Galactose-conjugated Antibodies in Cancer Therapy: Properties and Principles of Action

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ABSTRACT

Galactose conjugation of antibodies causes them to be recognized by the hepatic asialoglycoprotein receptor and therefore cleared very rapidly from the blood. In these investigations, some effector functions of galactose-conjugated antibodies were assayed, and several applications to experimental tumors in vivo were demonstrated. Galactose conjugation did not interfere with two antibody functions in addition to antigen binding, namely complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. This conjugation procedure was originally developed for its potential use in localized immunotherapy, such as i.p. injection of galactose-antibody conjugates i.e. demonstrated, more conclusively than other methods that have been used, that the presence of ascites causes prolonged retention of antibody in the peritoneal cavity and that this effect is correlated with the volume of ascites present. In mice bearing i.p. tumor xenografts, i.p. injection of galactose-antibody conjugates resulted in high tumor/nontumor ratios at 28 h after antibody injection, with values of 40:1, 43:1, 77:1, and 11:1 for the blood, kidney, lung, and spleen, respectively, although the ratio was only 4:1 for the liver. Control experiments demonstrated that i.p. injection of unconjugated antibody or a galactose-conjugated nonreactive antibody produced much lower tumor/nontumor ratios.

In investigations of possible systemic application of galactose-antibody conjugates, we found that injection of large amounts of an inhibitor that binds competitively to the hepatic receptor, asialo-bovine submaxillary mucin, can block clearance of galactose-conjugated antibodies for 2–3 days. In this way, high blood levels of antibody can be maintained for 2–3 days, thus allowing penetration and binding to solid tumors, followed by very rapid blood clearance. With this approach, using a human carcinoma growing s.c. in nude mice, high tumor/nontumor ratios were obtained 4 days after injection, with mean values of 43:1, 18:1, 17:1, and 15:1 for the blood, kidney, lung, and spleen, respectively, although the ratio for the liver was only 1.7:1. The blood level at this time was 0.04 ± 0.02% (SD) of the injected dose/g, while the tumor level was 1.69 ± 1.29% of the injected dose/g. In conclusion, galactose-conjugated antibodies appear to have diverse applications in regional or systemic immunotherapy.

INTRODUCTION

A method of galactose conjugation of antibodies was developed to allow manipulation of their blood clearance rate (1). Such conjugates bind to the ASGP receptor in the liver and are therefore cleared very rapidly from the blood within minutes. This property is potentially useful for localized immunotherapy, such as i.p. or intraventricular, since it essentially prevents antibodies from reaching any antigen-positive cells outside of the region injected. Moreover, for radioisotope-antibody conjugates, this modification could reduce irradiation of the whole body due to the presence of the isotope in the circulation. Retention of isotope by the liver appears not to be a major limiting factor, at least for 131I-conjugates, since more than 90% of this isotope was released from the liver, presumably in degraded form, within 1 h after liver uptake (1). Galactose-antibody conjugates were shown previously to have intact antigen-binding activity (1). In this report we determined whether two other functions of antibodies, namely complement-mediated cytotoxicity and antibody-dependent cell-mediated cytolysis, would be affected by galactose conjugation. We also investigated the retention of antibody in the peritoneal cavity after i.p. injection into mice having different volumes of ascites. In addition, we demonstrated that blood clearance of Gal-Ab conjugates can be prevented for prolonged periods by injection of an inhibitor that binds competitively to the ASGP receptor. By this mechanism, the blood concentration of antibody after i.v. injection can be maintained at a high level for 2–3 days, allowing penetration of solid tumors (2), followed by very rapid blood clearance after the inhibitor is consumed. We demonstrate several applications of galactose-antibody conjugates in nude mice bearing human tumor xenografts. In mice bearing i.p. tumors, high tumor/nontumor ratios were obtained 28 h after i.p. injection of galactose-antibody. Also, in mice bearing s.c. tumors, high tumor/nontumor ratios were obtained 4 days after i.v. injection of galactose-antibody, if uptake by the asialoglycoprotein receptor was inhibited for 2 days. In both cases, high tumor/nontumor ratios could be attributed primarily to very low blood levels at the time of dissection.

