Clinical and Immunological Significance of Human Melanoma Cytotoxic Antibody¹

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SUMMARY

The activity of a complement-dependent cytotoxic antibody in the sera of 21 melanoma patients was investigated using a microcytotoxicity assay. Heat-inactivated sera were caused to react against mechanically dispersed fresh tumor cells in the presence of exogenous blood group AB complement. Cytotoxicity was evaluated relative to pooled normal sera as a control. Sera were cytotoxic against autochthonous tumor cells in 9 of 10 patients with localized or regional melanoma and in 1 of 11 patients with disseminated metastases. Cytotoxicity of sera was unrelated to size of tumor burden. Six of 7 antibody-positive sera (autochthonous system) were noncytotoxic to between 2 and 7 different allogeneic melanoma tumor cell preparations. Immunological reactivity of the cytotoxic antibody-positive and -negative groups was similar with respect to their capacity to be sensitized to dinitrochlorobenzene, produce positive skin tests to microbial antigens, and produce antibodies to typhoid vaccination; serum immunoglobulins were comparable. These results support the reported findings of the presence of a cytotoxic antibody in the sera of melanoma patients without disseminated metastases.

INTRODUCTION

The existence of host immune response in human malignant melanoma (2, 3, 11) is now well established, indicating the presence of both cellular immunity and humoral antibodies. In 1969 Lewis et al. (4) reported on the presence of a specific autoantibody in the sera of human melanoma patients with localized or regional disease, but not in the sera of those with distant metastases. Others (9, 12, 15) have also demonstrated the presence of melanoma antibodies in human sera.

The purpose of this study was to determine the activity of serum antibody in malignant melanoma patients using a complement-dependent microcytotoxicity assay. Correlation with the presence of the antibody was sought for the clinical stage of disease, size of tumor burden, reactivity of the immune system, and response to therapy.

MATERIALS AND METHODS

In Vitro Study

Sera from melanoma patients were reacted against their own tumor cells in the presence of exogenous complement, with pooled normal sera as control using a modification of the microcytotoxicity assay described by Tagasuki and Klein (16).

Preparation of Sera. Blood collected from recurrent melanoma patients prior to surgical removal of tumor was allowed to clot; the sera were separated and heat inactivated at 56°C for 45 min. This provided the melanoma sera.

Sera from 6 healthy volunteers contributed to the normal serum pool. This was similarly heat inactivated.

Complement (C') source was a blood type AB Rh® donor previously screened and found to be noncytotoxic against melanoma cells. The same complement source was used for all assays. Complement and sera were stored in aliquots at −70°C and +4°C, respectively, until ready for use.

Processing of Tumor. Fresh tumor obtained from melanoma patients in the operating room was placed in modified Eagle's tissue culture medium supplemented with 20% FCS, 2 nonessential amino acids, penicillin, streptomycin, and kanamycin. The tumor was finely minced using scissors, and then forced through a No. 40 wire mesh sieve to produce a single-cell suspension. The freed tumor cells were then washed 3 times with media. Cell viability was assessed by the Trypan blue exclusion technique. Cell concentrations were next adjusted to 1 x 10⁶ and 5 x 10⁶ viable cells/ml.

Microassay Method. Falcon No. 3034 microtest plates were used for the microassay. A separate plate was used for each tumor concentration (500 or 1000 cells/reaction well).

Tumor cells were randomly pipetted into the reaction wells of the microtest plate by a 10-μl Centaur micropipet. To the tumor cells in each of 6 duplicate reaction wells, a 10-μl volume of the following undiluted sera and complement mixtures was added: (a) 5 μl control (pooled normal) sera + 5 μl C'; (b) 5 μl autochthonous sera + 5 μl C'; (c) 5 μl autochthonous sera + 5 μl heat-inactivated complement

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¹ The abbreviations used are: FCS, fetal calf serum; DNCB, dinitrochlorobenzene.
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(dC); (d) 5 µl allogeneic melanoma sera + 5 µl C': (e) 10 µl
20% FCS. Plates were gently shaken and then incubated for
40 hr in a 5% CO₂, humidified, 37° incubator and then for
4 hr in an inverted position. The adherent cells were then
washed twice with modified Eagle's medium, fixed in
Bouin's solution, stained with Giemsa, and examined visu-
ally under 100× magnification. The microtest plate show-
ing the better distribution of cells was then counted.

