Balb/c Mice as a Preclinical Model for Raltitrexed-induced Gastrointestinal Toxicity

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ABSTRACT

Raltitrexed (RTX) is an antifolate thymidylate synthase (TS) inhibitor used for the treatment of advanced colorectal cancer. RTX induces proliferating tissue toxicities that are largely confined to the intestine, with diarrhea being a severe side effect in a small but significant minority of patients. Similarly, weight loss and diarrhea were observed in BALB/c mice, and a maximum tolerated dose (MTD) was determined as approximately 5–10 mg/kg/day × 5 days. At an equivalent dose of 10 mg/kg/day × 5 days (d1–5), DBA2 mice lost considerably less weight, leading to a higher MTD (>500 mg/kg/day × 5 days), and there was no evidence of diarrhea. Histopathological consequences of damage, such as changes in small intestinal crypt architecture and villus atrophy induced by the 10-mg/kg/day dose, were greater and of longer duration in BALB/c mice. A higher dose of RTX (100 mg/kg/day × 5) induced weight loss and histopathological damage similar to that seen in BALB/c mice (10 mg/kg/day × 5) but was of later onset, nadir, and recovery. Small changes to the colon were only observed in BALB/c mice. Pretreatment levels of plasma thyminde, deoxyuridine (~1 μM), and folate (~40 ng/ml) were similar in both mouse strains. A single injection of radiolabeled RTX (5 mg/kg/day) did not lead to any marked difference 24 h later in the total drug concentration and distribution of polyglutamates (comprising 70–80% of drug extracted) in the liver, kidney, and intestinal epithelium (large and small intestine) between the two mouse strains. Further studies used a RIA to measure RTX polyglutamate formation in tissues at various times and drug doses. This led to the conclusion that, although there was a higher accumulation of RTX in BALB/c small intestinal epithelium (days 4–6), it may be an effect secondary to another undetermined cause of increased drug sensitivity. This model represents a vehicle by which the etiology and treatment of severe clinical toxicity induced by RTX may be evaluated.

INTRODUCTION

RTX (ZD1694; Tomudex) is a highly specific inhibitor of TS, which is now available for the first-line treatment of advanced colorectal cancer (1–5). In Phase I clinical studies, dose-limiting toxicities were reported to be gastrointestinal (diarrhea), myelosuppression, and malaise (6). Multicentered Phase II clinical studies demonstrated activity in several types of solid tumors that led to Phase III studies in advanced colorectal cancer (versus bolus 5-FU modulated with LV; Refs. 5, 7–9). RTX or bolus 5-FU/LV are generally considered equivalent in terms of response, survival, and palliative benefits. In addition, RTX is given in a more convenient administration schedule (a single 15-min infusion every 3 weeks) than bolus 5-FU/LV (usually daily bolus × 5 days repeated every 4–5 weeks). Furthermore, treatment with RTX is associated with a significantly lower incidence of mucositis and neutropenia. However, grade III/IV diarrhea has been reported in ~14% of patients treated with RTX, which may be life-threatening when there is coexisting neutropenia (8). This has been graphically demonstrated by a recent United Kingdom Medical Research Council study that compared RTX with two forms of infusional 5-FU. Although the overall survival difference was not statistically significant between the three arms, there was a 4% toxic death rate in the RTX arm, thought to be again principally attributable to the occurrence of severe diarrhea complicated by neutropenia (10). Although in the hands of clinicians experienced with using RTX such toxicities are generally manageable, these infrequent, but serious side effects are obviously important issues for the generalized use of this compound.

Preclinical studies had predicted that RTX would be active when given infrequently because it is an excellent intracellular substrate (K_m, ~1 μM) for FPGS (1, 11). This enzyme catalyzes the sequential addition of extra glutamates (up to five) to the γ-carboxyl of the glutamate ligand of the drug. RTX polyglutamates of high chain length (mainly tri-pentaglutamates) constitute the majority of intracellular drug (~80–98%) in preclin-
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In vitro and in vivo models (1, 12, 13). These polyglutamates are: (a) ~70-fold more potent TS inhibitors compared with the parent drug; and (b) not readily effluxed from the cell. Thus, retention of the drug in tumors (and some normal tissues) may result in TS inhibition continuing after the majority of the drug has been cleared from the plasma. In mice bearing the L5178Y lymphoma (i.m. implanted), high tumor: plasma RTX ratios have been measured several days after a single bolus injection of the drug (14). Similarly in the ascitic L1210:ICR tumor, TS was inhibited for at least 24 h after a single i.v. injection of RTX (1). High levels of polyglutamates are also formed in the liver and normal proliferating tissues, such as the intestinal epithelium (2).

The therapeutic efficacy of TS inhibitors is believed to be dependent on a number of factors, which include the relative inhibition of TS in tumor versus normal proliferating tissues and the relative time for induction of DNA damage and/or irreversible commitment to cell death. Adequate inhibition of TS by RTX to give a cytotoxic response, in tumor and normal proliferating tissues, is dependent on the level of expression of TS, and the enzymes responsible for formation and breakdown of the polyglutamates (the reduced-folate carrier, FPGS, and folylpolyglutamate hydrolase). TS inhibition may be circumvented altogether by an active thymidine (dThd) salvage pathway. Inhibition of TS, in the absence of significant dThd salvage, results in depletion of TTP and accumulation of cells in the early S-phase of the cell cycle (15–18). The highly cytotoxic nature of TS inhibitors, such as RTX, is believed to be attributable to, at least in part, the concomitant elevation in the level of dUTP that occurs when TTP is decreased (19). The large rise in the dUTP:TTP ratio leads to the misincorporation of uracil into DNA and damage during either synthesis or repair, or both (19–21). Cells eventually become committed to the apoptotic cell death pathway. Thus numerous factors, including those downstream of TS inhibition, may determine response to RTX (22–24).

