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# Modulation of the shedding of a rat tumor-associated antigen by growth regulation and anti-cancer drugs

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**CE7 antigen is shed from A3 cell surfaces by cells grown in medium containing a sufficient (10%) amount of fetal calf serum (FCS), but shedding of the antigen decreases with a decrease in FCS content in the culture medium. However, the cells contain similar amounts of antigen as evidenced by Western blotting, indicating that low FCS levels interfere with antigen shedding but not antigen synthesis. Antigen expression by A3 cells treated with mitomycin C gradually shifted from negative to a strong positive with time, and on day 2, two peaks corresponding to negative and positive cells within the population can be observed. In contrast, A3 cells treated with bleomycin and cyclophosphamide shifted as a whole from negative to weakly positive. When A3 cells in media containing 10% FCS were incubated at 4°C, although the cells did not proliferate, antigen expression could not be detected by flow cytometry.**

*Key words:* Antigen shedding, antitumor drugs, fibrosarcoma, tumor cell growth, Wistar King rat.

## Introduction

Neoplastic cell proliferation *in vivo* is normally controlled by either genetically regulated suicidal mechanisms such as apoptosis or immunological responses to stimulatory indicators which signal that the neoplastic cells have deviated from normal. These indicators include expression of tumor-associated antigens by tumor cells during and after the process of carcinogenesis. These tumor-associated antigens are not expressed by normal cells.[1] Expression of these antigens can lead to selective elimination by immune effector cells in much the same way as transplantation rejection occurs in immunocompetent hosts. However, because tumor cells comprise a heterogeneous population, and through *in vivo* selection mechanisms, some tumor cells which shed the antigen from the cell surface and therefore express the antigen only very weakly are able to escape host immunological surveillance.[2] In recent years, several investigators have reported antigen shedding by a variety of tumor cell types[3,4] and while some reports show that interferons (IFNs) can inhibit antigen shedding by melanoma cells *in vitro*,[5,6] other reports show that IFNs may actually enhance antigen shedding by adenocarcinoma cells.[7]

The rat fibrosarcoma KMT-17 has been used in our laboratory to study tumor immunology and tumor-induced immunosuppression, and variants of this tumor cultured *in vitro* were found to possess different immunogenic properties *in vivo*.[8] This variance in immunogenicity led investigators in this laboratory to show that parent KMT-17 and its *in vitro* clone, A3, shed CE7, a tumor-associated antigen, from the cell surfaces bound to the membranes of vesicles in a growth-related manner which is inhibited when cells are grown in medium depleted of fetal calf serum (FCS).[9] The expression of this antigen can be detected on the surface of tumor cells by a rat monoclonal antibody developed in our laboratory, after intradermal sensitization of rats with A3 cells grown in media depleted of FCS.[9]

In order to study CE7 shedding and ways for its inhibition, we are studying the effects of antitumor agents on CE7 shedding and have recently reported that bleomycin has inhibitory effects on CE7 antigen shedding *in vivo*; however, this property is not shared by its derivatives peplomycin and liblomycin, although all three agents have inhibitory effects on CE7 shedding *in vitro*.[10] These results indicated that although the shedding of CE7 antigen is growth-associated, the inhibition of tumor-associated antigen shedding by antiproliferation agents *in vivo* is not a simple case of inhibition of tumor cell growth. Antitumor agent-induced inhibition of tumor growth and increased expression of tumor-associated antigens *in vivo* would serve to keep in check tumor burden as well as stimulating antitumor effector cells. This study was

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carried out to study patterns of enhanced antigen expression by growth-regulatory conditions and antitumor agents *in vitro* as a preliminary step towards identifying mechanisms for tumor-associated antigen shedding and its inhibition by antitumor agents.

