR: Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J Immunol* (1988) **141**, 2629-2634.
- Rubin BY, Smith LJ, Hellermann GR, Lunn RM, Richardson NK and Anderson SL: Correlation between the anticellular and DNA fragmenting activities of tumor necrosis factor. *Cancer Res* (1988) **48**, 6006-6010.
- Kyprianou N, Alexander RB and Isaacs JT: Activation of programmed cell death by recombinant human tumor necrosis factor plus topoisomerase II-targeted drugs in L929 tumor cells. *J Natl Cancer Inst* (1991) **83**, 346-350.
- Shinagawa T, Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y and Takayanagi M: Apoptosis in cultured rat hepatocytes: The effects of tumor necrosis factor α and interferon γ . *J Pathol* (1991) **165**, 247-253.
- Dealtry GB, Naylor MS, Fiers W and Balkwill FR: DNA fragmentation and cytotoxicity caused by tumor necrosis factor is enhanced by interferon- γ . *Eur J Immunol* (1987) **17**, 689-693.
- Abe K, McKibbin JM and Hakomori S: The Monoclonal antibody directed to difucosylated type 2 chain (Fuc α 1 \rightarrow 2Gal β 1 \rightarrow 4 [Fuc α 1 \rightarrow 3] GlcNAc: Y determinant). *J Biol Chem* (1983) **258**, 11793-11797.
- Hiraishi K, Suzuki K, Hakomori S and Adachi M: Le^y antigen expression is correlated with apoptosis (programmed cell death). *Glycobiology* (1993) **3**, 381-390.
- Schwartz LM, Smith SW, Jones MEE and Osborne BA: Do all programmed cell deaths occur via apoptosis? *Proc Natl Acad Sci USA* (1993) **90**, 980-984.
- Woods KM and Chapes SK: Three distinct cell phenotypes of induced-TNF cytotoxicity and their relationship to apoptosis. *J Leukocyte Biol* (1993) **53**, 37-44.
- Grawunder U, Melchers F and Rolink A: Interferon- γ arrests proliferation and causes apoptosis in stromal cell/interleukin-7-dependent normal murine pre-B cell lines and clones *in vitro*, but does not induce differentiation to surface immunoglobulin-positive B cells. *Eur J Immunol* (1993) **23**, 544-551.
- Evans DL and Dive C: Effect of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. *Cancer Res* (1993) **53**, 2133-2139.
- Orita K, Fuchimoto S, Kurimoto M, Ando S and Minowada J: Early phase II study of interferon- α and tumor necrosis factor- α combination in patients with advanced cancer. *Acta Med Okayama* (1992) **46**, 103-112.

Received August 3, 1993; accepted August 30, 1993.