

THERAPY OF MURINE LEUKEMIA WITH MONOCLONAL ANTIBODY AGAINST A NORMAL DIFFERENTIATION ANTIGEN*

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The passive serum therapy of experimental and clinical tumors, while frequently attempted, has met with only very limited success (1). The elegant techniques of Köhler and Milstein (2), enabling the production of virtually unlimited amounts of homogeneous antibody, have led to a reconsideration of passive antibody treatment for malignant disease. This interest is based on the possibility that the use of monoclonal antibodies may be successful where serum was unsuccessful because (a) insufficient amounts of, or low titer serum was used; (b) antibody of ineffective isotype(s) was predominant; (c) the antibody was of low avidity; or (d) the antibody was of inappropriate specificity.

Monoclonal antibodies suitable for treatment would ideally be those specifically reactive with tumor cell surfaces. Since the search for true tumor-specific antibodies has been unsuccessful, an alternative approach has been taken using antibodies that are reactive with malignant and normal cells but that are not lethal or significantly toxic to the recipients (3-16). With this approach, we have been able to use monoclonal antibodies successfully against a normal murine differentiation antigen for the treatment of a transplanted T cell leukemia. Specifically, monoclonal antibodies against the Thy-1.1 differentiation antigen induced prolonged survival and cured a significant proportion of treated animals challenged with syngeneic AKR/J SL2 leukemia cells (4). In those studies, a comparison of antibodies of the immunoglobulin M (IgM), IgG_{2a}, and IgG₃ isotypes demonstrated that the IgG_{2a} antibody was most effective and the IgM antibody ineffective. In the present study, we further examined the antitumor activity of the IgG_{2a} anti-Thy-1.1 monoclonal antibody. We found that antibody therapy could consistently eliminate up to 3×10^5 transplanted syngeneic SL2 leukemia, and identified factors which limited the effectiveness of antibody. Specifically, metastatic disease resulted from the growth of Thy-1.1-negative leukemic cells, whereas development of a solid tumor nodule at the inoculum site resulted from failure of the host to eliminate anti-Thy-1.1 antibody-coated tumor cells.

Materials and Methods

Mice and Leukemias. AKR/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The transplantable AKR/J SL2 leukemia was derived from a spontaneous thymoma in an AKR mouse (17). The SL2 cells were maintained *in vitro* and *in vivo* by intraperitoneal

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inoculation of leukemic spleen cells. SL2 cells express high concentrations of Thy-1.1 both in vivo and in vitro. Tumors were implanted as single-cell suspensions of in vivo passaged cells given subcutaneously in a volume of 0.1 ml. The TD_{50} and TD_{100} for SL2 implanted subcutaneously in AKR/J mice are 10^2 and 10^3 cells, respectively (data not shown).

Monoclonal Anti-Thy-1.1 Antibody. The generation and characterization of the IgG_{2a} monoclonal anti-Thy-1.1 antibody designated 19-E12 have been previously described (3, 4). Hybrid cells secreting 19-E12 antibody were inoculated intraperitoneally into pristane-primed syngeneic (129 × BALB/c)F₁ mice for the production of ascites fluid. The ascites fluid was cytotoxic in the presence of rabbit serum as a source of complement against Thy-1.1⁺ AKR/J thymocytes and SL2 leukemic cells, but not against Thy-1.2⁺ AKR/Cu thymocytes or AKR/Cu SL1 leukemic cells (titer $<10^{-2}$). 19-E12 was also active in antibody-dependent cellular cytotoxicity with a titer of 5×10^{-5} using C57Bl/6 spleen cells (20% lysis end point) (4). The concentration of IgG in the ascites fluid was assayed by a solid-phase radioimmune assay (18). Briefly, rabbit anti-mouse IgG was fixed to wells of a microtiter plate and the concentration of 19-E12 ascites fluid that produced 50% inhibition of the binding of ¹²⁵I-labeled 19-E12 (purified by adsorption and elution from staph A [19]) to the anti-mouse IgG was compared with the concentration of a standard preparation of purified unlabeled 19-E12 (calculated from the absorbance at 280 nm assuming an extinction coefficient, $E_{1\%}^{1\text{cm}}$, of 14) that produced equivalent inhibition. Antibody was administered as unpurified pooled ascites adjusted to a complement-dependent cytotoxic titer against SL2 leukemic cells of 10^{-6} (50% lysis end point). The pooled, adjusted ascites contained ~4 mg/ml IgG.

Antibody was purified for therapy studies by adsorption and elution from a Staphylococcus protein A-Sepharose affinity column (Pharmacia Fine Chemicals, Uppsala, Sweden) (19). Column eluates were concentrated and dialyzed in phosphate-buffered saline (PBS)¹ using a PM-10 membrane (Amicon Corp., Lexington, MA). The concentrate contained 42 mg/ml of antibody and had a cytotoxic titer (50% lysis end point) against ⁵¹Cr-labeled SL2 leukemia cells of 2×10^{-7} . The concentrated antibody was diluted in PBS and administered in volumes of 200–400 μ l.

