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Effect of Doxorubicin on Mouse Hybridoma B Cells: Stimulation of Immunoglobulin Synthesis and Secretion[1]

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ABSTRACT

The purpose of these studies was to investigate whether the cell-differentiating effect of anthracyclines can trigger an over-production and secretion of molecules that may interfere with the tumor-host relationship. We exposed mouse hybridoma B-cells, which are devoted to immunoglobulin production, to doxorubicin (10–40 ng/ml). We found that most doxorubicin-treated cells secreted 3- to 5-fold higher amounts of immunoglobulin than untreated cells, along with an accumulation of 50% of them in the G2+M phase of the cell cycle. The antigenic specificity of the immunoglobulin and its size pattern as determined by polyacrylamide gel electrophoresis were similar whether or not cells were treated with doxorubicin. The enhancement of immunoglobulin secretion by doxorubicin was associated with an increase of the intracellular pool of heavy and light chains of the immunoglobulin. Furthermore, an elevated synthesis of immunoglobulin was observed. The synthesis of other proteins also appeared to be modified in these circumstances. These data suggest that doxorubicin can potentiate the biological functions of target cells when used at low concentrations, elevating the production and secretion of effector molecules that interfere with the tumor-host relationship.

INTRODUCTION

The anti-tumor activity of drugs used in cancer chemotherapy is due mainly to their specific DNA-damaging properties which interfere with the DNA replication and transcription processes and probably lead to the therapeutic effect observed (1–4). However, such drugs exhibit a large spectrum of effects on tumor cells which are not limited to DNA alterations. On the one hand, the appearance of a multidrug resistance which limits the successful use of these drugs in cancer patients is often observed (5–9). The underlying molecular events responsible for this phenomenon involve a surface glycoprotein whose expression is induced by the presence of the drugs (10, 11). On the other hand, it has also been reported that anti-cancer drugs induce differentiation of tumor cells (12, 13). The differentiation of leukemic (14–20), colon carcinoma (21), melanoma (22, 23), neuroblastoma (24, 25), and other tumor cells (26, 27) can be achieved upon exposure to a variety of such drugs. For instance, a differentiation-inducing effect of DOX,[3] an anthracycline antitumor antibiotic, has been shown in different cell lines (28–30).

This observation may have important consequences since one can expect that differentiation of tumor cells induced by the drugs *in vivo* can also contribute to their growth control and disappearance. Furthermore, the observed differentiating effect is not limited to cancer cells (31–33), and the drug-induced differentiation of cells from the immune system of the host could also contribute to the regression of the tumor. So far, it is unclear whether the appearance of fully differentiated anti-cancer effector cells could lead to an increased production and secretion of molecules, such as cytokines and antibodies, directly involved in the immune response of the host against the tumor. Reciprocally, an increase of the production and secretion by cancer cells themselves of autocrine growth factors or molecules inhibiting the action of anticancer cells could contribute to the expansion of the tumor. In fact, little is known about the effects of anticancer drugs on the production of such molecules, and, more generally, on their effects on protein synthesis (2, 34, 35). Thus, we investigated the effect of low doses of DOX on immunoglobulin production by hybridoma B-cells. These cells, like myeloma cells, produce and secrete high amounts of immunoglobulin and represent a powerful tool for studying the effects of anticancer drugs on cells already committed to a precise function. Furthermore, since these cells are cancerous, this experimental model makes it possible to study the effect of DOX on the secretion of effector molecules by cancer cells. Notably, since DOX is commonly used in the treatment of myeloma patients, one can expect that such a model will generate useful data on the production of myeloma proteins in presence of DOX, which could have important implications for *in vivo* treatments.

We report herein that DOX used at noncytotoxic concentrations can, in hybridoma B-cells, enhance the synthesis of the heavy (H) and the light (L) chains of the immunoglobulin. Furthermore, the synthesis of some other proteins was also found to be modified. The *in vitro* conditions for immunoglobulin increased production by DOX are reported and the differentiation-inducing potency of DOX discussed, as well as its interaction with cells from the host immune system.

MATERIALS AND METHODS

Cells. The UN2 and PC 140.4 hybridoma B-cells were kindly provided by Dr. M. D. Scharff (Albert Einstein College of Medicine, NY).

UN2 cells synthesize and secrete IgG_{2a},K antibodies directed to SRBC (36). Subclones have been derived in our laboratory and extensively characterized (37). Briefly, UN2.C3 cells produce IgG_{2a},K like the UN2 parental cells. Biochemical analysis revealed one H and two L chains with different mobilities in polyacrylamide gel electrophoresis. In contrast, UN2.C17 cells produce only the two K chains (37). PC140.4 cells secrete IgM,K antibodies to PC and have been previously characterized (38). These cells are grown in RPMI 1640 medium (GIBCO, Grand Island, NY) containing penicillin (100 units/ml), streptomycin (100 μg/ml), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 10% of heat inactivated FCS.