A wide program of research has been initiated in this laboratory to identify clinical determinants of response and toxicity to RTX. As part of this research, we have investigated the interesting observation that RTX has a significantly lower MTD in BALB/c (~10 mg/kg/day × 5 days) compared with DBA2 mice (>500 mg/kg/day × 5 days). This has now been ascribed to increased sensitivity of the gastrointestinal tract to RTX-induced damage, although the genetic factors responsible for this predisposition remain to be elucidated. Further investigation of this model system should contribute to an understanding of the underlying reason(s) for a small minority of patients experiencing severe, RTX-related gastrointestinal toxicity and provide a vehicle by which rescue procedures may be evaluated.

MATERIALS AND METHODS

Drug Administration and Body Weight Measurements. RTX was provided by Zeneca Pharmaceuticals as a powder and dissolved in 0.05 M NaHCO₃, and the pH was adjusted to ~9 by dropwise addition of 1 M NaOH. Solutions were sterile filtered by passing through a Minisart, 0.2-μm-pore filter size (Sartorius AG, Epsom, Surrey, United Kingdom), frozen, and protected from light. Mice were injected i.p. once a day for up to 5 days. Drug concentrations were calculated so that mice received 0.1 ml solution per 10 g of body weight. Control mice received solvent alone.

Male BALB/c and DBA2 mice were purchased when 5–6 weeks of age from either the Oxford Laboratory Animal Colonies (Oxford, United Kingdom) or the Medical Research Council animal laboratories (Mill Hill, London, United Kingdom) and maintained on a standard diet (RM1E; Special Diets Services, Witham, Essex, United Kingdom) and water ad libitum. They were randomly grouped into cages of five mice and used when 7–8 weeks of age. Daily weights of individual mice were recorded, and mice inspected for signs of distress. Mice were sacrificed if they could not freely access food and water and/or had >30% body weight loss.

Histopathology of the Small and Large Intestine. Mice were injected with 10, 100, or 500 mg/kg of RTX for up to 5 days (days 1–5), and five mice from each group were killed by cervical dislocation 24 h after each injection (days 2–6). In some experiments, mice given 10 mg/kg/day × 5 days were killed 48 and 72 h after the fifth dose (days 7 and 8). The whole length of the intestine was removed and flushed with Tris-HCl buffer (pH 10), using a 5-ml syringe and a pipette tip inserted through the proximal cut end of the intestine (junction with stomach). The specimen was placed in modified Methacarn consisting of 60% methanol, 30% Inhibisol, and 10% acetic acid, and left for at least 24 h. Tissues were fixed and embedded in paraffin, and sections stained with H&E were prepared. A graded scoring system was devised on a number of parameters. Villus atrophy was graded as follows: 0, normal; 1, clubbing; 2, atrophy +; 3, atrophy ++; 4, atrophy +++; and 5, tip necrosis. Edema of lamina propria, inflammatory cells, and crypt abscesses (necrotic cells in dilated crypts) were graded as either present or absent. The number of goblet cells were recorded as either normal or increased. The mitotic index was measured as the number of mitosis per five high-powered fields.

The architecture of the small intestinal crypts was included as an additional measure of cytotoxic effect (23). The number of cells/half crypt was measured by counting the number of cells from the crypt base to the crypt-villus junction down one side of an adequately sectioned crypt. Twelve crypts/mouse (60 per treatment group) were counted, two from each of six intestinal sections/mouse. More severe crypt damage was associated with cell loss and lower counts. In severely damaged sections, it was often difficult to find adequate crypts for cell counting; hence, this score was complemented by recording the number of “normal-looking crypts” per bowel section.

Hematology. Groups of five mice received injections of RTX: BALB/c mice (5 mg/kg) and DBA2 (5 and 100 mg/kg/day × 5 days). Control mice received 0.05 ml NaHCO₃. Blood for full blood count and differential white cell count was collected by open cardiac puncture under oxygen/halothane anesthesia (without recovery) into a 1-ml syringe and transferred to a 1.5-ml microcentrifuge tube containing 2% EDTA to give a dilution of 10 μl of anticoagulant per 100 μl of blood. Analysis was performed at the Royal Marsden Hospital Trust Clinic Hematology Laboratory.