## Materials and methods

### Tumor lines

The KMT-17 rat fibrosarcoma is a transplantable tumor induced in a Wistar King Aptekman/Hok (WKAH) rat by 3-methylcholanthrene. An *in vitro* clone of KMT-17 tumor is A3 which resembles the parent tumor in antigenicity and *in vivo* tumorigenicity. The origin of these lines has been described in detail elsewhere.[11]

### Drugs and reagents

Tumor cells were cultured *in vitro* in RPMI 1640 medium supplemented with various amounts of FCS (Flow Laboratories, Australia), sodium pyruvate (1 mM), L-glutamine (2 mM), glycine (0.1 mM) and L-serine (0.25 mM). Cell culture was performed at 37°C in a humidified 5% $CO_2$/air atmosphere with *in vitro* passage every 3 days. Examination for contamination with mycoplasma and viruses gave negative results. A monoclonal antibody to a tumor-associated antigen expressed on the tumor cell surface (CE7) was developed by intradermal immunization of syngeneic WKA rats with A3 cells grown in media containing low amounts of FCS (1%), which are highly immunogenic,[12] and isolation of an anti-CE7 antibody producing hybridoma according to standard procedures. The antitumor agents used in this study, bleomycin (BLM; Nihon Kayaku, Tokyo, Japan), mitomycin C (MMC; Kyowa Hakkoh, Tokyo, Japan) and 4-hydroxy cyclophosphamide (4-OH-CY; Shionogi Pharm. Co., Osaka, Japan), were dissolved in phosphate-buffered saline (PBS) immediately before use. The 4-hydroxy derivative of cyclophosphamide was used in our experiments because cyclophosphamide is inactive *in vitro*. All cell cultures were carried out in triplicate and determinations carried out on cells pooled from the triplicate cultures.

### CE7 antigen expression by A3 cells cultured in different concentrations of FCS

A3 cells cultured in media containing 10% FCS (10% FCS A3 cells) were allowed to grow in media containing various amounts of FCS over a period of 2–3 weeks, when cell growth appeared to be stable. Cells were then counted at several time points by the Trypan blue dye exclusion method to determine an average doubling time for the cells grown under such growth conditions. The cells were also stained with anti-CE7 monoclonal antibody for 40 min on ice and then washed extensively with cold PBS to remove unbound antibody. Cells were then stained with a fluorescein-conjugated goat anti-rat IgG antibody (heavy and light chain specific, Cappel Laboratory, Durham, NC, USA), at room temperature for 1 h. Cells were again washed with cold PBS and resuspended in a solution of pyridium iodide in PBS (PI, 2.0 $\mu$g/ml) to distinguish between living and dead cells. The cells were then scanned for positive staining with the antibody using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) after gating for living cells.

### Western blotting for CE7 antigen

A3 cells cultured in media containing 10% (10% FCS A3) and 1% (1% FCS A3) amounts of FCS were prepared as described above, and cells were treated with Nonidet P-40 for extraction of membrane and cytoplasmic proteins. Whole cell extracts were analyzed by SDS–PAGE, transferred to nitrocellulose membrane, and stained with anti-CE7 antibody and labeled anti-rat IgG antibody respectively. Bands were visualized by the ABC method and a molecular weight marker indicated that anti-CE7 antibody bound to a 42 kDa protein. Total protein content of the two cell types was determined by electrophoresis on polyacrylamide gels and stained with amido black.

### A3 cell treatment with antitumor agents

A3 cells cultured in media containing 10% FCS were seeded in 10 $cm^2$ plastic culture dishes at a cell density of $1 \times 10^6$ cells per 10 ml per dish and incubated at 37°C for 2 days. Cells were then exposed to various concentrations of either MMC for 1 h, BLM for 2 h or 4-OH-CY for 2 h. These

treatment conditions were indicated by results obtained from preliminary experiments on A3 cell sensitivity to a number of antitumor agents. Cells were then detached from the dishes by gentle agitation with warm EDTA and washed three times with warm PBS. Cells were then reseeded in plastic culture dishes as described above and the cell number was counted by the Trypan blue dye exclusion method every day for three consecutive days. Cells treated in this manner were also stained with the monoclonal antibody to CE7 and fluorescein-conjugated anti-rat IgG, and examined by flow cytometry after suspension in PI solution as described above every day for several days after treatment with the antitumor drugs. Untreated control cells maintained in medium containing 10% FCS were included in our experiments.

### Effects of low temperature on CE7 expression by A3 cells

A3 cells ($5 \times 10^5$) in medium containing 10% FCS were seeded in 10 $cm^2$ plastic culture dishes in 5 ml of medium per dish and cultured for 48 h at 37°C. Culture dishes were then transferred to a 4°C environment and cultured for a further 24 h. Control cell cultures were maintained at 37°C for the whole of the experiment. Cell growth was determined by counting the cell number 48, 60 and 72 h after the start of the experiment by the dye exclusion method. The expression of CE7 antigen was determined as described above 12 and 24 h after transfer of the cells to the cold environment by flow cytometry after staining of the cells with anti-CE7 antibody.

