DYE-SENSITIZED photoirradiation is a relatively recent addition to the arsenal of techniques used for the elimination of tumor cells from autologous bone marrow (BM) grafts. One photosensitizer, merocyanine 540 (MC 540), has been characterized extensively in preclinical models and is now being used in a phase I clinical trial for extracorporeal purging of autologous marrow grafts in patients with acute lymphocytic or nonlymphocytic leukemia, Hodgkin’s or non-Hodgkin’s lymphoma, or stage IV neuroblastoma.

The preclinical evaluation of MC 540 has shown that MC 540 has a low acute systemic toxicity and low mutagenic potential. It preferentially photosensitizes normal erythroid progenitor cells, leukemia/lymphoma cells, and neuroblastic cells. By contrast, pluripotent hematopoietic progenitor cells as measured by day 12 colony-forming unit-spleen (CFU-S) or CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) are quite resistant to MC 540-sensitized photoirradiation.

T (E+) and non-T (E−) subpopulations were separated by rosetting with 2-aminoethylisothiouronium bromide and 3% sheep red blood cells (RBC) as described. The T-cell populations greater than 95% E+ cells and the non-T-cell populations contained less than 5% E+ cells by rosetting.

Immune assays. Unless otherwise mentioned, the initial lymphocytes placed into culture were viable based on trypan blue exclusion after treatment. RPMI 1640 supplemented with penicillin-streptomycin, 4 mmol/L glutamine, and 10% fetal bovine serum (FBS; Hyclone, Logan, UT) was used for culture except for the mixed lymphocyte cultures. Human AB serum (10%) was used in the mixed lymphocyte cultures.

Viability after dye-mediated photoirradiation (DMP). Viability of the lymphocytes used for these assays was checked before culture and at 2, 6, and 24 hours after MC 540 DMP using trypan blue exclusion.

Lymphocyte separation. Peripheral blood mononuclear cells (PBMC) were purified by ficoll-hypaque density gradient centrifugation (F/H) of whole heparinized blood. T (E+) and non-T (E−) subpopulations were separated by rosetting with 2-aminoethylisothiouronium bromide hydrobromide-modified sheep red blood cells (RBC) as described.

MATERIALS AND METHODS

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T-cell helper activity could be abrogated by 90 minutes of treatment in cocultures containing untreated B cells. Purified B cells treated for 90 minutes cocultured with normal T cells did not produce Ig. Treatment of B cells completely inhibited Epstein-Barr virus-stimulated Ig synthesis. These data show that T- and B-cell immunity is suppressed by the MC 540-sensitized photoirradiation. Treatment of bone marrow with MC 540 and light may have profound effects on immune reconstitution in autologous marrow graft recipients. More provocative is the fact that the same immunomodulatory effects may be applicable to partially mismatched marrow transplant situations as a means of reducing graft-versus-host reactions.

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lymphocyte stimulation with Con A (10 μg/mL; Sigma, St Louis, MO) and phytohemagglutinin (PHA; 1:400 dilution of stock; Gibco, Grand Island, NY), or mixed lymphocyte cultures were measured by pulsing cultures with 25 μL of 6.5 Ci/mmol ³H-thymidine for 4.5 hours after the indicated culture intervals. All proliferation assays have been corrected for unstimulated background ³H-thymidine incorporation.

Induction of in vitro Ig secretion with pokeweed mitogen (PWM) and Epstein-Barr virus (EBV). The microcultures for culturing T and B cells after PWM or EBV stimulation have been previously described. Briefly, duplicate cultures stimulated with PWM were performed in round-bottom microwell plates containing various combinations of untreated or treated 5 X 10⁶ T and untreated or treated 5 X 10⁶ B cells (E−) as indicated, and the cultures stimulated with EBV containing 1.0 X 10⁶ untreated or treated B cells as indicated. PWM-stimulated cultures were plated for enzyme-linked immunosorbent assay (ELISA)-plaques after 6 days of culture and the EBV-stimulated cultures were plated for ELISA-plaques after 8 days of culture.

