Effect of Antiplatelet Antibody on the Development of Pulmonary Metastases following Injection of CT26 Colon Adenocarcinoma, Lewis Lung Carcinoma, and B16 Amelanotic Melanoma Tumor Cells into Mice

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ABSTRACT

Three different murine tumors, CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma, were injected into syngeneic mice (BALB/c and C57BL/6J) to test the effect of rabbit anti-mouse platelet antibody on the development of pulmonary metastases. Antiplatelet antibody, when injected i.p., decreased the platelet count from 1.5 × 10^6/μl to 0.12 × 10^6/μl at 6 hr, which remained at this level for 24 hr. Antiplatelet antibody given 6 hr pre- and 18 hr post-i.v. injection of tumor cells decreased the mean number of CT26 tumor nodules per lung by 57% (range, 47 to 65%) and decreased the mean nodule volume of tumor per lung by 37% (range, 0 to 71%) (124 experimental animals), when compared to the effect of nonimmune serum or irrelevant anti-immunoglobulin antibody in 136 control animals. With Lewis lung carcinoma, antiplatelet antibody decreased the mean number of tumor nodules by 62% (range, 57 to 78%) and decreased the mean nodule volume of tumors by 64% (range, 60 to 77%) using 48 experimental animals and 65 control animals. When tumor cells were given s.c., antiplatelet antibody given 6 hr pre-injection, 18 hr post-injection, and every 6 hr thereafter also decreased the mean number of metastases by 42% in 14 experimental and 15 control animals. With B16 amelanotic melanoma, antiplatelet antibody given 6 hr pre- and 18 hr post-injection decreased the mean number of tumor nodules by 85% and decreased the mean nodule volume of tumors by 66% using 9 experimental and 9 control animals. Similar results were obtained when all three tumors were injected 6 hr after the injection of antiplatelet antibody. However, negative results were obtained if antiplatelet antibody was injected 6 hr after the injection of tumor cells. Since antiplatelet antibody has its maximum effect at 6 hr, it is likely that platelets play their role in the development of pulmonary metastases during the first 12 hr of tumor inoculation.

INTRODUCTION

The lack of effect of prostacyclin (a potent inhibitor of tumor-induced platelet aggregation in vitro and thrombocytopenia in vivo) on the development of pulmonary tumor metastases following the i.v. injection of CT26, Lewis lung, or B16a tumor cells described in the previous paper (18) necessitated a reevaluation of the role of platelets in tumor metastases. Of the numerous reports in the literature suggesting a role for platelets in the development of metastases (4-17, 20, 22, 23, 26-31), the strongest evidence is the original observation of Gasic et al. (10) on the inhibition of pulmonary metastases with antiplatelet antibody. Gasic et al. pretreated CAF1/Jax mice with 0.05 ml of heat-inactivated, RBC-adsorbed antiplatelet serum 24 hr prior to the injection of 3.3 × 10^6 TAa ascites tumor cells and found a 65% decrease in the number of pulmonary metastases 14 to 16 days later. To our knowledge, this work has never been confirmed, nor have experiments been performed to rule out a direct toxic effect of rabbit anti-mouse platelet antiserum on TAa ascites tumor cells or to rule out the nonspecific effect of immune complex formation with an irrelevant antibody. The purpose of this communication is to report our experience with the effect of a rabbit anti-mouse antiplatelet antibody on the development of pulmonary metastases following the injection of CT26, Lewis lung, and B16a tumor cells into mice, using the above-mentioned controls.

MATERIALS AND METHODS

Tumor Cell Lines and Tissue Culture Media. CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma cells were maintained s.c. in vivo or grown in tissue culture, as described in the previous paper (18). Tumor cells which were maintained s.c. were grown in vitro 5 to 7 days prior to injection into syngeneic mice.

Enumeration of Tumor Cell Metastases. Animals were sacrificed at 21 days, and the number and volume of pulmonary metastases were determined as described in the previous paper (18).

Platelet Counts. Manual platelet counts were obtained, using a counting chamber and phase microscopy (19).