Chemicals. DOX was kindly provided by Les Laboratoires Roger Bellon (Paris, France). [^{35}S]L-methionine (111 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

Cell Analysis. UN2.C3 or UN2.C17 cells were grown for 72 h in presence of DOX or not. Cells were then washed and stained with PI as described (39). Flow cytometry analysis was performed with a

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[3] The abbreviations used are: DOX, doxorubicin; SRBC, sheep red blood cells; FCS, fetal calf serum; PI, propidium iodide; PFC, plaque forming cells; ELISA, enzyme-linked immunosorbent assay; TCA, trichloracetic acid; ID_{50}, dose of DOX that inhibits 50% of cell growth; PC, phosphorylcholine.

FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). Results of the analysis of the cell DNA content were computed with the Consort 30/DNA cell cycle analysis softwares (Becton-Dickinson) using the sum of broadened rectangle models. Although the staining of unfixed cells by PI may be affected by DNA-intercalating agents (40), major changes in the cell cycle distribution can easily be observed as DOX treatment only slightly decreases fluorescence by PI (41). Due to the G2+M accumulation of DOX-treated cells, light scatter properties were assessed on DOX-treated and untreated cells in G2+M as determined by PI staining (41).

Detection and Quantification of Secreted Immunoglobulin. Immunoglobulin secretion was evaluated using an indirect PFC assay and/or ELISA. PFC assays determine the number of cells producing a threshold amount of immunoglobulin that provokes SRBC lysis. Thus, the rate of immunoglobulin secretion by a single cell is evaluated by recording the size of the plaque after a given time of incubation (42). ELISA allows the quantitation of the cumulative amount of immunoglobulin present in the cell culture supernatants. These assays were performed as previously described (43–45).

Analysis of Immunoglobulin and Other Protein Synthesis. After a 72-h incubation in presence or absence of DOX and washing, 10^6/ml DOX-treated or untreated cells were labeled in absence of DOX for 2 or 8 h with 50 μCi/ml of [^{35}S]L-methionine. Cells were then lysed in Crumpton's lysis buffer (25 mM Tris-HCl, 50 mM NaCl, 10 mM NaN_3, 0.5% NP-40, 0.5% sodium deoxycholate, 3 mM phenylmethylsulfonyl fluoride). Culture supernatants, cell homogenates, and immunoprecipitates of these extracts obtained with rabbit anti-mouse IgG (H+L) were analyzed on 10% polyacrylamide gels in reducing conditions (37). In some experiments, DOX-treated cells were radiolabeled for 8 h in the presence or absence of DOX. Autoradiographic films (Kodak, Rochester, NY) were scanned by laser densitometry. TCA precipitations were performed by mixing 50 μl of culture supernatants or radiolabeled cell homogenates (10^6/ml) with 450 μl of phosphate buffered saline and 500 μl of 20% TCA. After incubation for 15 min on ice and centrifugation for 2 min with a minifuge (Bioblock, Illkirch, France), pellets were washed in 1 ml acetone (Merck, Darmstadt, FRG) and resuspended in the Econofluor scintillation cocktail (NEN, Boston, MA). Samples were counted in a liquid scintillation counter (LKB, Les Ulis, France) for the TCA precipitable radioactivity.

Evaluation of the Intracellular Pool of IgG. Following a 72-h treatment with various concentrations of DOX (10, 20, or 30 ng/ml) or not, cells were washed two times with drug-free medium in absence of FCS. Homogenates from 1.3×10^5 cells were obtained with Crumpton's lysis buffer. Intracellular pools of IgG (H and L chains) were evaluated using a dot-blot assay as previously described (46). Briefly, H (γ2a) and L (K) chains of immunoglobulin were revealed using ^{125}I-radiolabeled goat anti-mouse IgG_{2a} and anti-mouse K antibodies, respectively (Southern Biotechnology, Birmingham, AL). Autoradiographic films were scanned for semiquantitative analysis using the Ultroscan XL laser densitometer (LKB, Les Ulis, France).

RESULTS

Effect of Doxorubicin on the Cell Cycle and on the Cell Size. When 10^5/ml hybridoma B-cells (UN2.C3 and UN2.C17) were exposed to 20–40 ng/ml DOX for 72 h, cell growth was inhibited by 40 to 80% (ID_{50} = 25 ng/ml) while cell viability was similar to that of untreated cells. PC 140.4 cells treated with DOX were more sensitive (ID_{50} = 5 ng/ml) (Fig. 1). At higher concentration (100 ng/ml), trypan blue stained cells were up to 60%. After 72-h exposure of UN2.C3 or UN2.C17 cells to 40 ng/ml DOX, viable cells, isolated by Ficoll separation, were investigated for their DNA content by PI staining and flow cytometer analysis (Fig. 2). 54% of UN2.C3 DOX-treated cells were accumulated in the G2+M phase while only 21% were in the G1 phase. In contrast, 3 and 41% of untreated cells were respectively in the G2+M and G1 phases. Similar results were observed when UN2.C17 cells were treated in the same conditions. Moreover, the forward angle light scatter measurements of cells in G2+M phase revealed a significant increase in the cell size [proportional to forward angle light scatter (47)] in DOX-treated cells as compared to their untreated counterparts (Fig. 3).

