MOLECULAR AND CELLULAR BIOLOGY, Aug. 1985, p. 1814–1821
0270-7306/85/081814-08$02.00/0

Vol. 5, No. 8

Inhibition of Cell Growth by Monoclonal Anti-Transferrin Receptor Antibodies

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Received 25 February 1985/Accepted 25 April 1985

Five anti-murine transferrin receptor monoclonal antibodies have been characterized with respect to immunoglobulin class, effects on binding of transferrin, and effects on AKR1 lymphoma cell growth in vitro. The immunoglobulin M (IgM) antibodies, but not the IgG antibodies, prevent cell growth. We suggest that the profound effects of the IgM antibodies on cell growth are probably due to extensive cross-linking of cell surface receptors. In support of this, we are able to mimic the growth-inhibiting effects of the IgM antibodies by adding antiimmunoglobulin to an IgG antibody. By flow microfluorimetry, we show that an IgG antibody by itself induces up to a 10-fold downward regulation in the cell surface transferrin receptor, which is accompanied by accelerated receptor degradation. A similar downward regulation is seen in mutant cells resistant to growth inhibition by an IgM antibody, when grown in the selecting antibody. Wild-type cells grown in the presence of IgM antibody do not show receptor downward regulation. Inhibitory effects of antibody plus antiimmunoglobulin on mutant cells are also consistent with extensive cross-linking causing inhibition of growth.

Iron is incorporated into cells by binding the iron transport protein, transferrin, to specific cell surface receptors. The transferrin receptor in growing cells is continuously cycling from the cell surface to intracellular compartments and back to the cell surface (2, 4, 5, 7, 8). Furthermore, its expression is closely associated with cell proliferation (1, 9, 15, 16, 19, 20, 27). These properties of the transferrin receptor render growing cells particularly vulnerable to antibodies that might interfere with receptor function. Monoclonal anti-transferrin antibodies that inhibit iron uptake have been described in human (25) and murine (12, 24) systems. In vitro growth and subsequent differentiation of normal hematopoietic cells can be inhibited by these anti-transferrin receptor antibodies (14–16, 21; H. G. Ramensee, J. Lesley, I. S. Trowbridge, and M. Beven, Eur. J. Immunol., in press), as can the growth of murine (12, 24) and human (21, 25) cell lines. In addition, tumor growth in vivo has been inhibited by anti-transferrin receptor antibodies in model tumor systems (22, 26). However, not all anti-transferrin receptor antibodies inhibit cell growth (12, 21, 26). Although the anti-human transferrin receptor antibody 42/6, which inhibits cell growth, was selected by its ability to block transferrin binding (25), it is not clear that this is the basis of its inhibition of cell growth (R. Taetle, J. Castagnola, and J. Mendelsohn, submitted for publication).

We now have a total of five monoclonal antibodies of various immunoglobulin classes against the murine transferrin receptor. Two immunoglobulin M (IgM) antibodies completely inhibit in vitro growth of most cell lines, whereas three IgG antibodies slow growth moderately but allow the cells to continue growing. Multivalent IgM antibodies might inhibit transferrin receptor function by extensive cross-linking of the receptors. We present data here which support the hypothesis that extensive cross-linking of the transferrin receptor by multivalent antibodies is important for inhibition of cell growth by anti-transferrin receptor antibodies.

MATERIALS AND METHODS

Cells. AKR1, an AKR/J thymic lymphoma cell line (6), and its RI7 208-resistant mutant AKR1/RI7 208, which has been described previously (12), were cultured in 10% horse serum in Dulbecco modified Eagle medium.

Antibodies. RI7 208, a rat IgM, and RI7 217, a rat IgG, were described previously (12, 24). The other three anti-transferrin receptor antibodies are listed, along with their immunoglobulin classes, in Table 1. They were made by fusion of immunized BDIX rat spleen cells with the nonsecreting BALB/c myeloma cell line S194/5.XX0.BU.1 as previously described (12, 24). For REM 17, rats were immunized with BALB/c bone marrow cells. For RR24, rats were immunized with the thymic lymphoma R1.1 (18). For RL34-14, rats were immunized with L34, a leukemia of BALB/c/Nu/Nu mice, provided by G. Beattie, University of California, San Diego. IgG antibodies were typed by culturing hybridoma cells in [^{35}S]methionine and immunoprecipitating the supernatants with class-specific anti-rat immunoglobulins, followed by species-specific antiimmunoglobulins. IgMs were identified by the position of the reduced heavy-chain band on 10% polyacrylamide-sodium dodecyl sulfate gels and by cell surface immunofluorescence with an IgM-specific fluorescinated anti-rat immunoglobulin (Hyclone Laboratories). Antibodies were purified from pooled ascitic fluid and serum of BALB/c/Nu/Nu mice injected intraperitoneally with hybridoma cells. IgMs were purified by dialysis against low salt, and IgGs were purified by ion-exchange chromatography on DEAE-cellulose (12).

Anti-rat immunoglobulin was either a serum pool of repeated bleeds of five rabbits (immunized repeatedly with 4 to 7 mg of rat IgG [Miles Laboratories, Inc.]) or a serum pool of repeated bleeds of a goat (immunized repeatedly with 25 mg of rat IgG).

