Prolonged Binding of a Radiolabeled Monoclonal Antibody (B72.3) Used for the in Situ Radioimmunodetection of Human Colon Carcinoma Xenografts

David Colcher, Andrew M. Keenan, Steven M. Larson, and Jeffrey Schlom

ABSTRACT

Monoclonal antibody B72.3 binds to a glycoprotein complex with a molecular weight of 220,000 to 400,000. B72.3 reacts with approximately 50% of human mammary carcinomas and to 80% of the colon carcinomas tested but does not react appreciably with normal mammary tissue, with normal colon, or to a variety of normal adult human tissues tested using immunohistochemical techniques. B72.3 immunoglobulin G was purified and radiolabeled with \(^{125}\)I without significant loss in its reactivity to tumor extracts. The radiolabeled B72.3 immunoglobulin G was shown to efficiently localize human colon carcinoma xenografts in athymic mice. Tumor:tissue ratios of the localized antibody rose over the 7-day period studied, with tumor: liver, tumor: spleen, or tumor: kidney ratios of approximately 18:1 at Day 7 and a tumor: blood ratio of approximately 5:1 at Day 7. Tumor: muscle or tumor: brain ratios rose to over 100:1. The amount of radioactivity in the tumor increased for the first 2 days post inoculation of antibody and stayed constant over a 19-day period of study. Thus, there was no appreciable loss of radiiodine from the tumor over the study interval. No localization was seen in mice bearing a B72.3 antigen-negative human melanoma xenograft or with an isotype-identical control immunoglobulin G in mice bearing colon tumor xenografts. Gamma camera imaging with a pinhole collimator confirmed the ability of the radiolabeled antibody to detect the presence of colon carcinoma xenografts less than 0.5 cm in diameter over a 19-day period. The potential use of this system as a model for radioimmunotherapy will be discussed.

INTRODUCTION

Radiolabeled monoclonal antibodies that are reactive with the surface of human carcinoma cells may prove useful in several areas in the diagnosis and management of human cancer. In the case of mammary cancer, for example, only axillary lymph nodes removed at mastectomy are examined for tumor involvement for use in staging; the extent of nodal involvement in the internal mammary chain is not determined. The use of radiolabeled monoclonal antibodies in lymphoscintigraphy of the internal mammary chain as well as the axillary nodes may thus eventually increase the reliability of staging of nodal involvement as a prognostic indicator. The detection of occult metastatic lesions at distal sites via gamma scanning for many types of cancer could serve as an adjunct in determining which patients should receive adjuvant therapy, while subsequent scanning could reveal which tumors are responding to therapy.

We have developed a number of monoclonal antibodies that show a high degree of specificity to mammary and colon carcinomas (4). One of these monoclonal antibodies, B72.3, exhibits a high degree of specificity. B72.3 binds to approximately 50% of the human mammary carcinomas and to 80% of the human colon carcinomas tested using immunoperoxidase techniques (33, 39). Little or no reactivity was observed using a variety of apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes of the breast using immunohistochemical methods (33). A number of human cell lines were tested for surface binding to B72.3 using live cell RIAs;\(^{2}\) no reactivity was detected to the surface of sarcomas or melanomas or to any of the apparently normal human cell lines tested, but B72.3 did bind to the surface of the colon carcinoma cell line, LS-174T. The reactive antigen has been shown to be a M, 220,000 to 400,000 glycoprotein complex (5). As a prerequisite for clinical trials to detect human carcinomas in vivo, monoclonal antibody B72.3 was radiolabeled with \(^{125}\)I, and its suitability was determined for the radioimmunodetection of human colon carcinomas grown in athymic mice.

MATERIALS AND METHODS

Hybridoma Methodology. BALB/c mice were immunized with a membrane-encrusted fraction of a human breast tumor metastasis to the liver. The generation and the reactivities of the resulting monoclonal antibodies, including IgG1 B72.3, have been described in detail elsewhere (4, 33, 39).

