Delivery of Melanoma-associated Immunoglobulin Monoclonal Antibody and Fab Fragments to Normal Brain Utilizing Osmotic Blood-Brain Barrier Disruption

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ABSTRACT

Iodinated monoclonal antibodies (IgG 96.5 and two monomeric Fab fragments 96.5 and 48.7) to melanoma-associated antigens were administered after osmotic blood-brain barrier (BBB) opening in normal rats. Osmotic BBB disruption significantly increased monoclonal antibody delivery to the brain. Following BBB opening and intracarotid administration, there was no difference in the disrupted brain concentration integral area under the curve between Fab and IgG over the 72-h experimental period. However, Fab concentration in the disrupted brain was initially higher than IgG, and the clearance was more rapid (P < 0.001), decreasing 50% by approximately 4.5 h compared to 25.5 h for IgG. Plasma clearance was also more rapid for the Fab than for IgG. The levels decreased 50% by 1.5 h for Fab and 15 h for IgG. The route and timing of antibody infusion had a significant effect on delivery to the disrupted brain with the Fab fragments but not with the intact IgG. Antibody recovered from disrupted brain retained its immunological reactivity as measured by a cell binding assay for at least 24 h. IgG and Fab delivery to the ipsilateral brain after BBB disruption increased (P < 0.001) with increasing dose over a more than 3-log dose range. These data provide information applicable to the therapeutic use of monoclonal antibodies in brain tumor treatment.

INTRODUCTION

The emergence of monoclonal antibodies with a high degree of binding and specificity offers a unique immunological approach to brain tumor diagnosis and therapy (1-4). BBB disruption has been shown to increase the delivery of monoclonal antibodies, including IgM MAb and other high-molecular-weight proteins, to normal brain and cerebrospinal fluid (5-9). In a nude rat model of intracerebral human small cell lung carcinoma, BBB disruption increased the delivery of both albumin and methotrexate to the tumor and brain around the tumor (10). Although the BBB is abnormal in most CNS tumors, the permeability of the tumor and tumor-infiltrating surrounding brain is highly variable, and BBB disruption may be beneficial in obtaining the necessary therapeutic uptake of larger molecular weight proteins such as monoclonal antibodies (5, 6, 11, 12).

When applying the use of tumor-specific MAbs or antibody conjugates (3, 13-19) in brain tumor treatment, the effect of BBB disruption and MAb delivery, even in the absence of a target antigen, becomes an important consideration. In the present study in normal rats, an IgG and two monomeric Fab fragments with specificity to antigens present on human melanoma were administered following BBB disruption. The purpose was to assess the effect of BBB disruption on antibody delivery and to determine if the prolonged half-life and NSB to normal brain observed with a previously described IgM monoclonal antibody 7 could be decreased when divalent IgG antibody or monomeric Fab fragments are administered.

MATERIALS AND METHODS

Monoclonal Antibody. MAb IgG 96.5, Fab 96.5, and Fab 48.7, which bind two human melanoma antigens (a M, 97,000 protein and a proteoglycan) were prepared as previously described (2, 14, 15, 20-24). Iodination (123I, New England Nuclear) of each antibody was performed using the lactoperoxidase method as described previously (6, 7). Radiolabeled antibody was separated from unreacted radiiodine on a prepackaged disposable 9-ml Sephadex G-25 M column (Pharmacia, Piscataway, NJ). Following purification, an aliquot of the preparation was obtained, and protein binding of the radiolabel was determined using TCA precipitation. The TCA precipitation averaged 92.9 ± 1.2% among 14 separate iodinations. The specific activity was 2.4 to 7.4 µCi/µg. Preparations were stored at 4°C in 1% bovine serum albumin. The TCA precipitability was again determined prior to each study and averaged 87.9 ± 0.4% (range, 80.5 to 97.1%). Animals were given injections of 17 to 20 × 106 TCA-precipitable cpm (1.2 to 3.4 µg), unless otherwise stated.

Electrophoresis of IgG and Fab before following iodination was performed to evaluate for high-molecular-weight antibody aggregation. Nondenaturing gels were run with Triton X-100 to solubilize the protein (25). Determinations were made from gels at 4% (acrylamide plus bisacrylamide). All gels were prepared by dilution of a single stock solution of 40% (w/v) plus 0.14% (w/v) bisacrylamide and polymerized in a 0.1-× 8.6-× 10.0-cm slab. The gel was electrophoresed for 3 h at 12.5 mA in 1% Triton X-100 (40 mM Tris/20 mM sodium acetate, pH 7.4). Gels were stained with Coomassie blue or silver according to the method of Wray et al. (26). The results of the electrophoresis studies showed the antibodies migrated as a single band with no apparent antibody aggregation. The iodination procedure did not affect the migration distance of any of the monoclonal antibodies.