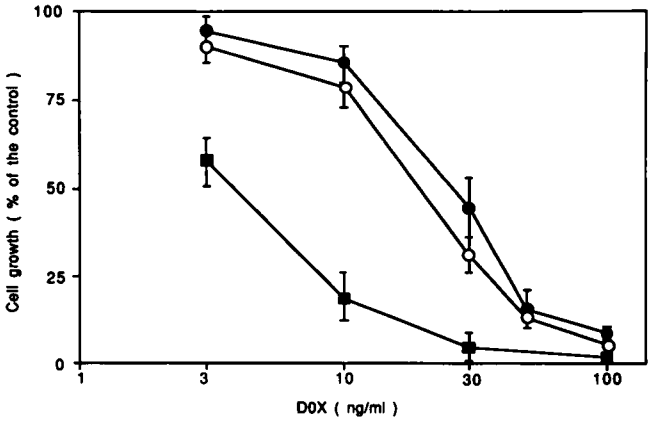

Fig. 1. DOX sensitivity of hybridoma B-cells. UN2.C3 (●), UN2.C17 (○), and PC140.4 (■) hybridoma B-cells were seeded (10^5/ml) in RPMI 1640 containing 10% FCS and various concentrations of DOX or not. After 72-h incubation at 37°C in a 5% CO_2 atmosphere, cells were harvested, counted, and evaluated for their viability with a trypan blue exclusion test.

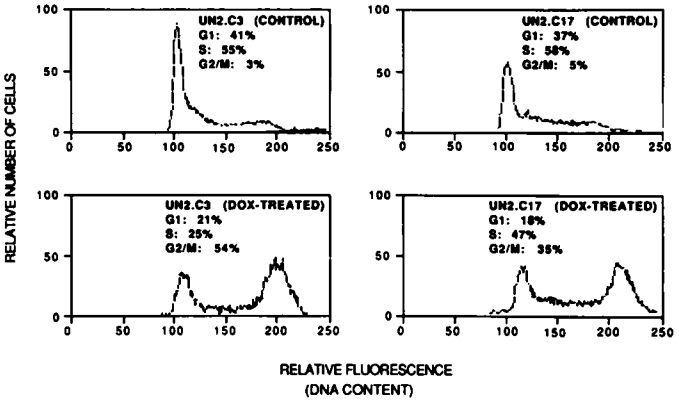

Fig. 2. Flow cytometry analysis of the cell cycle after DOX treatment. UN2.C3 and UN2.C17 cells (10^5/ml) were grown for 72 h in the presence (40 ng/ml) or in the absence of DOX. Viable cells were isolated by Ficoll separation and their DNA content determined by PI staining.

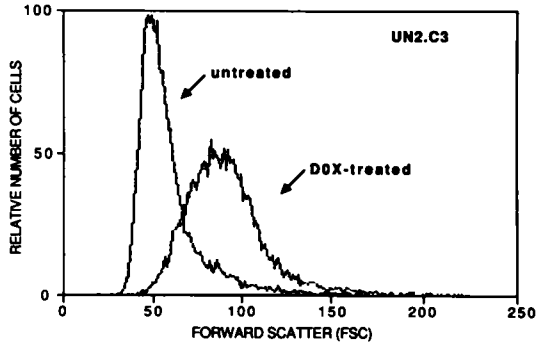

Fig. 3. Forward angle light scatter measurements of DOX-treated UN2.C3 hybridoma B-cells in the G2+M phase of cell cycle. Histograms of treated and untreated cells are overlaid. DOX treatment was the same as described in Fig. 2.

Increased Secretion of Immunoglobulin by Hybridoma B-Cells with Doxorubicin. UN2.C3 hybridoma B-cells (10^5/ml) were grown for 24, 48, or 72 h in the presence or absence of 40 ng/ml DOX. Cumulative IgG levels in culture media were analyzed by ELISA using a goat antimouse IgG_{2a} antiserum. In DOX-treated cells, a significant increase of IgG_{2a} secretion was ob-

served as compared to untreated cells, being the highest at 72 h (Table 1). In order to determine whether the continuous presence of DOX is necessary to maintain the increase of the IgG secretion, cells were exposed for 72 h to DOX, after which they were washed, seeded at 10^5/ml and incubated for 24 h further in drug-free medium. The immunoglobulin secretion by the cells remained greater (3473 ± 345 ng/ml per 10^5 cells) than that of untreated cells (1392 ± 184 ng/ml per 10^5 cells). However, following a further incubation of 24 h in drug-free medium, cell growth was recovered and the IgG concentration in the supernatant was similar to that of untreated cells (not shown).

