Enhanced Tumor Uptake of Macromolecules Induced by a Novel Vasoactive Interleukin 2 Immunoconjugate

Brian LeBerthon, Leslie A. Khawli, Mian Alauddin, Gordon K. Miller, Bishan S. Charak, Amitabha Mazumder, and Alan L. Epstein

Department of Pathology, University of Southern California School of Medicine, Los Angeles, California 90033

ABSTRACT

Low uptake of monoclonal antibodies (MAbs) in cancer lesions is a significant problem in cancer therapy. Recent studies have shown that antibody uptake in tumor is controlled in large part by the tumor blood flow and the vascular permeability of the tumor endothelium. We have hypothesized that these physiological properties of tumor vessels may be altered by pretreatment with vasoactive drugs or peptides linked to tumor-specific MAbs. To test this hypothesis, two MAbs, Lym-1 directed against human malignant lymphomas and B72.3 reactive with the TAG-72 antigen expressed in solid tumors, were chemically conjugated with human recombinant interleukin 2 (IL-2). IL-2 has been used in humans to activate lymphokine-activated killer cells for the treatment of cancer but is also known to produce a generalized vascular permeability by an unknown mechanism when used systemically. Chemical conjugation of IL-2 to MAbs appears to destroy its cytokine function as shown by T-cell proliferation studies in vitro. Despite this finding, MAb/IL-2 immunoconjugates retain their ability to produce an enhanced vascular permeability when injected i.v. into nude mice bearing relevant tumor models only. Biodistribution studies using 125I-labeled tracer Lym-1 have demonstrated that the Lym-1/IL-2 immunoconjugate can increase antibody uptake in tumor by a factor of 4 in a time (2.5-h pretreatment)- and dose (50 ìg/mouse)-dependent manner. In contrast, treatment of mice with free IL-2 and antibody showed this effect in all organs of the mouse including the tumor. Bidirectional crossover imaging studies in individual tumor-bearing nude mice showed improved uptake and decreased blood pool when the MAb/IL-2 immunoconjugates were used compared to controls. Finally, tumor blood flow and vascular permeability studies demonstrate that the physiological effect of the MAb/IL-2 is due to a reversible and specific vascular leakage at the tumor site. These studies indicate that pretreatment with this novel immunoconjugate may enhance the diagnostic and therapeutic potential of MAbs, drugs, and other macromolecules for the treatment of cancer.

INTRODUCTION

A major obstacle in the use of monoclonal antibodies for the treatment of cancer is the low percentage of injected dose of MAbs\(^1\) delivered to the tumor (1, 2). Dosimetric calculations obtained from clinical studies in humans have shown that only approximately 0.01–0.1% of the injected antibody dose actually binds and accumulates in tumor despite the use of high-avidity MAbs to tumor antigens (3–6). In an attempt to address this issue, we generated a novel immunoconjugate composed of a tumor-specific MAb (Lym-1 or B72.3) and IL-2 using chemical conjugation methods. IL-2 has been used clinically to generate LAK cells for the treatment of solid tumors (7–9) and is an important lymphokine of the T helper cell (10). When used systemically, however, IL-2 causes a capillary leak syndrome which is characterized by the extravasation of intravascular fluid in normal tissues (11, 12). While the exact cause of this syndrome is unknown, some studies indicate that IL-2 may have a direct effect on the morphology of capillary endothelial cells causing gaps between cells (13–15). Other studies have suggested that IL-2 may have an indirect action requiring the presence of activated LAK cells (16). In this study, MAbs directed against tumor antigens have been used to deliver IL-2 to the tumor site in order to produce a localized increased vascular permeability. Experimental studies using both biodistribution and imaging methods show that this novel immunoconjugate has both time- and dose-dependent effects and is active in two widely different tumor model systems.

