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STUDIES ON THE ABILITY OF MONOCLONAL ANTIBODIES TO SELECTIVELY MEDIATE COMPLEMENT-DEPENDENT CYTOTOXICITY OF HUMAN MYELOGENOUS LEUKEMIA BLAST CELLS[1]

EDWARD D. BALL,[2] JAMES M. KADUSHIN, BERNICE SCHACTER, AND MICHAEL W. FANGER

From the Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755 and the Department of Pathology, University Hospitals of Cleveland, Case Western Reserve University, Cleveland, OH 44106

A panel of monoclonal antibodies that bind to leukemic blast cells from patients with acute myelocytic leukemia and chronic myelocytic leukemia in blast crisis was studied for their ability to mediate complement-dependent lysis of a variety of cell populations from patients with leukemia, normal blood cells, and human leukemia cell lines. Several of these monoclonal antibodies were selectively cytotoxic to myeloid leukemia cells (AML-1-99, AML-1-211, AML-2-30, CML-75, CML-115, and CML-150). Although they were all capable of binding to normal cell populations, none of these hybridomas were cytotoxic to normal cells. Three of these antibodies (AML-1-211, CML-75, and CML-150) were cytotoxic to some leukemia cell samples only after dilution of the hybridoma supernatant, i.e., they showed a prozone. Binding of these three antibodies, as well as another, AML-1-201, as determined in a radioimmunoassay, also showed a prozone. Other monoclonal antibodies are described (AML-2-23 and AML-2-9) that mediate complement-dependent cytotoxicity to myeloid leukemia cells as well as selected normal cell types (monocytes and lymphocytes, respectively). The potential clinical utility of these monoclonal antibodies is considered in the context of recently encountered problems in the use of monoclonal antibodies to mediate leukemia cell lysis *in vivo*.

Although monoclonal antibodies that recognize tumor-specific antigens would be extremely useful in the diagnosis and treatment of human malignant diseases, it is as yet unclear whether truly tumor-specific antigens exist on human tumor cells (1). Nonetheless, it seems likely that quantitative differences in antigen expression will be found on tumor cells compared to normal cells, and such differences may be useful in demonstrating subgroups of selected tumor types or in mediating lysis of tumor *vs* normal cells.

In a previous paper,[3] we reported the preparation and properties of a panel of monoclonal antibodies that bind to myeloblasts from patients with acute myelocytic leukemia (AML)[4] or chronic myelocytic leukemia (CML) in blast crisis. An important finding was that the antigens determined by these antibodies were found to some extent on all cells tested, both normal and leukemic. Even so, we have determined that some of these antibodies can permit complement-mediated lysis of leukemic myeloblasts but not normal cells. This paper describes in detail the patterns of cytotoxicity of 20 different monoclonal antibodies to cells from patients with leukemia, normal individuals, and human leukemia cell lines. Several of these monoclonal antibodies appear exclusively cytotoxic to myeloid leukemia cells, whereas others mediate lysis of myeloid leukemia cells as well as subpopulations of normal cells. The potential clinical utility of this panel of monoclonal antibodies is considered.

MATERIALS AND METHODS

Hybridomas. Details of the production of these hybridomas and partial characterizations of the monoclonal antibodies are reported elsewhere.[3] Briefly, monoclonal antibodies were developed by immunizing BALB/c mice with human myelogenous leukemia cells and fusing spleen lymphocytes with cells from the P3-XAg63 murine myeloma cell line by using polyethylene glycol (m.w. 1000, J. T. Baker Chemical Co., Phillipsburg, NJ) according to the method of Köhler and Milstein (2). A panel of 20 different monoclonal antibodies were included in this study. All were IgM immunoglobulins except AML-2-23, which is an IgG of the γ_1 subclass. The hybridomas were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 5 mM HEPES[4] buffer, and gentamicin, 50 μg/ml. All medium components were obtained from K. C. Biologicals, Kansas City, MO, except gentamicin (Schering Corp., Kenilworth, NJ).