Preparation of Rabbit Anti-Mouse Antiplatelet Antibody. Platelets were obtained from 10 ml of EDTA-anticoagulated blood (10 μM final concentration) from BALB/c and C57BL/6J mice, mixed approximately 1:1. They were washed 4 times in 10 ml EDTA : 0.01 μM PBS, pH 7.4, followed by one wash in 1% ammonium oxalate and one wash in 0.01 μM PBS-10 μM EDTA, and stored frozen in 1 ml at -20°C. Platelets from 5 to 10 ml of mouse blood were emulsified in complete Freund’s adjuvant (1:1, by volume) and injected into a female 3.5-kg New Zealand White rabbit every 2 weeks for 4 injections. Rabbit serum was adsorbed twice with washed RBC [5 ml sera plus 1 ml packed RBC from both strains of mice (1:1) for 0.5 hr at 37°C] followed by adsorption of 5 ml of sera with 20 × 10^6 tumor cells. A partially purified globulin fraction was obtained by precipitation from a 20 to 50% saturated ammonium sulfate cut, dissolved in PBS in one-half its original serum volume and extensively dialyzed against PBS, and adsorbed with RBC and tumor cells, as above.

Effect of Rabbit Anti-Mouse Platelet Antibody on Platelet Count. Adsorbed sera (100 μl) or globulin fraction (50 μl), when injected i.p., decreased the platelet count to 7 to 10% of normal by 6 hr. This level remained below 15% for 48 hr (Table 1).

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2 To whom requests for reprints should be addressed.
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3 The abbreviation used is: PBS, phosphate-buffered saline.
Effect of Rabbit Anti-Mouse Platelet Antibody on Tumor Cells in Tissue Culture. Harvested subconfluent tumor cells (2.5 x 10^6/ml) were incubated in the presence of rabbit anti-mouse platelet antibody (adsorbed with RBC and tumor cells) and fresh mouse serum. Nonviable cells were evaluated after 30 min of incubation at 37°C, using trypan blue exclusion. Sparsely seeded tumor cell cultures (1 x 10^4 cells) were also grown in RPMI 1640 containing 5% calf serum in the presence or absence of a 5% globulin fraction of antiplatelet antiserum (or nonimmune serum) for 3 days and then harvested.

Effect of Rabbit Anti-Mouse Platelet Antibody and Rabbit Anti-Mouse IgG Antibody on Mouse Lymphocytes. Splenic cells from both C57BL/6J and BALB/c mice were each suspended in PBS and then applied to a Ficoll/Hypaque gradient. Lymphocytes were isolated and washed with PBS, and 10^6 cells were incubated with either PBS, 10% rabbit anti-mouse platelet antiserum, or 10% rabbit anti-mouse IgG antiserum for 0.5 hr at 37°C. Cells were then washed with PBS and incubated in 12.5% fresh rabbit serum (complement) for 0.5 hr at 37°C. Trypan blue (2%) was then added, and cytotoxicity was quantitated by trypan blue exclusion.

Experimental Protocol. One group of mice were pretreated with antiplatelet antibody i.p. 6 hr prior to the injection of tumor cells i.v. and 18 hr post-injection of tumor cells. Another group of mice were simply treated with antiplatelet antibody 6 hr prior to the i.v. injection of tumor cells. A third group of mice was first given injections of tumor cells i.v. and then treated with antiplatelet antibody 6 hr later; a fourth group was treated with antiplatelet antibody 18 hr after the injection of tumor cells. A fifth group of mice was pretreated with antiplatelet antibody 6 hr prior to the injection of s.c. tumor cells. These mice were then given injections of antiplatelet antibody, 18 hr later and every 48 hr thereafter. Suitable controls were given similar injections of either nonimmune serum or irrelevant antibody against mouse IgG.

RESULTS

CT26 Colon Carcinoma. Rabbit anti-mouse platelet antibody given 6 hr before and 18 hr after the i.v. injection of CT26 cells inhibited the number of pulmonary nodules by 47 to 65%, and (except for one negative experiment) inhibited the mean tumor nodule volume per lung by 41 to 71% compared to control animals that had been treated with nonimmune rabbit serum or irrelevant anti-mouse IgG antibody (Table 2). Similar results were obtained if animals were given antiplatelet antibody 6 hr prior to the injection of tumor cells (46% decrease in number and 41% decrease in mean nodule volume). No effect was noted on the number of pulmonary nodules if antiplatelet antibody was given 6 hr after the injection of tumor cells. However, mean nodule volume as well as total tumor mass did increase 68 and 39%, respectively.

