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Selection of Cell Lines Resistant to Anti-Transferrin Receptor Antibody: Evidence for a Mutation in Transferrin Receptor

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Some anti-murine transferrin receptor monoclonal antibodies block iron uptake in mouse cell lines and inhibit cell growth. We report here the selection and characterization of mutant murine lymphoma cell lines which escape this growth inhibition by anti-transferrin receptor antibody. Growth assays and immunoprecipitation of transferrin receptor in hybrids between independently derived mutants or between mutants and antibody-susceptible parental cell lines indicate that all of the selected lines have a similar genetic alteration that is codominantly expressed in hybrids. Anti-transferrin receptor antibodies and transferrin itself still bind to the mutant lines with saturating levels and K_d values very similar to those of the parental lines. However, reciprocal clearing experiments by immunoprecipitation and reciprocal blocking of binding to the cell surface with two anti-transferrin receptor antibodies indicate that the mutant lines have altered a fraction of their transferrin receptors such that the growth-inhibiting antibody no longer binds, whereas another portion of their transferrin receptors is similar to those of the parental lines and binds both antibodies. These results argue that the antibody-selected mutant cell lines are heterozygous in transferrin receptor expression, probably with a mutation in one of the transferrin receptor structural genes.

There is increasing evidence in support of the notion that transferrin receptor function is necessary for cell proliferation. Transferrin is an essential component of defined (serum-free) media for most in vitro-grown cells (1). It has been shown in several systems to be the major serum protein contributing to proliferation in vitro (31, 39). Transferrin receptor expression appears to be ubiquitous on dividing cells (8–11, 15, 17, 24, 25, 29, 33, 37). It is present on cells which are normally cycling in vivo such as hematopoietic progenitor cells (17, 25), on normal cells stimulated in vitro by mitogenesis (9, 15, 33), on some primary tumor cells (8, 10, 24, 29), and on virtually all cells proliferating continuously in vitro (11, 15, 33, 37). Antibodies that interfere with transferrin receptor function inhibit the uptake of iron (35, 37) and inhibit cell proliferation in a number of in vitro systems: growth of continuous cell lines (30, 35, 37, 38), growth and development of primary hematopoietic colonies (30; J. Lesley, unpublished data), and mitogen-stimulated proliferation of lymphoid cells (19, 20). Some of these antibodies also inhibit tumor growth in in vivo model systems in mice (35, 36).

The specific point(s) in the cell cycle at which iron, supplied to the cell by transferrin and transferrin receptor, is critical is not known. Most evidence is consistent with a purely nutritional requirement (e.g., see reference 14a), though the suggestion has been made that a growth signal may be provided by the interaction of transferrin and transferrin receptor (7, 32, 38).

To study the extent to which transferrin receptor is necessary for growth and the mechanisms of its expression and function, we have used an anti-murine transferrin receptor antibody (37) to select cell lines with altered transferrin receptors. We have obtained cell lines that are resistant to anti-transferrin receptor antibody inhibition of growth and have obtained evidence that their escape is due to a mutation in one of the two genes coding for transferrin receptor.

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MATERIALS AND METHODS

Antibodies. RI7 208 is an immunoglobulin M anti-murine transferrin receptor antibody that inhibits iron uptake and prevents cell growth in most murine cell lines (37). RI7 217 is an immunoglobulin G2a anti-murine transferrin receptor antibody (16, 17). It has only slight effects on cell growth (i.e., moderate slowing of growth, [J. Lesley, unpublished data]). RI7 208 was precipitated from pooled ascitic fluid and serum from BALB/c nu/nu mice (Microbiological Associates) (injected intraperitoneally with the hybridoma) by dialysis against low salt (37). RI7 217 was purified from ascitic fluid and serum of nude mice by precipitation with 50% saturated ammonium sulfate and ion-exchange chromatography on DEAE-cellulose. I3/2 monoclonal antibody against T200 glycoprotein, unrelated to transferrin receptor, was used as a positive control in immunoprecipitations (34). Culture supernatant from heavy cultures of the hybridomas was used for indirect radioimmune binding assays and immunoprecipitations, with medium alone for the negative control.

Cells. Four thymic lymphoma cell lines were used for the selection of transferrin receptor mutants: AKR1G.1 (AKR1) (Thy-1.1, 6-thioguanine resistant), BW5147.G.1.4 (BW5147) (Thy-1.1, 6-thioguanine resistant), S49.1TB.2.3 (S49) (Thy-1.2, bromodeoxyuridine resistant) (13), and EL4.BU.1.4 (EL4) (Thy-1.2, bromodeoxyuridine resistant) (22). All cells were grown in suspension in Dulbecco modified Eagle medium containing 10% horse serum. Hybridoma cell lines RI7 208 and RI7 217 were made as described in references 37 and 17, respectively, and were grown in Dulbecco modified Eagle medium containing 10% horse serum and 10^{-5} M 2-mercaptoethanol. Every effort was made to have all cells in comparable stages of exponential growth when they were used, since transferrin receptor expression varies with the state of cell growth (15).

