Circulating Immune Complexes and Tumor Cell Cytotoxins as Prognostic Indicators in Malignant Melanoma: A Prospective Study of 53 Patients


ABSTRACT

To evaluate the relationship between tumor burden and circulating immune complexes (IC) in malignant melanoma, we tested sera collected serially from 15 normal donors and 53 patients. Forty-eight of these had Stage III or IV disease at the outset of the study. The median survival time (MST) of ten patients with Stage IV disease whose sera contained C1q-binding IC at the outset of the study was 4.7 months; the MST of the 25 Stage IV patients whose sera were initially free of IC by this test was 8.65 months (p < 0.02). C1q-binding IC were not found in the initial serum samples from 13 patients with Stage III or 5 patients with Stage I disease. Abnormal C1q binding tests were measured in 4 of 67 sera (6%) from 13 patients who remained free of evident tumor for up to 41 months. IC were detected in 13 of 39 sera (33%) from 19 patients with progressively growing tumors and in 21 of 68 sera (31%) from 21 patients who were initially free of disease but developed recurrences later, or who had significant remissions of variable duration during follow-up. The MST of 31 patients whose serum samples remained free of C1q-binding IC was 15.8 months. Twelve patients whose sera were initially free of circulating IC later developed abnormal serum C1q-binding levels. Their MST was 10.3 months. The MST of ten patients with persistently abnormal serum IC levels was 4.7 months. C1q-binding IC were reciprocally related to the presence of complement-dependent antibodies, cytotoxic for cultured allogeneic malignant melanoma cells in sera from 29 of these patients (r = -0.491; p = 0.003). These results suggest that the appearance of circulating C1q-binding IC is pathophysiologically important in malignant melanoma. Measurement of C1q-binding IC may be useful in assigning prognosis in this disease.

INTRODUCTION

IC* have been found in sera of patients with lung cancer, leukemia, neuroblastoma, and many other cancers (2, 3, 5, 8–11, 13, 16–23). In some, the quantity of circulating IC appears to be related to the tumor burden (2, 3, 5, 9, 19, 24). In others, measurement of serum IC does not appear to relate to tumor burden or prognosis (8, 16, 17, 19, 24). There may be a number of reasons for these differences: (a) certain tumors may be poorly immunogenic; consequently, IC that appear in the serum may be formed in great antigen excess, causing aggregates of immunoglobulin so small that they escape detection by the majority of antigen-nonspecific methods; (b) in other cases, antigen may be shed only intermittently. One may fail to detect resulting IC unless these are sought repeatedly; (c) only certain of the techniques for measuring IC may be suitable for detecting complexes that reflect the host response to tumors. Other methods, although sensitive to the presence of heat-aggregated IgG or other laboratory standards, may preferentially detect IC that represent immune responses that have little if anything to do with the tumors. Indeed, few studies have correlated IC measurements with measurements of the humoral immune response to antigens commonly found in human tumors (18).

This is a report of a prospective study of 53 patients tested repeatedly for circulating IC by the fluid-phase C1q-binding test (26) and the Raji cell RIA (24) while undergoing treatment for malignant melanoma. Selected sera from these patients were assayed for complement-dependent antibodies cytotoxic for cultured allogeneic malignant melanoma cells (14, 15, 25) to evaluate whether the development of a tumor and the appearance of IC affect the normally hidden or blocked antitumor antibodies that are found in sera of many normal donors (14, 15).

MATERIALS AND METHODS

Recruitment of Patients. Fifty-three outpatients with histologically documented malignant melanoma were tested at monthly intervals until they were discharged from the clinic. Control sera were obtained from 179 normal blood bank donors and from 13 patients with rheumatoid factor-positive rheumatoid arthritis. Serial blood samples were also collected from 15 healthy laboratory and clerical workers at weekly intervals to evaluate how much fluctuation in serum immune complex levels normally occurs. Serum from 12 ml of blood was stored in 1-ml aliquots at −70°.