Other Antibodies. Fluorescein-conjugated 19-E12, monoclonal anti-H-H-2^k antibody, and fluorescein-conjugated anti-IgG_{2a} antibody were kindly provided by Dr. J. Ledbetter (Genetic Systems Inc., Seattle, WA). Monoclonal antibody 10.2, an IgG_{2a} directed against human lymphocytes, was kindly provided by Dr. P. Martin and Dr. J. Hansen (Fred Hutchinson Cancer Research Center). 10.2 was not cytotoxic to AKR/J thymocytes, SL2, or SL1 cells. Monoclonal murine anti-AKR-MuLV gp70 antibody 16B7 (20) and anti-Thy-1.1 alloantisera (AKR/Cu anti-AKR/J) were kindly provided by Dr. R. Nowinski (Genetic Systems Inc.).

Immunofluorescence Studies. Tissue samples were minced in RPMI 1640 supplemented with 5% fetal calf serum (5% RPMI) and filtered through 40- μ m nylon mesh. Viable cells were isolated on a Ficoll-Hypaque density gradient ($\Delta = 1.090$) and washed in 5% RPMI. Single-cell suspensions were incubated for 30 min with antibody-containing ascites fluid (at a 1:1,000 final dilution) or fluorescein-conjugated antibody in 5% RPMI at room temperature. The cells were then washed and, in indirect immunofluorescence studies, incubated with a 1:40 dilution of an affinity-purified fluorescein-labeled rabbit anti-mouse IgG or IgM serum (Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, MD) for 30 min on ice. Cells were examined by flow microfluorometry using the FACS II system (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) at a rate of 10^3 cells/s.

Results

We have previously demonstrated that the growth of a subcutaneous inoculum of $1-3 \times 10^5$ syngeneic SL2 leukemia cells could be prevented by the infusion of monoclonal IgG_{2a} anti-Thy-1.1 antibody (4). The treatment regimen used was an arbitrary one consisting of 100 μ l of ascites fluid containing anti-Thy-1.1 antibody given intravenously 1–2 h after the tumor was inoculated. This was followed by three or more doses of 50 μ l i.p. given at 3–4 d intervals. The following experiments were

¹ Abbreviations used in this paper: FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

TABLE I
Relationship of Antibody Dose and Antitumor Effects

Ascites fluid dose (mg anti-body)	Experiment				Total
	I	II	III	IV	
					%
0	0/5	0/5	0/4	0/5	0/19 (0)
0.4	4/5	1/5	—	2/5	7/15 (47)
1.2	1/5	0/5	4/5	0/5	5/20 (25)
3.2	5/5	5/5	1/4	4/4	15/18 (83)
6.4	—	—	2/4	0/5	2/9 (22)

The ability of increasing doses of ascites fluid containing 19-E12 antibody to inhibit the growth of 3×10^5 SL2 leukemic cells implanted subcutaneously in AKR/J mice was determined. 1–2 h after tumor cell inoculation, doses of ascites fluid (4 mg antibody/ml) containing 0.4, 1.2, 3.2, or 6.4 mg of antibody were infused via the retroorbital venous plexus. The number of animals surviving tumor-free >90 d and considered cured (numerator), and the number of animals treated (denominator) is shown for each of four experiments.

designed to determine the maximal anti-leukemia effect that could be achieved using monoclonal anti-Thy-1.1 antibody. First we examined the relationship of the dose of antibody infused and the number of subcutaneous leukemia cells that were eliminated, as well as the maximal number of leukemic cells that could be eliminated. Second, the effect of multiple vs. single doses of antibody was determined.

Relationship of Anti-leukemic Effect and Antibody Dose. To quantitate the anti-leukemic effect of monoclonal anti-Thy-1.1 antibody, we examined the ability of increasing amounts of antibody infused as a single, intravenous bolus to prevent leukemia in mice challenged with increasing doses of tumor cells. Specifically, AKR/J mice were challenged subcutaneously with 3×10^5 , 10^6 , or 3×10^6 SL2 leukemia cells. 1–2 h later, groups of these mice were treated by an intravenous infusion of 100, 300, 800, or 1,600 μ l of ascites fluid containing 4 mg/ml 19-E12 anti-Thy-1.1 monoclonal antibody (see Materials and Methods).

The results demonstrated that a significant proportion of mice challenged with 3×10^5 SL2 cells could be cured by a single dose of antibody (Table I). A portion of the mice treated with each antibody dose was cured, with the highest and most consistent rate (83%) seen in mice treated with ascites fluid containing 3.2 mg of antibody. Although a strict dose relationship was not observed in each experiment, lesser but significant effects were usually seen in mice treated with lower doses of 0.4 or 1.2 mg (cure rates of 47 and 25%, respectively). At the highest dose tested (6.4 mg antibody contained in 1,600 μ l ascites fluid) only a 22% cure rate was achieved. Because the decreased effectiveness of the highest dose of ascites fluid tested may have been due to the infusion of material other than antibody, the effectiveness of 19-E12 antibody purified by adsorption and elution from Staphylococcus protein A was tested. Treatment of mice with 1.5 mg purified antibody led to the inhibition of tumor growth in four of five mice, whereas the infusion of higher doses of antibody led to a lower cure rate. All mice treated with 16 mg developed leukemia (Table II). Similar results were obtained in a second experiment. To determine whether variability between animals in each treatment group was due to the faulty injection of antibody, serum was obtained from each mouse 24 h after the infusion of antibody and tested for cytotoxicity. Within each dosage group there was relatively uniform serum

TABLE II
Antitumor Effects of Purified Antibody

Antibody dose <i>mg</i>	Number cured/Number treated	Cytotoxic titer ⁻¹ (mean ± SD) × 10 ³
0	0/5	<0.1
0.5	3/5	1.2 ± 0.4
1.5	4/5	10.0 ± 2.4
8	2/5	66.8 ± 3.8
16	0/5	188.8 ± 39.6

3×10^5 SL2 leukemic cells were implanted subcutaneously in AKR/J mice. 1 h later antibody purified by adsorption and elution from Staphylococcus protein A (see Materials and Methods) was infused via the retroorbital venous plexus. The number of animals surviving tumor-free at 50 d (numerator) and the number of animals treated (denominator) is shown for each treatment group. Serum samples were obtained 24 h after the infusion of purified 19-E12 antibody and complement-dependent cytotoxic titers (50% lysis) against SL2 leukemic cells determined using a ⁵¹Cr-release assay. Mean titer ± SD is shown for each group.

