Inhibition of Hematopoietic Tumor Growth by Combined Treatment with Deferoxamine and an IgG Monoclonal Antibody against the Transferrin Receptor: Evidence for a Threshold Model of Iron Deprivation Toxicity

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ABSTRACT

Recent studies have suggested that iron deprivation may represent a useful new approach in cancer therapy, and several strategies for producing such deprivation are now under investigation. Thus, for example, we recently provided evidence that combined treatment with the iron chelator deferoxamine and an IgG monoclonal antibody against the transferrin receptor (ATRA) produces synergistic inhibition of hematopoietic tumor cell growth in vitro (J. D. Kemp, K. M. Smith, L. J. Kanner, F. Gomez, J. A. Thorson, and P. W. Naumann, Blood, 76: 991-995, 1990). The current study is an attempt to analyze the mechanisms responsible for the synergistic interaction. The data show that a single IgG ATRA can produce up to 75% inhibition of iron uptake while having little effect on DNA synthesis; this suggests that tumor cells either take up or have stored amounts of iron well in excess of that required to support immediate metabolic needs. When deferoxamine and the IgG ATRA are used together, the effects on iron acquisition and receptor down-modulation are either additive or subadditive but are clearly not synergistic. Overall, the findings suggest that the IgG ATRA produces an injury to iron uptake that is just below a critical threshold and that the additional effect provided by the iron chelator is sufficient to exceed that threshold and produce a rapid depletion of iron pools that are vital for short-term DNA synthesis. IgG ATRAs thus seem to be of even greater interest as therapeutic reagents, and further study of their properties and of how they interact with deferoxamine appears to be warranted.

INTRODUCTION

There is now a growing body of evidence, derived from studies in vitro and in vivo, suggesting that iron deprivation may represent an important therapeutic tool against tumors, especially those of hematopoietic origin (1-4). There are several means by which iron deprivation of tumors can be achieved, including treatment with gallium (which interferes with intracellular iron uptake) (5), deferoxamine (which chelates intracellular iron with very high affinity) (6), and anti-transferrin receptor antibodies (which interfere with receptor-mediated uptake of ferric transferrin) (7-10). Well-organized Phase I and Phase II trials with gallium have already shown promise (1), and monoclonal antibodies against the transferrin receptor are now being evaluated for clinical trials (11). We recently reported that the synergistic inhibition of the growth of several hematopoietic tumors could be observed in vitro when such tumors were treated concurrently with DFO and IgG ATRAs (3). These observations were significant because DFO, the only iron chelator with an extensive clinical experience base, had not been tested before in a combination strategy with ATRAs and because IgG ATRAs were not thought to be useful therapeutic agents. The observations were also of interest because they raised the possibility that the effective doses of DFO, the reagent with the most apparent risk of toxicity, might be significantly reduced in such combination treatment. We undertook the studies described here in order to learn more about the mechanisms involved in combination treatment with DFO and IgG ATRAs.

MATERIALS AND METHODS

Lymphoid Tumor Line. The EL4 T-cell tumor was originally obtained from Dr. Richard Lynch (University of Iowa, Iowa City, IA) and has been maintained in our laboratory for several years. Significant Reagents. The bacterial iron siderophore DFO is produced under the name Desferal by Ciba-Geigy, Inc. (Summit, NJ) and was obtained from the pharmacy of the University of Iowa Hospitals and diluted for use as previously described (3). The origin and means of purification of C2F2, a rat IgG2a monoclonal antibody directed against the mouse transferrin receptor, have also been previously described (11, 12).

Culture Conditions. Cells were cultured for the times noted at a density of 1.25 x 10^6 cells/mL in a standard RPMI-based medium, with 10% fetal calf serum, as previously described (3). Cultures destined for thymidine incorporation or iron uptake/acquisition assays were cultured in 24-well flat-bottomed plates from Corning (Corning, NY) or Costar (Cambridge, MA). Cultures destined for flow cytometric analyses were performed in T-25 or T-75 flasks.