Cells. Leukemic cells were obtained from patients with AML, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), and CML in blast crisis, and were separated from blood by Ficoll-Hypaque gradient centrifugation (3). Normal blood cells were obtained from the mononuclear cell populations of normal laboratory personnel. Monocytes were separated from the mononuclear cell fraction by adherence to plastic. T and B lymphocyte separations were accomplished by filtering lymphocyte preparations through nylon wool columns as described by Daynilovs *et al.* (4). T cells were collected in the initial filtrate while the adherent B cells were detached by vigorous agitation and rewashing of the column with medium. The human leukemic cell lines CCRF-CEM, KG-1a, HL-60, and U937 were also studied. CCRF-CEM, a lymphoblastoid cell line, derived from a patient with T-ALL (obtained from the American Type Culture Collection), was maintained in culture in RPMI 1640 containing 20% FCS (5). The KG-1a cell line, a subline of the KG-1 cell line derived from a patient with AML (6), was grown in Alpha medium (Flow Laboratories, Inc., Rockville, MD) containing 20% FCS and gentamicin. This line was a gift from Dr. P. Koeffler, Division of Hematology-Oncology, UCLA, Los Angeles, CA. The HL-60 cell line was derived from a patient with acute promyelocytic leukemia (7). This line was a gift of Dr. Robert Gallo, Laboratory of Tumor Immunology, National Institutes of Health and was maintained in RPMI supplemented with 10% FCS. The U937 cell line was derived from a patient with histiocytic lymphoma and has some functional and morphologic characteristics of macrophages (8). This line was a gift of Dr. Paul Guyre, Department of Physiology, Dartmouth Medical School, and was maintained in RPMI 1640 supplemented with 10% FCS.

Binding. Supernatants from hybridomas were serially diluted in DMEM containing 20% FCS and were added to wells coated with glutaraldehyde-fixed leukemic cells, normal lymphocytes, or normal monocytes and incubated for 2 hr at 37°C according to the method of Kennetrt (9). After washng off unbound antibody with phosphate-buffered saline (PBS) and

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[2] Address correspondence to: Edward D. Ball, M.D., Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755.

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[4] Abbreviations used in this paper: AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CML-BC, chronic myelocytic leukemia in blast crisis; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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^{125}I-anti-mouse κ-antibody was added and incubated for 2 hr at 37°C. After washing with PBS the wells were counted in a gamma counter (Beckman 4000).

Cytotoxicity. Cytotoxicity was tested by dye exclusion in microtiter trays according to the method of Amos (10). Cells from patients with leukemia and mormal peripheral blood were suspended in Veronal Buffer (Oxoid, K. C. Biologicals, St. Louis, MO) or calcium and magnesium free Hanks' balanced salt solution (M. A. Bioproducts, Walkersville, MD). Hybridoma supernatants were diluted in hybridoma culture medium and incubated with cells for 30 min at 22°C before a wash step and the addition of rabbit serum (diluted 1:4.2 in Veronal buffer) as a source of complement (Low-Tox H, Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada). Incubation with complement proceeded for 1 hr at 22°C. Trypan blue exclusion was used to estimate cytolysis. Controls included anti-β_2 microglobulin and anti-Ia antisera (Accurate Chemical and Scientific Corp., Westbury, NY). Negative controls included human AB serum, the myeloma parent supernatant, and heat-inactivated complement. Each experiment was done at least in duplicate and in many cases, in quadruplicate. The data reported are the means of replicate assays in which deviation from the mean was less then 10%.

RESULTS

Sixteen of the 20 monoclonal antibodies studied gave maximal binding to both leukemic and normal cells in undiluted or weakly diluted supernatant. The titer, defined as the dilution that gave 50% of maximal binding, ranged from 1:2 to 1:1024 for this group of monoclonal antibodies. The binding of several representative monoclonal antibodies to AML cells is seen in Figure 1*A*. Either a plateau or linear decline in binding was seen with decreasing concentration of antibody.

In contrast, four of the 20 monoclonal antibodies studied, AML-1-201, AML-1-211, CML-75, and CML-150, showed an increase in binding to AML cells as the hybridoma supernatant was diluted, with maximal binding observed between 1:8 and 1/32 dilution (Fig. 1*B*).