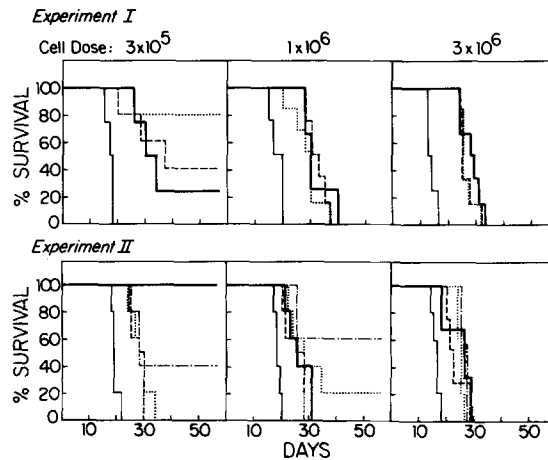


FIG. 1. Determination of maximal antitumor effect of antibody therapy. AKR/J mice were challenged subcutaneously with 3×10^5 (left), 10^6 (middle), or 3×10^6 (right) SL2 leukemic cells. 1–2 h later groups of 4–6 mice were treated with an intravenous dose of ascites fluid containing 0 (—), 0.4 (---), 1.2 (.....), 3.2 (— — —), or 6.4 (— · —) mg 19-E12 antibody. Upper and lower panels are two separate experiments.

cytotoxic activity (Table II). Furthermore, there were no differences in serum cytotoxic titer within each group between those animals that developed leukemia and those that did not (data not shown). This result suggested that the consistently observed differences within treatment groups may be caused by host factors.

The maximal number of tumor cells that could be eliminated by antibody treatment was also determined. In these experiments, antibody doses found effective in preventing leukemia due to inocula of 3×10^5 SL2 leukemic cells were tested for their ability to prevent the outgrowth of larger tumor doses. The results showed that infusion of amounts of ascites fluid containing 0.4–6.4 mg 19-E12 antibody inhibited the growth of inocula consisting of 3×10^5 SL2 leukemic cells and in some instances of 10^6 cells, but each dose level failed to cure any mice challenged with 3×10^6 leukemic cells (Fig. 1). We also determined whether the growth of an increased number of leukemic cells could be inhibited using purified antibody. In two studies, no cures were obtained

after challenge with 10^6 or 3×10^6 cells when groups of five mice each were treated with purified antibody using doses of 4, 8, or 16 mg per animal (data not shown). Thus, the maximal anti-leukemic effect that could be consistently achieved using a single bolus of up to 16 mg of antibody was the elimination of 3×10^5 cells. Furthermore, the few mice cured after challenge with 10^6 leukemic cells (4/67 in all experiments) were cured by lower antibody doses (0.4 and 1.2 mg). This lack of a dose-effect relationship suggests that 10^6 cells was the limiting dose curable by antibody because of factors other than antibody dose, e.g., participating host factors (see Discussion).

An analysis of the survival patterns of mice not cured of their tumors also revealed that the median survival for groups of antibody-treated mice challenged with a given tumor dose was essentially the same whether they were treated with 0.4, 1.2, 3.2, or 6.4 mg of antibody (Fig. 1). Thus, death, which resulted from metastatic disease in these mice, was equally delayed by lower as well as higher doses of antibody. The basis for this phenomenon is explored in later experiments that examine the reactions of the metastatic leukemic cells with 19-E12 antibody. Of further interest is the observation that the solid tumor that grew at the inoculum site often became significantly larger before death in the majority of treated mice as compared with untreated mice (data not shown). This was not a result of more rapid tumor growth in treated mice, because the appearance of a palpable nodule was delayed only slightly and because once a nodule was palpable the growth rate was similar in treated and untreated animals. Rather, it was due to the continued growth during the period of extended survival that resulted from the antibody treatment.

Therapeutic Effects of Single vs. Multiple Antibody Doses. We next determined whether there was an advantage in infusing multiple antibody doses. For this purpose, we compared the effects of a relatively high dose of 19-E12 antibody (3.6 mg) given as a single intravenous infusion on day 0 vs. a 3.6 i.v. dose on day 0 followed by four additional 1.8 mg doses given intraperitoneally every 3-4 d. These schedules were also compared with a regimen similar to that used in previous studies, i.e., 0.4 mg given intravenously on day 0 followed by four doses of 0.2 mg i.p. every 3-4 d. These regimens were used to treat mice challenged with 3×10^5 or 3×10^6 SL2 leukemic cells given subcutaneously. The results (Fig. 2) revealed a significant antitumor effect from 3.6 mg of antibody but no additional benefit from the subsequent 1.8-mg doses. Further, treatment with a single 3.6-mg dose was more effective than treatment with 0.4 mg followed by 0.2 mg in mice challenged with 3×10^5 cells. When mice were challenged with 3×10^6 cells there were no cures and survival was again prolonged to the same extent by all antibody regimens. Thus, the infusion of a single high amount of antibody on the day of tumor inoculation led to the maximal antitumor effect that could be achieved, i.e., the inhibition of tumor growth due to 3×10^5 cells.