Criteria for Analysis. The mean cell count for the 6
duplicate wells exposed to autochthonous sera plus C' was
compared to that for the control (pooled normal) sera plus
C'. Assuming the criteria of Lewis et al. (4), 20% or greater
reduction in the former was considered as positive for the
presence of the cytotoxic antibody. However, this was
accepted only if analysis by Mann-Whitney U test was
significant with p < 0.05.

The reaction wells exposed to autochthonous sera plus
ΔC, and to allogeneic sera plus C' served as controls for the
complement dependency and cross-reactivity, respectively,
of the reaction. The mean number of tumor cells counted in
the 6 duplicate wells exposed to 20% FCS were assumed to
represent an index of plating efficiency.

Cross-reactivity among melanomas was further explored
by reacting 7 autochthonous positive sera in the presence of
exogenous complement against 2 to 7 different allogeneic
melanoma tumor preparations using pooled normal sera as
control.

In Vivo Study

Clinical Staging. Melanoma patients were evaluated to
determine extent and distribution of malignant disease by
appropriate clinical studies. Patients were classified clini-
cally as regional disease if the tumor had not spread beyond
the primary lymph node draining area; patients with
extension of disease beyond the primary lymph node
draining area or visceral involvement were classified as
dissemi nated metastases.

Tumor Burden. Tumor burden (g) was estimated by
assuming the tumor density to be 1 and all lesions to be
spheroid. Volume (V) was then determined by the formulae
V = 4/3 π r³ or V = 4/3 π r₁ r₂ r₃.

Immune Status. The functional status of the immune
system was assessed by: (a) sensitization and subsequent
challenge with topical DNCB; (b) response to a battery of 6
microbial skin test antigens (purified protein derivative of
tuberculin, Candida, Trichophyton, mumps, histoplasmin,
and coccidiodin) at 48 hr; (c) typhoid O and H antibody
response between 14 and 21 days, after a single 0.5-ml i.m.
injection of typhoid vaccine; and (d) quantitation of serum
immunoglobulins.

Response to Therapy. Patients with metastatic melanoma
were treated by either conventional chemotherapy (with
dimethyltriazenoimidazole carboxamide or bischloroethyl-
nitrosourea plus vincristine) or investigational immuno-
therapy. Two modalities of immunotherapy were used. Patients
with intradermal disease received intralesional injection of
Bacillus Calmette-Guérin; those patients with s.c. or nodal
disease received intraarteral injections of irradiated autoch-
thonous tumor cells plus B. Calmette-Guérin. Responders
were classified as those patients with diminution of tumor
burden >50% and of more than 2 months' duration with
no evidence of new disease.

RESULTS

In Vitro Assay

Twenty-six melanoma patients were studied. The assays on
5 of these patients were not evaluable because of failure of
the tumor cells to plate adequately. Tumor suspension that
plated adequately showed a plating efficiency of approxi-
ately 10 to 20%. The results of the 21 evaluable patients
are shown in Table 1. Of these 21, 10 were positive for the
presence of the cytotoxic serum antibody (>20% reduction
in target cells relative to control, p < 0.05) and the other 11
were negative. The mean target cell count of wells exposed
to autochthonous sera plus heat-inactivated complement
were comparable to the counts for the controls (p > 0.05).
Similarly, the mean cell count of wells exposed to the
allogeneic sera was comparable to the controls (p > 0.05).

