Inhibition of Lymphoma Growth in Vivo by Combined Treatment with Hydroxyethyl Starch Deferoxamine Conjugate and IgG Monoclonal Antibodies against the Transferrin Receptor

J. D. Kemp, T. Cardillo, B. C. Stewart, E. Kehrberg, G. Weiner, B. Hedlund, and P. W. Naumann

ABSTRACT

Synergistic inhibition of hematopoietic tumor growth can be observed in vitro when the iron chelator deferoxamine (DFO) is used in combination with an IgG mAb against the anti-transferrin receptor antibody (ATRA). Our goal was to ascertain whether similar findings could be seen in vivo. A high molecular weight conjugate of deferoxamine, known as hydroxyethyl starch (HES) DFO or HES-DFO, was tested in conjunction with C2, a well-defined rat antimouse transferrin receptor mAb, against the 38C13 tumor in C3H/HeN mice. It was shown that while neither HES-DFO alone nor C2 alone produced consistent, significant inhibition of tumor growth, the combination of HES-DFO and C2 produced virtually complete inhibition of initial tumor outgrowth. The latter combination failed, however, to inhibit the growth of established tumors. It was then found that when C2 was used in conjunction with RL34, another IgG ATRA, the two ATRAs were themselves capable of causing synergistic inhibition of the growth of 38C13 in vitro. When the two IgG ATRAs were used together in vivo, regressions of established tumors were observed. Moreover, the addition of HES-DFO to the IgG ATRA pair then caused more frequent regressions. Although there was never any obvious toxicity seen with a single IgG ATRA, the use of the IgG ATRA pair was associated with sporadic mortality. In addition, although HES-DFO by itself was also not associated with any obvious toxicity, combined treatment with HES-DFO and a single ATRA resulted in death due to bacterial infection in about half of the mice after 10–15 days. Combined treatment with HES-DFO and the ATRA pair resulted in death attributed to infection in nearly all of the mice after 6 days. Thus, an iron deprivation treatment protocol with HES-DFO and IgG ATRAs produced both a significant antitumor effect and an increased risk of infection in a murine model system.

INTRODUCTION

There is steadily accumulating evidence that iron deprivation is a clinically useful tool in the treatment of cancer. Thus, for example, gallium nitrate, which is thought to deprive cells of iron by interfering with endosomal acidification, is known to have activity against several tumor types and has produced durable complete responses in clinical trials against transitional cell carcinoma (1). Impressive responses have also been observed when patients with neuroblastoma were treated with the iron chelator DFO® either as a single agent or in combination with other chemotherapeutic agents (2, 3). Moreover, there is experimental evidence which suggests that mAbs against the transferrin receptor (ATRAs) have significant activity against hematopoietic tumors in vitro and in vivo (4, 5). Finally, there is evidence that combinations of iron-depriving agents (i.e., an ATRA and a chelator or two ATRAs) produce stronger inhibitory effects in vitro and in vivo (6–9).

Our laboratory has focused on the evaluation of the effects of combined treatment with DFO and an IgG mAb against the transferrin receptor (ATRA; Refs. 7, 9, and 10). We have shown that this combination produces synergistic growth inhibition of a broad array of hematopoietic tumors in vitro (7). We have also suggested that the two reagents may jointly be capable of producing a critical degree of cellular iron deprivation that can rapidly deplete labile iron storage pools, halt DNA synthesis, and create cytotoxicity (9). We present here the results of a series of studies which show that lymphoma growth can be inhibited in vivo by treatment with an HES conjugate of DFO and IgG ATRAs.

MATERIALS AND METHODS

Iron Chelators. Deferoxamine mesylate (Desferal) was a gift from Ciba-Geigy Pharmaceutical Company (Summit, NJ). Desferal was obtained in lyophilized form and a 10-mg/ml (1.52 × 10⁻³ M) stock solution was prepared in distilled water and was further diluted with complete RPMI 1640 for use in tissue culture. An HES conjugate of DFO was supplied by Biomedical Frontiers, Inc. (Minneapolis, MN). HES-DFO was supplied as a 10% solution (w/v) in physiological saline in which the chelator concentration was 26 mM. The molecular weight of the conjugate ranged from 15,000 to 200,000. Seventeen percent of the conjugate weight is chelator. A 10% solution of HES without chelator was also provided as a control.

mAbs and Immunoglobulins. The IgG2a monoclonal rat antimouse ATRA C2 was derived by this laboratory as described previously (11). For these studies, C2 was purified from (Wistar Furth × LouJ)F male rat ascites fluid by a combination of 45% SAS precipitation, DEAE anion exchange chromatography, protein G affinity chromatography, and a selective nondenaturing precipitation of antibody from solution at pH 5.0.

