Significance of Clq-binding Macromolecules within the Head and Neck Cancer Patient

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ABSTRACT

Elevated levels of macromolecules, within the peripheral blood of head and neck cancer patients, capable of binding the first component of complement (ClqBM) in vitro have prognostic implication. Namely, elevated levels of ClqBM have been associated with nonresponse to induction chemotherapy. In this investigation, a series of in vitro studies regarding the biological properties of ClqBM were combined with a longitudinal analysis of 112 previously untreated head and neck cancer patients. Our purpose was to shed light on the biological significance of this circulating macromolecule, a substance composed, in part, of IgG and IgM. A potential confounding influence of ClqBM with induction chemotherapy, which could contribute to observed prognostic findings, was negated by two in vitro observations: the macromolecule failed both to bind the chemotherapeutic agents cisplatin, bleomycin, and 5-fluorouracil and to impede the cytotoxic effect of these same drugs on a cultured human head and neck cancer cell line.

The clinical relevance of ClqBM was reinforced by the observation that elevated levels predicted a high probability of death with disease (P = 0.005 by Cox’s proportional hazards model). The prognostic implication was independent of the use of induction chemotherapy, i.e., patients with high ClqBM levels treated with multimodality therapy did equally poorly. Thus, the prognostic significance of ClqBM in patients undergoing induction chemotherapy appears independent of drug effect and appears reflective of tumors that are more rapidly progressive and potentially less responsive to therapeutic intervention, including combinations of surgery, radiation, and/or chemotherapy.

INTRODUCTION

Initial studies have examined the relationship between circulating macromolecules, within the head and neck cancer patient which are capable of binding the first component of complement in vitro and response to IC2 (1, 2). Those patients with elevated levels of the substance failed to respond to IC, i.e., drug therapy applied before standard treatment consisting of surgery and/or radiation therapy (1). Furthermore, the increased lack of response was significant after accounting for standard staging techniques, thereby supporting the clinical relevance of measuring ClqBM within the peripheral blood (1). Similar results were independently shown by Blazar et al. (3) when quantitating the amount of a PEG precipitate derived from the blood of head and neck cancer patients, i.e., high levels of the precipitate (from which complement-binding substances are derived) portend a low probability of chemotherapeutic responsiveness.

The nature of ClqBM remains to be defined. Investigators have demonstrated the potential for numerous substances to bind complement in vitro, including cellular and subcellular membranes from heart, liver, and brain, complexed immunoglobulin or immunoglobulin aggregates, certain RNA tumor viruses, and complexes containing C-reactive protein, as well as mitochondrial membranes, most notably of cardiac origin (4–12).

It is not known why patients with ClqBM failed to respond to IC. One could first hypothesize an associated phenomenon. The substances may, in themselves, play no direct role. Rather, they may simply reflect large rapidly proliferating tumors undergoing necrosis, with subsequent release of cellular components into the peripheral blood. The antitumor effect of chemotherapy may simply fail to “keep pace” in such a scenario. In addition, the more rapidly proliferating cells of such a tumor may have enhanced DNA repair systems, thereby making them more resistant to DNA damage mediated by drugs such as cisplatin (13–15). Alternatively, a direct role for ClqBM may involve mechanisms such as binding of chemotherapeutic agents with resultant alteration of drug delivery or mechanisms directly impeding drug uptake (16–18).

Our purpose in this investigation was to address the nature of ClqBM and, specifically, its potential relationship to CIC. Furthermore, we addressed, through a series of in vitro experiments coupled with an in vivo longitudinal analysis, whether ClqBM has a potential confounding influence on chemotherapy, which would account for its association with negative response to such treatment.

MATERIALS AND METHODS

Patient Population and Controls

One hundred twelve patients with previously untreated squamous cell carcinoma of the upper aerodigestive tract form the basis of this report. Stage of disease was determined for each patient, using criteria of the American Joint Committee on Cancer (19). Disease stage was assigned by members of the Department of Head and Neck Surgery at our institution (The University of Texas M. D. Anderson Cancer Center), without their knowledge of the respective patient’s ClqBM level. All patients studied received definitive therapy at this institution. The median age of these 112 patients was 57 years (range, 21—85 years). The male to female ratio was 2:6:1.0. The primary disease included 29 oral cavity lesions, 49 pharyngeal cancers, and 34 laryngeal cancers. All blood obtained from these patients for ClqBM measurements was drawn by venipuncture before initiation of treatment.

