

THE ADDITIVE EFFECT OF CYTOKINES AND RETINOIC ACID ON THE GROWTH INHIBITION OF TRANSFORMED CELLS

Iris L. Sun, Leslie E. Sun, and Frederick L. Crane

Department of Biological Sciences

Purdue University

West Lafayette, Indiana 47907

ABSTRACT: Three cytokines, interferon- γ (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor- α (TNF- α) inhibit the growth of the transformed cells HL-60, HeLa, and RLT-28. Some increase in growth inhibition by these three cytokines was noted after adding 10^{-7} M *trans*-retinoic acid (*t*-RA). Maximum inhibition occurred at 10^{-5} M *t*-RA. 13-*cis*-retinoic acid is less effective. Structural specificity for *t*-RA at the receptor site is indicated. Cells proliferation was evaluated by both counting and flow cytometric analysis of the DNA and nuclear proteins. Inhibition of transplasma membrane diferric transferrin reductase (an enzyme involved in cell growth) by the cytokines is at 50% of maximum at 10 μ g/ml for IFN- γ , 40 μ g/ml for IL-2, and 1.2 ng/ml for TNF- α . These concentrations are similar to those required for growth inhibition. *t*-RA at 10^{-7} M inhibits transferrin reduction and causes additional inhibition when added with the cytokines. Thus, inhibition of plasma membrane redox activity is correlated with the growth inhibition of the cytokines. These results indicate a potential application for a combination of cytokines and *t*-RA for the treatment of malignant diseases.

KEYWORDS: Cytokines, growth inhibition, interferon-gamma, interleukin-2, retinoic acid, transmembrane electron transport, tumor necrosis factor-alpha.

INTRODUCTION

Cytokines are intercellular mediators which exert their effect through specific cell surface receptors (Hamblin, 1993a). Cytokines are important control agents in cell development, differentiation, proliferation, and death (Hamblin, 1993b). The target cells of cytokine action are very diverse. The actions of the cytokines on their target cells are suggested to be pleiotropic; *i.e.*, they have multiple biological activities on different target cells (Waksman, 1979). *Trans*-retinoic acid (*t*-RA) is known to be a potent mediator of cell differentiation and proliferation (Bollag, 1983; Breitman, *et al.*, 1981; Lotan, 1980; Sunkara, *et al.*, 1983) and is potentially a promising therapeutic agent for the prevention and treatment of cancer (Bollag, 1983; Lotan, 1980) and other diseases (Brinkerhogg, *et al.*, 1980; Sporn and Roberts, 1983). Sherman (1986) proposed that *t*-RA modifies membrane structure, and Cope and Boutwell (1983) as well as Sani (1979) have suggested that the *t*-RA ligand (receptor) is a component of the plasma membrane. Therefore, it is tempting to speculate on whether agents which share a similar target in the promotion of cell differentiation and in the establishment of anticellular effects through signals transduced

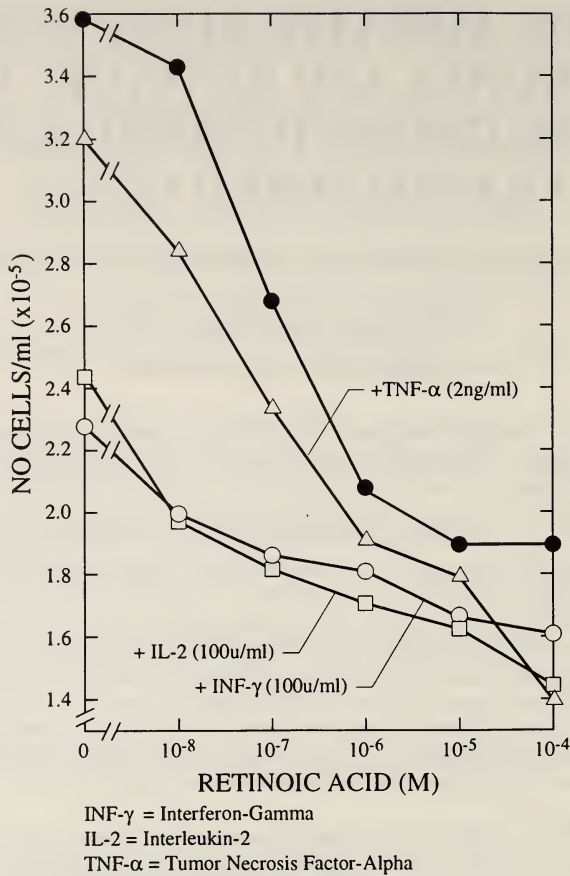


Figure 1. The synergistic effect of *t*-RA and the cytokines on the proliferation of HL-60 cells. The solid circles are *t*-RA only; the open symbols represent cytokines plus *t*-RA.

from cell surface receptors may work additively in suppression of the growth of certain types of cancer cells.

