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Progress has been made toward the first four of the five tasks listed in the statement of work. The most interesting results have come in pursuit in tasks one and two. We have discovered (and published) the fact that the breast cancer cell lines SK-BR-3 and MDA-MB-231 vary with respect to their sensitivity to iron deprivation treatment. Sensitivity to monoclonal anti-transferrin receptor antibodies appears to be correlated with the relative degree of receptor down-modulation seen. In addition, the degree to which the same monoclonal antibodies shift the deferoxamine dose-response curve to the left appears to be correlated with tumor growth rate. These findings indicate that breast tumors do respond to iron deprivation treatment in vitro and thus strengthen the rationale for proceeding on with the in vivo experiments as planned.

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John D Kemp 1.17.96

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Introduction

The problem being addressed is the need for improved therapy of metastatic breast cancer. By virtue of our prior studies which indicated that iron deprivation treatment was useful against hematopoietic lesions in vitro and in vivo, we began to perform testing in vitro with iron deprivation treatments and breast cancer cell lines.

Narrative

In relation to the pursuit of Specific Aim #1, we have quantified the sensitivity of two breast cancer cell lines (SKBR3 and MDA-MB-231) to treatment with deferoxamine (DFO), a pair of IgG monoclonal antibodies against the transferrin receptor (A27 and E2.3), and the combination thereof. We published this data in Pathobiology (63:65-70, 1995). There was some variability in response but both cell lines showed significant left-shifts in the DFO dose response curve. This finding supports the view that this reagent combination may show useful activity against some breast cancer cell lines. The level of DFO/Mab effect with breast cancer cell lines appears to be less impressive than that observed with lymphoma cell lines, however, and we are therefore now considering whether to expand the investigations outline in Specific Aim #5 to cover the interaction of iron deprivation with other established therapeutic agents such as Adriamycin and Taxol (in addition to Tamoxifen). We initially had some difficulty in getting the MCF-7 cell line to grow but have now overcome that difficulty. MCF-7 will therefore be the third cell line to be tested for sensitivity to DFO, the Mab pair, and the combination thereof, as planned for Specific Aim #1.

Thus, under Task 1, items A and B are nearly complete and item C has become essentially irrelevant.

In relation to Specific Aim #2, we have established for SKBR3 and MDA-MB231 that the IgG Mab pair does cause receptor down-modulation (Pathobiology paper). Moreover, it appears that the cells with higher initial density (SKBR3) undergo a greater degree of receptor down-modulation and exhibit a greater degree of growth inhibition as a result of antibody exposure. The results are consistent with model that we and others developed for Mab effects based on prior studies with lymphomas. Although we initially had some trouble getting MCF-7 to grow in vitro the difficulty has now been overcome and we expect to be able to further extend the studies with that cell line.

Thus, for Task 2, item B has been partially completed. We expect to be able to continue studies with item B over the next several months and to conduct the tests listed for item A. We hope to complete all of the work for Task 2 by the end of year two.

In relation to Specific Aim #3, we have evaluated the growth characteristics of MDA-MB-231 and SKBR3 in nude mice. Although we did not initially expect to encounter any problems in this regard, the initial intradermal inoculation experiments showed either extremely slow growth or no growth at all for both tumors. After reviewing more literature, we ascertained that co-injection with the basement membrane materials found in Matrigel (collaborative Medical Products, Bedford, MA) could enhance breast tumor growth in vivo. We then found that MDA-MB-231 will develop measurable tumors within two weeks at any injection site when $1X10^6$ cells are co-injected with 2.5 mg of Matrigel. Although SKBR3 will also grow in the presence of Matrigel, it definitely grows more slowly. This is consistent with our in vitro studies of the growth rates of the two tumors (Pathobiology

paper). We will therefore begin our in vivo therapeutic experiments with MDA-MB-231. We are currently expanding our stocks of the A27 and E2.3 monoclonal antibodies in preparation for these studies.

Thus, for Task 3, item A is complete, item B is nearly complete, and item C will be underway shortly.