Evaluation of Patients. Patients were staged as follows: Stage I, primary malignant melanoma of any thickness without dissemination; Stage II, primary tumor with one or more satellite lesions within 3 cm of the primary; Stage IIIA, in-transit metastases <3 cm from the primary; Stage IIIB, one or more regional node metastases; Stage IIIA/B, both in-transit and regional node metastases; and Stage IV, distant metastases beyond the regional nodes. In addition, when patients were first seen, they were classified as having or not having evident disease (ED...
versus NED) (19). On each subsequent visit, they were evaluated and categorized as having remained: (a) NED; (b) stable (no new lesions, <25% increase or 50% decrease in tumor size since last visit); or (c) progressed (a >25% increase in previously recognized tumor or development of one or more new lesions). Clinical evaluations were performed without knowledge of results of immune complex or antitumor antibody measurements.

**Laboratory Studies.** Soluble IC were measured by the C1q binding test (EDTA-treated sera) (26) and by the Raji cell RIA (24). Results were expressed as percentage of C1q bound (C1q binding test) or as μg equivalents of HAGG per ml in the Raji cell RIA. Duplicate or triplicate aliquots of each sample were tested. Each test included 30 to 60 sera, including samples from age-matched healthy donors (negative controls) and sera containing high levels of IC from patients with rheumatoid arthritis. In addition, serial dilutions of HAGG were tested to provide a standard curve (19, 24). In each run, unheated aliquots of sera tested previously for IC were tested again to be certain that results of the assay of that day were comparable to results obtained previously.

Serum alkaline phosphatase and glutamic-oxaloacetic transaminase levels were measured by established methods adapted to autoanalyzer.

**Cytotoxic Antibody to Malignant Melanoma Cells.** Sera from 29 patients were chosen from among the unheated samples remaining after IC measurements had been done. These sera encompassed a wide range of immune complex levels. They were analyzed for complement-dependent antibodies cytotoxic to malignant melanoma cells using the CAR 1 established tumor cell line as the target (14, 15). Antibody was measured by adding 25 μl of undiluted, heat-inactivated serum to 1 x 10⁵ tumor cells in 50 μl of MEM containing 25% FBS. Samples were incubated for 18 hr at 37° with 25 μl guinea pig complement previously titrated and used at a final dilution of 1:8 in MEM. During the final 30 min, 125 μl of Pronase at 5 mg/ml in phosphate-buffered saline (pH 7.4) were added to enhance cytolysis of damaged cells. Surviving cells were enumerated by hemocytometer in triplicate; percentage of cytotoxicity was calculated using:

\[
\frac{a - b}{a} \times 100
\]

where a is the number of living tumor cells in a sample that contained heat-inactivated guinea pig serum and b is the number of cells remaining after incubation in complement (15).

Some sera were tested for cytotoxic antibody with and without added CF II or ACF. ACF is an IgG anti-immunoglobulin that unblocks hidden complement-dependent cytotoxic antibodies in normal donor sera (15). In assays incorporating ACF or CF II, 25 μl of heat-inactivated test serum were mixed either with 25 μl of MEM containing 25% FBS or with 25 μl of a heat-inactivated serum containing ACF or CF II (14, 15). Tumor cells (1 x 10⁵) were then added in 50 μl of MEM with 25% FBS. Finally, 25 μl of appropriately diluted guinea pig serum were added as a complement source. The mixture was incubated at 37° for 90 min. Pronase was added, as above, for 30 min before counting the surviving cells. Samples to which heat-inactivated guinea pig serum had been added served as a negative control. In these tests, serum (diluted 1:8 in MEM with 25% FBS) from a patient with rheumatoid arthritis provided the ACF. By itself, ACF was not cytolytic for these tumor cells (15). CF II, as reported previously (14, 15), provided a source of hidden natural cytotoxic antibodies in complexes that contained anti-immunoglobulins that blocked the ability of these cytotoxins to react with the surface of the tumor cells. The CF II was added to sera from the malignant melanoma patients to see if these blocking anti-immunoglobulins would also interfere with the activity of the free cytotoxic antibodies in the sera of these patients.

**Statistical Comparisons.** Tests of statistical significance were carried out using the Clinfo project. The odds ratio was used to calculate the likelihood that an abnormal serum test was associated with tumor recurrence or progression. The odds ratio is an approximation of the relative risk that registers the occurrence of a risk factor in one group relative to its occurrence in another. The odds ratio is considered to be significant at the 5% level if the 95% confidence interval does not include 1.