Growth and clonal assays. Growth and clonal assays have been described previously (12). For clonal assays (Table 1), 24 wells of each of eight cell concentrations (between 6 and 0.5 cells per well) were plated in flat bottom, 96-well plates in the absence or presence of the indicated antibody at 10 μg/ml, and the proportion of negative wells at each concentration was plotted. The slope of the regression line fitted by the method of least squares was used to determine the cloning frequency. When the IgMs were used, serial twofold

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TABLE 1. Description of antibodies used in study[a]

Antibody	Class	Relative cloning frequency	Blocking of RI7 208	Complement-mediated cytotoxicity
RI7 208	IgM	5×10^{-5}	+	−
RI7 217	IgG2a	0.58	+	−
RL34-14	IgG2a	0.36	+	−
RR24	IgG2b	0.44	−	−
REM17	IgM	10^{-4}	+	+

[a] Antibody class and cloning frequency of AKR1 cells in each antibody were determined as described in the text. Cloning frequency is expressed relative to the cloning frequency of AKR1 cells in the absence of antibody assayed in the same experiment. Untreated AKR1 cells had cloning frequencies between 0.35 and 0.50 in several experiments. Blocking of ^{125}I-RI7 208 binding by other antibodies was determined as described in the text and in reference 12.

dilutions of cells, from 2×10^5 per well, were plated in antibody, and the frequency was estimated from the dilution at which positive wells appeared. Positive and negative wells were counted after 2 weeks.

Competition binding assays. Human transferrin (Pentex Biochemical) and purified RI7 208 were iodinated with chloramine T (13). Saturation binding assays for ^{125}I-human transferrin binding to AKR1 cells in the presence of 0, 25, 50, and 100 μg of RI7 208 or RI7 217 per ml were performed at 4°C in V-bottom microwells containing 10^6 cells per well as previously described (12). Each point was determined in triplicate (standard deviation was less than ±10%). Nonspecific binding in the presence of 100 μg of unlabeled transferrin per ml was determined for each concentration of ^{125}I-transferrin and subtracted to obtain the values shown in Fig. 1A. Saturation binding assays also were performed with ^{125}I-RI7 208 including competition with 100 μg of human transferrin per ml. For blocking experiments (see reference 12), dilutions of unlabeled antibody or diluent were added to cells in microwells, followed by the addition of a near-saturating concentration of ^{125}I-human transferrin (4 μg/ml, Fig. 1) or ^{125}I-RI7 208 (1.5 to 2.0 μg/ml, Table 1). Again, each point was determined in triplicate.

Flow microfluorimetry. Culture supernatants from hybridomas or culture medium alone were bound to AKR1 and AKR1/RI7 208 cells on ice for 30 min. After washes, a fluorescein-conjugated anti-rat immunoglobulin (Cappel Laboratories) was added. After a final incubation on ice and another set of washes, cells were resuspended in 5 μg of propidium iodide per ml to stain dead cells, and the cells were run on the Salk Institute flow microfluorimeter as described previously (11). In other experiments, well-washed cells (preincubated at 37°C in the absence of serum) were first incubated with human transferrin, followed by fluoresceinated anti-human transferrin (Cappel Laboratories).

Receptor degradation. To study the fate of the surface transferrin receptor in the presence of anti-transferrin receptor antibodies, AKR1 cells were surface ^{125}I-iodinated under sterile conditions with lactoperoxidase (17). Washed cells were then put into several replicate dishes in the presence or absence of 10 μg of RI7 217 or RI7 208 antibody per ml. At various times, cells from individual dishes were washed in phosphate-buffered saline (0.15 M NaCl, 10 mM NaH_2PO_4 [pH 7.2]) and solubilized in 1% Nonidet P-40. Nuclei were spun out (5 min at 850 × *g*), and the lysates were precipitated with hybridoma supernatants and anti-rat immunoglobulin (23). Precipitates were reduced and run on 10% acrylamide-sodium dodecyl sulfate gels.

RESULTS

Monoclonal antibodies against the murine transferrin receptor. Some of the properties of five anti-transferrin receptor monoclonal antibodies which we obtained are summarized in Table 1. As can be seen by the severe reduction in cloning frequency of the AKR1 lymphoma cells in RI7 208 and REM 17, the IgM antibodies completely inhibited the growth of almost all the cells. The cells that grew in the presence of the IgM anti-transferrin receptor antibodies, when studied in detail, appear to be mutants of the type previously described