In relation to Specific Aim #4, we are analyzing the toxicity of rat anti-mouse IgG Mabs against the transferrin receptor, HES-DFO, and the combination thereof. Insofar as the Mabs are concerned, we have now completed data on hematologic toxicity on day 10 in four experiments in which 3 doses of 3 milligrams of either a single Mab, or the Mab pair, are given on day, 0, 3, and 6. The basic findings are that red cell counts and hematocrits drop by about 30%, platelet counts are stable or rise slightly, and white counts are generally stable. An independent series of three experiments with a single injection of a single Mab have shown that reticulocyte counts drop by 80-90% within 24 hours after antibody injection and that there is a corresponding rise in serum iron levels and reduction in iron binding capacity. These results indicate that the major toxicity of Mab administration is inhibition of erythropoiesis and are in accord with expectations. A series of three experiments with HES-DFO alone indicate that it has little effect on red cell indices but can reduce white cell counts and platelet counts by 20-30%. With these data in hand, we are now in a position to analyze the hematologic effects of combined treatment and that is our next goal.

Thus, insofar as Task 4 is concerned, item A is accomplished and we are well into progress on item B.

In relation to Specific Aim #5, we undertook studies with the breast cancer line MCF-7. Although we initially had difficulty in obtaining stable growth in vitro, this difficulty has been overcome. We will therefore soon establish a basic dose/response curve for growth inhibition by Tamoxifen. We will then be prepared to undertake combined treatment with Tamoxifen, deferoxamine, and IgG Mabs and expect no particular difficulties in acquiring that data. Thus, insofar as Task 5 is concerned, we are ahead of schedule.

Conclusion

The principal conclusion is that the results obtained in the first year of work support the contention that iron deprivation will be of use in breast cancer treatment and thereby indicate that the work should continue as originally planned.

References

(See reprint in Appendix.)

Appendix

(See attached reprint.)

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Differing Sensitivity of Non-Hematopoietic Human Tumors to Synergistic Anti-Transferrin Receptor Monoclonal Antibodies and Deferoxamine in vitro

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Abstract
We tested non-hematopoietic human tumors for in vitro sensitivity to either a pair of synergistic IgG antitransferrin (Tf) receptor monoclonal antibodies (MAbs), deferoxamine (DFO) or the combination thereof. With an equimolar mixture of the two MAbs (A27.15, E2.3), two prostate tumors showed similar degrees of maximal growth inhibition (PC-3: 35%, DU 145: 38%), two breast tumors showed more variability (MDA-MB-231: 26%, SK-BR-3: 52%) and two neuroblastomas showed the most variability (SK-N-SH: 4%, SK-N-MC: 76%). When the MAbs were applied together with DFO, the D_{50} for DFO was reduced for all tumors (PC-3: 2.5×, DU 145: 3.7×; MDA-MB-231: 2.9×, SK-BR-3: 1.9×, and SK-N-SH: 2.6×, SK-N-MC: 7.0×). Sensitivity to MAbs was more closely correlated with the relative decrease in Tf receptor density resulting from antibody exposure than with initial receptor density. The degree of reduction of D_{50} for DFO resulting from the joint application with the MAbs was, however, most closely related to the growth rate of the tumors. Since some non-hematopoietic tumors exhibit sensitivity to the effects of a synergistic pair of IgG anti-Tf receptor MAbs and DFO, it appears that further preclinical studies with such tumors, especially those with higher Tf densities, would be of interest.

Indroduction

There is increasing interest in the therapeutic potential of iron deprivation as a tool in the treatment of cancer [1–14]. Several types of tumors are known to be sensitive to iron deprivation achieved by one or more methods. Thus, transitional cell carcinomas have been shown to be sensitive to gallium [12, 13] and neuroblastomas to deferoxamine (DFO) [2, 3] in clinical trials. Lymphomas have demonstrated some sensitivity to anti-transferrin (Tf) receptor monoclonal antibodies (MAbs) in experimental settings, with or without added DFO [4, 6–11]. Moreover, it is of particular interest to note recent studies showing that certain pairs of IgG anti-Tf receptor MAbs produce synergistic inhibition of hematopoietic tumors, as does the combination of DFO and an IgG anti-Tf receptor MAb [7, 8, 10].