In this study, three cytokines (IFN- γ , IL-2, and TNF- α) and *t*-RA are shown to act synergistically against the human promyelocytic leukemic cell line HL-60, the transformed human cervical cell line HeLa, and the rat liver transformed cell line RLT-28. The effect of the cytokines and *t*-RA was assessed using both direct cell counts and flow cytometric analyses of the DNA and nuclear proteins. The mechanism for their synergism may also be deduced — their additive cell-killing (as shown in the flow cytometric study) is correlated with their inhibition of the transmembrane oxidoreductase activities. Previously, transmembrane oxidoreductase activity was demonstrated to be closely related to cell growth (Avron and Shavit, 1963; Crane, *et al.*, 1985). Our results support the efficacy of combining cytokines with *t*-RA in the treatment of neoplastic diseases through inhibition of diferric transferrin reductase.

Table 1. Enhancement of cytokine-induced HL-60 cell growth inhibition by retinoic acid (*t*-RA = *trans*-retinoic acid (10^{-5} M); IFN- γ = interferon-gamma; IL-2 = interleukin-2; and TNF- α = tumor necrosis factor-alpha).

	Number of Cells/ML ($\times 10^{-5}$)	
	Without <i>t</i> -RA	With <i>t</i> -RA
Control	2.11	1.80
IFN- γ (25 μ g/ml)	1.82	1.59
IFN- γ (75 μ g/ml)	1.59	1.44
IL-2 (25 μ g/ml)	1.86	1.60
IL-2 (50 μ g/ml)	1.77	1.42
TNF- α (1 ng/ml)	1.82	1.58
TNF- α (3 ng/ml)	1.43	1.09

MATERIALS AND METHODS

All *trans*-retinoic acid (*t*-RA) and *cis*-retinoic acid (*c*-RA) were obtained from Sigma Chemical Company. HeLa cells were grown in α -modified minimal essential medium, HL-60 cells were grown in RPMI 1640 medium, and rat liver transformed (RLT-28) cells were grown in decarbonated minimal essential medium. All these media contained 10% fetal calf serum, 100 μ g penicillin, and 170 μ g of streptomycin per ml; all were maintained at pH 7.4. Cells were grown in a 37° C incubator in an atmosphere of 5% CO₂ and 95% air.

The effect of *t*-RA alone, the cytokines alone, or both acting together on cell proliferation was studied using a three-fold dilution of cells harvested during the exponential growth phase. After treatment with these agents for 48 hours at 37° C, the solution containing the treated cells was diluted 500 fold, and the cells were counted using a coulter counter. Flow cytometric analysis of the nuclear DNA and proteins was performed in a flow cytometer using propidium iodine (PI) and fluorescein isothiocyanate (FITC) (see Breitman and Keene, 1982).

The transmembrane electron activities of the whole cells were measured using internal cellular NADH to reduce external impermeable oxidants, such as diferric transferrin (Fe₂Tf) (Crane, *et al.*, 1985). The reduction of the iron in Fe₂Tf was assayed by the formation of an iron-chelate, ferrous-bathophenanthroline sulfonate (ferrous-BPS; a pink complex), which was detected spectrophotometrically at wavelength 535 nm minus 600 nm using the dual beam of a DW2a Aminco spectrophotometer having an extinction coefficient of 17 mM⁻¹cm⁻¹ (Avron and Shavit, 1963). Cells for assay were suspended in 2.8 ml TD buffer (NaCl = 8 g/l; KCl = 0.38 g/l; Na₂HPO₄ = 0.1 g/l; and trizma base = 3 g/l; pH = 7.4). Each assay was run using 10 μ M diferric transferrin and 10 μ M bathophenanthroline disulfonate (BPS) and 0.015 g cells (wet weight) at 37° C (Löw, *et al.*, 1986).