Details of longitudinal evaluation have been previously described (20). The length of the disease-free interval following therapy was calculated in months from the date of the patient’s initial treatment to the last noted physician contact at which the patient had no evidence of disease. The median follow-up for this population was 14 months. All patients were entered into the study before August 1, 1988. Follow-up occurred at 3-month intervals in the first year, 6-month intervals in the second year, and then annually. Patient disease status was assessed at these intervals by members of the Department of Head and Neck Surgery, again without knowledge of the ClqBM value. All data were
collected in a prospective fashion by a trained research nurse and transformed into a coding system suitable for computer entry and retrieval before the present analysis.

Measurement of Circulating Immune Complexes

Clq-binding Test. Sera were collected from clotted blood and then frozen at −70°C until tested, as we have previously described (1). Human Clq (Cytotech, Inc., San Diego, CA) was labeled with 125I by the iodobead method (Pierce Chemical Co., Rockford, IL). Five hundred μg of Clq were mixed with 1 mCi of Na125I in 0.01 m PBS, pH 7.4, in a final volume of 1 ml of ice. One iodobead was added to this mixture, and incubation was continued for 4.5 min. Labeled Clq was separated from free 125I on a 10-cm column of Sephadex G-25 (PD-10 column; Pharmacia, Piscataway, NJ). The Clq-binding test was performed as previously described, using EDTA-treated sera, according to the method of Zubler et al. (21). Results were expressed as μg/ml equivalents of heat-aggregated IgG, with reference to a standard curve made with purified IgG that was aggregated at 63°C for 20 min at a concentration of 3 mg/ml and diluted serially in heat-inactivated (56°C, 20 min) normal donor serum. Sera from healthy, age-matched, normal donors were used as negative controls, and sera from patients with previously determined elevated levels of ClqBM provided positive controls in each test run.

Polyethylene Glycol Precipitation of Serum Samples. The immune complexes were isolated from patient sera using a modification of the method of Zubler et al. (21). In brief, 1.0 ml of 0.2 m EDTA, pH 7.5, was added to 0.5 ml of patient serum, and the serum was incubated at 37°C for 30 min. The samples were then centrifuged for 10 min. Next, 0.5 μg of human Clq (Cytotech) in 5 ml of 0.005 m 5.5-ditythylbarbituric acid, pH 7.45, containing 0.145 m NaCl, 0.5 mM MgCl2, 0.15 mM CaCl2, and 1% bovine serum albumin was added to each sample. The samples were then made 2.7% PEG by adding 10 ml of 3% PEG made up in borate-buffered saline, pH 8.0. The samples were incubated at 4°C for 18 h and centrifuged at 1800 g for 10 min. The precipitates obtained from 0.5 ml of serum were finally dissolved in 2.0 ml of appropriate culture media or 0.01 m PBS containing 1% FCS and 0.01% thimerosal. The resuspended immune complexes were then used in cell culture or quantitated using an ELISA, described below.

