Optimization of Monoclonal Antibody Delivery via the Lymphatics: The Dose Dependence

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ABSTRACT

After interstitial injection in mice, antibody molecules enter local lymphatic vessels, flow with the lymph to regional lymph nodes, and bind to target antigens there. Compared with i.v. administration, delivery via the lymphatics provides a more efficient means for localizing antibody in lymph nodes. An IgG2a (36-7-5) directed against the murine class I major histocompatibility antigen H-2Kk has proved useful for studying the pharmacology of lymphatic delivery. The antibody specifically binds to most cells in Kk-positive strains of mice and to none in Kk-negative mice. At very low doses, most of the antibody remains at the injection site in Kk-positive animals. As the dose is progressively increased, most effective labeling occurs first in nodes proximal to the injection site and then in the next group of nodes along the lymphatic chain. At higher doses, antibody overflows the lymphatic system and enters the bloodstream via the thoracic duct and other lymphatic-venous connections. Once in the blood, antibody is rapidly cleared, apparently by binding to Kk-bearing cells. These findings indicate that the single-pass distribution of monoclonal antibodies in the lymphatics can be strongly dose dependent, a principle which may be of clinical significance in the improvement of immunolymphoscintigraphic imaging, especially with antibodies directed against normal and malignant lymphoid cells. Monoclonal antibodies directed against normal cell types in the lymph node may be useful for assessing the integrity of lymphatic chains by immunolymphoscintigraphy or, more speculatively, for altering the status of regional immune function. The results presented here indicate that a low or intermediate antibody dose may optimize the signal:noise ratio for imaging.

In Kk-negative animals, the percentage of dose taken up in the major organs was essentially independent of the dose administered; there was no evidence for saturable sites of nonspecific binding. These findings provide background for attempts to use antitumor antibodies via the lymphatic route. Specific binding to target cells (and any cross-reaction with normal tissues) would presumably be superimposed on the nonspecific pharmacology of the antibody in vivo.

INTRODUCTION

Monoclonal antibodies may have broad clinical applications when targeted to normal or pathological sites. Thus far, most in vivo studies have been directed toward i.v. administration of these agents, on the assumption that access to the target sites could best be achieved via the bloodstream. When targeting to lymph nodes, however, we have found s.c. delivery to offer several advantages over i.v. injection, including higher efficiency, lower dose, and faster localization (1, 2). Among the potential clinical uses of monoclonal antibodies, selective delivery in the lymphatics may be particularly effective for imaging ("immunolymphoscintigraphy"). The lymphatics provide a more direct route to lymph nodes and also limit exposure of the antibody to circulating antigen in blood or cross-reactive antigen on normal tissues. Clinical studies of this technique have been undertaken using both polyclonal (3, 4) and monoclonal (5) preparations. The experimental background and possible clinical applications have been reviewed elsewhere (6, 7).

In studying lymphatic delivery, we initially used antibodies directed against determinants on normal mouse cells in order to avoid the complexities and biological heterogeneity encountered in metastatic tumor systems. Presented here are studies using 36-7-5, a murine monoclonal IgG2a,x with private specificity for the mouse class I major histocompatibility antigen H-2Kk (8). This antigen is expressed to varying degrees on most cells of mouse strains genetically coding for it and is expressed on over 90% of the lymphocytes in such strains. Initial studies (1) indicated efficient labeling of lymph node cells in vivo. Here we examine the dose dependence of antibody biodistribution after s.c. injection for uptake by the lymphatics. In beginning these studies, we expected to see one of two patterns of distribution (or perhaps a mixture of the two): (a) lymph flow, stimulated by injection of fluid, carries IgG through the lymph node sinoids so rapidly that there is insufficient residence time in the node to permit binding of a large fraction of the antibody. In that case the fraction of dose taken up would be independent of the dose reaching the nodes; (b) residence time in each node is sufficiently long to permit binding of essentially all bindable antibody. In that case, a marked dose dependence of uptake would be seen. The results presented here indicate behavior largely, but not exclusively, of the second type. Containment of the antibody within the lymphatics and its optimal delivery to lymphoid targets are both dose dependent. In contrast, the distribution in Kk-negative animals proved to be independent of dose. These findings bear on the design of techniques for lymphoscintigraphic imaging of normal lymph node cell types (as a possible alternative to colloid lymphoscintigraphy). They also delineate pharmacologic principles important for lymphatic delivery to lymphoma cells in lymph nodes.

