Anti-Cancer Drugs, 3, pp. 659–664

Bleomycin but not its derivatives inhibits the *in vivo* shedding of a rat tumor-associated antigen

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Rat fibrosarcoma (KMT-17) cells and their *in vitro* clone, 10% FCS A3 cells, shed a tumor-associated antigen (TAA), CE7, from the cell surface under growth-promoting conditions. We treated cells with the antitumor agent bleomycin (BLM) and its analogs peplomycin and liblomycin *in vitro* and *in vivo* in an attempt to increase expression of this antigen and induce an antitumor response. Although all three agents enhance antigen expression *in vitro*, proportionate to their direct antiproliferative effects, only BLM enhances antigen expression *in vivo*. The *in vivo* regulation of CE7 expression appears not to be related to the direct cytotoxic effects of the antitumor agents but rather to the immuno-augmenting effects of BLM.

Key words: Antigen shedding, antitumor response, bleomycin.

Introduction

Antigenic tumors escape host immunological surveillance through a variety of ways. The overproduction and shedding of cell surface glycoproteins which may be highly antigenic if retained on the cell membrane has been previously described.[1–3] Antigen shedding is a phenomenon which can present many problems in the effective application of immunotherapy *in vivo*, even in the case of highly antigenic tumors. Tumor-specific forms of therapy such as the administration of immunotoxin, adoptive immunotherapies with tumor-induced cytotoxic T lymphocytes or tumor infiltrating lymphocytes and treatment with anti-idiotypic antibodies may be of no effective use under such conditions. Furthermore, the shedding of tumor-associated antigen (TAA) may also cause systemic immunosuppression if the antigen has immunosuppressive properties.[4]

One such antigen is CE7, a 42 kDa tumor-associated glycoprotein which, as we have previously reported, is shed from KMT-17 rat fibrosarcoma cells under favorable growth conditions. Shedding is decreased by inhibition of cell growth after deprivation of fetal calf serum (FCS) in the culture medium.[5,6] By the same reasoning, inhibition of cell growth by antitumor agents should also inhibit antigen shedding from tumor cell surfaces and induce an immunologically-mediated *in vivo* antitumor response, provided the antitumor agent applied is not by itself immunosuppressive. The extent of inhibition of antigen shedding is expected to be dependent on the severity of the antiproliferative effects of the antitumor agent. To test this hypothesis, we examined the *in vitro* and *in vivo* effects on CE7 antigen expression after treating 10% FCS A3 cells, cloned *in vitro* from the parent KMT-17 rat fibrosarcoma, with bleomycin (BLM), a well-known immuno-augmenting antitumor agent in the rat,[7–10] and its two derivatives peplomycin (PEP), which is also immuno-augmenting in the rat, and liblomycin (LIB), which is immunosuppressive in the rat.[10] It has previously been reported that, *in vitro*, LIB is more potent than BLM against a variety of tumor cell lines.[11,12] However, we have also recently reported that there is significantly increased production of immuno-regulatory cytokines such as interleukin-2 (IL-2), IL-6, interferon (IFN) and tumor necrosis factor (TNF) by rat spleen cells after treatment with BLM but not after treatment with PEP or LIB.[10] Furthermore, rats bearing KMT-17 tumors treated with BLM exhibited a 30% greater survival rate compared with those treated with LIB, a result

Supported in part by grants from the Japanese Ministry of Education, Science and Culture and from the Japanese Ministry of Health and Welfare.

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which correlates well with the enhanced cytokine release by rat spleen cells after treatment with BLM.[10]

In this study, we used flow cytometry to examine whether the direct *in vivo* antiproliferative effects of antitumor agents can enhance the expression of TAAs normally shed from the tumor cell membrane.

Materials and methods

Animals

Female Wistar King Aptekman (WKA) rats, 170–200 g in weight, were supplied by the Experimental Animal Institute, Hokkaido University School of Medicine, Sapporo, Japan. Animals were kept under conventional conditions until required for use in experiments.