ELISA-plaque assay. In brief, the petri dishes used to develop the ELISA-plaques were coated with a goat antihuman IgG or IgM solution overnight at 37°C in carbonate buffer (pH 9.6) and washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) the next morning. Duplicate microcultures were mixed, washed, resuspended in RPMI 1640 containing 10% FBS, and plated in duplicate on 35-mm Costar (3530) petri dishes (Cambridge, MA).

The lymphocytes were cultured on the petri dishes overnight at 37°C in a 5% CO₂ incubator. The next morning, the lymphocytes were discarded and the dishes washed with PBST and incubated with 2 mL of precipitated alkaline phosphatase-conjugated goat antihuman IgG or IgM (TAGO, Burlingame, CA) in 10 mmol/L phosphate buffer for 4 hours at 37°C. After incubation with the developing antibody, the dishes were washed with PBST and coated with 2 mL of 5-bromo-4-chloro-3-indolylphosphate in 2-amino-2-methyl-1-propanol (AMP) buffer in a 0.03% solution of gelling agarose at a final concentration of 1 mg/mL. Plaque forming cells (PFC) were identified as blue spots in the agarose and were counted with an image analysis system using a dissecting microscope (WILD M3Z; Leitz, Overland Park, KS), an MTI 65 video camera, and a 286 IBM compatible computer equipped with the BQ Meg IV System (R & M Biometrics, Nashville, TN). A representative area was counted and expressed as numbers of PFC/ml. All PFC data were corrected for unstimulated T- and B-cell cultures in the PWM-stimulated assays or for unstimulated B cells in the EBV-stimulated assays.

MC 540-sensitized photoinactivation. The MC 540-sensitized photoinactivation of cells was performed as previously. Briefly, cells were suspended at a concentration of 1 to 5 X 10⁶/mL in N-2-hydroxyethylpiperazine-N¢-2 ethanesulfonic acid (HEPES)-buffered (10 mmol/L, pH 7.4) α-modified minimal essential medium (α-medium, Sigma) containing 12% FBS and placed into polystyrene tubes (15-mm diameter; Corning Glass Works, Corning, NY). MC 540 (Kodak, Rochester, NY) was added from a 1 mg/mL stock solution in 50% ethanol to a final concentration of 15 μg/mL. The tubes were then mounted on a plexiglass disk that rotated at approximately 60 rpm between two banks of tubular white fluorescent light bulbs (five bulbs per bank; F20T12.CW, fluorescence rate at sample site approximately 70 W/m²; General Electric, Cleveland, OH) for the time intervals indicated. Dye-mediated photoinactivation was terminated by washing the cell suspensions twice with HEPES-buffered α-medium supplemented with 5% FBS. This and all subsequent operations were performed in the dark or under subdued lighting. Cell suspensions that received either no treatment or were exposed to MC 540 in the dark or to light only served as controls.

RESULTS

Viability after DMP. Incubation of lymphocytes with MC 540 alone in the dark or light alone for 90 minutes did not affect viability as measured by trypan blue exclusion.

Viability was greater than a mean of 90% after 6 hours of culture and greater than a mean of 75% after 24 hours of culture. After 30 minutes of MC 540 DMP, viability after 6 hours of culture was a mean of 97% and after 24 hours of culture was a mean of 92%.

In an earlier report, there were 15% less viable cells in long-term cultures of normal human BM treated for 30 minutes with DMP than in untreated long-term cultures. In the same report, burst-forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), and CFU-granulocyte-macrophage (CFU-GM) were assayed immediately after the dye-sensitized photoinactivation treatment of the human BM cell suspensions (data not shown). After 30 minutes of DMP, BFU-E, CFU-E, and CFU-GM were reduced from means of 6,250 to 2,300, 10,700 to 725, and 8,700 to 8,500 colonies, respectively. These data, obtained under the same experimental circumstances, show the relative sensitivities of each of the hematopoietic progenitor cells to DMP.