Binding of the majority of these monoclonal antibodies to mormal lymphocytes paralleled that to AML cells (Fig. 2). A notable exception was AML-2-23, which showed binding to lymphocytes only slightly above background while binding significantly to AML cells over a broad range of dilutions. The binding of monoclonal antibodies AML-2-23, AML-1-211, AML-2-9, AML-1-99, and CML-75 to monocytes is shown in Fig. 3. With the exception of monoclonal antibody AML-2-23, the binding patterns of these monoclonal antibodies to monocytes was similar to their binding to lymphocytes and AML cells. AML-2-23 showed similar binding to both monocytes and AML cells.

Several monoclonal antibodies selectively mediated complement-dependent lysis of leukemic myeloblasts (AML-1-211, AML-1-99, CML-75, CML-115, and CML-150). Three of these clones, AML-1-211, CML-75, and CML-150, mediated lysis of some leukemia cell samples only upon dilution of the supernatant, whereas other samples were lysed by both diluted and neat supernatant. Monoclonal antibody AML-1-211 permitted lysis of four different AML cell samples only when diluted between 1:8 and 1:128 (see Figs. 4 and 5 for representative cytotoxicity curves). Monoclonal antibody CML-75 permitted lysis of one of these AML cell samples at dilutions between 1:16 and 1:512 (Figs. 4 and 5). The dilutions of these two monoclonal antibodies that mediated cytotoxicity corresponded to those that yielded maximal binding. CML-150, another monoclonal antibody that had a prozone in binding to AML cells, was able to lyse one sample of leukemic myeloblasts in undiluted supernatant. However, in contrast to monoclonal antibodies AML-1-211 and CML-75, CML-150 was still not cytotoxic to the AML samples shown in Figures 4 and 5. Subsequently, other AML samples were lysed by hybridomas CML-75, AML-1-211, and CML-150 only at dilutions of super-

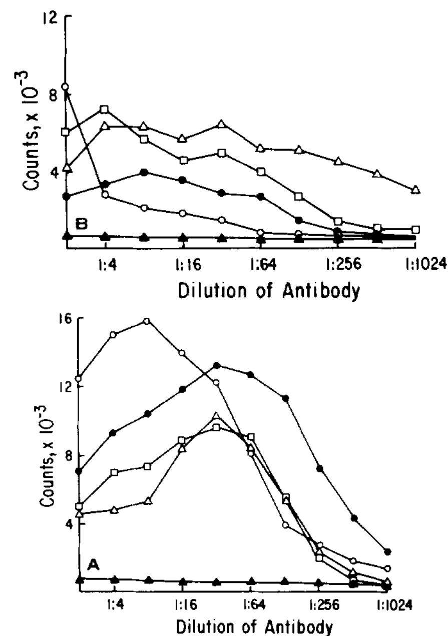

Figure 1. Binding of serially diluted hybridoma supernatant to 10^5 AML cells. *A*, Hybridomas represented are AML-2-17 (○), AML-2-23 (△), AML-1-22 (□), and AML-1-104 (●). *B*, Hybridomas represented are AML-1-201 (△), AML-1-211 (●), CML-75 (○), and CML-150 (□). The binding of the P3-X63Ag8 parent myeloma supernatant (▲) is also represented in both 1*A* and 1*B*. Supernatants were diluted with DMEM containing 20% calf serum and incubated for 2 hr at 37°C on glutaraldehyde-fixed leukemia cells. Bound monoclonal antibody was detected with a rabbit-anti-mouse kappa chain antibody labeled with ^{125}I. Each sample was counted for 2 min in a gamma counter.

three monoclonal antibodies (AML-1-211, CML-75, and CML-150) mediated lysis of some leukemic cells only upon dilution while other samples were lysed by neat supernatant. Moreover, a prozone was seen with some leukemic cells with only one or two of these monoclonal antibodies while another antibody permitted lysis in neat supernatant, yet the pattern was opposite for other cell samples. A summary of the collected cytotoxicity data for these monoclonal antibodies is shown in Table I. Neither AML-1-211, AML-1-99, CML-75, CML-115, or CML150 mediated lysis of any normal T and B lymphocytes or monocytes studied at any dilution of antibody (Figs. 6 and 7).