The IgG2a monoclonal rat antimouse ATRA RL34.14.2.5 has been previously described (12) and was kindly provided by Drs. Jayne Lesley and Ian Trowbridge of the Salk Institute (San Diego, CA). RL34.14.2.5 was purified from SCID mouse ascitic fluid by 45% saturated ammonium sulphate precipitation and protein G affinity chromatography or DEAE anion exchange chromatography.

Normal rat IgG (Ig131) was purchased from Sigma Chemical Company (St. Louis, MO).

Tumor Line. The mouse B cell lymphoma 38C13 is a well-described cell line that grows rapidly and consistently in immunocompetent C3H/HeN mice (13, 14).

Mice. C3H/HeN female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals 9–15 weeks of age were used in in vivo experiments and housed at least 1–2 weeks in in-house animal care facilities after shipment before use in any experiments. Animals were in the 20–25 g weight range in most experiments.

Tissue Culture Conditions. Routine culture and all experiments with 38C13 were conducted in RPMI 1640 medium with extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), L-2-mercaptoethanol (5 × 10⁻⁵ M), HEPES (10 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT). Cells in exponential growth with viabilities greater than 95% were used for all
experiments. Cultures were maintained at 37°C in 5% CO₂. Periodic screening for *Mycoplasma* infection was conducted using DNA staining with Hoechst dye 33258 (15).

**Proliferation Assays in Vitro.** Proliferation assays were conducted using [³H]thymidine incorporation. 38C13 tumor cells were harvested from log growth phase cultures, centrifuged 10 min at 300 × g, resuspended in fresh medium RPMI 1640, and counted by hemacytometer. A 1 × 10⁶ cell/ml suspension was prepared, and 0.1 ml was loaded by multichannel pipette into each well of Corning 96-well flat-bottomed plates. Test reagents were then added to the wells to achieve the desired concentrations and a final well volume of 0.2 ml and a cell density of 5 × 10⁴ cell/ml, with triplicate wells for each test condition.

Plates were cultured at 37°C in 5% CO₂ for approximately 42 h, after which 1 µCi [³H]thymidine (Amersham Corporation, Arlington Heights, IL) was added per well, in 10 µl, followed by another 6 h of incubation. Cells were then collected using a cell harvester (Skatron, Arlington, VA) onto glass filter mats. Upon drying, filter discs were placed into 7-ml polystyrene minivialis (RPI Corp., Mount Prospect, IL), and 5 ml saline solution (RPI) were added per vial. Counting was performed in a Beckman LS 7800 beta counter.

Concentrations of deferoxamine mesylate and HES-DFO are expressed as the molar value of chelator, while antibody levels are expressed as µg/ml. The hydroxyethyl starch control solution was used at the same percentage of starch concentrations as the HES-DFO.

**Initial Tumor Outgrowth Assays in Vivo.** In the first series of experiments, in which treatment with antibody and deferoxamine commenced on the same day as tumor implantation, 38C13 cells were collected from exponential growth phase culture. They were centrifuged 10 min at 300 × g, washed twice with DPBS (Sigma Chemical Co.), resuspended in DPBS, counted by hemacytometer, and adjusted to 1 × 10⁶ cells/ml for injection. Experimental groups contained no less than five C3H/HE female mice that were 9–15 weeks of age and 20–25 g in weight. They were anesthetized with approximately 0.15 ml of a 15.0 mg/ml ketamine HC1, 3.0 mg/ml Ace-Promazine solution (Ketaset; Aveco, Inc., Fort Dodge, IA)/25-g mouse given i.p. Mice were then closely clipped on the upper flank and 0.05 ml of the 1 × 10⁹ cell/ml suspension was injected (5 × 10⁵ cells) i.d. with a 1-ml syringe and 27-gauge ½-inch needle.

Upon recovery from anesthetic, animals received treatment by i.p. injection of 0.5 ml HES-DFO (26 mm for chelator) or HES control, and/or 0.5 ml (3 mg) antibody using a 3-ml syringe and 25-gauge ½-inch needle. Untreated mice received no injection. Subsequently, HES-DFO and HES control injections continued daily for 10 days at the same dose, while further antibody administration occurred on days 3, 6, and 9, also at the same dose.