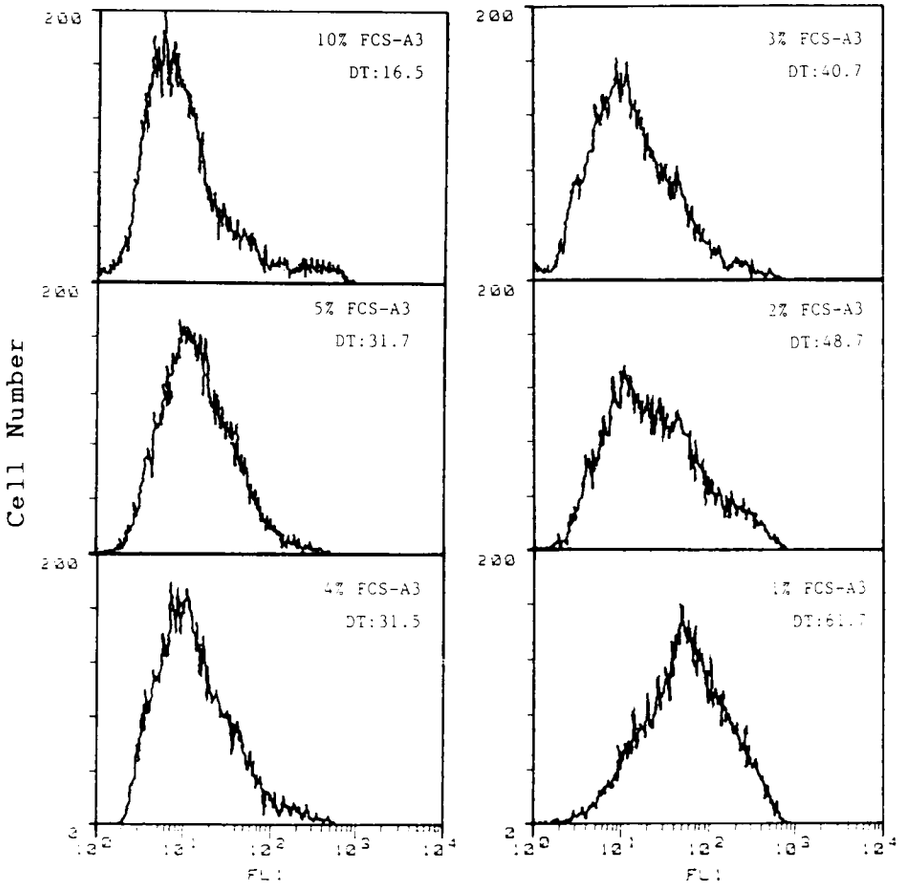


**Figure 1.** CE7 antigen expression by A3 cells grown in media containing decreasing amounts of FCS. Deprivation of FCS from 10 to 1% yields a corresponding increase in CE7 antigen expression. The percentage values shown indicate the amount of FCS in the medium and DT indicates the doubling time of the cells in hours.

## Results

### Effect of low concentrations of FCS on CE7 expression by A3 cells

Cytograms representing expression of CE7 antigen by A3 cells grown in media containing various concentrations of FCS are shown in Figure 1 along with the respective doubling times of the cells. As indicated in Figure 1, the doubling time of the cells increases in a dose-dependent manner with a decrease in the content of FCS in the culture medium. With an increase in doubling time, the fluorescence of the cells shifts to the right indicating an increase in the expression of CE7 by the cells with a decrease in FCS in the culture medium. The expression of CE7 antigen is therefore associated with a decrease in the growth rate of the tumor cells.

### Analysis of CE7 content by Western blotting

Western blotting of 10% FCS A3 cells and 1% FCS A3 cell extracts detected by anti-CE7 monoclonal antibody shows that although 10% FCS A3 cells do not express CE7 antigen on the cell surface, whereas 1% FCS A3 cells do so, cell lysates obtained from the two cell types contain similar amounts of the antigen, as shown by the band at 42 kDa in Figure 2. This indicates that 10% FCS A3 cells may be storing the antigen until releasing it into the surroundings. Chiba *et al.*[9] have previously reported that membrane extracts of 10% FCS A3 cells do not contain any CE7 antigen when determined by Western blotting, indicating that the CE7 content of 10% FCS A3 cells observed in our experiment must be located intracellularly. Separation of total protein by electrophoresis, also shown in Figure 2 indicates that the protein content of cells grown in media with varying FCS contents is not different.

