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Modulation of the interferon antiviral activity by adriamycin in human cells in culture

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Summary

The antiviral activity of exogenously applied HuIFN-α was significantly reduced in cells pretreated with non-toxic concentrations of adriamycin. With low concentrations of adriamycin (up to 0.125 μg/ml) the effect was reversible in all cells tested. In human foreskin (BG-9) cells, that are more resistant to adriamycin toxicity, higher nontoxic concentrations of adriamycin (0.625 μg/ml) caused an irreversible decrease in sensitivity to HuIFN-α. Under these conditions, adriamycin pretreatment irreversibly inhibited the rate of cellular protein synthesis in BG-9 cells by about 30%. In contrast, cellular RNA synthesis was inhibited by 80% in 4 h after exposure to the drug, but was restored to almost normal levels (97% of control) at day 4 after removal of the drug. These results suggest that the loss of sensitivity of adriamycin-treated BG-9 cells to HuIFN-α may be related to the inhibition of cellular protein synthesis.

interferon; adriamycin; protein synthesis

Introduction

Interferons (IFNs) and adriamycin are both used in the treatment of human cancer. Adriamycin is a broad spectrum antibiotic of the anthracycline group which was formerly considered to be cytotoxic by intercalating with cellular DNA [8,19]. More recent evidence, however, suggests that it primarily acts on the cell surface [22]. Regardless of its mechanism of action, adriamycin causes many membrane changes

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including lectin interaction [18], glycoprotein synthesis [15], and expression of hormone receptors [25]. IFNs on the other hand are proteins secreted by cells in response to a variety of inducers. Among the many biological activities exhibited by IFNs, the best known is their ability to confer on homologous cells a state of resistance against a wide variety of viruses [17,20]. Although the mechanism of this action is not clearly understood, it is known that interaction of the IFN with specific receptors on the surface, and a continued cellular macromolecular biosynthesis are essential [1,12]. Thus, any change in the composition or in the conformation of the cell receptor(s) or in the macromolecular biosynthesis could alter the response of the cell to IFN. For this reason, we thought it useful to study the effect of adriamycin on the antiviral activity of HuIFN-α in normal and neoplastic human cells in culture.

Materials and Methods

Interferon

Human leukocyte interferon (HuIFN-α) was induced by Sendai virus in peripheral white blood cells according to the procedure developed earlier [23]. It was purified to a specific activity of 10^7 units/mg protein according to our patented procedure [7].

Cell cultures

The four human cell lines used in this study were divided into two groups based on their sensitivity to HuIFN-α [9]. Group I, highly sensitive to IFN, consisted of GM-258, trisomic for chromosome 21 obtained from the American Type Culture Collection, Bethesda, MD, U.S.A., and BG-9, normal diploid fibroblast (human foreskin) isolated and characterized at RPMI [14]. Group II, moderately sensitive to IFN, consisted of human fibrosarcoma (HT-1080) and human lung adenocarcinoma (A549), kindly provided by Dr. E. Borden, University of Wisconsin and Dr. J. Fogh, Sloan Kettering Institute for Cancer, Rye, NY, U.S.A., respectively.

Assay for IFN activity

Antiviral activity of IFN was assayed on human foreskin (BG-9) cells according to the procedure of Finter [11] using VSV as the challenge virus. Antiviral titers are expressed as International Units (IU) based on standards provided by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

Determination of toxicity induced by adriamycin in cell cultures

Cells were grown in 24-well Linbro trays and exposed to varying concentrations of adriamycin (Adria Labs, Columbus, Ohio, U.S.A.) for 24 h at 37°C. Cells were then washed twice with medium to remove the drug, refed with medium without adriamycin and reincubated at 37°C. At daily intervals cellular toxicity was determined by the vital dye uptake procedure described by Finter [10]. The amount of dye uptake by cells treated with medium alone (control) represented 100% viability. Any reduction of dye uptake by cells pretreated with adriamycin was a reflection of the toxic effect induced by the drug.

