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SYNERGISTIC ANTIPROLIFERATIVE EFFECTS OF INTERLEUKIN-1α AND DOXORUBICIN AGAINST THE HUMAN OVARIAN CARCINOMA CELL LINE (NIH:OVCAR-3)

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Abstract—Interleukin-1α (IL-1α) exerts antiproliferative effects on a human ovarian carcinoma cell line, NIH:OVCAR-3, which is resistant to clinically relevant concentrations of doxorubicin (DOX) and other chemotherapeutic agents. This action of IL-1α depends on the presence of type I (80 kDa) receptors, although no quantitative relationship has been established between receptor occupancy and inhibition of cell growth. When NIH:OVCAR-3 cells were exposed to IL-1α and DOX in combination, a mutual potentiation of the antiproliferative effects of the two agents was observed. This synergistic effect was not due to IL-1 receptor expression up-regulation by DOX, and receptor-dependent internalization of the cytokine was also unaffected. The involvement of IL-1 receptors is supported by the observation that synergism between the two agents was diminished (but not abolished) in the presence of a specific IL-1 receptor antagonist at concentrations blocking more than 75% of IL-1α binding. DOX was found to significantly increase IL-1α accumulation by NIH:OVCAR-3 cells after long-term (48 hr) exposure to the cytokine at 37°, which might be due to increased nonspecific fluid phase uptake or to interference with cytokine degradation and/or release processes. The potent synergy of IL-1α and DOX against ovarian carcinoma cells *in vitro* suggests that this drug combination may be effective against this disease in the clinic.

Interleukin-1 (IL-1‡) is produced predominantly by activated monocytes and macrophages and possesses a wide range of biological activities. These include the ability to modulate T- and B-cell function, to induce prostaglandin E_2 synthesis and release, to stimulate proliferation of fibroblasts and mouse thymocytes, to stimulate the synthesis of other cytokines, such as interleukin-6 and tumor necrosis factor, and to evoke systemic responses such as fever and acute phase response [1–3]. Several studies suggest that IL-1 may act directly to inhibit the proliferation of certain tumor cells and also to exert antitumor activity indirectly, e.g. by enhancing cellular immune responses. IL-1 has been found to be cytocidal or cytostatic *in vitro* for the human melanoma cell line A375 [4–8] and for several human breast carcinoma cell lines [9, 10] and to exert antitumor activity *in vivo* against murine pancreatic cancer [11]. IL-1 binding to membrane receptors and its subsequent internalization seem to be essential to these antiproliferative effects; however, very little is known about the relationship between the antitumor effects of IL-1 and the activation of signaling pathways or other intracellular mechanisms triggered by IL-1 receptor occupancy. Two types of interleukin-1 receptor (IL-1R) have been identified [12–14]: type I being localized on T-cells and fibroblasts, and type II on B-cells macrophages and neutrophils. The severe host toxicity produced by IL-1α [1] limits its efficacy as a single agent in the clinic. Recently, combinations of cytotoxic agents and biological response modifiers, such as interferons and cytokines, have evolved as a potentially important modality for treating cancers [15, 16].

Studies from our laboratory on melanoma A375 cells have shown strongly synergistic antitumor effects of IL-1α combined with etoposide or doxorubicin (DOX) [6–8]. Our results indicated that modulation of IL-1R binding capacity is probably involved in the interaction between the cytokine and etoposide. However, studies of IL-1R function in A375 cells have a very low reproducibility, probably due to low levels of IL-1R expression in A375 cells [17]. Therefore, for the present study we used the NIH:OVCAR-3 human ovarian carcinoma cell line as a model to examine these processes for the following reasons: NIH:OVCAR-3 cells have been reported to express high levels of IL-1R (type I) and to respond to the antiproliferative action of IL-1α [18]. Furthermore, we chose to combine IL-1α with DOX because of the relative resistance demonstrated *in vitro* by NIH:OVCAR-3 cells against clinically relevant concentrations of DOX and other commonly used agents, such as cisplatin and melphalan [19].

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‡ Abbreviations: IL-1, interleukin-1; IL-1R, interleukin-1 receptor; IL-1RA, interleukin-1 receptor antagonist; DOX, doxorubicin; MTT, 3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl disuberate; and CI, combination index.

Ovarian cancer *in vivo* is often characterized by the rapid development of primary resistance to a broad spectrum of chemotherapeutic agents. Therefore, the possible synergism between IL-1α and DOX against this cell line might provide a strategy for the management of "difficult" clinical situations.

Our results confirmed the reported antiproliferative effect of IL-1α against NIH:OVCAR-3 cells and the involvement of type I (80 kDa) IL-1R in this action [18]. Combined treatment with IL-1α and DOX resulted in strongly synergistic cytotoxicity. However, in contrast to what was observed in A375 melanoma cells with VP-16 and DOX [7, 8], IL-1R up-regulation does not seem to play a major role in the interaction between IL-1α and DOX in NIH:OVCAR-3 cells, indicating a different mechanism for synergy in this cell line.