MATERIALS AND METHODS

Animals. B10.A and B10.BR mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and C57BL/10 ScN mice were obtained from an established animal colony at the Veterinary Resources Branch, Division of Research Services, NIH, Bethesda, MD. C57BL/10 mice differ from the other two at the K locus within the H-2 complex: B10.A and B10.BR strains are H2-A1, whereas C57BL/10 mice are H2-A1. Antibody. Murine monoclonal antibody 36-7-5 was purified from ascites fluid of BALB/c athymic mice (Small Animal Section, NIH, Bethesda, MD) by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) (9). Purified 36-7-5 was radiolabeled by the chloramine-T procedure using Na125I (New England Nuclear, Boston, MA). The reaction was quenched with N-acetylyserysine, and the iodinated protein was separated from unreacted iodine on a PD-10 Sephadex G-25 column (Pharmacia). For the studies in Figs. 2 and 3, the preparation had a specific activity of 8.3 × 10⁴ dpm/mmol. The equilibrium binding properties of a similar antibody preparation (specific activity, 7.6 × 10⁴ dpm/mmol) were assessed using the method of Segal and Hurwitz (10). Briefly, various concentrations of ¹³¹I-labeled antibody were incubated for 2 h with ¹³¹I-labeled spleen cells from Kk-
positive (B10.BR) or Kk-negative (C57BL/10) mice; at each concentration, cells and bound antibody were separated from unbound antibody by sedimenting triplicate 100-µl samples of incubation mixture through phthalate oils in 400-µl centrifuge tubes (Bio-Rad Laboratories, Richmond, CA); a 50-µl sample of the supernatant was used to assess the free antibody concentration in the fluid phase, and the tips of the tubes were clipped off for determination of the amount of antibody bound to sedimented cells. For these studies, sodium azide was not included in the binding medium.

Identity of Radioactivity in Lymph Nodes. To determine what fraction of the observed radioactivity in lymph nodes represented intact antibody, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was performed. Twenty mice were used and all were anesthetized with ether and then given injections. Fifty µl were administered s.c. in each hind footpad. Kk-negative (C57BL/10) mice were dissected from 18 Kk-positive (B10.A) mice. The nodes were collectively homogenized with a Teflon pestle tissue grinder and solubilized in buffer (2.3% sodium dodecyl sulfate in 0.0625 M Tris at pH 6.8). Slab gels containing polyacrylamide at concentrations of 9, 12, and 15% were used to run samples of solubilized node tissue, 125I-labeled monoclonal 36-7-5 antibody, and molecular weight standards (Bethesda Research Laboratories, Rockville, MD). The respective gel lanes were cut into 2-mm slices, and the radioactivity in each slice was determined using a gamma counter.

