Therapy of a Murine Sarcoma Using Syngeneic Monoclonal Antibody

Stephen J. Kennel, Trish Lankford, and Kathryn M. Flynn

INTRODUCTION

The advent of MoAb technology (15) has brought new hope for cancer detection and therapy (5, 19). Many of the current experimental applications of MoAb to cancer treatment involve their use as targeting agents for drugs (24-26) and toxins (23, 29). Immunotherapy by passive administration of MoAb would be less complicated and dangerous than using drug-antibody conjugates, particularly if syngeneic antibodies are available. Therapy of mouse leukemia with allogeneic MoAb to a naturally occurring glycolipid (31) or to lymphocyte differentiation antigens (3, 4, 14) has been successful in curing 30 to 100% of animals. One study using syngeneic MoAb to Rauscher leukemia virus-induced splenic leukemia (25) demonstrated effective eradication of spleen cell focus-forming units if therapy was begun within 3 days of virus injection. No data on long-term survival or multiple antibody treatments were reported, although a very sensitive dose-therapy relationship was established for single treatments. Antibodies were tested for therapeutic effect using three schedules of antibody injection. Injection i.p. of ascites fluid containing 244-19A MoAb given on Days -1, 0, and +1 relative to tumor cell injection increased life span significantly over that of control animals given injections (P3, immunoglobulin G, or MoAb 271-1A) and produced some seven of 19, one of five, and one of five long-term survivors in three separate experiments. Antibody given to animals with established tumors (4 days after implantation) also prolonged life span significantly and produced three of nine long-term survivors. Antibody given to animals with very large tumor burdens (10 days after implantation) did not prolong life span significantly. Optimal dose, schedule, and mechanism studies concerning this therapy are in progress.

ABSTRACT

Syngeneic monoclonal antibodies (MoAb) to Moloney sarcoma cells (MSC) were produced by fusion of spleen cells from MSC regressor mice to myeloma SP2/0. MoAb 244-19A, an immunoglobulin G2b, bound to MSC cells and did not bind to two other sarcomas (K-BALB and Ha2), a carcinoma (Line 1), a fibroblast (A31) or a fibroblast infected with C-type virus (A31-Moloney leukemia virus). In contrast, MoAb 271-1A bound to the MSC and Ha2 sarcoma and line 1 carcinoma as well as to the normal and infected fibroblast cultures. Antibodies were tested for therapeutic effect using three schedules of antibody injection. Injection i.p. of ascites fluid containing 244-19A MoAb given on Days -1, 0, and +1 relative to tumor cell injection increased life span significantly over that of control animals given injections (P3, immunoglobulin G, or MoAb 271-1A) and produced some seven of 19, one of five, and one of five long-term survivors in three separate experiments. Antibody given to animals with established tumors (4 days after implantation) also prolonged life span significantly and produced three of nine long-term survivors. Antibody given to animals with very large tumor burdens (10 days after implantation) did not prolong life span significantly. Optimal dose, schedule, and mechanism studies concerning this therapy are in progress.

MATERIALS AND METHODS

MoAb Production. BALB/c mice (Cumberland View Farms, Clinton, Tenn.) were given i.m. injections of 10^7 MSC cells. Tumors appeared and regressed within 2 weeks, and regressor animals were then challenged s.c. with 2 x 10^6 cells at Days 33, 41, and 58 and i.p. on Days 96 and 103 after the original injection. Spleen cells from these immunized mice were harvested and fused in 45% polyethylene glycol with BALB/c myeloma SP2/0 (27) obtained from Dr. R. Kennent, Philadelphia, Pa.

Fusion Products. Fusion products were selected by their ability to grow in hypoxanthine-aminopterinthymidine medium. To increase plating efficiency of hybridomas, normal peritoneal exúdate cells were used as a feeder layer as described by FraZeugas de St. Groth and Scheidegger (7), except Dulbecco-Vogt-modified Eagle's Minimal Essential Medium 21 containing 20% fetal bovine serum was used throughout. Hybridoma supernatant fluids were screened for antibody to MSC using glutaraldehyde-fixed cell monolayers. Radioiodinated, purified coat antibody to mouse IgG of approximately 1000 cpm/ng was used to detect binding (13).