Lewis Lung Carcinoma. Anti-mouse platelet antibody given 6 hr before and 18 hr after the i.v. injection of Lewis lung carcinoma cells decreased the number of pulmonary nodules by 57 to 78% as well as the mean nodule tumor volume per lung by 60 to 77% (Table 3). Similar results were obtained if animals were given antiplatelet antibody 6 hr prior to the injection of tumor cells (69% decrease in number and 42% decrease in volume; however, the decrease in volume was not statistically significant). No effect was noted on the number of pulmonary nodules if antiplatelet antibody was given 6 hr after or 18 hr after the injection of tumor cells. However, mean nodule volume increased 35 and 69%, respectively, and total tumor mass increased 5 and 75%, respectively. The effect at 18 hr was statistically significant. Lewis lung carcinoma cells were also injected s.c. 6 hr after the injection of antiplatelet antibody. If animals were then treated 18 hr post-injection of tumor cells and every 48 hr thereafter, the number of metastatic tumor nodules decreased by 42%, whereas the mean nodule volume was not altered.

B16 Amelanotic Melanoma. Anti-mouse platelet antibody given 6 hr before and 18 hr after the i.v. injection of B16 amelanotic melanoma cells also decreased the number and volume of metastatic tumor nodules by 85 and 66%, respectively.

DISCUSSION

These data clearly indicate a role for platelets in the development of murine pulmonary metastases, using 3 different syngeneic tumors (CT26 colon carcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma) and 2 different strains of mice (BALB/c and C57BL/6J). This work confirms and extends the original observations of Gasic et al. (10).

Antiplatelet antibody inhibited the number of CT26 tumor nodules per lung by 46 to 65% (except for one negative experiment) decreased the mean nodule volume of tumor per lung by 41 to 71%, using 124 experimental animals and 136 control animals for these studies. Similar results were obtained if the antibody was adsorbed with tumor cells ruling out the possibility that antiplatelet antibody may be tumoricidal or cross-reactive with tumor cell antigens. Results similar to the controls...
Table 2

Effect of antiplatelet antibody on development of pulmonary metastases following injection of CT26 colon carcinoma into BALB/c mice