MATERIALS AND METHODS

Animals and Cells. Female CF-1 mice (Charles River Laboratories, Wilmington, MA), 3–5 months of age, were used to assay blood clearance. To induce ascites in CF-1 mice, 0.2 ml complete Freund's adjuvant was injected i.p. weekly (3) until a large ascites developed, which required 3–7 injections for most mice. Spleen cells from BALB/c mice and human blood lymphocytes obtained from healthy volunteers were used for ADCC assays. The propagation of the ovarian carcinoma cell line HEY was described previously (4). The astrocytoma cell line U138 was obtained from Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center), and the carcinomas LoVo and HEP2 were obtained from the American Type Culture Collection (Rockville, MD). These were grown in tissue culture as described previously (5). To establish tumors in 4–6-week-old nude mice (Harlan Sprague Dawley, Indianapolis, IN), 10⁶ HEP2 cells were injected i.p., or 10⁶ LoVo cells were injected s.c. in the back. Radioimmunolocalization experiments were performed when LoVo tumors reached a diameter of 0.5–1.0 cm, which was 2–3 weeks after inoculation.

Antibodies. Three mAbs, MA103, TA99, and EPB-2 (Immu-L2), all IgG2a, and the method of purification on protein A-Sepharose were described previously (1, 6). MA88 is another IgG2a mAb that reacts with a widely distributed human cell surface antigen that has not been...
characterized biochemically. Normal rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO). Iodination with $^{125}$I was by the chloramine-T procedure, and the specific activity of IgG was in the range of 1.0-4.0 $\mu$Ci/\(\mu\)g. For galactose conjugation (1), cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-\(\beta\)-D-galactopyranoside (C-4141; Sigma) was dissolved in methanol at 0.1 M and mixed with 0.1 volume sodium methoxide (methanol sodium derivative; J. T. Baker Chemical Co., Phillipsburg, NJ), also 0.1 M in methanol. After 48 h at room temperature, this mixture was stored for weeks at 4°C. Varying volumes, as cited in the text, were evaporated dry and dissolved in 0.25 M sodium borate buffer, pH 8.5, containing IgG at 1.0 mg/ml. After 2 h at room temperature, the sample was dialyzed into phosphate-buffered saline. Since the conjugation ratio was not determined, batches are described in terms of the volume of activated sugar used per mg IgG. Each batch was tested routinely for rapid blood clearance and retention of antigen reactivity.

**Blood Clearance Rates and Radioluminidetection.** Iodinated antibodies were analyzed by SDS-PAGE, with or without reduction, followed by autoradiography. More than 95% of the radioactivity was associated with IgG chains, as demonstrated in previous studies (1). Samples containing 10$^6$cpm (0.25-1.0$\mu$g) in 0.3 ml DPBS (Gibco)-0.1% bovine serum albumin were injected i.v. with a 28-gauge needle into the suborbital plexus of the eye of groups of 3 mice anesthetized with Metofane (Pittman-Moore, Inc., Washington Crossing, NJ). All mice were bled from the tail approximately 30 s after injection, and subsequent tail bleedings were obtained at various intervals. For tail bleeding, mice were warmed under a heat lamp (not required for anesthetized mice), the tail was nicked with a scalpel, and 3-4 drops were collected into preweighed tubes. Excess bleeding was prevented by manual compression until clotting occurred. RAID was determined by methods described previously (7), using injections of 10 $\mu$Ci/mouse (2.5-10 $\mu$g) routinely, in groups of 3-4 mice. In experiments in which galactose-MA103 was injected i.v. (together with the inhibitor asialo-BSM), 2 injections of 10 $\mu$Ci were administered 24 h apart. In preliminary experiments, we found that IgG2a antibodies were cleared very rapidly in nude mice, making RAID experiments impossible to perform, but that this could be prevented by increasing the dose of IgG2a injected. Therefore, in all RAID experiments the injected $^{125}$I-labeled antibody was supplemented with 0.2 mg unlabeled EPB2.