Animals were monitored daily for tumor outgrowth, and due to the exceptional growth rate of 38C13 growth in untreated mice was usually evident in 4–5 days, and tumor size was measurable with Vernier calipers in 6–7 days. Once measurable, tumor sizes were assessed daily until termination of the experiment. Tumor size was estimated according to the following formula: size = (width, cm)² × (length, cm)² as per Talcet et al. (16).

**Established Tumor Growth Assays in Vivo.** In these experiments the same number of tumor cells were injected in the same manner, but they were allowed to grow for 6 to 9 days to a mean size of approximately 0.20 cm³ before treatment was begun. The tumor-bearing mice were then distributed into groups whose tumors exhibited equivalent mean size and SD. Treatment of animals and measurements of tumors then followed the same scheme as the initial outgrowth experiments, with the exception that the caliper measurements were performed in a “blind” manner (by an individual unaware of the treatment groups). Data on the tumor size for each mouse are plotted over time and are normalized to tumor size at the start of treatment. In groups receiving double antibody treatment, 1.5 mg of each antibody was administered i.p. for a total of 3.0 mg for each dose. The antibodies were given 1 h apart for the first dose but were given simultaneously for subsequent doses.

**38C13 Serum Idiotype Assay.** The serum concentration of the IgM antibody secreted by 38C13 was measured by a sandwich ELISA. Microtiter plates were coated with a previously characterized anti-idiotypic antibody (MS11G6; Ref. 17) at a concentration of 10 µg/ml overnight. Plates were blocked with 5% milk, and serial dilutions of test serum were added. The presence of bound idiotypic antibody was detected by the addition of biotin-labeled MS11G6 anti-idiotypic antibody followed by an avidin-alkaline phosphatase conjugate and a colorimetric substrate. Normal C3H serum to which a known concentration of purified 38C13 IgM was added served as a standard. Developed plates were read on a Microplate Autoreader (Biotek Instruments) at 405 and 595 nm.

**Blood Assays for HES-Ferrioxamine and HES-DFO.** Concentrations of HES-ferrioxamine and HES-DFO in mouse blood were measured using a minor modification of a standard colorimetric assay for ferrioxamine (18). HES-ferrioxamine was first measured directly and then the sum of the HES-ferrioxamine and HES-DFO concentrations was measured after saturating quantities of ferrous sulfate were added to convert HES-DFO to HES-ferrioxamine. The iron-free HES-DFO concentration was then obtained by subtracting the HES-ferrioxamine concentration from the sum.

A standard curve was generated in deionized water by making a series of 1:2 dilutions from a 1 µm HES-DFO stock solution into 1.5-ml microtube tubes (diluting into serum yielded an identical curve). Then, 500 µl of a given mouse whole-blood sample was loaded into each of two duplicate 1.5-ml microtube tubes. Twenty-five µl of freshly prepared 200 µm ferrous sulfate (deionized water) were added to all standard tubes and to one of the duplicate blood tubes. Twenty-five µl of water were added to the other duplicate blood tube. The tubes were vortexed and incubated at room temperature for 15 min. Twenty-seven and five-tenths µl of 100% (w/v) trichloroacetic acid were then added to each tube with immediate vortexing. All tubes were centrifuged at 5 min at 10,000 × g in an Eppendorf 5415C microtube followed by careful removal of supernatant and placement in fresh 1.5-ml microtube tubes. The supernatants were spun again for 10 min at 16,000 × g. One hundred sixty-three µl of each supernatant were then added to each of two replicate wells in a Corning 96-well flat-bottom plate into which 75 µl µl of sodium acetate (pH 5.0) have previously been added. Plates were read for absorbance at 450 nm using a Microplate Autoreader (Biotek Instruments). Sample absorbances were corrected for background by subtracting the absorbance of normal, untreated mouse blood.

**Serum ATRA Assay.** Serum ATRA levels were determined by indirect immunofluorescence flow cytometry. The 70-2 cell line, a pre-B-lymphocytic mouse cell line (ATCC, Rockville, MD), was used as a staining target. A standard curve was generated by spiking normal C3H sera with a known concentration of C2 antibody, and then making serial 1:2 dilutions in BSS (BBS + 2.5% FBS, 0.05% sodium azide). For staining, 5 × 10⁴ 70-2 cells from log phase cultures were spun at 4°C in 3.7- x 55-mm conical tubes at 300 × g for 10 min. Cell pellets were resuspended in 50 µl of appropriate standard or test sera dilutions in BSS and incubated for 30 min on ice. After this primary stain, 400 µl ice-cold BBS were added, and 100 µl µl µl of 0.05% sodium azide were underlayed in each tube. Tubes were spun for 10 min at 300 × g at 4°C, and, once aspirated, 50 µl of an appropriate dilution of FITC mouse F(ab')₂ antiser IgG (Jackson Immunoresearch, West Grove, PA) were added per tube with mixing. The tubes were then put on ice for 30 min, and this was then followed by another BBS/FBS underlay wash. Final cell pellets were resuspended in 500 µl 1.5% formaldehyde in DPBS, and were then analyzed on a B-D FACScan (Mountain View, CA). Using mean fluorescence from the C2 standard tubes, an estimation of the ATRA content in the test sera of the animals receiving injections could be made.