**RESULTS**

**Characteristics of the Study Population.** One hundred seventy-four sera collected from 53 patients over an average interval of 9.3 months (range, 2 to 41 months; median, 5 months) were analyzed for IC. Two serum samples from bleedings obtained more than 1 month apart were available from 24 patients; 3 samples spaced at greater than 1-month intervals were available from 14 patients, and multiple samples taken at these or greater intervals were collected from 15 patients. After collection of these sera, the patients were followed for an additional 2 years to evaluate the outcome of their disease. In age, sex, and prior treatment, these 53 patients appeared to be representative to the population with malignant melanoma evaluated at this referral center during the 2 years prior to this study.

Five of the 53 patients were Stage I, 13 were Stage III, and 35 were Stage IV when they entered the study. Three Stage I patients remained free of recurrence during 15 months of follow-up. Two developed recurrences during the 14th and 15th months of observation, respectively. Seventeen of the Stage III or IV patients were NED at the beginning, and 10 remained NED throughout the study. Nineteen patients had progressively growing tumors. Significant changes in tumor burden were observed in 21 patients. Nine were initially NED but developed recurrences. Four ED patients eventually became free of disease during treatment. The other 8 patients had temporary remissions, followed by recurrences for which they were treated again. In some cases, all evidence of tumor was removed, and they remained NED for the duration of the study. In others of this group, treatment only stabilized the tumor. In 11 of the 21 patients, recurrence or accelerated growth of the neoplasm was noted at the time of the last examination. These 21 patients were classified as having "fluctuating" or "changing" tumor burdens for the purposes of this study and were particularly noteworthy because in this group we could determine whether changes in serum IC corresponded in direction and magnitude to observed changes in tumor burden.

**Serial Measurements of IC: Assessment of Expected Within-Person Variation in Normal Donors.** IC measurements were made by the C1q binding test and Raji cell RIA on samples drawn at weekly intervals from 15 normal donors (Table 1). By the C1q binding test, there was little variation among samples from a single individual, but there was considerable variation among donors. Variation among C1q binding measurements from a single donor went from 0.8 to 4.0% (range); 0.3 to 1.8% (1 S.D.). The 95th percentiles for the range and S.D. were, respectively, 3.5 and 1.7%. The upper boundary for normal fluctuation in serum C1q-binding activity was considered to be 3.3%, 1.96 times 1.7%, the 95th percentile of the S.D. We concluded from these results that a change in serum C1q-binding activity between 3 and 4% was probably significant. Therefore, we chose to test the hypothesis that an increase or decrease in serum C1q binding >3% might represent biologically significant change when correlated with interval change in tumor burden.

In contrast to the experience with the C1q binding test, there was considerable variation within and among Raji cell RIA

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**Immune Complexes as Indicators in Malignant Melanoma**
Table 1

<table>
<thead>
<tr>
<th>Wk 1</th>
<th>Wk 2</th>
<th>Wk 3</th>
<th>Wk 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>% of C1q-binding activity</td>
<td>Raji cell RIA (µg HAGG equivalents)</td>
<td>% of C1q-binding activity</td>
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<tr>
<td>1</td>
<td>6.3</td>
<td>8.3</td>
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</tr>
<tr>
<td>2</td>
<td>5.5</td>
<td>14.4</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
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<tr>
<td>4</td>
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<tr>
<td>6</td>
<td>1.4</td>
<td>&lt;8</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td>5.2</td>
<td>&lt;8</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>2.9</td>
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<td>2.5</td>
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<td>9</td>
<td>4.4</td>
<td>&lt;8</td>
<td>2.6</td>
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<td>2.9</td>
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</tr>
<tr>
<td>12</td>
<td>5.2</td>
<td>&lt;8</td>
<td>3.0</td>
</tr>
<tr>
<td>13</td>
<td>6.2</td>
<td>49.6</td>
<td>3.1</td>
</tr>
<tr>
<td>14</td>
<td>1.7</td>
<td>&lt;8</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>2.9</td>
<td>&lt;8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Numbers in parentheses, range.
* ND, not done.
* C1q-binding activity 5.6 ± 4.2 ± 2.3 ± 1.7 ± 7.0 ± 3.7 ± 6.9 ± 2.2 ± 3.8 ± 1.8 ± 3.3 ± 4.9 ± 1.3 ± 2.6

results from single normal donors (Table 1). In 5 of the 15 normal donors, serial samples showed week-to-week fluctuations in Raji cell RIA results ≥27 µg HAGG per ml. This was not due to any lack of reproducibility in the Raji cell assay, since the variance of replicate samples in any test did not exceed 4% of the mean.

