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Cell Biology

Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro*

(iron starvation/leukemic cells)

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ABSTRACT **A murine hybridoma has been obtained that produces a monoclonal antibody against the human transferrin receptor. In contrast to previously characterized monoclonal antibodies that recognize the transferrin receptor, this antibody, designated 42/6, blocks the binding of transferrin to its receptor and inhibits the growth of the human T leukemic cell line, CCRF-CEM, *in vitro*. Inhibition of cell growth was dose dependent, and as little as 2.5 μg of purified antibody per ml had a detectable effect, even though transferrin was present in the tissue culture medium in large molar excess. Cells grown in the presence of antibody for 7 days accumulated in S phase of the cell cycle. The addition of iron to antibody-treated cultures in the form of ferric complexes or ferrous sulfate did not overcome the growth inhibitory effects of the anti-transferrin-receptor antibodies. This result suggests that either transferrin is the only means by which CCRF-CEM leukemic cells can be provided with sufficient iron *in vitro* or that other factors in addition to iron starvation are involved in the antibody-mediated growth inhibition. The inhibition of cell growth by 42/6 monoclonal antibody suggests that monoclonal antibodies against proliferation-associated cell surface antigens, such as the transferrin receptor, may be useful pharmacological reagents to modify cell growth *in vitro*.**

Iron plays an important role in cell growth and metabolism (1). Key reactions in energy metabolism and DNA synthesis are catalyzed by iron-containing enzymes, and it has been known for several years that transferrin, the major serum iron-transport protein, is an obligatory growth factor for cells growing *in vitro* (2–11). More recently, it has become apparent that cell surface receptors for transferrin are not only found in abundance on maturing erythroid cells and placental membranes but are expressed on proliferating cells *in vitro* and *in vivo* (12–16).

We and others have obtained monoclonal antibodies that react with the human cell surface receptor for transferrin and confirmed that the expression of transferrin receptors is regulated by the growth state of the cells (17–22). Although the anti-transferrin-receptor monoclonal antibody, which we obtained and designated B3/25, inhibits the growth of a human melanoma cell line in *nude* mice (23), it does not interfere with either transferrin binding or cell growth *in vitro*. In this paper, we report the identification of another murine monoclonal antibody against the human transferrin receptor that blocks transferrin binding and show that this antibody blocks the growth of a human T leukemic cell line *in vitro*.

MATERIALS AND METHODS

Cell Lines. CCRF-CEM, a human thymus-derived (T) leukemic cell line (24), was grown in RPMI 1640 medium supplemented with 10% horse serum. BW5147, a murine T lymphoma cell line (25) was grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum.

Monoclonal Antibodies. Monoclonal antibodies B3/25 and T56/14 directed against the human transferrin receptor and monoclonal antibody T29/33 specific for human T200 glycoprotein have been described (17, 18, 26). Monoclonal antibody 42/6, the principal subject of this report, was obtained by immunizing BALB/c mice with purified human transferrin-receptor glycoprotein isolated from CCRF-CEM cells by affinity chromatography on a B3/25 monoclonal antibody column (18). An initial subcutaneous injection of 50 μg of glycoprotein in complete Freund's adjuvant was given, followed by an intravenous injection of 50 μg of glycoprotein in saline 6 wk later. The mice were sacrificed 4 days later, and the immune spleen cells were hybridized with S194/5.XXO.BU.1 myeloma cells by standard procedures (27, 28). 42/6 monoclonal antibody was identified by testing the culture supernatants from the hybridomas thus obtained.

Transferrin Binding Inhibition Assay. Human transferrin (Miles) was ^{125}I-labeled as described (18). CCRF-CEM cells [50 μl containing 1–2 × 10^7 cells in 0.1% bovine serum albumin/0.15 M NaCl/0.015 M NaN_3/0.01 M Na phosphate buffer, pH 7.2 (albumin/phosphate buffer)] were incubated for 45 min at 4°C with 50 μl of hybridoma supernatant. Cells were then washed with albumin/phosphate buffer and incubated for 45 min at 4°C with ^{125}I-labeled transferrin [50 μl of protein at 2 μg/ml; specific activity, 1 μCi per μg (1 Ci = 3.7 × 10^{10} becquerels)]. After washing, the cell-bound radioactivity was determined in a Packard gamma counter. As a control, cells were preincubated with 50 μl of unlabeled human transferrin (15 μg/ml) in albumin/phosphate buffer.