We do not yet understand the range of tumors that might be susceptible to iron deprivation. The more common types of solid tumors have not been systematically

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investigated. The data that do exist suggest that such tumors are likely to be resistant to the effects of a single anti-Tf receptor MAb [14]. Therefore, we elected to undertake a study of the effects of a pair of synergistic IgG anti-Tf receptor MAbs, with or without DFO, on breast carcinoma, prostate carcinoma and neuroblastoma cell lines in vitro.

Materials and Methods

Materials. The cell lines producing murine MAbs A27.15 (IgG1) and E2.3 (IgG1) against the human Tf receptor [10] were kindly provided by Drs. S. White and I. Trowbridge (Salk Institute, La Jolla, Calif., USA). The antibodies were purified from Balb/c ascites fluid by protein G affinity chromatography. Deferoxamine (Desferal mesylate) was a gift from Ciba-Geigy (Summit, N.J., USA).

Cells. Human prostate carcinomas PC-3 and DU 145, human breast carcinomas SK-BR-3 and MDA-MB-231, and human neuroblastomas SK-N-SH and SK-N-MC were obtained from the American Type Culture Collection (Rockville, Md., USA).

Culture Conditions and Cell Growth Experiments. Medium RPMI 1640 containing extra *L*-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (15 m*M*) and supplemented with 10% of fetal bovine serum (HyClone Laboratories, Logan, Utah, USA) was used for cell maintenance and experiments. Cells were incubated in the medium at 37 °C in a humidified atmosphere of 5% CO_2. For growth experiments, cells were harvested employing trypsin (0.25%) + EDTA (0.1%) and then seeded into wells of 96-well plastic plates at 10^4 cells/100 μl of medium. Antibodies and/or DFO were added at various cencentrations as noted for particular experiments. A27.15 and E2.3 were always added in an equimolar mixture. This was done after preliminary experiments showed, as expected from the work of White et al. [10], that either MAb alone had little or no inhibitory effect and that an equimolar mixture yielded the greatest inhibitory effect. After 5 days in culture the number of living cells was estimated.

Cell Number Estimation. The number of living cells was determined by the photometric method of Mosmann [15] using 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide. The absorbance at 570 nm is directly proportional to the number of living cells [15–17].

Cell Surface Area Estimation. The mean surface area for a given cell line was calculated from the mean cell diameter. The latter was obtained, for cells in suspension, from the mean of 50 individual cell measurements per cell line with an ocular micrometer in a calibrated microscope.

Flow Cytometric Analysis of Tf Receptor Expression Levels. Cell harvesting was accomplished with EDTA (0.2%). Indirect immunofluorescence was employed to assess the expression of cell-surface Tf receptors. The anti-Tf receptor MAbs A27.15 and E2.3 were either used individually at a concentration of 20 μg/ml or as an equimolar mixture (with each at 10 μg/ml) as the primary staining agent. Fluorescein-conjugated goat $F(ab')_2$ antibody specific for mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) was used as a secondary staining reagent at 25 μg/ml. Prior studies have shown that, while a large portion of the surface receptors have antibodies bound if anti-Tf receptor MAbs are present in the culture (and are therefore detectable with the addition of a fluorescein-conjugated secondary only), an additional signal gain can be achieved by restaining with the same MAbs [7]. After the staining incubations, the cells were washed by centrifugation through fetal bovine serum. Stained cells were analyzed on a B-D FACSCAN (Mountain View, Calif., USA).

Tf Receptor Density Estimation. An index of receptor density was obtained by dividing mean fluorescence intensity by mean cell surface area and is expressed as arbitrary fluorescence units per square micrometer. In all cases, the value for mean fluorescence intensity was that obtained by staining with an equimolar mixture of A27.15 and E2.3.