**Other Proteins.** BSM was obtained from Sigma (M-4503) or from Worthington Biochemicals (Freehold, NJ). A different grade of BSM from Sigma (M-3895) was found to be consistently toxic to mice, with symptoms resembling those of endotoxin toxicity, so it could not be used in these experiments. The BSM was dissolved to 35.0 mg/ml in H$_2$O by gentle mixing for 30-60 min; then H$_2$SO$_4$ was added to 0.05 M. At this step, a large precipitate developed, which probably is composed of contaminating proteins. After a heating for 1 h at 80°C to desialylate the mucin (8), the sample was centrifuged at 6000 rpm for 30 min, and the pellet was discarded. The pH was then adjusted to 8.0 with 2.0 M NaOH, and the sample was dialyzed against DPBS. Using the Warren assay for sialic acid (9), this procedure resulted in 96% desialylation. BSM samples for injection were quantitated by the Lowry protein assay (10). However, BSM reads very low relative to albumin, desialylation. BSM samples for injection were quantitated by the Lowry protein assay (10). However, BSM reads very low relative to albumin.

**Antibody Functional Assays.** To assay complement-mediated cytotoxicity, trypsinized U138 astrocytoma target cells were plated at 300-800 cells/10 $\mu$l/well of Terasaki plates (Nunc, Thousand Oaks, CA) and incubated for 1-2 days. The plates were washed twice with DPBS containing 5% fetal calf serum, and dilutions of mAbs were added. After 45 min at 37°C, plates were washed twice with barbital-buffered medium (0.14 M NaCl-3 mM NaHCO$_3$-0.5 mM MgCl$_2$-15 mM CaCl$_2$-4 mM barbital, pH 7.3-0.1% glucose-0.2% bovine albumin), and in a complement source, 3-4-week-old rabbit serum (Pel-Freez, Rogers, AR) diluted 1:40 in barbital-buffered medium was added. After 3 h at 37°C, the plates were flooded gently with DPBS containing 2.0 $\mu$g/ml ethidium bromide and 0.2 $\mu$g/ml acridine orange and examined with an objective on a Leitz Dialux fluorescent microscope equipped with a 200-W mercury lamp and filter cube H. The percentage of dead cells (stained red, in contrast to green viable cells) was estimated at the nearest 10%. Controls included complement alone and antibody alone, both of which produced <10% lysis.

To assay ADCC, a clonogenic assay was used. Aliquots of 0.1 ml containing 2.5 x 10$^5$ HEY ovarian carcinoma cells were incubated with serial dilutions of antibody in microcentrifuge tubes for 20 min at 4°C. The cells were then washed, incubated for 16 h at 37°C with 2.5 x 10$^5$ mouse splenocytes or human peripheral blood lymphocytes (tumor cell:effector cell ratio, 1:50) in a total volume of 0.2 ml, and then assayed for colony formation in triplicate as described previously (11). Controls included HEY cells incubated alone, which was defined as 0% lysis, and effector cells without antibody. Both mouse and human effector cells were prepared by centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) for 30 min at 1500 rpm. Cells were collected from the interface and washed three times with RPMI 1640 (Gibco). For human effector cells, adherent cells were removed by plating the cell suspension in plastic tissue culture dishes for 1 h at 37°C. Viable cell counts were determined by trypan blue exclusion.

**RESULTS**

**Effector Functions of Antibodies.** Galactose conjugation of TA99 was described previously to have no effect on antigen binding activity (1). Complement-mediated cytotoxicity was assayed with the IgG2a mAb MA88 and the human astrocytoma cell line U138 as the target. As shown in Fig. 1A, this activity was unaffected by galactose conjugation. Fig. 1B demonstrates similar results for ADCC with mouse spleen effector cells and the human ovarian carcinoma cell line HEY as target, using the antibody MA103. Similar results were obtained with human peripheral blood effector cells. Fig. 1C shows the rapid blood clearance obtained with the same batch of galactose-conjugated MA88 used in the complement-mediated cytotoxicity experiments, and the galactose-MA103 conjugate used in the ADCC experiments had a similar rapid blood clearance. We conclude that these two antibody functions are unaffected under conditions in which very rapid blood clearance is obtained. We note that excessive galactose conjugation of antibody bodies can result in significant decreases in antigen reactivity as well as effector functions (data not shown).

