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Original Paper

Chronotolerance of Experimental Radioimmunotherapy: Clearance, Toxicity, and Maximal Tolerated Dose of ^{131}I-anti-carcinoembryonic Antigen (CEA) IgG as a Function of Time of Day of Dosing in a Murine Model

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The temporal variation in bone marrow proliferation has been used to help define the optimal time of day to dose with approximately 30 chemotherapeutic agents, so that treatment efficacy is maximised and toxicity is minimised. Since myelosuppression is also the dose-limiting toxicity for most forms of radioimmunotherapy, we hypothesised that time of day of administration might also influence tolerance for radioantibody therapy. Bone marrow proliferative activity in BALB/c mice was determined using cell cycle analysis of propidium iodide-stained bone marrow samples collected at 3 h intervals. Myelosuppression was determined at weekly intervals after a therapeutic dose of ^{131}I-NP-4 anti-CEA (carcinoembryonic antigen) intact IgG at either 0900 h (2 h after light onset [HALO]), 1300 h (6 HALO) or 1600 h (9 HALO). The highest bone marrow proliferative activity was noted between 20 HALO (0300 h) and 4 HALO (1100 h), and the lowest activity could be measured at 10–13 HALO (1700–2000 h). Seven days after a maximal tolerated dose (MTD) of radioantibody, granulocyte reduction was 50% at both 2 and 6 HALO and only 32% at 9 HALO ($P < 0.003$). Fourteen days after radioantibody therapy, an 87% granulocyte suppression was observed in mice treated at 2 HALO and only a 64% granulocyte loss was noted in the 9 HALO treated group ($P < 0.001$). The 2 HALO group recovered earlier than the 9 HALO group ($P < 0.013$; 22% loss from the 2 HALO dose and 40% loss from the 9 HALO dose) on day 28 post-radioimmunotherapy. The difference in magnitude of neutropenia, rather than duration, was critical for establishing the MTD. A 30% increase in the MTD was possible if mice were dosed at 9 HALO (320 μCi) versus 2 HALO (240 μCi). These studies suggest that principles of chronobiology may govern the magnitude of toxicity and the highest dose tolerated in radioantibody therapy in the same way that it does for cytotoxic drug therapy.

Key words: radioimmunotherapy, chronobiology, marrow, neutropenia, cell cycle, CEA
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INTRODUCTION

CIRCADIAN RHYTHMS have been demonstrated both in animals and in humans, in metabolically active normal tissues (cornea, gastrointestinal tract, bone marrow, gonads, epidermis), as well as in most experimental tumours (solid and ascites) and human cancers. Differences in cell division as a function of time of day account in part for the circadian variation in sensitivity of tissues to cytotoxic drugs (chronotoxicity/chronotolerance) [1]. For example, in mice peak activity of gastrointestinal mucosa occurs between 0200 h and 0700 h. Peak bone marrow activity occurs at 0200 h to 1200 h [2]. Peak activity for most spontaneous or transplantable carcinomas is between 1600 h and 2000 h. This phenomenon, primarily taken together with the chronopharmacology of anticancer drugs (e.g. circadian changes in pharmacokinetics, drug metabolism, production of glutathione) can

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account for the diurnal variability of tolerance and of antitumour activity [2, 3]. By charting rhythms in healthy and malignant tissues, it has been possible to find optimal times to deliver a dose of drug, such that it is most efficacious in killing cancer cells and least harmful to healthy cells. The tolerability of approximately 30 anticancer drugs can vary by 50% or more according to circadian rhythms in laboratory animals [4]. Indeed, therapeutic outcome has improved by several hundred per cent by treating rodents at the 'right' time under controlled laboratory conditions [5]. Clinical trials have confirmed the overall ability to deliver higher doses of chemotherapy and the improvement in clinical outcome by incorporating chronobiological principles [6].

Bone marrow toxicity is also the primary dose-limiting side-effect from most forms of low dose rate radiation associated with radioimmunotherapy (RAIT) [7–10]. In both preclinical and patient trials, prolonged (4–8 weeks) RAIT-induced granulocytopenia and thrombocytopenia have been documented with a single 2–3 Gy dose to bone marrow. The magnitude and/or duration of RAIT-induced bone marrow toxicity can be reduced by cytokine intervention [11], bone marrow transplantation (BMT) [12, 13] and haemoregulatory peptide (Hp5b) administration [14]. These three methods of haematopoietic rescue and/or protection allow for radioantibody dose intensification and/or increased dose frequency and improved therapeutic outcome in both localised and metastatic experimental tumour models.

In this study, we addressed the possibility that magnitude and duration of RAIT-induced myelosuppression could be influenced by chronobiological principles in a similar manner as has been documented for chemotherapy [4, 5]. The neutropenic effect of radioantibody administered at 2, 6, and 9 h after light onset (HALO) was determined and the results correlated with the bone marrow proliferative activity for that time of day. The maximal tolerated dose (MTD) was then determined at 2 and 9 HALO and the results correlated with induced toxicity at both times of dosing. The effect of dosing time on total body clearance and blood clearance of radioantibody was also determined.

MATERIALS AND METHODS

Animal model

Non-tumour-bearing male BALB/c mice from Taconic (Germantown, New York, U.S.A.; 5–6 weeks old or a minimum starting weight of 18 g) were used for these studies. The MTD for radioantibody is similar in BALB/c and tumour-bearing nude mice (unpublished data). Male mice were selected to avoid the influence of the oestrous cycle on tissue proliferative activity. The animals were placed in one of three 12:12 light:dark schedules as shown in Figure 1 for 3 weeks prior to the start of any study to allow for standardisation of chronobiological rhythms. Room 1 was dark from 1900 h to 0700 h, allowing sampling of the 3 HALO and 7 HALO animals at 1000 h and 1400 h, respectively. Room 2 was dark from 0300 h to 1500 h, allowing sampling of the 17, 20, and 23 HALO mice at 0900 h, 1200 h, and 1500 h, respectively. Room 3 was dark from 1000 h to 2200 h, allowing sampling of the 10 and 13 HALO points at 0800 h and 1100 h, respectively. Room temperature was kept at 21 ± 2°C. Animal care was provided in accordance with institutional guidelines.