MATERIALS AND METHODS

Reagents

Monoclonal antibody Lym-1 (IgG2a) was obtained from Techniclone International, Inc. (Tustin, CA). Monoclonal antibody B72.3 (IgG1) was a gift from Celltech, Ltd. (Slough, United Kingdom). Recombinant IL-2 was generously provided by Cetus Corporation (Emeryville, CA). All other chemicals such as chloramine-T, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate, N-hydroxysulfosuccinimide, stannous chloride, and glucoheptanoic 7-lactone were purchased from Sigma Chemical Co. (St. Louis, MO). 125I and 131I were obtained as sodium iodide in 0.05 n sodium hydroxide solutions (ICN Biomedicals, Irvine, CA). Sodium pertechnetate was obtained in saline solutions from Syncor (Van Nuys, CA).

Preparation of Antibody and IL-2 Immunoconjugate

IL-2 was radioiodinated by dissolving lyophilized recombinant IL-2 in water to a final concentration of 2 mg/ml. Fifty µl of IL-2 solution (100 µg), 100 µCi carrier free 125I, and 5 µl of chloramine-T (10 mg/ml) in water were added to 100 µl of 0.1 M sodium phosphate, pH 7.4, and the reaction was allowed to proceed for 1 min at room temperature. The reaction was quenched with 100 µl of anion exchange resin (AG 1-X8; Bio-Rad Chemical Division, Richmond, CA) in phosphate-buffered saline. After 1 min the suspension was withdrawn and filtered in a Spin-X centrifuge filtration unit (Costar, Cambridge, MA) to remove the resin. The radiolabeled IL-2 preparation was then diluted with phosphate-buffered saline to an appropriate volume for trace conjugation. The radiolabeled IL-2 was used in trace amounts in the reaction mixture to ascertain the binding of IL-2 to the antibody as noted below.

Coupling reactions were initiated by adding IL-2 to antibody (Lym-1 or B72.3), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate, and N-hydroxysulfosuccinimide in a 1:250:50 ratio by weight to give a total volume of 0.3 ml in phosphate buffer, pH 7.4. The reactions were incubated overnight at 4°C. After centrifugation, the soluble coupled antibody was chromatographed on a Sephadex G-100 column calibrated with blue dextran. The radioactivity and antibody peaks coeluted. From the antibody concentration and radioactivity, one or two molecules of IL-2 were calculated to be bound to each antibody molecule. Immunoconjugates retained a minimum of 75% of the antibody binding reactivity compared to the respective native antibody, as determined by an indirect live cell binding assay for Lym-1 (17) and an enzyme-linked immunosorbent assay using purified TAG-72 antigen for B72.3 (18).
To test the functionality of the IL-2 moiety, [3H]thymidine assays for the proliferation of IL-2-dependent cutaneous T-cell lymphoma cells (19) and LAK cell generation studies (20) were performed with the immunoconjugate in the concentration range of 800–1000 equivalent units of IL-2/ml. Both of these assays revealed that the MAb/IL-2 immunoconjugates did not have cytokine functionality when compared to free IL-2.

Radioiodination of Monoclonal Antibodies

All antibodies used in these studies were iodinated with [125I] and [131I] by modification of the chloramine-T method (21). Briefly, 400 μCi iodine and 10 μl of 43 mM aqueous solution of chloramine-T were added to a 5-ml test tube containing 100 μl of monoclonal antibody (10 mg/ml) in 100 μl PBS. The reaction was quenched after 3 min with 20 μl of 5% sodium metabisulfite. The radioiodinated antibody was purified by adding 100 μl of anion exchange resin (AG 1-X8; Bio-Rad) in 0.1 M PBS, pH 7.4, containing 1% bovine serum albumin. After 1 min the suspension was withdrawn and filtered in a Spin-X centrifuge filter unit (Costar) to remove the resin. The radiolabeled MAb was diluted with PBS for injection, stored at 4°C, and administered within 4 h after labeling.