**Retention of Antibodies in the Peritoneal Cavity.** We demonstrated previously that antibody injected i.p. into normal mice is transferred rapidly into the blood but that in mice with ascites this transfer appeared to be markedly delayed (1). However, in these earlier experiments, we did not consider the role of transfer from peritoneal cavity to blood and back to peritoneal cavity. This return to the peritoneal cavity might be substantial in mice bearing a large ascites, having a volume 2-5 times the total blood volume. Use of Gal-Ab conjugates can elucidate this situation, since rapid clearance by the liver prevents the return...
of antibodies from blood to peritoneal cavity. Therefore, mice bearing large ascites, induced by repeated injections of complete Freund's adjuvant, were given i.p. injections of $^{125}$I-galactose-MA103. At 24 h after injection, mice were killed, the ascites fluid was collected, and radioactivity was determined. As shown in Fig. 2, the presence of ascites did cause significant retention of antibody in the peritoneal cavity, but a large ascites volume was required to have a major effect. Mice with 9–13 ml ascites fluid retained approximately 30% of the $^{125}$I-Gal-Ab in their peritoneal cavity at 24 h. In contrast, mice with 4–5 ml ascites fluid retained only approximately 10%. The cpm retained in the peritoneal cavity was essentially all in solution, rather than bound to ascites cells, and analysis by SDS-PAGE of cpm recovered in ascites fluid demonstrated that it was essentially all associated with intact IgG. The development of ascites in mice induced by repeated complete Freund's adjuvant injections is due to a massive inflammatory response, which envelopes the lower surface of the diaphragm as well as other surfaces in the peritoneal cavity and probably obstructs the subdiaphragmatic lymphatics. In this respect, it appears similar to a tumor-induced ascites (12) and is likely to have a similar effect on protein transport.

Radioimmunodetection of i.p. Tumors in a Nude Mouse Model. Galactose-conjugated MA103 was injected i.p. into nude mice bearing the human carcinoma HEP2 growing i.p. This tumor cell line develops into multiple solid tumor masses throughout the peritoneal cavity, as well as ascites fluid containing tumor cells, and kills the host in approximately 3 weeks. Ascites becomes evident at approximately 2 weeks, and most of the mice used in these experiments had no evident ascites at the time of antibody injection. At dissection, 3 to 4 solid tumor masses were obtained from each mouse, comprising a range of sizes, from 0.02 to 0.5 g, to determine whether results would vary depending on the tumor size. However, no such variation was observed; therefore results with all tumor sizes were combined. Ascites fluid was collected from all mice at the time of dissection, but the total volume was <0.5 ml in most of the mice and the ascites fluid appeared bloody in all cases. Therefore, antibody binding to ascites cells was not analyzed.

Results of a biodistribution experiment are shown in Fig. 3 and Table 1. Since earlier experiments demonstrated that transfer from the peritoneal cavity to blood is fairly rapid, even in mice bearing i.p. tumor, dissection was performed at 28 h after injection of Gal-Ab i.p. Antibody was injected 13 days after tumor inoculation. As shown in Fig. 3A and Table 1, high tumor:non-tumor ratios were obtained for the kidney, lung, muscle, and blood, with a mean tumor: blood ratio of 40:1. Even the tumor: liver ratio was 4.3:1, despite the fact that any Gal-Ab entering the blood is rapidly bound by the liver. This can probably be attributed to the fact that the liver degrades galactose-protein conjugates very rapidly, with most of the $^{125}$I label being released from the liver within 1 h of uptake (1). These high tumor:non-tumor ratios required specific antibody binding, since they were not observed with the subclass-matched control Ab TA99 (Fig. 3A; Table 1). To demonstrate the importance of galactose conjugation, other groups of mice were given identical injections, except with unconjugated antibody (Fig. 3B). In these mice, the reactive Ab MA103 demonstrated substantial tumor localization compared to the control Ab TA99, but the tumor:non-tumor ratios were much lower than with the galactose conjugates. The difference, as anticipated, could be attributed primarily to the difference in blood levels, since the Gal-Ab had only 0.15% of the injected dose/g of blood at dissection, while 13 times more unconjugated antibody was present in the blood. The absolute amount of specific antibody bound to the tumor was quite similar in both groups, being 4.3 ± 2.6% ID/
experiment, with antibody injected i.v. and dissection at 5 days for galactose-MA103 and 5.2 ± 1.9% ID/g for unconjugated particularly for the blood. Bars, SD. 