We analyzed whether the increase of the immunoglobulin secretion in the presence of DOX occurs in all the cells or is due to some individual cells. We assessed the secretion of the cell population by an indirect PFC assay. This assay evaluates the rate of immunoglobulin secretion on a per cell basis, the size of the plaque being recorded after 30 min of incubation. While the number of plaques was similar in DOX-treated and untreated cells, their size was greater with treated cells (Fig. 4). This indicates that DOX stimulates the secretion of immunoglobulin on a per cell basis, with a great majority of the UN2 hybridoma B-cells affected.

Table 1 *IgG_{2a} secretion by DOX-treated or untreated UN2.C3 hybridoma B-cells*

UN2.C3 hybridoma B-cells (10^5/ml) were exposed to DOX (40 ng/ml) or not. Cells excluding trypan blue were counted and the amount of IgG_{2a} determined in triplicate from the supernatants by ELISA. The presence of DOX in the culture medium did not interfere with ELISA. In DOX-treated or untreated cultures, the cell mortality was less than 15% after 48 h and less than 20–30% after 72 h depending on the experiments.

Time (h)	DOX	Cells (× 10^5/ml)	IgG_{2a} (ng/ml)	IgG_{2a} (ng/ml per 10^5 cells)	Fold increase
24	–	3.5	4,575 ± 419[a]	1,308 ± 119	
	+	2.1	4,981 ± 506	2,377 ± 241	1.81
48	–	9.7	17,850 ± 1,842	1,844 ± 190	
	+	3.4	17,350 ± 1,288	5,107 ± 379	2.76
72	–	17.4	33,500 ± 4,023	1,928 ± 231	
	+	5.8	39,000 ± 4,484	6,638 ± 773	3.44

[a] Mean values of triplicates ± SD.

The PFC assay further demonstrates that IgG_{2a} secreted following 72 h DOX treatment still retain their anti-SRBC specificity. This possibility was also investigated with PC 140.4 cells (IgM anti-PC) exposed 72 h to DOX (10 ng/ml) and then incubated from 2 to 48 h in drug-free medium. The corresponding media were tested by ELISA using microplates coated either with goat anti-mouse IgM antibodies or with phosphorylcholine. All the IgM secreted by DOX-treated PC 140.4 cells still bound to PC (Table 2).

Effect of Doxorubicin on Protein Synthesis. To analyze whether the enhancement of immunoglobulin secretion is associated to an elevated immunoglobulin synthesis, UN2.C3 cells (10^5/ml at the initiation of the culture) were incubated for 72 h in the presence of 40 ng/ml DOX. After washing, 10^6 cells/ml were labeled for 2 or 8 h with [^{35}S]L-methionine (50 μCi/ml) in the absence of DOX. Cell culture supernatants and cell homogenates were then analyzed by 10% polyacrylamide gel electrophoresis and TCA precipitations. The latter demonstrated an enhancement of protein secretion (due to the enhanced secretion of immunoglobulin) after 2 and 8 h (Table 3). An increase of the protein synthesis was observed after 2 h, while no difference in the [^{35}S]L-methionine uptake between DOX-treated or untreated cells could be found after 8 h of incubation (Table 3).

The gel analysis of the cell culture supernatants (Fig. 5*A*) and of the H and L chains of immunoglobulin immunoprecipitated from cell homogenates (Fig. 5*B*) indicated that the synthesis of immunoglobulin was increased after DOX treatment. This increase ranged from 1.82-fold up to 3.37-fold, as determined by densitometric scanning analysis.

Interestingly, when UN2.C3 DOX-treated cells were labeled for 8 h either in presence or absence of DOX, an increase of immunoglobulin synthesis was only observed when the drug was present during the labeling period (Fig. 6). This further supported the data obtained with TCA precipitations, where no difference in the [^{35}S]L-methionine uptake was found when