For antibody selection, parental cells were cultured overnight in 1, 0.7, or 0.5 mg of ethyl methanesulfonate per ml, removed from the mutagen the following day, and observed for cell death. Dishes in which ca. 75% or more of the cells

died were chosen for selection. Approximately 7 to 10 days after mutagenesis, replicate dishes containing 1×10^6 to 2×10^6 cells and 10 μg of RI7 208 antibody per ml were set up. Most lines proliferated very slowly for a day or two and then began to die. After a week or more, growing cells began to appear in some of the dishes, and these cells were cloned in 10 μg of RI7 208 antibody per ml in 96-well flat-bottom cloning plates. Clones were picked into medium without antibody and tested for binding of RI7 208 and RI7 217 antibodies by an indirect radioimmune binding assay (37). Clones with the lowest levels of anti-transferrin receptor antibody binding were kept for further study (except in the case of EL4 for which a low- and a high-binding clone were kept).

Cell fusions. Cell fusions were performed as described previously (13). Usually, 10^7 cells of each line were pelleted and then mixed with 40% polyethylene glycol 1000 for 0.5 min. The polyethylene glycol was gradually diluted, and then the cells were washed once and plated in a 96-well cloning plate in HAT medium. Cell numbers were increased if the first fusions were not successful. Cells from individual wells were recloned if multiple colonies grew in each well.

Growth and clonal assays. For measuring cell growth, usually 10^5 cells were plated in 2 ml of medium, in duplicate, in 35-mm petri dishes (37, 38). At various days after plating, a sample was taken from each dish (after thorough mixing), diluted in phosphate-buffered normal saline (0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.2), and counted on a Coulter counter. For clonal assays, sequential twofold cell dilutions (eight wells of each cell dilution) were plated in 96-well cloning plates. An equal volume of medium was added either with or without 20 μg of RI7 208 antibody per ml. Positive wells were counted at several intervals after plating. Cloning frequency was determined by plating out 24 wells of each of five different cell dilutions (up to one cell per well), plotting the percentage of negative wells, and determining the slope of the regression line.

Immunological assays. Indirect radioimmune binding assays, used for screening selected clones, were performed as previously described (37). For direct binding assays, purified human transferrin (Pentex Biochemical), RI7 208, and RI7 217 were iodinated by the chloramine-T method (18). Specific activities for transferrin ranged from 2×10^6 to 5×10^6 cpm/μg in three preparations, and specific activities for the antibodies ranged from 7×10^5 to 4×10^6 cpm/μg. Saturation binding assays and blocking assays were performed in 96-well V-bottom microtiter plates containing 10^6 cells per well. For saturation binding assays, dilutions of ^{125}I-labeled antibody or ^{125}I-labeled transferrin were dispensed in triplicate. For blocking experiments, dilutions of unlabeled antibody or diluent were added to cells in triplicate, followed by the addition of a near-saturating concentration ^{125}I-labeled antibody to all of the wells. Plates were incubated at 4°C for 45 min and then centrifuged. Supernatants were removed, and the cells were washed three times in phosphate-buffered saline containing 0.05% bovine serum albumin. Cells were then transferred to tubes for radioactivity counting in a gamma counter.

Immunoprecipitation and clearing. Lactoperoxidase-catalyzed iodination of cell surface proteins, immunoprecipitations with fixed *Staphylococcus aureus* (Staph A, Pansorbin; Calbiochem-Behring); and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (21). ^{125}I-labeled membrane preparations from parental and mutant cells were each divided into three parts for preclearing with anti-transferrin receptor antibodies and for mock clearing. Samples were incubated with 100 to 200 μg of RI7 208 or RI7 217 or with diluent alone, followed by two incubations with anti-rat immunoglobulin sera and Staph A. The Staph A was removed by centrifugation after each incubation, and the final supernatants were used to prepare immunoprecipitates with anti-transferrin receptor and control antibodies. Clearing was also performed with Sepharose beads to which RI7 217 or RI7 208 antibody had been coupled to cyanogen bromide (33). This method also required two clearing steps and gave results identical to clearing with Staph A, but it resulted in more dilution of the ^{125}I membrane preparation. For determining the relative intensities of the iodinated bands on films of the gels, a Quick Scan R&D gel scanner from Helena Laboratories was used.

^{59}Fe uptake. Labeling of human transferrin with ^{59}Fe and measurement of cellular uptake of the label was performed as previously described (37).