In the cross-reactivity study (Table 2) 6 of 7 autochthon-
ous, cytotoxic, antibody-positive sera were noncytotoxic to
between 2 and 7 different allogeneic melanoma tumor
preparations (p > 0.05); the other sera were cytotoxic
against 3 different allogeneic tumor preparations (p <
0.05). The incidence of cross-reactivity in this study was
14%.

Clinical Correlations (Table 3)

When the clinical status of the 21 evaluable patients was
documented, 10 were classified as regional disease. Nine of
these 10 patients had a positive serum antibody assay. Of
the 11 patients classified clinically as disseminated metas-
tases, only 1 was positive for the antibody.

The mean tumor burden for the groups of patients
cytotoxic antibody-positive and -negative were 83 and 112 g,
respectively. Statistical analysis (Mann-Whitney U test)
showed no significant difference between the groups. Three
of 10 antibody-positive and 5 of 11 antibody-negative
patients were calculated to have modest tumor burdens of
20 g or less.

An analysis of the pretreatment status of the immune
system of the cytotoxic antibody-positive and -negative patient
groups showed no significant difference in the following
parameters: response to DNCB sensitization; delayed cutaneous hypersensitivity response to microbial
antigens; typhoid antibody response; and serum immuno-
globulin quantitative analysis (Table 3).

The presence of the cytotoxic antibody appeared to be a
discriminant in predicting response to immunotherapy.
Four of 6 antibody-positive patients responded to immuno-
therapy as opposed to 1 of 7 antibody-negative patients.
Only 1 of 4 antibody-positive and 0 of 4 antibody-negative
patients responded to chemotherapy. The cytotoxic anti-
body-positive patients showed an increased survival when
Table 1

Cytotoxic antibody assay

Autochthonous sera and exogenous complement (C'), autochthonous sera + heat-inactivated complement (ΔC), allogeneic melanoma sera + C', and pooled NHS° + C' were reacted against melanoma cell suspensions. Cell counts for 6 duplicate wells exposed to autochthonous sera + C' were compared to that for NHS + C' control by the Mann-Whitney U test. Cytotoxic antibody test considered positive if p < 0.05.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage of disease</th>
<th>Mean no. of cells in autochthonous sera + C'</th>
<th>Mean no. of cells in NHS + C'</th>
<th>Mean no. of cells in autochthonous sera + ΔC</th>
<th>Mean no. of cells in allogeneic sera + C'</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-6a</td>
<td>R</td>
<td>34 ± 16</td>
<td>76 ± 13</td>
<td>70 ± 14</td>
<td>67 ± 10</td>
<td>55</td>
</tr>
<tr>
<td>M-31</td>
<td>R</td>
<td>16 ± 14</td>
<td>84 ± 12</td>
<td>77 ± 8</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>M-57</td>
<td>R</td>
<td>25 ± 9</td>
<td>46 ± 7</td>
<td>45 ± 7</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>M-57</td>
<td>R</td>
<td>22 ± 6</td>
<td>46 ± 7</td>
<td>45 ± 7</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>M-59</td>
<td>M</td>
<td>32 ± 9</td>
<td>97 ± 9</td>
<td>93 ± 14</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>M-65</td>
<td>R</td>
<td>115 ± 19</td>
<td>174 ± 37</td>
<td>184 ± 30</td>
<td>164 ± 22</td>
<td>34</td>
</tr>
<tr>
<td>M-75</td>
<td>R</td>
<td>84 ± 11</td>
<td>160 ± 15</td>
<td>141 ± 12</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>M-75</td>
<td>5/73</td>
<td>91 ± 8</td>
<td>160 ± 15</td>
<td>141 ± 12</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>M-82</td>
<td>R</td>
<td>185 ± 23</td>
<td>245 ± 27</td>
<td>232 ± 26</td>
<td>218 ± 22</td>
<td>31</td>
</tr>
<tr>
<td>M-101</td>
<td>R</td>
<td>58 ± 5</td>
<td>84 ± 9</td>
<td>88 ± 7</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>M-124</td>
<td>R</td>
<td>34 ± 16</td>
<td>76 ± 13</td>
<td>70 ± 14</td>
<td>72 ± 12</td>
<td>55</td>
</tr>
<tr>
<td>M-196</td>
<td>R</td>
<td>118 ± 17</td>
<td>213 ± 24</td>
<td>207 ± 32</td>
<td>195 ± 14</td>
<td>45</td>
</tr>
</tbody>
</table>