Assay for 2',5'-oligo(A) polymerase activity

The enzyme 2',5'-oligo(A) polymerase polymerizes ATP into 2',5'-oligoadenylate. This enzyme activity in cell cultures was determined as follows. Cell extracts were prepared according to the procedure of Weber et al. [24]. Cells were trypsinized and washed in phosphate buffered saline (PBS), pH 7.2. Two packed cell volume of lysis buffer was added to the cell pellet and kept on ice for 10 min. The pellet was then homogenized and centrifuged at 12 000 × *g* for 4 min. Supernatant (cell extract) was collected, assayed for protein content by fluorocolorimetric assay [5] and stored at -70°C. The 2',5'-oligo(A) polymerase activity in the cell extracts was determined according to the procedure described by Baglioni [2]. Briefly, 70 µl of cell extract (10 mg/ml protein) was diluted in 200 µl of incubation mixture [2], applied to a poly(rI : rC)-agarose column and incubated for 17 h at 30°C. Radiolabeled 2',5'-oligoadenylate synthesized from radiolabeled ATP during incubation period was isolated by chromatography on DEAE-cellulose column. The enzyme activity is expressed as pmol of ATP polymerized to 2',5'-oligoadenylate in 17 h per mg protein [2].

Effect of adriamycin on cellular RNA and protein synthesis

Cultures of BG-9 cells in 24 well Costar trays were treated with 0.625 µg/ml of adriamycin or with medium (control) for 2 h at 37°C. All cultures were then washed twice with medium, refed with medium and reincubated at 37°C. At various times, adriamycin-treated and control cells were washed twice with PBS and labeled with 10 µCi/culture of [^{3}H]uridine or [^{3}H]amino acid mixture (New England Nuclear, Boston, MA, U.S.A.) for 30 min at 37°C. After the labeling period, cultures were again washed twice with PBS and trichloroacetic acid (TCA) insoluble counts determined as described previously [21]. It should be noted that a minimum of 2 h exposure of cells to adriamycin, followed by 18 h incubation at 37°C in the absence of the drug was sufficient to obtain maximum inhibition of the antiviral activity of HuIFN-α (see Results).

Analysis of proteins in adriamycin-treated cells

Cultures of BG-9 cells were treated with 0.625 µg/ml of adriamycin as described above. At 3 h after exposure to adriamycin, control and adriamycin-treated cells were washed with medium containing reduced methionine and 1% fetal bovine serum. The cells were labeled with 2.5 µCi/culture of [^{35}S]methionine (New England Nuclear, Boston, MA, U.S.A.) in methionine reduced medium for 3 h at 37°C. After the labeling period, the cells were washed twice with PBS and solubilized in 0.01 M Tris buffer, pH 7.4 containing 0.001 M EDTA, 0.01 M NaCl and 0.1% SDS [16]. The solubilized cell preparation was stored at -70°C prior to protein analysis. The proteins were separated on 7.5% SDS-polyacrylamide gel electrophoresis with appropriate standard markers as described previously [16].

Results

Adriamycin-induced toxicity in human cells in culture

To determine the concentrations of adriamycin that would be non-toxic to cells, triplicate cell cultures were exposed to various concentrations of adriamycin and examined for toxicity by the vital dye uptake procedure. The results showed that 4 days after removal of adriamycin, no toxicity was observed in any of the cells pretreated with up to 0.125 μg/ml of the drug. In BG-9 cells no toxicity was noted at 0.625 μg/ml of adriamycin. However, GM-258, HT-1080, A-549 and BG-9 cells showed toxicity at 1 μg/ml (data not shown).

The experimental protocol called for exposure of cells first to adriamycin for 24 h followed by 18 h treatment with HuIFN-α. In order to determine whether, under these conditions, HuIFN-α might affect expression of cellular toxicity, all four cell lines were sequentially exposed to adriamycin and HuIFN-α and tested for cell viability as described in the legend of Figure 1. No significant differences in cell viability were seen when cells were treated with adriamycin alone (90–95%) or in combination with HuIFN-α (88–92%, Figure 1).

Effect of adriamycin pretreatment on the antiviral activity of HuIFN-α

Confluent cell cultures were exposed to 0.062 or 0.125 μg/ml of adriamycin in the usual manner. All cultures were washed twice with medium and 2000 IU of HuIFN-α (based on assay in BG-9 cells) was assayed on each of the four cell lines. The results presented in Table 1 confirm our previous observation that different cells (BG-9, GM-258, A-549 and HT-1080) varied in their sensitivity to HuIFN-α [9]. However, pretreatment with adriamycin reduced the titer of HuIFN-α by 50–90% in all cells (Table 1).