RESULTS

Selection of cell lines. Mutants which were able to grow in the presence of 10 μg of anti-transferrin receptor antibody per ml were selected from a number of murine lymphoma cell lines by growing mutagenized cells in the presence of the anti-murine transferrin receptor antibody RI7 208, as described above. Cells which grew out were then cloned in 10 μg of RI7 208 per ml. There was always general cell death in cultures containing 10 μg of RI7 208 antibody per ml, but some lines produced growing cells more readily than others. AKR1 was growing vigorously 1 week after exposure to antibody. In contrast, only one of four dishes of the EL4 cell line had growing cells after 3 weeks. Cell lines picked after cloning in 10 μg of RI7 208 per ml were grown in the absence of antibody and then tested for transferrin receptor expression by radioimmune binding assays with two anti-transferrin receptor antibodies. All of the clones had reduced antibody binding relative to unselected cells, but all showed binding above the background binding of medium alone. Originally, clones selected for further study were those with the lowest levels of transferrin-receptor antibody binding after cloning. But clones so chosen gradually (over a period of about 2 months) increased their transferrin receptor expression to levels similar to those of their parental cell lines. When EL4 was cloned in antibody, a clone expressing high levels of transferrin receptor and one expressing low levels were studied. Their growth rates were initially quite different, with the line expressing less transferrin receptor growing much more slowly. But after several months, the two lines expressed very similar levels of transferrin receptor and grew at more similar rates.

Though the level of transferrin receptor expression (as detected by the binding of either the antibody used for selection, RI7 208, or a second antibody, RI7 217) increased when cells were grown in the absence of antibody, the ability to grow in the presence of RI7 208 antibody did not change. Most of the resistant cell lines have been growing for over 1 year in the absence of RI7 208 antibody, without any change in their resistance to growth inhibition by antibody. Growth of the parental lines, from which the resistant lines were derived, is completely inhibited by 10 μg of RI7 208 antibody per ml. Figure 1 shows growth curves of two of the selected lines and their parents. Several resistant clones of AKR1 (Fig. 1A) and of S49 (Fig. 1B) were assayed for growth in each of the experiments shown. All of the resistant clones tested had growth curves virtually identical to the ones shown in the figures.

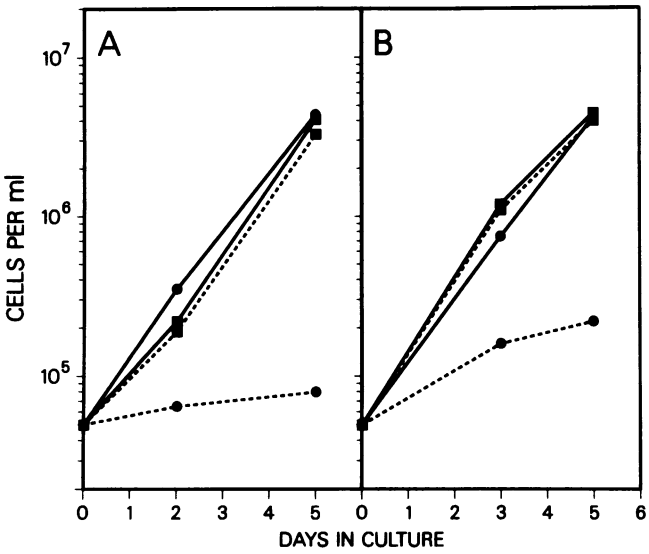

FIG. 1. Growth of parental and mutant cell lines in the presence or absence of RI7 208 antibody. Cells (10^5) were plated in duplicate 35-mm petri dishes containing 2 ml of Dulbecco modified Eagle medium with 10% horse serum. At the days indicated, cultures were mixed by pipetting, and a sample was removed and diluted for counting on a Coulter counter. (A) AKR1 parent (●) and AKR1/RI7 208.19 clone (■) cultured in the presence (– – – –) or absence (——) of 10 μg of RI7 208 antibody per ml. (B) S49 parent (●) and S49/RI7 208.2 clone (■) cultured in the presence (– – – –) or absence (——) of 20 μg of RI7 208 antibody per ml.

Short-term growth curves, however, do not reveal the extent of the difference between the parental and resistant lines, since growth of resistant lines is slightly retarded and parental lines may proliferate very slowly for a time. When AKR1 parental and resistant lines were plated in cloning plates at various numbers of cells per well in the presence of 10 μg of RI7 208 antibody per ml, no parental cells grew in wells containing up to 2×10^4 cells. Mutant AKR1 cells cloned in the presence of 10 μg of RI7 208 per ml produced colonies with a frequency ($f = 0.46$) indistinguishable from that of the parental line in the absence of antibody ($f = 0.48$).