Enzyme-linked Immunosorben Test. The ELISHA for IgG, IgA, and IgM found in the PEG precipitate was a modification of the assays developed by Baseler et al. (22) and Yarchoan et al. (23). The assay was a double-antibody solid-phase ELISA. First, 96-well capture microtiter plates (Immulon I; Dynatech, Chantilly, VA) were made by adding to the central wells (outside rows and columns not used) 150 μl of 0.1 m sodium bicarbonate buffer/0.01% thimerosal solution, pH 9.6, containing the IgG fraction of goat anti-human IgG, IgA, or IgM, at protein concentrations of 6.2, 3.1, and 6.2 μg/ml, respectively (Organon Teknika-Cappel, Malvern, PA). The plates were then incubated at 4°C in a humidified chamber for a minimum of 18 h. The plates were then washed 5 times with 0.01 m PBS, pH 7.2, containing 0.05% Tween 20 and 0.01% thimerosal. The test samples (100 μl) in 0.01 m PBS, containing 1% FCS and 0.01% thimerosal, were then added to the plate at the following dilutions: IgG, 1/45, 1/135, and 1/405; IgA, 1/5, 1/15, and 1/45; and IgM, 1/45, 1/135, and 1/405. Each plate contained six duplicate standard wells that received 100 μl of pure human IgG, IgA, or IgM (Organon Teknika-Cappel), ranging from 1.37 to 333 ng/ml. Six background wells containing 100 μl of 0.01 m PBS, containing 1% FCS and 0.01% thimerosal, were also included on each plate. The plates were then incubated for 2 h at room temperature and washed 5 times, as described above. All wells on the plate then received 100 μl of peroxidase-labeled IgG fraction of goat anti-human IgG, IgA, or IgM (Organon Teknika-Cappel) at dilutions of 1/4000, 1/8000, and 1/800, respectively, in 0.1 m PBS containing 1% FCS, 0.01% thimerosal, and 0.05% Tween 20. After a 2-h incubation at 22°C, the plates were washed 5 times as described. The following was then added to each well: 100 μl of 0.1 m citrate/0.1 m phosphate buffer, pH 4.0, containing 200 μg/ml 2,2'-azino-di(3-ethyl-benzthiazoline-sulfonate) and 1 μl/ml 3% hydrogen peroxide. Absorbance (410 nm) of each well was then determined on an ELISA plate spectrometer. The absorbance readings from each sample well were compared with the standard curve, and the concentration of CIC immunoglobulin was reported as total μg of immunoglobulin/0.5 ml of the original serum sample. The reagents were tested for cross-reactivity and none was observed.

Affinity Column Chromatography. The purpose of this procedure was to capture Clq-bound molecular complexes from the serum of patients and then determine whether these substances contained specific antibody isotypes. In brief, 90 μg of the IgG fraction of goat anti-human Clq (Organon Teknika-Cappel; catalogue no. 0201-0581) were diluted in 0.2 m sodium citrate buffer, pH 6.5, and attached to 6 g (dry weight) of washed CNBr-activated Sepharose 4B (Pharmacia), using the manufacturer’s instructions. There was greater than 95% binding of the IgG fraction.

Ten ml of patient serum were diluted to 30 ml with 0.01 m PBS, pH 7.2, containing 100 units of penicillin and 100 μg of streptomycin, and were passed through the 1.5- x 9.0-cm column for 24 h at 4°C, at a rate of 0.8 ml/min. The column was then washed with PBS buffer until the absorbance at 280 nm was reduced to less than 0.01. At this point, the bound fraction was eluted with 6 m urea in PBS buffer. The fractions containing the eluted peak were pooled and immediately dialyzed against PBS buffer. The amounts of IgG, IgA, and IgM in the eluted fraction were determined using the previously described ELISA.

Ability of ClqBM to Alter Serum Albumin Binding to Drugs

Drugs were obtained from the following sources: [S-methyl-1H]bleomycin A2 (44.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA); nonradioactive bleomycin A2 was the kind gift of Dr. Ted Sakai (University of Alabama, Birmingham, AL). Cisplatin was obtained from Bristol Laboratories (division of Bristol-Meyers Co., Syracuse, NY), and 5-FU was obtained from Roche Laboratories (division of Hoffmann-La Roche, Inc., Nutley, NJ). Drugs (12.5 or 50 μg) were added to 1-ml aliquots of human serum containing either isolated ClqBM or no added serum-derived products, i.e., control plasma. The samples were then placed into bags formed from standard dialysis tubing (Fisher Scientific, Pittsburgh, PA). The bags were tied and placed in 50-ml culture tubes. To each tube surrounding the bag was added 10 ml of 0.067 m phosphate buffer, pH 7.2, which contained 100% of the respective drug concentration placed in the serum compartment. The tubes were tightly sealed and gently agitated in a shaker-water bath at 37°C for 24 h.