Cell Growth Studies. Cells were plated in duplicate dishes at a final cell density of 2.5–10 × 10^4 cells per ml in RPMI 1640 medium supplemented with 10% horse serum. Antibodies were added either as hybridoma culture supernatant previously filtered through a 0.22-μm Millipore filter and diluted in Dulbecco's modified Eagle's medium containing 10% horse serum or as purified antibody isolated from ascitic fluid of tumor-bearing mice by ammonium sulfate precipitation and DEAE-cellulose chromatography (29). Cell growth was assessed by counting with a Coulter Counter, and the distribution of cells throughout the cell cycle was measured by flow cytometric analysis of fixed cells stained with mithramycin (30). Ferric–fructose complexes were prepared by mixing fructose with $FeCl_3$ in a molar ratio of 200:1 at pH 3.0, and ferric–nitrilotriacetic acid complexes were prepared by mixing the complexing agent with $FeCl_3$ in a molar ratio of 2:1 at pH 3.0 (6). Both were added to cultures to give a final concentration of 20 μM iron(III). Ferrous sulfate was dissolved in water at pH 3.0 and added (10 pmol/ml) daily to cultures.

Immunological and Biochemical Procedures. Trace ^{125}I-labeled antibody binding assays, cell surface iodination, immu-

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noprecipitation, and $NaDodSO_4$/polyacrylamide gel electrophoresis were carried out as described (17, 18).

RESULTS

Characterization of a Monoclonal Antibody Against the Human Transferrin Receptor that Blocks Transferrin Binding. To obtain a monoclonal antibody that blocks the binding of transferrin to its receptor on the surface of human cells, we employed the following strategy. Mice were immunized with purified human transferrin-receptor glycoprotein, and hybridomas were prepared between the spleen cells of the immune mice and S194/5.XXO.BU.1 myeloma cells. Culture supernatants of these hybridomas were then tested sequentially for the presence of antibodies that (*i*) bound to CCRF-CEM cells, (*ii*) immunoprecipitated labeled transferrin receptor from lysates of surface-iodinated CCRF-CEM cells, and, when preincubated with CCRF-CEM cells, (*iii*) inhibited the binding of ^{125}I-labeled human transferrin. This approach led to the identification of a monoclonal antibody designated 42/6 that gave positive results in each of these tests.

Inhibition of transferrin binding by preincubation of the cells with 42/6 tissue culture supernatant was dose-dependent (Fig. 1). At a dilution of 1:64, the hybridoma supernatant inhibited ^{125}I-labeled binding to the same extent as did 15 μg of unlabeled human transferrin per ml. In contrast, undiluted culture supernatants containing antibodies B3/25 and T56/14 to human transferrin receptor or antibody T29/33 to human T200 glycoprotein had no effect on transferrin binding (data not shown). Immunoprecipitation studies showed that 42/6 monoclonal antibody did not react with human transferrin, ruling out the possibility that inhibition of binding was the result of a direct interaction with transferrin. 42/6 monoclonal antibody was typed as IgA (k) by immunoprecipitation of antibody metabolically labeled with [^{35}S]methionine with isotype-specific antisera (Miles). The hybridoma producing 42/6 monoclonal antibody has been repeatedly cloned by limit dilution and is moderately stable for antibody production.

