**CONCISE REPORT**

**Amantadine Potentiates T Lymphocyte Killing by an Anti–Pan-T Cell (CD5) Ricin A-Chain Immunotoxin**

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The studies described in this report demonstrate that 1-adamantanamine hydrochloride (amantadine) is a potent enhancer of the cytotoxic activity of the anti–pan-T lymphocyte (CD5) T101 monoclonal antibody conjugated to purified ricin A-chain (T101-immunotoxin; T101-IT). We also demonstrate that T101-IT in the presence of amantadine does not induce immunotoxin-mediated cytotoxicity in nontarget cells such as human marrow hematopoietic progenitor cells. These results provide further knowledge for the improvement of ex vivo purification of human bone marrow from normal or leukemic T cells prior to allogeneic or autologous stem cell transplantation, respectively. Furthermore, since amantadine has long been employed safely in human therapy, its use in conjunction with immunotoxins might be exploited in vivo.

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**MATERIALS AND METHODS**

T101 ricin A-chain immunotoxin (T101-IT; SR 41322; batch RBM07) consists of the murine monoclonal IgG₄ antibody T101 (Hybritech, San Diego, Calif) chemically bound to purified ricin A chain.¹Other immunotoxins do have limitations, particularly in terms of potency and slow kinetics of cytotoxicity. These problems have been partly obviated in vitro by long incubations under conditions nonreproducible in vivo.¹²

In a systematic search for substances of potential clinical activity capable of improving A-chain translocation into the cytosol, we tested the symmetrical primary amine amantadine. This drug is selectively concentrated in lysosomes and other acidic organelles,¹³ is capable of elevating endosomal and lysosomal pH,¹⁰ and interferes with receptor recycling¹¹ at the level of compartment of uncoupling receptor and ligand.¹²

We report here that in vitro amantadine, at concentrations nontoxic to human hematopoietic progenitor cells, enhances the activity and accelerates the kinetics of the anti–pan-T cell (CD5) T101 ricin A-chain immunotoxin (T101-IT) against normal T lymphocytes.

Normal bone marrow cells were obtained by aspiration from the iliac crests of volunteers, who gave informed consent in accordance with institutional guidelines. Peripheral blood (PB) and bone marrow (BM) mononuclear cells were prepared by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Collected cells were washed 3 times with Hanks’ buffered saline solution (HBSS) before treatment with T101-IT. Ficoll-Hypaque-isolated mononuclear cells of 10⁷/ml (PB) or 2 × 10⁷/ml (BM) were suspended in HBSS containing amantadine hydrochloride (1-Adamantanamine HC1, Sigma, St. Louis, Mo) and/or ammonium chloride. The incubation medium was adjusted to pH 7.8 by addition of 2 N sodium hydroxide and contained 2% fetal calf serum (FCS) and 10 mmol/L HEPES buffer. After ten minutes at 37°C, incubation with T101-IT was initiated in ice for ten minutes and then continued at 37°C. At the end of incubation, cells were washed 4 times at 4°C with 1% FCS-HBSS and assayed according to the experimental protocol.

To measure lectin-induced polyclonal T cell proliferation, PB mononuclear cells were stimulated with 10 μg/mL phytohemagglutinin (PHA-P, Difco, Detroit, Mich). Mononuclear cells (10⁶ cells/well) were cultured in round-bottomed microtiter plates in 0.2 mL RPMI 1640 supplemented with 15% FCS, 10 mmol/L HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Triplicate cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere for 72 hours and then labeled with 1 μCi/well tritiated thymidine (³H-TdR). After eight hours, ⁹H-TdR incorporation was measured by standard scintillation counting techniques.