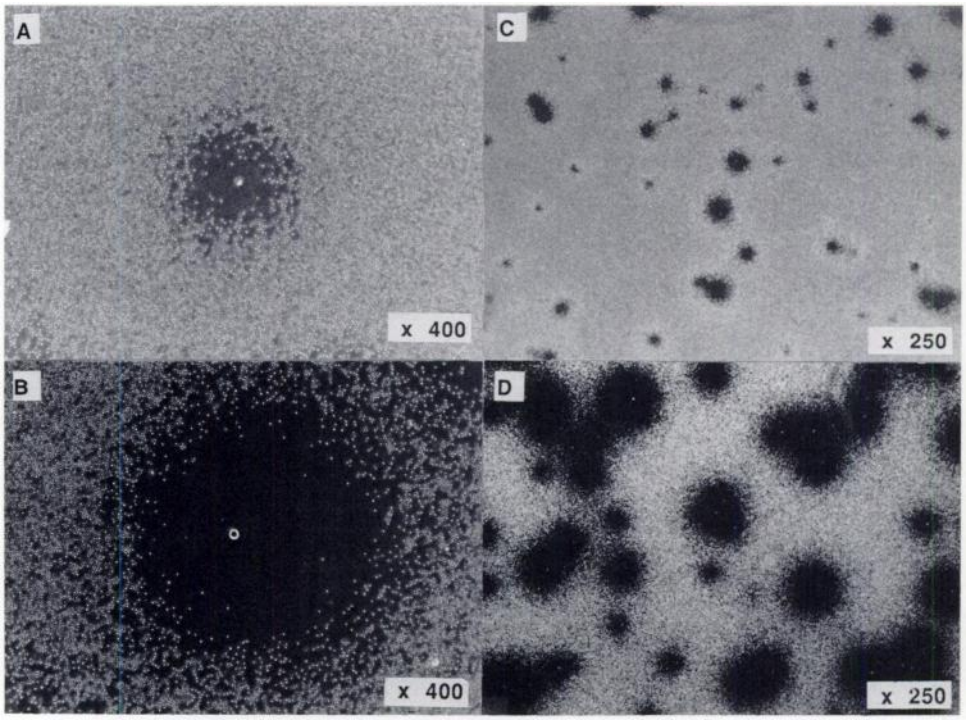

Fig. 4. PFC assays of DOX-treated or untreated UN2.C3 cells. After 72-h incubation in the presence of 40 ng/ml DOX or not, UN2.C3 cells were tested by PFC assays for their ability to lyse SRBC. *A* and *C*, PFC obtained with untreated cells; *B* and *D*, PFC obtained with DOX-treated cells; magnifications are 400 (*A* and *B*) and 250 (*C* and *D*). PFC were evaluated after a 30-min incubation.

Table 2 *Antigen specificity of immunoglobulin produced by DOX-treated PC 140.4 hybridoma B-cells*

PC 140.4 cells (10^5/ml) producing IgM anti-PC were cultured in absence or presence of 10 ng/ml DOX for 72 h. After washing, 10^5 cells/ml were cultured in fresh medium for 2, 4, 20, and 48 h. Supernatants were then tested in triplicate by ELISA. Concentrations indicated for 20 and 48 h culture supernatants are per 10^5 cells.

Culture period (h)	IgM (ng/ml)		IgM anti-PC (ng/ml)	
	Untreated	Treated	Untreated	Treated
2	50 ± 2.2[a]	136 ± 3.9	50 ± 2.9	142 ± 24
4	106 ± 4.2	306 ± 8.0	100 ± 3.6	256 ± 3.2
20	960 ± 68.9	1200 ± 52.8	930 ± 78.3	1320 ± 52.7
48	3660 ± 84.5	3390 ± 94.3	3150 ± 109.3	3030 ± 76.1

[a] Mean values of triplicates ± SD.

Table 3 *TCA precipitation of [^{35}S]L-methionine labeled proteins from DOX-treated or untreated UN2.C3 cells*

UN2.C3 cells were cultured for 72 h in the presence or absence of DOX as described in Table 1. They were then washed and equal number of cells (10^6/ml) were [^{35}S]L-methionine labeled in DOX-free medium for 2 or 8 h.

Labeling period (h)	TCA precipitation of culture supernatants		TCA precipitation of cell homogenates	
	Treated	Untreated	Treated	Untreated
2	3,478 ± 282[b]	2,300 ± 374	37,284 ± 8,794	28,331 ± 6,846
8	18,561 ± 864	12,451 ± 1,088	137,862 ± 7,636	134,096 ± 6,819

[b] Mean cpm values of triplicates ± SD.