For therapy and toxicity studies, male nude mice were used. At 6–8 weeks of age, the animals were implanted with the GW-39 human colonic tumour xenograft [15]. This tumour cell line was serially propagated subcutaneously in nude mice. Tumours of approximately 1 g were excised aseptically, and placed in Hank's balanced salt solution containing 0.08 mg/ml gentamicin. Tumours were then removed and first minced with scissors, and then transferred to a 40-mesh wire screen. The tumour was then physically pressed through the screen, and the screen was washed with phosphate buffered saline. The volume of phosphate buffered saline was adjusted to give the desired tumour cell concentration. For subcutaneous implantation, 0.2 ml of a 10% tumour cell suspension was injected. Tumours were allowed to grow for approximately 14 days or until they were 0.5–0.8 cm^3 before injecting radiolabelled antibodies.

Antibody production, radiolabelling and quality assurance

The MN-14 anti-CEA (carcinoembryonic antigen) IgG was purified from mouse ascites fluid by protein A (IPA-300, Repligen, Cambridge, Massachusetts, U.S.A.) and ion-exchange chromatography, as reported previously [16]. The purity was evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using reducing and non-reducing conditions, size-exclusion high-performance liquid chromatography (HPLC) and immunoelectrophoresis and found to be > 98% pure.

All labelled agents were prepared under sterile conditions and used on the day of preparation. The IgG was radioiodinated by the chloramine-T method, similar to the method

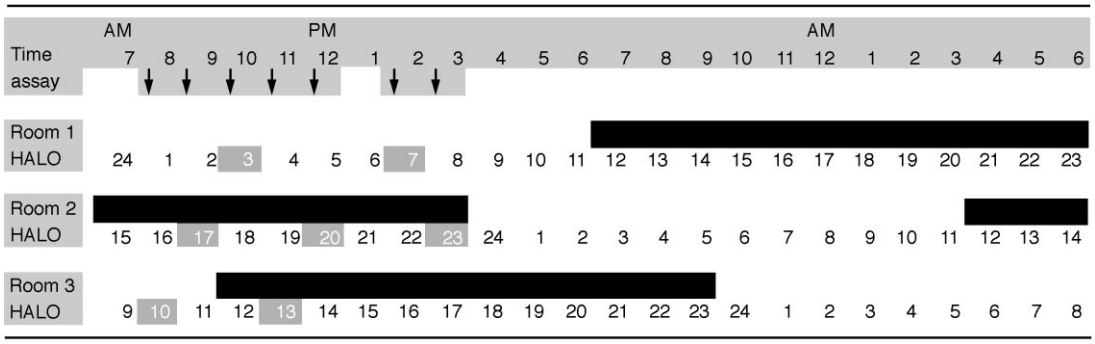

Figure 1. Light–dark cycles in three separate rooms within the animal facility that allowed for bone marrow sampling at each of seven time points (3, 7, 10, 13, 17, 20 and 23 h after light onset (HALO)) within the regular working day. All animals were on a 12:12 light:dark cycle with the 'on time' and 'off time' for lights varying between the three rooms to allow all seven HALO points above to be evaluated in an 8 h work day. Each HALO point came from one specified room as noted. Animals were kept in their respective rooms for 3 weeks prior to the start of the study in order to allow for the 12 h light:12 h dark synchronisation.

described previously [17]. The radioiodinated monoclonal antibody was separated from unbound iodine by passage over a PD-10 column (Pharmacia, Piscataway, New Jersey, U.S.A.) equilibrated in 0.04 M. Every antibody preparation was evaluated after labelling by size-exclusion HPLC and inverse thin layer chromatography (ITLC) to determine aggregates and unbound radionuclide. Immunoreactivity was assessed by determining the fraction of activity bound to a CEA-immunoadsorbent. All labelled agents were shown by HPLC to be > 95% of the native-sized material with less than 2% unbound radionuclide. The amount of unbound radionuclide was confirmed by instant thin-layer chromatography (Gelman) using acetone for iodine-labelled agents. Immunoreactivity was determined by passage of 0.1 ml of a dilution of the labelled product (approximately 100 000 cpm) over 2.0 ml of a CEA-immunoadsorbent. The CEA-immunoadsorbent was prepared by adding a partially purified preparation of CEA to 5 ml of Affigel-10 (BioRad, Richmond, California, U.S.A.) according to the manufacturer's instructions. After incubating the labelled products with the immunoadsorbent for 15 min, the column was washed with 10 ml of phosphate buffer (unadsorbed fraction) followed by 10 ml of 6.0 M guanidine–HCl and 10 ml of phosphate buffer (adsorbed fraction). The immunoreactivity of the ^{131}I-IgG was 72–84% for all preparations.

Quantitation of whole-body clearance and blood clearance

For total-body dose determination, animals were placed in a Deluxe Isotope Calibrator II (Nuclear Associates, Ramsey, New Jersey, U.S.A.) at defined intervals and the total dose remaining (mCi) in the animal was recorded. Blood clearance was determined by collecting 50 µl of whole blood by retro-orbital bleeding at defined intervals and counting the blood sample in a gamma counter with a window setting for ^{131}I. The counts per min (cpm) were normalised to the cpm injected and the percentage injected dose/g blood was determined for each animal. The average ± standard deviation (S.D.) of five mice was recorded.

Cell cycle analysis

Femoral bone marrow was collected from groups of four mice at each time point. Ice-cold 70% ethanol was added to the cell pellet and covered with parafilm and stored overnight at 4°C. The sample was centrifuged for 10 min at 3000 rpm to remove excess ethanol. One millilitre of permeablising buffer (6.05 g Tris buffer + 495 ml dH_2O + 4.5 g NaCl + 73.5 mg $CaCl_2$ + 23.8 mg $MgCl_2$ + 5 ml Nonidet P-40) was added to the tumour cell pellet and incubated for 10 min at room temperature. The sample was centrifuged at 1500 rpm and the supernatant decanted. The pellet was vortexed to resuspend it in the residual buffer. One millilitre (0.5 µM) Sytox Green (Molecular Probes, Eugene, Oregon, U.S.A., 0.1 ml of 50 µM Sytox Green + 9 ml permeablising buffer + 1 ml 1000 Kunitz RNAse A; Molecular Probes) was added and incubated for 15 min at room temperature with gentle rocking. Samples were spun at 3000 rpm for 10 min and the pellet washed twice with phosphate buffered saline (0.04 M) and resuspended in 1 ml sheath fluid with 1% formalin. The samples were analysed within 24 h by flow cytometry. The percentage of cells in each phase of the cell cycle was determined with the CellFit software provided by Becton Dickinson (San Jose, California, U.S.A.). In a direct comparison with the propidium iodide staining method, we have found that the Sytox Green method gives much less variability between samples and clearer delineation between G1, S, and G2/M. Points were fitted to a 24 h cosine by the method of least squares (cosinor analysis). This curve was used to determine the acrophase (time of highest value), mesor (rhythm-adjusted mean value) and amplitude (half of the peak–trough difference).