At the end of the equilibration period, aliquots were withdrawn from both the serum compartment and the dialysis fluid for determination of drug concentrations. Cisplatin levels were determined directly in the serum and dialysis fluid by measurement of the elemental platinum content, according to the method of Siddik et al. (24). Bleomycin A2 levels were determined by measuring radioactivity content, and 5-FU levels were determined by a high performance liquid chromatography assay, as described by Miller et al. (25).

Effect of ClqBM on Cytotoxicity of Anticancer Agents

An established cell line (183) of human squamous carcinoma cells was kindly provided by Dr. Peter Sacks (M. D. Anderson Cancer Center). Cells were grown in Dulbecco’s modified Eagle’s medium and high-glucose medium supplemented with 10% FCS and 2 mM L-glutamine (GIBCO Laboratories, Grand Island, NY), in a 5% CO2 atmosphere. Microtiter plates (96-well) were prepared containing 10,000 cells and 150 μl of medium/well. Cells were allowed to attach for 24 h before the medium was carefully removed by gentle aspiration. Fresh medium containing either the ClqBM material from individual patients or an equivalent volume of Hanks’ balanced salt solution (control) was then added to the wells.

At the end of a 48-h incubation period, filter-sterilized drug solutions (bleomycin A2, 5-FU, or cisplatin) were added at appropriate concentrations ranging from 0.01 to 100 μg/ml. After an additional 72 h of incubation with the drug, relative cell growth was determined by the method of Mossman (26). Growth in control cell cultures (non-drug treated but exposed to ClqBM material) was directly compared with the cultures that had been incubated with both the ClqBM material and the individual cytotoxic drugs.
RESULTS

ClqBM and Their Relationship to CIC. ClqBM from the peripheral blood of head and neck cancer patients with demonstrated elevated levels of this substance were captured on an anti-Clq antibody affinity column. The purpose of capturing and subsequently eluting these substances was to determine whether ClqBM represent, in part, antibody complexes containing IgG, IgA, and IgM. Results of three separate experiments, using different cancer patient serum for each assay, are presented in Table 1. Using the anti-IgA, -IgG, and -IgM ELISA system, ClqBM were noted to be, at least in part, composed of the antibody isotypes IgG, IgA, and IgM (Table 1).

The relationship of overall levels of these complexed antibody isotypes to overall levels of ClqBM found within the PEG precipitate was analyzed. Forty patients were assessed, based solely on the availability of sufficient quantities of sera so that a fresh aliquot could be thawed for each of the assays. Regression analysis showed that, as the level of ClqBM levels increased within PEG precipitates isolated from peripheral blood, so did levels of complexed IgM (r = 0.42) (P < 0.01) and IgG (r = 0.41 (P < 0.01). The same direction of association with aggregated IgA was identified (r = 0.25), but this correlation is not significantly different from zero with the sample size used. Results reveal that elevated ClqBM within the head and neck cancer patient is, at least in part, reflective of complexed immunoglobulins, principally of IgG and IgM isotypes.

ClqBM and Protein Binding of Drugs. The purpose of these experiments was to determine whether ClqBM bind with chemotherapeutic agents utilized in our clinical practice. Such binding may have a negative influence on IC. Ninety-eight % of the measured concentration of bleomycin, 96% of the cisplatin, and 95% of the 5-FU added to the dialysis systems were accounted for by drugs assayed from both sides of the dialysis system after equilibration. In control human serum, less than 10% protein binding was detected for either 5-FU or bleomycin A2. No difference in relative protein binding of 5-FU or bleomycin A2 was noted between the serum containing the ClqBM and control serum (data not shown).

At the end of the 24-h equilibration period, 87% of the elemental platinum in the dialysis system was associated with the serum compartment. These findings reflect the strong protein binding associated with aquated species of cisplatin. No difference was detected, however, between the relative cisplatin protein binding in the serum containing ClqBM and that in control human serum (data not shown).

ClqBM and Drug Cytotoxicity. Using a human head and neck squamous cell line, we observed that the ClqBM serum preparations made no significant difference in the cytotoxic properties of any of the three drugs tested (Table 2).