antibody i.p. resulted in a marked improvement in tumor/tumor ratios, antibodies resulted in little if any advantage, while use of galactose-conjugated 

0.34%; MA103 i.p., 5.15 ± 1.9%; TA99 i.p., 2.63 ± 1.08%; MA103 i.v., 2.5 ± 1.5%; TA99 i.v., 0.36 ± 0.34%: MA103 i.v., 1.30 ± 1.1; Liver 0.46 ± 0.3; Spleen 0.13 ± 0.09; Kidney 0.06 ± 0.01; Lung 0.08 ± 0.04; Muscle 0.15 ± 0.11 

Fig. 3. Biodistribution of 125I-labeled galactose-conjugated and control antibodies in nude mice bearing human carcinoma HEP2 growing i.p., after i.p. or i.v. injection. At the time of antibody injection, multiple solid tumor masses were present in the peritoneal cavity, with little or no ascites fluid. In all experiments, a reactive antibody, MA103 ( ), and a nonreactive antibody, TA99 ( ), were injected into groups of 4 mice, and the data shown are tumor/nontumor ratios. (Many of the open bars are barely visible in the figure, since the values are close to 1.0.) (A) Galactose-conjugated MA103 or galactose-conjugated TA99 injected i.p., with dissection at 28 h after antibody injection. (B) Unconjugated MA103 or TA99 injected i.p., with dissection at 28 h. (C) Unconjugated MA103 or TA99 injected i.v., with dissection at 5 days, a control included to show the localization obtained in a "conventional" biodistribution experiment using this antibody and tumor system. The absolute values of antibody localization to the tumor, in % ID/g, were: galactose-MA103 i.p., 4.26 ± 2.6%; galactose-TA99 i.p., 0.36 ± 0.34%; MA103 i.p., 5.15 ± 1.9%; TA99 i.p., 2.63 ± 1.08%; MA103 i.v., 2.5 ± 1.5%; TA99 i.v., 1.5 ± 0.5%. Use of i.p. rather than i.v. injection for unconjugated antibodies resulted in little if any advantage, while use of galactose-conjugated antibody i.p. resulted in a marked improvement in tumor/nontumor ratios, particularly for the blood. Bars, SD. 

g for galactose-MA103 and 5.2 ± 1.9% ID/g for unconjugated MA103, indicating that the antibody was binding efficiently to the tumor masses in the peritoneal cavity before being transported to the blood.

As an additional control to allow some basis for comparison of this antibody/tumor system with others reported in the literature, we performed a more conventional biodistribution experiment, with antibody injected i.v. and dissection at 5 days after antibody injection. In these experiments, the antibody was injected 9 days after tumor inoculation; therefore dissection was again performed on day 14 after tumor inoculation, as in the preceding experiments. As shown in Fig. 3C, MA103 produced relatively high tumor/nontumor ratios, with, for example, a tumor: liver ratio of 8.6:1 and tumor/lung ratio of 10:1:1, which was not seen with the control antibody. However, these ratios were, again, considerably lower than those obtained with galactose-MA103 injected i.p. We note that results of i.v. injection (with dissection at 5 days) and i.p. injection (with dissection at 28 h) of unconjugated antibodies were very similar. Although such results will vary depending on the time of dissection, these data seem to be consistent with the finding that antibody injected i.p. is rapidly transferred to the blood (1).