Positive cultures were cloned by limit dilution before expansion for large-scale antibody production. Ascites fluid containing MoAbs 244-19A, 271-1A, and P3 was induced by i.p. injection of 10^7 cells in mice pretreated for 1 to 4 weeks with 0.5 ml pristane. MoAb 244-19A (IgG2b) was purified from ascites fluid by Protein A-Sepharose (Sigma Chemical Co.) chromatography (11) followed by absorption and salt gradient elution from DEAE-cellulose. Myeloma protein P3 IgG was purified using DEAE-cellulose chromatography as described previously (12).

MoAb Characterization. Antibody concentrations in ascites fluid were estimated by radial diffusion, and class and subclass were determined by agarose double diffusion using class-specific antibodies (Liton Bio-technics, Kensington, Md.).

Purified IgG was analyzed on 10% polyacrylamide gels (13). Radioiodination of antibodies with chloramine-T, gel filtration, direct binding of [125I] antibodies to cells, and calculation of affinity constants were done exactly as described previously (13). Fibroblast and tumor cell lines used for these studies were propagated in McCoy's Medium 5A supplemented with 10% fetal bovine serum (Flow Laboratories), penicillin, and streptomycin (13). Cell lines included MSC, a fibrosarcoma of BALB/c mice (20); K-BALB, a non-virus-producing fibrosarcoma induced by MSV (1); Ha2, a virus-producing fibrosarcoma induced by MSV (8); clone A31 of BALB/c ST3 cells (2); clone A31 cells infected and chronically producing A31-MoLV; and Line 1 cells, an alveolar carcinoma of BALB/c mice (32).

Therapy Experiments. BALB/c mice (6 to 10 weeks old) were given i.m. injections of 1 to 5 x 10^6 tumor cells suspended in 100 μl phosphate-buffered saline (0.01 M sodium phosphate, pH 7.6) 0.15 M NaCl after scraping from 100-mm dishes. Each antibody treatment consisted of an

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1 The abbreviations used are: MoAb, monoclonal antibody; MSC, Moloney sarcoma cells; MoLV, Moloney leukemia virus.

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i.p. injection of 0.2 ml of syngeneic ascites fluid containing 244-19A MoAb (2 mg/ml), 271-1A MoAb (2 mg/ml), and P3-IgG (6 mg/ml). Animals were monitored daily for tumor growth and mortality. Statistical significance of results was analyzed using a death time function described by Mantel (18). Significance values (p) were calculated using a 2-sided consideration.

RESULTS

Hybridoma Production and MoAb Characterization. Hybridomas were produced by fusion of SP2/0 myeloma cells with spleen cells from BALB/c mice immunized with viable MSC cells. Approximately 20 of 800 primary cultures were positive for antibody to MSC cells at 10 days after fusion. Upon serial transfer and retesting, only one culture produced antibody binding to MSC cells which did not bind to the fibroblast line A31. One of the cultures which made antibody binding to both cell lines was also propagated. These cultures were cloned and expanded for growth in ascites form. Double diffusion analyses show that 244-19A secretes mouse IgG2b and that 271-1A secretes mouse IgG2a. Antibody 244-19A is capable of lysing MSC cells in vitro upon addition of exogenous rabbit serum as a complement source (data not shown).