ClqBM and Survival in Induction Chemotherapy Patients. We had previously reported that high levels of ClqBM within head and neck cancer patients were associated with a high probability of nonresponse to IC (1, 2). Since the initial patient was entered into that study, 36 patients with squamous cell carcinoma of the larynx, oral cavity, or pharynx have received IC and longitudinal follow-up. The relationship between ClqBM levels and the probability of death with disease was assessed. The median longitudinal follow-up in this group was 11 months (range, 3–29 months). Results revealed a direct relationship between ClqBM levels obtained before treatment and the likelihood of death with disease, i.e., those patients with highest ClqBM levels were at greatest risk (P < 0.05 by Cox's proportional hazards model). A change in ClqBM from 0 to 150 mg per ml equivalents of heat aggregated IgG represents an additional multiplicative risk of 4.8.

Our original assessment of association of ClqBM levels with response to IC also included an examination of the association between levels of ClqBM and response to radiation therapy in previously untreated patients (1). In contradistinction to chemotherapy results, no correlation with treatment could be identified in the latter population (1). Results raised the issue of a potential confounding influence between circulating ClqBM and the IC agents utilized. We, therefore, assessed patients undergoing treatment regimens other than chemotherapy for the relationship between ClqBM levels and clinical course. Differences in disease progression between IC patients and patients treated by other means may shed light on potential treatment-related biological interactions.

Table 3 reveals the stage of disease, the median ClqBM values, and the status of patients treated by four therapeutic regimens: surgery only, radiation therapy only, surgery plus chemotherapy, and induction chemotherapy alone. Differences in disease progression between IC patients and patients treated by other means may shed light on potential treatment-related biological interactions.


table 1
<table>
<thead>
<tr>
<th>ClqBM level (µg/ml equivalents)</th>
<th>Antibody isotype within ClqBM complex (µg)</th>
<th>Antibody isotype within serum (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>IgA</td>
<td>IgG</td>
</tr>
<tr>
<td>1</td>
<td>122</td>
<td>213</td>
</tr>
<tr>
<td>2</td>
<td>144</td>
<td>215</td>
</tr>
<tr>
<td>3</td>
<td>233</td>
<td>134</td>
</tr>
</tbody>
</table>

* Levels of ClqBM within respective patients were determined using serum samples previously frozen at −70°C and are expressed as µg/ml equivalents of heat-aggregated IgG.

CIC contained within ClqBM were captured by affinity column chromatography using goat anti-human Clq antibody, as described in “Materials and Methods.” The identification of immunoglobulins so captured within ClqBM was then established through the use of an ELISA for IgG, IgA, and IgM (see “Materials and Methods”). Values reported represent µg of the respective immunoglobulin isotype bound (mean ± 1 SD of 8–12 determinations for each respective test).

mg immunoglobulin per 100 ml of serum.

Table 2 Lack of effect of ClqBM on drug cytotoxicity

<table>
<thead>
<tr>
<th>IC50 (µg/ml)</th>
<th>Bleomycin</th>
<th>Cisplatin</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>3.9³</td>
<td>1.8</td>
<td>4.5</td>
</tr>
<tr>
<td>+</td>
<td>5.4 ± 2.7</td>
<td>2.1 ± 0.4</td>
<td>4.4 ± 0.9</td>
</tr>
</tbody>
</table>

* Data are presented as the mean of two experiments with eight replicates per experiment.

Data are provided as mean ± SD of IC50 values obtained from three experiments, using ClqBM material from three separate patients.