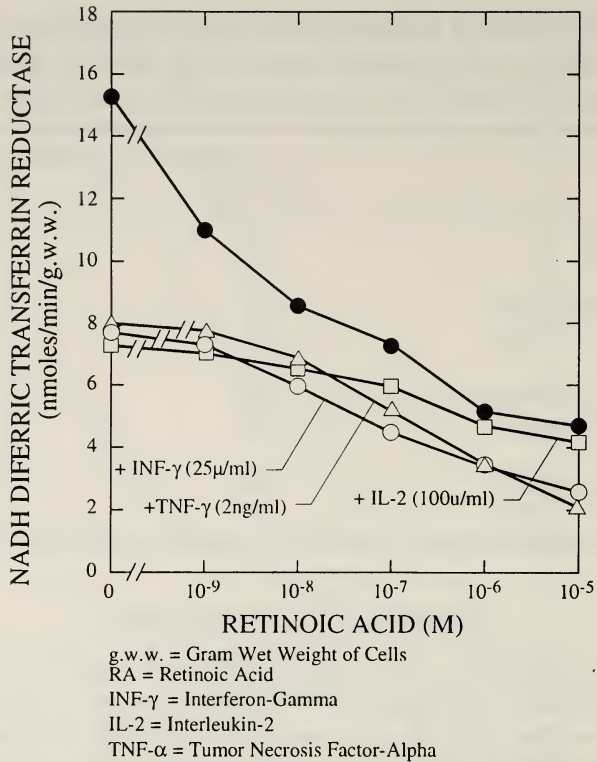


Figure 2. The additive inhibitory effect of *t*-RA and the cytokines on NADH-diferric transferrin reductase activity of HL-60 cells. The solid circles are *t*-RA only; the open symbols indicate cytokines plus *t*-RA.

RESULTS

The effect of *t*-RA on the proliferation of HL-60 cells shows a maximum inhibition of 47% at a 10⁻⁵ M concentration. A synergistic inhibitory effect on cell proliferation was also noted with the three tested cytokines; the maximum growth inhibition increased to 56%, 58%, and 61% in the presence of IFN- γ , IL-2, and TNF- α , respectively (Figure 1 and Table 1). With the addition of *t*-RA, cell-killing by the cytokines was enhanced ~10-15%.

t-RA inhibits the reduction of the iron in diferric transferrin by an oxidoreductase (as measured by the formation of ferrous-BPS). The maximum inhibition was 67% at a concentration at 10⁻⁵ M. The addition of the cytokines with *t*-RA causes an additional decrease in enzyme activity. The maximum inhibition of NADH-diferric transferrin reductase was increased to 89% with IFN- γ , 73% with IL-2, and 83% with TNF- α (Figure 2). Inhibition increased by ~12-20% after their association (Table 2).

The concentration of each of the cytokines required to reach one half maximum inhibition of cell proliferation is in the same concentration range as that which gives one half maximum inhibition of diferric transferrin reduction

Table 2. Enhancement of cytokine-induced inhibition of NADH-diferic transferrin reductase in HL-60 cells by retinoic acid (*t*-RA = *trans*-retinoic acid (10^{-5} M); IFN- γ = interferon-gamma; IL-2 = interleukin-2; and TNF- α = tumor necrosis factor-alpha).

	NADH-Diferic Transferrin Reductase (nmoles/min./gram wet weight)	
	Without <i>t</i> -RA	With <i>t</i> -RA
Control	18.5	6.0
IFN- γ (15 μ g/ml)	12.9	3.4
IFN- γ (25 μ g/ml)	6.5	2.1
IL-2 (25 μ g/ml)	15.4	5.1
IL-2 (50 μ g/ml)	10.3	2.1
TNF- α (1 ng/ml)	12.6	5.4
TNF- α (2 ng/ml)	6.6	3.0

(Table 3). Cytokine inhibition of the plasma membrane's electron transport system can thus be related to its inhibition of growth, because transmembrane oxidoreductase activity is related to cell growth (Avron and Shavit, 1963; Crane, *et al.*, 1985).