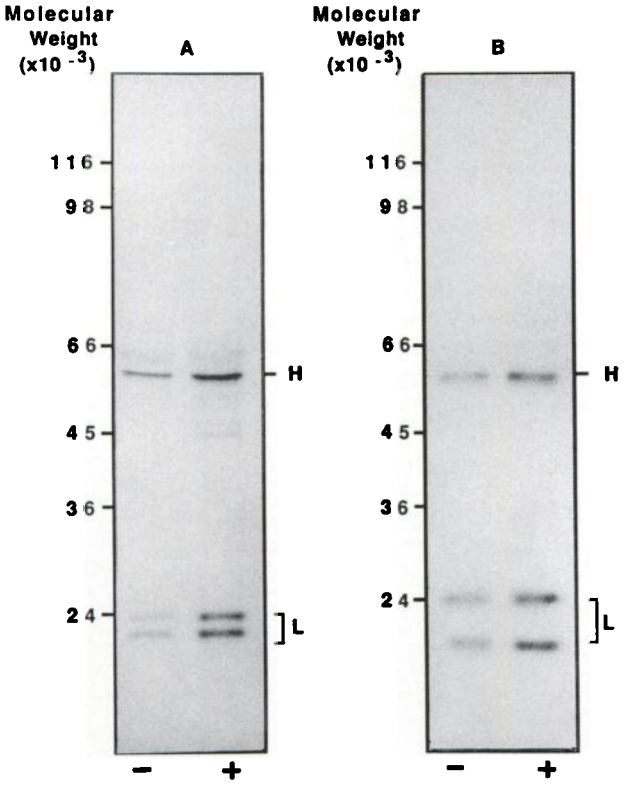

Fig. 5. Analyses of immunoglobulin synthesis and secretion by DOX-treated or untreated UN2.C3 cells. UN2.C3 cells were cultured with 40 ng/ml DOX for 72 h, washed and counted. 10^6 cells/ml were biosynthetically labeled for 2 h with [^{35}S]L-methionine. Whole cell culture supernatants (*A*) and IgG immunoprecipitated from cell homogenates with rabbit anti-mouse IgG (H+L)-Sepharose beads (*B*) were run on a 10% polyacrylamide gel in reducing conditions. +, DOX-treated cells; –, indicates untreated cells; *L*, light chains of immunoglobulin; *H*, heavy chain of immunoglobulin. Molecular weight markers are on the left. In this experiment, fold inductions were ×2.00 and ×2.03 in *A*, ×2.90 and ×3.37 in *B*, for the L chains ×1.82 *A*, ×2.32 in *B*, for the H chain, as detected by densitometric scanning analysis.

DOX-treated or untreated cells were labeled for 8 h in absence of DOX (Table 3). These results indicate therefore that the enhancing effect of DOX on immunoglobulin synthesis can be rapidly reversed. The densitometric scanning analysis of the

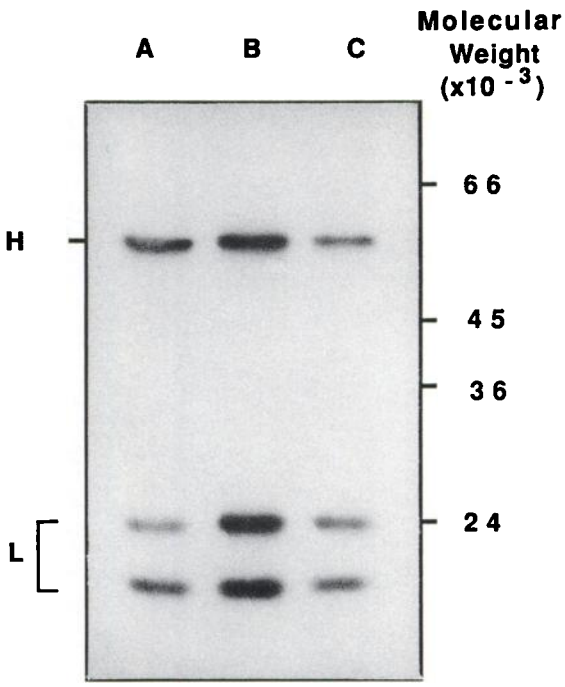

Fig. 6. Immunoprecipitation of IgG produced by UN2.C3 cells labeled with [^{35}S]L-methionine in presence or absence of DOX. DOX-treated cells were labeled for 8 h in presence of DOX (40 ng/ml) or not, in the same conditions as Fig. 5. IgG immunoprecipitated from the corresponding cell homogenates were compared to those obtained from untreated cells. Analyses were performed on a 10% polyacrylamide gel in reducing conditions: *Lane A*, IgG immunoprecipitated from untreated cells; *Lane B*, IgG immunoprecipitated from DOX-treated cells labeled in presence of DOX; *Lane C*, IgG immunoprecipitated from DOX-treated cells labeled in absence of DOX. Molecular weight markers are on the right. *L*, light chains of immunoglobulin; *H*, heavy chain of immunoglobulin.

gels on which the whole cell homogenates were run showed that the production of some proteins other than H and L chains of immunoglobulin also appeared to be slightly modified, being either enhanced or decreased (Fig. 7).

The increased immunoglobulin synthesis paralleled the increase of the intracellular pools of H and L chains, as detected by dot-blot assays (Fig. 8). Densitometric scanning analyses of the dot-blots indicated a stronger increase of the K light chains intracellular pool and synthesis than that of γ2a heavy chains (Fig. 8). For instance, when cells were treated with either 20 or 30 ng/ml DOX, 3.1- and 5.3-fold increases of the K chain cellular content were observed, respectively, while the increase of the γ2a cellular content reached 2.4- and 4.4-fold, respectively (Fig. 8). All of the data indicate that, at low concentrations, DOX transiently stimulates the synthesis and the secretion of IgG_{2a} by hybridoma B-cells.

DISCUSSION

In the present work, we analyzed the effect of low concentrations of DOX on mouse hybridoma B-cells. These cells are homogeneous and well-characterized tumor cells devoted to the production of large amounts of immunoglobulin. Thus, they represent a powerful tool to study the effect of DOX on protein synthesis and B-cell function. Such a study has been poorly documented so far, as most of the reports concern DOX effect on DNA and the appearance of drug resistance in targetted cells. Our results demonstrate that short-term DOX treatment of mouse hybridoma B-cells at nontoxic doses induces (*a*) a strong increase of immunoglobulin synthesis and secretion per cell and (*b*) the accumulation of the cells in the G2+M phase of cell cycle.