Plasma dUrd and dThd Measurements. Extraction and analysis of mouse plasma samples were by HPLC using a modification of a method described previously (25, 26). Briefly,
this involves a two-stage (separated by peak collection, lyophilization, and reconstitution in a small volume) HPLC isocratic method with UV detection. Briefly, 250 μl of plasma samples were treated with two volumes of acetonitrile, vortexed for 1 min, and placed on ice for 5 min, prior to centrifugation at 11,000 × g for 10 min. The supernatants were lyophilized (Savant SVC 200H refrigerated speed vacuum pump-IEC) and later reconstituted in 125 μl of the HPLC running buffer and centrifuged again for 5 min at 6,000 × g to remove particulate material prior to HPLC injection of 100 μl. An Apex C-18 column was used in the second HPLC run because this resulted in improved peak shape and separation as compared with the Apex ODS Symm 5 μm packing (Jones Chromatography, Ltd.) reported previously.

**Plasma Folate Estimation.** Blood samples were collected in pediatric (3 ml) Vacutainer bottles each containing 60 μl of EDTA and microcentrifuged, and the plasma was removed and stored at −20°C until analyzed. Red cell folate and plasma folate were measured using a Folate Radioassay kit [125I] obtained from Becton Dickinson, Ltd. (Oxford, United Kingdom).

**Measurement of [5-3H]RTX and Its Polyglutamate Forms in Mouse Tissues.** Radiolabeled RTX (19 Ci/mmol) was supplied by Zeneca Pharmaceuticals (Alderley Park, Macclesfield, Cheshire, United Kingdom). This was purified 24 h before use by a method described previously (13). Three mice were injected with 5 mg/kg RTX (1.1 Ci/mmol), and the tissues were removed 24 h later and prepared as described previously (2). Analysis was by HPLC (ion pairing), using synthetic polyglutamate standards to identify the peaks, and scintillation counting of collected fractions (13).

**Plasma and Tissue RTX (Polyglutamate) Levels Measured by RIA.** A polyclonal antibody raised to RTX was provided by Zeneca Pharmaceuticals. This was used to measure plasma RTX as described previously (14). This antibody cross-reacts equally with the polyglutamate forms of RTX and was therefore used to measure total tissue drug levels after injection of RTX to mice by a method described previously (14).

**RESULTS**

**Effect of RTX on Mouse Body-Weight Loss**

Neither male BALB/c nor DBA2 mice lost body weight or displayed any signs of toxicity after a single injection of 50 mg/kg RTX (data not shown). However, body weight loss was observed during and/or after completion of a course (≥2 days) of daily injections of various doses of RTX. At 10 mg/kg/day × 5 days, very little reduction in body weight was observed in mice of either strain over the first 48 h (controls gained some weight; Fig. 1). However, over the next 24 h, the BALB/c, but not DBA2, mice lost a significant amount of weight (day 4; ~93% of starting weight). Weight loss continued so that 24 h after completion of the course of five injections of 10 mg/kg (day 6), BALB/c mice weighed 79% of their initial weight. In contrast, DBA2 mice were ~95% of their initial body weight by this time. BALB/c mice continued to lose further weight up to a nadir of day 8 (74% of initial weight). About 10% of BALB/c mice receiving this 10-mg/kg/day dose became moribund and were culled (days 7–9). In addition, the majority of BALB/c, but not DBA2 mice, displayed evidence of diarrhea for 1–3 days (commencing on days 4–5). However, the rapidity of weight loss over the first 7 days was similar in those BALB/c mice that did not get diarrhea (data not shown).

The effect of other doses of RTX on mouse body weight are also given in Fig. 1. For example, the lower dose of 5 mg/kg/day × 5 days to BALB/c mice induced slightly less body weight loss (nadir day 7, rather than day 8), and all mice recovered. Increasing the dose to 50 mg/kg/day × 5 days induced severe weight loss such that the experiment was terminated on day 8. This and higher doses of RTX were tolerated by DBA2 mice, although the weight loss seen for the first 4–5 days was similar at all doses between 10 and 500 mg/kg/day × 5 days (and interestingly, less than in BALB/c mice seen at 10 mg/kg/day). After this time, DBA2 mice that had received the higher doses (100 and 500 mg/kg) of RTX lost increasingly more body weight with a nadir at day 9 (77% of initial weight). Comparison of these higher dose data for DBA2 mice with that of BALB/c mice injected with the lower 10 mg/kg/day × 5 days dose demonstrates similar weight loss, at their respective nadirs. However, the nadir was actually 1 day later for the DBA2 mice, and the rate of weight recovery was slower (starting weight regained by days 14–16 compared with ~day 12 for BALB/c mice). In fact, the length of time that DBA2 mice (100 or 500 mg/kg) weighed less than their starting weight was 2/3 days longer than for BALB/c mice (10 mg/kg). The MTD (defined here as the dose from which all mice recover) is ~5 and >500 mg/kg/day × 5 days for the BALB/c and DBA2 mice, respectively. Diarrhea was not observed in DBA2 mice at any dose or time.

Coadministration of thymidine (dThd; 500 mg/kg three times a day for 8 days) with RTX (5 mg/kg/day × 5 days) to BALB/c mice almost completely prevented the drug-induced weight loss (8% compared with 28% for RTX alone; data not shown).