MATERIALS AND METHODS

Reagents. *Escherichia coli*-derived recombinant human IL-1α (sp. act. 3×108 units/mg) was provided by Dr. P. Lomedico (Hoffmann-La Roche, Nutley, NJ). *E. coli*-derived recombinant human IL-1RA was purchased from R & D Systems (Minneapolis, MN). 3-[4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide and chloramine T were purchased from the Sigma Chemical Co. (St. Louis, MO). Disuccinimidyl disuberate (DSS) was obtained from the Pierce Chemical Co., Rockford, IL. Na-^{125}I (sp. act. 17.2 mCi/μg) was obtained from Amersham (Arlington Heights, IL). DOX was obtained from the Drug Development Branch, NCI, NIH Bethesda, MD.

Cell culture. NIH:OVCAR-3 cells (ATCC HTB 161; American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with an antibiotic mixture (5 mg/mL of penicillin, 5 mg/mL of streptomycin and 10 mg/mL of neomycin; Gibco) and 10% fetal bovine serum (Gibco) under standard culture conditions at 37° in a humidified CO_2 atmosphere. An NIH:OVCAR-3 cell subpopulation with reduced sensitivity to IL-1α was selected by prolonged culture in the presence of IL-1α according to the following protocol: 3 weeks at 6 D_{10} units/mL, 4 weeks at 18 D_{10} units/mL and 2 weeks at 36 D_{10} units/mL (1 D_{10} unit/mL corresponds to 0.2 pM IL-1α).

Preparation of ^{125}I-labeled IL-1α. Human recombinant IL-1α was labeled with ^{125}I using the chloramine-T reagent method as described by Palaszynski and Ihle [20]. The radiolabeled IL-1α had a specific activity of 1.8 to 2.2 Ci/mmol. No significant loss of biological activity was observed following iodination.

Cytotoxicity assays. The antitumor activities of the individual drugs and the combinations were measured by the MTT assay [21]. OVCAR-3 cells were plated onto 96-well plates (Costar, Cambridge, MA) at a density of 5000 cells/well and allowed to attach for 24 hr prior to drug treatment. Cells were exposed to the drugs for up to 5 days; for sequential administration, the first drug was removed after 24 hr and the cells were washed with drug-free medium before adding the second drug. When the interleukin-1 receptor antagonist (IL-1RA) was used, it was added to the wells 30 min before IL-1α. Cytotoxicity results were analyzed for synergism by the median dose/combination index method developed by Chou and Talalay [22]. According to this method, combination index (CI) values < 1.0 indicate synergism, values near 1.0 indicate additivity, whereas values > 1.0 represent an antagonistic interaction between the agents.

Receptor binding assay. Cells (0.5×10^6/well) were seeded into 12-well tissue culture cluster plates (Costar) and allowed to attach and grow for 24 hr; where appropriate, DOX was added at different concentrations for 24 hr. The culture medium was then replaced with 0.5 mL of RPMI 1640 supplemented with 25 mM HEPES (pH 7.2), 1 mg/mL bovine serum albumin and 1 mg/mL sodium azide (binding buffer) containing [^{125}I]IL-1α at the indicated concentrations, in the presence (nonspecific binding) or absence of a 100 nM concentration of unlabeled IL-1α. When IL-1RA was used, it was added to the cells 30 min before adding IL-1α. The cells were then incubated for 3 hr at 4° on ice with gentle shaking; the assay was terminated by aspirating the binding buffer and by washing the cells twice with ice-cold binding buffer. The washed cells were solubilized in 0.5% sodium dodecyl sulfate (SDS)

Table 1. Median dose values for the cytotoxic effects of IL-1α and DOX against NIH:OVCAR-3 cells*

Schedule	Single drugs		Combination†	
	IL-1α (pM)	DOX (nM)	IL-1α (pM)	DOX (nM)
Simultaneous exposure	1.6	60.0	0.42	8.4
Sequential exposure				
IL-1α 24 hr prior to DOX	33.9	78.4	0.6	11.43
DOX 24 hr prior to IL-1α	5.9	70.3	0.88	17.53

* Values represent the averages of three independent experiments.

† Values represent the calculated relative contributions of each agent to the median dose cytotoxic effect, based on the selection of a 1:20,000 molar ratio of IL-1α and DOX.