In addition to HL-60 cells, other transformed cell lines, such as HeLa cells and RLT-28 cells, also show that the combination of *t*-RA with a cytokine leads to a greater reduction in cell proliferation than treatment with either *t*-RA or a cytokine alone (Table 4). In the three tested transformed cell lines, 13-*cis*-RA (*c*-RA) is much less effective than *trans*-RA (*t*-RA) in reducing cell proliferation. Thus, the synergism between the cytokines and retinoic acid in inhibiting cell proliferation varies depending on which structural isomer of retinoic acid is used, indicating a clear structural specificity at the retinoic acid binding site.

The flow cytometric analysis of DNA and nuclear proteins of HL-60 cells also confirms the existence of a synergism between *t*-RA and the cytokines, which inhibits cell proliferation. Using fluorescein isothiocyanate on the flow cytometer (a procedure which can detect changes in DNA and nuclear protein simultaneously), a profound reduction in both DNA and nuclear proteins was found after the HL-60 cells had been treated with a combination of *t*-RA and the cytokines (Figure 3). A large reduction in fluorescence is seen in Figures 3b, 3c, and 3d (when the cells were treated with *t*-RA plus IFN- γ , *t*-RA plus IL-2, and *t*-RA plus TNF- α , respectively) in comparison to the control (Figure 3a). An instrumental count of the histogram constructed during flow cytometric analysis indicated 20,000 cells/ml for the control and 10,884 cells/ml, 13,232 cells/ml, and 6,382 cells/ml for *t*RA plus IFN- γ , *t*-RA plus IL-2, and *t*-RA plus TNF- α , respectively. The antiproliferative effect found using the flow cytome-

Table 3. A comparison of the cytokine concentration required for one-half maximum inhibition of growth and electron transport in HL-60 cells (IC_{50} = concentration at one-half maximum inhibition; IFN- γ = interferon-gamma; IL-2 = interleukin-2; and TNF- α = tumor necrosis factor-alpha).

Cytokine	Inhibition of Transferrin Reductase IC_{50}	Inhibition of HL-60 Cell Proliferation IC_{50}
TNF- α	1.2 ng/ml	1.1 ng/ml
IFN- γ	10 μ g/ml	20 μ g/ml
IL-2	35 μ g/ml	50 μ g/ml

ter was similar to the effect observed when using the coulter counter (Figure 1 and Table 1). A comparison of nuclear DNA content in different phases of the cell cycle for both the control and treated cells is presented in Table 5. The flow cytometric analysis demonstrates that DNA and nuclear proteins are markedly decreased when the cytokines and *t*-RA are administered in combination. The decrease is an indication that apoptosis (cell death) takes place when *t*-RA interacts with the cytokines.

DISCUSSION

IFN- γ (Müller, *et al.*, 1993; Vartanian, *et al.*, 1994; Watling, *et al.*, 1993) and IL-2 (Grimm, *et al.*, 1982) have been reported to induce cell death in various cell lines through binding at receptors that transduce signals to the target site of these cells. The actual mechanism for cell-killing is not known. TNF- α , on the other hand, induces oxygen radical generation, which is proposed as the basis for the induction of cell death (Wong and Groeddel, 1994). The antitumor effect of retinoids has been attributed to differentiation induction (Breitman, *et al.*, 1989; Chomienne, *et al.*, 1986) as well as to their direct influence on the rate of cell proliferation (Frey, *et al.*, 1991; Jetten, *et al.*, 1990). Recently, an additive antiproliferative effect of the combination of various retinoids with IFN- α on human transformed cell lines has been described (Frey, *et al.*, 1991). Our study demonstrates that the synergistic inhibition of cell proliferation in three transformed cell lines is not limited to the interaction of *t*-RA with interferon. Inhibition of growth also occurs with interleukin and tumor necrosis factor. The synergism is markedly influenced by which structural isomer of retinoic acid is present. *c*-RA is much less effective than *t*-RA, indicating that the receptor site for retinoic acid shows binding specificity.

Stimulation of plasma membrane oxidoreductase activities (the electron transport system) by the addition of external oxidants has been shown to increase cell proliferation in the HL-60 transformed cell line (Crane, *et al.*, 1995). Antitumor agents, such as adriamycin and retinoic acid, inhibit transmembrane electron transport at the same concentrations that inhibit cell growth

Table 4. The effect of retinoic acid (*cis* and *trans*) and cytokines on the growth of transformed cells (*t*-RA = *trans*-retinoic acid; *c*-RA = *cis*-retinoic acid; IFN- γ = interferon-gamma; IL-2 = interleukin-2; and TNF- α = tumor necrosis factor-alpha).