**RESULTS**

**38C13 Lymphoma Exhibits Synergistic Growth Inhibition in Vitro When Treated with an IgG mAb against the Transferrin Receptor and Either Low or High Molecular Weight Forms of DFO.** For the purposes of undertaking experiments in vivo, we considered that the 38C13 lymphoma of C3H mice would represent a well-characterized system to study (13, 14). Before beginning, however, it was critical to ascertain whether 38C13 would behave like other murine hematopoietic tumors and show synergistic growth inhibition in *vivo* when exposed to combination treatment with an IgG ATRA and DFO (7). Moreover, because it is known to be extremely difficult to maintain significant plasma DFO concentrations over time (19, 20), we elected to compare the effects of standard DFO (available as a low molecular weight mesylate salt from Ciba-Geigy) with the effects of a recently described high molecular weight DFO that exhibits improved pharmacokinetics (18). The latter is a
conjugate produced by chemical attachment of several DFO molecules to one molecule of hydroxyethyl starch (18).

Fig. 1A shows the effect of the addition of various doses of low molecular weight DFO mesylate, either alone or in combination with a fixed concentration of the IgG ATRA C2, on thymidine incorporation by the 38C13 tumor. Thus, while the half-maximal inhibitory dose of DFO alone was between 10 and 15 μM, the effect of the addition of the IgG ATRA was to shift the curve so that half-maximal inhibition occurred at a dose between 1 and 2 μM. The ATRA itself produced only very modest inhibition (13%). The results are entirely consistent with our prior observations (7) in that the 38C13 lymphoma is the sixth murine hematopoietic tumor tested, and that all six exhibit synergistic growth inhibition when exposed to combined DFO/ATRA treatment in vitro.

Fig. 1B shows the result obtained when the same type of comparison was carried out with HES-DFO instead of DFO. The first point to note is that, on a DFO molar equivalent basis, HES-DFO alone was a less effective inhibitor than DFO alone. Thus, the half-maximal inhibitory dose was increased approximately 20-fold, to about 200 μM. The addition of the ATRA, however, still produced a significant degree of left shift; i.e., the half-maximal inhibitory dose was reduced to between 2.5 and 25 μM. Thus, the synergistic growth inhibitory effect was still seen when the ATRA was added.

It is pertinent to note that the minimum dose of HES-DFO that was required to produce essentially complete inhibition of DNA synthesis by 38C13 cells in vitro in the presence of the ATRA was about 75 μM.

**Pharmacokinetics of HES-DFO and the IgG ATRA C2.** With the foregoing in vitro studies in mind, we asked whether we could achieve adequate blood chelator values in vivo using HES-DFO. We elected to evaluate a single daily i.p. injection protocol. This was based both on an earlier study showing that this route of injection was successful in producing iron excretion in Swiss-Webster mice (18) and on unpublished pharmacokinetic data. The results obtained are shown in Fig. 2.

Fig. 2A shows the effects of a single i.p. injection of 0.5 ml of a solution of HES-DFO that is 26 mm in terms of DFO equivalents. This corresponds to a dose of DFO of 295-mg/kg body weight. Twenty-four h after the injection, the total blood concentration of HES-DFO and HES-FO combined was approximately 800 μM. The value decreased by a little more than half at 48 h and then decreased again, by about half, at 72 h. The HES-FO value was approximately 300 μM at 24 h, remained stable at that value at 48 h, and then declined by a little less than half at 72 h. Free HES-DFO levels were therefore falling rapidly between 24 and 48 h after a single injection, and there was no free chelator available for further binding of iron by 48 h.

Fig. 2B shows the effect of sequential daily injections of HES-DFO. Total HES-DFO + HES-FO concentrations rose slowly to a value of about 1.2 mm on day 9, while HES-FO concentrations remained nearly stable around the 300 μM level. Free HES-DFO concentrations were therefore 500 μM or greater during the course of this daily injection protocol.