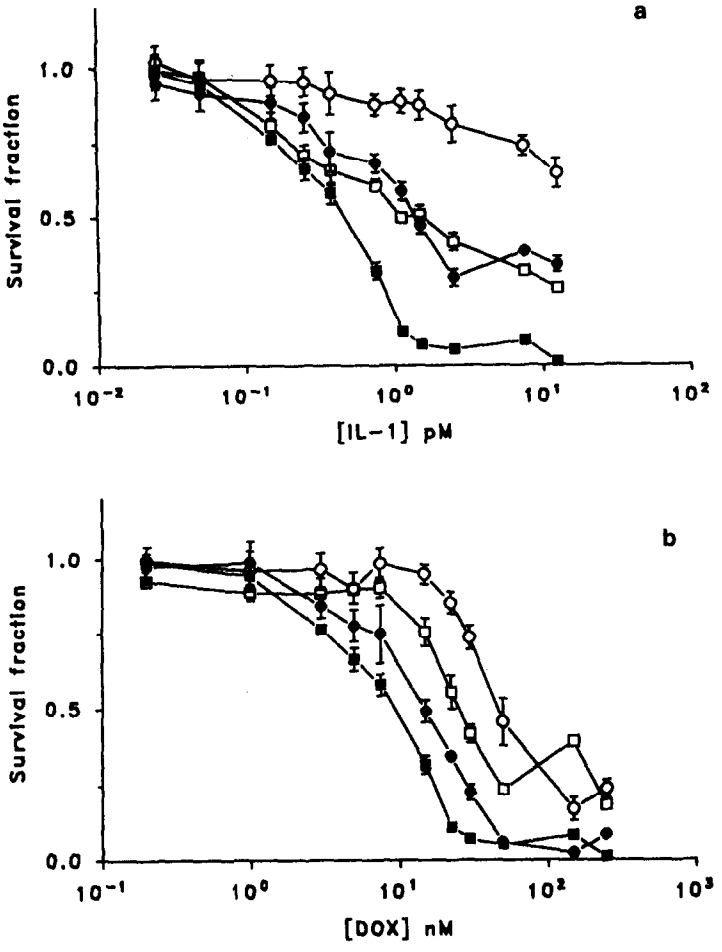

Fig. 1. Concentration–response curves of NIH:OVCAR-3 cells to IL-1α, DOX and their combination. (a) Effects of IL-1α exposure without DOX for 5 days (□) or for 24 hr (○) and with DOX during simultaneous (■) and sequential administration after 24 hr of IL-1α exposure (●). (b) Effects of DOX exposure without IL-1α for 5 days (□) or for 24 hr (○) and with IL-1α during simultaneous (■) and sequential administration after 24 hr of DOX exposure (●). The molar ratio between IL-1α and DOX was fixed at 1:20,000. Values are the means ± SD of 6 replications. Similar results were obtained in at least 6 independent experiments.

and counted for radioactivity using a Packard Tricarb 2000 CA liquid scintillation analyzer. Data from saturation binding studies were analyzed by the LIGAND program developed by Munson and Rodbard [23].

Receptor internalization studies. The cells (0.5×10^6/well) were plated onto 12-well tissue culture cluster plates, allowed to attach for 24 hr, treated with DOX (200 nM) for 24 hr (where appropriate) and incubated in the presence of 25 ng/mL of [^{125}I]IL-1α in 0.5 mL of binding buffer (without sodium azide) for 3 hr at 4° with gentle shaking. Unbound label was removed by washing the cells with ice-cold binding buffer; this washing before internalization eliminates 90% of fluid phase endocytosis, leaving primarily the receptor specific component [24]. Label-free ice-cold binding buffer (0.5 mL) was then added to each well and the cells were transferred to 37°. At the indicated times, released [^{125}I]IL-1α was determined in cell culture supernatants; surface-bound [^{125}I]IL-1α was determined by washing the cells for 8 min with ice-cold acid buffer (150 mM) NaCl, 50 mM glycine-HCl, pH 3.0) and by measuring the radioactivity present in the acid wash. Internalized [^{125}I]IL-1α was determined on acid-washed cells solubilized with 0.5% SDS as described in the previous section.

[^{125}I]IL-1α accumulation studies. The cells (0.5×10^6/well) were plated onto 12-well tissue culture cluster plates, allowed to attach for 24 hr, treated with different concentrations of DOX for 24 hr (where appropriate) and incubated with 25 ng/mL of [^{125}I]IL-1α in complete medium for 6–48 hr at 37°. At the indicated times, surface-bound and intracellular [^{125}I]IL-1α were determined as described in the previous section.

Affinity cross-linking studies. Cells from confluent flasks were harvested with EDTA (0.5 mM) and

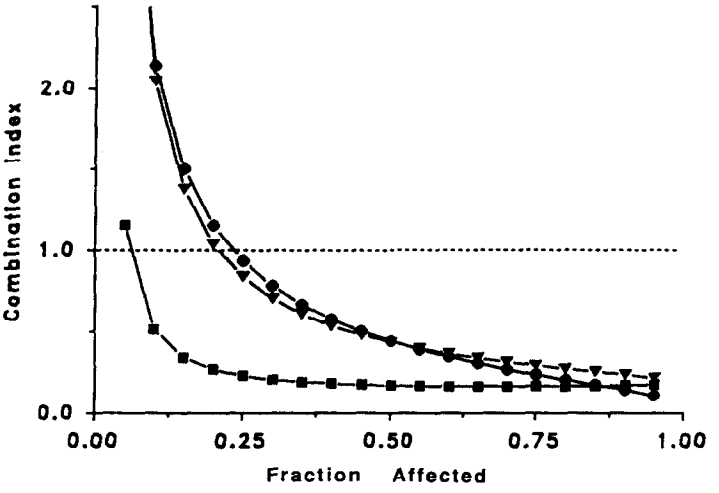

Fig. 2. Plots of the combination indices of IL-1α and DOX on NIH:OVCAR-3 cells during simultaneous exposure (●), IL-1α prior to DOX (■) and DOX prior to IL-1α (▼). The combination indices were calculated according to Chou and Talalay [22] and represent the average of at least 3 independent experiments.

