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ANTIBODY-MEDIATED TARGETING OF DIFFERENTIATION INDUCERS TO TUMOR CELLS: INHIBITION OF COLONIC CANCER CELL GROWTH *IN VITRO* AND *IN VIVO* A PRELIMINARY NOTE*

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Summary: A differentiation inducer (sodium butyrate) encapsulated in liposomes that are in turn covalently linked to an anti-Le^x monoclonal antibody, SH1 (IgG3 isotype), was successfully targeted to human colonic adenocarcinoma HRT-18 and HT29 cells expressing Le^x antigen *in vitro* as well as *in vivo* in athymic nu/nu mice. Tumor cell growth was significantly inhibited and was associated with changes in cell morphology and increases in membrane-bound alkaline phosphatase and γ-glutamyltranspeptidase, indicating the occurrence of butyrate-induced differentiation.

Antibody-mediated delivery of cytotoxic drugs or toxins to tumor cells, originally envisioned by Paul Ehrlich (1), has proven to be realistic since monoclonal antibodies (MoAbs) and their conjugates with toxins or with liposomes containing cytotoxic drugs have become available (2-7). However, there are major drawbacks to this approach, since the conjugates are rapidly taken up by macrophages and other components of the reticuloendothelial system before they can reach the tumor cells (8,9). Modification or deglycosylation of ricin carbohydrate chains lessens non-specific uptake of "immunotoxins" by macrophages (10-12), yet interaction of "immunotoxins" with normal cells is unavoidable, since truly tumor-specific antigens are virtually non-existent. Essentially all tumor cell populations in gastric, colonic, and lung cancer express aberrant glycosylation (13); hence, essentially 100% of populations in these tumors can be recognized by a combination of several different anti-carbohydrate antibodies (14,15). However, some of the antigens recognized by these anti-carbohydrate antibodies are also expressed in normal cells. Thus, cytotoxic antibody conjugates *in vivo* kill not only tumor cells but also, unavoidably, normal cells. In consideration of this situation, delivery of non-cyto-

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toxic differentiation inducers (DIs) conjugated with anti-carbohydrate antibodies would appear to be a novel system to suppress tumor cell growth, since DIs are known to modify tumor cell growth *in vitro* by inhibiting malignant properties such as invasive and metastatic abilities but have little effect on normal cells. Thus DIs inadvertently delivered to normal cells would not cause significant functional disorder. However, no studies have been reported on delivery of DIs mediated by MoAbs or any specific ligand directed to tumor cells *in vitro* or *in vivo*. This paper describes the effect of sodium butyrate encapsulated in liposomes that are coupled to an anti-Le^x MoAb on human colonic cancer cell lines grown *in vitro* as well as *in vivo*.

MATERIALS AND METHODS

Two human colonic cancer cell lines, HRT-18 and HT-29 (16), expressing Le^x antigen (Galβ1→4[Fucα1→3]GlcNAcβ1→R) on their surface were used as target cells throughout this experiment. Both of them were cultured in Dulbecco-modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum.

Single compartment liposomes were prepared and conjugated with the IgG3 anti-Le^x antibody SH-1 or an IgG preparation of normal mouse serum according to the method previously described (2,17). Briefly, lecithin, cholesterol, and ganglioside (GM_3) were mixed together in molar ratios of 1:0.75:0.1, dissolved in chloroform/methanol (2:1, v/v), and dried under a nitrogen gas stream. Subsequently, 10% sodium butyrate in 140 mM NaCl containing 15 mM phosphate buffer (PBS, pH adjusted to 6.5) was added to the lipid film (3 ml per 50 μM of lecithin), and sonicated for 3-6 hours in a bath sonicator at 25°C. The preparation was dialyzed against PBS (pH 6.5) to remove non-encapsulated sodium butyrate. The dialyzed liposomes were incubated with 8 mM sodium metaperiodate for 45 min in the dark on ice. Glycerol was added to a final concentration of 30 mM to stop the reaction. The preparation was dialyzed against PBS (pH 6.5) overnight. Either the purified anti-Le^x IgG3 antibody SH1 or purified IgG preparation from normal mouse serum was added (1 mg per 4 μM of lecithin) and incubated overnight at room temperature. Subsequently, sodium cyanoborohydride (8 μM per 50 μM of lecithin) was added and incubated at 4°C for 48 h. Single compartment liposomes encapsulating butyrate coupled to antibodies thus prepared were separated from free antibodies on a Sepharose CL-4B column, as shown in Fig. 1. Liposomes coupled to the antibodies were eluted in the void volume.