Inhibition of Cell Growth by Transferrin Blocking Monoclonal Antibody. The effects of 42/6 monoclonal antibody on the *in vitro* growth of the human T leukemic cell line CCRF-CEM was initially tested by growing the cells in the presence of various amounts of antibody-containing culture supernatant. After about 4 days in the presence of antibody, examination of cultures by phase-contrast microscopy showed a distinct morphological change (Fig. 2). Instead of the usual heterogeneity in cell size evident in exponentially growing cultures of CCRF-CEM cells, the antibody-treated cells appeared uniformly large. Cell growth was inhibited in a dose-dependent fashion by the antibody, and little or no increase in cell number was observed in cultures treated with the highest amount of antibody (Fig. 3). Control experiments showed that the monoclonal antibody did not inhibit the growth of the murine BW5147 T lymphoma cell line consistent with its lack of reactivity with the murine transferrin receptor. After 7 days of growth, cells from the same experiment shown in Fig. 3 were harvested, and their DNA content was analyzed by mithramycin staining. A striking difference was found in the cell cycle distribution of the antibody-treated cells and the control cells (Fig. 4). In the untreated cultures, 58% of cells were in G_1 phase, 16% in S and 26% in G_2 + M phases—values typical of exponentially growing cells. In cultures exposed to 42/6 monoclonal antibody, there was a progressive accumulation of cells in S phase and a corresponding drop in the proportion of cells in G_1. The effect was most marked at the highest amount of antibody used, and in this culture

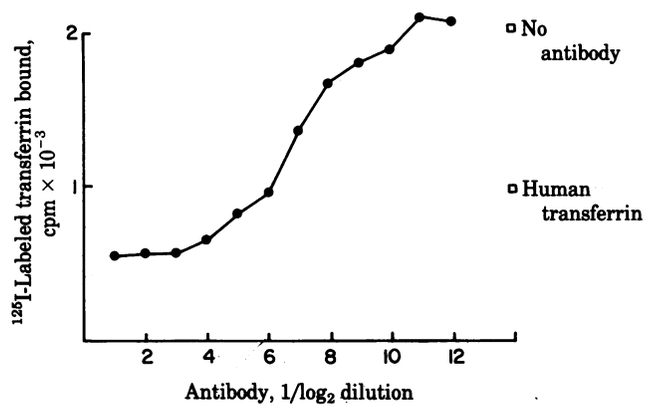

FIG. 1. Blocking of transferrin binding by 42/6 monoclonal antibody. CCRF-CEM cells were incubated with various dilutions of antibody-containing culture supernatant, washed, and then reincubated with ^{125}I-labeled human transferrin (●). Tissue culture medium was used as a negative control, and nonradioactive transferrin was used as a positive control for blocking. □, Controls.