Analysis of a separate control group of 179 sera from blood bank donors showed that 95% had C1q-binding activities <6.2%. A comparable value for the Raji cell RIA was <8 µg HAGG per ml.

Analysis of IC Measurements in Patients with Malignant Melanoma. The MST of patients whose sera contained C1q-binding IC at the beginning of the study was 4.7 months, whereas the MST of those whose sera lacked C1q-binding IC was 14.1 months (Chart 1; p = 0.004, generalized Wilcoxon test for comparing 2 life tables). The Raji cell RIA, in contrast, was not effective in identifying those with an especially poor prognosis (Chart 2). Moreover, with these patients’ sera there was no correlation between IC measurements obtained with the Raji cell RIA and those obtained with the C1q binding test (Chart 3). Consequently, only the C1q binding test was used in subsequent analyses.

All 10 of those who had circulating C1q-binding IC when they entered the study had Stage IV disease. Their MST (4.7 months) was significantly less than that of the other 25 Stage IV patients (8.65 months) whose serum C1q-binding activity was <6% (Table 2A). Notably, the MST of those whose serum C1q-binding activity remained <6% was 15.8 months (Table 2B). The MST of those whose serum C1q-binding activity later increased to abnormal (>6%) levels was 10.3 months, whereas the MST of those who persistently had circulating C1q-binding IC was 4.7 months (Table 2B).

Ninety-four % of sera from the 13 patients who remained NED did not contain C1q-binding IC. In contrast, 31% of samples from patients with changing tumor burdens and 33% of those from patients with progressively growing tumors had C1q-binding activities >6% (Table 3). An interval increase or decrease in serum C1q-binding activity >3% was also measured in one-third of the serial samples collected from patients with changing tumor burdens (Table 3, Group 2) and from nearly one-half of the sera from patients with progressively growing tumors (Table 3, Group 3). Treating these serial measurements as independent events, both the number of samples with abnormal C1q-binding activity and the number of pairs which differed by >3% were significantly increased in patients in Groups 2 and 3 as compared to those in Group 1 (Table 3) (χ² analysis, 2 d.f., p < 0.006).
Serial measurements of serum C1q-binding activity did not change necessarily in the same direction as tumor burden (Table 3). For example, in the 21 patients (Table 3, Group 2) whose tumors changed significantly in size during the study, a change in serum C1q-binding activity >3% was observed 16 times in 48 paired observations separated by 1 or more months. Ten of these changes in serum C1q-binding activity were associated with an increase, and 6 were associated with a decrease in tumor burden. In 6 cases, the direction of change in serum C1q-binding activity was opposite to the change in tumor burden. Notably, a >3% increase in C1q-binding activity was associated with a significant decrease in tumor burden on 4 of 6 occasions. Similarly, serum C1q-binding activity changed by >3% in only 10 of the 21 paired observations in patients with progressively growing tumors. During 6 of these intervals, the serum C1q-binding activity increased; in 4 cases, it decreased.

While a serum C1q-binding activity >6% or an interval change in this measurement of >3% was significantly associated with evidence of disease, it should be emphasized that the absence of these serological abnormalities did not necessarily assure a benign outlook. In patients with continually progressive disease or those whose tumor began to grow again after a remission, 66 to 69% of sera had C1q-binding activities of <6%; and in over one half of the paired observations, the interval change in C1q-binding activity was <3%.

**Measurements of C1q-Binding IC as a Prognostic Indicator in Malignant Melanoma.** The relationship between tumor burden and serum C1q-binding activity was also evaluated by calculating the likelihood of having an abnormal C1q binding test, given that there was residual tumor (Table 4). The odds ratio, measuring the association between a high (>6%) serum C1q binding test and evidence of residual tumor, exceeded 8 at the beginning of the study. Thereafter, the odds that subsequent changes in serum C1q-binding activity would deviate by >3% from the initial measurement were also highly associated with evidence of residual tumor (Table 4).