Table 1 shows single injection pharmacokinetic data for the IgG ATRA C2. The dose administered was adopted from the prior work of White et al. (8). Twenty-four h after a 3-mg dose of the ATRA was administered i.p., the plasma antibody concentration was slightly in excess of 1100 μg/ml. By 72 h, the value was still in the range of 700 μg/ml. Based on these findings, we concluded that plasma antibody concentrations would clearly exceed the doses used in vitro (20 μg/ml) when the mAb was given every 3 days, as per White et al. (8).

When the HES-DFO and IgG ATRA pharmacokinetic data were considered together, we concluded that the injection protocols tested were capable of producing relevant free chelator and antibody concentrations. We therefore now turn to the tumor treatment experiments that were undertaken with those protocols.

**Combined HES-DFO/ATRA Treatment Produces Synergistic Inhibition of Initial Tumor Outgrowth in Vivo.** For the in vivo experiments, 50,000 38C13 tumor cells were administered i.d. to C3H mice, and treatment was begun on the same day. The results of key experimental groups are shown in Fig. 3. The experiment shown in Fig. 3A is representative of two experiments undertaken with one thaw of 38C13 (with subsequent maintenance in vitro), and the experiment shown in Fig. 3B is representative of two experiments undertaken with...
not statistically significant when compared to the no-treatment control group. Treatment with C2 alone appeared to be slightly inhibitory, but the difference from control was also not significant. However, the combination of the two completely prevented any detectable outgrowth of tumor and was significantly different from the control.

In an effort to ascertain whether the tumor might have grown at locations other than the site of injection, an ELISA assay for the 38C13 idiotype was performed on serum samples from the combined treatment group in the experiment shown in Fig. 3A, and the results are shown in Table 2. In all cases, the serum idiotype levels were below the sensitivity limit of the assay. Moreover, none of the mice in the double treatment group in this experiment exhibited any grossly

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Table 1 Serum ATRA levels following a single 3-mg injection

<table>
<thead>
<tr>
<th>Time after injection (h)</th>
<th>Serum concentration (µg/ml)</th>
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<tbody>
<tr>
<td>24</td>
<td>1130</td>
</tr>
<tr>
<td>48</td>
<td>966</td>
</tr>
<tr>
<td>72</td>
<td>711</td>
</tr>
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* The IgG ATRA C2 was injected i.p. Values represent the average of two mice in a single experiment. Similar data were obtained in a second experiment.

Concentrations determined by flow cytometric assay as described in "Materials and Methods."

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**Fig. 2.** Single- and multiple-injection pharmacokinetics of HES-DFO. Blood samples were assayed for total HES-DFO + HES-FO concentrations and HES-FO concentrations as described in "Materials and Methods." A, concentration levels obtained at the time points indicated after a single i.p. injection at 0 h of 0.5 ml solution of HES-DFO that was 26 mM for chelator. B, concentration levels obtained at the time points indicated during the course of sequential single daily injections of the same dose of HES-DFO by the same route, beginning at 0 h. Four mice were used for each data point. The experiment shown is representative of several performed.

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**Fig. 3.** Effect of HES-DFO and C2 on initial tumor outgrowth in vivo. Fifty thousand tumor cells were injected i.d., and treatment was begun on day 0. The measurements presented were obtained on day 10 when the experiments were terminated. Treatment doses and schedules, as well as the calculation for estimating tumor size, were as described in "Materials and Methods." A, data from experiments performed with one thaw of the 38C13 tumor. B, data from experiments performed with a separate thaw, as noted in "Results." Data are presented as the group mean ±1 SD. At least five mice were used in each experimental group. Neither the HES control nor normal rat IgG, when given in equivalent amounts to HES-DFO and C2, respectively, produced any significant effect on tumor growth. HDFO, HES-DFO; NG, no growth; NO RX, no treatment.

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a separate thaw. Although the major conclusion was the same in both sets of experiments, there were some differences in detail.

In the experiments represented by the data in Fig. 3A, treatment with HES-DFO alone did not inhibit tumor growth. The data raised the possibility of a slight growth enhancement, but the difference was
have been expected from adding the effects of RL34 to the baseline established by C2, and are clearly similar to the synergistic interaction curves generated with C2 and either DFO or HES-DFO (see Fig. 1).

The data thus indicated that C2 and RL34 produced synergistic inhibition of the growth of 38C13 in vitro, and thereby provided a rationale for testing the antibody combination in vivo.