Added	HL-60 Cells	% Survivals HeLa Cells	RLT-28 Cells
Control	100	100	100
<i>t</i> -RA (10^{-5} M)	60.9	64.9	42.7
IFN- γ (100 μ g/ml)	56.8	54.6	31.8
IL-2 (100 μ g/ml)	62.3	67.5	32.3
TNF- α (2 ng/ml)	83.1	80.6	42.6
<i>t</i> -RA (10^{-5} M) + IFN- γ (100 μ g/ml)	48.3	45.8	20.8
<i>t</i> -RA (10^{-5} M) + IL-2 (100 μ g/ml)	51.1	41.9	25.1
<i>t</i> -RA (10^{-5} M) + TNF- α (2 ng/ml)	48.5	43.1	29.2
<i>c</i> -RA (10^{-5} M)	97.1	84.9	70.0
<i>c</i> -RA (10^{-5} M) + IFN- γ (100 μ g/ml)	60.6	58.9	43.4
<i>c</i> -RA (10^{-5} M) + IL-2 (100 μ g/ml)	65.2	58.7	50.5
<i>c</i> -RA (10^{-5} M) + TNF- α (2 ng/ml)	77.5	55.0	52.1

(Sun, *et al.*, 1988; Sun and Crane, 1990). Growth factors, such as epidermal growth factor (EGF) and bombesin, stimulate plasma membrane electron transport (Navas, *et al.*, 1992; Sun, Crane, and Löw, 1994). In this study, we have demonstrated the existence of a correlation between the inhibition (induced by the cytokines) of plasma membrane oxidoreductase activity (NADH-diferric transferrin reductase) and the inhibition of growth in transformed cells (Table 1); the concentration of cytokines is similar for the one half maximum inhibition of both NADH-diferric transferrin reductase and cell proliferation. Since there is evidence that inhibition of plasma membrane electron transport can also induce apoptosis (Morre, *et al.*, 1995), we propose that the inhibition of transmembrane oxidoreductase activities (or the plasma membrane's electron