**Effect of RTX on Mouse Intestinal Histopathology**

10 mg/kg/day. Comparative histopathological studies on the small and large intestine of both strains of mice were performed during and after administration of 10 mg/kg/day RTX × 5 days in both strains of mice. The small intestine was much more affected than the colon by the toxic effects of RTX. However, some evidence of minor damage (characterized by damage and shortening of the crypts) was seen in the colon of BALB/c, but not DBA2, mice (measured on day 6). There was also a reduction in the number of colonic crypt mitoses on days 3–7 in both mouse strains (nadir day 6; ~20% of day 1 mitoses; data not shown). These had recovered to above pretreatment levels by day 8 (72 h after the fifth injection). Minimal damage to the small intestine of both mouse strains was observed on day 2 (characterized by a small but statistically insignificant reduction in the number of crypt mitoses, some villus blunting, and minimal edema), which became more marked from day 3 (Fig. 2 and data not shown). Additionally, an inflammatory cell infiltrate in the lamina propria and crypt abscesses were observed from this time, with 100% of BALB/c and DBA2 mice showing evidence of the latter on days 3 and 5, respectively. Close examination of the number of “normal-looking” crypts/crypt circumference and the mean number of cells per hemicrypt revealed a significant reduction in the level of both parameters.
on days 3–6 (later times not measured) that was greater in the small intestine of BALB/c mice (data for day 6 given in Table 1). This damage was also characterized by a general villus atrophy that was apparent 24 h after the first injection of RTX and became progressively worse over the next 2 days (Fig. 2). This villus atrophy was greater in BALB/c compared with DBA2 mice from day 5 (statistical significance was only attained on days 5, 7, and 8). Sample histological sections taken from mice on day 7 are shown in Fig. 3. The difference between the two mouse strains was greatest on day 8 (72 h after the fifth injection) because of a significant improvement in the architecture of the villi of the small intestines of DBA2 mice at this time (and no evidence of crypt abscesses). Although the condition of the villi had not markedly improved in BALB/c mice, the number of BALB/c mice with inflammatory cells or crypt abscesses was reduced (~50%), and the crypt mitotic index had risen to above control levels (Fig. 2). Taken together, these data suggest that 10 mg/kg/day × 5 days induces more histopathological consequences of damage to the small intestine of BALB/c compared with DBA2 mice, and that on day 7, and more particularly on day 8, recovery was significantly more marked in the DBA2 mice. This recovery is coincident with some increase in body weight in this mouse strain, consistent with weight loss (and possibly diarrhea) being a result of gut damage.

**100–500 mg/kg/day.** The degree of villous atrophy and number of DBA2 mice with crypt abscesses or an inflammatory infiltrate on days 2, 5, and 6 after the 100-mg/kg dose was similar to that seen at 10 mg/kg and also to that in BALB/c mice after 10 or 100 mg/kg (data not shown). However, there was a significantly lower number of “normal-looking” crypts/crypt circumference and mean number of cells per hemicrypt at the

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**Fig. 1** Body weight loss in DBA2 and BALB/c mice after various doses of RTX given as an i.p. daily bolus for 5 days. **A**, DBA2 mice. ■, control; ▲, 10 mg/kg/day (also shown by a dotted line); ▼, 50 mg/kg/day; ●, 100 mg/kg/day; ○, 500 mg/kg/day. **B**, BALB/c mice. ■, controls; ▼, 1 mg/kg/day; ▲, 2.5 mg/kg/day; ●, 5 mg/kg/day; ○, 10 mg/kg/day (also shown as a dotted line). Mice were weighed daily, and their body weight was expressed as a percentage of their initial weight before administration of RTX. Bars, SE.
100-mg/kg dose compared with the 10-mg/kg dose in DBA2 mice (measured on day 6 only; Table 1). Nevertheless, the score at 100 mg/kg was equal to that of the BALB/c mice at both the 10- and 100-mg/kg doses. Similar results were seen when the number of crypt cells/half crypt were counted. Thus, 100 mg/kg/day × 5 days of RTX given to DBA2 mice produced historical damage to the small intestine similar to that seen at 10 and 100 mg/kg in BALB/c mice (day 6). However, DBA2 mice still lost less weight than BALB/c mice at this time. Although the small intestine of DBA2 mice showed some signs of recovery by day 8 (some improvement in villus atrophy score and a normal mitotic index), improvement was less than that observed in DBA2 mice that had received the lower dose of 10 mg/kg daily (data not shown). However, at the higher dose, histological consequences of damage (villus atrophy) on day 8 were still less in DBA2 compared with BALB/c mice at 10 mg/kg (similar body weight loss). Despite this improvement, DBA2 mice started to recover body weight later and less rapidly.

DBA2 mice tolerated (as measured by recovery of body weight and absence of moribundity) 500 mg/kg/day RTX, despite much greater small intestinal crypt “damage” and reduction in number of cells per crypt (day 6) than seen at the 100-mg/kg/day dose (Fig. 1 and Table 1). This damage was also greater than that seen in BALB/c mice after daily 10 or 100 mg/kg/day of RTX. However, villus atrophy was not increased at the 500-mg/kg dose, and there was significantly less weight loss in DBA2 mice over the first 4–5 days compared with BALB/c mice given 10 mg/kg. Furthermore, DBA2 mice did not get diarrhea at any dose of RTX.