Antibody 244-19A was purified from ascites fluid containing antibody (about 2 mg/ml) by Protein A-Sepharose chromatography. This procedure allows copurification of nearly all classes of mouse IgG present in the ascites fluid. Since MoAbs are homogeneous in charge and structure, they can be eluted as relatively sharp peaks from ion-exchange columns, while the majority of normal IgGs are spread out over a broad elution pattern. We used DEAE-cellulose chromatography with a shallow linear salt gradient to purify MoAb 244-19A from the majority of normal mouse IgG from ascites fluid. The monoclonal IgG eluted as a sharp peak in a salt gradient at about 0.1 M NaCl. Pooled fractions were concentrated by precipitation in 50% ammonium sulfate, redissolved, dialyzed, and stored frozen. Analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated homogeneous heavy- and light-chain bands with <5% contamination. Purified 244-19A IgG was radiolabeled with 125I using chloramine-T. Greater than 85% of the 125I was precipitable with goat antibody to mouse IgG. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the labeled antibody showed that >95% of bound 125I comigrated with heavy and light chains of 244-19A IgG (data not shown). Labeled MoAb was tested for direct binding to cells as described previously (13). Chart 1 shows that at saturation, approximately 80 ng of MoAb can be bound to 8 x 10⁵ MSC cells, while ~2 ng of 125I normal mouse IgG can be bound (data not shown). A carcinoma (Line 1) and a fibroblast (A31) culture also bound ~2 ng. This value represents about 4 x 10⁵ antibody binding sites/MSC cell. A double reciprocal plot of these binding data gives a linear relationship (Chart 2) showing an affinity constant (Kₐ) of 3 x 10⁸ liters/mol. In a similar experiment, the amount of 125I-244-19A IgG was held constant, and known amounts of unlabeled MoAb were added. Nearly identical binding curves resulted (Chart 2), yielding a Kₐ of 2.3 x 10⁸ liters/mol and demonstrating that the labeling procedure has little effect on the binding properties of 244-19A MoAb.

Specificity of 244-19A MoAb for other murine cell lines was tested using an indirect binding assay with 125I-goat antibody to mouse IgG (13). Antibody 244-19A shows strong binding to MSC cells but not to either of the other sarcomas K-BALB or Ha2 (Table 1). Experiments with fibroblast cultures A31 or A31-MoLV gave values slightly above control levels (ratio, ~1.5). Variations in nonspecific binding of this magnitude often occur with different preparations and/or subclasses of MoAb; however, they could represent significant binding to these fibroblast cultures, although at very low levels. MSC regressor serum and a nonspecific MoAb preparations and/or subclasses of MoAb; however, they could represent significant binding to these fibroblast cultures, although at very low levels. MSC regressor serum and a nonspecific MoAb (271-1A) obtained from a similar fusion procedure served as positive binding controls for all cells tested with the exception of K-BALB, which yields little if any specific binding of any antibodies we tested.

The fact that 244-19A does not bind to cell lines A31-MoLV and Ha2, which are both producers of C-type viruses, indicates that viral antigens do not mediate binding to MSC cells.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MSC</th>
<th>A31</th>
<th>A31</th>
<th>A31</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.3</td>
<td>0.8</td>
<td>0.9</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>23.2</td>
<td>1.9</td>
<td>5.0</td>
<td>7.2</td>
<td>18.6</td>
</tr>
</tbody>
</table>

* Approximately 10⁶ cells/well.

** Twenty-five μg of primary antibody.
Therapy. MSC cells injected i.m. at doses greater than $10^6$ cells/mouse grow progressively to produce tumors of about 1 cm in diameter by 4 to 6 days and nearly 2 cm by Days 11 to 13. Typical histology sections of these tumors are shown in Fig. 1. These large tumor burdens are tolerated relatively well, and animals survive for many weeks. A wasting condition (cachexia) precedes death at 6 to 8 weeks. Time of death varies significantly among groups of animals and the number of cells injected, and the age of the animals must be controlled carefully in each experiment to assure consistent results. Therapy experiments using ascites fluid containing MoAb according to 3 different treatment schedules were done: MoAb treatment starting before tumor cell injection (Days -1, 0, and +1); MoAb treatment of intermediate-sized tumors (daily starting at Day 4); and MoAb treatment of well-established tumors (daily starting at Day 10).