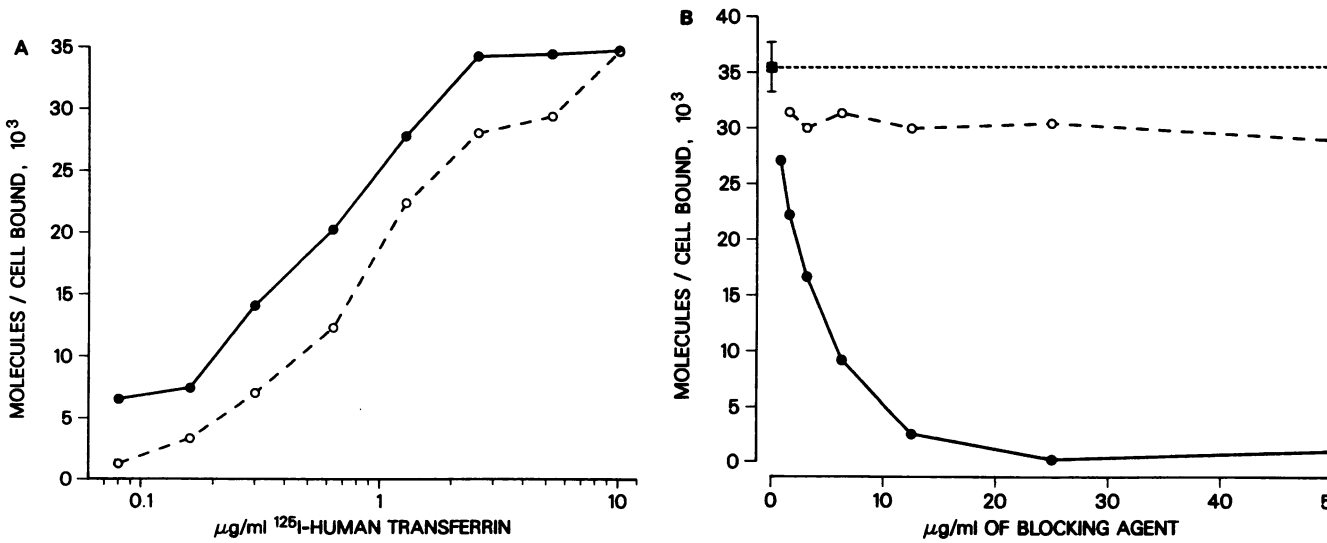

FIG. 1. Binding of ^{125}I-human transferrin to AKR1 cells. (A) Binding was assayed to 4°C, at the indicated ^{125}I-transferrin concentrations as described in the text and reference 12. Symbols: ●, control binding; ○, binding in the presence of 100 μg of RI7 208 per ml. Binding in the presence of 25 and 50 μg of RI7 208 per ml (not shown) was similar to that in 100 μg/ml. (B) ^{125}I-human transferrin at 4 μg/ml was bound to AKR1 cells in the presence of increasing concentrations of RI7 208 (○) or unlabeled transferrin (●) or in diluent only (■).

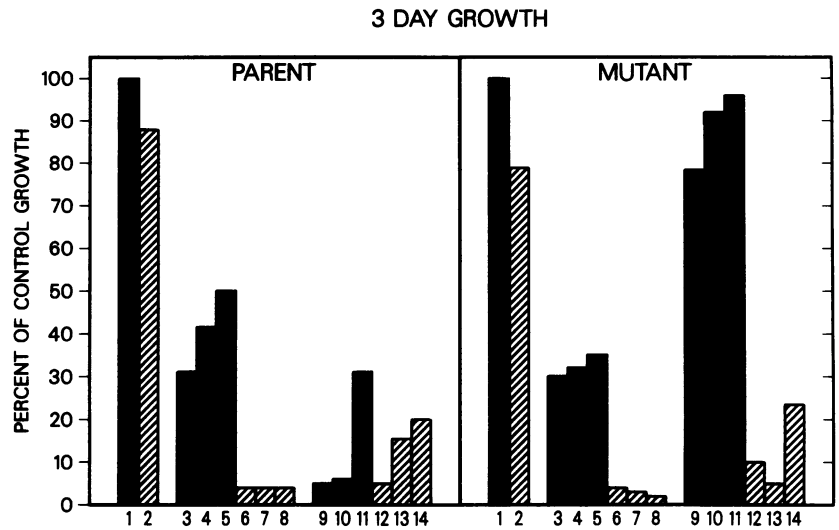

FIG. 2. AKR1 or mutant AKR1/RI7 208 cells were plated in two replicate 35-mm dishes at 5×10^4 cells per ml. Samples were counted at 3 days (when cells were still growing exponentially) on a Coulter Counter (Coulter Electronics, Inc.). Growth is expressed relative to untreated control cultures. Filled bars represent cultures without antiimmunoglobulins. Striped bars represent cultures including anti-rat immunoglobulin serum at a 1:100 dilution. Cultures 1 and 2 contain no anti-transferrin receptor antibodies. Cultures 3, 4, and 5 and 6, 7, and 8 contained RI7 217 at 10, 5 and 2 μg/ml, respectively. Cultures 9, 10, and 11 and 12, 13, and 14 contained RI7 208 at 10, 5 and 2 μg/ml, respectively.

(12). They are stably resistant to inhibition by the antibody used for selection and have lost the antibody-binding site on a fraction of their transferrin receptor molecules while retaining binding of the selecting antibody on another portion of their receptors. We previously interpreted this data as evidence for a mutation in one copy of the transferrin receptor structural gene (12).

In contrast to the results with the IgM antibodies, the cloning efficiency of the AKR1 line in the three IgG antibodies was only slightly reduced (0.3 to 0.6 relative to untreated controls). The appearance of macroscopic colonies was delayed in the presence of the IgG antibodies by about 1 week, indicating a reduction in growth rate. Nevertheless, AKR1 cells were able to grow indefinitely in the presence of the IgG antibodies.