**Effect of RTX on Peripheral Blood Elements**

The effect of 5 mg/kg RTX, given on days 1–5 inclusive, on peripheral blood elements was measured on days 5, 8, and 12. Some small but statistically significant changes were seen on day 5 (measured in BALB/c only), i.e., a ~50 and 20% reduction in the number of lymphocytes and platelets, respectively, and a 2-fold increase in the number of neutrophils. By day 8, the only blood element remaining different from controls was the platelets (~25% reduction in both strains; Table 2). None of these changes are large and are considered unlikely to have any biological consequence. Because DBA2 mice could tolerate 100 mg/kg/day × 5 days, the effect of this dose was measured in this strain. RTX induced significant neutropenia (95% reduction in neutrophils) and a fall in lymphocytes (37% reduction) and platelets (55%) on day 8 (Table 2). Recovery was evident by day 12, although the neutrophils were raised nearly 6-fold compared with controls, consistent with a rebound phenomenon (data not shown).

**Plasma dThd, dUrd, and Folate Levels**

Pretreatment levels of dThd and dUrd were ~1 μM in the plasma of both mouse strains. Twenty-four hour after administration of 5 mg/kg of RTX, there was a 50% fall in the level of plasma dThd in both strains (Fig. 4). However, 24 h after completion of the 5-day course of RTX (day 6), the level of dThd had actually returned to control level in BALB/c mice but remained 50% reduced in the DBA2 mice (P < 0.05). Interestingly, this had not recovered to pretreatment levels 7 days after treatment (day 12), and because of a small second fall in dThd in BALB/c mice, both strains did not have significantly different levels at this time. In both mouse strains, plasma dUrd increased 3- and 5-fold on days 2 and 6, respectively, consistent with TS inhibition, and had recovered to pretreatment levels by day 12.

Pretreatment plasma folate levels were not significantly different between BALB/c and DBA2 mice (42 ± 28 and 34 ± 22 ng/ml, respectively). Similarly, red cell folate was 678 and 761 ng/ml, respectively.

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### Table 1 Histopathological changes to the crypts of small intestines from DBA2 and Balb/c mice 24 h (day 6) after injection of RTX daily × 5 days (days 1–5)

<table>
<thead>
<tr>
<th>RTX dose mg/kg</th>
<th>No. of normal-looking crypts/circumference</th>
<th>No. of cells/half crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c 10</td>
<td>5.1 ± 1.3</td>
<td>14 ± 2.0</td>
</tr>
<tr>
<td>Balb/c 100</td>
<td>4.7 ± 2.6</td>
<td>14 ± 2.3</td>
</tr>
<tr>
<td>DBA2 10</td>
<td>9.5 ± 1.9</td>
<td>17 ± 1.6</td>
</tr>
<tr>
<td>DBA2 100</td>
<td>5.4 ± 2.6</td>
<td>13 ± 2.5</td>
</tr>
<tr>
<td>DBA2 500</td>
<td>0.4 ± 0.36</td>
<td>8.0 ± 1.8</td>
</tr>
</tbody>
</table>

*Balb/c controls = 59 ± 7.4 and DBA2 controls = 56 ± 15 (different experiment).

Balb/c controls = 20 ± 1.6; DBA2 controls = 23 ± 2.2 (different experiment).

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**Fig. 2**  Histopathological changes in the small intestines of DBA2 and BALB/c mice after injection of 10 mg/kg RTX daily × 5 days. A, mitotic index; B, villus atrophy. ▲, DBA2; ■, BALB/c. Villus atrophy was graded as follows: 0, normal; 1, clubbing; 2, atrophy +; 3, atrophy ++; and 5, tip necrosis. Bars, SD. *; statistically significant difference (P < 0.05).
Plasma and Tissue RTX Levels

Mice injected with 5 mg/kg [5-3H]RTX accumulated very high levels of drug in the tissues (kidney, liver, and small gut) relative to the plasma (≈10 nM) 24 h after treatment (Fig. 5). Highest levels were seen in the liver (0.7–0.9 nmol/g), and although there was no statistical difference, there was a suggestion of lower levels of the longer chain-length polyglutamate forms in DBA2 liver. Overall, polyglutamates (di-hexa) accounted for 70% of the total drug extracted. Small intestinal epithelium accumulated less drug (0.3–0.4 nmol/g), and 80% was recovered as polyglutamates (mainly tetra and penta). Similar results were observed in all sections of the intestine, includ-

Table 2  Effect of RTX on blood elements (day 8)

<table>
<thead>
<tr>
<th></th>
<th>Neutrophils$^a$</th>
<th>Lymphocytes$^a$</th>
<th>Platelets$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Balb/c</td>
<td>2.1 ± 0.57</td>
<td>7.1 ± 0.63</td>
<td>1210 ± 75</td>
</tr>
<tr>
<td>5 mg/kg Balb/c</td>
<td>2.1 ± 1.2</td>
<td>8.9 ± 4.3</td>
<td>911 ± 75$^c$</td>
</tr>
<tr>
<td>Controls DBA2</td>
<td>1.7 ± 0.92</td>
<td>6.7 ± 2.5</td>
<td>1449 ± 73</td>
</tr>
<tr>
<td>5 mg/kg DBA2</td>
<td>1.4 ± 0.77</td>
<td>4.1 ± 1.7</td>
<td>1100 ± 104$^c$</td>
</tr>
<tr>
<td>100 mg/kg DBA2</td>
<td>0.08 ± 0.10$^c$</td>
<td>5.0 ± 1.4</td>
<td>650 ± 71$^c$</td>
</tr>
</tbody>
</table>

$^a$ × 10$^{9}$/liter.  
$^b$ × 10$^{12}$/liter.  
$^c$ Statistically different from control mice.