C2/RL34 Antibody Pair Produces Regression of Established 38C13 Tumors: Effect Is Enhanced by HES-DFO. Having established that C2 and RL34 were synergistic growth inhibitors in vitro, we then asked whether they would affect established tumors in vivo. The 38C13 tumors were therefore allowed to grow for 6 to 8 days before any treatment was begun. Then, instead of injections of 3 mg C2 alone, the mice received 1.5 mg each of C2 and RL34. The dose and injection schedule for HES-DFO remained the same. The results of such treatment are shown in Figs. 5 and 6. Normalized tumor measurements over the course of several days are displayed for each mouse in four key groups in Fig. 5, and the means and SDs for the pooled data for the same groups in the same experiment are shown in Fig. 6.

As can be seen by comparing A and B in Fig. 5, treatment with HES-DFO alone (B) produced no obvious overall change in growth patterns when compared to the untreated control (A). In contrast, the pair of antibodies produced tumor regressions in 5 of 12 mice, and the tumors grew more slowly in the remainder (Fig. 5C). When HES-DFO treatment was added to the antibody pair, 8 of 11 mice showed no detectable tumor on day 6 of treatment, 1 mouse had a tumor that was regressing, and 2 mice had tumors that were static (Fig. 5D).

In Fig. 6 it can be seen that there was no difference between the means for the untreated and HES-DFO groups. The mean of the group treated with the ATRA pair alone was significantly different from the untreated control, and the mean of the group treated with both the ATRA pair and HES-DFO was significantly different from both the untreated control and from the ATRA-only group. Overall, in three similar experiments, the mean of the ATRA pair treatment group was always below that of the untreated control and was significantly different from the untreated control at the 0.05 level in two of the three

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**Table 2 Effects of HES-DFO and ATRA treatment on 38C13 serum idiotype levels**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Serum idiotype level (µg/ml)</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Animal 1 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal 2 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal 3 0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal 4 0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal 5 2.3</td>
<td></td>
</tr>
<tr>
<td>HES-DFO only</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>C2 only</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>HES-DFO + C2</td>
<td>&lt;0.1</td>
<td></td>
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</table>

*a* Serum samples were obtained from the animals described in Fig. 3A.

*b* Serum idiotype levels were determined by ELISA as described in “Materials and Methods.” Similar data were obtained in a second experiment.

detectable tumor at autopsy. We concluded that the tumor was not actively growing at the injection site or at other sites.

In the experiments represented by the data in Fig. 3B, treatment with HES-DFO appeared to cause some modest growth inhibition. The difference was sufficient to achieve significance at the 0.05 level using the t test. Treatment with C2 alone still failed to produce a statistically significant degree of growth inhibition. Once again, however, the combination of the two treatments produced profound inhibition of tumor outgrowth. Thus, 13 of 16 animals showed no detectable tumor growth and the remaining 3 showed only very small tumors.

Despite the fact that HES-DFO treatment achieved a statistically significant degree of growth inhibition in one set of experiments, our overall conclusion was that neither HES-DFO nor C2 alone was capable of producing consistent, impressive inhibitory effects. Combined treatment, however, produced consistent, synergistic, and nearly complete inhibition of tumor growth.

Subsequent observations indicated that tumor recurrence at the site of injection and in ipsilateral nodes occurred in almost all mice a few days after treatment was halted. Moreover, additional experiments indicated that the same treatment protocol had no significant inhibitory effect on established tumors; i.e., tumors allowed to grow for 6 to 7 days prior to the beginning of treatment (data not shown). Finally, although neither the ATRA alone nor HES-DFO alone produced any obvious toxicity, 40-60% of mice receiving 10 days of combined treatment exhibited ruffled fur and weight loss and would eventually die within a few days. Some of the stressed mice were euthanized and autopsy studies showed, in all cases, that they had developed disseminated bacterial infections with either Gram-negative rods or Gram-positive cocci thought to be staphylococci (data not shown).

A Pair of Rat AntiMouse IgG ATRAs Produces Synergistic Growth Inhibition of 38C13 in Vitro. In an effort to develop a protocol that would have an inhibitory effect on established 38C13 tumors, we decided to test other rat antihuman IgG ATRAs in combination with C2 in vitro. This was based on the prior work of White et al. (8), who showed that certain pairs of mouse antihuman IgG ATRAs could produce synergistic inhibition of hematopoietic tumor growth in vitro and in vivo. Three candidate antibodies, RR24 (12), RL34 (12), and H129 (21), were identified in screening tests with tissue culture supernatants, and the RL34 hybridoma was selected for expansion and protein purification. Dose-response curves were established for purified RL34, with and without a fixed concentration of C2, as shown in Fig. 4.