In previous studies, these melanoma antibodies were radiolabeled, and protein binding was determined using both TCA precipitation and electrophoresis (28). TCA precipitation consistently yielded 5% less iodine-antibody binding than electrophoresis. Thus, the values reported in these studies may be a slight underestimate of protein-bound radiolabel.

Osmotic BBB Disruption. Adult Sprague-Dawley rats (200 to 250 g) were anesthetized with sodium pentobarbital (50 mg/kg). BBB disruption in the rat was performed as previously described (7, 27). Animals were infused with either i.c. saline in control studies or i.c. mannitol (25%) in the barrier disruption studies (0.12 ml/s for 30 s). Prior to i.c. infusion of saline or mannitol, Evans blue (2%, 2 ml/kg) was administered i.v. Animals were infused with either i.c. saline or mannitol. Animals were sacrificed at 0.5, 1, 6, 24, or 72 h. One supplemental study compared the effect on MAb delivery to disrupted brain at 0.5 h when several different methods of MAb administration were used: (a) i.v. bolus immediately after mannitol; (b) i.c. bolus immediately after mannitol; (c) i.v. infusion over 15 min beginning 10 min before disruption; and (d) i.c. infusion over...
15 min beginning immediately after disruption (Fabs only). A second supplemental study evaluated antibody delivery to disrupted brain at 0.5 h after varying doses of i.e. bolus IgG and Fab were administered. The dose ranged from 0.049 to 250 µg.

At the time of sacrifice, Evans blue-albumin staining of the brain was macroscopically graded on a scale from 0 to 3+ as previously defined (7). All control animals had 0 staining, and all those sacrificed at 6 h or less postdisruption had 3+ staining. Those sacrificed at 24 or 72 h postdisruption had at least 2+ staining.

Sample Collection and Assay. In all of the animals a plasma sample was obtained prior to sacrifice. Intravascular radioactivity was eliminated by the i.v. infusion of 40 to 60 ml of normal saline warmed to 37°C with concurrent blood withdrawal from the i.c. catheter until cessation of the heart (in approximately 10 min). A plasma sample was obtained at the end of the perfusion. This technique resulted in an average of 96.4 ± 0.2% removal of radioactivity from the vascular compartment among all animals. Brain was divided into contralateral control (no staining) and ipsilateral disrupted (2 to 3+ staining) hemispheres and homogenized in 0.9% sodium chloride (2 to 5:1; v/w). Plasma and brain samples were counted to obtain cpm/g or ml and to determine TCA precipitability. All concentrations were corrected to exclude free 125I as determined by TCA protein precipitability.

Immunoactivity of MAb. The ability of iodinated IgG 96.5 and Fab 96.5 contained in disrupted brain tissue homogenate from in vivo studies to bind to paraformaldehyde-fixed H2669 melanoma cells (28) in vitro was evaluated. 125I-MAb was administered i.c. following barrier opening, and animals were sacrificed by perfusion after 1 or 24 h. Tissue samples were weighed and homogenized in 0.05 M PBS (2 to 5:1). Protein-bound 125I in the tissue homogenates was determined by TCA precipitation. The mean ± SEM of disrupted brain homogenates was 91.8 ± 2.0% for the Fab animals and 96.1 ± 0.7% for IgG animals. Sufficient quantity of brain homogenate to obtain approximately 1 ng of IgG or Fab (7 to 16 x 10^4 TCA-precipitable cpm) was mixed with 2 x 10^6 cells and incubated at room temperature for 30 min (in vivo CBA). Four ml of 0.05 M PBS were added, the sample was mixed and centrifuged, and the first supernatant was separated from the pellet. This step was repeated to wash the cell pellet, and a second supernatant was obtained. The three fractions were counted.

% of binding of radiolabeled antibody to cells

\[
\text{cpm in pellet} \times 100 = \text{TCA precipitability of sample}
\]

This calculation assumes any free 125I was in the supernatant fraction. Additionally, brain homogenate containing approximately 1 ng of antibody as described above was added to 4 ml of 0.05 M PBS (no tumor cells added) and centrifuged. The activity in the pellet fraction was considered to be the nonspecifically bound antibody to brain parenchyma tissue.