White blood cell quantitation

A heparinised specimen was collected by retro-orbital bleeding. Red blood cells were lysed with a 5 min incubation in 1.0 ml of lysing buffer (8.26 g/l ammonium chloride: 1 g/l potassium bicarbonate: 0.0379 g/l ethylene diamine tetraacetic acid (EDTA)). The sample was spun at 2000 rpm for 10 min, and the supernatant was aspirated. The cell pellet was washed in 1.0 ml phosphate buffered saline (pH 7.2) and resuspended in 1.0 ml of phosphate buffered saline + 1% formalin for counting on a Becton Dickinson FACScan at a flow rate of 1 µl/sec. This flow cytometry system records total cells counted per unit time and forms a scattergram. The cell size (forward scatter) and internal cellular complexity (side scatter) are delineated, allowing lymphocytes (small simple cells) and granulocytes (larger more complex cells) to be enumerated. In studies that included three time points (2, 6, and 9 HALO only), the results were analysed by multiple-way ANOVA and pairwise comparisons.

Determination of the MTD

Mice were treated at either 2 HALO or 9 HALO with radioantibody. Dose escalation studies were conducted at 40 µCi increments beginning at 200 µCi and increasing to 360 µCi. The MTD was equal to the highest dose that resulted in zero lethality and less than 20% body weight loss ($n = 5$ per group).

Tumour therapy

Subcutaneous tumour growth studies were performed by determining size in three dimensions using a digital caliper immediately before radioantibody injection and at weekly intervals thereafter. Subsequent measurements of tumour size were compared with the initial measurement and the average change in size for the treatment group was recorded over time. These data were used to calculate area under the curve (AUC) and time for the tumour to double (TDT), or triple (TTT) or quadruple (TQT). Comparisons were then made between treatment groups.

Statistical analysis

In studies having 6–7 HALO points, points were fitted to a 24 h cosine by the method of least squares [18] (cosinor analysis) to find the best fit cosine curve. These curves allowed the determination of the acrophase, mesor and amplitude. For the analysis of cell counts (white blood cells, granulocytes, lymphocytes), descriptive statistics (mean, S.D.) were calculated for each treatment group at days 7, 14, 21 and 28. To compare changes due to treatment applied at the three different times (2 HALO, 6 HALO, 9 HALO), the standard *t*-test was applied. Because the response variables were counts and the quantity of interest was change in ratio (thus multiplicative effect), logarithmic transformation was used. Specifically, for day 7, 14, 21 or 28, the *t*-test was used to compare if the treatment difference (due to RAIT) applied at 2 HALO was different from that at 9 HALO. Such tests were repeated

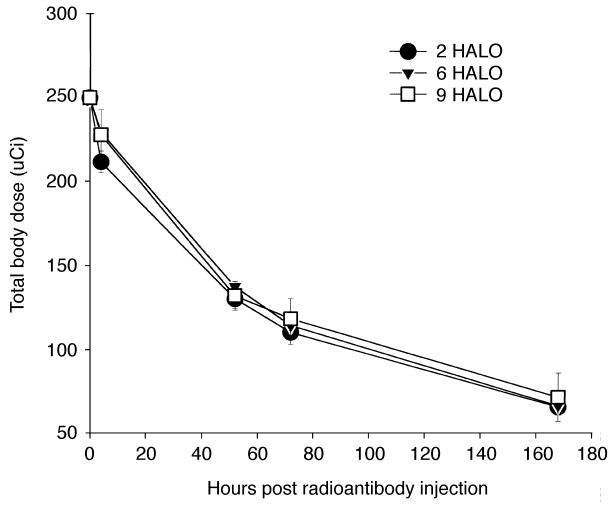

Figure 2. Whole-body clearance of intravenous injected radioantibody (240 μCi ^{131}I-NP-4 anti-carcinoembryonic antigen (CEA) IgG) into groups of five male BALB/c mice dosed at either 2, 6 or 9 h after light onset (HALO). Mice were dose calibrated immediately after injection, and again after 1, 48, 72 and 168 h. The mean ± standard deviation (S.D.) for each group of five mice was recorded.

for comparisons of 2 HALO versus 6 HALO and of 6 HALO versus 9 HALO. All the *P* values are based on two-sided tests, which are more conservative than their one-sided counterparts. For therapy studies, tumour sizes were scaled by their initial sizes. AUCs were used as the endpoints to measure treatment efficacy. Analysis was performed at days 28, 42 and 84 post-treatment. Both parametric (*t*-test) and non-parametric (Wilcoxon) tests were used.