Tumors

KMT-17 rat fibrosarcoma is a highly antigenic tumor induced by 3-methylcholanthrene in a WKA rat and maintained *in vivo* by intraperitoneal passage every 3 days. An *in vitro* clone of the parent tumor, 10% FCS A3, resembles the parent tumor in tumorigenicity, sensitivity to BLM and antigenicity, and also expresses and sheds CE7 antigen from the cell surface. The characteristics of this tumor model have been extensively described elsewhere.[13]

Drugs and reagents

Antitumor agents were kindly supplied by Nihon Kayaku Co. Ltd (Tokyo, Japan) in lyophilized form and dissolved before use in phosphate-buffered saline (PBS). RPMI 1640 medium was supplemented with 10% heat-inactivated FCS (Filtron, Australia), L-glutamine and sodium pyruvate, and used throughout these experiments for *in vitro* cell culture. Anti-CE7 monoclonal antibody was produced in our laboratory by immunizing rats intradermally with highly antigenic A3 cells grown under low FCS conditions.[6] Fusion and selection of anti-CE7 antibody producing hybridomas was carried out according to standard procedures.[14] Titrations were carried out to determine appropriate dilutions for staining tumor cells.

In vitro assays

The 10% FCS A3 cells, in their log phase of growth *in vitro*, were treated with various doses of BLM or its derivatives for 2 h at 37°C in culture dishes. Cells were then washed free of drug with large volumes of PBS and reseeded at 1×10^5 cells per 60 mm^2 culture dish in triplicate and incubated at 37°C in a humidified atmosphere. The number of surviving cells was determined by the trypan blue exclusion method every day for three consecutive days after treatment. Experiments were carried out three times with similar results.

In vitro CE7 antigen expression

The 10% FCS A3 cells were treated with BLM and its derivatives as described above, and cultured at 37°C in a humidified 5% CO_2 atmosphere. Cells were detached from the culture dishes by incubation in a 0.25% solution of EDTA in PBS. After washing twice with large volumes of cold PBS, 1×10^6 cells were spun down to a pellet in 15 ml centrifuge tubes and incubated with 40 μl of an appropriate dilution of anti-CE7 monoclonal antibody at 4°C for 40 min. After completion of the incubation period, cells were washed free of unbound antibody with large volumes of cold PBS and, after spinning down for a second time, incubated with fluorescein isocyanate-conjugated goat anti-rat IgG in the dark for 30 min. After thorough washing, cells were resuspended in pyridium iodide solution to distinguish between living and dead cells, and examined for positive staining with a fluorescent cell sorter (FACS Systems; Becton-Dickinson, Mountain View, CA). Antigen expression was examined for three consecutive days after treatment with the antitumor agents.

In vivo CE7 expression

Rats were subcutaneously injected with 1×10^5 KMT-17 cells on day 0 of an experiment and treated in groups of three with equivalent doses of either BLM (5 mg/kg), PEP (5 mg/kg) or LIB (2.5 mg/kg), intraperitoneally, every day for 5 days from day 8 to 12 after tumor challenge. BLM, administered according to this protocol, produces the best antitumor results, as previously reported by our group.[7] On the 13th day after tumor challenge, rats were sacrificed and their tumors

asceptically removed. The tumors were minced with scissors and incubated at 37°C in a 0.2% solution of collagenase in PBS for 45 min. A single cell suspension was obtained by passage of the crude treated tumor tissue through several layers of sterile stainless steel mesh. Cells were washed free of cellular debris and stained with anti-CE7 antibody as described above. CE7 antigen expression after treatment with BLM, as compared with that in untreated rats, was carried out twice with similar results being obtained.

In vitro exposure of 10% FCS A3 cells to IFN-γ

The 10% FCS A3 cells were seeded in 10 cm^2 culture dishes at a cell density of 1×10^6 cells per dish in medium containing IFN-γ at three separate doses of 500, 1000 and 2000 IU/ml of medium for 24 h. Cells were then detached with EDTA and, after incubation with anti-CE7 antibody, goat anti-rat IgG and pyridium iodide, examined for positive staining as described above.

Statistical analysis

All *in vivo* experiments were performed on groups of three rats and *in vitro* experiments were performed on triplicate samples with similar results being obtained. Statistical determinations, where applied, were calculated using the Student's *t*-test.