The failure of antibody to consistently eliminate $>3 \times 10^5$ cells at a subcutaneous site could have resulted from either the lack of access of antibody to the tumor, a failure to eliminate antibody-coated tumor cells, or the escape of Thy-1.1-negative tumor cells. In the following experiments each of these issues was examined.

Ability of Antibody to Bind to Tumor Cells Within a Subcutaneous Nodule. Binding of infused anti-Thy-1.1 antibody to subcutaneous tumor cells in vivo was directly examined using quantitative immunofluorescent techniques. For this purpose 3×10^6 tumor cells were inoculated subcutaneously and, when a palpable tumor nodule was

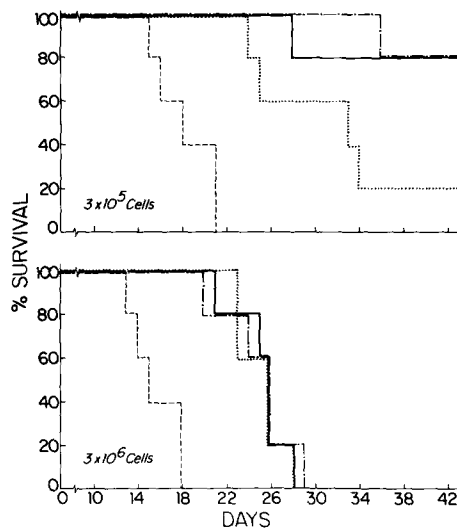


FIG. 2. Antitumor effect of multiple vs. single antibody doses. Groups of five AKR/J mice each were implanted subcutaneously with 3×10^5 (top) or 3×10^6 (bottom) SL2 leukemic cells. 1–2 h later, mice were treated with ascites fluid containing 4 mg/ml 19-E12 antibody. Treatment regimens were (a) no antibody (---), (b) a single intravenous dose of 3.6 mg (—), (c) 3.6 mg i.v. on day 0 followed by 1.8 mg i.p. on days 3, 7, 11, and 14 (-·-·-), (d) 0.4 mg i.v. on day 0 followed by 0.2 mg i.p. on days 3, 7, 11, and 14 (.....).

present (10-mm average diam), ascites fluid containing 19-E12 antibody was infused intravenously. At various times thereafter, the tumor nodules were removed and a single-cell suspension was obtained. The cell surfaces were then examined for the presence of the infused antibody by staining cells with fluorescein-conjugated anti-mouse IgG antibody. The amount of fluorescence obtained was compared with the maximum amount that could be achieved if all available Thy-1.1 cell surface sites were coated with antibody. For this purpose, aliquots of the tumor cells were incubated in vitro with excess 19-E12 antibody before incubation with the fluorescein isothiocyanate (FITC)-anti-mouse IgG antibody. A third aliquot of cells was incubated with FITC-anti-IgM to determine nonspecific fluorescent binding. After incubation, cells were examined by flow microfluorometry using the FACS-II, and the location of the fluorescence histogram peaks was determined. Thus, the proportion of cell surface Thy-1.1 sites that were coated in vivo with the infused IgG antibody (percent in vivo saturation) could be determined from the histograms according to the formula:

$$\frac{[\text{Peak anti-IgG-FITC}] - [\text{Peak anti-IgM-FITC}]}{[\text{Peak (in vitro 19-E12 + anti-IgG-FITC)}] - [\text{Peak anti-IgM-FITC}]} \times 100.$$

We first examined the specificity and time course of antibody binding. 100 μ l of ascites fluid containing 0.4 mg 19-E12 antibody or, as a control, an equivalent amount of antibody of the same isotype but irrelevant specificity (antibody 10.2) was infused intravenously. Tumor nodules from five to seven individual mice were then excised and examined at 12, 24, and 48 h after antibody infusion (Fig. 3). Binding of 19-E12 was maximal at 24 h with $12 \pm 3\%$ (mean \pm SE) of the available Thy-1.1 sites coated with IgG antibody at 12 h, $39 \pm 7\%$ at 24 h, and $18 \pm 2\%$ at 48 h. The binding was

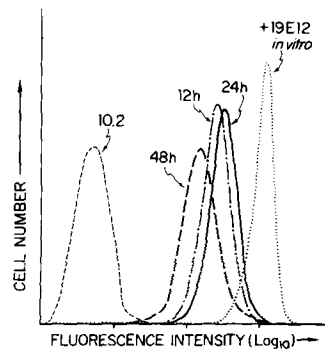


FIG. 3. Kinetics of in vivo antibody binding to tumor cell surfaces. AKR/J mice with palpable SL2 subcutaneous tumor nodules (0.5–1.0 cm diameter) were given intravenous infusions of ascites fluids containing 100 μ l 0.4 mg 19-E12 or 10.2 (an IgG_{2a} antibody of irrelevant specificity) antibody 12, 24, or 48 h before analysis. Excised nodules were minced and viable cells isolated on a Ficoll-Hypaque density gradient ($\Delta = 1.090$). Cells were then tested by indirect immunofluorescence after incubation with (a) FITC-rabbit anti-mouse IgG (to determine antibody bound in vivo); (b) 19-E12 ascites fluid (1:1,000 dilution) and FITC-rabbit anti-mouse IgG (to determine the maximal amount of anti-Thy-1.1 antibody that can bind to the cell surface); or (c) FITC rabbit anti-IgM (to determine nonspecific binding). Fluorescence was analysed with the FACS-II and a composite of representative fluorescence histograms is shown. Not shown is that light scatter (size) patterns in each case demonstrated that a uniform cell population was being analysed. Furthermore, location of control FITC-anti-IgG fluorescence peaks with cells from untreated animals were always within 10% of FITC anti-IgM peaks and at least two orders of magnitude less than 19-E12 + FITC anti-IgG₁ peaks.

specific, since IgG was not detected on tumor cells from mice treated with the control antibody.