RESULTS

Chronotolerance can encompass both rhythms associated with toxicity and rhythms associated with pharmacokinetics and metabolism. In the first set of studies, we evaluated the whole body clearance and blood clearance of a dose of radioantibody administered at 2 HALO, 6 HALO or 9 HALO in BALB/c mice. Figure 2 demonstrates that the time of day of dosing does not influence the total body clearance. Forty-eight hours after an intravenous dose of ^{131}I-NP-4 IgG (240 μCi), the average remaining body dose was 52.2% (130 ± 6 μCi), 54.8% (137 ± 6 μCi) and 52.4% (132 ± 9 μCi) of the injected material when administered at 2, 6 and 9 HALO, respectively. Total-body doses continued to overlap for the three groups, with an average of 26.2% (66 ± 5 μCi), 26.6% (66 ± 6 μCi) and 28.6% (71 ± 14 μCi) of the total injected dose remaining after 168 h. Similarly, the early and late blood clearance phases were not significantly affected by time of day of radioantibody dosing (Figure 3). One hour after radioantibody injection at 2, 6 and 9 HALO, 37.8%, 40.2% and 37.9% injected doses/g blood, respectively, were observed. By 24 h after radioantibody dosing, 19.3%, 20.3% and 21.3% of the total dose was still found in the blood compartment for each HALO dose, respectively. Therefore, the time of day of dosing does not significantly affect either blood or whole-body kinetics of the intact radioiodinated antibody. Since the three blood clearance curves overlapped, we used a single set of data to derive the dose rate (Figure 3 insert). During the first hour after a dose of ^{131}I-IgG, the dose rate was 63.5 rads/h. The rate declined to an average of 33.3 rads/h from 1 to 4 h after dosing and further declined to 17.5 rads/h during hours 5–8. Thus, even though the total dose during the first hour was low, the dose rate was very high. The extent of injury to bone marrow from this high initial dose rate may be influenced by the proliferative activity of the progenitor/stem cells.

The effect of time of day of dosing on the primary dose-limiting toxicity of RAIT was evaluated next. Other investigators have demonstrated circadian variations in bone marrow proliferation [19]. Their observations made in other murine models (e.g. BDF1 and CD2F1) were largely confirmed in the BALB/c mouse model (Figure 4) used in many of our toxicity studies. The peak percentage of bone marrow

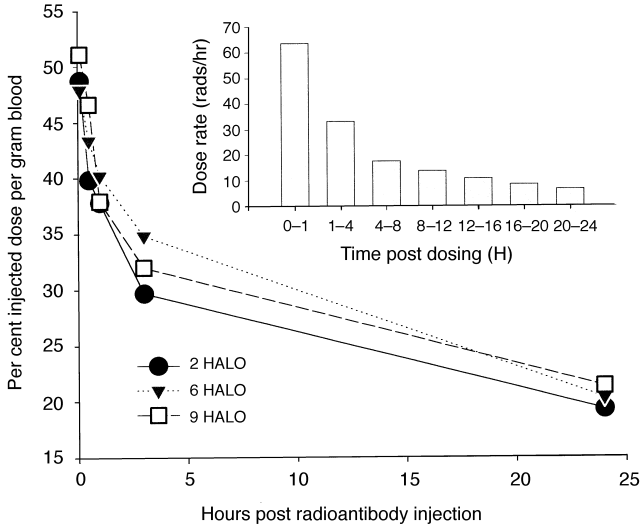

Figure 3. Blood clearance of intravenous injected radioantibody (10 μCi ^{131}I-NP-4 IgG) into groups of five male BALB/c mice dosed at either 2, 6, or 9 h after light onset (HALO). Mice were bled at 5 min, 30 min, 1 h, 4 h and 24 h after injection from the retro-orbital sinus and 50 μl samples were collected and counts per min (cpm) of ^{131}I determined in a gamma counter. The results were normalised to the total cpm injected (percentage of injected dose) and the average of five mice per group was recorded. Blood clearance data were used to calculate the dose rate (rads/h) as a function of time after the injection of radioantibody (Inset). The mean dose rate was determined for blocks of 4 h to show the exponential decline after the initial 4 h period.

cells in S phase was higher in BALB/c mice (approximately 31.5% with a mesor of 26.8%) compared with other mouse strains (18% S phase). Peak activity occurred between 20 HALO and 3 HALO, with an acrophase of 23.3 h (− 349°). The amplitude of the curve was 8.7. The activity curve showed significant variability, with a nadir of activity at 10 HALO equal to 17.5% S phase. Thus, it was possible to select several time points with distinct differences in bone marrow proliferative activity to determine how time of day of dosing, i.e. bone marrow activity, influences the magnitude of toxicity in response to a fixed dose of radioantibody (in which radioantibody clearance was not a factor).

A 240 μCi dose of ^{131}I-NP-4 IgG was administered either at 2 HALO when bone marrow has nearly 30% of cells in S phase, at 6 HALO when approximately 26% of bone marrow cells are in S phase or at 9 HALO when bone marrow is nearly at the nadir of quiescence with only 19% of cells in S phase (Figure 5). The dose given at 2 HALO resulted in a 50% loss in peripheral white blood cells on day 7 post-RAIT (Figure 5a; 1181 ± 150 cells in treated mice versus 2376 ± 544 cells in untreated mice). When the same dose was given at 9 HALO, only a 32% loss in peripheral white blood cells on day 7 after RAIT was observed (2110 ± 633 cells in treated mice versus 3110 ± 803 cells in untreated mice; $P < 0.017$). The difference in granulocyte suppression (Figure 5b; 46% at 2 HALO and 21% at 9 HALO) was also significant ($P < 0.006$). By day 14 post-RAIT (Figure 5d), an 87% loss in peripheral white blood cells was observed for the 2 HALO dose (406 ± 150 versus 2322 ± 431) and a 75% (590 ± 317 versus 2332 ± 443) and a 64% loss in peripheral white blood cells (979 ± 185 versus 2727 ± 410) was observed for the 6 and the 9 HALO dose groups, respectively ($P < 0.001$ for the 2 HALO versus 9 HALO and $P < 0.045$ for 2 HALO versus 6 HALO). On day 14, the loss of granulocytes which comprise 76–78% of the total peripheral white blood cells (approximately 96 + % are neutrophils) was comparable to the results for total blood cells, with an 81%, 71% and 61% loss in cells observed at 2, 6 and 9 HALO, respectively (Figure 5e). In contrast, the induced lymphocytopenia was statistically comparable at all times of dosing, with an 89% and an 87% loss at 2 and 9 HALO, respectively, on day 14 after RAIT (Figure 5f). The absence of a relationship between HALO for dosing and lymphocytopenia may be the result of the lymphocytes being a more radiosensitive subpopulation, and/or peak lymphopoiesis occurring 3–5 h after peak myelopoiesis. Since neutropenia is of far greater clinical concern than lymphocytopenia, we believe the granulocyte populations should be the focus in this evaluation. Thus, bone marrow proliferative activity governs the magnitude of myelotoxicity from RAIT, in much the same way as it does for chemotherapy.