Suppression of mitogen-stimulated lymphocyte proliferation. Mitogen-induced proliferative responses of PBMC were progressively inhibited by increasing doses of light at a constant dose of MC 540 (15 μg/mL). Figure 1A shows the data from one of six representative experiments where treatment with MC 540 and increasing doses of white light decreased the ability of PBMC to incorporate ³H-thymidine when stimulated with PHA. Figure 1A shows that PHA-stimulated ³H-thymidine incorporation was inhibited by greater than 80% and 98% after 30 and 60 minutes of treatment, respectively.

In the six experiments performed using PHA, the mean PHA-stimulated proliferation for control (untreated) PBMC was 29,362 cpm with a range from 3,366 to 68,129 cpm. MC 540 treatment alone did not alter proliferation. PHA-stimulated proliferation was inhibited by a mean of 70% ± 8% (±1 SD), 98% ± 2%, and 98% ± 3% after 30, 60, and 90 minutes of treatment, respectively. The range of inhibition of the PHA-stimulated control ranged from 61% to 79% after 30 minutes, 96% to 100% after 60 minutes, and 92% to 100% after 90 minutes.

Similarly, MC 540 and light treatments progressively inhibited Con A-induced proliferation of control PBMC with increasing doses of light exposure. Figure 1B shows representative data from one of four experiments. Figure 1 shows that Con A-induced lymphocyte proliferation was inhibited by greater than 90% and 99% after 30 and 60 minutes of treatment, respectively.

The mean Con A-stimulated proliferation for control (untreated) PBMC for four experiments was 22,845 cpm with a range from 2,148 to 55,375 cpm. Con A-stimulated proliferation was inhibited by a mean of 75% ± 18% (±1 SD), 96.5% ± 3%, and 94% after 30, 60,
and 90 minutes of treatment with MC 540 and light, respectively. The inhibition of the Con A-stimulated control ranged from 50% to 94% after 30 minutes, 92% to 100% after 60 minutes, and 92% to 97% after 90 minutes. MC 540 treatment alone did not alter Con A-stimulated proliferation.

In summary, after only 30 minutes of MC 540 and light, PHA and Con A-stimulated proliferation was inhibited a mean of 70% and 75%, respectively. By 90 minutes of treatment, PHA and Con A-stimulated proliferation was inhibited greater than 90%.

**Effects of MC 540 and light on PWM-induced Ig synthesis.**

Cocultures of T and B cells were performed to determine the effects of MC 540 and light on T-cell helper function and on B-cell function in the T-cell-dependent PWM-stimulated Ig synthesis system. Figure 2A and B shows two representative experiments of five that demonstrate the effects of the MC 540 and light treatment on PWM-stimulated IgG and IgM synthesis as measured by an ELISA-plaque assay. The results are reported as the total number of IgG and IgM PFC per 10^6 non-T cells cultured after background corrections for unstimulated PFC. Control cultures containing PWM-stimulated T and B cells produced 6,800 PFC and 14,700 PFC/10^6 B cells cultured (Fig 2A and B, respectively). T cells (T_c) or non-T cells (B_c) treated with only MC 540 or MC 540 and light for 30 minutes (T_m30 or B_m30) did not inhibit the number of PFC detected after 6 days of culture. Sixty minutes of MC 540 and light treatment had almost no effect on T-cell helper activity in the normal subject in Fig 2A but inhibited T-cell helper activity in the second subject in Fig 2B nearly 90%. On the other hand, treatment of non-T-cell populations with MC 540 and light for 30 minutes completely inhibited Ig synthesis stimulated by PWM.