Measurements of serum glutamic-oxaloacetic transaminase and alkaline phosphatase also give useful information concerning prognosis in malignant melanoma (5). In comparison with these serum enzyme measurements, the C1q binding test

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**Table 2**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Initial serum C1q-binding activity</th>
<th>No. at stage of disease</th>
<th>Median survival (mo.)</th>
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<tr>
<td></td>
<td>&lt;6%</td>
<td>&gt;6%</td>
<td>MST (mo.)</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>5</td>
<td>0</td>
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<td></td>
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<tr>
<td>III</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>35</td>
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<td></td>
<td>35</td>
<td>25</td>
<td>0</td>
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</tbody>
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**Table 3**

<table>
<thead>
<tr>
<th>Tumor burden groups</th>
<th>No. of patients</th>
<th>Follow-up (mos.)</th>
<th>Total</th>
<th>No. with C1q binding activity &gt;6%</th>
<th>Total</th>
<th>Change in serum C1q binding activity</th>
<th>No. of paired observations at intervals &gt;1 mo.</th>
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</thead>
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<tr>
<td>Remained NED</td>
<td>13</td>
<td>18.1 ± 12.7^c (2-41)^c</td>
<td>67</td>
<td>4 (8)%</td>
<td>54</td>
<td>&lt;3%</td>
<td>48</td>
</tr>
<tr>
<td>Changing tumor burden</td>
<td>21</td>
<td>7.6 ± 5.0 (2-17)</td>
<td>68</td>
<td>21 (31)</td>
<td>48</td>
<td>&gt;3% increase</td>
<td>16</td>
</tr>
<tr>
<td>Increase</td>
<td>16</td>
<td>4.6 ± 5.4 (2-32)</td>
<td>39</td>
<td>13 (33)</td>
<td>21</td>
<td>&gt;3% decrease</td>
<td>11</td>
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<tr>
<td>Decrease</td>
<td></td>
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<tr>
<td>Progressive tumor growth</td>
<td>19</td>
<td>4.6 ± 5.4 (2-32)</td>
<td>39</td>
<td>13 (33)</td>
<td>21</td>
<td></td>
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</tr>
</tbody>
</table>

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^c Z = 2.065; p < 0.02.
^d Difference between Groups I and II: Z = 0.895; p = 0.190.
^e Difference between Groups II and III: Z = 2.396; p = 0.009.
^f Difference between Groups I and III: Z = 2.805; p = 0.003.
Table 4
Comparison of the C1q binding test and the serum enzymes, GOT* and APT, as prognostic indicators in malignant melanoma

<table>
<thead>
<tr>
<th></th>
<th>ED</th>
<th>NED</th>
<th>Sensitivity a/(a+b)</th>
<th>Specificity c/(c+d)</th>
<th>False negatives b/(a+b)</th>
<th>False positives c/(c+d)</th>
<th>Predictive values</th>
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<tr>
<td>C1q binding</td>
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<td></td>
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</tr>
<tr>
<td>&gt;6%</td>
<td>9 (a)</td>
<td>1 (c)</td>
<td>29</td>
<td>95</td>
<td>71</td>
<td>5</td>
<td>90</td>
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<td>&lt;6%</td>
<td>22 (b)</td>
<td>21 (d)</td>
<td>65</td>
<td>42</td>
<td>35</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>GOT*</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>&gt;20 miU</td>
<td>17 (a)</td>
<td>11 (c)</td>
<td>81</td>
<td>37</td>
<td>19</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>&lt;20 miU</td>
<td>9 (b)</td>
<td>8 (d)</td>
<td>81</td>
<td>37</td>
<td>19</td>
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<tr>
<td>APT*</td>
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<td>&gt;64 miU</td>
<td>21 (a)</td>
<td>12 (c)</td>
<td>81</td>
<td>37</td>
<td>19</td>
<td>63</td>
<td>64</td>
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<td>7 (d)</td>
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<tr>
<td>&gt;3%</td>
<td>11 (a)</td>
<td>2 (c)</td>
<td>41</td>
<td>92</td>
<td>59</td>
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<td>85</td>
</tr>
<tr>
<td>&lt;3%</td>
<td>16 (b)</td>
<td>24 (d)</td>
<td>78</td>
<td>48</td>
<td>22</td>
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<td>GOT*</td>
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</tr>
<tr>
<td>&gt;20 miU</td>
<td>14 (a)</td>
<td>13 (c)</td>
<td>89</td>
<td>44</td>
<td>11</td>
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<td>&lt;20 miU</td>
<td>4 (b)</td>
<td>12 (d)</td>
<td>89</td>
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<td>&gt;64 miU</td>
<td>16 (a)</td>
<td>14 (c)</td>
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<td>11 (d)</td>
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<td>2 (c)</td>
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<td>85</td>
<td>44</td>
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<td>11 (d)</td>
<td>62</td>
<td>50</td>
<td>38</td>
<td>50</td>
<td>53</td>
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</tr>
<tr>
<td>&gt;20 miU</td>
<td>8 (a)</td>
<td>7 (c)</td>
<td>62</td>
<td>50</td>
<td>38</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>&lt;20 miU</td>
<td>5 (b)</td>
<td>7 (d)</td>
<td>62</td>
<td>50</td>
<td>38</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>APT*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;64 miU</td>
<td>12 (a)</td>
<td>5 (c)</td>
<td>92</td>
<td>64</td>
<td>8</td>
<td>36</td>
<td>71</td>
</tr>
<tr>
<td>&lt;64 miU</td>
<td>1 (b)</td>
<td>9 (d)</td>
<td>92</td>
<td>64</td>
<td>8</td>
<td>36</td>
<td>71</td>
</tr>
</tbody>
</table>