Monoclonal antibodies AML-2-9 and AML-2-23 were cytotoxic to a number of leukemia cell samples as well as selected normal cell populations. AML-2-23 mediated lysis of eight of 13 myeloblast samples as well as all normal monocytes studied (Fig. 6), but was not cytotoxic to any lymphocytic leukemia cell samples or normal lymphocytes (Fig. 7). In contrast, AML-2-9 mediated lysis of four of 12 myeloid leukemias as well as some lymphocytic leukemias and normal lymphocyte samples.

One monoclonal antibody, AML-1-201, mediated lysis of nearly every cell population studied, both normal and leukemic. The exception was the cell line Daudi, which does not express class I HLA antigens or β_2-microglobulin on the cell surface (11, 12). This antibody appeared specific to β_2-microglobulin as determined by an enzyme-linked immunosorbent assay with purified human β_2-microglobulin (a gift of Dr. George Bernier, Department of Medicine, Dartmouth Medical School). Finally, a number of monoclonal antibodies did not lyse any cell population studied despite the demonstration that significant binding occurred and the fact they were IgM immunoglobulins.

Several monoclonal antibodies (AML-1-22, AML-1-99, AML-

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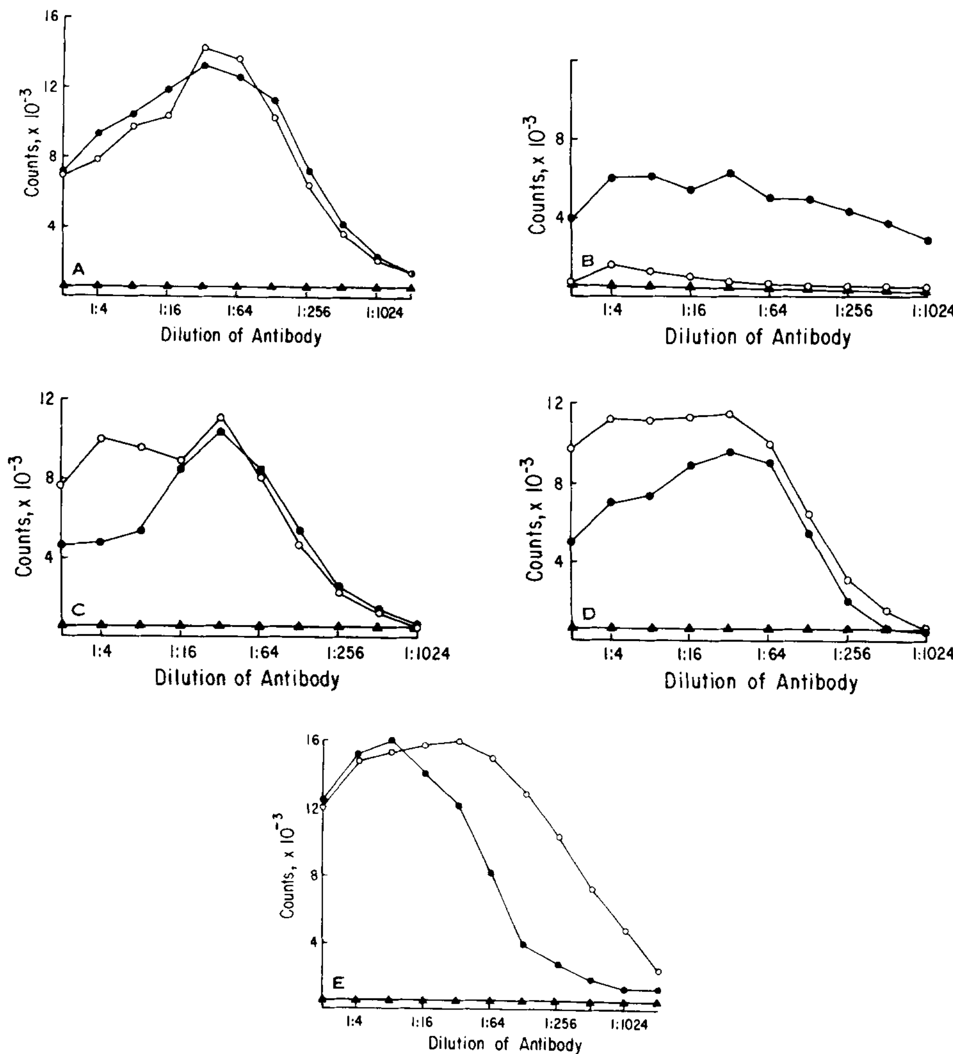