All but one of the monoclonal anti-transferrin receptor antibodies blocked binding of ^{125}I-RI7 208 in a competition binding assay (Table 1), indicating binding at a site adjacent to the RI7 208-binding site. This suggests that the region of the transferrin receptor to which the antibody binds is not the determining factor in growth inhibition by these antibodies. It also suggests that this region is a major antigenic determinant of the receptor. Though the binding sites of three of the other antibodies are near the RI7 208-binding site, they are not identical to it. RI7 208-resistant mutant cells lack RI7 208-binding sites on a portion of their transferrin receptor surface molecules. These mutant molecules, however, still bind the four other anti-transferrin receptor antibodies. This has been shown in sequential immunoprecipitations by the failure of RI7 208 to remove all of the transferrin receptor from an ^{125}I-labeled mutant cell lysate. Subsequent immunoprecipitation detects a mutant receptor molecule (not present in parental cells) which is still recognized by other anti-transferrin receptor antibodies but not by RI7 208 (12). Only the REM 17 IgM antibody is cytotoxic to cells in the presence of (rabbit) complement. It does not cause complement-mediated lysis, however, under the conditions in which growth inhibition is assayed.

Effects on transferrin binding. Inhibition of growth and iron uptake by anti-transferrin receptor antibodies might be the result of competition between antibody and transferrin for the transferrin-binding site of the receptor. Binding experiments with ^{125}I-human transferrin receptor showed minimal inhibition of transferrin binding by either RI7 208 (Fig. 1) or RI7 217 (data not shown). There was a slight reduction in transferrin binding at below and near saturating concentrations (less than 10 μg of transferrin per ml) in the presence of RI7 217 (not shown) and RI7 208 antibody (Fig. 1A and B), but this did not increase with increasing concentrations of RI7 208 (Fig. 1B). The levels of transferrin in cultures inhibited by RI7 208 are well above saturation (about 250 μg/ml in 10% horse serum). In reciprocal experiments, the binding of ^{125}I-RI7 208 and ^{125}I-RI7 217 was unaffected by the presence of 100 μg of human transferrin per ml or by up to 25% horse serum, fetal bovine serum, or calf serum. None of the anti-transferrin receptor antibodies described in Table 1 competed with transferrin for its binding site on the transferrin receptor when fluorescein-labeled anti-transferrin antibody and flow microfluorimetric analyses were used to detect transferrin binding to cells in the presence of these antibodies (data not shown). We conclude, therefore, that growth inhibition is not the result of competition for the transferrin-binding site of the transferrin receptor.

Cross-linking antibodies with anti-rat immunoglobulin. Since we speculated that the growth inhibition by RI7 208 and REM 17 might be due to the multiple valency of the IgM antibodies, we attempted to mimic this effect by cross-linking the IgG anti-transferrin receptor antibodies with anti-rat immunoglobulin. The effects on cell growth when anti-rat immunoglobulin serum was added to cultures in the presence of anti-transferrin receptor antibodies are shown in Fig. 2. The RI7 217 antibody, which by itself reduces cell yield after 3 days to about 40% of control cultures (columns 3 to 5), in the presence of anti-rat immunoglobulin reduces cell yield by more than 95% (columns 6 to 8). Anti-rat immunoglobulin alone does not affect cell growth (column 2). In clonal assays (described above; Table 1), RI7 217 plus anti-rat im-

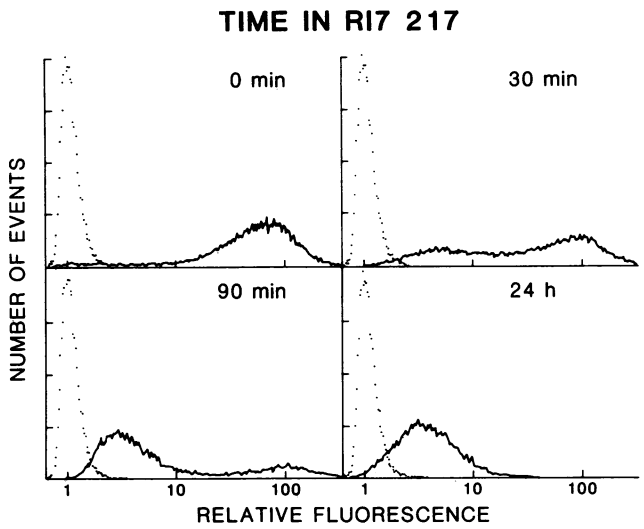

FIG. 3. Fluorescence histograms of AKR1 lymphoma cells cultured in 10 μg of RI7 217 per ml. At the indicated times, cells were chilled to 4°C, washed twice in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Dulbecco modified Eagle medium containing 0.15% polyvinylpyrrolidone and 2 mM sodium azide, and then restained at 4°C with RI7 217 culture supernatant and fluorescein-conjugated anti-rat immunoglobulin. The dotted curve represents background staining of AKR1 cells incubated with medium alone and fluorescein anti-rat immunoglobulin. Background mean fluorescence is normalized to 1.0 U.

munoglobulin reduced the cloning frequency of AKR1 cells to about 10^{-4}, which is comparable to the frequencies seen with the IgM anti-transferrin receptor antibodies. Two other rat antibodies against cell surface determinants of AKR1 cells, anti-T200 and anti-Thy-1, either alone or in combination with anti-rat immunoglobulin, did not reduce the cloning frequency of these cells by more than fourfold (i.e., cloning frequency of 0.25 relative to control).