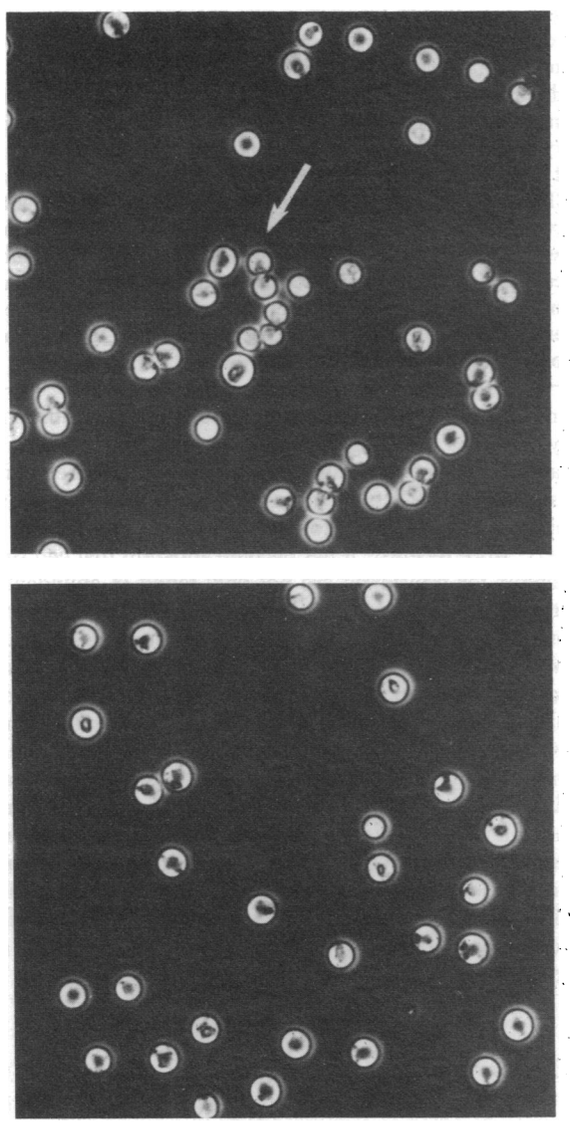

FIG. 2. Phase-contrast microscopy (magnification, ×200) of cells grown in 50% (vol/vol) 42/6 monoclonal antibody supernatant for 4 days (*Lower*) and of control cells (*Upper*). Note the heterogeneity in size of the control cells compared to the antibody-treated cells.

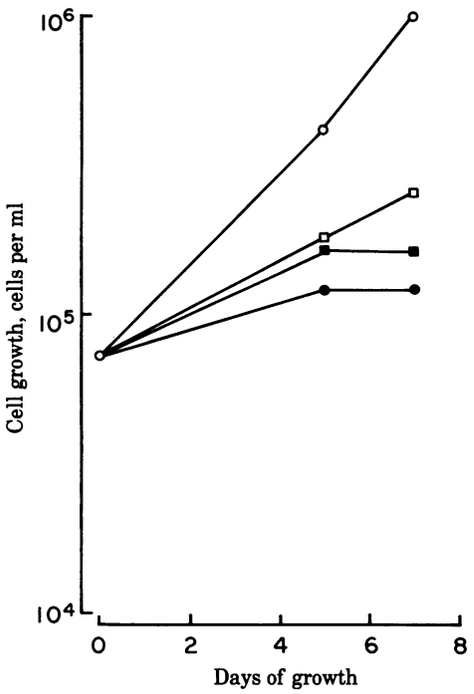

FIG. 3. Transferrin-blocking anti-transferrin-receptor monoclonal antibody inhibits cell growth *in vitro*. CCRF-CEM cells were set up at 7×10^4 cells per ml in medium supplemented with 10% horse serum. Each culture contained 5 ml of RPMI 1640 medium and 5 ml of Dulbecco's modified Eagles medium (control) or 5 ml of 42/6 hybridoma supernatant serially diluted with Dulbecco's modified Eagle's medium. ○, Control (no antibody), □, 12.5% antibody; ■, 25% antibody; ●, 50% antibody.

≈63% of the cells were in S phase of the cell cycle as judged by mithramycin staining.

Growth Inhibition by the Transferrin-Blocking Anti-Transferrin-Receptor Antibody is Not Overcome by Fe^+. The most likely explanation to account for the growth inhibitory effect of 42/6 monoclonal antibody was that by interfering with the binding of transferrin to its cell surface receptor, the antibody-treated cells were being deprived of iron. Indeed, the uptake of iron from human [^{51}Fe]transferrin is completely inhibited by the antibody (unpublished results). If so, then we thought it should be possible to overcome the inhibitory effects of the antibody by providing the cells with iron in the form of soluble ferric–fructose complexes or by daily addition of ferrous sulfate. For these experiments we used purified 42/6 monoclonal antibody. As little as 2.5 μg of monoclonal antibody per ml produced significant inhibition of cell growth (Fig. 5). However, the effects of the antibody could not be overcome by daily addition of 10 pmol of ferrous sulfate per ml. In similar experiments 20 μM ferric–fructose and 20 μM ferric–nitrilotriacetic acid complexes were also ineffective in preventing antibody-mediated growth inhibition, although in a few experiments in the presence of ferric complexes, the proportion of cells found in S phase in antibody-treated cultures was reduced, and a small increase in cell number was observed relative to cultures to which antibody but not ferric complexes were added.