Inhibition of Blood Clearance. In previous experiments we demonstrated that rapid blood clearance of Gal-Ab could be inhibited by injection of asialofetuin, but not by intact fetuin (1), however, such inhibition was for a very brief period, less than 1 h. We attempted to delay clearance for much longer periods by injecting larger amounts of inhibitors. A variety of inhibitors was tested. Our preliminary experiments demonstrated that relatively large amounts of inhibitors were required to block blood clearance of Gal-Ab for prolonged periods. The inhibitors were injected either i.v., together with 125I-labeled galactose-MA103, or i.p. 20 min before i.v. injection of the tracer, since we had previously shown that injected proteins pass rapidly after i.p. injection into the blood (1). Initial experiments utilized asialo-α1-acid glycoprotein, which from earlier studies appeared to be among the most strongly binding ligands to the ASGP receptor (13, 14). Asialo-α1-acid glycoprotein (6.5 mg) produced considerable inhibition of the clearance of Gal-Ab, but although 12.5% ID/g blood was present at 1 h, only 4% ID/g blood was present at 3 h, and the clearance rate did not have an evident 2-phase pattern. More explicitly, the desired result was slow clearance followed by very rapid clearance, as would be expected if the inhibitor binds more avidly to the ASGP receptor than Gal-Ab, but this was not observed. Galactose-conjugated normal rabbit IgG (0.5 ml activated sugar/mg) was comparable in inhibitory activity to asialo-α1-acid glycoprotein. We then tested 20 mg each of asialo-BSM and asialofetuin. Both produced strong inhibition; asialo-BSM was most effective, and the results are shown in Fig. 4. Blood clearance was slow for approximately 14 h, followed by very rapid clearance. In similar experiments, a single injection of 10 mg asialo-BSM inhibited clearance for approximately 8–10 h. Therefore, we can estimate that asialo-BSM was cleared from the blood at a rate of approximately 1.4 mg/h, and it appears that Gal-Ab is cleared efficiently only after the inhibitor is consumed. In other experiments we varied the time interval between i.p. injection of the inhibitor and i.v. injection of Gal-Ab, between 30 min and 4 h. There was no significant difference in results depending on the intervals tested; therefore in subsequent experiments we used an interval of 1 h. The blood clearance rate in the presence of the inhibitor, although relatively slow, was always faster than the clearance rate of unconjugated IgG2a (Fig. 4), suggesting that inhibition of uptake by the ASGP receptor was not complete. The slowest τo, observed, in the presence of the largest amount of inhibitor tested, was approximately 12 h.

To maintain high blood levels for 2–3 days, which is the time
GALACTOSE-CONJUGATED ANTIBODY

DISCUSSION

Although galactose conjugation of antibodies was developed initially for the purpose of regional immunotherapy, we believe at this time that its use for i.v. injection together with an inhibitor, which can be applied to all types of cancer, seems likely to be its most valuable application. With certain forms of immunotherapy, it is desirable to remove antibody from the...
blood rapidly after the antibody has bound to the tumor cells. Perhaps the best example is the use of enzyme-antibody conjugates, in which the enzyme can convert a prodrug to an active form. This approach has been reported by 2 laboratories (15, 16), with impressive therapeutic results in animal studies. In these experiments, it was necessary to allow normal blood clearance mechanisms to reduce the level of circulating antibody to a sufficiently low level before infusion of the prodrug. Clearly, the strategy described herein would result in more timely and efficient blood clearance. This approach also may be advantageous for therapy with radioisotope-antibody conjugates, in order to minimize toxicity from the circulating radionuclide. A high affinity antibody that is retained on cells for at least 24 h after binding to target cells is clearly a major advantage in applications of this type.

Our method of inhibiting ASGP receptor-mediated clearance is based on earlier experiments of Ashwell and Morell (13), who demonstrated that different ligands are cleared at different rates via the ASGP receptor. In particular, Fig. 5 of Ashwell and Morell (13) shows that, under conditions in which the ASGP receptor is saturated in vivo, some ligands are cleared more rapidly than others. Such variation seems likely to be due to varying avidity for the receptor. It is important for our purpose that, despite the fact that binding and uptake are so rapid, the receptor does exhibit some selectivity between ligands. The affinity of a panel of ligands for the rabbit ASGP receptor in vitro was determined by Sarkar et al. (14), who reported that the most strongly binding ligand tested was asialo-BSM. This result was unexpected at that time, considering that the terminal sugar in asialo-BSM is galactosamine rather than galactose, but was consistent with free sugar inhibition data. Therefore, our finding that asialo-BSM was the most effective inhibitor in vivo was predictable. It is probably essential that the inhibitor bind more strongly to the ASGP receptor than Gal-Ab in order to obtain effective inhibition. For this reason, it is important to use Gal-Ab conjugates that contain as little galactose as possible while still producing very rapid blood clearance. A more efficient way to block the function of the ASGP receptor may be to use an antibody reacting with it (17), and preparation of such an antibody to the mouse ASGP receptor is in progress. We have not investigated the effect of asialo-BSM injection on the clearance rate of unconjugated IgG, since there is little apparent advantage in lengthening the normally long half-life. However, we note that there is no evidence that normal clearance of IgG is via the ASGP receptor, and in fact the normal physiological role of the ASGP receptor is unknown.