We next determined the relationship of antibody dose and the amount of antibody bound to the tumor cell surfaces. Animals with tumor nodules received 0.4, 1.2, or 3.2 mg of 19-E12 intravenously and cells from the nodules were examined 24 h later. The proportion of available Thy-1.1 sites coated in vivo by antibody (percent saturation) was found to increase when larger amounts of antibody were infused. Specifically, infusion of 0.4 mg led to the coating of $27 \pm 13\%$ (mean \pm SE) of available Thy-1.1 sites, 1.2 mg to $51 \pm 5\%$ saturation, and 3.2 mg to complete saturation. Moreover, 24 h after antibody infusion, a linear relationship between the log of the serum complement-dependent cytotoxic titer against SL2 leukemic cells in vitro (as a measure of unbound anti-Thy-1.1 antibody in serum) and tumor cell surface antibody was observed (Fig. 4). Thus, antibody saturation of tumor cell surface antigenic sites could be accurately predicted from the analysis of free serum antibody content. Further, only the presence of very high amounts of antibody in the serum (titer $\geq 10^{-5}$ after infusion of 3.2 mg of antibody) led to sufficient infiltration of the subcutaneous space by the infused antibody to achieve maximal cell surface binding.

Antigenic Nature of Transplanted Leukemia Cells that Escape Antibody Therapy. Because the infused antibody was capable of binding to tumor cells in the subcutaneous space and saturating surface antigenic sites, we examined whether the failure of antibody therapy to eliminate $>3 \times 10^5$ cells was a result of the failure of the host to eliminate antibody-coated cells or of alterations in expression of the target Thy-1.1 antigen by the leukemic cells. For this purpose, mice were challenged with 3×10^6 SL2 leukemic cells and, 1–2 h later, received an intravenous infusion of ascites fluid containing 3.6

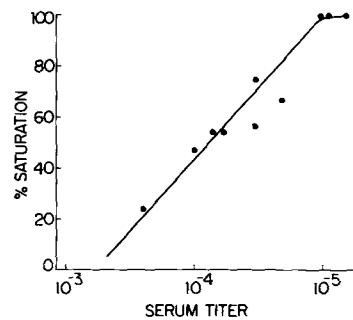


Fig. 4. In vivo binding of anti-Thy-1.1 antibody to subcutaneous leukemic cells: relationship with antibody dose. AKR/J mice with palpable subcutaneous SL2 tumor nodules were treated intravenously with doses of ascites fluid containing 0.4, 1.2, or 3.2 mg of 19-E12 antibody. 24 h later the nodule was excised and the amount of infused antibody bound to the leukemic cell surfaces was determined by quantitative immunofluorescence (see text). The serum cytotoxic titer at the time of nodule excision was determined by a complement-dependent ^{51}Cr -release assay (50% lysis end point).

mg 19-E12 antibody, followed by intraperitoneal injections of 1.8 mg on days 3, 7, 10, and 14. 12 d after the last antibody dose, when the treated mice had developed tumor nodules at the inoculum site and enlarged spleens, they were killed and leukemic cells from their spleens and subcutaneous nodules were obtained. As in the previous experiments, the amount of cell surface IgG was determined by direct immunofluorescence using fluorescein-conjugated anti-mouse IgG antibody, and the expression of the target Thy-1.1 antigen was determined by indirect immunofluorescence using 19-E12 followed by the fluoresceinated anti-Ig reagent.

Evaluation of leukemic cells from the nodules of five treated mice revealed that the cell surfaces were coated with IgG and that only a small increase in fluorescence was seen when the cells were incubated in vitro with 19-E12. Assuming that all of the in vivo bound surface Ig represented infused antibody bound to Thy-1.1 sites, the analysis of the fluorescence histograms revealed that $87 \pm 4\%$ of the available Thy-1.1 sites had been coated with antibody in vivo. Representative results from one mouse are shown in the left-hand column of Fig. 5. Further, the level of Thy-1.1 expression by subcutaneous tumor cells from treated mice, determined by quantitative immunofluorescence, was comparable to that seen with tumors from untreated mice. Thus, the growth of leukemic cells in the subcutaneous nodule occurred despite the presence of high levels of anti-Thy-1.1 antibody on their cell surfaces over a prolonged period of time and may be attributed to a failure of host mechanisms to eradicate antibody-coated leukemic cells from the subcutaneous space.