The RI7 208-resistant mutant, whose growth is unaffected in the presence of RI7 208 antibody alone (Fig. 2, mutant columns 9 to 11) is inhibited by RI7 208 plus anti-rat immunoglobulin (Fig. 2, mutant columns 12 to 14). The mutant is similar to the parent AKR1 cell line in its sensitivity to RI7 217 and anti-immunoglobulin. It should be noted that the concentrations of the anti-transferrin receptor antibody and anti-rat immunoglobulin in these experiments are critical.

The ability of IgG anti-transferrin antibody to inhibit growth in the presence of anti-rat immunoglobulin supports the hypothesis that the cross-linking of receptors is involved in growth inhibition. Inhibition of the RI7 208-resistant mutant with RI7 208 plus anti-rat immunoglobulin, as well as with RI7 217 plus anti-rat immunoglobulin, shows that it is still susceptible to inhibition by the cross-linking of receptors. Both of these treatments were used to select additional mutants, again probably in the transferrin receptor structural gene, similar to those previously selected with RI7 208 alone (12). These mutants will be described in detail elsewhere (manuscript in preparation).

Cell surface expression of receptor in the presence of anti-transferrin receptor antibodies. Flow microfluorimetry with fluorescent anti-rat immunoglobulin to detect the cell-associated anti-transferrin receptor antibody (11) was used to study the surface expression of the transferrin receptor in the presence of anti-transferrin receptor antibodies. AKR1 cells were cultured at 37°C in the presence of 10 μg of RI7 208 or RI7 217 antibody per ml for 30, 60, 90, 120, and 180 min and for 24 and 48 h. The effects of antibody on the surface receptor at some of these times are shown (Fig. 3 and 5). The cells were restained with the antibody in which they were cultured, but the same results were seen without restaining (due to antibody which was bound in culture) or after restaining with a different anti-transferrin receptor antibody. Cells were cultured for the indicated times in RI7 217 and then restained with RI7 217 to detect all surface transferrin receptors (Fig. 3). The upper left panel (Fig. 3) shows cells stained with RI7 217 without any preincubation in the antibody, representing the control level of transferrin receptor. After 30 min at 37°C, a portion of the cells have reduced their surface receptor to a lower level, and with time, more cells assume this lower level of receptor. By 180 min (not shown), all but about 9% of the cells have reduced receptor levels. At 24 h, all the cells have a lower receptor level. The mean relative fluorescence of the cell population at 24 h is 3.17 U above the background (1.0 U) as compared with a mean relative fluorescence of 38.25 U before culture in antibody (0 time). This indicates an average 10-fold reduction in cell surface transferrin receptor. Repeated experiments show 5- to 10-fold average reductions in the transferrin receptor after culture for 24 h in the presence of RI7 217. This result is supported by measurements of ^{125}I-transferrin binding after overnight culture in RI7 217 (not shown here), in which three- to eightfold reductions in transferrin binding were seen. At 48 h, the lower levels of transferrin receptor are still maintained.

The rapid reduction in surface antibody and receptor seen in the presence of RI7 217 is correlated with an increase in degradation of receptor (Fig. 4). AKR1 cells were surface

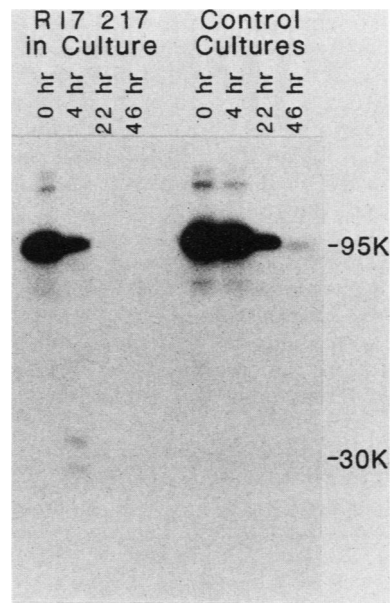

FIG. 4. Autoradiogram of 10% acrylamide-sodium dodecyl sulfate gel of immunoprecipitated transferrin receptor from ^{125}I-surface iodinated AKR1 cells cultured in the presence and absence of 10 μg of RI7 217 per ml. Lactoperoxidase ^{125}I-labeled cells were cultured for the indicated times and solubilized in 1% Nonidet P-40, and the membranes were immunoprecipitated with RI7 217 and anti-rat immunoglobulin.

iodinated with lactoperoxidase, and then samples were returned to culture in the presence and absence of anti-transferrin receptor antibodies RI7 208 and RI7 217. At various times after labeling, cell lysates were prepared, immunoprecipitated with several anti-transferrin receptor antibodies, reduced, and run on 10% acrylamide gels containing sodium dodecyl sulfate. Control and RI7 217 cultured cells precipitated with RI7 217 are shown in Fig. 4. The 95,000-molecular-weight band representing the intact transferrin receptor subunit decreases in intensity in the control cultured cells with a $t_{1/2}$ of 10 to 12 h. Degradation of the band is accelerated in the presence of RI7 217 to the extent that the intact receptor remaining at 4 h is comparable to what is seen at 22 h in the control cultures ($t_{1/2}$ of about 2 h). Two degradation products with molecular weights of ca. 30,000 are detected in the RI7 217 cultures at 4 h. These bands can sometimes be seen in overexposed gels from 4-h control cultures. The amounts of ^{125}I-transferrin receptor precipitable from RI7 208 cultured cells at these times were indistinguishable from those in control cultures (not shown). Thus, culture in RI7 208 does not result in a detectable increase in degradation over controls.