DISCUSSION

We describe here a murine monoclonal antibody against the human transferrin receptor that interferes with transferrin binding to cells and inhibits cell growth *in vitro*. These properties distinguish this antibody from other monoclonal antibodies to human transferrin receptor described earlier (17–22) and

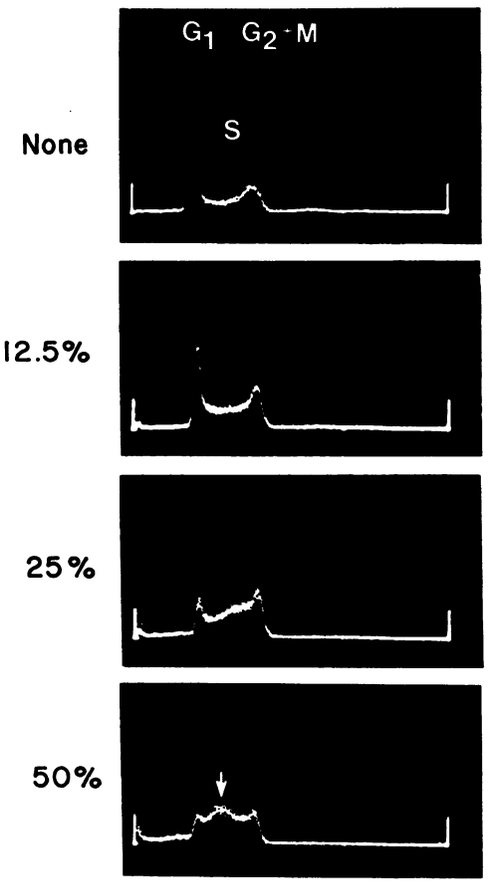

FIG. 4. Cell cycle analysis of CCRF-CEM cells, grown for 7 days in the presence of various amounts (shown on the left) of 42/6 monoclonal antibody; flow cytofluorimetric analysis of mithramycin-stained cells. The arrow indicates cells in S phase in the antibody-treated cell population.

provide new possibilities for studying the growth requirements of both normal and malignant cells *in vitro*. The detailed mechanism of action of the antibody remains to be established.

A reasonable interpretation of the data is that the antibody binds to a site close to but not identical with the transferrin binding site on the transferrin receptor and, thus, inhibits binding of transferrin. Preliminary data suggests that the 42/6 monoclonal antibody has a higher affinity for the transferrin receptor than transferrin itself and inhibits transferrin binding in a competitive manner (unpublished results). This would explain how growth inhibition by the monoclonal antibody is observed, even in the presence of the substantial molar excess of transferrin derived from the serum supplement to the tissue culture medium.

However, other more complex mechanisms involving interference with recycling of the receptors by 42/6 antibody or some other mechanism involving loss of the receptors from the cell surface cannot be ruled out. Crossblocking experiments with 42/6 monoclonal antibody and B3/25 monoclonal antibody (17, 18, 23), which neither inhibits transferrin binding nor cell growth *in vitro*, shows that each antibody partially interferes with the binding of the other, suggesting that the antigenic sites recognized by the two antibodies are juxtaposed although not identical. Thus, a significant factor in the difference between the two antibodies in their ability to block transferrin binding may be that B3/25 monoclonal antibody is an IgG, whereas 42/6 monoclonal antibody is a sterically more bulky IgA.

There is considerable evidence suggesting that the primary role of transferrin as a growth factor *in vitro* is to provide iron