Results

In vitro antiproliferative effects

Cell viability, after treatment with the three antitumor agents *in vitro*, was reduced by various degrees. Cell proliferation, as shown in Figure 1, recovered 48 h after treatment with BLM. However, treatment with LIB produced a dose-dependent cytotoxic effect on the cultured cells; the cells did not regain their original high proliferation rates and this result confirms that this clone is more sensitive to LIB than it is to BLM.

In vitro CE7 expression

The results in Figure 2 show CE7 antigen expression by 10% FCS A3 cells 2 days after exposure to the antitumor agents for 2 h. Table 1 shows the relative expression of the antigen on three consecutive days after treatment. CE7 antigen expression after treatment with the three antitumor agents *in vitro* correlates well with the inhibition of

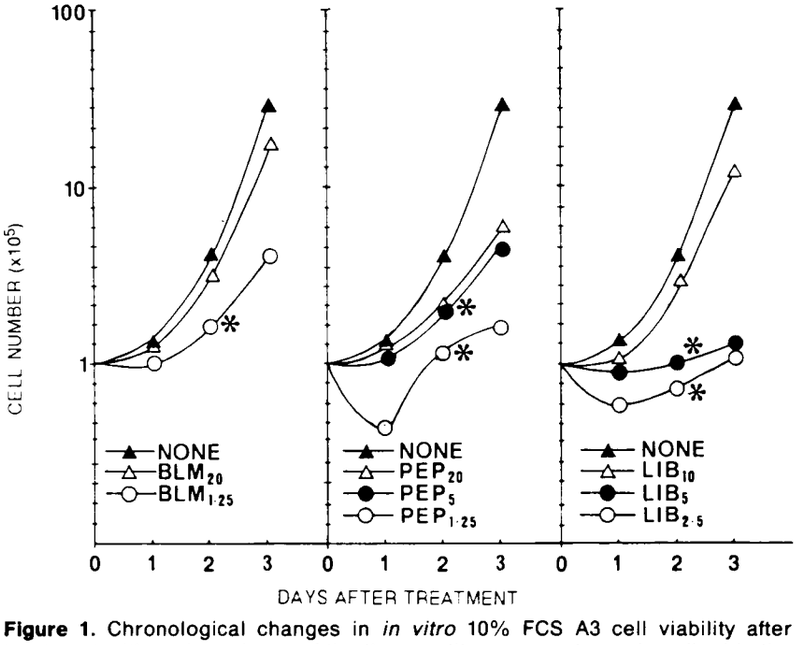

Figure 1. Chronological changes in *in vitro* 10% FCS A3 cell viability after treatment with various doses of the three antitumor agents as described in the text. The numbers suffixed to the drug abbreviations indicate the drug concentrations (μg/ml medium). $^*p < 0.01$ versus the untreated cells.

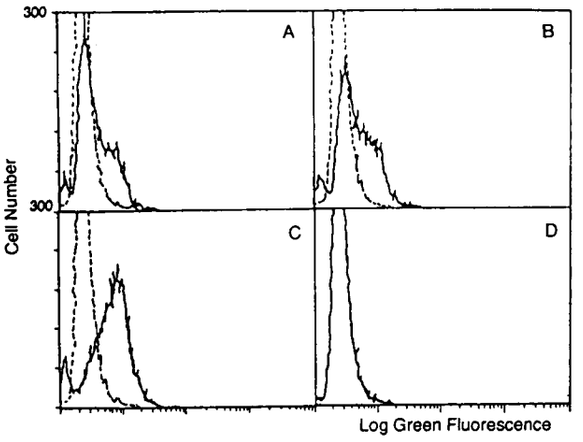

Figure 2. *In vitro* CE7 antigen expression by 10% FCS A3 cells 2 days after treatment for 2 h with (A) 20μg/ml BLM, (B) 20μg/ml PEP or (C) 10μg/ml LIB as described in the text. The dashed line represents expression of CE7 antigen by the untreated (D) cells for comparison.

in vitro cell proliferation shown in Figure 1. Treatment with BLM produced a moderate, short-lived increase in CE7 expression on day 2 after treatment, corresponding to the anti-proliferative effects of the antitumor agent (Figure 1). PEP and LIB both produced a longer-lasting, dose-dependent strong expression of CE7 antigen by 10% FCS A3 cells, corresponding to the more potent cytostatic effects of the two agents. After treatment with BLM, CE7 expression was observed to diminish by day 3, as the cells quickly regained their normal high proliferation rates.