Culture of AKR1 cells in the presence of RI7 208 (Fig. 5) does not result in downward regulation of cell surface receptor. Though some cells (about 30% in this experiment) reduce their receptor levels to almost background by 60 min, most retain the higher receptor levels seen at 0 time. After 24 h, many cells have actually bound more antibody than was bound initially. Smaller increases in fluorescence of the positive cells could also be seen at 180 min. There appears to be an accumulation of antibody on the cell surface in many of the cells. This result was seen in numerous experiments, with some variation in the proportion of cells in the receptor-negative population. In several experiments, 15 to 40% of the cells appeared negative at 24 h, whereas the positive cells were always more fluorescent than were those in untreated controls. At 48 h, the fluorescence of the positive cells was even brighter and negative cells were fewer. Many cells were dying by this time. In this experiment, the mean relative fluorescence of AKR1 cells stained with RI7 208 at 0 min was 15.13 U. At 24 h, the mean fluorescence of the positive cells (fluorescence above the background peak) was 28.24 U; at 48 h, it was about 60 U. Binding of ^{125}I-human transferrin to cells incubated overnight in the presence of RI7 208 (data not shown) gave variable results that may be explained by the heterogeneity of the cells and the variability in the proportions of the negative and positive cells. Some experiments showed slight reductions in ^{125}I-transferrin binding relative to controls, but others showed no decrease or even increases in transferrin binding. Immunoprecipitations, such as those shown in Fig. 4, of surface ^{125}I-labeled transferrin at different times after labeling and culture in RI7 208 indicated no increase in degradation of receptor over control cultures in the absence of RI7 208.

In pulse exposures to antibody (45 min at 4°C), followed by washing and incubation at 37°C, both RI7 208 and RI7 217 were rapidly cleared from the cell surface. When cells were cultured in RI7 208 for 24 h, then removed from antibody, and cultured, cell surface RI7 208 gradually disappeared (about half the cells were free of antibody after an additional 24 h), but upon restaining with anti-transferrin receptor antibodies new receptor could be detected on the cell surface. These cells grew normally after about a 1-day lag. After 3 days in antibody, the surviving cells could be recovered with little effect on cloning efficiency, but their growth did not resume for 2 to 3 days. After 7 days in antibody, no viable cells could be recovered. Thus, antibody had to be present for several days to irreversibly inhibit cell growth.

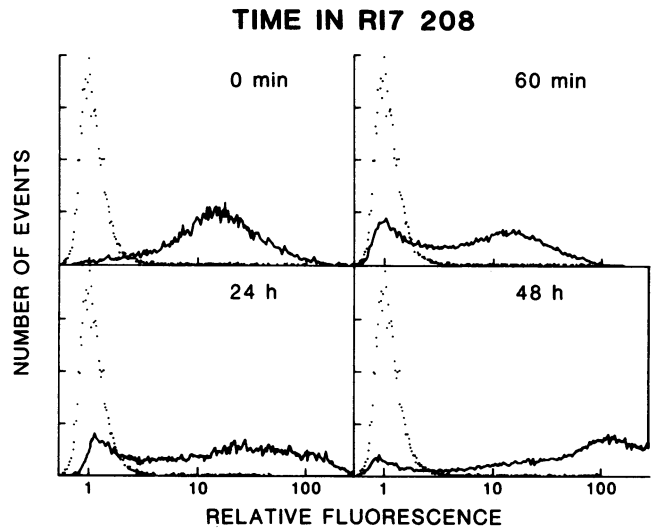

FIG. 5. Fluorescence histograms of AKR1 lymphoma cells cultured in 10 μg of RI7 208 antibody per ml. At the indicated times, cells were chilled, washed as in Fig. 3, and then stained at 4°C with RI7 208 culture supernatant and fluorescein-conjugated anti-rat immunoglobulin. The dotted curve represents background staining of AKR1 cells incubated with medium alone and fluorescein anti-rat immunoglobulin. Background mean fluorescence is normalized to 1.0 U.

We previously showed by sequential immunoprecipitation that mutant cells selected by growth in RI7 208 antibody have two types of transferrin receptor molecule on their surface (12). Probably due to a mutation in one copy of the transferrin receptor structural gene, half of the transferrin receptors of the mutant cells can no longer be precipitated by RI7 208, whereas they remain precipitable by all of the other anti-receptor antibodies. Half of the receptors are wild type and bind RI7 208 as well as the other antibodies. RI7 208-resistant mutant AKR1 cells cultured overnight in the presence and absence of RI7 208 antibody and restained with RI7 208 and RL34-14 are shown (Fig. 6). Whereas parental cells show identical results regardless of what antibody is used for restaining, the two types of transferrin receptor molecule can be detected on these mutant cells. After overnight culture in RI7 208, the mutant cells show a reduced level of RI7 208 binding, with a mean relative fluorescence of 1.6 (background of 1.0) compared with control cultured cells, which have a mean fluorescence of 5.0 times background. When these cells are restained with RL34-14, another set of receptors is seen which does not bind RI7 208 but is still abundantly expressed on the cell surface. When cultured in the presence of RI7 208, the RI7 208-bearing, wild-type receptors are modulated to a lower level of receptor expression. The mutant receptors, which bind RL34-14 but not RI7 208, are expressed at normal levels on the cell surface (this staining is also seen with RI7 217, RR24, and REM 17). The reduction in intensity of RL34-14 staining is attributable to the downward regulation of wild-type receptors which also bind RL34-14.