In Vivo Dose Dependence Studies. Various doses of 125I-labeled antibody were prepared in phosphate buffered saline containing crystallized bovine serum albumin (1.0 mg/ml; Miles Laboratories, Inc., Elkhart, IN). Groups of three Kk-positive (B10.A) mice were briefly anesthetized with ether and then given injections. Fifty µl were administered s.c. in each hind footpad. Kk-negative (C57BL/10) mice were inoculated similarly, but groups of four mice were used and the injection volumes were 30 µl. Other studies (results not shown) indicate that injection volumes between 20 and 50 µl give similar results. The animals were sacrificed by ether overdose 2 h after injection, their organs were dissected, and the radioactivity in each specimen was then determined using a gamma counter. After injection, a cotton swab was applied to the footpad to catch any small amounts of antibody leaking during injection. The swabs were routinely counted, and the results used to arrive at a value for the actual dose. Separate experiments showed that there was little leakage from the footpad at later times. Because the data suggested a proportional error model, the results are expressed as geometric means with standard error of the geometric mean. The contribution from blood in the organs has not been subtracted. Preliminary studies (1) showed that almost all of the radioactivity found in the hind feet had simply remained there after injection; only a minuscule amount had arrived via the bloodstream. Similarly, almost all of the radioactivity in the popliteal nodes had come directly from the ipsilateral foot via the lymphatics, rather than by way of the bloodstream. Therefore, the dose to one foot (rather than both) was used as the denominator when calculating percentage of dose in each foot and each popliteal node.

RESULTS

Characterization of 125I-labeled Antibody in Lymph Nodes. Samples of 125I-labeled monoclonal 36-7-5 antibody gave a single stained band in the M, 150,000-200,000 range in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reflecting the purity of the injection material. Solubilized lymph nodes from animals sacrificed 2 h after injection also showed a band at M, 150,000-200,000 representing 73 to 89% of recovered 125I counts. The node samples also gave detectable 125I counts at M, 8,000 to 12,000 and at the ion front. These studies are described in more detail elsewhere. 2

Binding of 36-7-5 Antibody. Fig. 1 shows the results for binding assays at 4°C and 37°C. Nonspecific binding to Kk-negative (C57BL/10) cells was minimal. The affinity constant and saturation value for B10.BR cells were 5 × 10⁹ M⁻¹ and 2.9 × 10⁴ molecules/cell, respectively, at 4°C (as estimated by nonlinear regression using a single-site, quasimonomvalent model of the binding). Dower et al. (11) obtained similar results when monoclonal 36-7-5 antibody was incubated at 4°C with either B10.A or B10.BR spleen cells. Modest increases in the apparent affinity and saturation value to 9 × 10⁹ M⁻¹ and 3.8 × 10⁴ molecules/cell, respectively, resulted when the incubations were conducted at 37°C. The contribution of cellular processes to the apparent "binding" at 37°C requires further evaluation. The bindable fraction of counts with the labeling procedure used was routinely in the range 80–90%.

In Vivo Dose Dependence Studies. Several dose dependence relationships are evident in Fig. 2 for B10.A (Kk-positive) mice. At low doses, over 70% of the injected antibody remains in the feet. As the dose is increased, the percentage of dose there decreases and that in the popliteal lymph nodes appears to increase. In terms of percentage of dose, labeling of these nodes is optimal at doses below 0.7 µg, and there is the suggestion of a peak at 0.3 µg. At doses in excess of 0.7 µg, antibody appears to traverse the popliteal nodes and bind more effectively in lumbar nodes, which are farther along the lymphatic chain. Optimal lumbar node labeling (in terms of percentage of dose) occurs near 1.0 µg, and smaller percentages localize there at higher doses. The cervical nodes, which are not in the drainage pattern from the hind footpads, contained very little label at any dose.

At doses greater than 0.7 µg, dramatically larger amounts of antibody begin to reach the liver, spleen, lung, and kidney. Only a small rise in blood labeling is apparent. Uptake by the thyroid is minimal at this early time point (2 h).

The findings for lumbar and popliteal nodes in Kk-positive mice are replotted in Fig. 3 in terms of labeled antibody concentration in the node (µg antibody/g lymph node), rather than percentage of dose. This format demonstrates clearly the plateau which suggests saturation of binding sites. However, the two curves also show a continued increase (with slope greater than that explainable as nonspecific background on the basis of data from the Kk-negative animals). Fig. 4 shows the data for the systemic organs in terms of concentration. There is an initial "lag" at low dose and no clear evidence of saturation at high doses.