59Fe Loading of Transferrin. 59FeCl3 was obtained from Amersham (Arlington Heights, IL), and human apotransferrin was obtained from Sigma (St. Louis, MO). Loading was accomplished by means of an 59Fe-nitrilotriacetic acid complex intermediate following the method described by Bates and Wernicke (13). The degree of iron saturation of the preparation utilized is 62% unless otherwise noted.

Iron Uptake/Acquisition Assays. Cells were cultured for 48 h as noted above, except that the medium contained only 1% fetal calf serum and was supplemented with 59Fe-loaded human transferrin at 30 µg/mL. Human transferrin is known to have a higher affinity interaction with the murine transferrin receptor than does bovine transferrin, and 1% fetal calf serum inhibits human transferrin binding by no more than 5% (14). Cells were harvested and pelleted through a 1:1 mixture of n-butyl phthalate and dioctyl phthalate oils from Fisher Chemical Co. (Chicago, IL) and Aldrich Chemical Co. (Milwaukee, WI), respectively. The portion of each tube containing the pellet was cut away with a razor blade and placed in a separate tube for placement in a Gamma 5500 gamma counter from Beckman (St. Louis, MO).

Thymidine Incorporation Assays. Cells were cultured as noted above for the Fe uptake/acquisition assays, except that transferrin loaded with 59Fe was added to the culture 24 h before harvest. The cells were harvested, washed, and resuspended in complete medium, and then triplicate 250 µL samples were applied to Terasaki wells. Thymidine incorporation was measured as described above. Each experiment was performed in triplicate.

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4 The abbreviations used are: DFO, deferoxamine; ATRA, anti-transferrin receptor antibody.
cold iron (59% saturated) was utilized at the same concentration. During the terminal 6 h of the cultures the cells were pulsed with 1 µCi of [3H]thymidine and were then harvested and analyzed as previously described (3).

Flow Cytometric Analysis. Flow studies were performed as previously described (3), with the exception that some data were obtained with a FACSCAN and CONSORT 30 software from Becton-Dickinson (Mountain View, CA).

RESULTS

An IgG ATRA Causes Significant Inhibition of Iron Uptake with Little Concomitant Inhibition of DNA Synthesis. Since our earlier study indicated that IgG monoclonal antibodies against the transferrin receptor could interact with the iron chelator deferoxamine to synergistically inhibit lymphoid tumor growth (3), we wished to learn more about the mechanisms involved and, in particular, we wished to learn about the effects of both reagents on tumor cell iron uptake. The uptake of exogenous iron is known to be crucial to DNA synthesis (15), and the inhibition of DNA synthesis is known, in turn, to be a very sensitive indicator of cellular iron deficiency (16). We began the studies by first investigating the effects of an IgG ATRA alone, i.e., in the absence of DFO. The data presented in Fig. 1 reveal that a single IgG ATRA can inhibit 59Fe uptake by the EL4 tumor by as much as 75%. Although single IgG ATRAs have been observed to inhibit iron uptake in previous work (10), an effect of this magnitude appears to be uncom-mon (4). Of at least equal interest, however, is the fact that such a substantial degree of inhibition of iron uptake can be coupled with such a minimal effect on DNA synthesis. This confirms and extends an observation made earlier with a multivalent IgM ATRA (17). Such findings suggest that cells may take up, or have available in somewhat labile pools, amounts of iron that are well in excess of that required to meet the short-term requirements for DNA synthesis.

Simultaneous Exposure to an IgG ATRA and DFO Results in Subadditive Inhibition of Iron Acquisition. Having established that the IgG ATRA alone had a remarkable inhibitory effect on iron uptake, it was of interest to ascertain whether the combination of DFO and the IgG ATRA would produce a synergistic inhibition of iron acquisition in a manner that would correlate with the synergistic inhibition of DNA synthesis. The data shown in Fig. 2 (A and C) further confirm the effect of the antibody alone on iron uptake and, in addition, show that there is a dose-dependent net reduction of cellular iron acquisition produced by DFO in the absence of antibody. When the lowest dose of DFO is then combined with the IgG ATRA, the effects on iron acquisition appear to be additive. As one increases the dose of DFO, however, it appears that the combined effect becomes modestly subadditive, especially at the higher doses of the ATRA.