Iron uptake. Uptake of ^{59}Fe from transferrin was measured in parental and resistant cell lines in the presence and absence of RI7 208 antibody. Figures 2A and B show uptake by AKR1 and EL4 lines, respectively. The RI7 208 antibody reduced iron uptake in the parental lines by greater than 90%. There was a partial effect on the mutant lines, but substantial iron uptake still occurred. The relative resistance of mutant cells to inhibition of iron uptake by the antibody suggested that resistance was in fact due to some alteration in their iron uptake mechanism rather than in other aspects of iron metabolism such as storage or utilization. However, there is clearly still some effect of antibody on iron uptake in the resistant cells.

Hybrid cells. The genetic basis of resistance was investigated by making hybrids between independent mutant cell lines and between mutant and parental cell lines. Different Thy-1 allotypes in the fusion partners enabled us to test the cells that grew in HAT medium for the presence of both Thy-1 allotypes. After cloning and verifying that the clones were indeed hybrids by Thy-1 typing and mithramycin staining for DNA content per cell, several clones of each fusion combination were tested for growth in the presence of RI7 208. Histograms of cells recovered after 3 days of growth in antibody for one of the fusion combinations are shown in Fig. 3.

In two sets of fusions, EL4 × BW5147 (not shown) and AKR1 × S49 (Fig. 3) (each done both ways), the fusions between a sensitive parental line and an antibody-resistant line produced hybrids that were intermediate in their sensitivity to inhibition by RI7 208 antibody. That is, resistance appeared to be codominant. Many more fusions were done between two different mutant lines, and all showed resistance to antibody comparable to that of the mutant lines themselves. There was no indication of complementation between mutants. These results indicated that the basis of the resistance was indeed genetic and that the alteration may be in a structural gene involved in iron uptake, perhaps in the transferrin receptor structural gene itself.

Antibody and transferrin receptor binding studies. Transferrin receptor expression in parental and mutant cell lines was quantitated by determining saturation binding curves of ^{125}I-labeled human transferrin, ^{125}I-labeled RI7 208, and ^{125}I-labeled RI7 217. These results are summarized in Table 1. The binding studies show that the RI7 208-resistant mutant cell lines still bind transferrin and anti-transferrin receptor antibodies at values very similar to those of their parental lines. This indicates that mutant cells continue to express apparently normal levels of transferrin receptor and continue to bind the antibody used for selection. There is no clear difference between parental and mutant cells in either the number of molecules of antibody or transferrin bound or the apparent binding constants, with the possible exception of the AKR1 lines. AKR1 parental and mutant lines show a

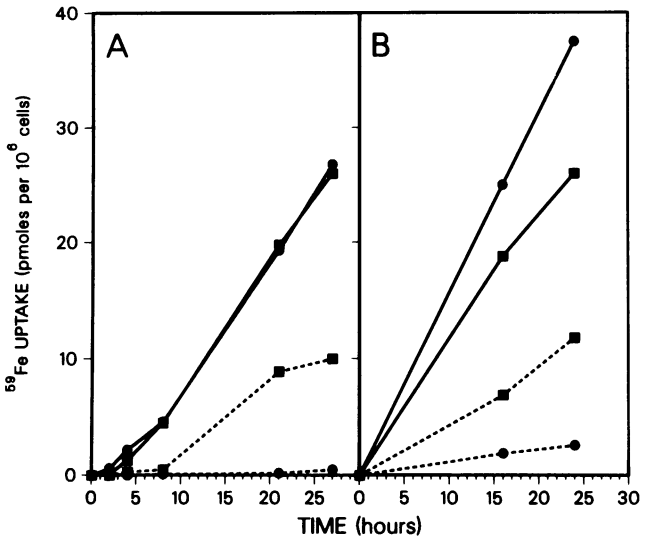

FIG. 2. Iron uptake of parental and mutant cell lines in the presence or absence of RI7 208 antibody. Cells (10^6), washed three times in serum-free medium, were plated in 35-mm petri dishes containing 1.0 ml of Dulbecco modified Eagle medium without serum and 5 μg of ^{59}Fe-labeled human transferrin per ml (37). RI7 208 antibody was added to half of the cultures. At the indicated times, duplicate dishes were harvested into microfuge tubes, and cells were spun out and washed three times in phosphate-buffered saline containing 0.05% bovine serum albumin and 2 mM sodium azide. Cells were then transferred to new tubes for radioactivity counting in a gamma counter. (A) AKR1 parent (●) and AKR1/RI7 208.19 (■) cultured in the presence (– – – –) or absence (——) of 30 μg of RI7 208 antibody per ml. (B) EL4 parent (●) and EL4/RI7 208.12 (■) cultured in the presence (– – – –) or absence (——) of 20 μg of RI7 208 antibody per ml.