Figure 2. Binding of serially diluted hybridoma supernatant to 10^5 normal lymphocytes and 10^5 AML cells. Figure 2*A* shows the binding of AML-1-211 to lymphocytes (○) and AML cells (●). The P3-X63Ag8 myeloma cell line supernatant is also represented (▲) in Figures 2*A*, *B*, *C*, *D*, and *E*. Figure 2*B* shows the binding of AML-2-23 to lymphocytes (○) and AML cells (●). Figure 2*C* shows the binding of AML-1-201 to lymphocytes (○) and AML cells (●). Figure 2*D* shows the binding of CML-150 to lymphocytes (○) and AML cells (●). Figure 2*E* shows the binding of CML-75 to lymphocytes (○) and AML cells (●). Samples were counted for 2 min.

of one or more of the CCRF-CEM, KG-1a, HL-60, or U937 cell lines (Table I). Only AML-1-99 and AML-1-201 were capable of lysing all cell lines while AML-2-9 and AML-1-22 permitted lysis of CCRF-CEM and AML-1-116 permitted lysis of KG-1a cells. Both AML-2-9 and AML-2-23 permitted lysis of the HL-60 cell line.

DISCUSSION

We presented data previously that described the binding of a panel of monoclonal antibodies to leukemic and normal cell populations. None of these hybridomas were specific for any leukemia cell type, yet significant quantitative differences in antigen expression on leukemic cells compared to normal cells were shown with several of these antibodies. In spite of a lack of absolute binding specificity, however, several antibodies are capable of selective complement-dependent cytotoxicity of leukemic, and, in some cases, myeloid leukemia cells. The present report documents these observations and demonstrates that some monoclonal antibodies can, under appropriate conditions, express specific cytotoxic activities that were not evident in the initial screenings.

Monoclonal antibodies AML-1-99, AML-1-211, AML-2-30, CML-75, CML-115, and CML-150 all demonstrated cytotoxicity to myeloid leukemias exclusively while sparing normal cell populations. AML-1-211 mediated lysis of seven of 13 myeloid

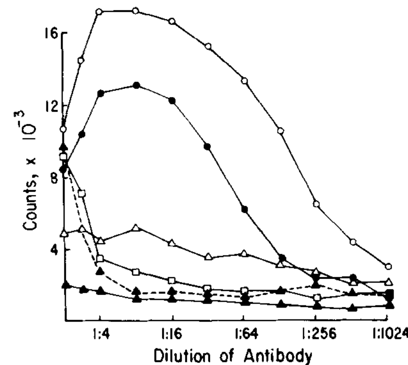

Figure 3. Binding of serially diluted hybridoma supernatant to 10^5 normal monocytes. Hybridomas represented are AML-1-99 (□), AML-1-211 (●), AML-2-9 (▲---▲), AML-2-23 (△), and CML-75 (○). The P3-X63Ag8 myeloma cell line supernatant is also represented (▲). Each sample was counted for 2 min.

four of these AML samples that coincided with the binding behavior of the hybridoma supernatant. Lysis of normal lymphocytes or monocytes was not observed with this antibody at any dilution. Monoclonal antibody CML-75 was able to cause complement-dependent lysis of five of 13 myeloid leukemia samples but was not cytotoxic to normal lymphocytes and monocytes despite the demonstration of large amounts of CML-75 antigen on these cells. Antibodies AML-1-99, CML-115,

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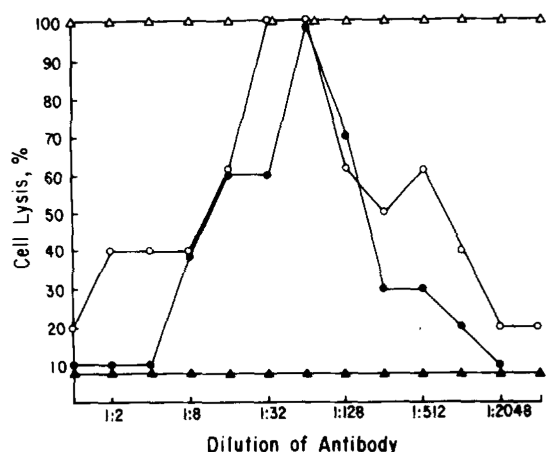