Table 3 Characteristics of head and neck cancer patients treated by diverse therapeutic regimens

<table>
<thead>
<tr>
<th>Treatment group (no. of patients)</th>
<th>ClqBM level*</th>
<th>Median follow-up (month)</th>
<th>Disease stage (no. of patients)</th>
<th>Status (no. of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC (36)</td>
<td>28 (0–162)</td>
<td>11</td>
<td>I II III IV</td>
<td>14 19 24 14 4 13</td>
</tr>
<tr>
<td>Surgery plus XRT² (31)</td>
<td>30 (0–125)</td>
<td>19</td>
<td>1 2 12 12 20 1</td>
<td>3</td>
</tr>
<tr>
<td>Surgery (19)</td>
<td>18 (0–125)</td>
<td>19</td>
<td>6 5 5 2 17 1</td>
<td>3</td>
</tr>
<tr>
<td>XRT (30)</td>
<td>34 (1–217)</td>
<td>18</td>
<td>6 12 5 6 24 1</td>
<td>3</td>
</tr>
<tr>
<td>Total population (112)</td>
<td>31 (0–217)</td>
<td>14</td>
<td>13 20 36 41 75 7 19</td>
<td>3</td>
</tr>
</tbody>
</table>

* Clq binding levels were determined using serum samples previously frozen at −70°C and are expressed as µg/ml equivalents of heat-aggregated IgG. All values are expressed as the median (range).

² Stage of disease was determined by American Joint Committee on Cancer staging system. Two of the total 112 patients were excluded from disease staging because previous diagnostic biopsies precluded specific stage classifications.

³ NED, no evidence of disease; AWD, alive with disease; DWD, dead with disease.

⁴ XRT, radiation therapy.
radiation therapy, and IC plus surgery and/or radiation therapy. Likewise, the median follow-up time for the four groups is indicated. No significant differences could be identified in ClqBM values, although the surgery only population had the lowest values.

An assessment was made as to the distribution of staging between the four groups. Results reveal that the surgery only and the radiation therapy only groups had significantly lower disease stages than the remaining two populations ($\chi^2 = 47$, with 12 degrees of freedom; $P < 0.001$). No differences in staging between the surgery plus radiation therapy group and the IC group, however, existed. Thus, the groups described in Table 3 are not entirely comparable, although the IC group and the surgery plus radiation therapy group are nearly equivalent in the assessed parameters.

Each group was then evaluated for the relationship between the ClqBM levels and the risk of subsequent death with disease, again using Cox's proportional hazards model. Fig. 1 shows the individual values for the patients, either alive or dead with disease, as categorized by the four treatment plans. None of the patients treated with surgery alone have died with disease, thus precluding analysis. No relationship between ClqBM and death with disease could be identified in the radiation therapy only group ($P = 0.93$); however, as noted in the patients treated with IC, a significant correlation could be identified in patients treated with surgery plus radiation therapy ($P < 0.01$) (a multiplicative risk of 7.2). Overall hazard of death with disease for the 112 patients, regardless of treatment, was directly related to increasing levels of ClqBM ($P = 0.005$ by Cox proportional hazards model) (a multiplicative risk of 4.2). Thus, given that patients in advanced stages, with elevated levels of ClqBM, treated by surgery and radiation therapy did poorly, one cannot conclude that the prognostic implication of ClqBM was primarily due to an interaction between ClqBM and the chemotherapeutic agents utilized.

It is evident from Table 3 that earlier staged patients (stage I or stage II disease) tended to be treated by single modality therapy only, either surgery or radiation therapy. In contrast, the majority of patients with advanced stage disease were treated by multimodality therapy. Given that the measurement of ClqBM levels failed to provide prognostic implication in patients treated by surgery or radiation therapy alone, we questioned whether ClqBM levels simply reflected prognostic implications provided in standard staging, rather than having independent significance.

Patients were categorized in four standard stages, as designated by the American Joint Committee on Cancer standards, and assessed for survival by Kaplan-Meier methods (27). The disease-specific survival of the patients in the four American Joint Committee on cancer stages was compared using the log rank test. There were no significant differences between stages I, II, and III; the stage IV survival was worse than that in any of these stages. The lower three stages were consequently combined into a single group (Fig. 2). Using the Cox proportional hazards model, stage IV patients had a multiplicative risk of 13.3 of death with disease, as compared to the combined stage I, II, and III patients.