### CE7 expression after treatment with antitumor agents

Figure 3(A–C) represents 10% FCS A3 cell survival after exposure to MMC, BLM and 4-OH-CY, as described in the text. As can be observed from Figure 3, 10% FCS A3 cell growth is inhibited by all the drugs tested, however, MMC has the greatest effect on cell survival. Cell growth appears to recover after treatment with either BLM or

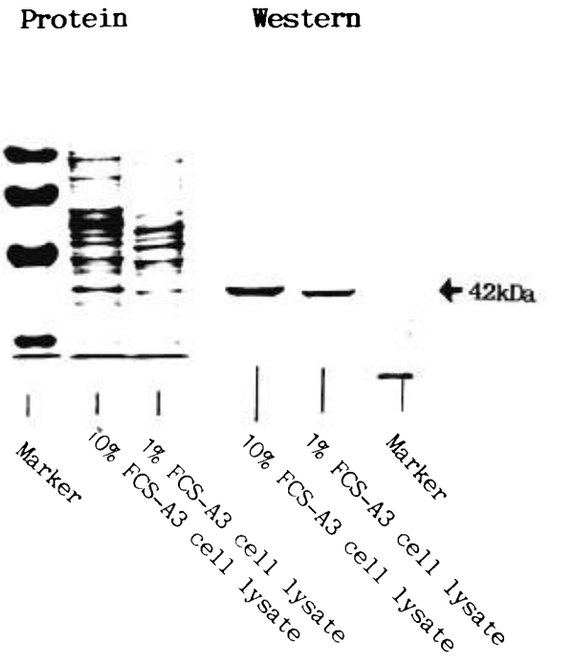


**Figure 2.** Western blotting of 10% and 1% FCS A3 cell lysates by SDS–PAGE. Localization with anti-CE7 monoclonal antibody indicated that although 10% FCS A3 cells do not express the 42 kDa antigen on the cell surface, a large amount of the antigen is present intracellularly. Total protein content of the two cell types, indicated by the title Protein on the left-hand side, was similar.

4-OH-CY, but not after treatment with MMC. Exposure of A3 cells to MMC for only 1 h drastically reduced cell survival, which did not recover even after extensive washing of the cells. Table 1 shows the chronological changes in relative expression of CE7 antigen by 10% FCS A3 cells with time after treatment with the antitumor agents. The strongest expression of antigen could be observed after treatment with MMC, corresponding with the potent growth inhibitory effects of the antitumor agent, and the weakest effects were observed after treatment with 4OH-CY. For comparison purposes, Figure 4 shows a cytogram representing CE7 antigen expression by A3 cells, 2 days after treatment with the indicated doses of antitumor agent. Of interest is the pattern of the cytograms, especially when contrasted with the cytotoxic effects of the antitumor agents. Treatment with MMC produces two cell populations as indicated by the two peaks in the cytogram, one comprising cells with do not express CE7 on the left and another peak comprising cells which express CE7 on the right. In contrast, A3 cell