Further experiments were conducted to determine the minimum adriamycin exposure time required for inhibition of the antiviral activity of HuIFN-α. Confluent monolayers of BG-9 cells were exposed to 0.125 μg/ml of adriamycin for various

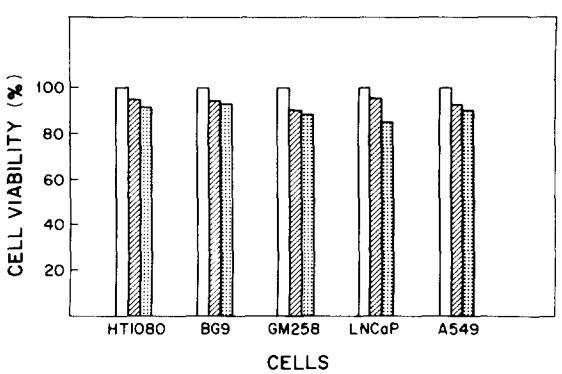

Fig. 1. Effect of sequential exposure of cells to adriamycin and HuIFN-α on cellular toxicity. Cells were exposed to 0.125 μg/ml of adriamycin for 24 h followed by 2000 IU of HuIFN-α. At the end of 18 h of HuIFN-α exposure, cell viability was determined by dye uptake method of Finter [10]. □, control; ▨, adriamycin alone; ▩, adriamycin and HuIFN-α.

TABLE 1

Effect of adriamycin pretreatment on the antiviral activity of HuIFN-α

Cells[1]	IFN titer (IU/ml)[2] (adriamycin pretreatment, μg/ml)		
	0	0.062	0.125
BG-9	2000	700 (65)[3]	360 (82)
GM-258	8500	2550 (70)	1275 (85)
A-549	1500	750 (50)	420 (72)
HT-1080	540	150 (72)	55 (90)

[1] Cells were pretreated with adriamycin for 24 h prior to IFN assay.
[2] Represents the average of three separate experiments.
[3] Percent inhibition of IFN titer.

periods of time (0.25, 0.5, 1, 2, 4 h) at 37°C and then washed and reincubated at 37°C for an additional 18 h prior to assay for HuIFN-α activity. Results showed that exposure of cells to adriamycin for 1–2 h at 37°C was sufficient to obtain maximum inhibition of the antiviral activity (80%) of HuIFN-α in BG-9 cells (data not shown).

Reversal of the effect of adriamycin pretreatment of cells on antiviral activity of HuIFN-α

Cell cultures (BG-9, A-549 and HT-1080) were treated with various concentrations of adriamycin in the usual manner. At day 1, 2 and 4 after removal of the drug, 5000 IU of HuIFN-α (based on an assay in BG-9 cells) was assayed in adriamycin-treated and control cells. Interferon titers obtained in cultures at different times after removal of the drug are presented in Table 2. The results show that cells pretreated with adriamycin (up to 0.125 μg/ml) progressively regained their antiviral sensitivity to HuIFN-α. Maximum recovery (70–90%) was noted at 4 days after removal of the drug.

From preliminary studies it was noted that BG-9 cells were significantly more resistant to the toxic effects of adriamycin (up to 0.625 μg/ml) than A-549 and HT-1080 cells (unpublished observation). It was, therefore, of significance to determine whether BG-9 cells would regain the antiviral sensitivity to HuIFN-α when treated with higher but nontoxic concentrations of adriamycin. Results presented in Table 2 show that cells treated with 0.625 μg/ml adriamycin regained only 5% of the sensitivity to HuIFN-α at day 4 after removal of the drug. These results show that in BG-9 cells the reversibility of adriamycin effect on antiviral activity of HuIFN-α is dependent on its concentration.

Effect of adriamycin on cellular RNA and protein synthesis

The observation that BG-9 cells exposed to a high but nontoxic concentration (0.625 μg/ml) of adriamycin were unable to regain significant sensitivity to HuIFN-α four

TABLE 2

Reversal of the effect of adriamycin pretreatment on the antiviral activity of HuIFN-α

Cells	Adriamycin pretreatment[1] (μg/ml)	IFN titer (IU/ml)[2] (days after removal of adriamycin)		
		1	2	4
BG-9	0	5000	4550	4900
	0.062	1250	2050	4410
		(75)[3]	(55)	(10)
	0.125	600	910	4410
		(88)	(80)	(10)
	0.310	250	680	1710
		(95)	(85)	(65)
	0.625	<10	<10	250
		(100)	(100)	(95)
A-549	0	3750	3800	3675
	0.062	1125	2355	2940
		(70)	(38)	(30)
	0.125	937	2280	2570
		(75)	(40)	(20)
HT-1080	0	1250	1320	1400
	0.062	256	595	1050
		(80)	(55)	(25)
	0.125	185	435	980
		(85)	(67)	(30)