Recovery from RAIT-induced neutropenia occurred earlier for the 2 HALO and 6 HALO groups than the 9 HALO group ($P = 0.073$ for 2 HALO versus 9 HALO; $P = 0.003$ for 6 HALO versus 9 HALO; and $P < 0.013$ for 2 HALO versus 6 HALO), with a 22% loss in granulocytes from the 2 HALO group (1543 ± 170 versus 1970 ± 175) and a 40% loss from the 9 HALO group (949 ± 205 versus 1555 ± 196) on day 28 post-RAIT (Figure 5k; $P < 0.028$). The slower recovery at 9 HALO was probably due to a reduced bone marrow proliferation at that time of day, compared with proliferation at 2 and 6 HALO. We postulate that this difference, based on dosing as a function of HALO, could potentially influence the frequency at which redosing with radioantibody is possible at different HALO points. Based on these data, more frequent dosing is potentially feasible at 2 and 6 HALO than at 9 HALO. This issue requires experimental confirmation.

Since a comparable dose of radioantibody resulted in less myelotoxicity when given at 9 HALO compared with mice dosed at 2 HALO, it seemed likely that the animals would be able to tolerate a higher dose later in the day when bone marrow proliferation was approaching its nadir. Dose escalation

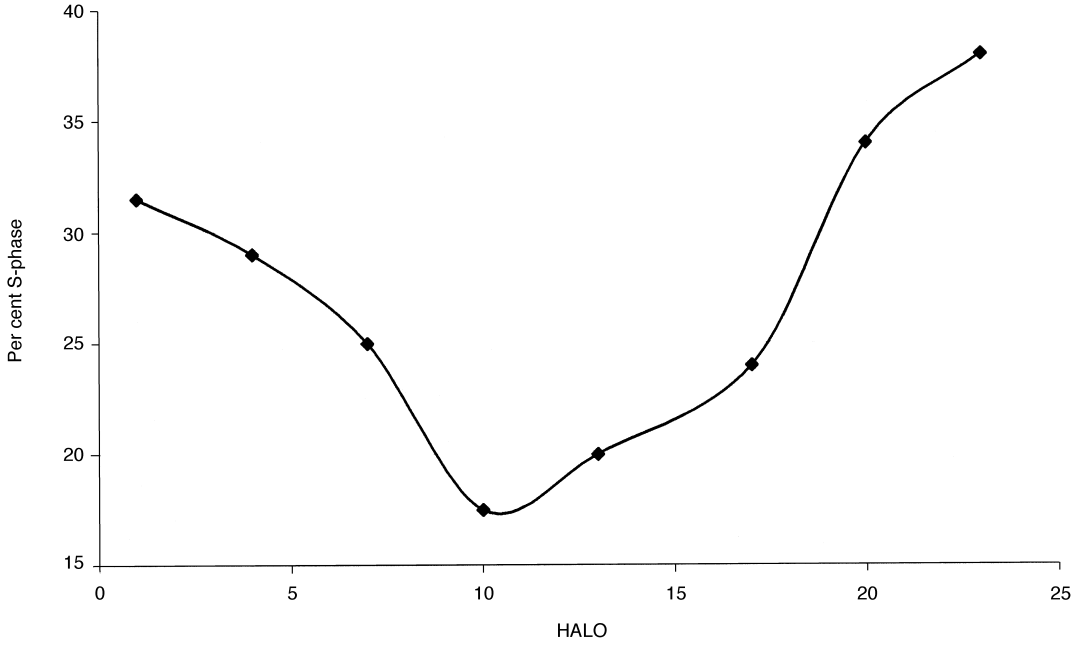

Figure 4. Bone marrow proliferation was determined using Sytox Green staining of femoral bone marrow samples (n=4 per time point) and FACScan determination of fluorescence. Data were analysed using CellFit software and the percentage of cells in S phase determined. The average was recorded at each of eight time points (1, 3, 7, 10, 13, 17, 20 and 23 h after light onset (HALO). Cosinor analysis was used to calculate acrophase, amplitude and mesor.

studies (Figure 6) at 40 μCi increments from 240 to 320 μCi revealed that the MTD for ^{131}I-NP-4 IgG was 240 μCi when administered at 2 HALO and 320 μCi when administered at 9 HALO (Figure 6). Mice given 280 μCi at 2 HALO or 360 μCi at 9 HALO all died within 21–28 days. Therefore, dosing with radioantibody when bone marrow is less mitotically active permits a 30% increase in the dose that can be tolerated, comparable with what has been observed when using other forms of intervention to ameliorate RAIT-induced myelosuppression [12, 20]. When body weight was used as an endpoint (Figure 7) we found that the two MTDs at 2 HALO and 6 HALO resulted in fairly comparable weight