washed twice with ice-cold phosphate-buffered saline (PBS, Gibco), prior to resuspending them (2×10^7 cells/sample) in 200 μL of the binding buffer (see above), containing [^{125}I]IL-1α (2 nM) with or without a 200 nM concentration of unlabeled IL-1α. After a 3-hr incubation at 4° with gentle shaking, unbound [^{125}I]IL-1α was removed by washing the cells in ice-cold binding buffer. The cells were then resuspended in cross-linking buffer (PBS with $MgCl_2$, 1 mM, pH 8.3) and incubated for 1 hr at 4° (with shaking) in the presence of 200 μg/mL of DSS. At the end of this period, the cells were washed with ice-cold PBS and resuspended in lysis buffer Tris–HCl, 50 mM, pH 7.5; NaCl, 300 mM; NP-40, 1%; phenylmethylsulfonyl fluoride, 50 μg/mL; aprotinin, 1 μg/mL; pepstatin, 1 μg/mL) for 30 min; lysates were centrifuged at 10,000 g for 10 min to remove nuclei and other cell debris. The lysates were electrophoresed under reducing conditions on 4–20% SDS–polyacrylamide gradient gel in the presence of molecular weight markers (Gibco BRL, Gaithersburg, MD). Affinity labeled protein was detected by autoradiography of the dried gels.

RESULTS

Synergism between IL-1α and DOX. Table 1 shows the median dose values obtained with NIH:OVCAR-3 cells treated with IL-1α and DOX as single agents or in combination. Preliminary experiments using different IL-1α:DOX dose ratios showed that a maximally synergistic effect could be achieved when the two agents were present at a molar ratio of 1 IL-1α:20,000 DOX (data not shown). Therefore, this ratio was adopted for all the experiments presented in this study.

A 5-day exposure to IL-1α as a single agent yielded an IC_{50} value of 1.57 pM, which was reduced by about 4-fold (0.42 pM) by the simultaneous treatment with DOX (Fig. 1a). The IC_{50} obtained following a 5-day exposure to DOX alone (60 nM) was also reduced (8.4 nM) by the combination with IL-1α (Fig. 1b). Sequential exposure to the two agents resulted in a synergistic cytotoxic interaction. The CI plots obtained with simultaneous and sequential exposures to IL-1α and DOX are shown in Fig. 2. A greater synergism was observed when IL-1α treatment (24 hr) preceded DOX (4 days) than for simultaneous exposure. However, this was probably due to the fact that IL-1α and DOX were much less effective when used as single agents according to this sequential schedule (Table 1). Maximal cell kill was achieved when both agents were present simultaneously throughout the 5-day period.

By prolonged culture of NIH:OVCAR-3 cells in the presence of IL-1α, we obtained cells with reduced sensitivity to IL-1α (IC_{50} = 43 pM); however, these cells retained DOX sensitivity comparable to the parental line (IC_{50} = 21 nM). A strong synergism between IL-1α and DOX was also observed in this subpopulation (Fig. 3).

Characterization of IL-1R and effects of DOX on IL-1R. As previously reported by Kilian *et al.* [18], saturation binding studies with [^{125}I]IL-1α and the corresponding Scatchard plot showed the presence of a single class of IL-1 binding sites, with a K_D of about 280 pM, and a maximal binding capacity of about 2900 molecules/cell. Furthermore, cross-linking studies with DSS showed the presence of a single class of receptors of about 80 kDa, corresponding to type I IL-1 receptors (data not shown), confirming published results of Kilian *et al.* [18].