Figure 4. C-dependent cytotoxicity mediated by monoclonal antibodies to cells from a patient with AML. Equal volumes of hybridoma supernatant and cells at 2×10^6/ml were incubated for 30 min at 22°C in microtiter wells. Rabbit C was added and incubation continued for 60 min. Cytolysis was estimated by Trypan blue exclusion. Hybridomas represented are AML-1-211 (●), AML-2-23 (△), and CML-75 (○). The P3-X63Ag8 myeloma supernatant is also represented (▲). All assays were done in duplicate.

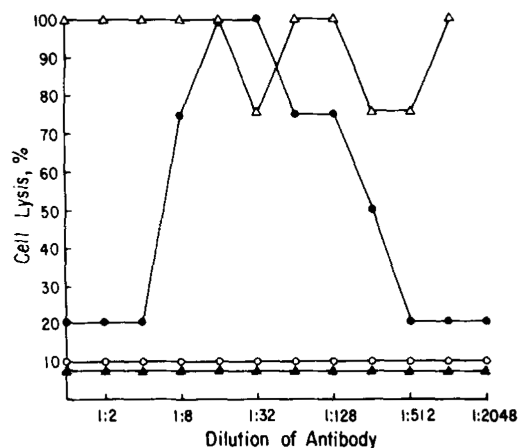

Figure 5. C-dependent cytotoxicity mediated by monoclonal antibodies to cells from a patient with AML (a different patient from that shown in Fig. 6). Hybridomas represented are AML-1-211 (●), AML-2-23 (△), and CML-75 (○). The P3-X63Ag8 myeloma supernatant is also represented (▲). All assays were done in duplicate.

and CML-115 each permitted lysis of one of the AML samples studied and CML-150 permitted lysis of three AML samples.

Monoclonal antibodies AML-2-9 and AML-2-23 were cytotoxic to some myeloid leukemias as well as some normal cell types. AML-2-9 permitted lysis of some samples of normal lymphocytes but not of normal monocytes. AML-2-23 was highly active in killing monocytes and six of eight AML and three of five CML-blast crisis cell samples but was not cytotoxic to lymphoid cells. The specificity of this antibody is similar to that of monoclonal antibodies reported by Linker-Israeli *et al.* (13) and Ugolini *et al.* (14) that also were capable of mediating lysis of monocytes (13) and some myelomonocytic leukemia cells. Todd *et al.* (15) reported the production of a monoclonal antibody, Mo2, that binds to normal monocytes and some leukemic myeloblasts. This antibody does not bind to the HL-60 cell line, which suggests that AML-2-23 and Mo2 bind to different antigenic determinants because AML-2-23 both binds to and permits complement-dependent lysis of this cell line.

The explanation for the prozone effect in the binding of some of these IgM monoclonal antibodies is not clear. One possibility is that when excess antibody is present, binding may be divalent or trivalent binding. More easily dissociable complexes would be formed that would result in some loss of antibody by washing during the assay. As less antibody is presented to the cell surface more cross-linking of receptors might occur, leading to a more stable complex. Although this behavior was noted for four of the 20 antibodies studied, all of which were IgM immunoglobulins, 15 other IgM monoclonal antibodies showed either a plateau or a nearly linear decrease in binding upon dilution. Differences in antigen density or distribution and/or that some of the monoclonal antibodies were of a different IgM subclass may also contribute to these results. The monoclonal antibodies that showed a prozone were among those with the highest binding to a variety of cells under standardized conditions, whereas none of the monoclonal antibodies recognizing less densely expressed epitopes had a prozone in the binding assay. The prozone effect noted in the cytotoxicity assay may have the same explanation because a wash step is included in the assay.