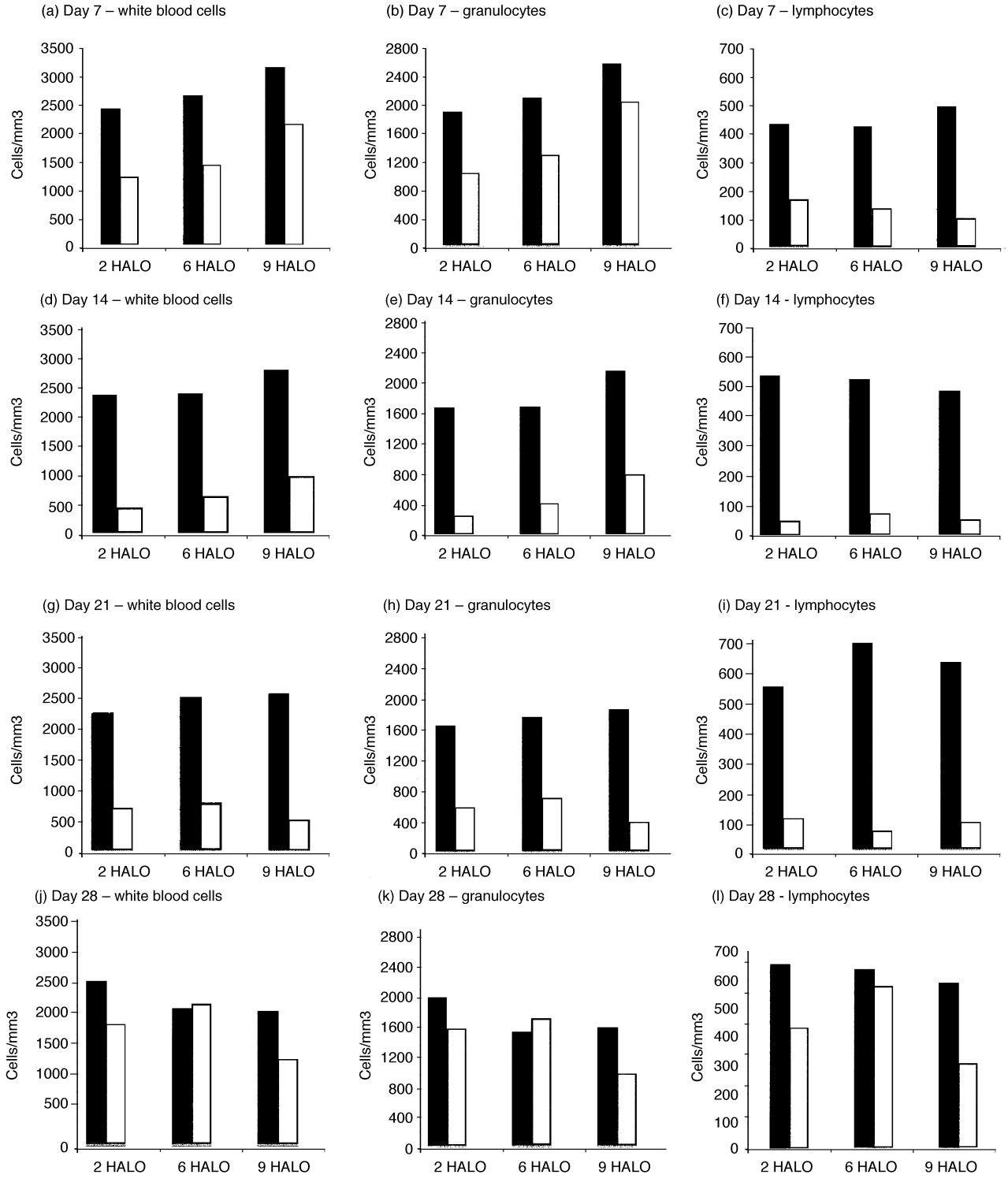

Figure 5. Total peripheral white blood cells, granulocytes, and lymphocytes for mice at 7, 14, 21 and 28 days after a single dose of 240 μCi ^{131}I-IgG administered intravenously at either 2 h after light onset (HALO) (0900 h), 6 HALO (1300 h) or 9 HALO (1600 h). The average of 10 mice was recorded (standard deviations are noted within the results section). ■, untreated; □, radioimmunotherapy.

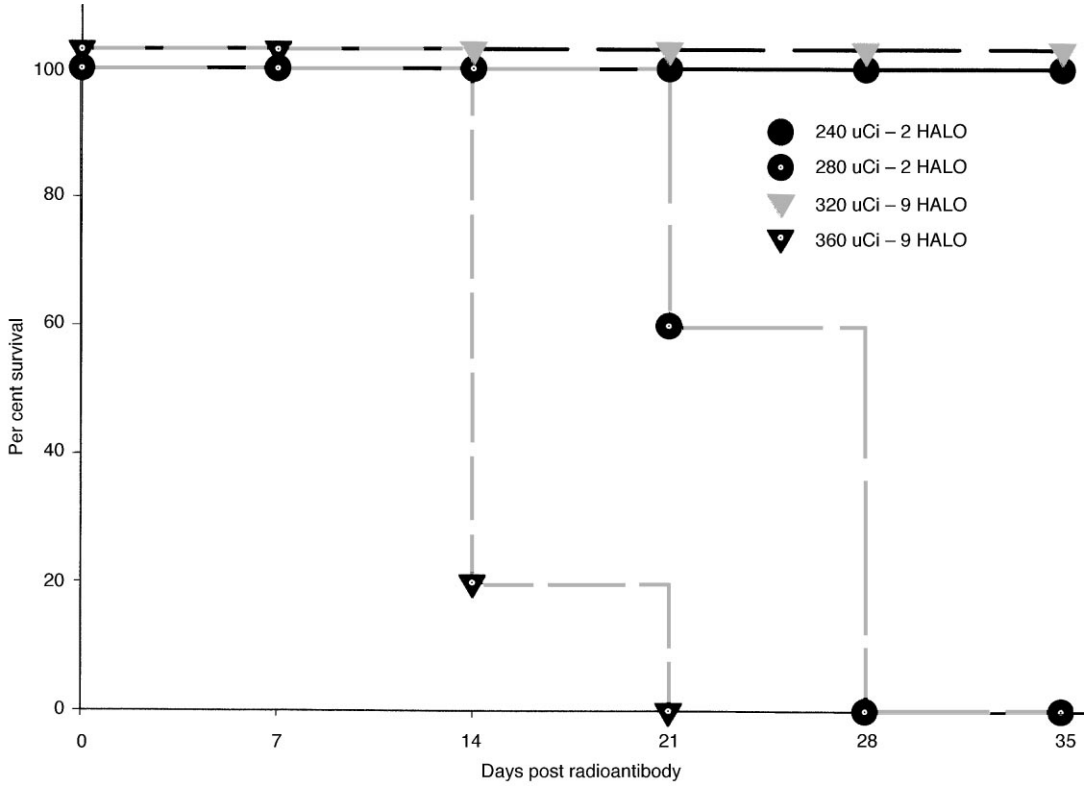

Figure 6. Maximal tolerated dose (MTD) at 2 h after light onset (HALO) and 9 HALO as determined by survival analysis. Groups of 10 BALB/c mice were given one of four doses at 40 μCi increments: 240, 280, 320 or 360 μCi ^{131}I-IgG.

losses (1.5–2.2 g), whereas the next highest dose at each HALO resulted in a significantly larger body weight loss (4.5–5.5 g). This observation suggests that gastrointestinal and bone marrow proliferation share a similar periodicity.