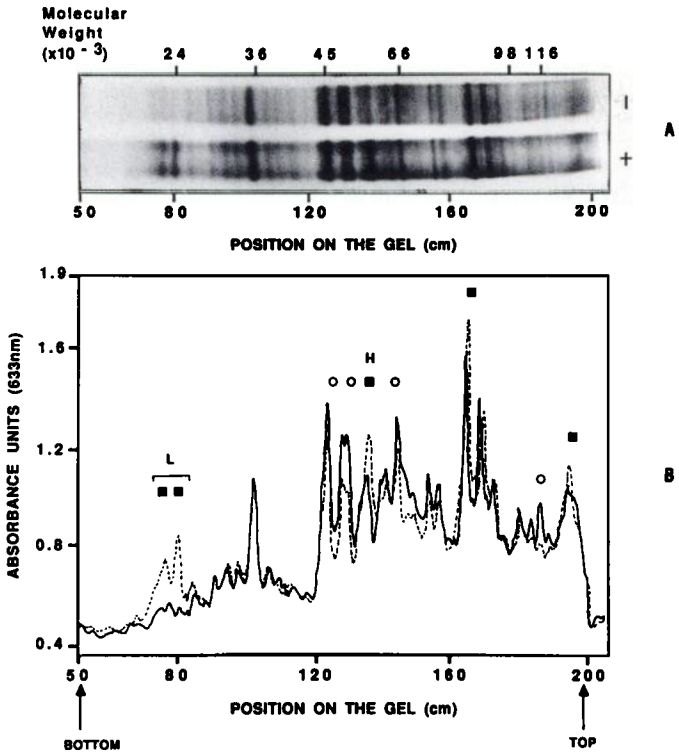

Fig. 7. Densitometric scanning analysis of biosynthetically labeled cell homogenates of DOX-treated or untreated UN2.C3 cells. *A*, autoradiogram whose scanning profiles are pictured in *B*; +, DOX-treated cells; −, untreated cells. Molecular weight markers are indicated above the autoradiogram; scanning profiles of whole cell homogenates of DOX-treated or untreated cells are overlaid in *B*; ■, band intensity elevated by DOX treatment; O, band intensity diminished by DOX treatment.

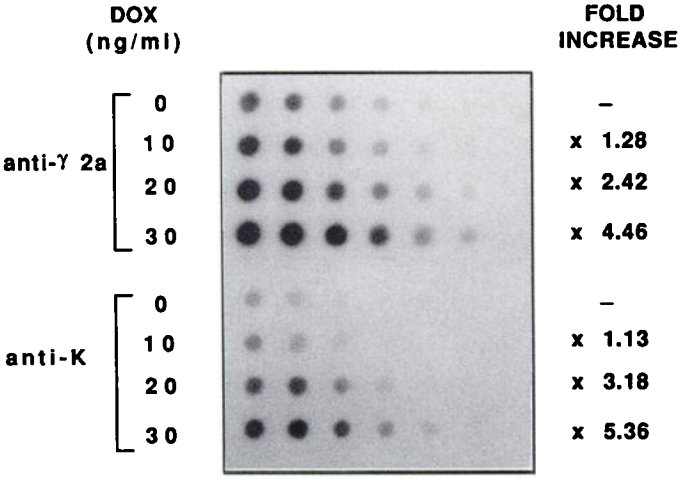

Fig. 8. Analysis of the intracellular pools of IgG (γ2a and K) in DOX-treated or untreated UN2.C3 cells. Cells were cultured for 72 h in presence of various concentrations of DOX or not. Dot-blot assays were performed using twofold diluted cell homogenates from the same number (1.3×10^5) of untreated or DOX-treated cells. Doses of DOX used are indicated on the left; the fold increase in γ2a and K contents is shown on the right.

The higher amount of immunoglobulin detected after DOX treatment in cell culture supernatants is not due to the release of intracellular immunoglobulin by cells killed by DOX. Cell mortality remains low under our experimental conditions and PFC assays allow the detection of viable cells still able to secrete immunoglobulin. Furthermore, biosynthetic labeling of DOX-treated cells showed an enhanced immunoglobulin production. It should be stressed that the rate of secretion relative to synthesis is not affected in DOX-treated cells, with a 2.5- to 5-fold increase in both cases depending on the experiments. Thus, the higher amount of immunoglobulin found in DOX-treated cell culture supernatants reflects a major increase in immunoglobulin synthesis.