These studies illustrate that for selected monoclonal antibodies, binding and/or effector functions can either be underestimated or undetectable if screenings are limited to undiluted hybridoma supernatants. This has implications for both the initial selection of hybridomas and attempts to demonstrate specificity of hybridomas as it is clear that cytotoxicity can be demonstrated for some monoclonal antibodies only at critical antibody concentrations. Furthermore, this behavior needs to be considered in trials of serotherapy of human leukemia.

TABLE I

Cytotoxicity of leukemia cells and cell lines mediated by monoclonal antibodies in the presence of complement

Monoclonal Antibody	Leukemia Cell Type[a]				Cell Line[b]			
	AML	CML-BC	ALL	CLL	KH-1a	CCRF	HL-60	U937
AML-1-99	1/7	0/4	0/3	0/4	++	++	++	++
AML-1-201	8/8	4/4	3/3	3/4	++	++	++	++
AML-1-211	6/8	1/5	0/3	0/4				
AML-2-9	2/8	2/4	1/3	1/4		++	++	
AML-2-23	5/8	3/5	0/3	0/4			++	
AML-2-30	1/5	0/4	0/2	0/4				
CML-75	4/8	1/5	0/3	0/4				
CML-115	2/8	0/4	0/3	0/3				
CML-150	2/8	1/4	0/3	0/3				

[a] Cells were obtained from patients with a variety of leukemias. The numerator refers to the number of different individuals whose cells showed positive reactions and the denominator refers to the number of individuals whose cells were tested. Positive is defined as >50% lysis of leukemia cells by hybridoma supernatant.

[b] ++ refers to >50% lysis of the cell line mediated by monoclonal antibody. No score indicates that no lysis was observed.

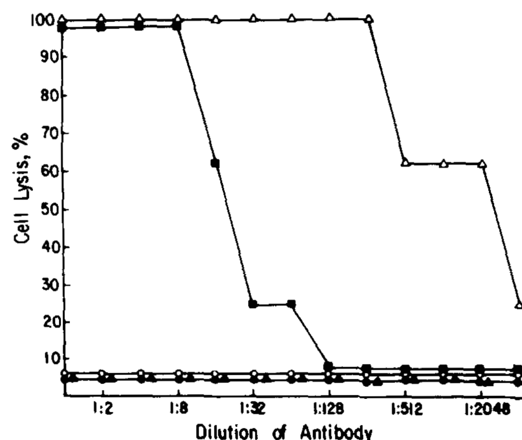

Figure 6. C-dependent cytotoxicity mediated by monoclonal antibodies to normal human monocytes. Hybridomas represented are AML-1-201 (□), AML-1-211 (●), AML-2-23 (△), and CML-75 (○). The P3-X63Ag8 myeloma supernatant

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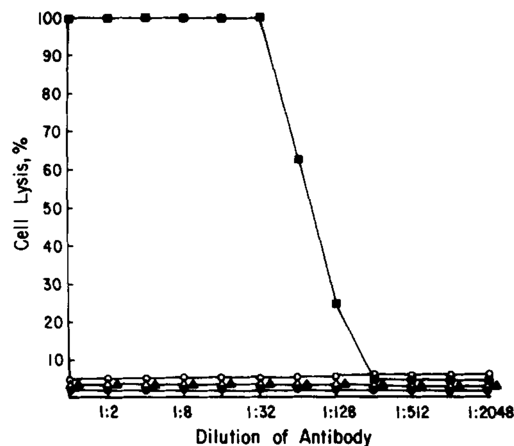

Figure 7. C-dependent cytotoxicity mediated by monoclonal antibodies to normal human lymphocytes. Identical results were obtained with unfractionated peripheral blood lymphocytes, T lymphocytes, and B lymphocytes. Hybridomas represented are AML-1-201 (■), AML-1-211 (●), AML-2-23 (△), and CML-75 (○). The P3-X63Ag8 myeloma supernatant is also represented (▲). All assays were done in duplicate.

Optimal conditions for leukemia cell lysis may vary with the monoclonal antibody used and the leukemia cell population.