The final study was designed to determine the efficacy of tumour therapy using 240 μCi at 2 HALO or 320 μCi at 9 HALO. If the tumour shared the same proliferation rhythm as normal tissue, then the two groups would give comparable therapy. If tumour proliferation had a unique rhythm from bone marrow or gastrointestinal tissue and proliferation at 9 HALO was equal to or greater than proliferation at 2 HALO, then the 320 μCi dose would result in improved growth

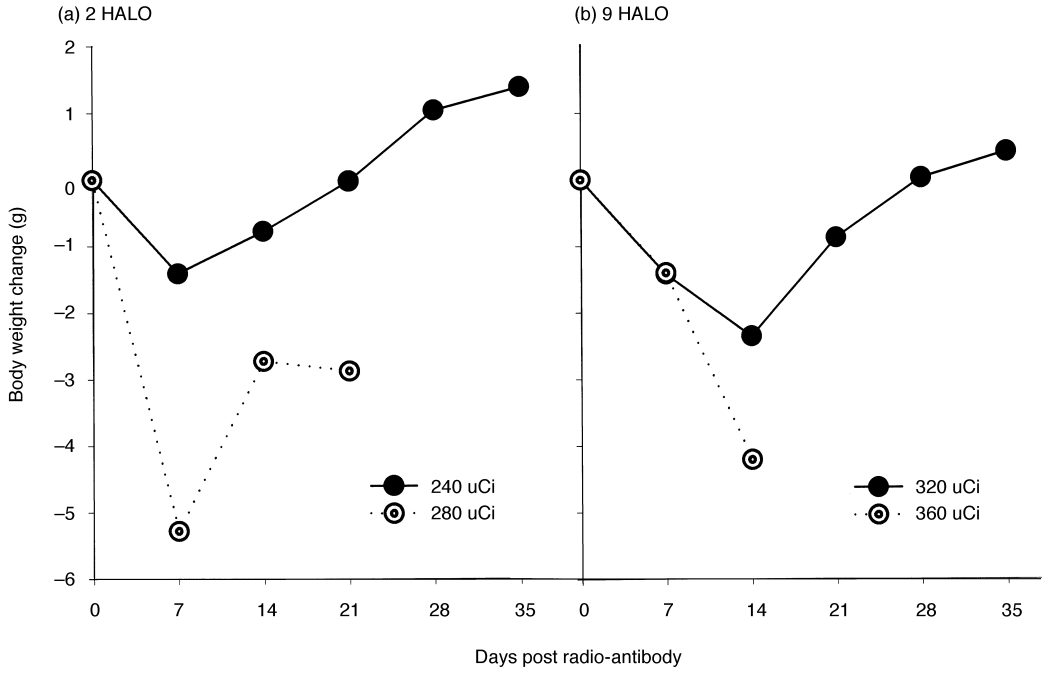

Figure 7. Maximal tolerated dose (MTD) at 2 h after light onset (HALO) and 9 HALO as determined using body weight loss as the endpoint (10 mice/group).

control. Figure 8 depicts the growth of GW-39 subcutaneous tumours in both treatment groups compared with a matched untreated control group. Using day 28, day 42 and day 84 as endpoints, the 320 μCi group administered at 9 HALO provided better growth control than the 240 μCi dose administered at 2 HALO ($P = 0.034$; $P = 0.025$; $P = 0.015$ on days 28, 42 and 84, respectively).

DISCUSSION

Potential life-threatening drug-induced side-effects to bone marrow have been reduced by proper timing of chemotherapy (chronotherapy) according to circadian stages. The temporal variation in bone marrow proliferation has been used to help define the optimal time of day to administer many chemotherapeutic agents, such that treatment efficacy is maximised and toxicity is minimised [4]. Outcomes have been improved significantly by treating at the optimal time [5]. Clinical trials have confirmed the overall ability to deliver higher doses of chemotherapy and the improvement in clinical outcome by incorporating chronobiological principles [6]. For example, in phase I/II trials, the dose of 5-fluorouracil (5-FU) could be increased by 75% if the dose was delivered at 2100–2200 h instead of at 0300–0400 h. In phase III clinical trials, less toxicity and improved survival were achieved when the time of day of dosing with doxorubicin and cisplatin was adjusted. In general, intravenous administration of antimetabolites, cytoskeleton poisons, intercalating agents and alkylating agents are best tolerated when administered in the evening hours, when DNA synthesis in bone marrow and gut is at a minimum [4].

The results presented here are the first to document that radioantibody conjugates are governed by chronobiological principles in a similar way as has been reported for chemotherapeutic agents. Dosing mice at a time of day when bone marrow is mitotically less active permits a 30% increase in the MTD of a radioiodinated intact antibody. If we used our standard GW-39 human colonic xenograft as a model, this dose intensification would be equivalent to an additional 740 rads (total 3210 rads) delivered to the tumour using the NP-4 anti-CEA antibody. When using drug therapy, the proliferative activity of tumour cells and circadian changes in susceptibility (e.g. enzymatic activity, metabolic processes) of tumour cells to the drug must also be considered [2, 3, 21]. However, tumour cell activity would not impact on the optimal time of day for radioantibody dosing, since the tumour residence time of radioantibody is so much longer than it is for chemotherapy. We have found that approximately 80% of the total radiation dose is delivered during the first 7 days after dosing and 42% of it is deposited between days 3 and 7.

