Variables Affecting the Killing of Cultured Human Neuroblastoma Cells with Monoclonal Antibody and Complement

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ABSTRACT

To determine incubation conditions that result in optimal in vitro killing of human tumor cells with monoclonal antibody and complement, variables affecting the killing of cultured human neuroblastoma cells with monoclonal antibody 6-19 and baby rabbit complement were studied. Neuroblastoma target cells were stained with the fluorescent dye Hoechst 33342, which enables rapid, sensitive detection of surviving cells in conjunction with trypan blue exclusion. Maximal cell lysis was observed at an antibody concentration of 5-10 μg/ml. Greater than 5 logs of cell kill were obtained with appropriate treatment conditions. No antigenic modulation was detected. Complement activity was found to be the factor which limited the extent of cell kill. Specific cell lysis increased with decreasing concentration of complement. As the reaction with antibody coated cells proceeded, complement activity was depleted. This resulted in the greatest cell kill occurring during the first 10-20 min of treatment. Generation of factor(s) inhibitory to complement activity was also found. However, the effect of inhibition on the limiting extent of cell kill was much less significant than the effect of complement depletion. When compared to a single incubation of the same total duration, the reduction in complement activity with duration of incubation resulted in greater killing by multiple brief incubations with fresh complement. The depletion of complement was found to increase at a greater cell concentration. This resulted in greater proportional survival as neuroblastoma cell concentration increased. Depletion of complement activity by aggregated monoclonal antibody was found to decrease cytotoxicity. This evaluation may provide a framework for optimization of target cell destruction using complement and other monoclonal antibodies and target cells.

INTRODUCTION

BMT* is used widely in the treatment of a variety of diseases. Elimination of malignant cells from harvested bone marrow prior to autologous BMT might allow more cancer patients to benefit from BMT. Similarly, elimination of T-cells from harvested bone marrow prior to allogeneic BMT may decrease the incidence and severity of graft-versus-host disease. One technique used to eliminate nucleated cells (tumor cells or T-cells) selectively from harvested bone marrow is antibody dependent, complement mediated cytolysis (1-3).

Antibody dependent, complement mediated destruction of cells in bone marrow has been analyzed using rat leukemic cells (4), 31Cr-labeled cultured human cells (5), and cultured human cells in a clonogenic assay (6). The 31Cr release assay is limited in detecting fewer than 2 logs of cell kill. The clonogenic assay is more sensitive, allowing detection of 4-5 logs of cell kill; but it is tedious, labor intensive, and requires clonogenic cells, and results are not immediately available. We have shown that the technique of staining cultured human neuroblastoma cells with the fluorescent dye, Hoechst 33342, in conjunction with trypan blue exclusion, allows rapid determination of rare viable cells with a sensitivity equal to or greater than that of a clonogenic assay (7). In this study, we have used a murine IgG2a McAb that binds to a wide variety of tumor cells (8) to investigate the conditions that affect killing of cultured human neuroblastoma cells with this antibody and complement. We demonstrate that the major factor limiting complete cytolysis is depletion of complement activity during incubation of target cells with antibody and complement.

MATERIALS AND METHODS

Neuroblastoma Cell Line. Cultured human neuroblastoma cells, SK-N-MC (9), were grown in 50% Dulbecco's modified Eagle's medium-50% Ham's F12 (Flow) in a humidified environment containing 5% CO2. The medium was prepared as described by Bottenstein and Sato (10) and contained 15% fetal bovine serum, penicillin, and streptomycin (DF-15). For use in cytotoxicity assays, subconfluent cells were suspended from culture dishes with phosphate-buffered saline containing 0.1% trypsin and 0.02% EDTA, washed with DF-15, and resuspended in P-15.

Antibody. The murine hybridoma producing McAb 6-19 has been described (8). McAb 6-19 was purified from hybridoma ascites by means of protein A-Sepharose affinity chromatography (11) in the presence of 0.01% sodium azide, dialyzed against sterile phosphate-buffered saline, and stored at 4°C at a concentration of 2 mg/ml.

RPC-5 is a murine myeloma IgG2a McAb with no known binding specificity (12). It was purified from ascites by affinity chromatography as above.

Complement. Sterile, low-endotoxin, newborn rabbit complement (Pel-freez, Lot 2516) was selected for lack of toxicity to clonogenic human bone marrow progenitor cells as described previously (8) and stored at -70°C.