**Note:** All values shown x 100 as a percentage.

* a 2x2 contingency table test; d.f. = 1; >3.84 is significant at the 5% level.

---

provided a more specific, although less sensitive prognostic test. At the initial testing, this IC assay appeared to be the most useful of these 3 measurements. During the second and subsequent months of the study, the sensitivity of the C1q binding test improved with little loss in specificity (Table 4). The serum alkaline phosphatase also became a better indicator of progressive or recurrent disease in later follow-up periods.

**Serum Cytotoxins for Malignant Melanoma and Circulating IC.** To further evaluate the biological significance of an abnormal C1q binding test, we measured the relationship between IC and the presence in sera of antibodies that are cytotoxic for allogeneic malignant melanoma cells. We postulated that the C1q binding test might reflect perturbations in the levels of one or the other component of a naturally occurring 26 to 28S immune complex (14, 15). This complex is frequently found in normal donor sera. It contains antibodies cytotoxic for a number of cultured human tumor and fetal cells as well as blocking antibodies which interfere with the antitumor activity of these cytotoxins (14, 15). The cytotoxic activity of sera from 29 patients (Chart 4) correlated reciprocally with their C1q-binding activity (r = -0.491; p = 0.003, Spearman rank correlation test).

We attempted to dissociate the IC in these sera to determine whether those that had high IC levels and little or no cytotoxic activity were unreactive or contained hidden or blocked antibodies to the malignant melanoma cells. Serial dilution of such sera increased their cytotoxic activity significantly (Chart 5). Concurrently, their C1q-binding activity fell, suggesting that dilution had resulted in dissociation of low-avidity complexes containing hidden or blocked cytotoxins.

To establish that we were indeed dealing with hidden cytotoxins and not suppression of complement-dependent cytotoxicity caused by anti-complementary IC, we added ACF, an unblocking reagent (15), to sera containing relatively low levels of cytotoxic antibody. Nine of the 11 sera tested had C1q-binding activities >6%. The cytotoxic activity of 7 of these sera increased significantly, i.e., by >10% (14, 15) when ACF was added, as compared to the activity of paired samples containing an equal quantity of MEM (Table 5). Notably, only 2 of the sera that lacked antitumor activity prior to adding ACF failed to kill the malignant melanoma cells after this unblocking agent was added.