Solid-Phase RIAs. Immunoreactivity of monoclonal antibody B72.3 was determined by solid-phase RIA using cell extracts (6). Five \(\mu\)g (in 50 \(\mu\)l) of the cell extracts were added to each well of 96-well microtiter polystyrene plates and allowed to dry. To minimize nonspecific protein adsorption, microtiter wells were treated with 100 \(\mu\)l of 5% BSA in PBS and incubated for 1 hr at 37°. The BSA was removed, and varying amounts of radiolabeled antibody (in 25 \(\mu\)l) were added. After a 1-hr incubation at 37° or overnight at 4°, the unbound immunoglobulin was removed by washing the plates with 1% BSA in PBS. The bound cpm were detected by cutting the individual wells from the plate and measuring the radioactivity in a gamma counter.

Purification of IgG. B72.3 IgG was purified from ascitic fluid after clarification by centrifugation at 10,000 \(\times\) g for 10 min. The supernatant was adjusted to 0.1 M Tris-HCl (pH 7.5), and the immunoglobulin was precipitated by the addition of an equal volume of saturated ammonium sulfate, pelleted by centrifugation at 10,000 \(\times\) g for 10 min, dissolved in 5 ml of 10 mM sodium phosphate buffer (pH 7.5), dialyzed against the

\(^{2}\) The abbreviations used are: RIA, radioimmunoassay; PBS, phosphate-buffered saline (Na\(_2\)HPO\(_4\), 1.2 g/liter; KH\(_2\)PO\(_4\), 0.2 g/liter; KCl, 0.2 g/liter; NaCl, 8.0 g/liter; MgCl\(_2\), 6 H\(_2\)O, 0.1 g/liter; CaCl\(_2\), 0.1 g/liter); BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.
same buffer, and applied to an ion-exchange column (DE52, 15 ml). The column was washed with 10 mM sodium phosphate buffer (pH 7.5) and the antibody was eluted with a salt gradient (300 ml) of 10 to 100 mM sodium phosphate, pH 7.5. Solid-phase RIAs using an extract of a human mammary tumor metastasis as antigen were used to localize the antibody. The appropriate fractions were pooled and dialyzed against PBS. The protein concentration was determined by the method of Lowry et al. (27).

Control immunoglobulins were used to demonstrate the specificity of the B72.3 antibody binding. These include: (a) MOPC-21, a myeloma Ig31 which was purchased from Litton Bionetics, Inc. (Rockville, MD); and (b) normal murine immunoglobulin which was purified from sera of BALB/c mice. Methods for the purification of these immunoglobulins were as described for B72.3.

**Labeling of Antibody.** The monoclonal antibody B72.3 was labeled with Na\(^{125}\)I using iodogen. Forty \(\mu\)g of the antibody were adjusted to 0.1 mM sodium phosphate buffer (pH 7.2) and added to a 12- \(\times\) 75-mm glass tube coated with 20 \(\mu\)g of iodogen (Pierce Chemical, Rockford, IL) followed by 0.5 mCi of Na\(^{125}\)I (New England Nuclear, Boston, MA). After a 2-min incubation at room temperature, the protein was removed from the insoluble iodogen, and the unincorporated \(^{125}\)I was separated from the antibody by gel filtration through Sephadex G-25 (10-ml column). The labeled antibody in the void was pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.2) containing 5 mM NaI. The iodination protocol yielded labeled IgG with a specific activity of 5 to 15 \(\mu\)Ci/\(\mu\)g (approximately 8 to 25 \(\times\) \(10^6\) cpm/\(\mu\)g), with up to 80% of the input \(^{125}\)I bound to the protein. MOPC-21 was radiolabeled using the same methodology. The labeled antibodies were analyzed on discontinuous SDS-polyacrylamide gels (25, 40).

**Cell Lines.** The LS-174T cell line (41) was obtained from the American Type Culture Collection and was grown in Eagle's minimum essential medium with nonessential amino acids supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). The A375 human melanoma cell line was obtained from Dr. S. Aaronson (National Cancer Institute). The A375 cell lines were grown in Dulbecco's modified Eagle's medium with the same supplements as the LS-174T cell line. The cells were removed from culture flasks with 0.1% trypsin containing 0.5 mM EDTA and washed twice in growth medium without serum before inoculation. All cell lines were tested for the presence of Mycoplasma species and were negative.