Inhibition of the ASGP receptor of the liver by large amounts of protein could theoretically result in toxicity, although none was observed in this study. There are several potential mechanisms of toxicity: (a) assuming that the normal function of the ASGP receptor is to clear certain glycoproteins from the blood, which is not proven, inhibition of this function might result in toxicity. However, it seems likely that inhibition of such a function for 2–3 days would not have major consequences; (b) the large amounts of inhibitor required may have unanticipated toxic effects. However, we note that the inhibitor is of course present in the animal at high levels for only a few days. Although we have not directly investigated the processing of asialo-BSM in this study, the fact that 125I-labeled Gal-Abs are eventually cleared, with a delay dependent on the dose of asialo-BSM injected, strongly suggests that the inhibitor has been consumed. Also, we (1) and others (18, 19) have previously shown that glycoproteins bound by the ASGP receptor are rapidly catabolized, within hours. Also, many choices of inhibitor are available that seem unlikely to produce toxicity, including desialylated human α₁-acid glycoprotein, partially deglycosylated human mucin (leaving only a single sugar, galactosamine, linked to serine or threonine), galactose-conjugated human albumin, or synthetic polymers containing multiple terminal galactose or galactosamine residues, although some of these may not be as effective inhibitors as asialo-BSM.

The inhibition method described here is similar, in its potential applications, to the second antibody method of manipulating the clearance rate of a xenogeneic antibody. In this method, for example, a goat anti-mouse IgG is injected a few days after injection of a hamster with mouse antibodies (7), in order to induce rapid clearance. This method has been shown to improve radioimmunodetection results in some cases (7). However, the ASGP receptor inhibition method has several advantages over the second antibody method. First, the clearance of circulating antibody is much more orderly, involving uptake and degradation at only one site, the liver. In contrast, the second antibody method would be expected to result in immune complex formation not only in the blood, but also in interstitial fluid. While immune complexes formed in the blood are likely to be cleared rapidly by the spleen and liver, immune complexes in the interstitial fluid seem likely to be degraded much less efficiently and to persist in many tissues for prolonged periods. Also, the second antibody method may induce immune complex disease symptoms, particularly if high doses of the first antibody are used, as would be required to saturate antigenic sites on tumors (2).

The data presented here demonstrate that Gal-Abs retain effector functions as well as antigen-binding activity. Such results are consistent with those of Krantz et al. (20), who found that galactose-conjugated enzymes, which bind avidly to the ASGP receptor, retain their enzymatic activity. This is true despite the fact that high galactose conjugation ratios are required to achieve efficient binding to the ASGP receptor. Such excellent retention of functional activity can probably be explained by the fact that the charge on the lysine residues, to which galactose is conjugated, remains unchanged at physiological pH. The biological effector functions of antibodies are potentially important in the i.p. use of Gal-Abs. Thus, Gal-Abs may be able to lyse i.p. tumor cells by complement activation or ADCC, while antigen-positive cells outside the peritoneal cavity would not be attacked. In contrast, these effector functions are not relevant to the use of Gal-Abs i.v., since, if it is desired to utilize them, the optimal approach is to maintain high levels of circulating antibody for prolonged periods.