Staining Cells with Hoechst 33342. Hoechst 33342 (Calbiochem) was added to SK-N-MC cells (2 x 106/ml) in P-15 at a concentration of 5 μM, and the cells were incubated 60 min at room temperature. The cells were pelleted and resuspended in fresh P-15 for subsequent use. Fluorescent cells were examined with an American Optical epifluorescence microscope (50-W mercury vapor lamp and bandpass 365-nm excitation filter). The number of viable cells remaining following treatment with the McAb and complement was determined by enumerating the fluorescent cells as described previously (7). Treated aliquots of cells were pelleted and resuspended in 20 μl of P15/0.25TB, and the entire aliquot was placed on a microscope slide and covered with a coverslip.

Fluorescent cells were counted by carefully scanning the entire coverslip at 100X magnification. Alternatively, the cells were resuspended in a larger volume of P15/0.25TB and examined in a hemocytometer.

Treatment with Monoclonal Antibody and Complement. Conditions were varied in order to determine the optimal conditions for maximal cytolysis. In general, Hoechst 33342 stained SK-N-MC cells were suspended in P-15 at a known concentration and aliquoted into microfuge tubes. McAb 6-19 was added to a predetermined concentration. Next, complement (baby rabbit serum) was added to yield various vol%. The samples were mixed, incubated at 37°C for 10-60 min, and then...
pelleted by centrifugation at 13,000 × g for 45 s. Cells subjected to multiple treatments were resuspended in fresh P-15, and the procedure was repeated with fresh antibody and complement for a second or third incubation. After the final treatment, the cells were suspended in P15/0.25TB, and the viable cells were enumerated as above. Survival was calculated as the total number of viable cells after treatment divided by the number of viable cells prior to cytotoxic incubations. All survival data are expressed as the mean ± SE. Significance of differences between results was evaluated statistically using the t-test, 2 tailed.

Indirect Immunofluorescence/Flow Cytometry. SK-N-MC cells were suspended from culture by trypsinization, washed in DF-15, and resuspended at a concentration of 1 × 10^6/ml in P-15AZ. McAb was added, and the cells were incubated for 30 min at 0°C. The cells were washed 3 times with P-15AZ, resuspended in affinity purified fluorescein conjugated goat anti-mouse anti-serum (Cappel), and incubated for 30 min at 0°C. Following three additional washes with P-15AZ, the cells were filtered through 20-μm nylon mesh and examined in a fluorescence activated cell sorter (EPICS C, Coulter Electronics). Excitation was achieved with the 488-nm line of an Argon laser at 500 mW power. The logarithm of the intensity of the fluorescent light emitted was measured after passage through 560-nm dichroic and 525 ± 20-nm bandpass filters. The peak channel number of the fluorescent intensity (logarithmic scale) of the resulting cytohistogram was recorded.

RESULTS

Effect of Antibody Concentration. SK-N-MC cells (1-1.5 × 10^6/ml in P-15) were incubated with increasing concentrations of McAb 6-19, followed by addition of complement to 20 vol% and incubation at 37°C for 30 min. At a concentration of 10 μg/ml killing was maximal (Fig. 1), and survival following a single treatment was 0.05 ± 0.03%. The optimal antibody concentration with 3 treatments was similar with 0.0009 ± 0.0007% of cells surviving. Because subsequent studies revealed greater cell survival at higher cell concentrations, the effect of McAb concentration was examined at a lower (7 × 10^5/ml) cell concentration. Maximal cell kill was also achieved with a McAb concentration of 5-10 μg/ml (Fig. 2). In all of these experiments, concentrations of McAb higher than 10 μg/ml killed fewer cells. To determine whether McAb aggregates might be responsible, McAb was clarified immediately before use by centrifugation at 100,000 × g for 30 min at 4°C in an air-driven ultracentrifuge (Beckman). Clarified or unclarified McAb was added to SK-N-MC cells at a concentration of 5 and 100 μg/ml, and cytotoxicity was assayed after a single 30-min incubation at 37°C with 20 vol% complement. Cell survival was decreased at the higher antibody concentration following ultracentrifugation (0.11 ± 0.04% versus 1.40 ± 0.54%; P < 0.01) and was equal to the cytotoxicity at the lower concentration of unclarified antibody (Table 1). Clarification had no effect on cytotoxicity of the antibody at 5 μg/ml and did not significantly decrease the antibody concentration, as measured by absorbance at 280 nm. This suggests that at high McAb concentrations, McAb aggregates decrease the cytotoxic effects of complement.