Examination of the metastatic leukemic cells from the spleens of treated animals yielded contrasting results. As shown in the right-hand column of Fig. 5, there was a failure to detect either surface IgG or Thy-1.1 sites after staining with a fluoresceinated anti-mouse IgG antibody or with 19-E12 antibody followed by the fluoresceinated anti-mouse IgG reagent. A similar result was found for the other mice in this experiment. The antigenic nature of the metastatic leukemic cells in antibody-treated mice was further examined in an additional experiment. A lower dose (0.4 mg) of antibody was administered intravenously on day 0 followed by 0.2 mg intraperitoneally every 3-4 d on a continuous basis. As shown in Fig. 6, the infusion of lower

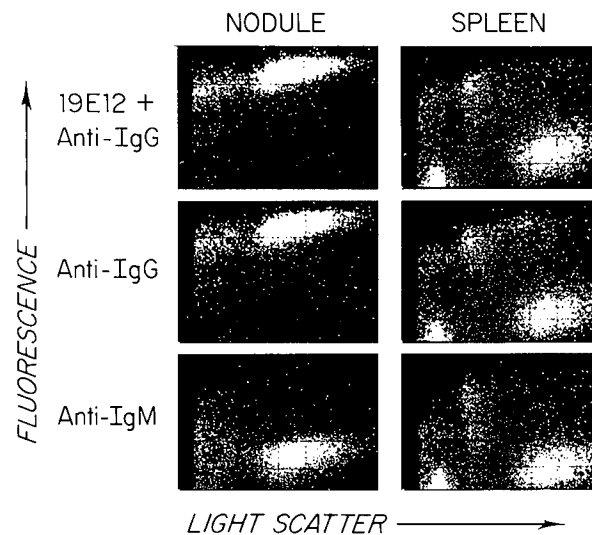


FIG. 5. Thy-1.1 antigen expression by leukemic cells from mice treated with anti-Thy-1.1 antibody. AKR/J mice were challenged subcutaneously with 3×10^6 SL2 leukemic cells and treated with ascites fluid containing 3.6 mg 19-E12 antibody on day 0 and 1.8 mg intraperitoneally on days 3, 7, 11, and 14. On day 26, when palpable tumor nodules were present at the inoculum site and significant spleen enlargement was apparent, individual leukemic cells were isolated from the nodule and the spleen. Cells from the nodule (left) and spleen (right) were incubated with 19-E12 antibody followed by fluorescein conjugated anti-IgG antibody (to measure unbound Thy-1.1 sites), fluorescein conjugated anti-IgG antibody alone (to measure *in vivo* binding of the infused anti-Thy-1.1 antibody), or FITC anti-IgM (control). Each plot represents 4,000 cells analyzed with logarithmic amplification.

amounts of antibody also led to metastatic growth of cells that lacked surface Thy-1.1 antigenic sites. This failure to detect surface antigen was not due to blocking of the antigenic sites since surface IgG_{2a} was not detected. The failure of Thy-1.1 expression was selective since surface expression of H-2^k and gp70 was maintained at levels comparable to SL2 cells from untreated leukemic animals. In addition, to examine the possibility that only the epitope on the Thy-1 molecule detected by 19-E12 was not expressed, we also examined the leukemic spleen cells using an anti-Thy-1.1 alloantiserum. Again, surface binding of the anti-Thy-1.1 serum was not detected. In an additional study, the intravenous infusion of even a single dose of 19-E12 (3.6 mg) 1–2 h after subcutaneous inoculation of 3×10^6 SL2 cells was able to prevent the metastatic spread of leukemic cells expressing the Thy-1.1 antigen, while allowing the metastatic invasion of the spleen by Thy-1.1 negative leukemic cells (data not shown).

This absence of Thy-1.1 on metastatic cells could have resulted from modulation of the antigen in the presence of antibody or from the selection of antigen-negative variants. To differentiate these possibilities, five antigen-negative tumors were sequentially passaged five times through normal syngeneic hosts and then examined for the reexpression of Thy-1.1, as well as the continued expression of gp70. The passaged tumor continued to lack detectable Thy-1.1, whereas gp70 expression was maintained (Fig. 7). Thus, metastatic disease in treated mice resulted from growth of stable antigen-negative variants.