Results

Growth Inhibition by a Pair of Synergistic Anti-Tf Receptor MAbs

Representative dose-response curves for an equimolar mixture of IgG anti-Tf receptor MAbs (A27.15 and E2.3) are shown in figure 1. Half-maximal inhibition occurred in all cases at about 1 μg/ml and near-maximal inhibition occurred at about 5 μg/ml. The degree of maximal growth inhibition ranged from 4 to 76%, as shown in the second column of table 1. The two prostate carcinomas (DU 145, PC-3) exhibited similar, intermediate degrees of growth inhibition (38 and 35%, respectively). The two breast carcinomas (SK-BR-3, MDA-MB-231) exhibited more variation (52 and 26%, respectively) and the two neuroblastomas (SK-N-MC, SK-N-SH) exhibited the most variation (76 and 4%, respectively). When these data are compared to the growth rate data shown in the first column of table 1, it can be seen that while the neuroblastoma cell line with the higher growth rate was more sensitive to the MAbs, the reverse was the case for the breast carcinoma cell lines.

Sensitivity to Anti-Tf Receptor MAbs and Tf Receptor Downregulation

The differences in sensitivity to MAb-mediated inhibition raised two questions. The first immediate question was if each of the cell lines actually reacted with each of the MAbs? It is clearly possible for tumors to exhibit some variation in the Tf receptor epitopic display [18] and lack of binding by either of the reagents would clearly lead one to expect little, if any, inhibition. However, each of the cell lines reacted with each MAb. Moreover, for each cell line, the relative intensity of the fluorescence signal generated by each MAb was similar (data not shown).

It was therefore of interest to ascertain whether the differences in sensitivity might be related to the level of Tf receptor expression. This question arose as a result of a hypothesis put forth by Lesley and Schulte [5] in an effort