Fig. 3  Histological sections of small intestine from BALB/c and DBA2 mice 48 h (day 7) after injection of 10 mg/kg RTX daily × 5 days. A, BALB/c controls (×10). B, DBA2 controls (×10). C, BALB/c: RTX (×10); marked blunting and shortening of villi with interstitial edema and karyomegaly of epithelial cells. D, DBA2: RTX (×10); some blunting and shortening of villi with interstitial edema and karyomegaly of epithelial cells. E, BALB/c: RTX (×25); marked inflammatory cell infiltrate in the villus cores; marked interstitial edema and loss of epithelial cells. F, DBA2: RTX (×25); marked inflammatory cell infiltrate in the villus cores; some interstitial edema and loss of epithelial cells.
ing the colon (data not shown). A significantly higher \( (P < 0.05) \) concentration of total drug was measured in DBA2 kidney (0.51 compared with 0.34 nmol/g), attributable to a higher level of each polyglutamated form. A similar experiment was carried out on day 4 (24 h after three injections of 5 mg/kg; data not shown). There was a \(-3\)-fold increase in total drug present compared with that on day 2 in the liver and small intestinal epithelium. Although there was a trend toward higher drug levels in the liver and gut of BALB/c compared with DBA2 mice, no statistical significance could be attached to this because of the small number of mice examined (two/group). Similarly, the distribution of polyglutamates could not be considered different.

Using RIA, additional studies were performed where plasma RTX and tissue RTX (including polyglutamates) were measured in BALB/c and DBA2 mice during and after a course of 10 mg/kg RTX daily \( \times \) 5 days. One day after the first injection, the plasma level (measured by RIA) was similar in both strains (0.007 and 0.014 nmol/ml for BALB/c and DBA2 mice, respectively; \( P = 0.06 \)), and the small intestinal epithelium and liver levels were \(-30-\) and \(-200-\) fold higher, respectively \( (-0.3 \) and \( 2.0 \) nmol/g; Fig. 6). However, no significant difference in these levels was apparent between strains. By day 3 (24 h after two injections), levels in the plasma, liver, kidney, and small intestinal epithelium of DBA2 mice had risen slightly \( (-2\) -fold higher in the epithelium compared with day 2). However, a similar increase was not seen in BALB/c mice, leading to a statistically significantly higher drug level \( (-2\) -fold) in most of the tissues of DBA2 mice on day 3. However, by day 4, because of an increased accumulation of drug in the tissues of BALB/c but not DBA2 mice, the level in the tissues was similar in both mouse strains with the exception of BALB/c intestinal epithelium, which had a statistically significantly higher level \( (-2\) -fold) at this time. Further accumulation occurred in this tissue of BALB/c mice, leading to an \(-4\) -fold higher level compared with DBA2 epithelium on days 5 and 6. In addition, a statistically significantly higher concentration of RTX was measured in the plasma of BALB/c mice on days 4–6. Coincident with the peak level seen in the plasma (day 6), there was a significantly higher drug concentration (2-fold) in the kidney of BALB/c mice. No significant difference was seen in the liver drug levels at any time. Two days after completion of the 5-day course of injections (day 7), RTX still persisted in all tissues, although some small reductions in drug levels were observed. However the reduction in the plasma, small intestine, and kidneys was relatively greater in BALB/c compared with DBA2 mice, which had the effect of reducing the difference in drug levels between the two strains.

BALB/c mice that displayed evidence of diarrhea had a higher concentration of RTX in the small intestinal epithelium (day 5) compared with those that did not (3.8 \( \pm \) 2.2 and 2.2 \( \pm \) 0.7 nmol/g, respectively), although this did not quite reach statistical significance \( (P = 0.07) \). Also there was a nonsignificant trend for mice with more severe weight loss (day 5) to have higher RTX levels in the mucosa (day 5). The weight of scraped mucosa per unit length of small intestine was reduced significantly in BALB/c mice after treatment with RTX so that on day 7, the mean weight was 7.5 \( \pm \) 3.4 mg/cm compared with 18.5 \( \pm \) 3.4 mg/cm (five mice/group). However, the protein content per gram of wet tissue did not change (55 \( \pm \) 29 and 50 \( \pm \) 17 mg/g, respectively). This suggests that the increased drug level measured in BALB/c mice is not a direct result of decreased water content.

After administration of a 10-fold higher dose of RTX (100 mg/kg), the plasma concentration increased by \(-5-\)10-fold in both mouse strains. Liver and small intestinal epithelia drug levels rose \(-1-2\) -fold and \(-2-8\) -fold, respectively (Fig. 7 and data not shown) compared with those seen after 10 mg/kg. Otherwise, the time course and pattern of changes were similar to those seen at 10 mg/kg. Comparison of the drug accumulation data for the small intestinal epithelium at 10 mg/kg/day \( \times \) 5 in BALB/c mice with that at 100 mg/kg/day in DBA2 mice (Fig. 7) demonstrated that the RTX level in DBA2 epithelia was \(-2-7\) -fold higher on days 2 and 3 and comparable on days 4 and 5 (although \(-2\) -fold lower on day 6). Furthermore, DBA2 given 500 mg/kg/day \( \times \) 5 days had small intestinal drug levels (measured on days 4–6) \(-5\) -fold higher than those found in the small
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discussed. Consistent with both of these observations is the fact that dThd phosphorylase-treated mice are more sensitive to folate-based TS inhibitors (29). Otherwise, repeated injections of TS inhibitors such as CB3717 and RTX are required to induce an antiproliferative effect in mice, which may be attributable to, at least in part, the need to reduce the plasma concentration of dThd (27).