**Results**

**Purification and Iodination of B72.3 IgG.** Monoclonal antibody B72.3 IgG was purified as described in "Materials and Methods" and analyzed on SDS-polyacrylamide gels (Fig. 1A). Scans of the gels demonstrated that the IgG peaks accounted for greater than 95% of the stained protein on the gel. The purified IgG exhibited a limited distribution when analyzed by isoelectric focusing with major bands at isoelectric points of approximately 6.13 and 6.32 (Fig. 1B). The IgG was radiolabeled with Na\(^{125}\)I using a variety of techniques including chloramine-T, lactoperoxidase, Bolton-Hunter, and iodogen (7). The IgG was efficiently radiolabeled using all of the methods, but the immunoreactivity of the antibody was greatly reduced in all cases. After adjusting the 3 major parameters of the iodogen method, i.e., ratios of immunoglobulin protein, iodine, and iodogen, a protocol was obtained (40 \(\mu\)g of IgG, 0.5 mCi of Na\(^{125}\)I, 20 \(\mu\)g of iodogen (see "Materials and Methods")) that yielded a labeled antibody that would bind over 80% of its radioactivity to tumor extracts as measured in sequential solid-phase RIAs. The iodinated antibodies were then analyzed for purity by SDS-polyacrylamide gel electrophoresis, with and without disruption by \(\beta\)-mercaptoethanol, to determine the size of the IgG after labeling and to examine the distribution of the \(^{125}\)I in the heavy and light chains (Fig. 1C). After labeling, the IgG maintains its molecular weight of approximately 150,000. Upon disruption with \(\beta\)-mercaptoethanol, the \(^{125}\)I label migrates at \(M\), 50,000 and 25,000, consistent with the size of the heavy and light chains of IgG. The iodine was distributed between the heavy and light chains as expected, based on their molecular weights.

**Immunoreactivity of the Radiolabeled B72.3.** The radiolabeled B72.3 was used in a solid-phase RIA to determine its immunoreactivity after iodination. Extracts from a variety of tumors and apparently normal liver were dried at varying concentrations onto a solid-phase RIA plate, and iodinated B72.3 was added and allowed to bind to the extracts at 4\(\circ\) overnight. The iodinated monoclonal antibody bound well to the breast tumor metastasis that was used as the immunogen (Chart 1) while showing no reactivity to another breast tumor metastasis (Chart 1), which was shown previously (4) to lack the M, 220,000 to 400,000 glycoprotein that is bound by B72.3. No binding of B72.3 was observed to apparently normal liver or a human lymphoid cell line (Chart 1). The radiolabeled antibody was then tested for reactivity to a number of human tumor lines in order to develop a model for radioimmunodetection studies with the B72.3 antibody. As can be seen in Chart 1, 125I-B72.3 exhibits substantial reactivity with a colon carcinoma tumor (LS-174T) grown in athymic mice. No binding was observed to extracts of the A375 melanoma xenograft (from athymic mice) which was shown previously (4) to lack the antigen bound by B72.3 (Chart 1). Extracts of the LS-174T cell line exhibit low levels of binding with the 125I-B72.3 IgG, while extracts of LS-174T tumors show considerable binding of the labeled antibody. Tumors derived from the cell line were passaged in athymic mice by implantation of 0.1-cm pieces s.c. The tumors grew rapidly, and the levels of the antigen detected by MAbs B72.3 in the early passages of the LS-174T tumors were comparable to those found in the tumors derived directly from the cell line. Slight decreases in antigen content were noted after 2 passages, but after 13 passages the level of the antigen bound by B72.3 was signifi-
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cantly reduced as compared to that of the tumor derived directly from the cells. Therefore, all subsequent experiments were performed using tumors derived from the inoculation of LS-174T cells from culture.