Cytometric analysis of the DOX-treated cells indicated a significant cell enlargement with about half of the cells in G2+M phase of the cell cycle. This only partial accumulation is likely to be due to the low doses of DOX used. A previous report indicates that G2+M accumulation can reach up to 90% of leukemic cells after treatment with a higher dose of DOX (1.6 μg/ml) (41). The enhanced synthesis of immunoglobulin may therefore be a consequence of this accumulation in G2+M phase and/or of cell enlargement. However, these phenomenons may also be unrelated. Results of the PFC assay, which analyzes events on a per cell basis, support the second hypothesis. Almost every DOX-treated hybridoma B-cell in the cultures secreted an increased amount of IgG whereas only one half were in the G2+M phase. Furthermore, the amount of immunoglobulin produced by B-cells is not related to the cell enlargement that occurs in the G2+M phase of cell cycle since more than 60% of immunoglobulin production takes place in late G1 (48). Thus, if immunoglobulin production following DOX treatment is related to the accumulation of hybridoma B-cells in G2+M, one can expect a decrease of this production and not the reverse phenomenon.

DOX treatment affects heavy and light chains synthesis and secretion with no detectable change in their molecular weights or in the antibody specificity. Although the occurrence of rare variants cannot be ruled out, most of the immunoglobulin produced in excess by short-term DOX-treated cells are identical to those produced by untreated cells, as they retain their ability to bind the antigen (Table 2 and Fig. 4). In our experiments, the light chain increase was always more important than the heavy chain increase as evaluated by scanning gel autoradiograms (Fig. 5) and dot blots (Fig. 8).

Cell enlargement induced by DOX treatments suggests profound changes in cell metabolism. The enhancement in protein synthesis was not restricted to immunoglobulin and in fact involved at least two other proteins. A decrease in the intensity of four bands was also observed (Fig. 7). One can hypothesize that the proteins whose synthesis rate is also affected by DOX treatment are either related to the immunoglobulin production and secretion processes or are proteins whose expression is either up- or down-regulated during the terminal differentiation of B-cells. Alternatively, DOX could act by modulating the expression or the binding ability of DNA-binding proteins that specifically recognize DNA sequences implicated in the regulation of different genes.

Our data contrast with previous reports indicating no change or even slight decreases in protein synthesis of DOX-treated cells from various cell lines (2, 34, 35). We exclude however that our observations are related to a particular cell line since other hybridoma B-cells producing other immunoglobulin classes and subclasses showed an identical increase in immunoglobulin production (not shown). One must consider that the amounts of drugs used in the previous reports are far higher than the one used herein (ranging from 0.5 to 10 μg/ml). The exposure times are also much shorter (2, 34, 35). In fact, in these experiments, DOX is used at short-term toxic doses that lead to the blockade of DNA replication and transcription, the latter phenomenon probably inducing a decrease in the incorporation of radiolabeled amino acids.

A recent report by Sherr *et al.* (49) indicated that only human B-cell hybridomas from patients with common variable immunodeficiency produce larger amounts of immunoglobulin when

treated with the differentiation-inducing agent retinoic acid. Although we cannot totally rule out a terminal differentiation of the DOX-treated hybridoma B-cell in a true plasma cell secreting higher amounts of immunoglobulin, several points must be stressed. Our data indicate that the immunoglobulin production can quickly go back to a normal level after removal of DOX from culture medium. [^{35}S]L-methionine labeling for 8 h instead of 2 h in DOX-free medium of 72-h DOX-treated cells shows that immunoglobulin synthesis is back to normal levels (Fig. 6). This implies that if cell differentiation is induced by DOX treatment, it is rapidly reversible. Furthermore, in contrast to the human hybridoma B-cells, UN2 mouse hybridoma B-cells secrete important amounts of immunoglobulin while expressing extremely little membrane immunoglobulin (37), thus reflecting a more mature stage of differentiation than their human counterparts. Whether this possible terminal differentiation of DOX-treated hybridoma B-cells is the consequence of an up-regulation of particular proteins induced by DOX remains to be established.

Our data have important consequences in term of the effects of *in vivo* DOX treatment: we can expect that low concentrations of DOX arising during the clearance of the drug will enhance the production and the secretion of cytokines and immunoglobulin produced by tumor and normal bystander cells of the immune system of cancer patients. Ehrke *et al.* (31) reported that mouse treatment with DOX induced a diminished *in vitro* antibody response 5 days later but an increased antibody response if the spleen cells were obtained 7 days later. This could be related to a large decrease of DOX concentrations in the animals between Days 5 and 7, leading to a situation as observed in our *in vitro* experimental system. In addition, these authors observed a correlation between the augmentation of Interleukin 2 induced by doxorubicin and that of cell-mediated cytotoxicity (32). They reported more recently that liposome encapsulation of DOX extended the duration of DOX potentiation of murine natural killer activity (33). Preisler *et al.* (50) reported that a wide range of doxorubicin plasma levels was found in patients with acute nonlymphocytic leukemia 3 h after the drug was administered. High plasma cells were correlated with death during remission induction therapy or long remissions (50). Thus, the enhancing effect of DOX treatment due to its low serum concentrations following clearance could provoke either a clinical improvement by potentiating the activity of effector antitumor cells or a devastating effect by allowing tumor cells to produce more molecules such as autocrine growth factors or factors antagonizing the immune system response. The evolution of cancers could depend on the balance between these two opposite situations rather than being due strictly to the killing of tumor cells by DOX.

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