Addition of CF II, in contrast, decreased the direct cytotoxic activity of 3 of the 4 sera that killed >10% of the tumor cells when incubated alone with the tumor cells and complement. Paradoxically, the addition of CF II to 4 of the sera that contained high levels of C1q-binding IC resulted in a significant increase in the percentage of tumor cells killed, similar in magnitude but not entirely parallel to the increase in complement-dependent cytotoxic activity seen when ACF was added to these same samples (see Table 5; Percentage of tumor cells killed; Patients 7, 9, 10, and 11). As reported previously (15, 426...
Immune Complexes as Indicators in Malignant Melanoma

Table 5

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum C1q-binding activity</th>
<th>25 μl MEM added</th>
<th>25 μl ACF added</th>
<th>25 μl CF II added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.3</td>
<td>13</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5.4</td>
<td>16</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7.7</td>
<td>20</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.9</td>
<td>17</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
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<td>7</td>
<td>11.1</td>
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<tr>
<td>10</td>
<td>18.6</td>
<td>7</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>20.4</td>
<td>3</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 6

<table>
<thead>
<tr>
<th>Change in tumor burden during past mo.</th>
<th>Total no. of patients</th>
<th>No. of patients with cytotoxic antibodies</th>
<th>No. of patients IC</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NED</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>A B C D</td>
</tr>
<tr>
<td>Tumor evident but stable</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Progressively growing tumor</td>
<td>17</td>
<td>8</td>
<td>7</td>
<td>10 5 0 2</td>
</tr>
</tbody>
</table>

* ≥10% of tumor cells killed in the presence of active complement.
* Serum C1q binding activity >6%.
* Treatment A, none at this time; Treatment B, combined chemotherapy and immunotherapy; Treatment C, immunotherapy alone; Treatment D, chemotherapy alone.

without evident tumor and 10 of the 17 patients with progressively growing malignant melanoma were not receiving any therapy. Indeed, we could not demonstrate any relationship between these serological measurements and treatment.

**DISCUSSION**

IC have been detected previously in sera of patients with malignant melanoma, and other tumors have been detected by means of the C1q binding test, the Raji cell RIA, and other antigen-nonspecific tests (5, 10, 19, 24, 26). The design of the present study, nevertheless, afforded some novel insights into the relationship between tumor burden and the presence of circulating immune complexes and allowed us to evaluate whether repeated measurements of IC were useful for estimating prognosis in patients with this tumor. A previous evaluation of the relationship between tumor burden and serum C1q-binding IC measured after heat inactivation of the serum samples at only one time point in the evolution of this disease had not shown a relationship between circulating IC and prognosis (19).

The initial plans for this investigation called for measurement of circulating IC by at least 2 independent techniques. However, the discovery that there was considerable within-person variation in the results of the Raji cell RIA from week to week in tests of sera from normal donors and the failure of this method to identify patients with particularly unfavorable prognoses led us to abandon further use of this assay. The lack of correlation between results obtained with the C1q binding and...
those from Raji cell tests indicate that the 2 methods must
detect very different IC in sera of these patients.

In contrast to the experience with the Raji cell RIA, serial
testing in normal donors by the C1q binding test showed very
little within-person variation. Consequently, it appeared that
changes of as much as 3 to 4% in the serum C1q-binding
activity might be significant. In fact, in the patients with mali-
gnant melanoma, interval changes of ≥3% in serum C1q-binding
activity were seen mainly in those with progressively growing
or changing tumor burdens.

All but 5 of the patients had Stage III or IV disease when they
entered the study, but 21 of the 53 subjects had no evidence of
residual tumor at that time. The initial measurement of serum
C1q-binding activity was helpful in identifying Stage IV patients
with a particularly unfavorable prognosis. In the 13 who re-
mained NED, abnormally elevated C1q binding tests and
changes in C1q-binding activity >3% occurred no more fre-
quently than in normal controls. Serial IC measurements in-
creased the sensitivity but did not diminish the specificity of
this diagnostic test. Calculation of the odds ratio to evaluate
the relationship between recurrent or progressive disease and
change in serum C1q-binding activity >3% suggested that
these IC measurements may be more useful than was mea-
surement of glutamic-oxaloacetic transaminase and alkaline
phosphatase (4) in detecting patients likely to have occult
tumor.