In concurrent in vitro control studies, 125I-MAb (TCA precipitability of 90.1% for IgG and 91.5% for Fab) diluted in 1% bovine serum albumin was used to spike brain homogenate from untreated animals. The spiked sample was incubated with cells as in the above CBA or washed with 0.05 M PBS after incubation to determine in vitro NSB. Values were calculated as described above.

Statistical Analysis. Values are expressed as cpm/g of tissue or cpm/ml of plasma, or as the percentage of uptake (cpm per g/total cpm dose). Mean values ± SEM were calculated to summarize the data. All values are corrected to exclude free 125I.

The overall experimental design tested the effects of the following main factors on MAb levels in the ipsilateral hemisphere: (a) presence or absence of BBB disruption; (b) IgG versus Fab; and (c) time. A multiple regression analysis was conducted using these experimental factors as independent variables. Logarithmic transformations of MAb concentrations and time were performed to achieve linearity. First and second order interaction effects among the main experimental factors were also examined. Supplementary analyses were conducted to examine specific relationships among selected variables as indicated below (29).

### RESULTS

Delivery of Monoclonal Antibody to Normal Brain. Regression analysis showed that there was no significant difference in delivery to the brain between Fab 48.7 and Fab 96.5. The mean concentration ± the SEM in disrupted brain was 118,374 ± 24,011 cpm (n = 18) and 121,321 ± 26,230 cpm (n = 15), for these two fragments, respectively. Thus, the two groups were combined for analysis in which IgG and Fab were compared.

125I-MAb concentration in brain and plasma samples was corrected to exclude any free radiolabel as determined by TCA precipitation. Table 1 shows the percentage of TCA-precipitable counts in disrupted brain and plasma at various times after the infusion of IgG or the Fab fragments. At all time points, disrupted brain contained greater than 92% protein-bound radiiodine for both IgG and Fab. The radioactivity in plasma was 97 to 98% bound in the IgG animals but less stable with the Fabs, decreasing to 53 to 66% at 6 h.

In all experimental designs, paired t tests demonstrated a significantly higher (P < 0.0001 in all cases) MAb level in the ipsilateral disrupted hemisphere compared to the contralateral hemisphere. The results of overall multiple regression analysis indicate that the delivery of MAb to ipsilateral brain over the 72-h experimental period was increased significantly (P < 0.0001) in BBB-disrupted animals compared to saline-infused controls. Over 72 h, the mean concentration (cpm/g) in the infused hemisphere was 112,052 ± 11,283 (n = 57) following i.c. mannitol and was 3,184 ± 464 (n = 37) following i.c. saline. There was an overall pattern of decrease of MAb concentration in the brain with increasing time that was linear in a log-log scale (P < 0.0001).

Fig. 1A shows that the rate of clearance of radiolabeled IgG and Fab from the ipsilateral disrupted hemisphere is more rapid in the Fab group than in the IgG group (P < 0.001). In this case, the Fab level is initially 2 times higher than the IgG level but is 4 times lower by the end of the experiment. Fab concentration in disrupted brain decreased 50% by approximately 4.5 h, whereas approximately 25.5 h were required for IgG. The result was an insignificant difference between IgG and Fab delivery to disrupted brain over time. The mean cpm/g for the experimental period was 119,714 ± 17,436 for Fab (n = 33) and 101,517 ± 12,127 for IgG (n = 24). The brain exposure integral (area under the curve) was 8.56 x 10^6 and 7.92 x 10^6 cpm for Fab and IgG, respectively. The ratio of the integrals is 0.92.

The clearance of Fab and IgG from plasma is shown in Fig. 1B. IgG plasma concentration was higher than Fab at all time points. Fab cleared from plasma at a faster rate than IgG, decreasing 50% by 1.5 h compared to 15 h for IgG. At 72 h

<table>
<thead>
<tr>
<th>Time after Disruption (h)</th>
<th>Disrupted</th>
<th>Plasma n</th>
<th>Disrupted</th>
<th>Plasma n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>95.7 ± 3.0*</td>
<td>96.7 ± 0.2</td>
<td>14</td>
<td>92.3 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>94.3 ± 0.8</td>
<td>96.9 ± 0.3</td>
<td>6</td>
<td>93.4 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>97.3 ± 0.3</td>
<td>96.7 ± 0.3</td>
<td>5</td>
<td>95.2 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>97.5 ± 0.2</td>
<td>96.6 ± 0.3</td>
<td>5</td>
<td>96.0 ± 0.4</td>
</tr>
<tr>
<td>72</td>
<td>97.5 ± 0.2</td>
<td>98.1 ± 0.2</td>
<td>6</td>
<td>92.5 ± 1.2</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
MAb DELIVERY ACROSS THE BBB