Relationship to Antibody Binding. To compare the optimal antibody concentration for complement mediated cytotoxicity with the optimal antibody concentration for antibody binding, saturation of antibody binding was examined by indirect immunofluorescence. RPC-5 antibody was used to determine the non-specific binding. Maximal binding of fluorescein-conjugated anti-serum was achieved following initial incubation of the cells with clarified McAb 6-19 at a concentration of 1 μg/ml, after one (O) or three (•) 30-min incubations with 20 vol% complement. Points, mean of replicate samples from 2-4 experiments; bars, SE.

**Table 1** Effect of McAb aggregates on complement mediated cytotoxicity

<table>
<thead>
<tr>
<th>Antibody concentration (μg/ml)</th>
<th>Non-clarified McAb</th>
<th>Clarified McAb</th>
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<tbody>
<tr>
<td>5</td>
<td>0.18 ± 0.12</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>100</td>
<td>1.40 ± 0.54</td>
<td>0.11 ± 0.04</td>
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SK-N-MC cells in P-15 at 1 × 10^6/ml were incubated with McAb 6-19 and 20 vol% complement at 37°C for a single 30-min incubation. Mean ± SE.
ml or greater (Fig. 3). This represents 10–20% of the McAb concentration required for maximal cell kill.

Antigenic Modulation. In order to determine whether modulation of the 6-19 antigen enables cells to avoid the cytolytic affect of complement, SK-N-MC cells were incubated with McAb 6-19 at 4°C or 37°C for 30 min prior to the addition of complement. Twenty vol% complement was then added, and the cells were incubated at 37°C for 30 min. Cell survival was similar regardless of prior incubation condition (Table 2). Similarly, when indirect immunofluorescence was assayed under the same conditions, there was no decrease in fluorescence intensity (data not shown), suggesting that no modulation of antibody occurs with McAb 6-19.

Complement Concentration. To determine the complement concentration that gives maximal cell killing, McAb 6-19 (5 µg/ml) was added to SK-N-MC cells (1-2 × 10⁶/ml in P-15), and the cells were incubated once at 37°C for 30 min with various concentrations of complement. Greater concentrations of complement resulted in increased cell kill, with no maximum detected when 30 vol% complement or less was used (Fig. 4A). Nonspecific toxicity of the complement to SK-N-MC cells also increased with increasing complement concentration. With multiple incubations with complement and antibody greater cytolsis was achieved, and the differences between complement concentrations became less pronounced (Fig. 4B). When survival was assessed at a lower initial cell concentration, the effect of complement concentration on cell survival was similar (Fig. 5).

Duration of Incubation. The time course of complement killing of nucleated cells was studied. McAb 6-19 was added to SK-N-MC cells (1.5–2.5 × 10⁶/ml in P-15) at a final concentration of 5 µg/ml, and complement was added to 20 vol%. The cells were then incubated at 37°C for 10–60 min. Multiple incubations of varying duration were also studied. Cell survival following 1, 2, or 3 incubations is shown in Fig. 6. Although there is additional cell kill during longer incubations, most occurred in the first 20 min of incubation. Multiple incubations resulted in more cytotoxicity than single incubations of the same total duration. With multiple incubations, prolonging each incubation more than 20 min resulted in no additional cell kill (Fig. 6).

Depletion of Complement Activity. To investigate whether the decreasing rate of cell kill with duration of incubation correlated with lowered complement activity, McAb 6-19 (5 µg/ml) was added to SK-N-MC cells (1 × 10⁶/ml in P-15), complement was added to 20 vol%, and the aliquots were incubated at 37°C for 10–30 min. The cells were pelleted and assayed for survival, and the supernatant was removed for further assay. Complement activity in the supernatants was assayed by addition of fresh antibody and SK-N-MC cells. After incubation at 37°C for 30 min, cell survival was determined. During the first incubation, cell kill increased with longer treatment (Table 3). When the corresponding supernatant was assayed in a second incubation, the cytolytic activity decreased as the duration of the first incubation increased (Table 3). Thus, longer incubation

<table>
<thead>
<tr>
<th>Antibody concentration (µg/ml)</th>
<th>% of cell survival with initial incubation at:*</th>
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<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>0.40 ± 0.19*</td>
</tr>
<tr>
<td>10</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>100</td>
<td>0.66 ± 0.21</td>
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*SK-N-MC cells (1 × 10⁶/ml) were suspended in P-15, and McAb 6-19 was added. Some samples were incubated for 30 min at 4°C or 37°C. Complement was then added to 20 vol%, and the cell suspensions were incubated for 30 min at 37°C.