These longitudinal studies of patients with malignant mel-
oma also illuminated the biological significance of a naturally
occurring system of IC previously identified in normal donor
sera (14, 15). These complexes contain complement-depend-
et cytotoxins that react with a wide array of cultured human
tumor cells and certain fetal tissues, but only after the cytotox-
ins have been dissociated from specific anti-immunoglobulins
that interfere with their ability to react with the tumor cell
surface (14, 15). Previous studies have demonstrated that
these blocking anti-immunoglobulins have many of the attri-
butes of anti-idiotypes (15). Measurement of the complement-
dependent cytotoxic activity of sera from the patients in the
present study revealed that many were able to kill some of the
cells of the index tumor cell line (used in previous studies)
without first having to remove any blocking antibodies. In fact,
there was a significant reciprocal correlation between the C1q-
binding activity of these sera and the numbers of cultured
tumor cells that were killed in the presence of complement.

Further studies of samples with IC showed that hidden tumor
cytotoxins could be disclosed in some of these by either of 2
unblocking strategies: simple dilution; or addition of allogeneic
sera, previously shown to contain anti-immunoglobulins (ACF)
that unblock the hidden cytotoxins in normal donor sera (14,
25). Addition of pooled human IgG in the form of CF II de-
creased the spontaneous cytotoxic activity of 3 of the 4 sera
that killed high percentages of these cultured melanoma cells.
This result is consistent with previously assembled evidence
that suggests that normal donor IgG contains an excess of the
anti-immunoglobulins that block the cytotoxic activity of the
naturally occurring antitumor antibodies in normal donor sera
(14, 15). In 4 of 5 sera that contained high levels of C1q-
binding IC and were not cytotoxic when tested alone, addition
of CF II significantly increased the percentage of malignant
melanoma cells killed (Table 5). Conceivably, these patients'
sera may have contained anti-immunoglobulins similar to ACF
that can unblock cytotoxins in normal donor IgG pools such as
CF II (14, 15). Notably, the unblocking anti-immunoglobulins in
sera that have ACF activity can reveal cytotoxins in allogeneic
but not in autologous donor samples (15).

These results suggest that the apparent lack of antitumor
activity in sera with C1q-binding IC, when tested without dilu-
tion, did not indicate a lack of antibodies to the tumor but rather
indicated the presence of novel IC as effective at hiding the
activity of these antibodies as those present in normal donor
sera (1, 6, 7, 10-12, 14, 15).

The presence of unblocked or “free” cytotoxins in sera that
contained little if any C1q-binding activity suggests that the
donors of these samples may have expanded their reservoir of
naturally occurring antitumor antibodies but not their pool of
complementary blocking antibodies. Immunological stimuli pro-
vided by the patients’ tumor may conceivably have tipped a
normally balanced equilibrium between antitumor and blocking
antibody production in favor of the synthesis and release of
antibodies that react with antigens expressed by the tumor.

This ascendency of free tumor-reactive antibodies in sera of
some patients may be brief, however, inasmuch as the expan-
sion of clones that produce anti-idiotypes or an increase in
antigen shedding by the tumor provides additional materials
that can also block these antibodies (6, 7, 10). Unless the rate
of clearance of IC from the circulation can be accelerated,
either event would increase the quantity and therefore the
frequency of detection of IC and would decrease the apparent
anti-tumor cell activity in these sera. Presently, it is not clear
whether circulating tumor antigens, increased production of
blocking anti-idiotypes, or decreased clearance of naturally
occurring IC is responsible for the increased C1q-binding ac-
tivity demonstrated in these sera.

In conclusion, it should be noted that the C1q binding test is
an antigen-nonspecific test for IC and as such can also become
abnormally elevated by conditions that are irrelevant to the
progression of malignant melanoma (18, 20–22). Similarly, the
test for antibodies that are cytotoxic for malignant melanoma
cells used in these investigations may not reflect changes in
the repertoire of specific humoral immune responses that de-
velop in individual patients towards unique antigens expressed
on their own tumors (1, 7). Nevertheless, it is noteworthy that,
even with tests that undoubtedly do not reflect such individual-
specific responses, one can demonstrate that humoral immune
responses to antigens commonly displayed by malignant mel-
anoma cells are a prominent feature of the host response to
this tumor.

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the preceding 2 years by means of the search of the data base program of the
Department of Epidemiology, The University of Texas System Cancer Center.

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Immune Complexes as Indicators in Malignant Melanoma


Circulating Immune Complexes and Tumor Cell Cytotoxins as Prognostic Indicators in Malignant Melanoma: A Prospective Study of 53 Patients


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