Tumor Distribution Studies. Radiolocalization studies were performed using athymic mice bearing human colon carcinomas (LS-174T) in comparison with a human melanoma xenograft (A375) as an antigen-negative control for nonspecific uptake of immunoglobulin. Athymic mice were given s.c. injections of 4 x 10^6 cells. Tumor growth was rapid with a doubling time of approximately 2 to 3 days for the LS-174T cells. After 7 to 10 days when the tumors were approximately 0.3 to 0.5 cm in diameter, the mice were given i.v. injections of approximately 1.5 µCi of ^125I-B72.3 IgG or ^125I-MOPC-21 IgG (control antibody of the same isotype). The ratio of cpm radioactivity:mg of tissue in the LS-174T tumor in comparison with that of various tissues was examined over a 7-day period. The tumor:tissue ratio (Chart 2) rose over this period with tumor: liver, tumor: spleen, or tumor: kidney ratios of approximately 18:1 at Day 7. Tumor: blood ratios also rose during this time, resulting in ratios of 5:1 at Day 7. There was no specific uptake of ^125I-B72.3 IgG in any of the normal organs examined including brain, muscle, stomach, intestines, uterus, or ovary in addition to the organs specified in Table 1. Approximately 10% of the injected dose per g reached the tumor at Day 2 postinoculation of the radiolabeled antibody (Table 1). The amount of radiolabel at the tumor stayed constant over the first 4 days; the activity on a per g basis then began to drop as the tumor progressed in size. The increased tumor: tissue ratios result primarily from the clearance of labeled IgG from the blood pools. The absolute amount of radioactivity in the tumor rose over the first 2 days and then remained constant through Day 7. Athymic mice bearing melanomas (A375, a tumor line that shows no reactivity with B72.3 in live cell RIAs) were used as controls; no specific uptake of ^125I-B72.3 was observed in the tumors of these control animals (Chart 2). Similarly, no localization was observed in athymic mice bearing the colon carcinoma cell line when using ^125I-MOPC-21 IgG as a control antibody (Chart 2).

Imaging of Athymic Mice Bearing Human Tumors. Studies were then undertaken to determine whether localization of the ^125I-labeled B72.3 was sufficient to detect by gamma camera scanning. Athymic mice bearing colon carcinomas or melanomas were given injections of approximately 70 µCi (approximately 5 µg) of ^125I-B72.3 IgG; the higher dose was necessary to minimize imaging time. The mice bearing the human colon carcinomas demonstrated significant tumor uptake at early time points with most of the activity in the area of the tumor (Fig. 2A). The remaining activity in the mice was detected primarily in the area of the heart and lungs. No significant activity was seen in the liver, kidneys, bladder, or stomach. The lack of activity in these organs indicates that there is no significant breakdown of the
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Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Time (hr)</th>
<th>% of injected dose/g</th>
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<td>Colon carcinoma</td>
<td>18</td>
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<tr>
<td></td>
<td>48</td>
<td>10.75</td>
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<td></td>
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<td></td>
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<td></td>
<td>166</td>
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<td></td>
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Table 2

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<th>cpm in total body</th>
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DISCUSSION

Radioiodinated antibodies to a variety of tumor-associated antigens have been used to detect tumors in both experimental animals and humans by gamma scintigraphy. The majority of the antibodies used for clinical trials were constituents of goat or rabbit antisera and were directed against antigens such as carcinoembryonic antigen (8, 11, 14, 28), alpha fetoprotein (12, 15, 21), ferritin (34), and human chorionic gonadotropin (13, 16). In some studies, the immunoglobulins have been partially purified using affinity chromatography with an increase in immunoreactivity of the IgG (35). With the development of hybridoma technology, homogeneous populations of monoclonal antibodies (22, 23) which bind tumor-associated antigens can now be utilized in radioimmunodetection studies. There are many groups who have recently used radioiodinated monoclonal antibodies generated against a variety of immunogens to detect human tumor xenografts (1-3, 9, 18, 19, 32, 37, 42, 43). Many of these studies used antibodies reactive against colon carcinomas, melanomas, milk fat globules, and carcinoembryonic antigen. Some of these monoclonal antibodies exhibit a broad range of reactivity to normal tissues but still show some utility as imaging agents in the clinical setting (10). Other antibodies with a more restricted range of reactivity have been used successfully to localize melanomas (26) and colon carcinomas (29, 30). The majority of the clinical localization studies were performed using antibodies radiolabeled with 131I, while some work is being done with 123I (10), 111In (36), and 99mTc (31).