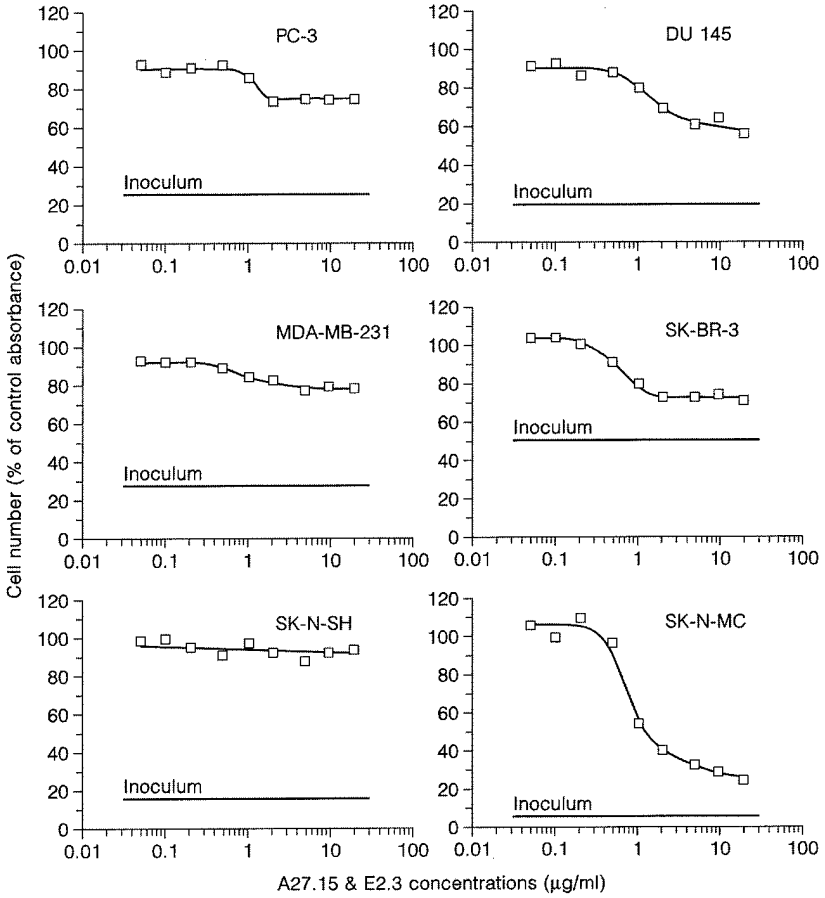

Fig. 1. Effect of equimolar concentrations of MAbs A27.15 and E2.3 against the human Tf receptor on the growth of PC-3, DU 145, MDA-MB-231, SK-BR-3, SK-N-SH and SK-N-MC cells. Each point represents the mean of eight separate cultures after 5 days in culture beginning with an inoculum of 10^4 cells/100 μl of medium in the well. The SEM was always less than 5%. The number of cells was estimated by the photometric method of Mosmann [15] and is expressed as a percentage value of the absorbance at 570 nm of control cells grown without any treatment. The relative value of the absorbance of the initial inoculum of cells is denoted by a bar. Equivalent protein concentrations of normal mouse IgG had no significant inhibitory effect.

Table 1. Effects of MAbs A27.15 + E2.3 on the growth and on DFO growth inhibition of non-hematopoietic human tumors

Cell line	Multiplication during incubation period[a]	Maximun effect of A27.15 + E2.3[b], % inhibition	D_{50} of DFO[c], μ*M*		Decrease in DFO D_{50}[e]
			without antibodies	with A27.15 + E2.3[d]	
PC-3	4.1 ×	35	3.2	1.3	2.5 ×
DU 145	5.1 ×	38	3.3	0.9	3.7 ×
SK-BR-3	2.3 ×	52	2.1	1.1	1.9 ×
MDA-MB-231	4.3 ×	26	3.2	1.1	2.9 ×
SK-N-SH	5.2 ×	4	0.8	0.3	2.6 ×
SK-N-MC	16.2 ×	76	2.8	0.4	7.0 ×

[a] Relative increase in the number of untreated control cells (10^4 cells/100 μl of medium) after 5 days of culture.
[b] Maximum growth inhibitory effect achieved by 5 μg of A27.15 + 5 μg of E2.3/ml after 5 days of culture, relative to untreated control. Two to four similar dose-response experiments were performed for each cell line.
[c] Dose of DFO producing 50% growth inhibition after 5 days of culture.
[d] 5 μg of A27.15 + 5 μg of E2.3/ml.
[e] Relative decrease in DFO D_{50} resulting from simultaneous treatment with DFO and A27.15 + E2.3.

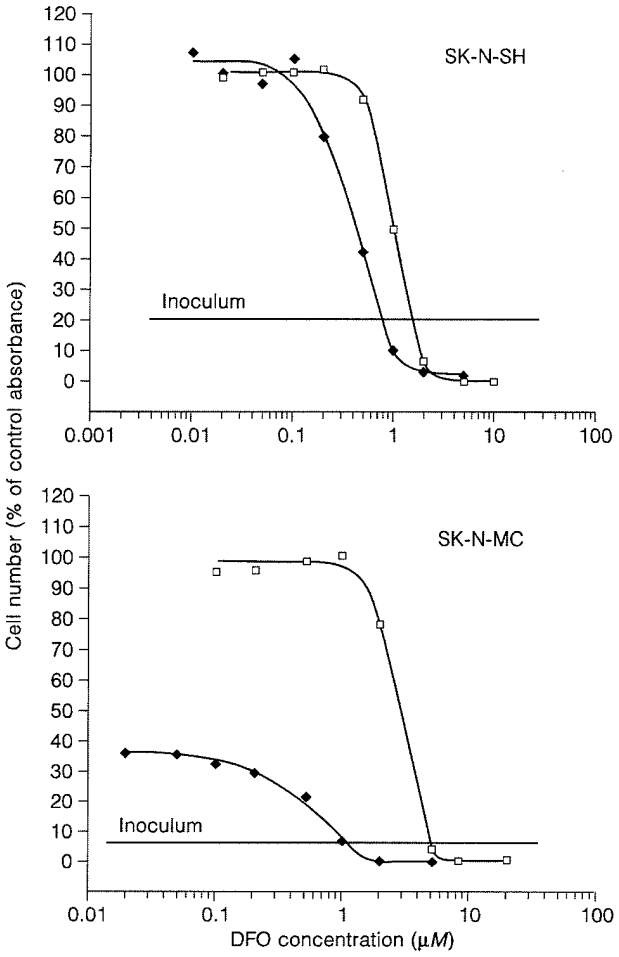

Fig. 2. Effect of DFO, without the presence (squares) and with the presence (closed diamonds) of equimolar concentrations (5 μg/ml) of MAbs A27.15 and E2.3 against the human Tf receptor, on the growth of SK-N-SH cells and SK-N-MC cells. For further details, see the legend to figure 1.