It is interesting to note that [^{125}I]IL-1α binding to the NIH:OVCAR-3 subpopulation, obtained by prolonged culture in the presence of IL-1α, was found to be reduced by about 50% (data not shown), suggesting that continuous exposure to IL-1α

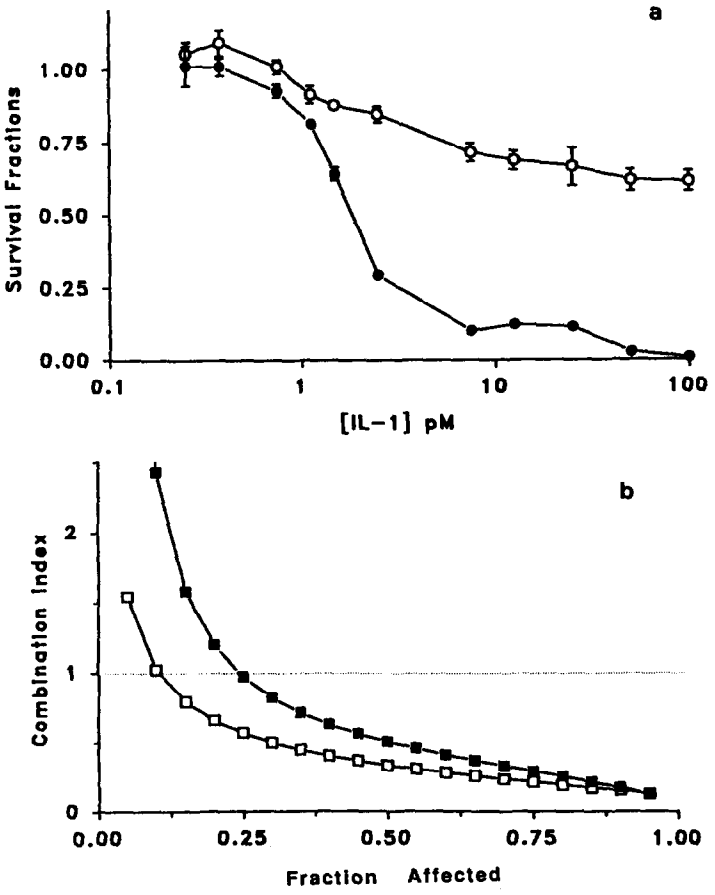

Fig. 3. Antiproliferative effects of IL-1α and DOX on an NIH:OVCAR-3 IL-1-resistant subpopulation obtained by prolonged culture in the presence of IL-1α. (a) Typical concentration–response curves to IL-1α alone (○) and with DOX in simultaneous exposure (●) for 5 days. Values are the means ± SD of 6 replications. Similar results were obtained in 2 independent experiments. (b) Combination index plots in the NIH:OVCAR-3 parental line (□) and with the IL-1α-selected subpopulation (■).

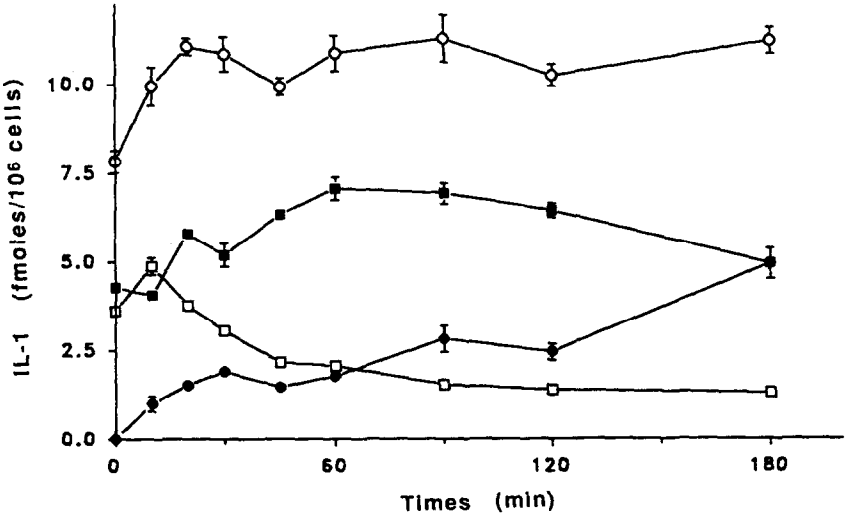

Fig. 4. Internalization of [^{125}I]IL-1α by NIH:OVCAR-3 cells. NIH:OVCAR-3 cells were incubated with [^{125}I]IL-1α (25 ng/mL) for 3 hr at 4° and then transferred to 37° (zero time point) after removal of unbound [^{125}I]IL-1α by washing with cold binding buffer. Total (○), released (●), surface-bound (□) and intracellular (■) [^{125}I]IL-1α are represented as the means ± SD of triplicate determinations; similar results were obtained in 2 independent experiments.