The utility of this technique will be enhanced using monoclonal antibodies with a high degree of selective reactivity for tumor cells. We chose to examine the use of monoclonal antibodies for localization studies with one such antibody developed in our laboratory, B72.3. Monoclonal antibody B72.3 has been shown to have an extremely high degree of selective reactivity for human breast and colon carcinomas versus any normal human tissue tested (4, 33, 39). This makes it especially desirable for subsequent clinical trials. The studies presented here demonstrate that monoclonal B72.3 can be radioiodinated with significant loss of immunoreactivity. The antigen that B72.3 recognizes appears unusual in that it is expressed at higher levels in vivo as compared to cell lines propagated in tissue culture. When the iodinated B72.3 was tested for binding to extracts of the LS-174T cell line, lower levels of reactivity were observed as compared to those observed with extracts from xenografts. The nature of this
change in the expression of this tumor-associated antigen is under investigation.  

The studies reported here demonstrate that radiolabeled B72.3 can be used for the radiolocalization of human colon carcinoma xenografts in athymic mice. The specificity of this localization has been shown both by the detection of the colon carcinomas by radioiodinated B72.3 without any significant accumulation in the antigen-negative melanoma and by the inability of the control antibody, of the same isotype, to localize in colon carcinoma xenografts. The specificity of B72.3 for the tumor was also shown by the increasing tumor:tissue ratios obtained. Tumor:blood ratios rose to approximately 5:1 at 7 days postinjection of the antibody, while tumor:lung, tumor:spleen, and tumor:kidney ratios rose to 18:1 over the same period of time. Tumor: muscle or tumor:brain ratios also rose to over 100:1. The increase in these ratios was primarily due to the clearance of the radiolabeled antibody from the blood. The activity in the vital organs decreased rapidly with a t1/2 of approximately 7.5 days, resulting in 40% of the remaining activity in the tumor at 19 days postinjection of the radioiodinated antibody (Table 2). There was no significant accumulation of the radiolabel in the liver, spleen, kidneys, bladder, or stomach at any time in the athymic mice bearing either the LS-174T colon carcinoma or the A375 melanoma.

The long retention of the radiolabeled B72.3 in the LS-174T tumor is highly desirable for clinical trials. In previous reports using monoclonal antibodies to other tumor-associated antigens, the interval from the time of the monoclonal antibody injection to optimal tumor localization was 3 to 7 days (1–3, 9, 18, 19, 32, 37, 42, 43). The studies reported here demonstrate (Fig. 3; Table 2) that specific B72.3 localization (i.e., percentage in tumor) is increasing at Day 7 and indeed continues to rise through Day 19. This is important because the increasing tumor:background ratios at late (post-Day 7) time intervals make radioiodinated B72.3 an excellent candidate for therapeutic studies, since the time when the monoclonal antibody is bound to the tumor becomes an important factor in determining the overall radiation dose received by individual tumor cells.

The results reported here are similar to those obtained with another monoclonal antibody developed in this laboratory, B6.2 (6), in that there was no evidence of significant uptake of radiolabel in any of the vital organs; this indicates that there was no significant breakdown of radiolabeled antibody or deiodination of antibody. In fact, the absolute amount of the radioiodinated B72.3 that was detectable in the colon tumors increased over the first 2 days postinoculation and stayed essentially the same over the 19-day period studied (Table 2). The stability of the antibody is further demonstrated by the lack of redistribution to other areas of the progressing tumor. These results are in contrast to previously published articles in which a significant deiodination of antibody was reported. It has been stated that labeling with other isotopes such as 111In using metal chelates is better (17, 38). The stability of the radiolabeled antibody used in the studies reported here and in another study from our laboratory (6) may in part be due to the systematic development of labeling conditions that maintained the immunoreactivity of the radiolabeled antibody. The conditions needed for labeling different monoclonal antibodies vary dramatically between the different monoclonal antibodies, and one therefore must optimize the labeling method and conditions for each antibody. When 125I-B6.2 was used to localize the LS-174T tumors, similar tumor:tissue ratios were obtained; the absolute amount of the injected dose to reach the tumor, however, was less than that obtained with B72.3. There was also a decrease in the amount of B6.2 at the tumor with time. This demonstrates that the ability of the radiolabeled B72.3 to stay at the LS-174T tumor is a function of both the tumor and the B72.3 antibody. The ability of B72.3 to detect small tumors less than 0.5 cm in diameter and the ability of the radiolabeled B72.3 to stay in the tumor must be examined in other systems and may not be representative of what will happen in clinical trials with this antibody.