Table 2. Effect of MAbs A27.15 + E2.3 on expression of cell-surface Tf receptor by non-hematopoietic human tumors

Cell line	Tf receptor density[a]		Decrease in Tf receptor density[c]
	incubated without antibodies	incubated with A27.15 + E2.3[b]	
PC-3	0.91	0.22	4.1×
DU 145	0.42	0.12	3.5×
SK-BR-3	3.61	0.59	6.1×
MDA-MB-231	0.93	0.57	1.6×
SK-N-SH	0.75	0.51	1.5×
SK-N-MC	2.20	0.36	6.1×

[a] Cell-surface Tf receptor density after a 48-hour incubation in the presence or absence of the antibodies. The values are arbitrary units of mean fluorescence intensity per unit (μm^2) of cell area. Mean fluorescence intensity and cell surface area were determined as described.

[b] 5 μg of A27.15 + 5 μg of E2.3/ml.

[c] Relative decrease in Tf receptor density after the incubation with A27.15 + E2.3 when compared with the incubation without the antibodies.

to account for the characteristics of growth inhibition mediated by anti-Tf receptor MAbs. That hypothesis states that anti-Tf receptor MAbs must cross-link receptors in order to cause downmodulation and degradation, and that MAb effects will be a function of antibody valence and receptor density. Since antibody valence was a constant factor in our experiments, we compared the Tf receptor densities before and after exposure to the MAbs. Data from this experiment are shown in table 2.

Taking the neuroblastoma pair first, it can be seen that the more sensitive neuroblastoma cells SK-N-MC exhibited a higher receptor density prior to treatment and a greater relative decrease in density after treatment than the more resistant neuroblastoma cells SK-N-SH. The breast cancer pair was similar in that the more sensitive SK-BR-3 cells exhibited a higher density prior to treatment and a greater relative decrease after treatment than the more resistant MDA-MB-231 cells. Although prostate cells PC-3 exhibited a higher density than the prostate cells DU 145 prior to treatment, the tumors were nevertheless nearly equal in terms of growth inhibition. Interestingly, however, the tumors were also nearly equal in terms of the relative decrease in receptor density that resulted from treatment.

Enhancement of the DFO Effect by Anti-Tf Receptor MAbs

A summary of half-maximal inhibitory doses (D_{50}) of the iron chelator DFO is presented in the third column of table 1. The D_{50} for the cell lines fell within the range of 0.8–3.3 μ*M*. Specifically, we asked what degree of left shift might occur in the DFO D_{50} values when the antibody pair was also present at near-maximal inhibitory concentrations. Representative dose-response curves for the two neuroblastomas are shown in figure 2. The absolute D_{50} values obtained with combination treatment are shown in the fourth column of table 1 and the relative changes are shown in the fifth column.

Insofar as the absolute DFO D_{50} values are concerned, it can be seen that combined treatment resulted in reductions in all cases (1.9×–7.0×). Insofar as the relative degrees of change are concerned it can be observed that, for each tumor pair, the tumor with the higher initial growth rate exhibited the greatest decrease in D_{50}. It is interesting to note that, for the pair of breast carcinomas, the relative sensitivity to the MAb effect alone did not predict the relative decrease in D_{50} produced by combined treatment.