C57BL/10 (Kk-negative) mice give quite different results. At low dose, only one-half as much of the injected material (35%) remains at the injection site after 2 h, and increasing the dose does not affect the percentage of dose remaining there. Localization in nodes is very slight, and the fraction of dose in each of the internal organs appears to be nearly independent of the

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Footnotes:
LYMPHATIC DELIVERY OF ANTIBODIES

Fig. 2. Dose dependence of 125I-anti-K\(^4\) distribution 2 h after injection in the hind paws of K\(^4\)-positive and K\(^4\)-negative mice. The results for K\(^4\)-positive mice suggest sequential overflow from injection sites to regional nodes to systemic organs. In K\(^4\)-negative mice, distribution was essentially independent of dose over a 30-fold range of dose. Bars, SE of the geometric mean for groups of 3 animals.

Fig. 3. Dose dependence of 125I-anti-K\(^4\) IgG in lymph nodes of K\(^4\)-positive mice 2 h after injection in the hind paws. Uptake partially saturates at high dose. Bars, SE of the geometric mean for groups of 3 animals.

Fig. 4. Dose dependence of 125I-anti-K\(^4\) distribution in systemic organs of K\(^4\)-positive mice 2 h after injection in the hind paws. A, full dose range (0.1–10 \(\mu\)g); B, 0.1–1 \(\mu\)g dose range. All organs abruptly increase in uptake at approximately the same dose, about 0.7 \(\mu\)g, consistent with sequential overflow. Bars, SE of the geometric mean for groups of 3 animals.

amount injected, over a 30-fold range of doses. The observed tissue localization includes a contribution from antibody in blood within the tissue. Hence, when corrections are made for blood levels, the differences in organ uptake between negative and positive strains become even greater than they appear in Fig. 2, since residual blood in the tissues of C57BL/10 mice accounts for 5- to 10-fold more of the observed labeling than it does in tissues of B10.A mice.

In these experiments, the negative control was provided by use of the same antibody (i.e., 36-7-5) in K\(^4\)-negative mice. In other studies\(^2\) an isotype-matched control antibody (27-11-13 anti-D\(^\delta\)/\(^4\)) was used in B10.A mice (which are D\(^\delta\)). The results were qualitatively and quantitatively consistent with the negative controls used in the present study.
LYMPHATIC DELIVERY OF ANTIBODIES

DISCUSSION

The studies summarized in Fig. 2 demonstrate a marked dose dependence in the biodistribution when monoclonal antibodies directed against determinants on normal cells are injected s.c. By choosing an appropriate dose, effective containment of antibody within the lymphatics (i.e., maximal percentage uptake by the lymph node cells) can be achieved. Monoclonal antibody 36-7-5 has specificity for determinants on almost all cells of B10.A mice, including those at the injection site; hence, at very low doses, much of the antibody is effectively sequestered. Larger doses appear to saturate local binding capacity near the injection site, enter the lymphatics, and bind first in the proximal lymph nodes, then in more distant ones.

When the results for popliteal and lumbar nodes are replotted in terms of $\mu$g of labeled antibody per g of tissue (Fig. 3), the apparent saturation becomes more obvious. However, the curves also show a continued increase at high doses, more than can be accounted for by the nonspecific background seen for K$^+$-negative animals. Whether this behavior reflects a second, less accessible population of antigen-bearing cells, secondary processing of antigen, or some other phenomenon will be best answered by projected flow cytometry experiments using fluorescently labeled antibodies.