In the first treatment schedule, animals were treated at 24 hr prior to cell injection, 3 hr after cell injection, and 24 hr after cell injection (i.e., Days -1, 0, and +1). Encouraging results were obtained in a preliminary experiment in which survival time of 244-19A MoAb-treated animals was increased ($p < 0.1$) relative to control (P3 ascites-treated) animals (data not shown). One of the 5 treated animals was cured (survival, >200 days tumor free). Chart 3 shows the survival of larger groups of mice treated with ascites fluid containing MoAb 244-19A or P3 IgG. Animals in the group tested with 244-19A ascites fluid had a significant increase in survival time ($p < 0.01$) relative to those in the group treated with P3 ascites fluid. Seven of 19 animals in the 244-19A group have survived tumor free for >200 days. In order to determine the importance of MoAb specificity for therapy, a MoAb (271-1A) which binds to normal cells as well as tumor cells (Table 1) was used. In this experiment, the same treatment schedule was instituted (5 animals/group) but this time comparing treatment with MoAb 244-19A to treatment with MoAb 271-1A. Results in Chart 4 show that treatment with specific antibody 244-19A prolongs survival ($p < 0.05$) relative to treatment with the nonspecific MoAb 271-1A. One animal from the group treated with 244-19A MoAb survived and remained tumor free.

Realistic therapy schedules must be effective after tumors have been diagnosed. To test the utility of MoAb 244-19A for this kind of therapy, a different treatment schedule was used. Mice inoculated with progressive doses of MSC cells develop palpable tumors as early as 2 days following injection. At Day 4, tumors approach 1.0 cm in diameter. Antibody therapy (9 animals/group; i.p. injection of 0.2 ml ascites fluid) was started at this time (Day 4) and continued daily for 14 days with subsequent treatments at Days 23, 28, 33, and 43. Data plotted in Chart 5 show that survival of 244-19A-treated animals is significantly greater than that of P-3-treated controls ($p < 0.01$). Of the 4 antibody-treated animals surviving past 100 days, one died of sarcoma on Day 145, and the remaining 3 are alive and tumor free (Day 200).

The specificity of therapeutic effect of MoAb 244-19A is demonstrated by the lack of therapeutic effect of this antibody on tumor growth and animal survival of another sarcoma cell line (K-BALB) that does not bind the antibody. Data plotted in Chart 6 show that therapy started 4 days following injection of K-BALB cells is not effective in prolonging survival. Antibody-treated animals die from tumors at the same rate as do control animals with no significant difference ($p > 0.5$).
Transplanted tumors may not become established adequately for many days. The effect of antibody therapy on a well-established tumor was assessed in an experiment starting therapy at 10 days following injection. At this time, tumors are nearly 2 cm in diameter and completely displace leg muscle (Fig. 1C). Data in Chart 7 show that treated and control survival curves differ in average time of death, but overall, no significant increase in life span is demonstrated \((p > 0.05)\). Treatment was discontinued when the first death occurred. The slightly longer survival time of both groups of animals in this experiment relative to previous ones is probably due to the fact that younger animals (6 weeks), which tend to be more resistant to tumor growth, were used.

**DISCUSSION**

Most applications of MoAb to cancer therapy have been directed at the use of these antibodies as targeting agents for cytotoxic drugs (23–26, 29). MoAbs have not been studied as carefully for direct therapeutic potential; this is probably due to the relative inefficiency of passive serum therapy in the past. Previous failure using passive serum therapy has been attributed to antibody interference with cellular effector mechanisms (10) which inhibit tumor elimination (16) as well as complications due to circulating tumor antigens or immune complexes. It is possible that such problems could be avoided using MoAb of specific subclasses directed at appropriate cell surface antigens.

Recent studies have shown that MoAb may have therapeutic potential. Studies in humans are preliminary (mostly Phase 1) and have pointed out problems of antigenic modulation (31) and antibody responses to mouse immunoglobulin (6, 22, 28). The data for experimental animals pertain to leukemias or lymphomas and involve antibodies to viral antigens (25, 26) or differentiation antigens (3, 4, 14). In this report, we demonstrate that MoAb directed at the use of these antibodies as targeting agents for cytotoxic drugs (23–26, 29). MoAbs have not been studied as carefully for direct therapeutic potential; this is probably due to the relative inefficiency of passive serum therapy in the past. Previous failure using passive serum therapy has been attributed to antibody interference with cellular effector mechanisms (10) which inhibit tumor elimination (16) as well as complications due to circulating tumor antigens or immune complexes. It is possible that such problems could be avoided using MoAb of specific subclasses directed at appropriate cell surface antigens.