There are now several human leukemia cell lines available for *in vitro* studies of leukemia cell biology (16). It is of interest that few of the monoclonal antibodies from the panel reported here mediated lysis of the myeloid leukemia cell lines, KG-1a or HL-60. Only one of the hybridomas that showed selective complement-dependent cytotoxicity for fresh leukemia samples from patients, AML-1-99, was cytotoxic to both of these cell lines despite evidence that significant binding occurred with all hybridomas but AML-2-23. Antibody AML-1-99, however, mediated lysis of the KG-1a, HL-60, U937, and CCRF-CEM cell lines, suggesting that the AML-1-99 antigen is expressed similarly on these cell lines. Because these cell lines are of diverse lineage, their susceptibility to lysis by AML-1-99 may possibly be due to binding to an antigen expressed on rapidly proliferating cells, a "division antigen." Monoclonal antibody AML-2-23, which mediated lysis of 8 of 13 myeloid leukemia cell populations, also mediated lysis of the HL-60 cell line but not of another myeloid cell line, KG-1a. It is of interest that AML-2-23 did not permit lysis of the U937 cell line. These cells seem to require activation by medium conditioned by mixed lymphocyte cultures to assume some of the functional characteristics of monocytes (17). The failure of AML-2-23 to bind to and permit complement-dependent lysis of this cell line in its unstimulated state also shows that at least one normal surface marker of normal monocytes is absent. Studies with stimulated U937 cells to determine if the AML-2-23 antigen is expressed on stimulated cells are planned.

In contrast, the expression of the AML-2-23 antigen on cells of the HL-60 cell line shows that these cells express an antigen characteristic of mature monocytes. We have also found that cells of the HL-60 cell line express an antigen characteristic of polymorphonuclear leukocytes that is recognized by a monoclonal antibody, PMN-29, developed in our laboratory (unpublished observation). The expression of two different antigens on these cells in the native state that correlate with specific states of differentiation is interesting in light of the demonstrated ability of these cells to differentiate into either mature granulocytes or monocytes with appropriate chemical inducers (18). The present report suggests that certain markers of differentiated cells are present before induction of differentiative changes, if any, of these specific markers on cells stimulated to differentiate are in progress.

Although the monoclonal antibodies reported here did not detect leukemia-specific antigens, there are several reasons to consider their utility in leukemia treatment. Some experimental evidence exists that monoclonal antibodies directed toward normal cell surface antigens can be used to effectively treat animal tumors that bear that antigen without necessarily causing untoward effects. The studies of Bernstein *et al.* (19) using a monoclonal anti-Thy-1.1 antibody to treat a Thy-1.1-positive murine leukemia *in vivo* have shown efficacy in the elimination of tumor metastases while demonstrating no serious toxicity as a result of normal T lymphocyte binding and lysis. The use of monoclonal antibodies directed toward antigens that are expressed more, quantitatively, on leukemic cells may be equally efficacious and yet not seriously deleterious to normal host cells. It has also been shown that under appropriate conditions subpopulations of tumor cells can develop that do not express certain surface antigens (20). Thus, monoclonal antibody therapy directed toward a given single antigen could result in selection of a subpopulation of tumor cells. Finally, modulation of cell surface antigens can occur as a result of monoclonal antibody binding (18).

It seems likely that successful treatment of human tumors may require the use, in combination, of panels of monoclonal antibodies such as AML-1-99. AML-1-211, AML-2-9, AML-2-23, AML-2-30, CML-75, CML-115, and CML-150. In this manner, the problem already encountered in trials of serotherapy in humans, that of antigen modulation (21-23), may be circumvented, as multiple determinants are used as targets for antibody-mediated leukemia cell lysis. Studies utilizing the panel of monoclonal antibodies described in this report are being conducted to evaluate tumor cell escape from antibody-mediated lysis and its prevention.

Several of these monoclonal antibodies have immediate diagnostic value based on their ability to selectively lyse leukemic myeloblasts but not leukemic lymphoblasts. This panel of monoclonal antibodies may also be useful in defining subsets of myeloid leukemia cells based on variable binding to and lysis of cells from individual patients. Such data can be correlated with cellular morphology and histochemical staining. It may be possible to show characteristic "fingerprints" of certain subtypes of leukemia defined by binding to panels of monoclonal antibodies that could aid in both diagnosis and possibly in determining optimal therapy.

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