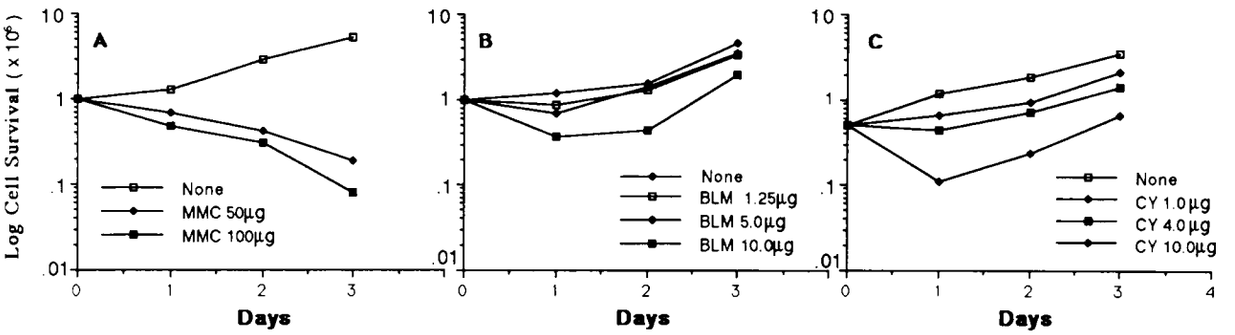


**Figure 3.** A3 cell viability after treatment with the antitumor agents *in vitro*. The 10% FCS A3 cells were treated with various doses of MMC for 1 h, or BLM or 4-OH-CY for 2 h, and cultured for 3 days. The number of viable cells were counted on every day of culture after treatment with the antitumor agents by the Trypan blue exclusion method. The concentration of antitumor agent per milleliter of medium are suffixed to the abbreviated antitumor agent.

**Table 1.** Modulation of CE7 antigen expression by 10% FCS A3 cells on consecutive days after treatment with antitumor drugs[a]

| Treatment | Dose (μg/ml) | CE7 antigen expression | | |
|---|---|---|---|---|
| | | day 1 | day 2 | day 3 |
| None | | − | − | − |
| MMC | 50.0 | ± | + | ++ |
| | 100.0 | ± | + | ++ |
| BLM | 1.25 | − | + | − |
| | 2.5 | − | + | − |
| | 5.0 | − | + | − |
| | 10.0 | − | + | − |
| | 20.0 | − | + | − |
| 4-OH-CY | 2.0 | − | − | − |
| | 4.0 | − | + | + |
| | 5.0 | − | + | + |
| | 10.0 | − | + | + |

[a] Tumor cells were exposed to either MMC for 1 h, BLM for 2 h or 4OH-CY for 2 h, washed extensively and examined by flow cytometry for CE7 expression on three consecutive days after treatment.

populations treated with BLM and 4-OH-CY shift as a single peak to the right, indicating that all the cells express CE7 antigen even though at a weaker intensity than cells treated with MMC, as evidenced by the shift in channel number.

### Effects of low temperature on CE7 expression by A3 cells.

Proliferation of 10% FCS A3 cells after transfer to 4°C was observed to be completely inhibited by low temperature as depicted in Figure 5. The tumor cells, which when grown in medium containing 10% FCS have a doubling time of about 15 h, did not increase in number after 24 h of culture. Figure 6 shows that although 10% FCS A3 proliferation was completely inhibited by culture at low temperature, CE7 antigen expression was not detected on the surface of the cells, indicating that although CE7 expression is related to a decrease in growth rate, as shown by an increase in antigen expression with increase in doubling time of A3 cells grown under low FCS conditions, a decrease in cell proliferation is not always accompanied by an increase in antigen expression.

## Discussion

In our studies on tumor cells and their immunosuppressive mechanisms, we have encountered CE7 antigen, produced and shed from cell membranes in a growth-related manner by A3 cells derived from the KMT-17 fibrosarcoma. Rats bearing the parent KMT-17 fibrosarcoma, which also sheds the antigen, were observed to be defective in inducing cytotoxic T lymphocytes early after subcutaneous injection of tumor cells.[13] Mitogen-stimulated spleen cells obtained from KMT-17 tumor-bearing rats were also found to produce significantly lower amounts of immunoregulatory cytokines than similarly stimulated cells obtained from normal rats.[14] We have no evidence to show that CE7 antigen is the factor which is causing immunosuppression in the KMT-17 tumor-bearing rats; however, KMT-17 cells do not produce transforming growth factor (TGF-$\beta$) and neither do they produce appreciable amounts of prostaglandin $E_2$. It would seem that inhibition of tumor-associated antigen shedding would be desirable because even if the antigen itself is not immunosuppressive, enhanced expression of antigens on tumor cells