Table 1. Chronological changes in CE7 expression by 10% FCS A3 cells[a] after treatment *in vitro* with BLM and its analogs

Antitumor agent	Dose (μg/ml)	CE7 Expression		
		day 1	day 2	day 3
None		−	−	−
BLM	20.0	−	+	−
	5.0	−	+	−
PEP	20.0	−	++	+
	10.0	−	++	+
	5.0	−	+	+
	1.25	−	±	−
LIB	10.0	±	+++	+
	5.0	±	+++	+
	2.5	±	+	−
	1.25	−	±	−

[a] The 10% FCS A3 cells (1×10^6) were seeded in 10 cm^2 dishes 2 days before treatment with various concentrations of the antitumor agents. Cells were reseeded in culture plates and examined for CE7 expression on three consecutive days after treatment.

In vivo CE7 expression

Tumor-associated CE7 antigen expression by *in vivo* KMT-17 tumor cells harvested from untreated rats was not detected, as shown in the right lower quarter of Figure 3. After treatment with BLM, expression of CE7 antigen could be detected on the cell surface, as also demonstrated in Figure 3. Treatment with an equivalent dose of PEP only negligibly increased CE7 expression, as indicated by a slight shift to the right of the peak for CE7 expression shown in Figure 3 as compared with the untreated controls. Figure 3 shows that LIB does not affect CE7 expression *in vivo*, in contrast to the expression of CE7 by 10% FCS A3 cells after exposure to the three antitumor agents *in vitro*.

In vitro exposure to IFN-γ

The expression of CE7 antigen by 10% FCS A3 cells after exposure to IFN-γ is shown in Figure 4. Comparison with the untreated controls clearly shows that IFN-γ does not modify CE7 expression by 10% FCS A3 cells even at the relatively high concentration of 2000 IU/ml of medium. Similar results were obtained after exposure to TNF and IL-2 under the same experimental conditions.

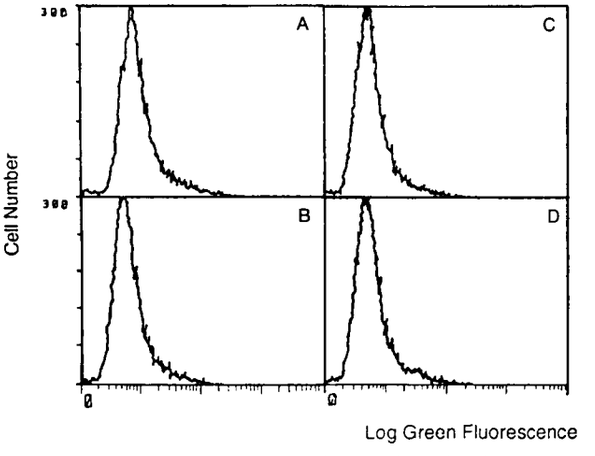

Figure 3. *In vivo* expression of CE7 antigen by the tumor cells within the solid KMT-17 tumor after treating rats with equivalent doses of (A) BLM, (B) PEP or (C) LIB, every day for 5 days, 8 days after subcutaneous injection with tumor cells as described in the text; (D) untreated cells.

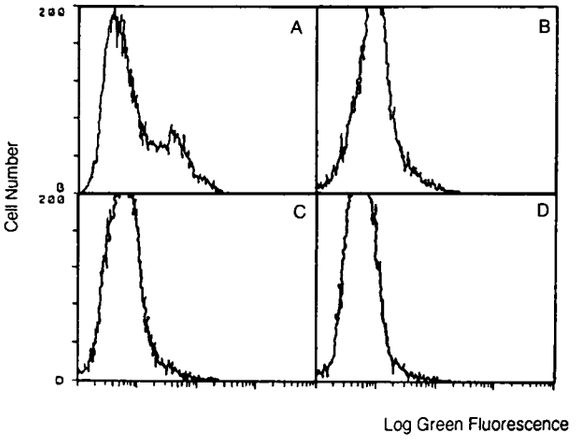

Figure 4. *In vitro* negative expression of CE7 antigen by 10% FCS A3 cells after exposure to (A) 0, (B) 500, (C) 1000 and (D) 2000 U/ml rat IFN-γ for 24 h. Similar results were obtained after treatment with TNF and IL-2 under the same experimental conditions.