[1] Cells were treated with adriamycin for 24 h.
[2] Represents the average of three separate experiments.
[3] Percent inhibition of IFN titer.

days after removal of the drug (Table 2), prompted us to examine the effect of this concentration of adriamycin on cellular RNA and protein synthesis. The experiment was conducted as described in Materials and Methods. The results presented in Figure 1 show that adriamycin markedly inhibited host RNA synthesis (80%) by 4 h after cells were exposed to adriamycin. This level of inhibition was maintained for at least the next 4 h. However, the cellular RNA synthesis recovered almost completely with only 3% inhibition observed 96 h after removal of adriamycin (Figure 1). Maximum inhibition of cellular protein synthesis (30%) was achieved by 2 h after exposure of cells to adriamycin, and was subsequently maintained for 96 h (Figure 1). These results show that the inhibitory effect of adriamycin on cellular RNA synthesis is rapidly reversible whereas the effect on protein synthesis is unchanged after removal of the drug.

Analysis of cellular proteins in adriamycin-treated BG-9 cells

Since the inhibition of antiviral activity of HuIFN-α in adriamycin-treated cells may be related to its ability to inhibit host protein synthesis, it was of interest to determine changes in the profile of cellular proteins synthesized in adriamycin-treated cells. Four

cultures of BG-9 cells were treated with adriamycin (0.625 μg/ml, maximum nontoxic concentrations; Table 2) and four with medium (control) and labeled with [^{35}S]methionine between 3 and 6 h after exposure to the drug and the proteins analyzed on polyacrylamide gel electrophoresis. It should be noted that there was an average of 40% inhibition of the incorporation of [^{35}S]methionine into TCA-insoluble counts in adriamycin-treated cells as compared to the control cells. Figure 3 shows that there was no significant change in the overall profile of the radiolabeled cellular proteins. However, a 56 K protein which appeared consistently in all control cultures (Fig. 3A, see arrow) was markedly reduced in adriamycin-treated cells (Figure 3B).

Effect of adriamycin pretreatment on the induction of 2',5'-oligo(A) polymerase by HuIFN-α

IFNs have been shown to induce in cells an enzyme designated 2',5'-oligo(A) polymerase which polymerizes ATP into 2',5'-oligoadenylate [3,4]. This enzyme activity is believed to play a role in the development of antiviral state in IFN-treated cells [2–4]. The following experiment was conducted to determine the effect of adriamycin pretreatment on the induction of 2',5'-oligo(A) polymerase by HuIFN-α. Confluent BG-9 and HT-1080 cell cultures were treated with 0.062 μg/ml of adriamycin for 24 h at 37°C. The cultures were then washed in the usual manner and exposed to 2000 IU of HuIFN-α for an additional 18 h at 37°C. The induced 2',5'-oligo(A) polymerase activity was determined by incorporation of labeled [^{3}H]ATP. The results presented in Table 3 show that in both cell lines treatment with HuIFN-α significantly enhanced 2',5'-oligo(A) polymerase activity as indicated by the enhanced incorporation of [^{3}H]ATP into 2',5'-oligo(A) nucleotides. However, when these cells were pretreated with adriamycin prior to exposure to HuIFN-α, the enzyme activity was significantly suppressed (Table 3).

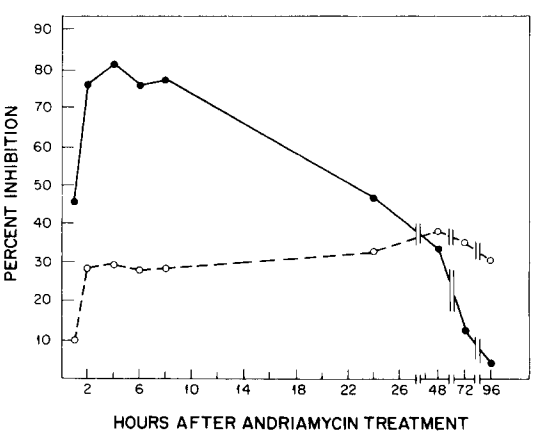

Fig. 2. Effect of adriamycin on BG-9 cellular RNA (●——●) and protein (○---○) synthesis. The average radioactivity incorporated into TCA insoluble counts in control cultures was 9745 and 10 752 for [^{3}H]uridine and [^{3}H]amino acid mixture, respectively.