When a proportional hazards model relating ClqBM and survival was fit separately to the early (stages I, II, and III) and late stages (stage IV), the multiplicative factor relating hazard to ClqBM level was 3.7 times higher in the late stage than in the early stages. For combined stages I–III, there was no evidence of a relation between ClqBM and survival ($P > 0.05$), although there is strong evidence in the late stage ($P < 0.005$). Thus, the prognostic implication of ClqBM is dependent upon stage of disease. The level of ClqBM within peripheral blood provided no significant information in patients with early stage disease. In contrast, stage IV patients with elevated ClqBM values fared significantly worse than similarly staged patients with low ClqBM values. Results show that the quantitation of ClqBM provides information beyond the clinical staging of disease alone.

**DISCUSSION**

The purpose of this study was to investigate mechanisms underlying previous observations related to elevated levels of ClqBM in head and neck cancer patients and, specifically, to investigate the mechanisms that may contribute to lack of response to IC (1–3). We questioned whether ICQBM directly interfered with chemotherapeutic agents, thus acting as a confounding influence in the effectiveness of a chosen therapeutic modality. Should this prove correct, clinical investigations would need to consider therapeutic approaches that account for this interaction. Conversely, should no interaction be identified, then one could conclude that high levels of ClqBM may simply be an associated phenomenon regarding lack of response to IC. The macromolecules may play no direct role but simply reflect properties inherent in the tumor, which would dictate resistance to bleomycin, cisplatin, and/or 5-FU, the drugs most commonly utilized for treating head and neck cancer. The intrinsic biolog-

![Fig. 1. Individual ClqBM levels in patients treated by either surgery alone, radiation (XRT) alone, surgery plus radiation, or induction chemotherapy. Patients are characterized as alive following respective therapy (O) or dead with disease (•).](image1)

![Fig. 2. The relationship between pretreatment disease stage, as classified by American Joint Committee on Cancer methods (19), and survival time in patients with head and neck cancer. Only death with disease was considered. O, Stage I; •, stage II; ◦, stage III; ▲, stage IV.](image2)
ical tumor properties reflected in elevated ClqBM levels would, furthermore, likely be associated with a worse prognosis, regardless of treatment applied. Our data, including both the in vitro and the in vivo prognostic studies, support the latter hypothesis.

Several features militate against a potential confounding influence on treatment response induced by the interaction of the drugs with ClqBM. First, we compared the relative ability of ClqBM to alter the usual drug-binding characteristics of serum for cisplatin, bleomycin, or 5-FU. Cisplatin has repeatedly demonstrated its ability to bind protein within peripheral blood, with resultant alteration in drug clearance and diminished cytotoxic capacity (16–18). Results showed no alteration in the relative serum protein binding of these drugs in the presence of added ClqBM. These findings, however, were not unexpected. Cisplatin is highly protein bound, so that if in the presence of serum for more than 8 h. There is, however, little specificity in its protein binding, and it will essentially bind to many if not most proteins that contain sulfhydryl groups (28); it probably binds to ClqBM as well. However, the percentage of cisplatin bound by ClqBM, relative to that bound by all other serum proteins, is undoubtedly extremely small.

Second, further potential confounding influence exerted by these complement-binding molecules was evidenced by their lack of effect when examined for chemotherapy-mediated cytotoxicity against human squamous cell carcinoma cell line 183. The ClqBM had no apparent influence on the properties of any of the three drugs examined.

It should be emphasized, of course, that such in vitro analyses may not always reflect in vivo circumstances. To test directly the significance of ClqBM levels in patients, one could remove these substances from the patient's bloodstream and determine whether such a plasmapheresis procedure improved chemotherapeutic efficacy. Terman et al. (29) have demonstrated such results in a canine breast cancer model, which involved plasmapheresis and infusion of 1-β-D-arabinofuranosyl cytosine. In that study, the two procedures combined were more effective in reducing tumor burden than either would have been alone. It should be emphasized, however, that alternative explanations may exist to account for antitumor responses observed in the canine model, such as activation of complement cascade by reinfused enterotoxins from the Staphylococcus aureus column utilized. We have recently initiated similar plasmapheresis studies in patients with advanced recurrent head and neck cancer, that is recalcitrant to other therapeutic modalities including chemotherapy, with elevated levels of ClqBM. To date, although our experience is limited, no dramatic results have been identified.