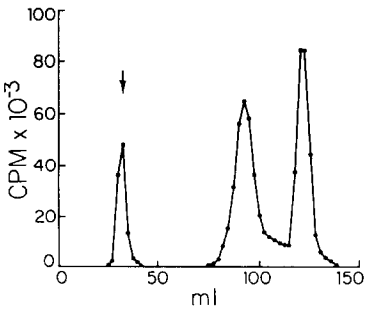


Fig. 1. The elution pattern of ^{125}I-labeled antibody from Sepharose 4B column (1.5 x 90 cm). Liposome-associated antibody was eluted in the void volume (shown by arrow).

RESULTS AND DISCUSSION

The effect of butyrate-encapsulated liposomes conjugated with the anti-Le^x antibody SH1 or with normal mouse serum IgG on HRT-18 cell growth and the experimental design are shown in Fig. 2. Only SH1-conjugated liposomes were effective in inhibiting the growth of tumor cells. Furthermore, cells treated with SH1-conjugated liposomes showed clear morphological changes, i.e., cells were flattened, the cell boundary became discrete, and cell growth behavior became similar to that of normal cells, i.e., contact-inhibited cell growth appeared (data not shown).

In *in vivo* experiments, 5 x 10^6 cells were inoculated into athymic nu/nu mice. When the tumors reached 0.5-1.0 cm in diameter, unconjugated liposomes, liposomes conjugated with SH1, or liposomes conjugated with normal mouse IgG (liposomes labeled with ^{14}C-cholesterol in each case) were intravenously injected in order to observe the distribution of liposomes in various tissues and in tumors (details of experimental design are described in the legend for Fig. 3). The accumulation of SH1-conjugated liposomes, normal mouse IgG-conjugated liposomes, and unconjugated liposomes was nearly identical in liver, spleen, lung, kidney, and heart (as shown in Fig. 3A-E). However, in tumors, the accumulation of SH1-conjugated liposomes was 3-4 times higher than that of unconjugated liposomes 48 h after injection of liposomes (Fig.

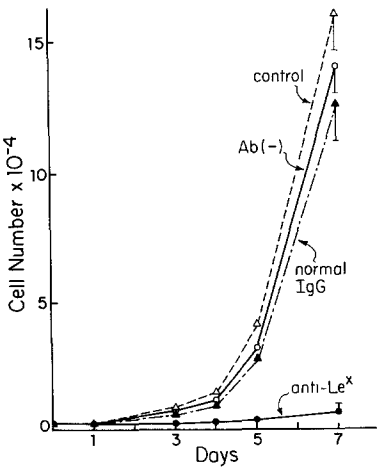


Fig. 2. HRT-18 cells cells were seeded in 24-well Falcon plates (2,000 cells/well). Subsequently, 10 μl of one of the following four preparations was added: (i) liposome suspension containing 5 μmol of sodium butyrate conjugated to 10 μg of anti-Le^x antibody (•); (ii) liposome suspension containing 5 μM sodium butyrate conjugated to 10 μg of normal mouse IgG (▲); (iii) liposome suspension containing the same amount of sodium butyrate without antibody conjugate (○); or (iv) PBS, pH 7.4 (△). The number of cells (ordinate) was counted after trypsinization at the indicated time (abscissa).