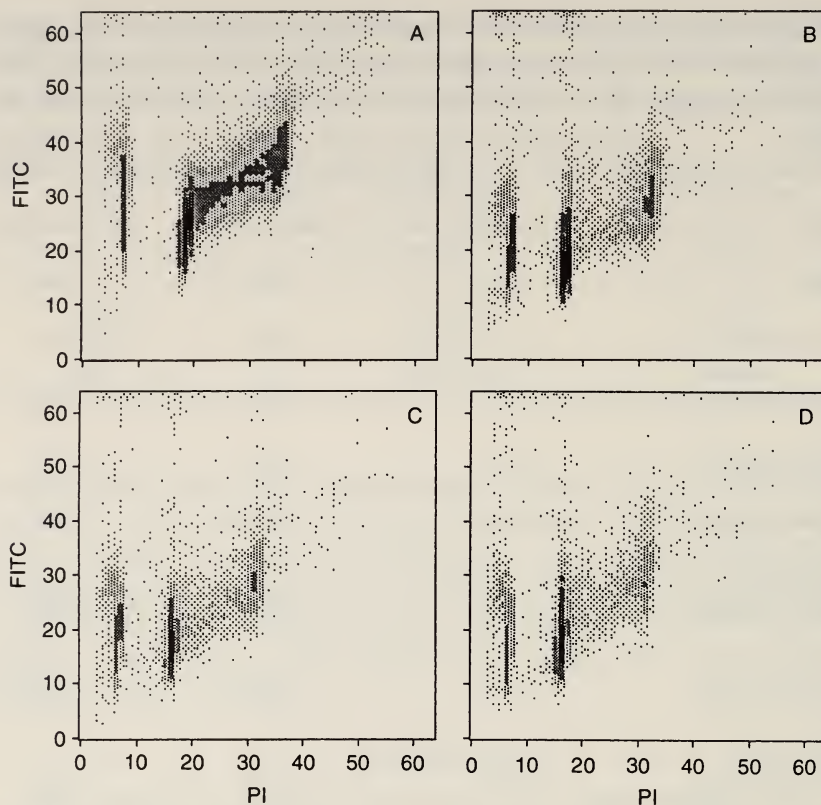


Figure 3. The flow cytometric analysis of changes in the DNA and nuclear proteins in control cells and *t*-RA-cytokine treated cells: A) control; B) *t*-RA plus IFN- γ (100 μ g/ml); C) *t*-RA plus IL-2 (100 μ g/ml); and D) *t*-RA plus TNF- α (2 ng/ml). The *t*-RA concentration was 10^{-5} M; PI is used to measure DNA; and FITC is used to measure proteins.

transport system) accounts for the induced antiproliferative effect of *t*-RA and the cytokines on these three tested transformed cell lines. Our results strongly suggest that the combination of cytokines with *t*-RA may represent a new approach for the treatment of neoplastic diseases, thereby opening a new era in cancer chemotherapy.

LITERATURE CITED

- Avron, M. and N. Shavit. 1963. A sensitive and simple method for determination of ferricyanide. *Anal. Biochem.* 6: 549-554.
- Bollag, W. 1983. Vitamin A and retinoids: From nutrition to pharmacotherapy in dermatology and oncology. *Lancet* 1: 860-863.
- Breitman, T.R., S.J. Collins, and B.R. Keene. 1981. Terminal differentiation of human promyelocytic leukemia cells in primary culture in response to retinoic acid. *Blood* 57: 1000-1004.

Table 5. A comparison of the nuclear DNA content in different phases of the cell cycle of control and treated HL-60 cells (*t*-RA = *trans*-retinoic acid).

Treatment/DNA Content	G ₁ G ₀ (%)	S (%)	G ₂ M (%)
Control	51.7	29.0	10.3
<i>t</i> -RA + IFN- γ (100 μ g/ml)	37.4	1.25	0.1
<i>t</i> -RA + IL-2 (100 μ g/ml)	44.2	1.5	0.2
<i>t</i> -RA + TNF- α (2 ng/ml)	6.1	0.15	0.0

- _____ and B.R. Keene. 1982. Growth and differentiation of human promyelocytic cell line HL-60 in a defined medium. In: G.H. Sato, A.B. Pardee, and D.A. Sirbasku (Eds.), *Growth of Cells in Hormonally Defined Media*, pp. 691-702, Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1214 pp.
- _____, S.E. Selonick, and S.J. Collins. 1989. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Nat. Acad. Sci. USA* 77: 2936-2940.
- Brinkerhogg, C.E., R.M. McMillan, J.M. Dayer, and E.C. Harris. 1980. Inhibition by retinoic acid of collagenase production in rheumatoid synovial cells. *New Engl. J. Med.* 303: 432-436.
- Chomienne, C., N. Baslitrang, H. Cost, L. Degos, and J.P. Abita. 1986. Structure-activity relationships of aromatic retinoids on the differentiation of the human histiocytic lymphoma cell line U-937. *Leukemia Res.* 10: 1301-1305.
- Cope, F.E. and R.K. Boutwell. 1983. Retinoic acid binding to mouse brain plasma membrane. *Fed. Proc.* 42: 1312-1317.
- Crane, F.L., C. Grebing, and H. Löw. 1985. Transplasma-membrane redox systems in growth and development. *Biochem. Biophys. Acta* 811: 233-264.
- _____, I.L. Sun, E.E. Sun, and R.A. Crowe. 1995. Plasma membrane redox and regulation of cell growth. *Protoplasm* 184: 3-7.
- Frey, J.R., R. Peck, and W. Bollag. 1991. Anti-proliferative activity of retinoids, interferon α and their combination in five human transformed cell lines. *Cancer Lett.* 57: 223-227.
- Grimm, E.A., A. Mazumder, H.Z. Zhang, and S.A. Rosenberg. 1982. Lymphokine-activated killer cell phenomenon. *J. Exp. Med.* 155: 1823-1841.
- Hamblin, A.S. 1993a. Cytokines. In: D. Rickwood and D. Male (Eds.), *Cytokines and Cytokine Receptors*, p. 1-19, Oxford Univ. Press, New York, 90 pp.
- _____. 1993b. Cytokines in the activation of T cells, B cells and macrophages. In: D. Rickwood and D. Male (Eds.), *Cytokines and Cytokine Receptors*, pp. 45-54, Oxford Univ. Press, New York, 90 pp.
- Jetten, A.M., J.S. Kim, P.G. Sacks, J.I. Rearick, D. Lotan, W.K. Hong, and R. Lotan. 1990. Inhibition of growth and squamous-cell differentiation markers in human head and neck carcinoma cells by β -cell *trans* retinoic acid. *Int. J. Cancer* 45: 195-202.
- Lotan, R. 1980. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* 605: 33-91.
- Löw, H., I.L. Sun, P. Navas, C. Grebing, F.L. Crane, and D.J. Morre. 1986. Transplasmalemma electron transport from cells is part of a diferric transferrin reductase system. *Biochem. Biophys. Res. Commun.* 139: 1117-1123.
- Morre, D.J., P.J. Chueh, and D.M. Morre. 1995. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc. Nat. Acad. Sci. USA* 92: 1831-1835.
- Müller, M., J. Briscoe, C. Laxton, D. Crushin, A. Zlemlecki, O. Silvernoinen, A.G. Harpur, A.G. Barblerl, B.A. Witthuhn, C. Schindler, S. Pellegrini, A.F. Wilks, A.F. Ihle, J.N. Stark, G.R. Kerr, and L.M. Kerr. 1993. The protein tyrosine kinase JAK1 complements defects in interferon- α/β and - γ signal transduction. *Nature* 366: 129-135.
- Navas, P., F.J. Aleain, F. Buron, J.C. Rodrigney, A. Guilira, J.M. Villalba, D.M. Morre, and D.J. Morre. 1992. Growth factor-stimulation *trans* plasma membrane electron transport in HL-60 cells. *FEBS Lett.* 299: 223-226.
- Sani, B.P. 1979. Retinoic acid binding protein: A plasma membrane component. *Biochem. Biophys. Res. Cummun* 91: 502-507.
- Sherman, M.I. 1986. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. In: M.I. Sherman (Ed.), *Retinoids and Cell Differentiation*, pp. 161-186, CRC Press, Boca Raton, 198 pp.