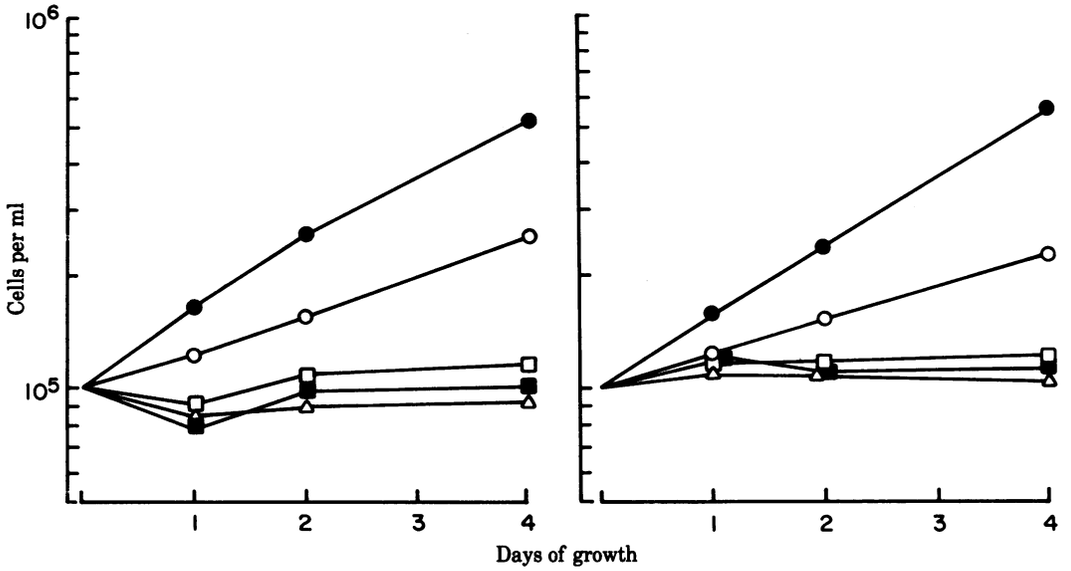

FIG. 5. Inhibition of cell growth *in vitro* by purified 42/6 monoclonal antibody with (*Right*) or without (*Left*) addition of iron(II). CCRF-CEM cells were set up at 1×10^5 cells per ml and grown in the presence of various concentrations of purified 42/6 monoclonal antibody with or without daily additions of ferrous sulfate [−10 pmol/ml; stock solution, 0.1 M in H_2O (pH 3.0)]. ●, None; ○, 2.5 μg/ml; □, 5 μg/ml; ■, 10 μg/ml; △, 20 μg/ml.

(6, 7, 31) and that iron is essential for cell growth (32–37). Consequently, we thought it likely that 42/6 monoclonal antibody inhibited growth by blocking transferrin binding and, thus, depriving the cells of iron. This conjecture was supported by the fact that CCRF-CEM cells treated with antibody accumulated in S phase of the cell cycle, mimicking the effect of picolinic acid, an iron chelator on some cell types (35, 37). However, addition of ferric complexes or ferrous sulfate to cultures of CCRF-CEM cells did not overcome the inhibitory effects of the antibody. Thus, if the growth of CCRF-CEM were being limited by the availability of iron, then neither of these compounds would completely replace the requirement for transferrin iron. This contrasts to earlier studies of Chinese hamster V79 lung fibroblasts (6) and human embryonic lung fibroblasts (31) showing that the iron requirements of these cells could be met by addition of either ferrous sulfate or ferric–fructose complexes. It is possible that different cell types have different iron requirements, and, in the case of CCRF-CEM cells, that only transferrin can provide iron efficiently. Another explanation is the CCRF-CEM cells also may require other trace metal ions that are transported by transferrin and not needed by all cells. A third, less likely, possibility is that the interaction of 42/6 antibody with the transferrin receptor may produce a growth inhibitory signal unrelated to the transport function of the receptor. However, other monoclonal antibodies such as B3/25 and 56/14 bind to the transferrin receptor yet do not inhibit growth *in vitro* under the same conditions.

Previously, attention has been focused upon the therapeutic use of monoclonal antibodies to target covalently-bound drugs or toxins to tumor cells selectively expressing specific surface antigens (23, 38–42). Monoclonal antibodies against T-cell differentiation antigens also have been used in attempts to eliminate tumor cells by immunological effector mechanisms (43–46). A third novel possibility raised by the results reported in this paper is that monoclonal antibodies against proliferation-associated cell surface antigens, such as the transferrin receptor, may be used as pharmacological reagents to modify cell growth directly. Although the experiments we have reported here are concerned exclusively with the effects of the 42/6 anti-transferrin-receptor antibody on cell growth *in vitro*, it is possible that similar inhibition of tumor cell growth might be achieved *in vivo*. Certainly, it seems unlikely that the high concentration of transferrin in serum will interfere with the inhibitory effects of the antibody, unless there is a large difference in the efficiency with which horse and human transferrin can provide iron to human cells. However, even if 42/6 monoclonal antibody were shown to inhibit tumor growth *in vivo*, its therapeutic value would also depend upon its effects on normal tissues.

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