To measure lymphocyte proliferative response to allogeneic cells in mixed lymphocyte culture (MLC), responder PB mononuclear cells (5 × 10⁵) were cultured with 5 × 10⁴ irradiated (2,500 rad) stimulator cells (1:1 mixture of allogeneic PB mononuclear cells from two unrelated individuals). Triplicate cultures were performed in supplemented RPMI 1640 with 15% FCS in round-bottomed microtiter plates at 37°C in a 5% CO₂ humidified atmosphere. After 120 hours, plates were pulsed with ³H-TdR 1 μCi/well. After an additional 24 hours, ³H-TdR incorporation was measured by standard scintillation counting techniques.

The frequency of clonal T lymphocytes in BM samples was evaluated by a limiting dilution microculture assay in PHA-IL 2 feeder cell containing medium according to Kernan et al with minor modifications as previously described. T lymphocyte frequency was determined by the minimum chi-square method from the Poisson distribution relationship between the cell number seeded per well and the logarithm of the percentage of negative culture wells.

The frequency of multipotential (CFU-GEMM), erythroid (BFU-E), and granulomonocytic (CFU-GM) hematopoietic progenitor cells was evaluated by clonal assays as previously described.

RESULTS

Unless otherwise specified, 10 mmol/L T101-IT was employed throughout the experiments presented here. In previous studies, this concentration was found to be optimal in terms of high immunotoxin activity and lack of cytotoxicity to human hematopoietic progenitors.

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Viability of PB as well as BM mononuclear cells was assessed by Trypan blue dye exclusion test at the end of four hours incubation. Treatment with 0.1 to 1.5 mmol/L amantadine and T101-IT did not affect cell viability. Higher concentrations, ie, 1.75 and 2 mmol/L amantadine either with or without T101-IT, reduced the number of total viable cells to 84.75% ± 13.36% and 27.45% ± 9.68% of medium controls, respectively.

In the absence of enhancer molecules, four-hour treatment with T101-IT did not change the mitogenic response of PB T lymphocytes. In contrast, the addition of amantadine to the incubation medium enhanced the activity of T101-IT in a dose-related manner (Fig 1). The most effective nontoxic concentration was 1 mmol/L amantadine which in the presence of 10 mmol/L T101-IT resulted in the inhibition of T lymphocyte mitogenic response to 2.18% ± 2.50% of medium controls (n = 4). The same amantadine concentration potentiated also the T101-IT inhibition of PB T cell response to allogeneic cells in MLC (11.72% ± 1.26%, 12.23% ± 1.32%, and 12.98 ± 1.40 of medium, amantadine, and T101-IT controls, respectively; n = 1).

In parallel experiments, T101-IT in the presence of 10 mmol/L ammonium chloride reduced the PB T cell proliferative response to PHA and to allogeneic cells in MLC to only 41.23% ± 21.60% (n = 3) and 32.5% ± 5.38% (n = 1) of controls, respectively. T101-IT in the presence of 20 mmol/L ammonium chloride, or 10 mmol/L methylamine, or 100 μmol/L chloroquine reduced the T cell proliferative response to PHA to 20.04% ± 9.70%, 42.30% ± 11.30%, and 49.00% ± 9.83% (n = 2) of controls, respectively.

The kinetics of the inhibition of PB T lymphocyte proliferative response to PHA by T101-IT in the presence of 1 mmol/L amantadine or 20 mmol/L ammonium chloride, is shown in Fig 2. Amantadine sped the rate at which T101-IT inhibited T lymphocyte proliferation more dramatically than did ammonium chloride. In addition, the overall enhancement of T101-IT activity was markedly higher with amantadine than with ammonium chloride. A two-hour incubation with T101-IT and 1 mmol/L amantadine was sufficient to inhibit lymphocyte proliferative response to 1.29% ± 0.41% (n = 2) of control medium.

To address the possibility that amantadine is toxic to an accessory cell(s) necessary for the in vitro T cell activation and proliferation, PB mononuclear cells treated with T101-IT and amantadine were mixed at different ratios with autologous medium-treated PB mononuclear cells and assayed for PHA proliferative response. The observed rate of 3H-TdR incorporation corresponded to the expected values calculated on the basis of dilution in the cell mixtures, thus ruling out a toxic effect of amantadine plus T101-IT on an accessory cell(s) (data not shown).