Cytotoxic antibody-positive patients (p < 0.05)

Cytotoxic antibody-negative patients (p > 0.05)

* NHS, normal human sera; R, regional disease (tumor confined to primary lymph node draining area); M, disseminated metastases (extension beyond primary lymph node draining area or visceral metastases).

% cytotoxicity = (mean no. of cells in NHS + C' — mean no. of cells in autochthonous sera + C' x 100)/(mean no. of cells in NHS + C').

The presence of a complement-dependent cytotoxic antibody in the sera of 9 of 10 melanoma patients without visceral metastases confirms the initial findings of Lewis et al. (4). The negative cytotoxic antibody assay in one of the above group of patients was obtained from a patient with extensive chest wall recurrence, who subsequently developed visceral metastases and died within 2 months of the date of assay. At the time of assay this patient probably had microscopic visceral metastases not detectable by clinical investigation. This phenomenon of a negative antibody assay preceding the development of clinically detectable visceral metastases has been previously described (5).

The lack of correlation of the presence of antibody with size of tumor burden argues against the concept of the mass in disseminated tumor acting as an antigenic "sponge" soaking up all available antibody. In patients with regional disease, antibody was present through a wide range of tumor burdens from 2 to 200 g. In addition, only 3 of 8 patients with tumor burdens of 20 g or less were antibody positive. These findings support the concept that the presence of antibody was related to clinical stage of disease and not to size of tumor burden. The inability to detect the cytotoxic antibody in the sera of patients with disseminated metastases has been shown to be due to the production of a blocking substance causing inactivation of the tumor-specific antibody (7).

A number of workers (8, 9, 13) have demonstrated prognostic significance for melanoma patients capable of expressing a delayed hypersensitivity response. In this investigation.
Table 2

**Cross-reactivity cytotoxic antibody**

Previously tested autochthonous positive sera were reacted against different allogeneic melanoma tumor cell suspensions in the presence of exogenous complement (C'). Cell counts for 6 duplicate wells exposed to autochthonous positive sera + C' were compared to that for pooled normal human sera + C' control by the Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Autochthonous positive sera</th>
<th>Tumor cell suspension</th>
<th>Mean no. of cells in autochthonous serum + C'</th>
<th>Mean no. of cells in pooled normal human serum + C'</th>
<th>Mann-Whitney U (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-57</td>
<td>M-65</td>
<td>164 ± 22</td>
<td>174 ± 37</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-57</td>
<td>M-129</td>
<td>84 ± 19</td>
<td>93 ± 15</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-59</td>
<td>M-31</td>
<td>46 ± 14</td>
<td>84 ± 12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M-59</td>
<td>M-65</td>
<td>103 ± 11</td>
<td>174 ± 37</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M-59</td>
<td>M-82</td>
<td>158 ± 16</td>
<td>245 ± 26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M-65</td>
<td>M-6b</td>
<td>71 ± 20</td>
<td>70 ± 14</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-65</td>
<td>M-57</td>
<td>47 ± 13</td>
<td>46 ± 12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-6b</td>
<td>66 ± 9</td>
<td>70 ± 14</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-57</td>
<td>42 ± 10</td>
<td>46 ± 12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-80</td>
<td>120 ± 12</td>
<td>118 ± 13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-115</td>
<td>50 ± 3</td>
<td>43 ± 5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-118</td>
<td>113 ± 7</td>
<td>121 ± 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-119</td>
<td>114 ± 6</td>
<td>123 ± 9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-75</td>
<td>M-196</td>
<td>195 ± 14</td>
<td>213 ± 24</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-82</td>
<td>M-6b</td>
<td>63 ± 5</td>
<td>70 ± 14</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-82</td>
<td>M-57</td>
<td>45 ± 7</td>
<td>46 ± 12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-82</td>
<td>M-80</td>
<td>116 ± 5</td>
<td>118 ± 13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-82</td>
<td>M-132</td>
<td>104 ± 17</td>
<td>108 ± 22</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-101</td>
<td>M-6b</td>
<td>88 ± 15</td>
<td>89 ± 25</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-101</td>
<td>M-57</td>
<td>46 ± 7</td>
<td>46 ± 12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-101</td>
<td>M-80</td>
<td>120 ± 3</td>
<td>118 ± 13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-101</td>
<td>M-115</td>
<td>49 ± 9</td>
<td>43 ± 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-101</td>
<td>M-119</td>
<td>117 ± 7</td>
<td>123 ± 9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-124</td>
<td>M-6b</td>
<td>67 ± 10</td>
<td>70 ± 14</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-124</td>
<td>M-57</td>
<td>38 ± 9</td>
<td>46 ± 12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-124</td>
<td>M-80</td>
<td>114 ± 8</td>
<td>118 ± 13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-124</td>
<td>M-82</td>
<td>218 ± 22</td>
<td>244 ± 27</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>M-124</td>
<td>M-132</td>
<td>110 ± 27</td>
<td>108 ± 22</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Table 3