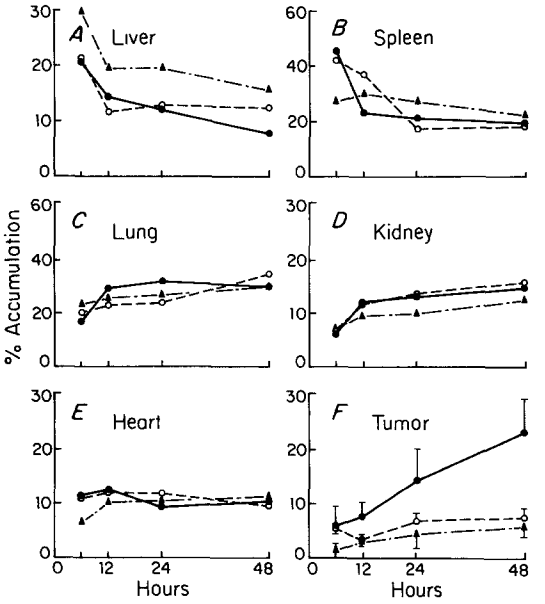


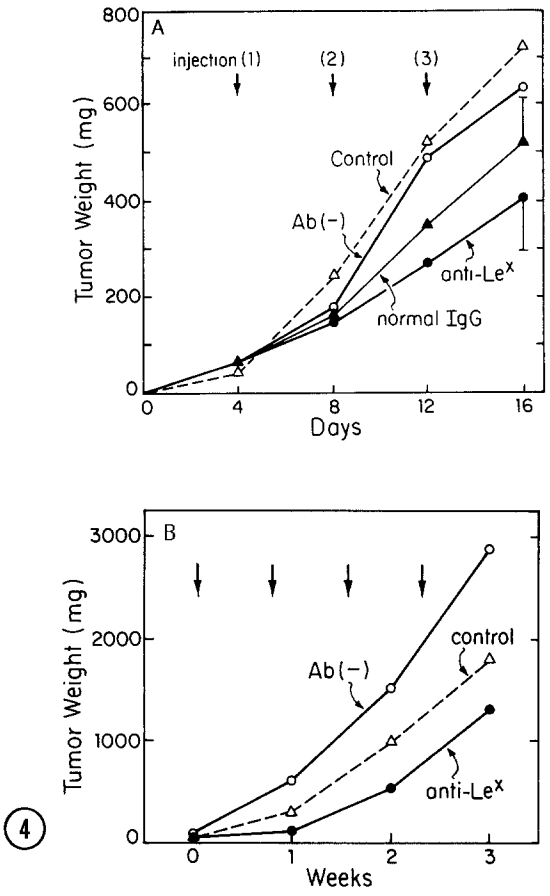
Fig. 3. Nude (nu/nu) mice were inoculated subcutaneously with 5 x 10^6 HRT-18 cells/mouse. When the tumors reached 0.5-1.0 cm in diameter, the mice were injected intravenously with 0.2 ml of anti-Le^x antibody-conjugated liposome suspension (•), normal IgG conjugated liposome suspension (▲), or unconjugated liposome suspension (○) in which ^{14}C-cholesterol was a component of the liposome. The concentration of liposome injected was 1 μM/ml. The mice (3-4/group) were sacrificed at 6, 12, 24, and 48 h after the injection of the liposome suspension. Livers, spleens, lungs, kidneys, hearts, and tumors were removed and weighed. Cholesterol was extracted from each type of tissue according to the method previously described (24) and resuspended in ethanol. Radioactivity was measured by liquid scintillation counter. Accumulation of liposomes was determined according to the following formula:

$$\% \text{ accumulation} = 100 \times \frac{\text{cpm/weight (specific organ)}}{\text{cpm/weight (total of all organs)}}$$

Standard deviations are indicated by vertical bars.

3F). These results clearly demonstrate that the accumulation of liposomes in tumors clearly depends on antibody conjugation, and are consistent with the observation of Ballou et al. (18) that labeled anti-Le^x antibody accumulates in Le^x-containing tumor cells preferentially over Le^x-containing normal tissues.

The *in vivo* effect of liposomes or their conjugates on tumor cell growth is shown in Fig. 4 (experimental details described in the legend). Growth of both HRT-18 and HT-29 tumors in athymic mice was clearly inhibited in the group treated with anti-Le^x antibody-conjugated liposomes containing sodium butyrate. The tumor growth was not inhibited in the group treated with liposomes alone and was less effectively inhibited in the group treated with liposomes bound to non-specific mouse IgG. These results



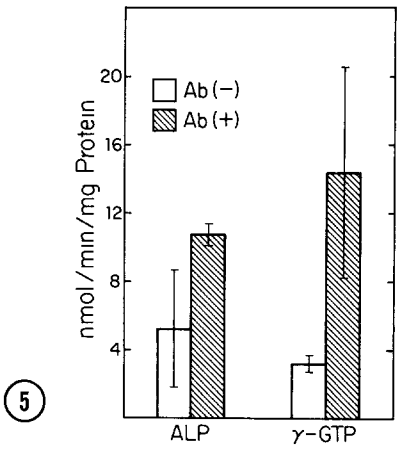


Fig. 4. The effect of liposomes encapsulating sodium butyrate with or without conjugation with anti-Le^x antibody SH1. Panel A, effect on HRT-18 tumor growth; Panel B, effect on HT-29 tumor growth. Athymic mice were inoculated subcutaneously with 5 x 10^6 HRT-18 or HT-29 cells. When the tumors reached 0.3 to 0.5 cm in diameter, the mice (10-15 mice/group) were injected intravenously every four days with 0.2 ml of one of the following: (i) sodium butyrate-containing liposome suspension conjugated with anti-Le^x antibody (•); (ii) sodium butyrate-containing liposome conjugated with non-specific mouse IgG (▲); (iii) sodium butyrate-containing liposome without antibody (○); or (iv) PBS, pH 7.4 (△). The concentration of liposome injected was 1 μM/ml. The tumor size was measured, and tumor weight was estimated as follows: tumor weight (mg) = 0.5 x length (mm) x width^2 (mm^2). Ordinate, tumor size; abscissa, days after intravenous injection of liposomes. Arrows indicate day of injection. Experimental error for each group, $p < 0.005$.