Fig. 1. Concentration (cpm/g) of i.c. administered 125I-MAb (IgG 96.5, Fab 96.5, and Fab 48.7) in ipsilateral brain (A) and plasma (B) measured over 72 h following BBB disruption. Bars, SEM. Fab delivery was initially higher than IgG and cleared more rapidly (P < 0.0001). The area under the curve for Fab and IgG is 8.56 x 10^9 and 7.92 x 10^9 cpm, respectively. IgG plasma concentration was higher than Fab at all time points and was cleared from plasma at a slower rate. A 50% decrease in concentration was reached at 15 h for IgG and 1.5 h for Fab.

postdisruption, the Fab plasma concentration was less than 10% of the IgG level. The plasma integral for the experimental period was 2.00 x 10^7 and 7.85 x 10^7 cpm for Fab and IgG, respectively. Thus, the plasma concentration of Fab is considerably lower than the IgG plasma level, while the disrupted brain integrals are quite similar. The ratio of the brain AUC to plasma AUC after BBB disruption is 0.43 for Fab and 0.10 for IgG.

The effect of i.v. or i.c. MAb administration and the effect of a bolus or slower infusion on antibody delivery to disrupted brain were evaluated 0.5 h after BBB disruption (Fig. 2). In the animals in which Fab was given as a bolus infusion immediately after mannitol, the i.c. route of administration resulted in a 2-fold greater delivery than the i.v. route (253,584 ± 28,571, n = 6; and 144,125 ± 21,406, n = 6, respectively). The i.c. bolus administration was also better than either of the two slower infusion protocols evaluated. At the slower infusion rate, i.v. infusion (105,724 ± 14,031, n = 5) was higher than i.c. infusion (66,818 ± 5701, n = 5). In the animals in which IgG was given in association with barrier disruption, there were no significant effects of route and duration of infusion on delivery to ipsilateral brain.

A separate analysis considered the effects of varying dose on MAb delivery to ipsilateral brain (Table 2). Fab or IgG, at various doses ranging from 0.049 to 250 µg, was administered i.c. bolus following barrier disruption. Animals were sacrificed 0.5 h after MAb administration. A two-way analysis of variance indicated an increased (P < 0.001) delivery to disrupted brain with increasing dose. Even though there was a more than 3-log dose range, the percentage of the dose/g of brain and the disrupted brain:plasma ratio were fairly constant, indicating a linear relationship with the dose. The percentage per g of brain of the total administered dose was 1.0 to 1.4% for Fab and 0.7 to 1.0% for IgG. These data suggest that the delivery to brain following barrier opening did not saturate over this dose range.

Table 2 Percentage of delivery per gram of brain of the total administered IgG or Fab dose following BBB disruption

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Contralateral control hemisphere</th>
<th>BBB-disrupted hemisphere</th>
<th>Disrupted brain:plasma ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG dose (µg)</td>
<td>0.049 ± 0.003* (4)*</td>
<td>0.02 ± 0.01</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>Fab dose (µg)</td>
<td>0.34 ± 0.13 (5)</td>
<td>0.01 ± 0.01</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>249.5 ± 1.7 (3)</td>
<td>0.03 ± 0.01</td>
<td>0.99 ± 0.10</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

** Numbers in parentheses, number of animals studied.
of the antibody in a cell binding assay (Table 3). NSB was determined with brain homogenate from untreated animals that was spiked with iodinated antibody (*in vitro*) and from brain homogenate from barrier-disrupted animals (*in vivo*). These NSB samples were treated the same as the samples for the CBA except melanoma cells were not added. The *in vitro* NSB averaged 20% for Fab and 16% for IgG. The *in vivo* NSB was 20% for Fab and 25% for IgG.

*In vitro* CBA controls were performed in which brain homogenate from untreated animals was spiked with 125I-MAb prior to incubation with cells (CBA) or without cells (NSB). The mean percentage of binding of the controls was 46% for Fab and 57% for IgG.