Viable CT26 colon carcinoma cells (25,000) were injected i.v. into BALB/c mice, animals were sacrificed on Day 21, and the number and volume of pulmonary metastases were determined. Control animals were given i.p. injections of nonimmune sera, and experimental animals were treated with 100 μL of antiplatelet antibody adsorbed with RBC at the time intervals indicated, pre- and post-injection of tumor cells. Tumor mass was calculated by multiplying mean number of tumor nodules by mean volume of tumor nodules. When p values are given, the first refers to the difference between mean number of nodules per lung, and the second refers to the difference between mean volume of nodules per lung.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean no. of nodules/lung</th>
<th>Mean volume of nodules/lung</th>
<th>Tumor mass</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>52 Controls</td>
<td>21.4 ± 2.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.56 ± 0.25</td>
<td>33.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>54 Controls</td>
<td>9.0 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89 ± 0.17</td>
<td>8.0</td>
<td>0.05</td>
</tr>
<tr>
<td>15 Controls&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.2 ± 5.1</td>
<td>2.24 ± 0.41</td>
<td>83.3</td>
<td>0.001</td>
</tr>
<tr>
<td>11 Controls&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.5 ± 4.1</td>
<td>1.70 ± 0.41</td>
<td>70.6</td>
<td>0.001</td>
</tr>
<tr>
<td>12 Controls&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.6 ± 0.9</td>
<td>1.01 ± 0.24</td>
<td>41.56</td>
<td>79.82</td>
</tr>
<tr>
<td>24 Controls</td>
<td>18.7 ± 2.3</td>
<td>1.10 ± 0.17</td>
<td>20.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25 Controls</td>
<td>9.9 ± 1.3</td>
<td>1.09 ± 0.25</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>14 Controls</td>
<td>41.4 ± 3.0</td>
<td>2.16 ± 0.19</td>
<td>89.4</td>
<td>0.001</td>
</tr>
<tr>
<td>12 Controls&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14.1 ± 1.8</td>
<td>0.62 ± 0.09</td>
<td>71</td>
<td>8.7</td>
</tr>
<tr>
<td>20 Controls</td>
<td>28.9 ± 4.6</td>
<td>1.83 ± 0.37</td>
<td>52.9</td>
<td>0.05</td>
</tr>
<tr>
<td>21 Controls&lt;sup&gt;f&lt;/sup&gt;</td>
<td>15.7 ± 2.8</td>
<td>1.08 ± 0.25</td>
<td>41</td>
<td>17.0</td>
</tr>
<tr>
<td>22 Controls&lt;sup&gt;g&lt;/sup&gt;</td>
<td>16.0 ± 2.4</td>
<td>1.61 ± 0.19</td>
<td>25.8</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>25 Controls</td>
<td>13.3 ± 1.9</td>
<td>2.70 ± 0.43</td>
<td>68</td>
<td>35.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> N, number of animals in each group.
<sup>b</sup> Mean ± S.E.
<sup>c</sup> Viable CT26 cells (50,000) used.
<sup>d</sup> Irrelevant anti-IgG antibody used.
<sup>e</sup> Antiplatelet antibody adsorbed with RBC.
<sup>f</sup> Antiplatelet antibody adsorbed with tumor cells and RBC.

Table 3

Effect of antiplatelet antibody on development of pulmonary metastases following injection of Lewis lung carcinoma or B16 amelanotic melanoma into C57BL/6J mice

Viable Lewis lung cells (150,000) or B16a tumor cells (25,000) were injected i.v. into C57BL/6J mice, and the number and volume of pulmonary metastases were determined on Day 21. Protocol was the same as that described in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean no. of nodules/lung</th>
<th>Mean volume of nodules/lung</th>
<th>Tumor mass</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis lung carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 Controls</td>
<td>14.5 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.62 ± 0.64</td>
<td>52.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>36 Controls</td>
<td>6.2 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.44 ± 0.39</td>
<td>8.9</td>
<td>0.05</td>
</tr>
<tr>
<td>15 Controls</td>
<td>16.7 ± 2.5</td>
<td>3.25 ± 0.40</td>
<td>54.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12 Controls&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 0.4</td>
<td>0.74 ± 0.25</td>
<td>77</td>
<td>2.7</td>
</tr>
<tr>
<td>6 Controls</td>
<td>12.0 ± 3.0</td>
<td>2.60 ± 0.80</td>
<td>31.2</td>
<td>0.05</td>
</tr>
<tr>
<td>6 Controls</td>
<td>3.7 ± 0.4</td>
<td>1.52 ± 0.52</td>
<td>42</td>
<td>5.6</td>
</tr>
<tr>
<td>21 Controls</td>
<td>20.6 ± 2.8</td>
<td>2.64 ± 0.51</td>
<td>54.4</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>24 Controls&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.1 ± 1.3</td>
<td>3.56 ± 0.51</td>
<td>57.3</td>
<td>+5</td>
</tr>
<tr>
<td>27 Controls</td>
<td>21.1 ± 3.3</td>
<td>2.64 ± 0.43</td>
<td>55.7</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>19 Controls&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.8 ± 2.5</td>
<td>4.46 ± 0.76</td>
<td>97.2</td>
<td>+75</td>
</tr>
<tr>
<td>15 Controls</td>
<td>9.9 ± 0.77</td>
<td>1.01 ± 0.02</td>
<td>10.0</td>
<td>0.01</td>
</tr>
<tr>
<td>14 Controls&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.7 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.04 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42</td>
<td>5.9</td>
</tr>
</tbody>
</table>

| B16 amelanotic melanoma |
| 9 Controls | 15.5 ± 2.5 | 0.41 ± 0.10 | 6.4 | <0.001 |
| 9 Controls | 2.3 ± 0.39 | 0.14 ± 0.03 | 66 | 0.3 | 0.05 |