The phrase "net iron acquisition" is used here because, as DFO chelates and removes iron from the cell, the cell responds as expected by up-regulating its receptor number and taking up more transferrin-bound iron in an effort to compensate for the ongoing loss. While this happens to be consistent with the current understanding of transferrin receptor gene regulation, we know that it occurs in the particular case under study because we have confirmed our earlier observation that DFO does cause receptor up-regulation in the EL4 tumor (Ref. 3; Fig. 3). Moreover, we have also measured short-term iron uptake after washing out the DFO and found that it increases by 50–60% at the highest doses of DFO.

The contrast between the patterns of the iron acquisition data and thymidine uptake data in paired cultures can be appreciated by inspection of Fig. 2 (B and D). As expected, the ATRA alone shows little inhibition. When the ATRA is combined with DFO, however, a synergistic inhibitory effect is readily apparent, especially between the doses of 100 and 1000 ng/ml of the IgG ATRA. These results are in close agreement with our prior observations (3).

The subadditive effect on iron acquisition seen at the higher doses of DFO was somewhat surprising and raised the question as to whether the degree of antibody-induced receptor down-modulation might also be decreasing.

Deferoxamine Does Not Accelerate the Relative Rate of Receptor Down-Modulation that Occurs as a Result of Exposure to an IgG ATRA. In our recent paper, we surmised that the synergistic inhibition of DNA synthesis that occurs with simultaneous treatment with DFO and an IgG ATRA might arise because DFO either accelerates the rate of transferrin receptor turnover or causes an altered intracellular trafficking pattern.
such that the receptors are more likely to be rapidly degraded when bound by an IgG ATRA (3). In an effort to address this idea, and especially in view of the preceding iron acquisition studies, we conducted flow cytometric kinetic studies of IgG ATRA-associated receptor down-modulation resulting from exposure to either the ATRA alone or the ATRA plus DFO.

The data shown in Fig. 3 reveal that receptor levels, expressed as a percentage of baseline, appear to rise for a brief period of time after exposure to the IgG ATRA and then undergo a steady process of down-modulation; the end result is consistent with expectations arising from earlier work that we and others have performed (8, 11). Although not shown, similar data have been obtained for two other tumors. One way to approach the data obtained in each of the experimental groups is to compare the point at which the ATRA-treated group crosses the baseline defined by the normal rat IgG control group. In the first experiment it can be seen that, in the absence of DFO, the cross-over occurs at approximately 40 to 45 min. In the presence of 10 μg/ml of DFO the cross-over occurs at about 75 min, and in the presence of 20 μg/ml of DFO the cross-over occurs after 90 min. In the second experiment the control group shows a cross-over at about 60 min, and the DFO-treated group does not clearly drop below baseline until after 120 min. It would appear that the relative rate of receptor down-modulation is no faster in the presence of DFO and may well be slower.

**DISCUSSION**

The purpose of the current studies has been to further elucidate the physiological mechanisms involved in the synergistic inhibition of tumor cell growth that is seen when such cells are exposed simultaneously to an IgG ATRA and DFO. The focus has been on studies of iron uptake and the modulation of surface expression of the transferrin receptor. The specific intent has been to test a model that we put forth in a previous paper (3).

The model proposed that the presence of DFO would either accelerate the turnover or alter the intracellular trafficking pathway of transferrin receptors in such a way that an IgG ATRA would become progressively more effective in causing receptor down-modulation. More specifically, the model predicted that both receptor down-modulation and inhibition of tumor cell growth that is seen when such cells are exposed simultaneously to an IgG ATRA and DFO. The focus has been on studies of iron uptake and the modulation of surface expression of the transferrin receptor. The specific intent has been to test a model that we put forth in a previous paper (3).

In particular, the rate of IgG ATRA-mediated receptor down-modulation certainly appears to be no faster in the presence of DFO and may well be slower. These data are most easily explained by the idea that DFO either has no effect on or...
decreases the rate of receptor turnover. In order for the current data to be consistent with a hypothetical acceleration of turnover rate induced by DFO, there would have to be a concurrent increase in resistance to the process of antibody-mediated down-modulation that was equal to or greater than the increase in receptor turnover. This seems intrinsically unlikely, but further work is required to definitively exclude this possibility. If further work supports the idea that DFO treatment actually diminishes the turnover rate, then it may be of some interest to attempt to define the underlying mechanism.