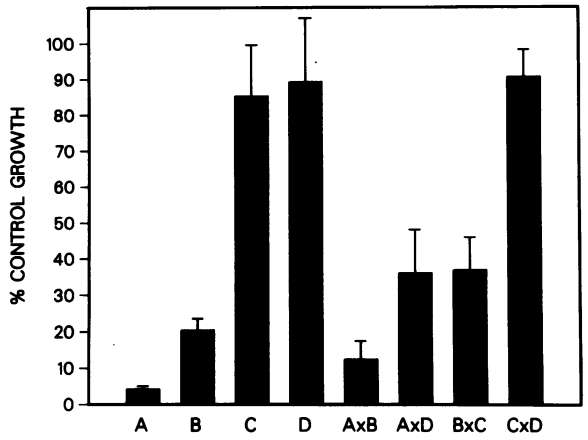

FIG. 3. Growth of parental, selected, and hybrid cell lines in the presence of RI7 208 antibody. Each cell line was cultured in the presence or absence of 10 μg of RI7 208 antibody per ml. At days 2, 3, and 4, cells were mixed, a sample was removed for dilution and its cell number was counted on a Coulter counter. The figure shows day 3 growth in the presence of RI7 208 antibody as a percentage of growth in the absence of antibody: % Control growth = (cells per milliliter in presence of 10 μg of RI7 208 per ml, day 3/cells per milliliter in absence of 10 μg of RI7 208 per ml, day 3) × 100. Cell lines are: A, AKR1 parent; B, S49 parent; C, AKR1/RI7 208. 19; D, S49/RI7 208. 2; and hybrids between these lines as indicated. Each bar represents the mean and standard deviation of the following numbers of samples: A, 2; B, 3; C, 3; D, 3; A×D, 3; A×B, 4; B×C, 4; C×D, 15.

discrepancy in the relative amount of transferrin receptor detected by RI7 217 or transferrin binding compared with the amount of transferrin receptor detected by RI7 208 binding. Whereas mutant AKR1 cells reproducibly bound up to twice the levels of RI7 217 and transferrin bound by the parent, RI7 208 binding was slightly higher with parental cells. This discrepancy suggested that up to one-half of the transferrin receptor of the mutant AKR1 line may not be binding RI7 208 antibody. The lower RI7 208 binding to BW5147 and S49 mutant lines relative to other measures of transferrin receptor (Table 1) also suggests this possibility. The saturation binding studies reveal that, if there are any differences between parental and mutant cells in antibody binding, such differences require more sensitive techniques for their demonstration. Antibody competition and clearing experiments, described below, were performed to verify the presence of an altered transferrin receptor molecule in the mutant cells.

Blocking of ^{125}I-labeled antibody binding. The possibility that some of the transferrin receptor molecules of the mutant cells do not bind RI7 208, although still binding RI7 217 and transferrin, was studied by measuring the ability of unlabeled RI7 208 antibody to block binding of ^{125}I-labeled RI7 217 antibody to parental and mutant cells (Fig. 4). In reciprocal competition studies (blocking the binding of an iodinated antibody with an unlabeled antibody), we had found that RI7 217 blocks RI7 208 binding (Fig. 4A and B) and vice versa (Fig. 4C), indicating that these antibodies bind at identical or spatially nearby sites of the transferrin receptor molecule. However, although unlabeled RI7 208 antibody (85% inhibition) was almost as effective as RI7 217 antibody (91% inhibition) at blocking ^{125}I-labeled RI7 217 binding to parental AKR1 lymphoma cells (Fig. 4C), it only partially blocked the binding of ^{125}I-labeled RI7 217 to mutant cells (Fig. 4D). In this experiment, inhibition by RI7 208 plateaued at 32% below control, unblocked binding of ^{125}I-labeled RI7 217. In four blocking experiments with AKR1 parental and mutant cells, homologous blocking of ^{125}I-labeled RI7 217 by unlabeled RI7 217 resulted in 93.4 ± 2.5% (mean ± standard deviation) inhibition. Reciprocal blocking of ^{125}I-labeled RI7 217 binding by RI7 208 resulted in 88.7 ± 3.5% inhibition with the parental cell line and 43.8 ± 9.9% inhibition with the mutant cells. This pattern was found on the parental and mutant sets of the other three lymphoma lines as well, with RI7 208 inhibition of ^{125}I-labeled RI7 217 binding to mutant cells plateauing at 60, 58, and 53% for BW5147, S49, and EL4 mutants, respectively. RI7 208 inhibition of ^{125}I-labeled RI7 217 binding to parental cells was 85, 85, and 86%, respectively. Variation of the control, unblocked binding of ^{125}I-labeled RI7 208 and ^{125}I-labeled RI7 217 was less than ±10% for triplicate determinations in all the experiments. As a negative control, ^{125}I-labeled RI7 217 and ^{125}I-labeled RI7 208 binding varied ±8.4% from that of the unblocked control in the presence of dilutions of human transferrin from 25 to 0.4 μg/ml or of horse serum from 25 to 0.4%. (Transferrin does not compete with RI7 208 binding to the receptor [Lesley, unpublished data].)