DISCUSSION

In this report, we examined the effects of five anti-transferrin receptor antibodies of several immunoglobulin

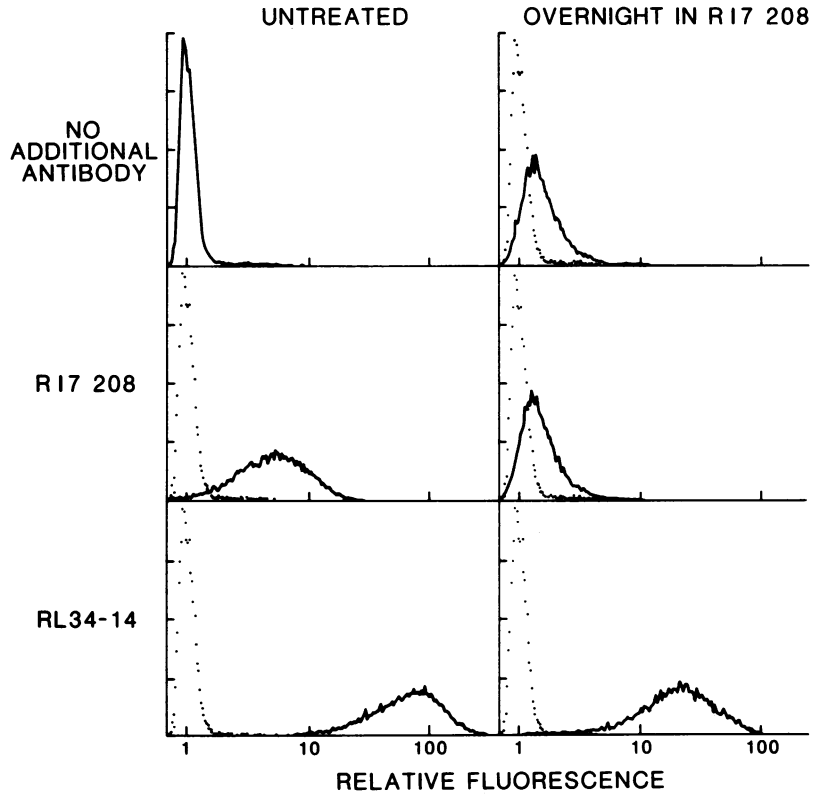

FIG. 6. Fluorescence histograms of AKR1/RI7 208 mutant cells cultured overnight in the absence and presence of 10 μg of RI7 208 antibody per ml. Cells were chilled, washed as in Fig. 3, and then stained with medium only (top two panels), RI7 208 culture supernatant (middle panels), or RL34-14 culture supernatant (bottom two panels), followed by fluorescein-conjugated anti-rat immunoglobulin. The dotted curve insert represents background staining of untreated cells as shown in the upper, untreated panel. Staining of RI7 208-treated cells without additional antibody (upper right panel) is due to antibody bound in culture.

classes on transferrin binding, cell growth (yield and cloning efficiency), and cell surface transferrin receptor distribution. Although none of the antibodies significantly affected transferrin binding (Fig. 1), the IgM antibodies had a qualitatively different effect on cell yield and cloning efficiency (12, 24; Table 1 and Fig. 2). Inhibition of growth by IgM antibodies, but not by IgG antibodies, does not correlate with other properties of the antibodies such as the region of binding on the receptor molecule or cytotoxicity in the presence of complement. This correlation of growth inhibition with the IgM class of anti-transferrin receptor antibodies extends to in vivo tumor models where RI7 208 and REM 17 (IgMs), but not RI7 217 (IgG), retard growth of the tumor line SL2 in mice (22, 26). Interestingly, the only anti-human transferrin receptor monoclonal antibody that inhibits growth of cell lines in vitro is of the IgA class, which is also multivalent (14, 25). Furthermore, the serum of a patient with iron-deficient anemia caused by autoantibodies to the transferrin receptor contains IgM antibodies that inhibit iron uptake in the human erythroid cell line K562 (10). Both the monoclonal IgM anti-murine transferrin receptor antibody RI7 208 (12, 24) and the IgA anti-human transferrin receptor monoclonal antibody (25) have been shown to inhibit iron uptake of cultured cell lines.

We suggest that the profound effects of the IgM antibodies on cell growth are due to extensive cross-linking of cell surface receptors. In support of this idea is the ability to mimic the growth-inhibiting effects of the IgM antibodies by adding antiimmunoglobulin to an IgG anti-transferrin receptor antibody (Fig. 2). (Other antibodies against cell surface molecules of the AKR1 lymphoma [anti-Thy-1 and anti-T200] do not inhibit cell growth when coupled with antiimmunoglobulin.) The escape of RI7 208-resistant mutant cell lines through a reduction (by half) in the surface density of antibody-binding sites (12) is also consistent with this proposal.