The mean number of PWM-stimulated PFC/10^6 B cells cultured in five experiments was 19,132 ± 8,816 (±1 SD) with a range from 7,000 to 30,160 PFC/10^6 B cells cultured. When T cells were treated with MC 540 and light, the numbers of PFC produced were progressively inhibited by greater light exposure intervals. Control T- and B-cell cultures for all five experiments were inhibited by a mean percent of 6 ± 31 (±1 SD), 52 ± 27, and 57 ± 32 after 30, 60, and 90 minutes of MC 540 and light treatment, respectively. On the other hand, when B cells were treated with MC 540 and light for 30, 60, and 90 minutes, the mean percent (±1 SD) inhibition of control T- and B-cell cocultures was 77 ± 20, 96 ± 3, and 96 ± 6, respectively. In summary, greater than 50% inhibition of T-cell helper activity occurred after 60 minutes of treatment, whereas greater than 77% inhibition of B-cell function occurred after 30 minutes of treatment.

**Inhibition of EBV-stimulated polyclonal Ig synthesis by MC 540 and light treatment.**

Enriched B cells were treated with MC 540 and light followed by stimulation with EBV to determine if the treatment would directly affect B-cell Ig secretion in a T-cell-independent Ig synthesis system. In these experiments, 1 × 10^6 non-T cells (designated B cells) were stimulated with EBV for polyclonal IgG and IgM
Fig 2. (A and B) PWM-stimulated Ig synthesis as measured by PFC per 10^6 B cells (E-) cultured after 6 days of culture. Various coculture combinations of T (5 x 10^6) and B cells (5 x 10^5) treated with MC 540 alone (T_a or B_a) or MC 540 and light at various exposure intervals (T_m, B_m, etc) are shown. The numeric subscripts indicate duration of treatment. The cultures have been corrected for unstimulated background IgG and IgM PFC after 6 days of culture.

Fig 3. EXP 1 and EXP 2 show enriched B-cell (1 x 10^6 E-) subpopulations from two normal subjects stimulated with EBV to induce polyclonal IgG and IgM PFC after 8 days of culture. B cells (B) did not receive any treatment; B_m received MC 540 incubation in the dark for 90 minutes; B_m30, B_m60, and B_m90 were treated with MC 540 and light for 30, 60, and 90 minutes, respectively. All values have been corrected for unstimulated background PFC after 8 days of culture.

DISCUSSION

This investigation shows that treatment with MC 540 and light has major effects on T- and B-lymphocyte functions. MC 540 DMP inhibits T-cell proliferation, the generation of T-cell helper activity, B-cell differentiation, and MLC. Some dye-sensitized photoirradiation effects on T and B cells may be related to differential loss of specific lymphoid subpopulations as well as a reduction in cell numbers. However, more interesting are the effects of MC 540 DMP on the remaining viable cells. In the latter circumstance, there are several mechanisms that may explain impaired T- and B-cell functions. One explanation is that there may be delayed death of the treated cells, ie, T and B cells may be irreversibly damaged by the treatment yet continue to exclude trypan blue and then go on to die 2 or 3 days later. A more interesting alternative explanation is that the remaining T and B cells were inhibited in their ability to function due to DMP. The inhibition of T-cell helper functions seen in the mitogen-induced T-cell proliferation and Ig biosynthesis suggest that MC 540-sensitized photoradiation may affect a variety of T-cell functions. Some of these impaired T-cell functions may alter the rate of hematopoietic engraftment. In all of the functions tested, T-cell helper function in
MC 540 + LIGHT INHIBIT IMMUNE FUNCTIONS

Table 1. Effects of MC 540 and Light on Allogeneic MLC (net cpm $\times 10^3$)

<table>
<thead>
<tr>
<th>Light (min)</th>
<th>1 (A x B*)</th>
<th>2 (A x C*)</th>
<th>3 (B x A*)</th>
<th>4 (B x C*)</th>
<th>5 (C x A*)</th>
<th>6 (C x B*)</th>
<th>Mean ± 1 SD</th>
<th>% Control</th>
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<tr>
<td>No MC 540</td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>8.3</td>
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<td>18.8</td>
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<td>3.1</td>
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<td>0.4</td>
<td>0</td>
<td>0.9±1.0</td>
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Columns 1 through 6 show six MLC combinations between subjects A, B, and C. Responder PBMC $(1 \times 10^5)$ were stimulated with $1 \times 10^5$ irradiated $(2,500$ rad) PBMC. All values have been corrected for autologous background MLC; responder cells were left untreated (control), exposed to 90 minutes of light alone, or treated with MC 540 doses and the light exposure times (min) indicated.