The data indicate that, even at a dose of 10 µg/ml, RL34 alone produced very modest growth inhibition (maximum of 19%). C2 alone also produced very modest inhibition at 10 µg/ml (see Fig. 1). Both results were typical of those seen previously with single IgG ATRAs (4, 12, 22). Of interest was the effect of adding the same doses of RL34 to C2 when the latter was at a fixed concentration of 10 µg/ml. The effect of the mixture was clearly much greater than would

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**Fig. 4. Effect of two IgG ATRAs on growth of 38C13 in vitro. Tissue culture of 38C13 cells and thymidine incorporation assays were carried out as described in ‘Materials and Methods.’ RL34 concentrations are as noted. When present, C2 was always at a fixed concentration of 10 µg/ml. The experiment shown is representative of several performed.**
COMBINED IRON DEPRIVATION TREATMENT OF LYMPHOMA

Fig. 5. Effect of HES-DFO and the two IgG ATRAs C2 and RL34 on the growth of established 38C13 tumors. Treatment was delayed until day 6 after tumor inoculation as described in “Materials and Methods.” Tumor sizes for individual mice are plotted over time as ratios relative to the value of 1 assigned to a given animal’s tumor size on day 0 (day 0 of treatment). A, no treatment control group. B, HES-DFO only group. C, C2 + RL34 treatment group. D, combined treatment group with C2 + RL34 and HES-DFO. The experiment shown is one of three closely related experiments.

experiments. The mean for the ATRA plus HES-DFO group in three similar experiments was always significantly below the untreated control and was also always below the ATRA-only group. Although the difference between the pooled means of the latter two groups was significant at the 0.05 level in only one of three experiments, the combined treatment group always showed more mice with no visible tumor or regressing tumors.

We concluded that the antibody pair was capable of causing regression of established tumors and that the effect was further enhanced by HES-DFO. The regressions were not curative, however, as tumor recurrence eventually developed in surviving mice (data not shown).

Treatment with the antibody pair alone was associated with death of about 10% of the mice without any obvious preceding signs of stress and whose cause(s) is not yet clear. Combined treatment produced signs of stress after 6 days in more than 90% of the mice (similar to that seen in about 50% of the mice in the initial outgrowth experiments), and, as in the initial outgrowth experiments, the stressed mice would eventually die. Preliminary autopsy studies of euthanized animals from combined treatment groups revealed hepatic bacterial foci consistent with septicemia in 75% of the cases studied (data not shown).

DISCUSSION

Prior work indicated that combined treatment with DFO and an IgG ATRA produced synergistic inhibition of the growth of five of five hematopoietic tumors in vitro (7). The work presented here shows that this synergism also occurs with a sixth new tumor in vitro, and therefore confirms and extends the earlier studies. It seems increasingly likely that some degree of synergistic inhibition will be observed with most hematopoietic tumors. We have proposed that the synergism arises from the fact that the two agents together are capable of creating a critical degree of iron deprivation that neither is capable of creating alone (9).

The primary goal of the work presented in this article was to ascertain whether combined DFO/IgG ATRA treatment would also inhibit hematopoietic tumor growth in vivo. A problem that had to be confronted early on was the fact that it is difficult to sustain satisfactory plasma levels of DFO in both mice and humans (23). We therefore undertook an evaluation of HES-DFO, a high molecular weight HES conjugate of DFO (18), both in vitro and in vivo. Despite the fact that it took about 10–20-fold more DFO (in the form of HES-DFO) to produce the same degree of inhibition as a given quantity of the low molecular weight DFO mesylate in vitro, the pharmacokinetic studies indicated that blood chelator levels in excess of that required for synergy in vitro could be maintained in vivo in mice by daily i.p. injections. This new information thus extends our
understanding of the experimental pharmacokinetics of HES-DFO, an iron-chelating agent that may be of interest in several areas of medicine (24, 25).

Combined treatment with HES-DFO and a single IgG ATRA resulted in a synergistic and nearly complete inhibition of the initial outgrowth of 38C13 tumor inocula in vivo. Tumor regrowth occurred after the cessation of treatment, however, and this suggests that the treatment protocol was predominantly cytostatic. Since cytotoxicity is demonstrable in vitro with such treatment (7), and since both reagents appeared to be at adequate concentrations in the blood, the lack of cytotoxicity in vivo might be explained if the actual concentrations of one or both of the reagents were suboptimal in the extravascular interstitial tissue spaces. The same line of reasoning might also account for the absence of a cytostatic effect against established solid tumors since diffusion would be expected to be further reduced in a tumor bed (26).