- Sporn, M.B. and A.B. Roberts. 1983. Role of retinoids in differentiation and carcinogenesis. *Cancer Res.* 43: 3034-3040.
- Sun, I.L. and F.L. Crane. 1990. Transmembrane electron transport and growth of transformed cells. In: F.L. Crane, D.J. Morre, and H.E. Löw (Eds.), *Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport, Volume I*, pp. 141-170, CRC Press, Boca Raton, 318 pp.
- _____, _____, and H. Löw. 1994. Bombesin stimulates transplasma-membrane electron transport by 3T3 cells. *Biochim. Biophys. Acta* 1221: 206-210.
- _____, W. Toole-Simms, F.L. Crane, D.J. Morre, H. Löw, and J.Y. Chou. 1988. Transformation with SU40 virus prevents retinoic acid inhibition of NADH diferric transferrin reductase in rat liver cells. *J. Bioenerg. Biomemb.* 20: 383-391.
- Sunkara, P.S., N.J. Prakash, G.D. Mayer, and A. Sjoerdsma. 1983. Tumor suppression with a combination of α -difluoromethylornithine and interferon. *Science* 219: 851-853.
- Vartanian, T., Y. Li, M. Zhau, and K. Stefausson. 1994. Interferon-g induces programmed death of oligodendrocytes. *Mol. Biol. Cell* 5: 153a.
- Waksman, B.H. 1979. Overview: Biology of the lymphokines. In: S. Cohen, E. Pick, and J.J. Oppenheim (Eds.), *Biology of the Lymphokines*, pp. 585-616, Acad. Press, New York, 626 pp.
- Watling, D., D., Cruschin, M. Müller, O. Silvernoinen, B.A. Witthuhn, F.W. Quelle, N.C. Rogers, C. Schindler, G.R. Stark, J.N. Ihle, L.M. Kerr, and G.R. Kerr. 1993. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon- γ signal transduction pathway. *Nature* 366: 166-170.
- Wong, G.H.W. and D.V. Groeddel. 1994. Induction of oxygen radical by TNF- α . *J. Immunol.* 152: 1751-1755.