1Exposure time at 70 W/m².

PWM-stimulated Ig synthesis was more resistant to MC 540 DMP than mitogen- or alloantigen-induced proliferative responses. We hypothesize that pathways for cell proliferation may be more sensitive to MC 540 and light treatment than pathways responsible for lymphokine synthesis. Additional studies are being performed to further dissect the effects of MC 540 DMP on the molecular mechanisms involved in T-cell activation for helper function and alloreactivity.

The dose titrations used in this study were performed at doses equal to or less than those used in the phase I clinical trial for extracorporeal purging of autologous marrow grafts from patients with acute leukemias, lymphomas, or stage IV neuroblastoma. Therefore, it is possible that the prolonged neutropenia or delayed engraftment in some patients receiving extracorporeally purged autologous marrow grafts was due to either reduced numbers of T cells in the marrow graft or attenuated T-cell functions in the marrow grafts. Alternatively, delayed engraftment or failure of engraftment in this group of patients may have been related in part to decreased numbers of stem cells secondary to multiple courses of chemotherapy the patients received for the treatment of their malignancy.

The inhibition of mitogen-induced and alloantigen-induced T-cell proliferation is remarkable. PHA-induced or Con A-induced proliferative responses were consistently inhibited greater than 70% after 30 minutes of treatment. The ability of B cells to secrete Ig in PWM- or EBV-stimulated Ig secretion was inhibited. MC 540-sensitized photoradiation of PBMC completely blocked allogeneic MLC responses in all six combinations studied. These studies show that MC 540 at a dose of 15 $\mu$g/mL and white light (70 W/m²) exposure for 90 minutes could inhibit MLC greater than 90%. We emphasize that these studies were performed on viable lymphocytes after the MC 540 DMP.

It is not clear whether these MC 540 and light doses could be used in clinical situations to inhibit reactions such as graft-versus-host disease (GVHD). These situations include cases where: (1) a related donor and recipient are mismatched for two antigens; or (2) an unrelated donor and the recipient are phenotypically mismatched for one antigen. These are situations in which T-cell depletion or
modulation may markedly reduce the incidence and the overall severity of GVHD. Mixed lymphocyte culture studies between family members with known HLA differences may provide information on optimal doses of dye and light needed to decrease the incidence and severity of acute GVHD in partially mismatched transplants. If such studies are successful, MC 540 DMP of the allogeneic marrow inocula would provide a tool for modulating GVHD and expand the use of marrow grafting to patients with unrelated or partially mismatched marrow grafts as an alternative to other means of modulating the marrow inoculum to prevent acute GVHD.

Finally, the direct effect of MC 540 DMP on B cells may be clinically important for several reasons. MC 540 DMP may provide information on optimal doses of dye and light needed to decrease the incidence and severity of acute GVHD. Mixed lymphocyte culture studies between family members with known HLA differences may markedly reduce the incidence and the overall severity of GVHD. If such studies are successful, MC 540 DMP of the allogeneic marrow inocula would provide a tool for modulating GVHD and expand the use of marrow grafting to patients with unrelated or partially mismatched marrow grafts as an alternative to other means of modulating the marrow inoculum to prevent acute GVHD.

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The immunoregulatory effects of merocyanine 540 on in vitro human T- and B-lymphocyte functions

LG Lum, M Yamagami, BR Giddings, I Joshi, SL Schober, LL Sensenbrenner and F Sieber