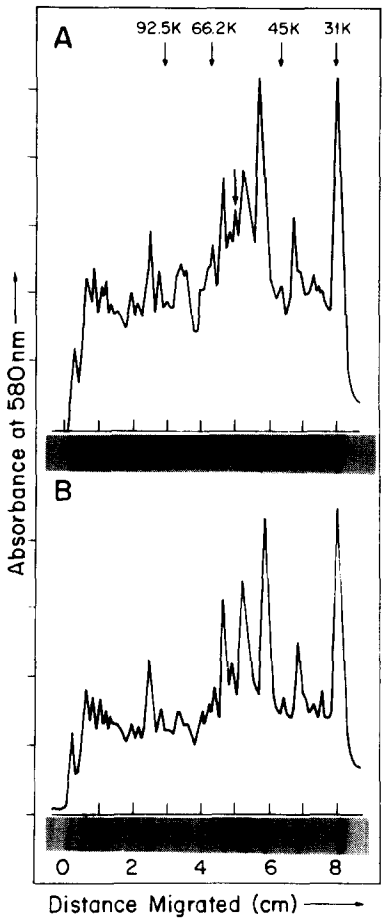

Fig. 3. Autoradiogram and densitometer tracings of radioactive polypeptides synthesized in BG-9 cell cultures treated with medium alone (control, A) or with medium containing 0.625 μg/ml of adriamycin (B).

Discussion

The purpose of this study was to evaluate the effect of adriamycin pretreatment of cells on the antiviral activity of HuIFN-α. The results presented suggest that sequential treatment of cells with adriamycin and HuIFN-α, significantly reduces the ability of both normal and neoplastic cells to develop IFN-induced antiviral state. However, this effect of adriamycin is transient. Cells treated with up to 0.125 μg/ml of adriamycin progressively regained their sensitivity to HuIFN-α upon removal of the drug (Table 2).

The mechanism(s) by which adriamycin alters the ability of the cells to respond to the antiviral effects of HuIFN-α is not known. This is primarily because the mecha-

TABLE 3

Effect of adriamycin on the induction of 2',5'-oligo(A) polymerase by HuIFN-α

Cells	Treatment	[^{3}H]ATP incorporation in 2',5'-oligo(A) nucleotides (cpm)	2',5'-oligo(A) polymerase activity (pmol/h per mg protein)
BG-9	Control	3470	0.76
	HuIFN-α[1]	12120	2.60
	Adriamycin and HuIFN-α[2]	4956	0.66
HT-1080	Control	3370	0.45
	HuIFN-α[1]	8848	1.12
	Adriamycin and HuIFN-α[2]	4624	0.61

[1] Cells were treated with 2000 IU of HuIFN-α for 18 h at 37°C.

[2] Cells were first treated with 0.062 μg/ml of adriamycin for 24 h at 37°C and then with 2000 IU of HuIFN-α for an additional 18 h at 37°C. The cpm represent an average of three experiments.

nism of action of adriamycin and the mechanism by which IFN induces the antiviral state in cells are not fully understood. However, this study provides possible explanations with regard to the observed changes seen in IFN response in adriamycin-treated cells in vitro. The overall reduction of protein synthesis in adriamycin-treated cells (Figure 2), including marked inhibition of a 56 K protein (Figure 3), may contribute in part to the reduced ability of HuIFN-α to induce antiviral state in adriamycin-treated cells. The decrease in the level of induction of 2',5'-oligo(A) polymerase in adriamycin-treated cells in response to HuIFN-α in cells may also be correlated with the inhibition of host protein synthesis.

It is interesting to note that IFN has been shown to affect intracellular levels of calmodulin, a major Ca^{2+} receptor which has been shown to regulate a large number of cellular enzymes such as protein kinase and 2',5'-(A) polymerase [6]. Since adriamycin affects IFN-induced 2',5'-(A) polymerase activity (Table 3), the possibility cannot be excluded that the effect of adriamycin on the IFN system in cell cultures may be affected with the Ca^{2+}/calmodulin dependent processes.