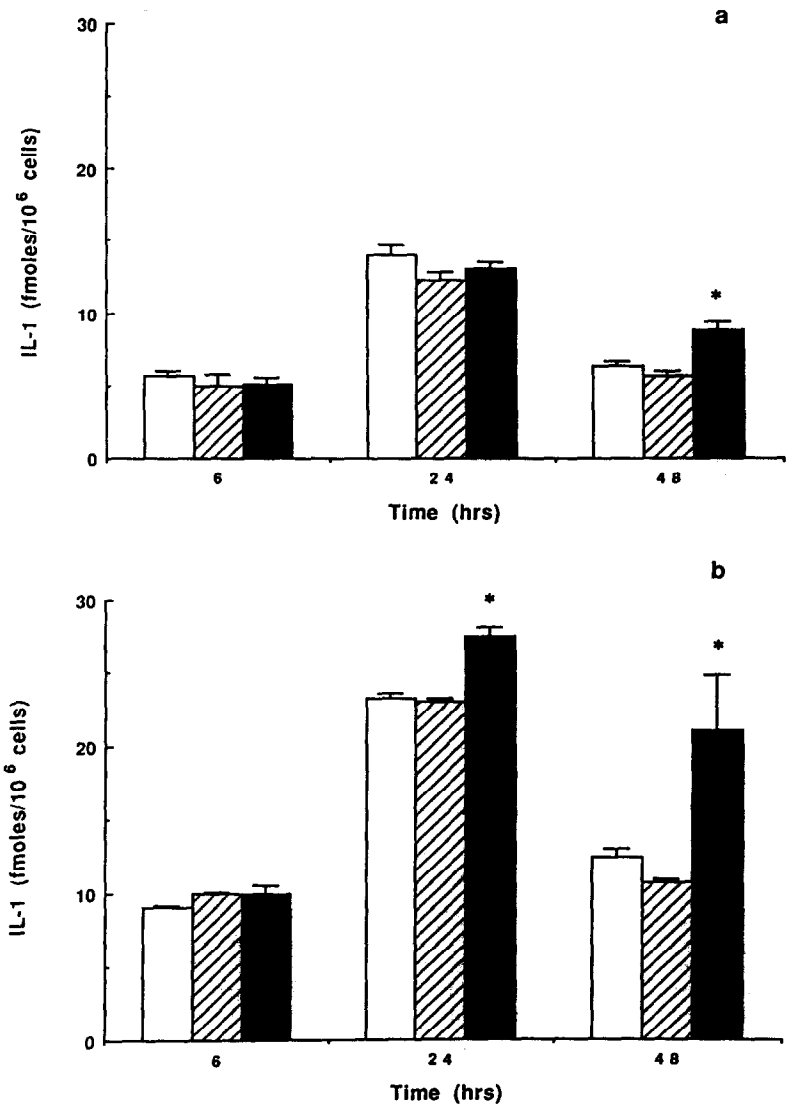

Fig. 5. Cellular accumulation (surface, panel a) and internalization (panel b) of [^{125}I]IL-1α at 37° by control NIH:OVCAR-3 cells (□) and by cells pretreated with DOX at 50 (▨) and 100 (■) nM. Values are the means ± SD of triplicate determinations; similar results were obtained in 2 independent experiments. *$P < 0.05$ vs controls.

causes receptor down-regulation, as reported by Matsushima *et al.* [25].

A 24-hr preincubation with DOX concentrations up to 100 nM did not affect IL-1α binding significantly (data not shown).

Effects of DOX on intracellular accumulation of IL-1α. The time course of [^{125}I]IL-1α internalization by NIH:OVCAR-3 cells is shown in Fig. 4. Intracellular IL-1α concentration was found to peak between 60 and 90 min after transferring the cells to 37°. At later times, a significant amount of the cytokine was released and could be recovered in the culture medium. The relative distribution of IL-1 among the released, surface-bound and internalized pools was not affected significantly by 24-hr pretreatment of the cells with 200 nM DOX (data not shown). In contrast, when the cells were incubated with [^{125}I]IL-1α at 37° for longer times (up to 48 hr), DOX pretreatment (24 hr) significantly increased the amount of IL-1α accumulated intracellularly (Fig. 5).

Effects of IL-1RA on IL-1α binding, antiproliferative action and synergy with DOX. When [^{125}I]IL-1α binding experiments on NIH:OVCAR-3 were carried out in the presence of a 200-fold excess of IL-1RA, about 75% of specific IL-1α binding was effectively blocked. This same IL-1RA excess, which is devoid of intrinsic effects on cell proliferation and does not affect cell sensitivity to DOX, significantly inhibited IL-1α antiproliferative effects by about 90% (IC_{50} = 1000 pM; Fig. 6a). However, as shown in Fig. 6b, IL-1α was still