Immunoprecipitation and clearing by antibodies. To show the presence of two types of transferrin receptor molecule with different antibody binding properties on mutant cells, cell surface transferrin receptor was precipitated from surface-iodinated membrane preparations with RI7 208 antibody and RI7 217 antibody. The cleared membranes were then precipitated again with both anti-transferrin receptor antibodies and with anti-T200 (34) as a control. Polyacrylamide gel electrophoresis of the second immunoprecipitates is shown in Fig. 5. Parental AKR1 cell membranes show no transferrin receptor molecules precipitable by RI7 217 or RI7 208 after clearing with RI7 208 (Fig. 5, lanes G and H). Mutant AKR1 cells, on the other hand, show a residual band of RI7 217-precipitable molecules after clearing with RI7 208 (Fig. 5, lane T).

TABLE 1. Binding to transferrin receptor[a]

Cell line	Molecules per cell		
	RI7 208	RI7 217	Human transferrin
AKR1 parent	9.8×10^{3}[b]	2.0×10^{4}[b]	4.1×10^{4}[b]
AKR1/RI7 208	8.8×10^{3}[b]	3.9×10^{4}[b]	6.4×10^{4}[b]
EL4 parent	2.3×10^{4}[b]	1.3×10^{5}[c]	3.9×10^{4}[b]
EL4/RI7 208.2	2.1×10^{4}[b]	1.2×10^{5}[c]	3.6×10^{4}[b]
EL4/RI7 208.12	2.6×10^{4}[b]	1.5×10^{5}[c]	4.0×10^{4}[b]
BW5147 parent	8.6×10^{3}[b]	2.9×10^{4}[c]	1.9×10^{4}[b]
BW5147/RI7 208	6.2×10^{3}[b]	2.7×10^{4}[c]	1.8×10^{4}[b]
S49 parent	7.6×10^{3}[c]	ND[d]	1.6×10^{4}[c]
S49/RI7 208	5.2×10^{3}[c]	ND	1.4×10^{4}[c]

[a] ^{125}I-labeled RI7 208, RI7 217, and human transferrin were used to determine saturation binding curves for each of the cell lines. Scatchard analysis (23) of the data for six points on each curve was used to calculate the molecules bound per cell and the apparent dissociation constant. The K_d values for RI7 208 binding ranged from 1.2×10^{-10} to 1.2×10^{-9}, for RI7 217 binding from 1.7×10^{-10} to 2.5×10^{-9}, and for transferrin binding from 4.8×10^{-9} to 4.8×10^{-8}. Variation in K_d values was less than twofold between parental and mutant lines in the same experiment.

[b] Values given are averages of two experiments. Although variation between experiments was up to threefold, variation between parental and mutant cell lines within an experiment was always less than twofold with the exception of RI7 217 and transferrin binding to AKR1 cells. See text.

[c] Value determined in one experiment only.

[d] ND, No data.

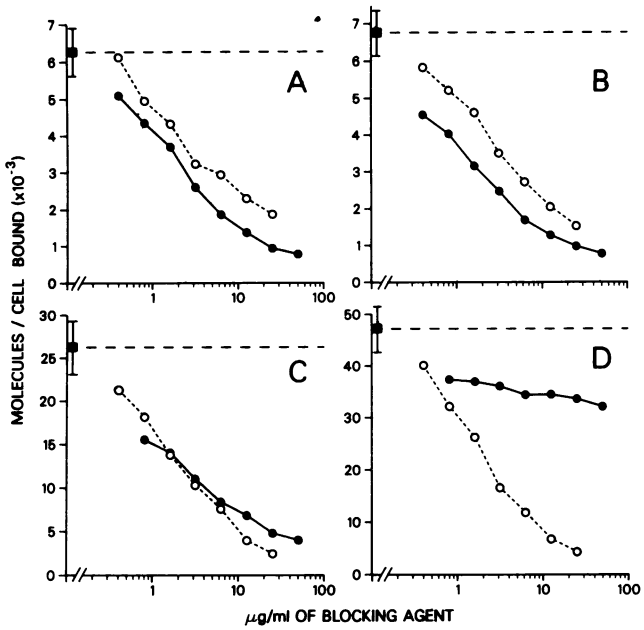

FIG. 4. Reciprocal anti-transferrin receptor antibody blocking of binding to AKR1 parent and AKR1/RI7 208 mutant cells. Near-saturating concentrations of ^{125}I-labeled RI7 208 (1.3 μg/ml) (A and B) and ^{125}I-labeled RI7 217 (0.5 μg/ml) (C and D) were incubated with 10^6 AKR1 parent or 10^6 AKR1/RI7 208.19 mutant cells in the presence of various concentrations of unlabeled RI7 208 (●) or RI7 217 (○) antibody or with diluent only (■- - - - -). In (A), AKR1 parental cells were labeled with ^{125}I-labeled RI7 208; in (B), AKR1/RI7 208 mutant cells were labeled with ^{125}I-labeled RI7 217; in (C), AKR1 parent cells were labeled with ^{125}I-labeled RI7 217; and in (D), AKR1/RI7 208 mutant cells were labeled with ^{125}I-labeled RI7 217. Standard deviations of triplicate determinations were ±10% or less.