* Mean ± SE.
of cells with complement and McAb resulted in a greater decrease of complement activity. This is consistent with either depletion of complement activity or production of inhibitors of complement mediated cytotoxicity. To ascertain whether production of inhibitors or depletion of complement occurred, 10 vol% complement was initially incubated at 37°C for 30 min with buffer alone, antibody alone, cells alone, or cells and McAb. The cells were pelleted, and the respective supernatants were assayed for residual complement activity by addition of fresh SK-N-MC cells and McAb, incubation for 30 min at 37°C, and enumeration of surviving cells. The supernatants were also assayed for inhibition of cytotoxicity by addition of fresh complement with fresh cells and antibody and measurement of cell survival. Reduced complement activity remained after incubation with antibody coated cells, as demonstrated by the increase in survival of cells incubated in supernatant previously incubated with both cells and antibody (18% versus 2.9%; P < 0.01) (Table 4). Little or no loss in complement activity was seen in supernatants incubated with cells or antibody alone. Slight inhibition of the cytolytic activity of freshly added complement also occurred (1.03% survival with Supernatant D plus 10 vol% complement versus 2.9% with 10 vol% fresh complement; P < 0.001). The effect of cell concentration on depletion of complement activity was also examined. The first incubation of complement with McAb 6-19 and SK-N-MC cells was performed at 5 × 10^6/ml SK-N-MC cells and 2 × 10^6/ml SK-N-MC cells. Greater depletion of complement and greater production of inhibitory factors was seen at the higher cell concentration when the respective supernatants were assayed for complement activity with fresh cells (Table 5).

**Target Cell Concentration.** To determine the effect of cell concentration on cell survival, SK-N-MC cells were suspended at various concentrations (3.6 × 10^4–2.5 × 10^6/ml) in P-15 with McAb 6-19 (5 μg/ml) and complement (20 vol%). The cell survival was determined following a single 30-min incubation at 37°C. Proportional cell kill decreased with greater cell concentration (Fig. 7). Statistical analysis yields the linear regression relationship.

\[
\text{Survival} \% = 10^{-0.24} (\text{cells/ml})^{1.24}
\]

with a correlation coefficient of 0.959. Similar evaluation of the cell concentration effect at 10 vol% complement (data not shown) yielded:

\[
\text{Survival} \% = 10^{-0.79} (\text{cells/ml})^{1.38}
\]

with a correlation coefficient of 0.903. The larger exponent on cell concentration at 10 vol% complement versus 20 vol% complement (1.38 versus 0.82; P < 0.0001) is consistent with the greater depletion of complement at higher cell concentrations. If complement were not rate limiting, depletion by antibody coated cells would be negligible, the exponent on cell concentration would approach zero, and percentage of survival would be constant regardless of cell concentration.