The above-mentioned results would suggest that the clinical significance of ClqBM, in relationship to nonresponse to chemotherapy, is an associated phenomenon. Thus, one would suspect that potentially similar results, i.e., adverse clinical outcome associated with nonresponse to chemotherapy, would likewise be identified in patients treated with other regimens. Such a consideration is consistent with a growing consensus that response to chemotherapy and associated prolonged survival (or the converse in instances of nonresponse) is more reflective of the biology of the disease than the impact of the chemotherapeutic agents (30). Our in vivo prognostic data again confirm, in part, the latter consideration. Patients with advanced stage disease and elevated levels of ClqBM receiving multimodality therapy did poorly, regardless of whether chemo-

therapy was part of their regimen. Thus, elevated ClqBM reflect a more aggressive disease in head and neck cancer patients, which appears independent of the treatment applied. It is, however, noteworthy that in this study the prognostic implication of quantitative measures of these substances became less apparent in early stage disease, disease stages that are principally treated by single modality therapy consisting of either surgery or radiation therapy. The reasons for this discrepancy are not clear but may relate to the specificity of the assay. ClqBM, although noted to be composed of circulating IgG and IgM immune complexes, may be composed of numerous substances (4–12). Within the head and neck cancer patient, we have noted that elevated ClqBM is principally a reflection of tumor burden (1). Thus, ClqBM levels, when elevated in those patients with minimal tumor burden, may be reflective of a different disease process, which bears no relation whatever to head and neck cancer progression. Indeed, the patient treated by surgery alone who had the highest level of ClqBM (135 μg/ml, as shown in Fig. 2) had severe degenerative arthritis. Thus, the nonspecificity of the Clq-binding assay represents a current degree of limitation in its application. Future efforts, which define tumor-related antigens within the complement-binding substances, may help to resolve this issue.

The finding in this study that ClqBM represent a close relationship with levels of circulating IgG and IgM complexes, complexes composed of either antibody-antibody components, antibody-antigen complexes; or free antibody capable of binding Clq, must also be considered in assessing their prognostic implications. It, therefore, must be considered in light of CIC assays in other disease processes. Evidence related to the prognostic implication of CIC assays, including the ClqBM assay, in cancer has been conflicting (31–36). The results generated here raise the possibility that the prognostic implication of elevated levels of ClqBM may depend upon a particular neoplastic process. Thus, the implication of ClqBM or, for that matter, immune complexes which are measured by any current methodology may be different when considering melanoma and breast cancer, for instance, as compared with head and neck cancer. Distinct from melanoma and breast cancer, the latter tumor process appears to be less aggressive biologically, i.e., metastases and death from disease tend to occur much later in the course of the disease (37–40). Indeed, the risk of distant metastases in head and neck cancer generally becomes apparent only when tumors reach dimensions exceeding 4 cm, i.e., T3 or greater lesions according to American Joint Committee on Cancer staging, and have associated advanced lymph node disease. In contrast, any melanoma with a volume less than 50% that size is almost universally fatal, with prognostic implications being quantified by dimensions in millimeters rather than centimeters (37). The significance of ClqBM within the head and neck cancer patient, apparent principally in later stage disease, may reflect cell death commonly identified in large fungating head and neck cancers with necrotic centers. In addition, it may become apparent in advanced head and neck cancers which massively infiltrate and destroy normal surrounding structures, such as bone, cartilage, and muscle. With tissue necrosis, regardless of its source, subcellular components may be shed into the bloodstream and contribute to elevated levels of ClqBM when quantitated in vitro. Indeed, similar circumstances have been identified in patients undergoing myocardial infarction (11, 12). Under circumstances of chronic persistent tumor necrosis or in circumstances of relatively acute massive cellular destruction, the capacity of the host to remove these complement-binding substances may be exceeded. Therefore,
the prognostic implications of nonspecific measures of CIC are more likely to reflect tumor processes that metastasize late in their course and that are characterized by death from uncontrolled local/regional disease, sites of disease more consistently associated with cellular destruction.

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Significance of C1q-binding Macromolecules within the Head and Neck Cancer Patient

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