Immunoreactivity of Radiolabeled Tracers

The in vitro immunoreactivity of the F(ab′)2 fragment of Lym-1 was tested by a live cell binding assay (17) using 106 Raji cells/tube. Briefly, Raji cells resuspended in 100 μl of 1% bovine serum albumin in PBS were pipetted into each test tube. One hundred μl of radiolabeled antibody were added to each triplicate set of test tubes (100,000 cpm/tube), and the tubes were incubated for 30 min at room temperature with continuous mixing using an orbital shaker apparatus (Belo Glass, Inc., Vineland, NJ) set at medium speed. Following incubation, the cells were washed 3 times with 1% bovine serum albumin in PBS by spinning the tubes at 1000 rpm for 5 min, decanting the supernatants, and resuspending the cells in 200 μl PBS. After completion of the washes, bound antibody was detected by measuring the radioactivity using a 1282 Compugamma counter (LKB Instruments, Pleasant Hill, CA). Using these methods, the radiolabeled antibody preparations were found to be more than 80% immunoreactive.

Tumor Models

Raji lymphomas were grown in the left thigh of 6-week-old female athymic nude mice by s.c. injection of 0.2 ml inoculum consisting of 4 × 107 Raji cells and 4 × 106 human fetal fibroblast feeder cells established in our laboratory. Three days prior to injection, the mice were treated with 400 rads using a cobalt irradiator to ensure a high take rate of implanted cells. The tumors grew to 1-2 cm in diameter in approximately 12-15 days.

Biodistribution Studies

Time and Dosage Dependence Studies. In the time dependence study, five mice/group were given i.v. injections of 30 μg of Lym-1/IL-2 immunoconjugate. At 48, 24, 5, and 2.5 h after injection, each group was given an injection of 20 μg of F(ab′)2 fragment of Lym-1 labeled with 50 μCi of [125I]. In the dosage dependence study, five mice/group were given injections of 0, 15, 22, 30, 45, 60, and 90 μg of Lym-1/IL-2 conjugate (a value of 0 μg of conjugate represents treatment with 30 μg of Lym-1 alone). At 2.5 h after injection, each group was injected with 20 μg of F(ab′)2 labeled with 50 μCi of [125I]. In all experiments, animals were sacrificed 72 h after biodistribution analyses as described previously (21). Briefly, groups of mice were sacrificed and the major organs were removed and weighed using an analytical balance. The samples were then counted in a gamma counter. For each mouse, data were expressed as percentage of dose/g of organ. From these data, the means ± SD were calculated for each group.

Comparative Pharmacokinetic Studies. Five mice/group were given i.v. injections of 30 μg of Lym-1/IL-2, 30 μg of Lym-1, or 30 μg Lym-1 in combination with 12 μg of free IL-2. At 2.5 h after injection, each group received 50 μCi of [125I]-labeled F(ab′)2 fragment of Lym-1 (20 μg) as tracer. Animals were sacrificed 72 h later for biodistribution analyses as described above. For each mouse, data were expressed as cpm/g of tumor:cpm/g of organ and percentage of dose/g of organ. From these data, the means ± SD were calculated for each group.

Vascular Volume and Permeability Studies

Vascular volume and vascular permeability were determined as previously described (22). Thirty μg of monoclonal antibody (Lym-1 or B72.3) and IL-2 immunoconjugates were injected into two groups of mice (n = 5). At 2.5 h after injection, each mouse was given an i.v. injection of 1.2 μg of stannous chloride and 1.2 μg of glucoheptanoic γ-lactone followed 30 min later with 50 μCi of 99mTcO4-. Within 30 min, more than 95% of 99mTc in the blood was bound to the RBC. Vascular permeability was determined using 4 μCi of [125I]-labeled mouse serum albumin which was injected i.v. immediately after the 99mTcO4- injection. At the time of sacrifice, the mice and organs were weighed, and the samples were then counted in a gamma counter to determine the 99mTc activity. 99mTc counts were adjusted for crossover from the 125I channel. Following sufficient time had passed for the total decay of 99mTc, the organs were recounted for their content of 125I. For each mouse, data were expressed as ml of blood/g of organ. From these data, the means ± SD were calculated for each group.