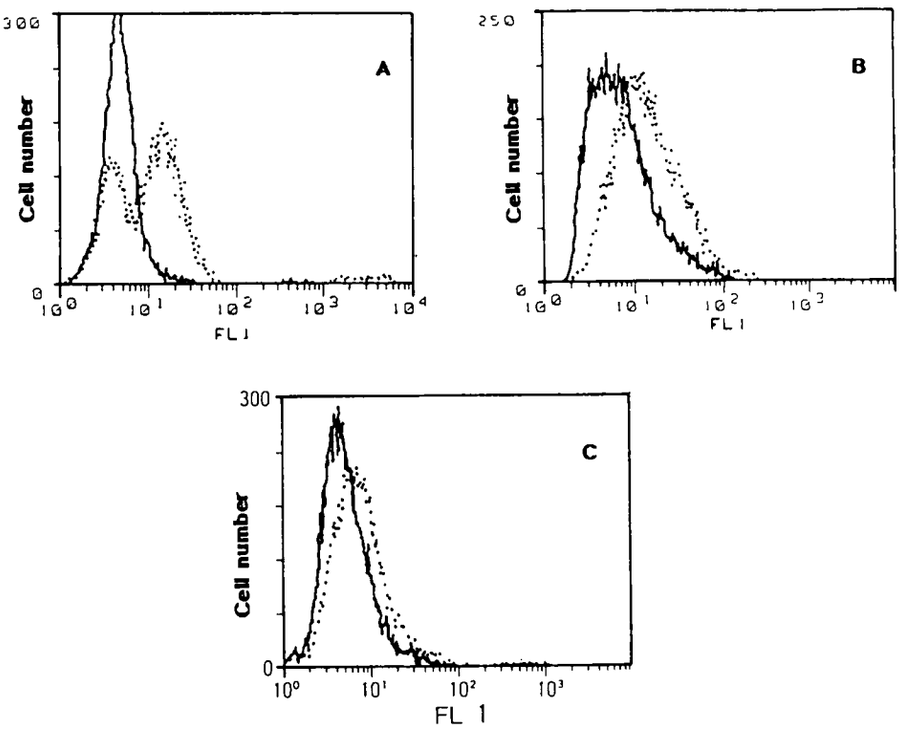


**Figure 4.** CE7 antigen expression 2 days after treatment with MMC (A), BLM (B) and 4-OH-CY (C) as described in the text. The solid line represents a flow cytogram of CE7 expression by untreated 10% FCS A3 cells and the dotted line represents the expression by cells treated with MMC (50 μg/ml), BLM (1.25 μg/ml) and 4-OH-CY (5.0 μg/ml) in A, B and C, respectively.

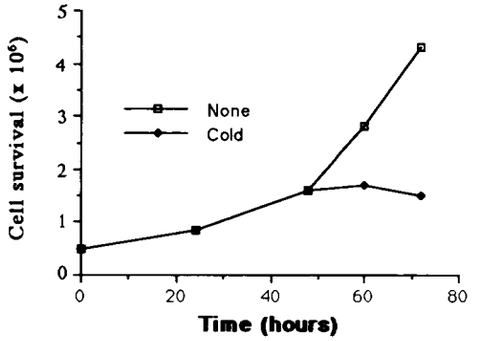


**Figure 5.** Growth of 10% FCS A3 cells after exposure to low temperatures. Cells in their logarithmic phase of growth were transferred to a cold (4°C) environment and the numbers of viable cells were counted after 12 and 24 h by the Trypan blue exclusion method. Cells transferred to the cold environment failed to grow but control cells cultured at 37°C maintained a high proliferation rate.

would probably assist the host in mounting an effective immunologically-mediated antitumor response. Recent work in this area focussed on antigen shedding by melanoma cells and its inhibition by cytokines and dimethyl sulfoxide.[5,6] This work has added meaning when one considers that some success has been achieved in treating melanoma with tumor infiltrating lymphocytes,[15] whose cytotoxic effects are antigen dependent.

Another area of interest in our laboratory is the 'xenogenization' of tumor cells.[16-18] Xenogenization is a term which refers to the foreignization of tumor cells to the host by chemical, viral and other means.[19] We consider the inhibition of CE7 antigen shedding by antitumor agents as a form of chemical xenogenization, with potential use for the development of monoclonal antibodies applied in studies on immunotherapy.[20] Xenogenization itself is a means for the development of tumor cell vaccines, since X-irradiated or murine Friend leukemia virus infected A3 cells were found to protect immunized rats from lethal injections of the parent KMT-17 tumor cells.[21]

A characteristic of tumor cells is proliferation at a rate which is generally higher than that of normal cells and this high proliferation is often accompanied by a high turnover of protein products. Protein synthesis may be a direct result of the process of growth or a result of uncontrolled