Preclearing with RI7 217 removed all the material precipitable by both anti-transferrin receptor antibodies from both parental and mutant cell lines (Fig. 5, lanes K, L, V, and W). Preclearing did not affect precipitation of T200 (Fig. 5, lanes I, M, U, and X). Scanning of the intensities of the two bands (equivalent to lanes P and T in Fig. 5) at several different exposures indicated that the amount of RI7 217-precipitable transferrin receptor remaining in RI7 208-cleared membrane preparations was about one-fourth of the total RI7 217-precipitable material in control (mock-cleared) mutant membrane preparations (0.23 ± 0.04 relative to an intensity of 1.0 for the mock-cleared band). Clearing of ^{125}I-labeled membrane preparations from hybrids between parental and mutant cell lines, followed by immunoprecipitation, also showed a residual band which was precipitable by RI7 217 antibody after clearing with RI7 208 antibody.

DISCUSSION

We have presented evidence that murine lymphoma cells selected for their ability to grow in the presence of an anti-transferrin receptor antibody that inhibits cell growth have a mutation in one of their transferrin receptor genes. The properties of these selected lines satisfy the criteria for genetic alteration (26). (i) The resistance is stable in the absence of selection for over a year of continuous culture. (ii) The frequency of resistant cells is increased by mutagenesis (by about eightfold in the case of AKR1 [Lesley, unpublished data]). (iii) There is a change in the transferrin receptor protein, i.e., its antibody binding properties are altered. (iv) The resistance is inherited in somatic hybrids in a codominant fashion.

The suggestion that the alteration in the mutant is in the transferrin receptor structural gene itself is supported by the following evidence. (i) Resistant lines show a change in iron uptake in the presence of selecting antibody, iron uptake

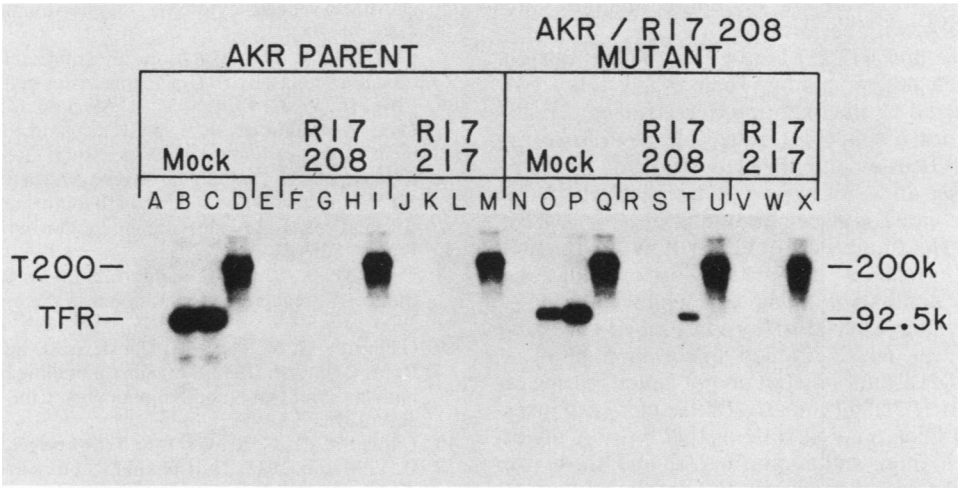

FIG. 5. Polyacrylamide gel electrophoresis of AKR1 parental and mutant transferrin receptors after mock, RI7 208; and RI7 217 preclearing. Cells were surface iodinated with ^{125}I with lactoperoxidase. The solubilized cell preparations were each divided into three parts for preclearing with RI7 208, RI7 217, or diluent (mock). After material precipitated in the clearing step was removed, the remaining samples were again precipitated with anti-transferrin receptor (anti-TFR) antibodies (RI7 208 and RI7 217), with control antibody I3/2 (anti-T200), or no antibody, and the precipitates were electrophoresed on 10% acrylamide-sodium dodecyl sulfate gels. Lane E contains the molecular weight standards, unlabeled. Lanes A through D and F through M are parental cells. Lanes N through X are AKR1/RI7 208.19 mutant cells. Lanes A through D and N through Q were mock cleared. Lanes F through I and R through U were precleared with RI7 208 antibody. Lanes J through M and V through X were precleared with RI7 217 antibody. Final immunoprecipitates were with medium alone (negative control) (lanes A, F, J, N, R); culture supernatant from RI7 208 hybridoma (lanes B, G, K, O, S, V); culture supernatant from RI7 217 hybridoma (lanes C, H, L, P, T, W); and culture supernatant from I3/2 (anti-T200) hybridoma (lanes D, I, M, Q, U, X). k, $\times 10^3$.