References

1 Ankel, H., Krishnamurti, C., Besancon, F., Stefanos, S. and Falcoff, E. (1980) Mouse fibroblast (type I) and immune (type II) interferons: pronounced differences in affinity for gangliosides and in antiviral and antigrowth effects on mouse leukemia L-1210R cells. Proc. Natl. Acad. Sci. USA 77, 2528–2532.

2 Baglioni, C. (1979) Interferon induced enzymatic activities and their role in antiviral state. Cell 17, 255–264.

3 Baglioni, C. and Maroney, P.A. (1980) Human leukocyte interferon induces 2',5'-oligo(A) polymerase and protein kinase. Virology 101, 540–544.

4 Baglioni, C. and Maroney, P.A. (1980) Mechanism of action of human interferons. J. Biol. Chem. 255, 8390–8393.

5 Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Fluorimetic assay of proteins in the nanogram range. Arch. Biochem. Biophys. 155, 213–220.
6 Bourgeade, M.F., Besancon, F. and Thang, M.N. (1983) Interferon affects intracellular calmodulin levels. Biochem. Biophys. Res. Commun. 111, 430–437.
7 Chadha, K.C. and Sulkowski, E. (1984) Method for the production and purification of human leukocyte interferon. U.S. Patent 4,485,038, November 1984.
8 Di Marco, A. and Arcamone, F. (1975) DNA complexing antibiotics: Daunomycin, adriamycin and their derivatives. Arzneimittel Forsch. 25, 368–375.
9 Dinolfo, E. and Chadha, K.C. (1983) Antiviral and antiproliferative activities of three molecular components of leukocyte-derived human interferon. Microbiol. Immunol. 27(7), 589–599.
10 Finter, N.B. (1969) Dye uptake method of assessing viral cytopathogenicity and their application to interferon assays. J. Gen. Virol. 5, 419–425.
11 Finter, N.B. (1973) The assay and standardization of interferon and interferon inducers. In: Interferon and Interferon Inducers (Finter, N.B., ed.), pp. 135–170, North Holland Publ. Co., Amsterdam.
12 Friedman, R.M. (1977) Antiviral activity of interferons. Bacteriol. Rev. 41, 543–567.
13 Giles, R.E. and Ruddle, F.H. (1973) Production of Sendai virus for cell fusion. In Vitro 9, 103–107.
14 Horoszewicz, J.S., Leong, S., Ito, M., Diberardino, L. and Carter, W.A. (1978) Aging in vitro and large scale production by 15 new strains of human diploid fibroblasts. Infect. Immun. 19, 720–726.
15 Kessel, D. (1979) Enhanced glycosylation induced by adriamycin. Mol. Pharmacol. 16, 306–312.
16 Laemmli, V.M. and Favre, M. (1973) Maturation of the head of bacteriophage T4. DNA packaging events. J. Mol. Biol. 80, 575–600.
17 Marcus, P.I. and Salb, J.M. (1966) Molecular basis of interferon action: inhibition of viral RNA translation. Virology 30, 502–516.
18 Murphree, S.A., Cunningham, L.S., Hwang, K.M. and Sartorelli, A.C. (1976) Effects of adriamycin on surface properties of sarcoma 180 ascities cells. Biochem. Pharmacol. 25, 1227–1231.
19 Neidle, S. (1979) The molecular basis for the action of some DNA-binding drugs. Prog. Med. Chem. 16, 152–221.
20 Sonnenfeld, G. and Merigan, T.C. (1979) The role of interferon in viral infections. Springer Semin. Immunopathol. 2, 311–338.
21 Thacore, H.R. and Youngner, J.S. (1975) Abortive infection of a rabbit cell line by vesicular stomatitis virus: conversion to productive infection by superinfection with vaccinia virus. J. Virol. 16, 322–329.
22 Tritton, T.R. and Yee, G. (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science 217, 248–250.
23 van Oss, C.J., Bronson, P.M., Dinolfo, E.A. and Chadha, K.C. (1981) Two methods for removal of erythrocytes from buffy coats for the production of human leukocyte interferon. Immunol. Commun. 10, 549–555.
24 Weber, L.A., Femen, E.R. and Baglioni, C. (1975) A cell free system from Hela cells active in inhibition of protein synthesis. Biochemistry 14, 5315–5320.
25 Zuckier, G.N., Tomiko, S.A. and Tritton, T.R. (1981) Increased number of ^{125}I epidermal growth factor binding sites in HeLa cells grown in the presence of adriamycin. Fed. Proc. Fed. Am. Soc. Exp. Biol. 40, 1877.