Of interest has been the observation that BALB/c mice are more susceptible than DBA2 mice to the toxic effects of RTX with MTDs of -5 and >500 mg/kg, respectively, when given in a daily × 5 regimen. This very large difference appears to be attributable to an increased sensitivity of BALB/c mouse intestine to RTX. At a dose of 10 mg/kg/day × 5, greater weight loss, evidence of diarrhea, and low tolerance (moribundity) was associated with more marked histopathological changes to the small intestine of BALB/c mice compared with DBA2 mice. The effects observed are related to inhibition of TS, as shown by the fact that coadministration of dThd largely prevented the majority of the weight loss induced by RTX in BALB/c mice. Increasing the dose of RTX administered to DBA2 mice to 100 mg/kg/day × 5 led to weight loss and histological consequences of damage (inhibition of proliferation and/or tissue degeneration attributable to cell death) that was comparable with that seen in BALB/c mice at the lower dose. However, the weight loss was less rapid, had a later nadir (day 9 rather than day 8), recovered more slowly, and was not associated with diarrhea. Furthermore, there was evidence of a greater improvement in the villus architecture of DBA2 compared with BALB/c mice by day 8. This suggests that toxicity to another organ may be contributing to the slow recovery of weight in DBA2 mice at 100 mg/kg. Similarly, the reason for the higher 100-mg/kg dose not being tolerated in BALB/c mice (small intestinal histology similar at both doses) may relate to the combined effects of a higher susceptibility of these mice to intestinal damage and the toxic effects of RTX to another organ. This toxicity could be, for example, to another part of the gastrointestinal tract, giving rise to malabsorption or anorexia, or to the bone marrow. Indeed, at 100 mg/kg daily × 5, severe neutropenia was seen in DBA2 mice (day 8).

Thus, the relevant features of this mouse strain toxicity difference to be explained is the earlier weight loss, the presence of diarrhea, and the greater histopathological evidence of damage at an equivalent 10 mg/kg/day dose in BALB/c mice. Interestingly, DBA2 mice given 500 mg/kg/day × 5 had greater crypt changes and equivalent villus atrophy (day 6) in the small intestine compared with BALB/c mice given 10 mg/kg/day × 5. Nevertheless, this high dose was tolerated and not associated with early weight loss or diarrhea, suggesting that: (a) small intestinal damage, as measured by histopathological changes, in BALB/c mice is accompanied by, but not directly linked to, severe functional damage to this organ in this mouse strain at the cellular level; and/or (b) the small intestine is not the only area of the intestinal tract to be more sensitive to damage induced by RTX in BALB/c mice. Consistent with the latter is the observation that in BALB/c mice given 10 mg/kg/day × 5, mild histopathological changes to the colon were observed that were not seen at any dose in DBA2 mice. Thus, it may be these types of damage to the gut that account for the differences in toxicity between these two strains.

### DISCUSSION

RTX is a potent cytotoxic antitumor drug that specifically targets TS. However, inhibition of this enzyme also leads to some toxicity to normal proliferating tissues, particularly gut and bone marrow in mice, dogs, and humans (6, 8, 14). A single injection of RTX to either BALB/c or DBA2 mice induced some mild histological changes to the small intestinal epithelium, which did not manifest as toxicity using parameters such as body weight loss or diarrhea. Previous studies identified the high concentration of plasma dThd in this species, affording a protective effect for TS inhibitors (27). Pressacio et al. (28) have also demonstrated induction of nucleoside transport and thymidine kinase activity after TS inhibition in tumors, which may be a common feature of proliferating tissues. Consistent with both of these observations is the fact that dThd phosphorylase-treated mice are more sensitive to folate-based TS inhibitors (29). Otherwise, repeated injections of TS inhibitors such as CB3717 and RTX are required to induce an antiproliferative effect in mice, which may be attributable to, at least in part, the need to reduce the plasma concentration of dThd (27).

Fig. 5 Mouse tissue level of RTX and its polyglutamate forms 24 h after a single i.p. injection of 5 mg/kg [5-3H]RTX. Mouse tissues were removed and rapidly frozen 24 h after administration of 5 mg/kg [5-3H]RTX (1.1 Ci/mmol). After the drug and polyglutamates were extracted, quantification was by HPLC (ion pairing), fraction collecting, and scintillation counting using synthetic polyglutamate standards. Results are represented as the means (n = 3 mice); bars, SE. Total RTX levels (inset) are means; bars, SD.

intestine of BALB/c mice after 10 mg/kg (Fig. 7). This does not necessarily reflect the distribution of polyglutamate forms.
of effects, which when combined with the small intestinal effects common to both mouse strains, that produces the severe nontolerated consequences in BALB/c mice.