The iron acquisition studies also show no evidence of a synergistic interaction and are subadditive at some doses. This might best be explained by considering one or both of two possible effects. On the one hand, it may be due in part to a decline in the rate of ATRA-mediated receptor down-modulation. On the other hand, the data are also consistent with the idea that any reduction in iron uptake resulting from ATRA-mediated transferrin receptor degradation correspondingly reduces the size of the potentially chelatable pool of labeled iron. These two effects are not mutually exclusive, and it seems plausible to suppose that both may be operative. This interpretation is consistent with (and the studies thus provide new support for) the commonly held views that (a) transferrin-mediated uptake is the principal means by which most cells obtain iron, and (b) DFO chelates iron only after it is released from transferrin.

Given the somewhat unexpected nature of the findings, how can one account for the highly reproducible appearance of what certainly appears to be synergistic inhibition of DNA synthesis? Part of the explanation could include the idea that a given tumor exhibits a characteristic critical threshold value of iron deprivation that has to be exceeded before any significant effect on DNA synthesis can be observed. Implicit in such a notion is the idea that there is not a one-to-one correspondence between short-term iron uptake and immediate metabolic utilization. A necessary corollary idea is that the cells must either have significant iron stores in reserve or routinely take up and secrete iron well in excess of their needs. The latter ideas were suggested in an earlier study as one way to explain why one tumor might, in a qualitative sense, show sensitivity to ATRA treatment while another was resistant (17). That study did not, however, provide any quantitative evidence for the conversion of a resistant tumor into a sensitive one by incremental increases in the degree of inhibition of iron acquisition.

In the present case, it would appear that higher doses of the IgG ATRA can produce an injury to iron uptake that approaches or just barely exceeds the postulated threshold. The degree of injury is, however, still apparently insufficient to cause a rapid collapse of DNA synthesis. The addition of a modest dose of DFO may result in a rapid reduction of the iron pools that directly support DNA synthesis in the short term. It is worth noting in this context that the injury to cellular iron stores caused by DFO treatment may well be underestimated in the present studies. We have measured only the radioactive iron that has been recently imported via transferrin and, since it is believed that DFO can chelate not only free iron (such as would be found in an endosome after release from transferrin) but also that iron which is stored in ferritin (6), it seems possible that DFO is also draining existing unlabeled iron pools. Concomitant drainage of other iron pools may explain why, for a given degree of inhibition of recent iron uptake, DFO appears to produce a greater degree of inhibition of DNA synthesis than does the IgG ATRA.

While this seems to be the simplest approach to the data, there are alternative possibilities that could be considered. Thus, one could suppose that DFO interferes with DNA synthesis either by binding some metal other than iron or that the effects seen are the indirect result of the removal of iron from another site, such as the mitochondrial electron transport chain. The arguments against these ideas are based on the extraordinary specificity of DFO for iron (6) and on the fact that, at the limiting doses we have used, DNA synthesis is the cellular function that has been shown to be the most sensitive to treatment with DFO (16).

It was not anticipated that the IgG ATRA would produce such significant inhibitory effects on tumor cell iron uptake since it appears that many (perhaps most) IgG ATRAS are poor inhibitors of iron uptake when evaluated one at a time (4). The fact that a single IgG ATRA can exhibit such impressive effects is, however, encouraging from a therapeutic point of view because it suggests that further study will allow the identification of other such reagents with equal or greater potential. It should be remembered, however, that the specific properties that determine whether an ATRA will be a good inhibitor, such as the epitope recognized and the affinity with which it reacts, are still not well understood (18). Although a fair amount of effort may ultimately be required to fully understand the rules governing the process of ATRA-mediated inhibition of iron uptake, it would nevertheless appear that the justification for such effort is steadily increasing.

All of the available data continue to support the idea that iron deprivation strategies may provide a set of interesting new therapeutic tools for use in the treatment of neoplasia (19). Combination DFO/IgG ATRA therapy appears to be an interesting example of such a strategy, particularly insofar as hematopoietic tumors are concerned, and further work aimed at understanding the mechanisms involved appears to be warranted.

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