Discussion

In order for anti-TF receptor MAbs to be most effectively utilized as antitumor agents, it is clearly important to develop a better understanding of both the range of potential tumor targets and of the physiological basis for anti-Tf receptor MAbs effects. Although prior preclinical investigations with an IgA anti-Tf receptor MAb suggested that solid tumors would be relatively resistant to the effects of anti-Tf receptor MAbs [14], the recent discovery that pairs of IgG anti-Tf receptor MAbs could produce synergistic growth inhibition [10] provided a rationale for undertaking new studies with IgG anti-Tf receptor MAbs and solid tumors.

The data presented show that human solid tumors varied in their sensitivity to the effects of an IgG anti-Tf receptor MAb pair in vitro, with growth inhibition ranging from 4 to 76%. Since hematopoietic tumors often show greater than 90% growth inhibition when treated with the same pair [10], the findings remain consistent with the view that solid tumors are generally more resistant to the treatment with anti-Tf receptor MAbs [14].

The reasons for this apparent difference in sensitivity are still not clear, however. It does not appear likely, for example, that the response of a given tumor to anti-Tf receptor MAbs can be predicted solely on the basis of the histogenetic origin. Thus, only the two prostate cell lines exhibited similar degrees of inhibition. The two breast carcinoma cell lines differed from each other and from the prostate cell lines, with one line showing less inhibition than the prostate lines and one showing more. The two neuroblastoma cell lines presented the most extreme cases, with one exhibiting the lowest inhibition overall and the other exhibiting the highest. It may well be that if enough tumors of a given type are tested, a broad spectrum of the sensitivity to anti-Tf receptor MAbs will be encountered for each type.

The data provide new, but qualified, support for the view that the growth inhibitory effects of IgG anti-Tf receptor MAbs are somehow related to Tf receptor density of a given tumor. The observations with the neuroblastoma and breast carcinoma tumor pairs are consistent with the hypothesis that the MAb effect is related to initial Tf receptor density [5], but the data with the prostate tumor pair suggest a modification. In the latter case, the initial receptor densities were different but the degrees of growth inhibition were similar. Interestingly, however, the values for the relative decreases in receptor density resulting from antibody treatment were fairly close for the two cell lines. It may be that the relative decrease in receptor density resulting from MAb exposure, rather than the initial receptor density per se, will come closer to predicting growth inhibition by anti-Tf receptor MAbs.

When the anti-Tf receptor MAb pair was combined with DFO, there was a left shift in the DFO dose-response curve in all cases. The effect was noted even in the case where the MAb pair seemed to have virtually no growth inhibitory effect by itself (SK-N-SH). These observations are consistent with prior studies of hematopoietic tumors that utilized a single anti-Tf receptor MAb with DFO [7, 8]. Those studies showed that the MAb alone could inhibit a substantial fraction of iron uptake without a significant inhibitory effect on growth. It was proposed that a relatively high threshold of iron uptake inhibition had to be crossed before substantial growth inhibition would occur [8].

Interestingly, the degree of anti-Tf receptor MAb induced left shift in D_{50} appears to be predicted by the initial tumor growth rate for each member of the tumor pairs and not by the inhibitory effect observed when the MAbs are used alone (SK-N-SH, MDA-MB-231). It is not clear why this should be, but this type of observation might make more sense if faster growing cells responded to a given dose of DFO with larger rises in Tf receptor expression level. Alternatively, Tf-independent iron uptake [19–24] might also be involved. If DFO were to preferentially inhibit this process, then the cells might become more dependent on their Tf receptors and thus become more sensitive to anti-Tf receptor MAb effects.

In summary, the studies presented have shown that synergistic IgG anti-Tf receptor MAbs can produce differing degrees of growth inhibition of non-hematopoietic tumors. Moreover, as might have been predicted from earlier work with hematopoietic tumors [7, 8], the MAbs can enhance the effect of DFO even when they appear to be noninhibitory by themselves. These findings raise the possibility that combination treatment with synergistic

anti-Tf receptor MAbs and DFO may have some value in the treatment of solid tumors. In vivo studies may be productive because preliminary work with murine lymphoma has suggested that the effects of a pair of synergistic IgG anti-Tf receptor MAbs against established tumors can be enhanced by high molecular weight DFO [25].

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