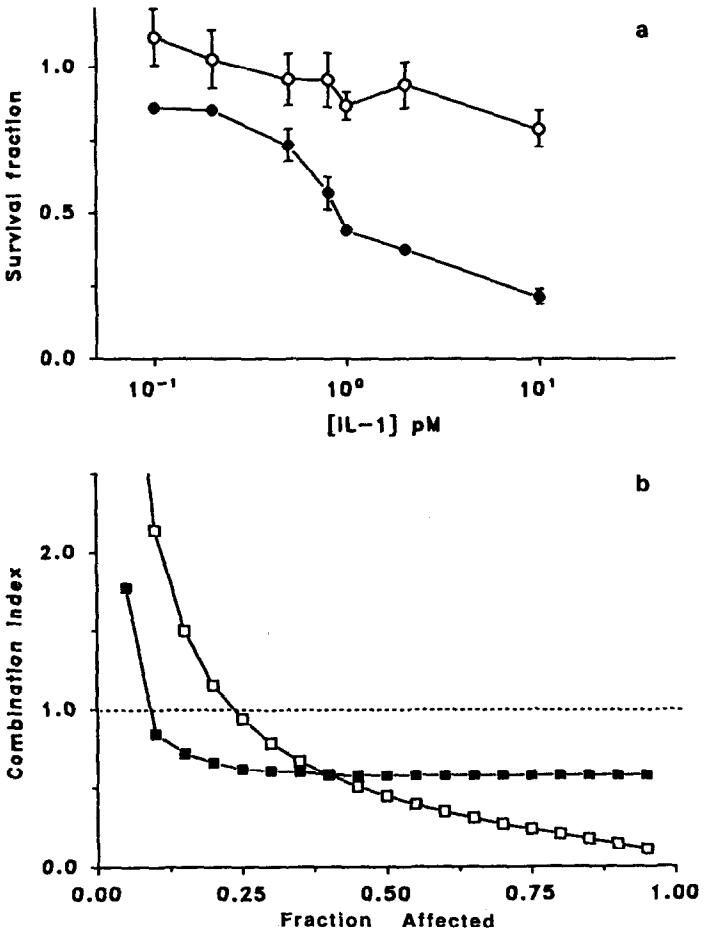

Fig. 6. Effect of IL-1RA on the antiproliferative effect of IL-1α on NIH:OVCAR-3 cells and on the synergism between IL-1α and DOX. (a) Concentration–response curves to IL-1α alone (○) and with DOX in simultaneous exposure (●) for 5 days in the presence of a 200-fold excess of IL-1RA. The antagonist was added to the plates 30 min before the other agents. Values are the means ± SD of 6 replications. Similar results were obtained in 4 independent experiments. (b) Combination index plots obtained with IL-1α and DOX in the absence (□) and in the presence (■) of a 200-fold excess of IL-1RA.

synergistic with DOX, even in the presence of a significant receptor block (75%). However, this synergistic interaction was somewhat diminished in the presence of IL-1RA as the IC_{50} values of 1.7 pM for IL-1α and 20.5 nM for DOX were higher than those in the absence of the antagonist with an IC_{50} of 0.42 pM for IL-1α and 8.4 nM for DOX, respectively (Table 1). These results suggest either that IL-1 receptors are not involved in the synergistic interactions of IL-1α and DOX or, alternatively, and more likely, that only a small percentage of receptors is required for the actions of IL-1. Since NIH:OVCAR-3 cells express 3000 receptors/cell, around 700 receptors would still be available in the presence of a 200-fold excess IL-1RA over IL-1α concentration.

DISCUSSION

The human ovarian carcinoma cell line NIH:OVCAR-3 was established from the malignant ascites of a patient with progressive papillary adenocarcinoma of the ovary after combination chemotherapy. Ovarian cancer is clinically noted for the rapid development of primary drug resistance characterized by a broad cross-resistance pattern; accordingly, this cell line has been found to be resistant *in vitro* to clinically relevant concentrations of melphalan, cisplatin and DOX [19]. NIH:OVCAR-3 cells differ from several other human ovarian carcinoma cell lines in that they do not produce endogenous IL-1 [26], express relatively high levels of IL-1R (80 kDa), and respond with growth arrest to exposure to this cytokine [18].

The results of the present study confirm these observations. In our hands, concentrations of DOX approximately corresponding to one-tenth of the peak-achievable plasma levels—assumed to reflect clinically relevant drug doses [27]—inhibited NIH:OVCAR-3 cell growth by 50%. IL-1α was

found to inhibit cell proliferation rather than exert a cytotoxic effect, and this effect appears to require interaction of the cytokine with specific surface receptors.

As reported by Kilian *et al.* [18], we found that NIH:OVCAR-3 cells express a single class of IL-1R; based on the molecular mass (80 kDa), this receptor was tentatively identified as the type I form, which is typical of T-cells and fibroblasts [12–14]. No direct relationship was established between its antiproliferative effect and receptor occupancy by IL-1α. In fact, in the presence of a 200-fold excess of IL-1RA, 75% of [^{125}I]IL-1α specific binding was abolished, whereas the growth inhibitory effect was only reduced by 60%. In the NIH:OVCAR-3 subpopulation obtained by prolonged culture in the presence of IL-1α, the antiproliferative effect of IL-1α was reduced by more than 20-fold, even though receptor binding was only reduced by 50% as compared with the parental cell line. On the other hand, IL-1α has been shown to elicit dramatic antiproliferative effects on cell lines expressing low levels of IL-1R, such as the human melanoma cell line A375 (less than 500 molecules/cell [17]), and human T-cells with 50 or less receptors/cell respond to low levels of IL-1α. Thus, it is possible that IL-1-resistant OVCAR-3 cells have other post-receptor alterations that decrease their response to IL-1α.