<sup>a</sup> N, number of animals in each group.
<sup>b</sup> Mean ± S.E.
<sup>c</sup> Antiplatelet antibody adsorbed with tumor cells as well as RBC.
<sup>d</sup> Tumor cells (500,000) injected s.c. into flank, with animals sacrificed on Day 21.

were also obtained if an irrelevant anti-IgG antibody was used, ruling out the possibility that nonspecific immune complex formation or macrophage activation (1) might be responsible for the decrease in tumor metastases. In addition, since platelets and lymphocytes share the la antigens, it was necessary to determine whether lymphocytoxicity may have been responsible for the results. This was shown not to be the case since both antiplatelet antibody and the irrelevant anti-IgG control antibody gave similar complement-dependent lymphocytoxic responses with murine lymphocytes. An additional consideration is the possible cross-reactivity of antiplatelet antibody with endothelial cells, which might increase metastases due to endothelial cell damage. However, this would serve to decrease the inhibitory effect of antiplatelet antibody on metastases rather than be responsible for it. Similar results were obtained with Lewis lung carcinoma where antiplatelet antibody inhibited the number of tumor nodules per
lung by 57 to 78% and decreased the mean nodule volume of tumor per lung by 60 to 77%, using 48 experimental animals and 65 control animals. Antiplatelet antibody also inhibited the development of metastases from s.c. inoculation of tumor cells (42% decrease in number whereas mean nodule volume was not affected). Antiplatelet antibody also inhibited the number of metastatic B16 amelanotic melanoma tumor nodules per lung and mean tumor nodule volume per lung by 85 and 66%, respectively, using 9 experimental animals and 9 controls.

Of particular interest was the observation that a single injection of antiplatelet antibody given 6 hr prior to tumor inoculation prevented metastases, whereas a single injection of antiplatelet antibody given 6- or 18 hr post-inoculation of tumor cells did not decrease metastases. Since antiplatelet antibody has its maximum effect at 6 hr, it is likely that platelets play a role during the first 12 hr of tumor inoculation and spread and have no effect on the development of metastases from Day 2 to Day 21. The precise role of platelets during this early initiation of metastases has not yet been defined. Several possibilities are suggested. Platelets may enhance implantation of tumor cells by adhering to them in the form of a mixed-platelet tumor thrombus which can then lodge in a capillary or arteriole (31). Platelets may stabilize the initial interaction between the tumor cell and the subendothelial matrix after endothelial cell damage or separation, which may occur following the interaction of tumor cells with the endothelial cell lining (23, 31). Platelets may release platelet-derived permeability factor (24, 25) or platelet-derived growth factor (3, 4, 13, 21) which may enhance extravascular tumor cell penetration and colony formation. In this regard, it is of interest to note that antiplatelet antibody had an effect on tumor volume as well as number of tumor nodules, suggesting a possible role for platelet-derived growth factor in the contribution of platelets to metastases. The paradoxical increase in mean nodule volume per lung noted when antiplatelet antibody was given 6 and 18 hr post-injection of tumor cells for Lewis lung and 6 hr post-injection for C576 remains unexplained. It is possible that this may be due to the release of platelet-derived growth factor induced by antiplatelet antibody after the tumor has had an opportunity to lodge in the vasculature. Platelets may also shield tumor cells from cytotoxic killer cells by coating the tumor cells and blocking their antigenic determinants or receptors which are necessary for reactivity with cytotoxic killer cells.

The possible clinical therapeutic usefulness of these observations remains to be established. However, several experimental modalities should be considered. These include: the treatment of patients with agents which can safely lower their platelet counts to levels which are antimitastatic yet hemostatic; the use of antiplatelet agents or combination of antiplatelet agents which might render the platelet inert in its role as a metastatic promoter; and the use of monoclonal antibodies directed against the precise contribution of platelets to the development of metastases, possibly anti-growth factor antibody or anti-Von Willebrand factor receptor antibody. Experiments of this nature are currently in progress.

REFERENCES

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