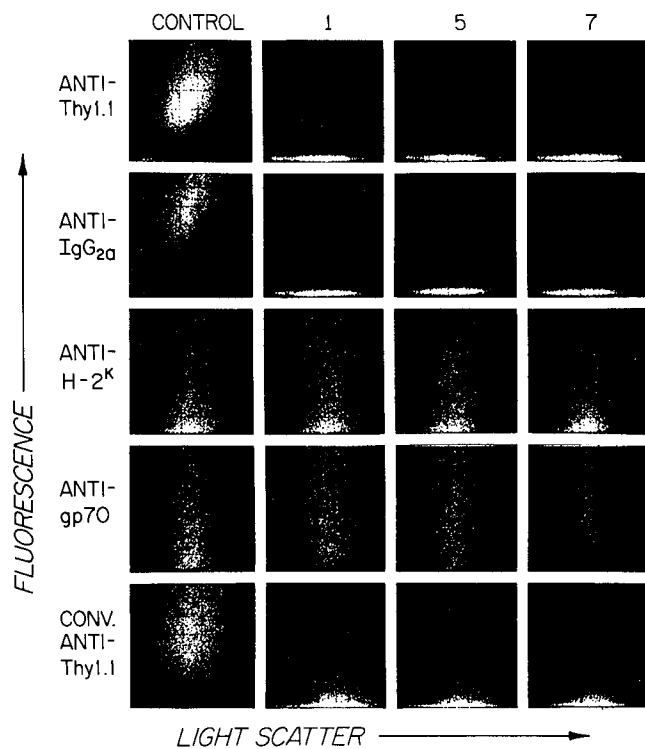


FIG. 6. Antigen expression by metastatic spleen cells. AKR/J mice were challenged with 3×10^6 SL2 leukemic cells subcutaneously and remained untreated or were treated with ascites fluid containing 0.4 mg 19-E12 antibody on day 0, and 0.2 mg i.p. twice weekly thereafter. When splenic enlargement was apparent, the metastatic leukemic spleen cells were obtained and incubated in vitro with FITC-19-E12 (top row), FITC-rabbit anti-mouse IgG_{2a} antibody (second row), anti-H-2^k followed by FITC-anti-IgG (third row), anti-gp70 followed by FITC-anti-IgG (fourth row), or a conventional anti-Thy-1.1 alloantiserum followed by FITC-anti-IgG (bottom row). Spleen cells from an untreated control and three treated animals (1, 5, and 7) are shown. In second box from top in the first column, control leukemic cells were preincubated with 19-E12 antibody before addition of the anti-IgG_{2a}-FITC reagent. Each plot represents 4,000 cells analyzed with linear amplification.

Discussion

Our previously reported studies of monoclonal antibody therapy of transplanted leukemia have demonstrated the ability of antibody to cure mice challenged with up to 3×10^5 syngeneic SL2 leukemia cells (3-5). In the current studies we examined varying antibody regimens and determined the maximal number of leukemia cells present in a subcutaneous inoculum that could be eliminated by antibody. IgG_{2a} anti-Thy-1.1 antibody administered as a single intravenous bolus, in doses of ascites fluid ranging from 0.4 to 6.4 mg of antibody or 0.5 to 16 mg of purified antibody, could prevent the outgrowth of 3×10^5 SL2 leukemia cells in a portion of treated mice. The maximal and most consistent effect was achieved by the infusion of ascites fluid containing 3.2 mg of antibody which cured 83% of the mice challenged with 3×10^5 SL2 leukemia cells. This relatively high dose was necessary to achieve maximal antibody binding to most or all of the target antigen expressed by subcutaneous tumor cells. Presumably, lower doses were less effective because fewer antibody

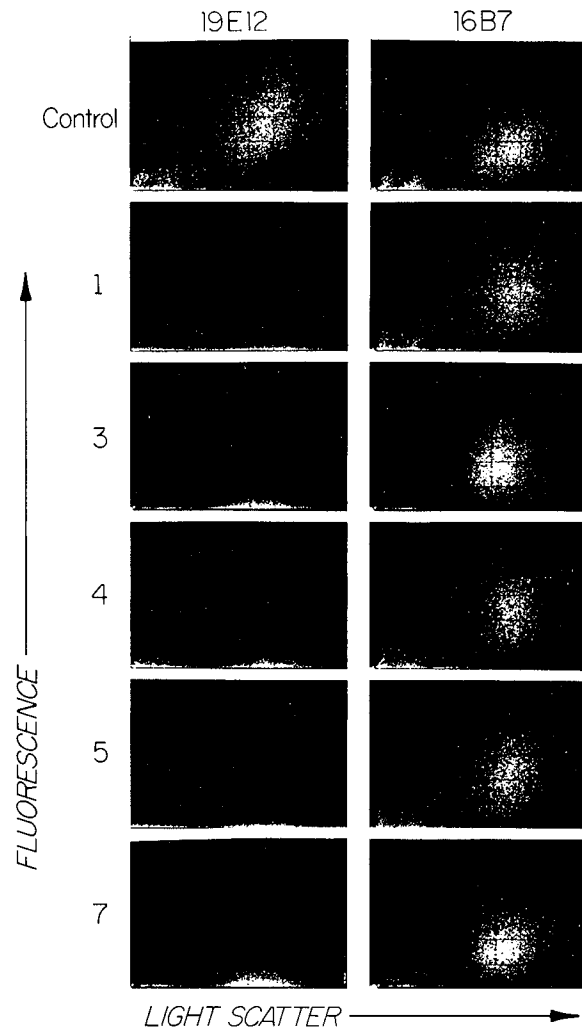


FIG. 7. Failure of Thy-1.1 expression by metastatic leukemic cells from treated mice is due to emergence of stable antigen-negative variants. Five Thy-1.1-negative leukemias established from metastatic splenic leukemic cells in mice treated with anti-Thy-1.1 antibody (animals 1, 3, 4, 5, and 7) were passaged intraperitoneally five times each in untreated syngeneic mice. The ability of these cells to reexpress the Thy-1.1 antigen was examined by incubating the cells *in vitro* with 19-E12 + FITC-anti IgG (to determine Thy-1.1 expression). In addition, alterations in the expression of AKR-MuLV gp70 were examined by staining the cells with 16B7 followed by FITC-anti-mouse IgG antibody. Results were compared with those obtained with passaged SL2 leukemic cells from untreated mice (control). Each plot represents 4,000 cells analyzed with linear amplification.

molecules fixed to the tumor cell surfaces or because antibody concentrations were maintained in the subcutaneous space for an insufficient length of time, thereby not allowing for optimal host effector activity (e.g., macrophages).