being the function of transferrin receptor. (ii) Resistant lines show a change in the antibody binding properties of their transferrin receptor glycoprotein. (iii) Resistance is codominantly expressed in somatic hybrids, arguing against *trans*-acting or post-translational regulatory or processing changes (such as changes in glycosylation).

Antibody blocking studies indicate that resistant cell lines have lost RI7 208 antibody binding in about one-half of their surface transferrin receptor molecules, while retaining it in another portion of their transferrin receptor molecules. This is similar to the α-amanitin-resistant mutants of rat myoblasts (27) and human fibroblasts (3), in which both mutant and wild-type RNA polymerase II enzymes are expressed. This observation can be explained by postulating that there has been a mutation in one of the two transferrin receptor structural gene copies and that the mutation is expressed codominantly with wild-type transferrin receptor in the antibody-resistant mutant cell lines. The proposal of a single mutation step, affecting only one transferrin receptor gene copy, is also consistent with the frequency of antibody-resistant cells observed in clonal assays of nonmutagenized cells. This frequency is on the order of 10^{-5} in AKR1 parental cells, rather than 10^{-10} to 10^{-8}, as would be expected for a recessive alteration requiring two mutation steps in a diploid cell (2, 14).

Transferrin receptor is a dimer on the cell surface consisting of two identical subunits linked by a disulfide bond (29, 33). Resistant cells could have mixed dimers, as well as pure mutant and pure wild-type receptors, only if the mutant molecules were able to dimerize with the wild type. The existence of mixed dimers is suggested by the ratio of material precipitated by RI7 217 in mock-cleared and RI7 208-cleared samples. After clearing with RI7 208, only about one-fourth of the transferrin receptor remained precipitable by RI7 217 when compared with RI7 217 precipitation of mock-cleared preparations. This would indicate that only one-fourth of the transferrin receptor is in the form of pure dimers, rather than one-half as would be expected if mixed dimers did not form. Nevertheless, more direct methods are required to show this with certainty.

Although RI7 208 and RI7 217 bind close enough to each other to block each others' binding reciprocally, only RI7 208 binding is affected by the mutation to resistance. Transferrin binding is not blocked by either of the transferrin receptor antibodies (Lesley, unpublished data), and transferrin receptor binding does not appear to be affected by the mutation. Similar antibody-specific mutations in *H-2*K^k were selected by Holtkamp et al. (12) by flow cytometry, using a protocol essentially designed to select mutations against a specific antibody binding site while retaining a nearby antibody binding site. Mutations described by Chapdelaine et al. (4) in *H-2*D^b, which affect binding of a monoclonal anti-*H-2*D^b antibody but do not affect binding of other antibodies to *H-2*D^b or anti-*H-2*D^b cellular responses to the mutant cell lines, may also be of this type. A major difference between these mutations in *H-2* and those we describe here is that selection against *H-2* determinants was carried out in heterozygous cells against only one of the two *H-2* allotypes, whereas we selected in cells that are homozygous for the gene being selected against. Other systems in which cell lines were rendered heterozygous at a particular locus by selection for partial resistance to a drug have been described by Chasin (5) and Jones and Sargent (14) for the adenine phosphoribosyl transferase (APRT) locus of Chinese hamster cells, by Clive et al. (6) for the thymidine kinase (TK) locus of L5178Y mouse lymphoma cells, and by Bourgois and Newby (2) for the glucocorticoid receptor of WEHI 7 murine thymona. In these systems, the mutation appears to result in the loss of expression of one copy of the gene, though an undetected nonfunctional gene product may be present.

We cannot, as yet, show any structural change in the transferrin receptor glycoprotein of the mutants other than the failure to bind RI7 208 antibody. One-dimensional sodium dodecyl sulfate-acrylamide gels of the intact receptor and of trypsin and Staph A protease digests (28) revealed no differences in the migration of peptides. Mutant cells invariably grew more slowly than the parental lines. This could be the result of a defect in transferrin receptor function due to the mutation affecting RI7 208 antibody binding, but it may also be a consequence of mutagenesis unrelated to transferrin receptor. The mechanism of inhibition of growth by RI7 208 antibody is not known, but it does not interfere with transferrin binding. It may disrupt internalization or recycling of the receptor, and we are currently studying this question.

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