In an effort to identify a more effective treatment protocol, we sought to identify a pair of rat antimouse IgG ATRAs that might themselves interact to create synergistic growth inhibition, as had been described for mouse antihuman ATRAs by White et al. (8). We were able to identify another ATRA (RL34) that produced synergistic, and complete, growth inhibition of 38C13 when used with C2 in vitro, and we proceeded to test the C2/RL34 combination in vivo. Since a fraction of the established tumors regressed when mice were treated with both antibodies, the data clearly suggest that antibody synergy occurred in vivo. This not only confirms some important aspects of the work of by White et al. (8), but also extends that work in an important respect. Specifically, our studies indicate that an antitumor effect can be achieved with IgG ATRAs even when normal mouse tissue transferrin receptors are available to compete for antibody binding. This point could not be approached in the heterologous human tumor/nude mouse model used by White et al. (8).

Since the antibody combination produced virtually complete cytotoxicity in vitro, but regression of only some tumors in vivo, the question is again raised as to whether antibody concentrations might have become limiting in the interior of the tumor masses. That such might have been the case is further suggested by preliminary studies showing that the antitumor effect of the antibodies is sensitive to modest dose reductions. The idea that intact IgG mAbs may not diffuse easily in solid tumor beds has been raised previously (26) and has led to the investigation of smaller molecular weight antigen-binding moieties such as single-chain Fvs (27). Similar reagents, directed against the transferrin receptor, might therefore be of some interest in this context. They would presumably still have to be either bivalent and/or bispecific (28, 29), however, since all available evidence suggests that receptor cross-linking is critical to the process of receptor down-modulation and degradation (12, 28). Bispecific antibodies that cross-link transferrin receptors to other antigens that are expressed at higher densities on tumor cells than on normal tissues (29) might produce less toxicity.

Another observation that could also reflect lower antibody concentrations in the tumor tissue is the lower incidence of long-term remissions in our study as compared to White et al. (8). They observed such remissions in 60% of their mice while we have observed eventual tumor recurrence after tumor regression in our protocols. In their study, however, the mice were given twice as much antibody per injection and received two or three times as many injections. We are currently planning additional experiments with higher and more frequent doses of the antibodies without HES-DFO in an effort to ascertain whether long-term remissions of the 38C13 tumor can be produced with administration of antibodies only.

When HES-DFO was combined with the antibody pair, a further significant enhancement of the antitumor effect was observed, and almost all of the tumors regressed. This indicates that HES-DFO is capable of diffusing into the solid tumor mass and that it is once again producing the synergistic growth inhibitory effect observed in vitro and in the initial outgrowth experiments. The fact that an occasional tumor escaped, however, still suggests that either the antibodies, or HES-DFO, or both were at suboptimal concentrations in the tumor beds.

We have not yet attempted to increase the dose of either the ATRAs or the HES-DFO in the combination treatment group because we encountered a high incidence of bacterial infection in that group. An increased risk of infection might arise due to hematopoietic toxicity, gastrointestinal toxicity, or a combination of the two. We are currently investigating the origin of these infections and the nature of the organisms responsible for them in more detail. We are also attempting to ascertain whether the administration of antibiotics will prevent the infections. While it is not necessarily the case that combined treatment would also produce infections in human beings, a clear understanding of their pathophysiology and dose-response relationships in mice will nevertheless be important to consider in the design of clinical trials.

It may also be important to note that both of the rat antimouse IgG ATRAs used in the studies described were of the IgG2a subclass. It is conceivable that some or all of the toxicity observed arose as a result of effector functions characteristic of that subclass and that mouse antihuman ATRAs from other IgG subclasses might not produce similar toxicity.

These studies have provided the first evidence that a synergistic growth inhibitory effect can be seen in vivo with combined HES-DFO/IgG ATRA treatment. In addition, we have confirmed and extended the findings of White et al. (8) with respect to synergistic pairs of IgG ATRAs. Since both an ATRA (30) and HES-DFO have shown little toxicity as single agents in human beings, some form of combined treatment may progress to clinical trials. Overall, this work and other recent work provide further support for the view that iron deprivation treatment may be a useful tool in cancer treatment, and thereby suggest that further preclinical investigation is warranted.

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Inhibition of Lymphoma Growth in Vivo by Combined Treatment with Hydroxyethyl Starch Deferoxamine Conjugate and IgG Monoclonal Antibodies against the Transferrin Receptor

J. D. Kemp, T. Cardillo, B. C. Stewart, et al.


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