Fig. 5. Four days after the fourth injection (described in the legend for Fig. 4), mice were sacrificed and tumors removed. Each tumor was homogenized using a Teflon glass homogenizer and centrifuged at 100,000 g for 1 h to obtain the membrane fraction. Alkaline phosphatase and γ-glutamyltranspeptidase activity of the membrane fraction from tumors was determined using assay kits from Sigma Chemical Co. (St. Louis, MO). The protein concentration was measured according to the method of Bradford (25). Open bar, tumors from the control group injected with liposome-encapsulated sodium butyrate without antibody conjugation. Shaded bar, tumors from a experimental group injected with liposome-encapsulated sodium butyrate conjugated to SH1 anti-Le^x antibody. ALP, alkaline phosphatase; γ-GTP, γ-glutamyltranspeptidase. Values represent mean ± S.D. (indicated by vertical bar).

demonstrate specific inhibition of tumor growth by liposomes containing sodium butyrate conjugated with SH1.

Since the activity of some membrane-associated enzymes in colonic tumor cells was reported to be greatly enhanced in association with induced differentiation (16), the level of membrane-associated enzymes in tumors grown in mice treated with SH1-conjugated liposomes was determined. The levels of two enzymes, alkaline phosphatase and γ-glutamyltranspeptidase, as determined by the procedure described in the legend for Fig. 5, were found to increase 1.7 fold and 3.8 fold, respectively, in the tumors of mice treated with SH1-conjugated liposomes as compared with those of mice in the control groups. Thus, the inhibition of tumor growth *in vivo* may also be a result of differentiation induction.

Many tumor cells cultured in the presence of DIs show an induction of contact inhibitability, reduction of saturation density, and become incapable of forming colonies in soft agar (16,19-21), although susceptibility of tumor cells to DIs varies greatly and the mechanism of differentiation induction is essentially unknown. Oral administration of retinoids has been reported to have some effect on tumorigenesis (22). However, it has been difficult to ensure that a sufficient concentration reaches the tumors without negative side effects. Results of the present studies clearly indicate that sodium butyrate was targeted efficiently to Le^x-expressing tumor cells mediated by an anti-Le^x IgG3 antibody. Antibody-mediated targeting of cytotoxic drugs and toxins to tumor cells has been an increasingly popular idea (2-9,12), although there are serious drawbacks, including (i) lack of antibodies truly specific to tumor cells, (ii) uptake of antibody-liposome conjugates by macrophages and the reticuloendothelial system, and (iii) heterogeneity of tumor cells in their expression of antigens. The third problem above can be overcome by application of a combination of multiple (5 or 6) anti-carbohydrate antibodies, since essentially 100% of gastric and colonic tumor cell populations in a single tumor have been shown to express multiple carbohydrate antigens in a clear mosaic pattern (14,15). The first two drawbacks in the use of cytotoxic drug conjugates described above can be overcome by the approach presented in this paper. A large proportion of liposomes containing butyrate conjugated with an anti-Le^x antibody was shown to accumulate in various tissues and organs when administered intravenously; nevertheless, the animals appeared perfectly normal, since DIs have no serious cytotoxic effects. Only tumor cells specifically targeted with butyrate showed reduced growth and could be converted to a benign phenotype. Such "benign" tumors can be surgically removed or treated by radiation more easily than highly malignant tumors.

The major drawback of the present approach is that not all tumor cells are susceptible to DIs. Therefore, the approach may be limited to certain types of tumors that are susceptible to DIs. Further intensive studies on DIs may greatly increase the spectrum of tumor cell susceptibility to such inducers. Since leukemia or lymphoma cells are highly susceptible to DIs, this approach may be very useful for treatment of leukemia or lymphoma. This approach combined with other non-toxic reagents such as glycosylation inhibitors, including castanospermin, swainsonine, and N-methylnojirimycin, which are known to alter tumor cell phenotypes (23), may also be effective.

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