Unlike antibodies to common acute lymphocytic leukemia antigen (21), melanoma P97 (30), or carcinoembryonic antigen, it does not react with all of the tumors of a given type and as such is relatively tumor specific. However, the best test of specificity is to measure thecirculation half-life in whole animals (28). We do not have these data for 244-19A, but the fact that this MoAb is therapeutic indicates that any reaction with normal cells in vivo must be limited. At least one other MoAb with specificity for an individual sarcoma has been reported (17), but there are no published data concerning its therapeutic potential. Antibody 244-19A is therapeutic in contrast to MoAb 271-1A, which is of the IgG2a class and binds to fibroblasts as well as to MSC cells. In Chart 7 show that treated and control survival curves differ in average time of death, but overall, no significant increase in life span is demonstrated \((p > 0.05)\). Treatment was discontinued when the first death occurred. The slightly longer survival time of both groups of animals in this experiment relative to previous ones is probably due to the fact that younger animals (6 weeks), which tend to be more resistant to tumor growth, were used.

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(a) MoAb 244-19A is relatively restricted in specificity. It binds only to MSC cells and does not react significantly with fibroblasts, a carcinoma, or 2 other BALB/c sarcomas tested. Although it has a relatively high number of binding sites per cell \((4 \text{ to } 8 \times 10^4)\), it apparently does not react with C-type viral antigens or histocompatibility antigens displayed on other sarcoma cell lines.

(b) The affinity constant and dose of antibody are probably important. MoAb 244-19A \((K_a = 3 \times 10^6 \text{ liters/mol})\) requires \(>1 \mu g\) of added MoAb to saturate binding sites on \(10^6\) MSC cells in vitro. It is probable that relatively large doses of MoAb in vivo are necessary for effective therapy (studies now in progress). The fact that 244-19A is of BALB/c origin (syngeneic with the host) allows multiple treatments with minimal complications due to host immune responses to the MoAb.

(c) Although the effector mechanism is not known, it is likely to be dependent upon the class and subclass of the antibody. For example, MoAb 244-19A is an IgG2b, which is an effective fixer of complement and may act in vivo through this mode. Alternatively, efficient antibody-dependent, cell-mediated cytotoxicity may be mediated specifically by this subclass in a syngeneic system. In the mouse leukemia system, there is still controversy about the importance of antibody class (3, 4, 14), and the mechanisms of these interactions between the host and passively administered antibody are likely to be complex.

(d) A major factor may be the antigenicity of the MSC tumor. The natural immune response to injection of \(10^6\) MSC cells is an influx of lymphoid cells and serum resulting in eradication of the tumor and subsequent induction of increased resistance to tumor cell challenge (9). Inoculation of tumorogenic doses \((10^6\) MSC cells) in MoAb-primed animals may simply lower the inoculated dose to manageable levels. However, it is unlikely that MoAb therapy is operating solely through this mechanism, since animals bearing larger tumor burdens (Chart 5) also showed significant response to MoAb therapy. Large established tumors (Chart 7) did not respond as well to MoAb therapy; however, this may be due to increased vascularization or cell necrosis and may not reflect a simple increase in tumor burden. It has been shown that certain antibodies in regressor animals can "de-block" a paralyzed cellular immune response by interaction with effector cells (10). Since 244-19A MoAb binds specifically to cell surface components, it is unlikely to be active in this mode.

We have attempted similar therapy experiments in a mouse lung carcinoma system (13) which were not successful, although the MoAb was of similar class and of comparable affinity constant. It is of interest to note that unlike the sarcoma system described here: (a) the MoAb was not syngeneic; (b) the antigen was shared among several tumors of the same class; and (c) the tumor was very weakly antigenic. Thus, it is necessary to work out the mechanism(s) by which MoAb can act as cancer therapy agents. Elucidation of these mechanisms should allow better design of MoAb and therapy schedules for human cancers once syngeneic MoAb becomes available.

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REFERENCES


Fig. 1. H & E stain of paraffin sections of MSC tumors growing progressively in BALB/c mice. Samples taken 1 hr after injection of $10^6$ cells (A), 4 days after injection (B), and 10 days after injection (C).
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