125I-MAb contained in brain homogenate from *in vivo* studies had a mean percentage of binding of 47% for Fab and 64% for IgG. The *in vitro* NSB was subtracted from the *in vitro* CBA, and the *in vivo* NSB was subtracted from the *in vivo* CBA to determine the net specific binding of antibody to melanoma cells. At 1 and 24 h postdisruption, antigen binding for both Fab and IgG contained in brain homogenate from the *in vivo* studies was essentially identical to the *in vitro* controls, indicating that the immunoreactivity of the 96.5 IgG and Fab was maintained *in vivo* for at least 24 h.

**DISCUSSION**

The current BBB disruption studies were performed to characterize the delivery to brain of IgG and two Fab fragments, which bind melanoma-associated antigens, and to evaluate nonspecific binding and clearance from normal brain. Since melanoma shares antigens with gliomas, the antibodies in this report may also be applicable to primary brain tumors (24, 30–35). These results point to several factors related to the potential of monoclonal antibodies in the diagnosis and therapy of brain tumors. One is that BBB disruption markedly enhances immunologically reactive MAb delivery to the brain, a finding similar to results obtained with other IgG and IgM MAbs (6, 7). This may be particularly significant in the case of relatively impermeable tumors or tumor-infiltrated normal brain (8).

Another important finding was that Fab cleared rapidly from normal brain, decreasing 50% by 4.5 h as compared to 25.5 h for IgG and more than 3 days for IgM (7). The initial greater delivery to brain combined with the more rapid clearance of Fab versus IgG resulted in an overall AUC that was virtually the same for both antibodies.

The reason for the more rapid clearance of Fab is unclear.

One possible explanation is that Fab is smaller and more diffusible into CSF. However, previous studies of two very differently sized proteins (M, 68,000 and 1,000,000) showed delivery to CSF following BBB disruption to behave very similarly (6). Others have described higher tissue clearance rates and markedly reduced background of nonspecific and tumor-specific monoclonal immunoglobulin fragments compared with whole immunoglobulin (21, 36, 37). These studies suggest that the Fab fragment offers significant pharmacokinetic advantages, which may permit increased delivery after BBB opening to tumor and to tumor-infiltrated surrounding brain, yet is rapidly cleared from otherwise normal brain. Conversely, the Fab fragments bound 37% less to the antigen than the intact immunoglobulin (see Table 3).

In addition, the delivery and percentage of the total dose per gram of brain were initially higher for Fab than IgG, but the overall brain exposure was essentially the same. The difference between Fab and IgG may relate to the Fc portion of the molecule or molecular size. Evidence that the plasma concentration is not entirely responsible for a higher level of Fab in brain is the 4-fold higher brain:plasma AUC ratio for Fab than for IgG and the fact that Fab plasma levels were always lower than IgG.

A second observation relates to the optimal method of MAb infusion which results in the greatest delivery to disrupted brain. After evaluation of various methods of Fab administration with BBB opening, clearly i.c. bolus infusion compared with i.v. bolus or a slower infusion resulted in the greatest delivery to brain. Longer infusion times were not evaluated because previous studies have shown a size-dependent decrease in permeability with time after BBB opening and thus a theoretical advantage to administration at times close to the osmotic procedure (7, 9). There was no such effect with IgG which correlates with studies by Fenstermacher (38) who predicted no advantage to intraarterial administration when the rate of transformation or excretion is very low. These findings correlate with studies by Bullard et al. in which there was no difference in the delivery to brain after barrier opening between i.c. or i.v. administration of a glioma-specific IgG MAb (5).

In a recent pilot study of three patients with melanoma metastatic to the CNS, there was no uptake of either a nonspecific antibody or tumor-specific antibody after i.v. administration (8). After BBB disruption, there was a marked increase in antibody delivery to the tumor-bearing hemisphere, and serial brain scans showed that the effective half-life of radiolabeled Fab antibody in disrupted brain ranged from 12 to 30 h. An important observation in this initial study was that only one patient had any evidence of specific increased uptake in the region of the tumor, and the clearance from the tumor region was the same as the surrounding apparently normal brain. However, immunohistological studies indicated that only a fraction of the antigen binding sites on tumor cells were bound with antibody, and the lack of persistent localization may have been the result of giving an inadequate dose of antibody.

Studies are currently under way in nude rats with human tumors implanted intracerebrally and s.c. (10). These studies are addressing MAb dose (2, 15); antibody affinity or avidity; type and dose of radionuclide (4, 20, 39, 40); the delivery of different tumor-specific IgG, Fab, and F(ab')2 antibodies (14, 21); and other parameters, such as tumor size (41), which may increase the degree and persistence of localization of monoclonal antibodies to tumor (42, 43).
REFERENCES


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