In order to demonstrate that the MAb/IL-2 immunoconjugate must bind to the tumor to produce its effect on the tumor vasculature, a control experiment was performed in which groups of tumor-bearing nude mice were pretreated with either relevant or irrelevant MAb or MAb/IL-2 immunoconjugates. In this crisscross experiment, 3 groups of mice bearing Raji tumors and 3 groups bearing LS174T tumors were pretreated with 30 μg of either (A) relevant MAb alone, (B) irrelevant MAb/IL-2 immunoconjugate, or (C) relevant MAb/IL-2 immunoconjugate (see Table 1) 2.5 h prior to the administration of 5 μCi of 125I-labeled mouse albumin as tracer. Twenty-four h later, all mice were sacrificed and dissected as above to quantitate the vascular permeability of the tumors. Activity was expressed as the percentage of injected dose/g of tumor.

Table 1 Percentage of injected dose/g of tumor in 6 groups of mice, pretreated with (A) relevant MAb (Lym-1 or B72.3), (B) irrelevant immunoconjugate, and (C) relevant immunoconjugate

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Lym-1</th>
<th>B72.3</th>
<th>B72.3/IL-2</th>
<th>Lym-1/IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Relevant MAb alone</td>
<td>0.31 ± 0.04</td>
<td>0.31 ± 0.04</td>
<td>0.40 ± 0.02</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>B. Irrelevant immunoconjugate</td>
<td>1.20 ± 0.12</td>
<td>1.20 ± 0.12</td>
<td>1.69 ± 0.07</td>
<td>1.69 ± 0.07</td>
</tr>
<tr>
<td>C. Relevant immunoconjugate</td>
<td>3.77 ± 0.15</td>
<td>3.77 ± 0.15</td>
<td>3.77 ± 0.15</td>
<td>3.77 ± 0.15</td>
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* Mean ± SD.
RESULTS AND DISCUSSION

Nude (athymic) mice heterotransplanted with Raji human B-cell lymphomas were given an injection of an IL-2 immunoconjugate of the anti-lymphoma mouse monoclonal antibody Lym-1 (17, 24, 25) (designated Lym-1/IL-2), Lym-1 alone, or Lym-1 plus free IL-2 in an amount equivalent to that in the immunoconjugate dose. Each treatment was applied to five mice, and each mouse was given a coinjection of the $^{125}$I-labeled F(ab')$_2$ fragment of Lym-1 as a tracer. The biodistribution of the labeled fragment was determined 48 h later and expressed as a percentage of the injected tracer dose/g of tissue (Fig. 1). With the injection of antibody alone, the tumor received on average 1.4% of the tracer dose/g. Treatment with the combination of free IL-2 and antibody or with Lym-1/IL-2 resulted in significantly higher tumor uptakes of 2.82 and 5.68% of tracer/g, respectively ($P < 0.025$ by Student's two-tailed t test. Figure 1 also details the biodistribution data for several selected organs. Following free IL-2 and antibody treatment, several key normal organs, such as lung, spleen, liver, and kidney, had significantly increased tracer uptakes, consistent with the generalized permeability induced by free IL-2 described in other studies (11, 12). Conversely, treatment with Lym-1/IL-2 did not increase tracer uptake in these tissues above that seen with treatment by antibody alone. By increasing uptake of tracer in tumor without increasing that of normal tissues, Lym-1/IL-2 caused an increase in the tumor/organ ratio of tracer localization. Fig. 2 details tumor uptake of tracer with respect to that of normal organs. For each organ surveyed, the average ratio of uptake in Lym-1/IL-2-treated animals was higher than that in mice treated with either antibody alone or with the antibody plus IL-2 combination.

Further characterization of this effect showed that it is both time and dose dependent. A standard dose of the immunoconjugate was administered at various times in advance of tracer to determine the time necessary to express the permeability effect fully. Fig. 3 shows the percentage of injected tracer/g of tumor resulting from varying the immunoconjugate injection time with respect to tracer administration. The greatest increase in tumor uptake of tracer was seen when the immunoconjugate was given 2.5 h before tracer, indicating that the biological response to the immunoconjugate is maximal after 2.5 h and diminishes thereafter. Various doses of the immunoconjugate were then administered 2.5 h before the tracer (Fig. 4). The tumor uptake showed a dosage dependency and was maximal at a dosage of 30 µg of immunoconjugate.