Though the mechanisms by which cells may process anti-transferrin receptor antibodies are unclear, our working model based on the studies presented here is as follows. (i) Antibodies cross-link transferrin receptors. (ii) Cells downward regulate receptor density to eliminate cross-linking. (iii) When cross-linking is extreme, downward regulation either does not occur or is not adequate to eliminate cross-linking; then cells die because transferrin receptors do not perform their function. (iv) When cross-linking is extreme, mutant cells can be selected which have reduced the density of antibody-binding determinants on their surface. The evidence in support of these points is discussed below.

Evidence for statements (i) and (ii) (above) is as follows. The fluorescent experiments on the distribution of cell surface transferrin receptor show that in the presence of IgG anti-transferrin receptor antibody AKR1 lymphoma cells downward regulate their receptor 5- to 10-fold (Fig. 3). Downward regulation is accompanied by increased receptor degradation (Fig. 4). Similarly, RI7 208-resistant mutant cells downward regulate the wild-type portion of their receptors, which bind RI7 208, in the presence of RI7 208 (IgM) antibody, whereas cell surface expression of receptors which lack the RI7 208-binding site is unchanged (Fig. 6). In both of these cases, binding of antibody results in downward

regulation of antibody-associated receptors. Enns et al. have shown that monovalent Fab anti-transferrin receptor antibody fragments, unlike intact antibody, do not induce receptor internalization (4). Thus, we suggest that limited cross-linking of receptors by antibody may provide a signal for induction of downward regulation, perhaps by triggering changes in intracellular routing of cross-linked receptor compared with normally cycling receptor. Under these conditions, though cell growth may be slowed, receptor function is maintained.

Evidence for statement (iii) is as follows. RI7 208 (IgM) binding to wild-type AKR1 cells, RI7 208 plus anti-immunoglobulin binding to RI7 208-resistant mutant cells, and RI7 217 (IgG) plus anti-immunoglobulin binding to wild-type and mutant cells result in growth inhibition. Here, cross-linking of receptors is increased by multivalent reagents or complexes of reagents. Fluorescent studies of receptor distribution in AKR1 cells grown in RI7 208 consistently show a bimodal population with either background levels of staining or high levels of surface receptor. Though there are cells present with intermediate receptor levels, there is no accumulation of cells with lowered receptor levels, as is seen in the presence of the IgG antibody RI7 217. With increasing time, most of the surviving cells express increasingly higher levels of surface receptor. Though some of the cells remove essentially all antibody (and receptor) from their surface initially, the number of these negative cells gradually declines (Fig. 5), either through death or re-expression of surface receptor. Cells with high levels of antibody-complexed receptors (such as those seen at 48 h [Fig. 5]) may be largely unable to clear antibody from their surface.

The reason for failure of transferrin receptor function (i.e., failure of iron delivery [12, 24, 25]) in the presence of extensive cross-linking is unclear. Two possible explanations for this suggest themselves, and both may apply. One possibility is that the receptors complexed with antibody are not being internalized but simply remain on the cell surface. The accumulation of receptor on the surface with time may indicate this. Alternatively (or in addition), this accumulation may reflect a regulatory mechanism such as is seen in the presence of the intracellular iron chelator desferroxamine, which causes an increase in surface receptor expression requiring ongoing protein synthesis (3). Taetle et al. (submitted for publication) have presented evidence that transferrin internalization is greatly reduced in human cells cultured in 42/6, the IgA anti-human transferrin receptor antibody which inhibits cell growth. The second possibility is that the transferrin receptors with bound antibody and transferrin are internalized but because of cross-linking by antibody are sorted inappropriately so that iron is not delivered. Electron microscopy or subcellular fractionation may be necessary to determine the intracellular localization of antibody and receptor in growth-arrested cells.

Evidence for statement (iv) (above) is as follows. RI7 208-resistant mutant cells are not inhibited by RI7 208 antibody alone. Though they express a substantial population of mutant transferrin receptor which does not bind RI7 208 antibody (Fig. 6; 12), they can still be inhibited by RI7 208 plus antiimmunoglobulin (Fig. 2). This suggests that it is not the presence of mutant receptors but the reduction of RI7 208-binding (wild-type) receptors and the resulting inability of IgM antibody to effectively cross-link that allow their escape from growth inhibition by RI7 208 alone. Other mutant cell lines have been cloned from several different murine lymphoma lines by selection in RI7 208 (12) and RI7 217 plus antiimmunoglobulin (manuscript in preparation). All of these lines have lost the antibody-binding site for the selecting antibody on a portion (about half) of their cell surface receptors. This has been shown by sequential immunoprecipitation and reciprocal blocking studies such as those described in reference 12.

Cross-linking of the surface receptor is a function of both antibody valence and receptor density, as our observations show. A noninhibiting antibody can be made inhibitory by increasing its valence (by complexing it with antiimmunoglobulin). An inhibiting antibody (IgM) can be rendered noninhibitory by a reduction in cell surface antibody-binding determinants (in RI7 208-resistant mutant cells). Thus, the effects of anti-transferrin receptor antibodies on different cells may be expected to vary with differences in receptor density, which in turn are related to cell growth.

ACKNOWLEDGMENTS

We thank R. Hyman and I. Trowbridge for reviewing the manuscript.

This work was supported by National Cancer Institute grant CA-25893.

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