As the dose is raised above 0.7 $\mu$g, there is a dramatic increase in the percentage of dose appearing in the major systemic organs (liver, spleen, lungs, and kidneys) of B10.A mice, consistent with the concept of "overflow" from the lymphatics into the systemic circulation. Further experiments will be required to determine directly the processes responsible for removing bindable antibody from the bloodstream. However, it seems likely that antigen on circulating cells, on endothelial surfaces, and on parenchymal cells (especially in organs with sinusoidal blood supply) all play roles. At higher absolute doses, the percentage of dose in blood does not show as dramatic an increase as do the organs, probably because nonbindable 125I counts contribute significantly at low dose. The increase is also less pronounced for thymus, heart, and thyroid, but the uptake in those organs is so low (less than 0.3%) that nonbindable and blood-borne counts obscure the pattern that might otherwise be seen for pure bindable antibody in the tissues. The low uptake in thymus, despite the presence of antigen there, agrees with the findings of Houston et al. (12) using an antibody against Thy-1 injected i.v.

The distribution of 36-7-5 in vivo is undoubtedly related to the binding characteristics of the antibody, to its metabolic handling, and to the fate of the $^{125}$I label. Fig. 1 shows "equilibrium binding" profiles at 4°C and 37°C for 36-7-5 in K$^+$-positive and K$^+$-negative mice. The incubations were for 2 h, a period sufficient to approach equilibrium (or steady state) at all but the lowest concentrations. Other experiments (not shown) yielded forward rate constants at 4°C of about $3 \times 10^4$ M$^{-1}$ min$^{-1}$. The change from 4°C to 37°C would be expected to alter intrinsic binding properties and to introduce a variety of new cellular processes. However, the profiles in Fig. 1 indicate only a modest temperature dependence. Whether this near-equivalence represents a balance of opposite effects or an absence of major change remains to be determined.

Whereas 36-7-5 anti-K$^+$ represents the logical extreme of almost universal binding in K$^+$-positive strains, it binds specifically to no cells in K$^+$-negative mice. In the negative case, the percentages of dose shown in Fig. 2 appear essentially independent of dose (i.e., the pharmacokinetics are linear). No changes of even as much as a factor of 2 take place over a 30-fold range of doses. Nonspecific uptake in popliteal and lumbar nodes is very much lower than specific uptake at all doses, in agreement with our previous findings (1).

In the hind paw injection site, there is a very good match between the level of nonspecific counts in K$^+$-negative animals and the apparent plateau observed at the highest doses in K$^+$-positive ones; 35–40% of the dose remains at the injection site nonspecifically 2 h after injection. This observation is in good agreement with full time-course experiments performed with the same antibody preparations. Uptake of radioactivity by blood and systemic organs at low dose is greater in K$^+$-negative mice than in K$^+$-positive ones. This finding is consistent with the interpretation that, in K$^+$-positive mice, much of the dose is sequestered at the injection site and in regional nodes before reaching the systemic organs. In other words, a pattern of "sequential overflow" is seen in K$^+$-positive mice.

The lymphatic route is currently under consideration for a number of clinical applications, including the detection of lymph node metastases of breast, lung, colon, and prostate tumors. However, when the target is a solid tumor, we do not expect to see a pharmacology dominated by the pattern of "sequential overflow." Also, attempts to image solid metastases will probably be complicated, at all but the earliest stages, by distortions or blockage of lymph flow and by poor penetration of the solid masses (13). However, the present study does indicate the high efficiency with which antibody can be delivered to accessible nodes, and it establishes a base line for anticipated background effects of IgG molecules that do not find the target antigen.

The clinical relevance of these studies with anti-K$^+$ is most direct when we consider immunolymphoscintigraphy with antibodies directed against normal cell types for nonspecific assessment of lymph node status (1). A possible alternative to colloid lymphoscintigraphy (14) is lymph node metastases of a solid tumor. Science (Wash. DC), 222: 423–426, 1983.


Weinstein, J. N., Parker, R. J., Holton, O. D., III, Keenan, A. M., Covell, D. G., Black, C. D. V., and Sieber, S. M. Lymphatic delivery of monoclonal antibody conjugates to normal cells near the tumor. When antigens on lymphoma or leukemia cells are the target, choice of the right dose would maximize the signal/noise ratio for imaging and would reduce toxicity if therapy were attempted with toxin, drug, or radionuclide conjugates of the antibody.

REFERENCES


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