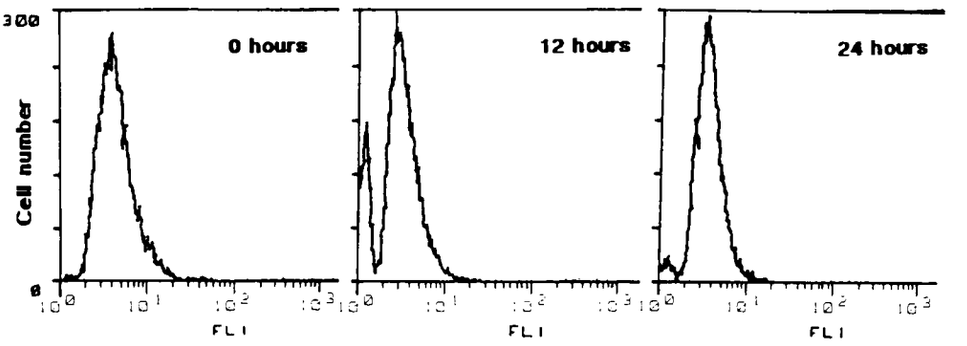


**Figure 6.** CE7 antigen expression by 10% FCS A3 cells incubated at low (4°C) temperatures. Cells were examined for CE7 expression by flow cytometry after staining with anti-CE7 monoclonal antibody 12 and 24 h after transfer to the cold environment. Although cell growth was completely suppressed by the low temperatures, CE7 antigen could not be detected on 10% FCS A3 cell surfaces.

intracellular signal transduction brought about by disruption of normal regulatory networks inside the cell during the process of carcinogenesis. Therefore, although some protein synthesis may be inhibited by growth-regulatory conditions, some synthesis may occur independently of cell growth if stimulatory biochemical pathways are not disrupted. Amongst several products of tumor cells are gangliosides which, if shed into the surroundings, may have immunosuppressive effects on the host,[2,22] and other cell membrane components which if retained on the cell surface may render the tumor cell liable to lysis by immune effector cells.[23,24]

A3 cells growing in media containing different amounts of FCS were found to contain similar amounts of CE7 antigen indicating that 10% FCS A3 cells may be storing the antigen intracellularly. MMC was observed to cause a different pattern of CE7 expression on treated cells than BLM and 4-OH-CY. The cytogram for CE7 expression obtained after treatment with MMC indicated two cell populations, one expressing and the other not expressing the antigen. Because CE7 is shed from cell surfaces bound to vesicle membranes, BLM and 4-OH-CY are thought to disrupt vesicle production by the majority of treated cells soon after exposure to the antitumor agents. MMC appears to cause cell damage but CE7 expression, and indirectly vesicle production, are affected in only about half of the cells. This difference in the patterns of enhanced antigen expression between these antitumor agents probably reflects different cytotoxic mechanisms of the antitumor agents. This observation is important when rapid and effective augmentation of tumor-associated antigen expression on tumor cells by antitumor drugs is desired. The fact that CE7 expression does not increase at low temperatures, even though cell proliferation is completely inhibited, shows that the effect of antitumor agents on CE7 expression is probably through disruption of biochemical pathways.

We are now attempting to establish appropriate protocols to study the mechanisms of vesicle production by A3 cells, and questions which remain unanswered are what exactly are the contents of these vesicles being shed by the tumor cells and what effect do they have on tumor cell growth.

## Conclusion

Tumor-associated CE7 antigen shedding is a phenomenon which is growth-associated but not growth-dependent and directly linked to the vesicle-producing mechanisms. Data show that inhibition of shedding of this antigen by antitumor agents can either affect all the cells in a tumor cell population early after treatment, even though the effect is slight, such as after treatment with BLM and 4-OH-CY, or it can initially affect only part of the population, although strongly, such as after treatment with MMC. This, we believe, is directly linked to the inhibitory effects of the antitumor agents on intracellular biochemical pathways rather than antiproliferative effects through DNA damage. However, we cannot at this point speculate which type of inhibition of antigen shedding will give the best antitumor results *in vivo*. In conclusion, use of appropriate antitumor drugs not only inhibits tumor cell growth, but by preventing tumor-associated antigen shedding, may also indirectly induce an immunologically-mediated antitumor response.

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