Based on the results of the experiments in which PB lymphocytes were the target of the immunotoxin cytotoxicity, human BM mononuclear cells at 20 × 10⁶/mL were incubated for two hours with T101-IT alone or T101-IT plus either amantadine or ammonium chloride. The frequency of BM T lymphocytes was evaluated by a limiting dilution microculture assay which is the most sensitive method for the detection and enumeration of very low numbers of residual BM T cells.

In the presence of 1 mmol/L amantadine, a two-log depletion of BM T lymphocytes was achieved. In contrast, when 10 mmol/L ammonium chloride was employed instead of amantadine, only one log reduction of BM T lymphocytes was achieved. In all instances, the treatment with T101-IT and/or either enhancer molecule did not significantly alter the recovery of CFU-GEMM, BFU-E, and CFU-GM hematopoietic progenitor cells (Table 1).

**DISCUSSION**

The data presented in this report demonstrate that amantadine enhances T101-IT cytotoxic effect and that it is
superior to ammonium chloride both in terms of rapidity of action and level of T101-IT cytotoxicity against PB T lymphocytes. In 1984, Casellas et al reported that the degree of enhancement of T101-IT cytotoxicity against T leukemia CEM cells by 1 mmol/L amantadine is inferior to 100 μmol/L chloroquine, 10 mmol/L ammonium chloride, or 10 mmol/L methylimidazole. The same authors chose ammonium chloride in their subsequent work. The present study shows that when targets of T101-IT cytotoxicity are normal T cells, the most effective potentiator is amantadine followed by ammonium chloride, methylimidazole, and chloroquine. The diversity of our results compared to those of Casellas et al supports the notion that A-chain immunotoxins exhibit variability in target cell toxicity.

The immediate application of these data is the use of amantadine as optimal potentiator of T101-IT for ex vivo depletion of mature T lymphocytes from bone marrow grafts prior to allogeneic transplantation. Recently, Kernan et al have shown that the number of clonable residual T lymphocytes measured by a limiting dilution assay correlates with the development of GVHD in 32 leukemia patients following transplantation of histocompatible donor T cell-depleted BM. In particular, these authors found that 24 patients who received a median of 3.72 × 10⁶ (0.92 to 16.0 × 10⁶) residual BM T cells per kg of recipient body weight did not develop GVHD. Of the 8 patients who received a median of 23.99 × 10⁶ (18.63 to 43.93 × 10⁶) T cells per kg, 4 had no GVHD, 4 developed grade I-II (skin only) GVHD, and 4 developed grade III-IV GVHD.

Employing the same limiting dilution assay for quantitation of residual BM T cells, we show in this report that T101-IT in the presence of amantadine induces a 2 log reduction of BM T lymphocytes without adversely affecting multipotential hematopoietic progenitor cells. Typical untreated BM grafts contain 1.0 × 10⁹ (0.7 to 1.4 × 10⁹) clonable T lymphocytes. Following a 2 log T cell reduction, an average 50 kg patient would receive 2 × 10⁶ (1.4 to 2.8 × 10⁶) T cells per kg. Thus, based on Kernan’s studies, data presented here suggest that depletion of BM T cells by T101-IT plus amantadine may be effective in preventing severe grade III-IV GVHD.

Amantadine is a licensed drug employed for prophylaxis of influenza A virus infections in humans. The in vitro concentrations found to be active in present experiments cannot be achieved in the blood of patients treated with amantadine. For this reason we are now investigating whether amantadine analogues will prove active at clinically occurring concentrations. T101-IT in the presence of non-toxic amantadine concentrations prompts its immediate use for a more efficient ex vivo depletion of human bone marrow T lymphocytes.

ACKNOWLEDGMENT

We are grateful to Groupe Sanofy, Centre de Recherches Clin Mdy, Montpellier, France, for supplying T101-IT. We are also indebted to Dr Nancy A. Kernan for helpful discussions.

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