The use of monoclonal antibodies coupled with isotopes decaying via high-energy transfer with short-range radiation could also prove useful as radiotherapeutic agents. A number of isotopes can be used to radiolabel antibodies that can kill several cell diameters (44); therefore, only one cell in a cluster of tumor cells need express the target antigen. This approach could be quite useful in light of the heterogeneity of the antigen expression in some tumors (4, 20, 33, 39). Furthermore, a monoclonal antibody coupled to the appropriate isotope need not be internalized by the tumor cell for its therapeutic potential to be realized. Indeed, it has been shown that some of the carcinoma-associated antigens detected by monoclonal antibodies are stable components of the cell membrane; thus, antigen: antibody complexes formed at the surface of the cell were shown not to internalize (24). The amount of the initial activity that reaches the tumor and the fact that this activity is stable at the tumor indicates the potential of this antibody as a therapeutic agent in the treatment of advanced disease or as adjuvant therapy after surgical resection of the primary tumor. Unlike chemotherapeutic agents in which the amount that reaches the tumor is of primary importance, the use of radiolabeled monoclonal antibodies involves several equally important parameters: (a) amount bound to the tumor cells; (b) time of binding of the monoclonal antibody to the tumor cell surface (i.e., the longer the binding of the radiolabeled antibody to the cell surface, the higher the radiation dose delivered); and (c) the half-life and energy of the isotope. Because of the high uptake of B72.3 in tumors and its prolonged binding to the tumor, studies on the therapeutic efficacy of this antibody in model systems are in progress.

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REFERENCES

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Fig. 1. Gels of B72.3 IgG. Monoclonal antibody B72.3 IgG was purified and tested by SDS-polyacrylamide gel electrophoresis for purity. The unlabeled antibody was run on a 5 to 20% gradient gel (A). Lane 1, markers, conalbumin, M, 84,000; BSA, M, 68,000; ovalbumin, M, 47,000; lactate dehydrogenase, M, 36,000; carbonic anhydrase, M, 30,000; myoglobin, M, 17,000; and cytochrome c, M, 11,700. Lane 2, disrupted with β-mercaptoethanol. Lane 3, without disruption. The unlabeled antibody was analyzed by isoelectric focusing (B). Lane 1, markers (pH is identified), Lane 2, B72.3 IgG. The radiolabeled antibody was also analyzed by SDS-polyacrylamide gel electrophoresis on a separate gel and analyzed by autoradiography (C). Lane 1, disrupted with β-mercaptoethanol. Lane 2, without disruption.

Fig. 2. Gamma camera scanning of athymic mice bearing human tumors. Athymic mice bearing LS-174T colon carcinomas (A to C) or A375 melanoma (D to F) were inoculated with approximately 70 μCi of 125I-B72.3 IgG. The mice were scanned after 1 (A, D), 2 (B, E) and 3 (C, F) days until 100,000 cpm were detected in the field. The images are displayed without blood pool corrections or contrast enhancement, and all are scaled to a common maximum value. T, tumor.
Fig. 3. Gamma camera scanning of athymic mice bearing human colon carcinomas. Athymic mice bearing LS-174T colon carcinomas were inoculated with approximately 70 µCi of noble-β2 IgG. The mice were scanned after 7 (A), 11 (B), 14 (C), and 19 (D) days until 50,000 cpm were detected in the field. The images are displayed without blood pool corrections or contrast enhancement, and all are scaled to a common maximum value.
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