The advantage of dose escalation should result in improved therapeutic outcome. We have previously evaluated the effect of dose escalation (using BMT) on therapeutic outcome [12]. Using a subcutaneous tumour model, a 30% increase in MTD resulted in an additional 7 weeks of growth control. With the micrometastatic intrapulmonary model, median survival increased by 8 weeks with a 30% increase in dose delivered. The application of autologous bone marrow support for clinical RAIT is now being studied [9]. Press and colleagues recently studied high-dose RAIT of relapsed B-cell lymphoma patients using re-infused autologous purged bone

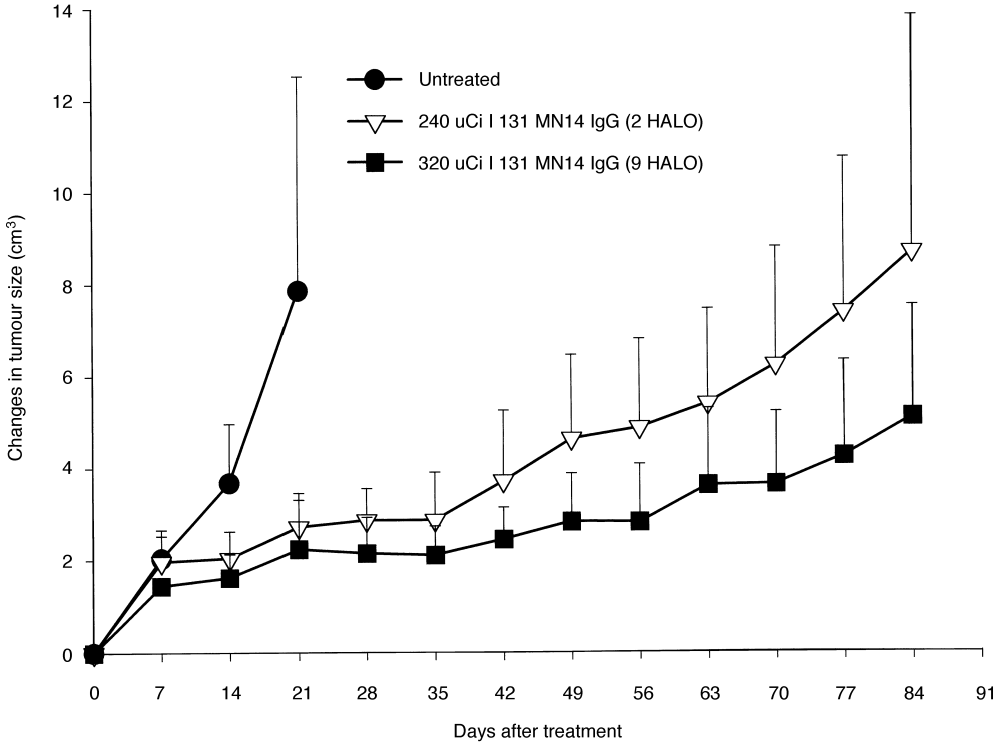

Figure 8. Tumour growth curves of GW-39 tumours grown as subcutaneous xenografts in nude mice. Groups of 10 mice were given either the maximal tolerated dose (MTD) of 240 μCi at 2 h after light onset (HALO) or the MTD of 320 μCi at 9 HALO. Three-dimensional caliper measurements were made and the tumour size (cm^3) was determined weekly and the mean ±standard deviation (S.D.) was calculated for each group of mice.

marrow. BMT permitted the use of approximately 3.2-fold higher (myeloablative) initial radioantibody doses (mean = 487 ± 154 mCi), resulting in a greater number of remissions and a longer duration for remissions. This high escalation of radioantibody dose achieved in humans is of even greater significance, because humans have a higher sensitivity to whole-body radiation than mice. Therefore, the 30% dose difference achievable in mice because of chronobiological principles may prove to be a greater difference in humans, just as was the case when BMT methods were employed preclinically and clinically.

We have shown that the ability to dose escalate radioantibody was due to differences in normal tissue proliferation and not to temporal differences in the clearance rate of the antibody conjugate. This is an important consideration, since drug metabolism also has recognised chronobiological rhythms with time-dependent variations in hepatic drug biotransformation [22], which has explained the chronopharmacokinetic and chronohepatotoxicity of some drugs or toxic agents. The increase in MTD of ^{131}I-NP-4 IgG appears to be the direct result of variability in bone marrow proliferation, the primary dose-limiting toxicity for radioantibody therapy. These fluctuations in bone marrow proliferation have been well documented, with rhythmic changes identified for cell number, cell activity, cytokine production, immunomodulating hormone production and cellular and humoral activity in humans and in other species [23–28]. Recently, it has been noted that the response of bone marrow progenitor colony forming unit-granulocyte macrophage to colony-stimulating factors (interleukin-3, granulocyte-macrophage colony stimulating factor, granulocyte-colony stimulating factor) is also governed by circadian rhythms, with peak stimulation occurring at 3 HALO for all factors [29].

Much of our past work has focused on optimising the use of radioantibodies in tumour-bearing experimental animal models; i.e. maximising the therapeutic effect and minimising the toxic side-effects [8, 17, 30, 31]. We have employed cytokine intervention, BMT and the use of a haemoregulatory peptide to reduce RAIT-induced myelosuppression and permit dose escalation [11–14]. Using these approaches, a 25–50% increase in the dose of RAIT was possible, depending on the radionuclide and antibody form. Optimising the time of day of dosing as a function of HALO represents a new approach to maximising dose and minimising toxicity. The possibility of combining cytokines or BMT with chronobiological principles may afford even greater improvements in dose intensification.

Although rhythms in bone marrow have been reasonably well defined, with peak bone marrow activity occurring between 0300 h and 0800 h [2], there may be differences within the population, for example, as a function of patient age and previous therapy. Since it is not possible to sample bone marrow from patients at regular intervals to determine when activity is lowest, one could instead sample patient blood. Cell traffic rhythms for T, B, natural killer cells, neutrophils and platelets in peripheral blood have been identified [32, 33]. Peak neutrophils occur at 9–12 HALO (1600–1900 h) and peak lymphocytes occur at 17–19 HALO (0000–0200 h) in humans [19], and are approximately 8–10 h out of phase of activity in bone marrow. Thus, blood cell counts may possibly be used to predict bone marrow activity and optimal dosing time as a function of HALO on an individual basis.

In summary, chronobiological principles exist for RAIT with ^{131}I-IgG in a murine model. The optimum time of dosing was at 9 HALO, when bone marrow proliferative activity is at a minimum. The magnitude of myelosuppression was more important at governing the MTD than the duration of myelosuppression, since the white blood cell loss was greatest when dosing was carried out at 2 HALO, whilst the duration was longest when dosing was carried out at 9 HALO and the MTD of ^{131}I-IgG was higher at 9 HALO. These results are similar to those observed both preclinically and clinically with a variety of chemotherapeutic agents.

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