The highest antibody doses, whether given in the form of ascites fluid or purified Ig, led to suboptimal effects. A decrease in therapeutic effects with supra-optimal doses has also been observed in other experimental systems (21). It is possible that the inhibitory effects of very high doses of antibody may result from the inhibition of

effector cell function by high concentrations of free antibody. The occurrence of this phenomenon suggests the need for careful dose titrations when antibody is used for therapy. Although infusion of multiple doses of antibody did not prove advantageous in this study, it is possible that prolonged therapy may be of benefit in situations where the tumor has a longer doubling time than that of the SL2 leukemia used in this study (doubling time <1 d). In those situations, additional effector cells may localize at the tumor site and kill the antibody-coated cells before significant tumor growth occurs.

The maximal number of leukemic cells that could be consistently eliminated was 3×10^5 . The results of this study identified two factors potentially responsible for this limitation. First, the failure of antibody to mediate the elimination of higher numbers of subcutaneous tumor was shown to result from a failure of the host to eliminate antibody-coated cells. This failure was most likely a consequence of inadequate participation of host effector cells. It was not due to lack of access of antibody to the tumor because saturation of available tumor cell surface Thy-1.1 sites *in vivo* by the infused antibody was documented. Participating host effector mechanisms have already been identified as a limiting factor in serum therapy (22, 23). Presumably, the participating host factors consist of effector cells (23–25) and not lytic complement because the AKR/J recipients in the present experiments are deficient in the fifth component of complement. It may also be argued that a limiting factor in this antibody therapy model may be that the target antigen is widely expressed by normal T cells and that the host effector activity against opsonized leukemic cells may be diluted by the antibody-coated lymphocytes. However, studies with both antiserum and monoclonal antibodies with less or no reactivity with normal cells have suggested that the maximal antitumor effect obtainable with antibody is the elimination of $\leq 10^6$ cells (10, 24, 26, 27). Importantly, it has been shown that this limitation applies to individual sites, and that antibody treatment can prevent the growth of multiple implants of 10^5 cells in a single mouse (27).

Although the number of antibody-coated leukemia cells which could be eliminated from a subcutaneous site was relatively small, there are situations where this effect could prove therapeutically useful. In situations where minimal residual disease is present, e.g., when leukemia in remission, antibody alone may eliminate the few tumor cells that remain following conventional therapy. We have successfully used this latter approach to prolong chemotherapy-induced remissions in AKR/J mice with spontaneous T cell lymphomas (5). Where larger masses of extravascular tumor are involved, the potential of antibody therapy will only be realized if it is directly inhibitory to tumor cells or if it is used as a carrier for toxic agents. However, the relative localization of antibody to tumor vs. other normal tissues must first be studied to determine to what extent these agents can be localized to tumor as compared with nonantigen-bearing target cells.

The second factor found to limit antibody therapy was the emergence of variant leukemic cells that did not express the target Thy-1.1 antigen. Antibody was, however, highly effective in eliminating antigen-positive metastasizing cells and significantly delayed the occurrence of metastatic disease and death. Even when relatively large doses of tumor cells and therapeutically suboptimal amounts of antibody were administered, antibody therapy completely prevented the spread of Thy-1.1-bearing tumor cells. This effect most likely resulted from the efficient removal of antibody-

coated cells by the reticuloendothelial system. Alternatively, it is possible that surface antibody prevented tumor cells from establishing themselves at an appropriate site.

The problem of the emergence of antigen-negative variants as observed in these studies potentially can be overcome by using combinations of antibodies directed at different antigens, because the frequency of variants lacking multiple unrelated determinants should be vastly reduced from that of variants lacking only one antigen. The effectiveness of antibody in preventing metastatic disease also suggests that the infusion of antibody during complete or partial resection of solid tumor masses may prove useful in preventing hematogenous dissemination of malignant cells that are shed during surgery.

Summary

The ability of monoclonal antibodies against the Thy-1.1 differentiation antigen to inhibit the growth of transplanted syngeneic AKR/J SL2 leukemic cells has been previously demonstrated. In the present study we further examined therapy with monoclonal antibody of the IgG_{2a} isotype, which was the most effective isotype studied. Intravenous infusion of ascites fluid containing the anti-Thy-1.1 monoclonal antibody 19-E12 1–2 h after tumor implantation led to inhibition of the growth of 3×10^5 but not 3×10^6 syngeneic SL2 leukemic cells. The achievement of the maximal therapeutic effect required the infusion of a dose containing 3.2 mg of antibody, which inhibited the growth of a subcutaneous inoculum of 3×10^5 SL2 leukemic cells in 83% of treated mice. Multiple doses of antibody were no more effective than a single dose given shortly after tumor implantation. The infusion of this relatively large 3.2-mg dose of antibody was required to infiltrate the subcutaneous space and saturate surface Thy-1.1 sites on leukemic cells in a subcutaneous tumor nodule.

The failure of antibody to inhibit larger numbers of tumor cells was investigated. Growth of a subcutaneous tumor nodule in mice challenged with more than 3×10^5 cells resulted from the growth of Thy-1.1-bearing cells in spite of the presence of the infused anti-Thy-1.1 antibody on their surfaces. In contrast, metastatic growth was due to the emergence of variant leukemic cells lacking the Thy-1.1 antigen. Thus, treatment of transplanted T leukemic cells with an IgG_{2a} anti-Thy-1.1 monoclonal antibody was effective in eliminating 3×10^5 antigen-bearing leukemic cells from the subcutaneous space and was very effective in preventing metastasis of leukemic cells expressing the target Thy-1.1 antigen. Therapy was limited by the failure of host mechanisms to eliminate larger numbers of subcutaneous leukemic cells coated with the infused antibody and by the emergence of variant leukemic cells lacking the target antigen.

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