When the cells were exposed to a combination of IL-1α and DOX, a synergistic potentiation of the effects of the two agents was observed, with both simultaneous and sequential exposures; a similar synergistic effect has been observed in A375 cells with IL-1α and etoposide and has been related to modulation of IL-1R activity by the cytotoxic drug [17]. Therefore, the effect of DOX on IL-1α binding in NIH:OVCAR-3 cells was investigated. However, DOX pretreatment did not increase this parameter significantly, and a small decrease in IL-1α binding was actually observed at high DOX concentrations, thus ruling out the possibility of IL-1R up-regulation by DOX.

We proceeded next to investigate how DOX affected the IL-1α internalization processes. Internalization following receptor binding is an essential step in the mechanism of action of polypeptide hormones that do not cross the plasma membrane or gain access to the cell via pinocytic and endocytic pathways, as these generally involve lysosomal delivery and probably protein degradation. In fibroblasts and T-cells, internalized IL-1α was shown to accumulate in the nucleus and no appreciable degradation of the cytokine was observed up to 6 hr after transferring the cells to 37° [24, 28]. In NIH:OVCAR-3 cells, DOX did not affect receptor-dependent IL-1α internalization; the only difference observed between DOX-treated and untreated cells after 6 hr at 37° concerned the absolute amounts of cytokine in the different compartments (released, surface-bound and internalized). However, the lower amounts recovered in treated cells can be accounted for by the reduction of IL-1α binding during the "loading phase" in cells pretreated with high DOX concentrations. Four hours after transferring the cells from 4 to 37°, a substantial amount of [^{125}I]-IL-1α was recovered in the culture medium, which may result from release of intracellular IL-1α (either as the intact peptide or as its degradation products) and/or from dissociation of the cytokine/receptor complex. This was an unexpected finding, given the reported stability of the IL-1/80 kDa receptor complex in fibroblasts and T-cells [24, 27, 29]. The reasons for this rapid release are not clear at this time and suggest that receptors belonging to the same class may behave differently in different cell lines.

Interestingly, when cells were incubated for longer time periods in the presence of [^{125}I]IL-1α at 37°, significant differences became apparent between control and DOX-pretreated cells (Fig. 5). First, after 24 hr the surface-bound component of cell-associated [^{125}I]IL-1α was found to decline in both control and treated cells, possibly due to receptor down-regulation, as described by Matsushima *et al.* [25] in a large granular lymphocyte cell line, YT, also expressing the 80 kDa receptor. DOX pretreatment seems to reduce or to slow down this process, since at 48 hr surface-bound IL-1α was significantly higher in treated than in control cells. However, the most remarkable effect of DOX pretreatment concerns IL-1α accumulation, which was enhanced significantly in treated cells after 24 and 48 hr. Maintenance of receptor function during long-term exposure to IL-1α by DOX may be involved in this effect, and it is also likely that nonspecific fluid phase endocytosis plays a role. Qwarnstrom *et al.* [24] reported that in fibroblasts, which also express type I IL-1R, receptor-dependent IL-1α internalization reaches a plateau after 2 hr, whereas nonspecific uptake increases linearly with time and predominates at later times. DOX may synergize with IL-1α in NIH:OVCAR-3 cells by increasing its nonspecific intracellular accumulation, in contrast to what has been observed in the murine T cell line EL-4 [29]. In NIH:OVCAR-3 cells IL-1α by itself, and not the IL-1/IL-1R complex, could have been responsible at least for some of the growth inhibition produced by the cytokine. Since modulation of gene expression has been proposed as the ultimate mechanism by which IL-1α elicits its multiple effects [30, 31], it is likely that free intracellular IL-1α interacts with nuclear sites. However, the direct interaction of IL-1α with the nucleus has been ruled out for EL-4 cells, where no nuclear receptors for IL-1 were detected [29]; at present, we have no clue as to their existence in NIH:OVCAR-3 cells.

Other mechanisms by which DOX could produce the observed increase in intracellular IL-1α accumulation would involve interference with IL-1α degradation and/or release processes. It is possible that DOX and IL-1α have specific molecular targets for their synergistic interaction. None of these possibilities was specifically addressed in the present study; further investigations are in progress to clarify these points and to elucidate the signal transduction pathway leading to the antiproliferative effect of IL-1α in NIH:OVCAR-3 cells.

IL-1α has not been studied extensively in humans, but its hematologic effects and antitumor activity make it an attractive candidate for use in conjunction with conventional cytotoxic chemotherapy. IL-1α

has already been demonstrated to decrease some of the acute side effects produced by DOX in mice [32]. Because IL-1 has been reported to stimulate growth and differentiation of bone marrow stromal cells, it would be possible to give patients larger doses of a cytotoxic drug like DOX, resulting in a better clinical response. Determination of the antitumor activity of IL-1α and DOX in combination in an animal model of human ovarian cancer will be the next step in determining whether this combination will be clinically useful against ovarian carcinoma.

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