In order to demonstrate that these results indicated a quantitative increase in the tumor uptake of tracer and not merely an increase in tumor blood flow, dual tracer studies were performed to compare tumor vascular permeability to vascular volume using standard methods (22). For these studies, an IL-2 immunoconjugate was also prepared with B72.3, a murine MAb specific for a variety of mucin-producing human aden-
carnomas (18, 26), to demonstrate that the observed vascular changes were not unique to the Raji tumor model. Experimentally, four mice bearing LS174T human colon carcinoma (B72.3 target) and four mice bearing Raji tumors (Lym-1 target) were given injections of 30 μg of B72.3/IL-2 or Lym-1/IL-2, respectively, 2.5 h before the injection of 125I-labeled mouse albumin to quantitate vascular permeability. For the tumor blood flow studies, mouse RBC were labeled intravascularly with 99mTc also 2.5 h following the injection of the immunoconjugate. The results of these studies (Fig. 5) indicate that while tumor blood flow remained at control levels, tumor vascular permeability increased with immunoconjugate pretreatment in both tumor models. These results were obtained despite the fact that the LS174T model was found to have an intrinsically 10-fold higher level of tumor blood flow compared to that of the Raji model. The increase in tumor uptake of albumin confirms that the mechanism of immunoconjugate action is that of increased vascular permeability. Fig. 6 illustrates that the immunoconjugate-induced increase in permeability is localized to the tumor in both models and that normal organs do not show any significant change. Crosssection control experiments using Lym-1 and B72.3 immunoconjugates in relevant and irrelevant tumor models demonstrated that the immunoconjugate must bind to the tumor in order to produce the observed enhanced tumor vascular permeability (Table 1).

To demonstrate the effects of the immunoconjugate pretreatment on the radioimmunodetection of heterotransplants in tumor-bearing nude mice, a bidirectional crossover study was performed using individual mice imaged on two separate occasions with either antibody pretreatment (control) (Fig. 7, A and D) or immunoconjugate pretreatment (Fig. 7, B and C). These studies show that, regardless of the order of treatment, the clarity and intensity of the tumor images were enhanced by the immunoconjugate compared to antibody alone.

Previous in vitro studies by Biddle et al. (27) and in vivo studies by Gill et al. (28) using the Raji lymphoma model in nude mice have shown that when used in combination with IL-2, Lym-1 is more effective in generating an enhanced antibody-dependent cellular cytotoxicity and antitumor response. Similarly, Schultz et al. (29) using a murine lymphoma model report on the augmentation of MAH therapy by the concurrent administration of IL-2 or LAK cells. In our studies, the immunocon-
jugates generated by chemical coupling methods were shown to be devoid of IL-2 reactivity with respect to T-cell proliferation and LAK cell generation (see Fig. 1) yet were found to still produce vascular permeability changes in the tumor. It thus appears that LAK cell generation is not a prerequisite for the vascular permeability changes quantitated in these models. To test the effects of an immunoconjugate with functional IL-2 moieties, our laboratory is currently producing a genetically engineered immunoconjugate that would not require the use of chemical conjugation methods.

This is the first report of an immunoconjugate that specifically enhances tumor localization of blood-borne macromolecules. The mechanism of action for these immunoconjugates is that of increased tumor vascular permeability and not of increased tumor blood flow. Immunoconjugate pretreatment appears to increase the amount of radiolabeled antibody binding to tumor by 4-fold with no concomitant increase observed in other organs. These findings have important implications for the use of monoclonal antibodies in the radioimmunodetection and therapy of cancer. In addition, immunoconjugate pretreatment may potentially be used to improve the delivery of anti-neoplastic drugs or biologically important molecules to the tumor site. The demonstration of the success of this new approach opens the door to the development of other immunoconjugates composed of tumor-specific monoclonal antibodies and vasoactive, proinflammatory, or bioregulatory molecules linked by chemical or genetic engineering methods which can alter the microenvironment of tumors.

REFERENCES


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