The fact that the concentration of peripheral blood elements was not suppressed more in BALB/c than DBA2 mice at the 10-mg/kg/day dose of RTX is consistent with the difference in drug sensitivity between the two strains being confined to the gastrointestinal tract. Furthermore, it seems unlikely that systemic factors such as baseline plasma dThd or folate levels are directly responsible, because measurement of pretreatment levels of dThd or folate levels demonstrated no difference. dThd fell by ~50% (and dUrd increased 3-fold) after the first treatment in both strains, suggesting that TS inhibition was occurring (not necessarily to an equal degree). This reduction in salvageable dThd is believed to be attributable to the inhibition of TS in proliferating tissues, which both reduces the amount of dThd nucleotides/nucleosides produced and increases the salvage of dThd from the plasma. In DBA2 mice, the dThd level remained at this reduced level after the fifth injection of RTX (day 6). However, the dThd level in RTX-treated BALB/c mice had returned to the pretreatment level. These data are contrary to what might have been predicted from the increased toxicity seen in BALB/c mice, but it could be argued that increased intestinal tissue damage was contributing toward increased plasma dThd in the BALB/c strain. Alternatively, it could indicate a higher rate of dThd salvage in the DBA2 mouse intestinal epithelium, which in turn could be responsible for the difference in tolerance to RTX. The reduced dThd level in both strains at day 12, when both body weight and dUrd had recovered, could be attributable to increased proliferation and hence demand for DNA synthesis during this period.

RTX is rapidly eliminated from the plasma of mice, although a prolonged terminal phase of elimination is evident (14). However, polyglutamate retention in tissues such as liver, kidney, and intestinal epithelium leads to high tissue:plasma ratios that persist for several days (2, 14). This accounts for the delay in the recovery from the toxic effects of RTX after completion of 5 days of injections. Potentially, differences in drug uptake and/or polyglutamation in the intestinal epithelium between mouse strains could explain the differences observed in drug sensitivity. Indeed, increased weight loss and more marked changes to histology in
BALB/c compared with DBA2 mice at the 10-mg/kg daily dose was associated with (but not necessarily caused by) increased drug levels in the small intestinal epithelium of BALB/c mice between days 4 and 7. However, it is highly relevant to note that on day 3, the gut epithelium from BALB/c mice did not have higher drug levels than from DBA2 mice (in fact, the reverse was true). Nevertheless, greater histopathological changes were observed on day 3 and more notably on day 4, consistent with greater damage in BALB/c small intestine. This was associated with greater weight loss in BALB/c mice by day 4. We cannot, therefore, eliminate the possibility that the higher drug levels measured at later times are an “effect” rather than a “cause” of greater damage. For example, reduced proliferation/more damage to the small intestine of BALB/c mice may result in higher drug levels per gram of tissue. Alternatively, drug-induced functional changes that may lead to diarrhea, coupled with dehydration and weight loss, may compromise renal function, leading to retention of the drug in the circulation and further uptake into the intestinal tissue.

Relevant is the fact that over the first 3 days of treatment, the drug levels were found to be higher in the small intestinal epithelium of DBA2 mice given 100 mg/kg/day than in BALB/c mice given 10 mg/kg/day, but this was not coupled with histopathological differences (measured on days 2, 5, and 6). Perhaps the most convincing evidence that higher drug accumulation in the small intestine is not linked to diarrhea/moribundity/early weight loss in BALB/c mice comes from the observation that DBA2 mice given 500 mg/kg/day RTX not only accumulated significantly higher (5-fold) small intestinal epithelial drug levels (measured on days 4 – 6 only) compared with BALB/c mice given 10 mg/kg/day RTX but had comparatively more “crypt damage” to the small intestine (although there was no evidence of increased villus atrophy). Nevertheless, there was less rapid weight loss and no evidence of diarrhea.

In summary, it appears that the gastrointestinal tract of BALB/c mice is susceptible to an “early phase” toxicity induced by RTX that may not be related to increased drug accumulation. Instead, this may be a colon/small intestinal dysfunction/dam-
age, leading to early weight loss and diarrhea which, when combined with the later effects induced by higher doses, causes an intolerable amount of damage and a low MTD. The causative factor may be attributable to metabolic differences, such as a reduced dThd salvage capability in BALB/c mouse intestinal epithelium, attributable to, for example, reduced thymidine kinase expression. Studies outside of the scope of this report did not show a significant difference in TS or FPGS mRNA expression or TS protein levels. These studies will be expanded to activity measurements and determining the relative contribution of the de novo and dThd salvage pathways to thymidylate synthesis in these two mouse strains. An alternative explanation and continuing area of investigation is that BALB/c mouse intestinal epithelial cells (small intestinal and/or colonic) may sense drug-induced cellular damage more readily, leading to more rapid engagement of cell death pathways. Indeed, ongoing studies suggest increased susceptibility of BALB/c small intestinal epithelium to induction of apoptosis, not only 24 h after treatment with RTX (30), but also 4.5 h after ionizing radiation (31). Regardless of the underlying mechanisms of this strain difference, a BALB/c mouse model has been described that appears to be highly relevant to the study of RTX-induced gastrointestinal toxicity in humans. Indeed, it has proved to be a useful model for studying the effects of potential rescue agents such as dThd and LV (2).

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