**Clinical correlation for presence of cytotoxic antibody**

The relationship between the cytotoxic antibody-positive and -negative patients is shown for the clinical parameters studied.

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>Antibody-positive</th>
<th>Antibody-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regional</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Disseminated</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Tumor burden (g)</td>
<td>83</td>
<td>112</td>
</tr>
<tr>
<td>Mean</td>
<td>2-200</td>
<td>4-500</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNCB (positive)</td>
<td>9/10</td>
<td>11/11</td>
</tr>
<tr>
<td>Microbial antigens (positive)</td>
<td>5/9</td>
<td>8/11</td>
</tr>
<tr>
<td>Typhoid antibody (positive)</td>
<td>7/10</td>
<td>6/10</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I_G</td>
<td>3/10I</td>
<td>1/111/3II</td>
</tr>
<tr>
<td>I_A</td>
<td>2/10I</td>
<td>1/111/1II</td>
</tr>
<tr>
<td>I_M</td>
<td>2/10F</td>
<td>2/10F</td>
</tr>
<tr>
<td>Responders to treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>4/6</td>
<td>1/7</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>1/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

In this study, the presence of the cytotoxic antibody was unrelated to the functional status of the immune system of the patients studied. However, the patients who were cytotoxic antibody positive showed an increased survival. This was not an unexpected finding since the antibody-positive patients were shown to represent the patient group with nonvisceral metastatic disease.

The reported cross-reactivity of the cytotoxic antibody demonstrated in malignant melanoma patients has varied with the method used. In a study of membrane immunofluorescence Lewis et al. (4, 6) demonstrated a lack of significant cross-reactivity with other melanoma sera, whereas with cytoplasmic immunofluorescence (14) multiple cross-reactivity occurred between the sera and cells of several patients. Other investigators (8, 12) have shown cross-reactivity using both cytoplasmic and membrane immunofluorescence techniques. In this limited study using a complement-dependent cytotoxicity assay, a low incidence of cross-reactivity was found. Only 1 of 7 tested sera cross-reacted with other melanomas. The specificity of the cytotoxic antibody reaction requires confirmation by testing against patients with other types of cancer and pigmented skin lesions. However, other workers (1, 10) have shown a high degree of specificity for the cytoplasmic antigen in malignant melanomas in studies of patients with pigmented and nonpigmented skin lesions or cancers other than melanoma.
REFERENCES


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