**DISCUSSION**

*In vitro* treatment of bone marrow with McAb and complement to eradicate malignant cells prior to autologous BMT or to deplete T-cells prior to mismatched allogeneic BMT has been pursued by several BMT centers (1–3). Conditions used for *in vitro* treatment with McAb and complement have varied, and model systems have documented >2-4 log reduction in viable "target cell" concentration (5, 6, 13). We investigated the treatment conditions to optimize cell killing with McAb and complement using a more sensitive system in which more than 5 logs of cell kill could be achieved and accurately measured. Also, because it was demonstrated that there is no significant modulation of the neuroblastoma antigen with which McAb 6-19 reacts, evaluation of the other treatment variables can be performed without consideration of antigen negative cells escaping complement mediated lysis.
The optimal McAb 6-19 concentration for complement mediated cytotoxicity (regardless of initial cell concentration) was found to be 5–10 µg/ml. Based on saturation of cell surface antigens as detected by flow cytometric analysis, LeBien et al. (6) also used McAb BA-1,2, and 3 at 10 µg/ml each. The optimal McAb 6-19 concentration for cytotoxicity is 5–10-fold higher than the concentration of McAb that results in saturation of antigenic sites when examined by indirect immunofluorescence and fluorescence activated flow cytometry. This difference may be related to antigenic sites that are bound with low affinity by the antibody. The antibody bound with low affinity could be removed during washing steps prior to exposure of cells to the secondary antibody. However, in the presence of excess antibody, such low affinity sites may still be effective in complement activation, resulting in a greater number of lytic complexes per cell. Alternatively, the difference may also reflect differences in the nature of recognition of bound McAb by secondary antibody (goat anti-mouse anti-serum) versus the components of the complement system. A decrease in cytotoxicity at a 50–100 µg/ml McAb concentration was noted. The decrease appears to result from greater activation of complement by McAb aggregates in solution, because the high concentration of McAb was as effective as the optimal concentration following removal of aggregates by ultracentrifugation. McAb aggregates may result in reduction of complement activity available for activation at the cell membrane and effecting cytolysis.

Several reports have demonstrated greater efficacy of multiple treatments with antibody and complement when compared to single treatments (4–6, 14). We have confirmed this observation and documented that most of the cytolyosis occurs in the initial 10–20 min of incubation. The reason that multiple incubations with complement are more cytotoxic is that complement activity is rapidly depleted during incubation of cells with antibody and complement.

Depletion of complement activity, which occurred as the reaction with antibody coated cells proceeded, limited the cytotoxicity that could be obtained. Increased cytotoxicity occurred with increasing complement concentration, whereas an optimal antibody concentration was obtained. Although nonspecific cytotoxicity also increased with complement concentration, the increase in specific, antibody dependent toxicity was much greater. Also, the decreased relative cytotoxicity observed at higher cell concentrations appears to result from a greater depletion of complement activity. Thus, for this target cell-McAb system (SK-N-MC cultured human neuroblastoma and McAb 6-19) it is likely that the factor limiting the extent of cell kill is the complement activity. The significant decrease in cytolytic activity of complement was primarily (but not exclusively) the result of utilization of complement component(s) rather than production of inhibitors.

The increase in complement depletion with increased target cell concentration would be predicted by the multi-hit mechanism of complement mediated lysis of nucleated cells (15). At higher cell concentrations, the lytic complexes of complement are distributed into a greater number of cells and, under limiting conditions, a greater percentage of cells will have a subthreshold number of lytic complexes. Therefore, proportional survival would increase, as was demonstrated. It has been suggested that the reason for reduced complement mediated cytotoxicity in the presence of bone marrow cells is the depletion of the third component of complement due to the presence of heterophile antibodies in rabbit serum that react with bone marrow cells (16). Most of the complement activity depletion may be secondary to insertion of complement components into cells that are lysed. In addition, Campbell and Morgan (17) have recently shown that nucleated cells may resist complement attack by removal of the lytic complex via formation of membrane vesicles that are either internalized or shed by the cell. This process may also contribute to complement activity depletion.

The concentration of complement used by others examining in vitro nucleated cell kill has been about 10–20 vol% (1/10–1/5 dilution). There has not been excessive marrow toxicity using baby rabbit complement in this concentration range with McAb 6-19 (18), and reengraftment has been documented by others following one, two, or three treatments with 10–20 vol% complement (1, 3). Therefore, the range of concentrations studied appears to be practical for use in the treatment of bone marrow. The use of higher complement concentrations results in the nonspecific destruction of many normal bone marrow cells. Investigation of the depletion of complement activity by antibody coated cells may suggest ways to supplement complement activity without increasing nonspecific cytotoxicity.

Initial success with BMT utilizing bone marrow that has been processed in vitro with McAb and complement (1–3) has stimulated efforts to improve the existing techniques. The results of the studies presented in this report document differences in cytotoxicity at levels of cell survival from 0.001–0.01%. To determine the clinical utility of this McAb, further studies are necessary. They will document the extent of cell kill obtainable in the presence of excess bone marrow cells. However, the results obtained with cultured human neuroblastoma-McAb 6-19 may be applicable to other target cell-McAb systems. Also, the use of Hoechst 33342/trypan blue in other target cell-McAb systems should enable rapid determination of the effect of particular variables affecting complement mediated cytolysis for those target cells.

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REFERENCES

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