Considering the high tumor:non-tumor ratios obtained after i.p. injection of Gal-Ab (Fig. 3), radioimmunotherapy in this system seems feasible. An important consideration is that Gal-Ab bound by the liver is degraded rapidly and >90% of 125I bound to the liver is released within 1 h (1). This approach would not be appropriate for isotopes retained by the liver, such as most metals. Some of the effect of Gal-Ab injected i.p. may be non-antibody specific, since, with any galactose-125I-protein conjugate, the radiation would be virtually confined to the peritoneal cavity after i.p. injection, with any radioisotope entering the blood being rapidly catabolized by the liver, released, and excreted. However, the use of reactive antibodies would be expected to improve antibody retention in the peritoneal cavity (by binding to tumor cells) and to increase the contact of radioisotope with tumor. We have attempted ra-
dioimmunotherapy of i.p. HEP2 with 131I-galactose-MA103, using doses up to 1 mCi/mouse, which was the highest nonlethal dose that could be injected, but no significant protective effect was observed. In these experiments, the antibody was injected 10–17 days after tumor inoculation, at a time when little or no ascites was present. Possible approaches to improve the therapeutic effect include: (a) use of isotypes with a shorter half-life than the 8 days of 131I; (b) use of a tumor model in which a large ascites volume develops 1–2 weeks before death of the animal, so that antibody can be injected into mice bearing a large ascites; and (c) use of isotypes emitting higher energy β particles, to improve penetration of solid tumors.

Other laboratories have attempted to treat i.p. tumor with i.p. antibody conjugated to various toxic substances (21–25). A basic uncertainty in such experiments is whether antibody binds to tumor cells directly in the peritoneal cavity or whether antibody transported to the blood plays a critical role. Use of galactose conjugates can aid in dissecting the mechanisms involved, since Gal-Ab conjugates cannot move from the peritoneal cavity to the blood and then return to the peritoneal cavity. The experiments described here demonstrate that a substantial amount of antibody can bind to i.p. tumor masses after i.p. injection, before entering the blood. The advantage of i.p. over i.v. injection of unmodified antibody for RAID of i.p. tumors was investigated in several studies (21, 24, 25). While our experiments do not include enough time points to fully evaluate the difference between i.p. and i.v. injection, the data appear consistent with the idea that i.p. injection does provide an advantage at early time points. However, as seems clear that such results will strongly depend on the tumor burden, the presence of ascites, the amount and volume of antibody injected, and other factors. We note that, in general, the transport rate from peritoneal cavity to blood appears to be inversely related to the size of the animal (26–28); thus use of a small animal model such as a mouse may be a considerable disadvantage in such experiments.

We suggest that the major obstacle to effective therapy of i.p. tumors by i.p. injection of antibodies is the lack of penetration of solid tumor from the surrounding fluid. Our experiments (2) and those of others (29) indicate that antibody injected i.p. does not penetrate solid tumors significantly from the peritoneal cavity; the penetration that does occur is via the blood, since antibody injected i.p. passes quite rapidly from peritoneal cavity to blood. The reason for this poor penetration is probably the fact that the normal direction of flow is in the opposite direction, from blood to interstitial fluid to peritoneal cavity to diaphragmatic lymphatics to blood. In this regard, we note that there is not an absolute physical barrier to penetration of solid tumors in the peritoneal cavity. Even the mesothelium, which envelops all normal tissues within the peritoneal cavity, except the ovaries, and also envelops many solid tumors growing i.p., is permeable to serum proteins, being somewhat more permeable than capillary endothelial cells (30). Therefore, agents that increase permeability will probably not be useful to increase solid tumor penetration. What would be effective, but may not be possible, is to reverse the direction of fluid flow. In any case, antibodies injected i.p. should bind efficiently to floating ascites cells, and this may be useful in some situations.

In conclusion, galactose conjugation provides a method to manipulate the clearance rate of antibodies and other injected proteins with a precision that is not possible for most low molecular weight therapeutic drugs. This approach can produce a localized high concentration of the injected protein, or can generate a 2-phase blood clearance rate in which high blood concentrations can be maintained for a period of hours or days, followed by very rapid clearance. We have demonstrated that high tumor:non tumor ratios can be obtained by Gal-Ab in both i.p. and systemic model systems. This strategy may be advantageous in the clinical use of a variety of therapeutic proteins.

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