Discussion

In this study, we present a possible method for overcoming the problems caused by the *in vivo* shedding of TAAs. This is not achieved by the direct suppression of tumor cell growth through the administration of high doses of antiproliferative agents, which may have immunosuppressive effects on the host, but by the administration of BLM, an antitumor antibiotic, which is well-known for its immuno-augmenting effects.[7–11] This immuno-augmentation, together with increased expression of antigen by the tumor cells, is certainly favorable to tumor rejection by the host.

Our results show that LIB, which exhibits the same cytotoxic mechanisms as BLM and is a more potent anti-proliferative agent than BLM, does not share the immuno-augmenting effects of BLM[11] and does not increase the *in vivo* expression of CE7 antigen. This indicates that the mechanism for increased CE7 expression is not direct inhibition of cell proliferation. We are now investigating whether the increase in antigen expression induced by treatment with BLM is due to actual inhibition of its shedding by changes in the production of regulatory growth factors, enhanced expression of the antigen due to increased production of stimulatory cytokines and/or inflammatory factors by immune effector cells or some other mechanism. It is possible that more than one of these mechanisms is responsible for the increase in tumor antigen expression. We have recently reported that BLM, but not PEP or LIB, significantly increases the production of cytokines such as IL-2, IL-6, IFN and TNF[10,15] by mitogen-stimulated spleen cells after treatment *in vivo*. Other investigators have reported that BLM also increases the production of tumor growth factor-β (TGF-β) by rat alveolar endothelial cells.[16] These regulators of cell growth may play a role in the increased expression of CE7 antigen; however, direct treatment of 10% FCS A3 cells with IFN-γ *in vitro* failed to increase CE7 antigen expression. Similar results, not presented in this report, were obtained after treatment with IL-2 and TNF. Since we only examined antigen expression after exposure to one cytokine at a time, it remains possible that exposure to a combination of the cytokines and/or growth factors, such as TGF-β, might increase antigen expression. Treating rats with BLM increases macrophage random migration and invasiveness,[15] and induces infiltration of tumor by lymphocytes in tumor-bearing rats (results not shown). Cytotoxic factors such as the cytotoxic nitrite anion, secreted by tumor infiltrating macrophages, or direct effector cell–tumor cell interaction after treatment with BLM may also be involved in increased antigen expression.

Another possibility is that BLM might accumulate within the tumor tissue at higher concentrations than either PEP or LIB and as a result induce greater antiproliferative effects on the tumor cells. Biosensitivity assays for the three agents after intraperitoneal administration *in vivo* indicate, however, that the three agents have overlapping bioavailability patterns (data not shown). Therefore, a difference in the bioavailability of the three agents cannot explain their effects on tumor-associated antigen expression. We are now further studying what factors inducable by BLM might explain the increase in antigen expression.

In this study, we have only examined *in vivo* antigen expression at one point in time after treatment. It is possible that increased antigen expression also occurs in rats treated with PEP or LIB in the later stages of tumor burden; however, it is our opinion that the early increase in tumor antigen expression brought about by treatment with BLM is a determining factor in effective immunologically-mediated antitumor responses.

Conclusion

This study presents some preliminary results which indicate that an antitumor antibiotic can increase the expression of a tumor-associated antigen which

is shed from the tumor cell surface under normal growth conditions. The mechanism for the enhanced expression of this antigen after treatment with BLM is still under investigation but is thought to be related to the immuno-augmenting effects of BLM rather than the direct antiproliferative effects of the drug. LIB, which has more potent *in vitro* cytotoxic effects on the tumor cells, does not cause any change in antigen expression at the time point examined. It is possible that the high concentrations of antitumor agents inside the tumor tissue required for increased expression of shed antigen by antiproliferative mechanisms cannot be achieved without severe toxic effects. Our result provides an alternative method by which enhanced TAA expression is achieved.

Acknowledgements

The authors would like to thank Professor N Takeichi, Dr J Hamada and Dr F Okada for many helpful discussions during this study, and Dr I Chiba for the production of the monoclonal antibody to CE7 antigen.

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(Received 1 September 1992; accepted 14 September 1992)