The Anti–Human Leukocyte Antigen-DR Monoclonal Antibody 1D09C3 Activates the Mitochondrial Cell Death Pathway and Exerts a Potent Antitumor Activity in Lymphoma-Bearing Nonobese Diabetic/Severe Combined Immunodeficient Mice

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Abstract

The fully human anti-HLA-DR antibody 1D09C3 has been shown to delay lymphoma cell growth in severe combined immunodeficient (SCID) mice. The present study was aimed at (a) investigating the mechanism(s) of 1D09C3-induced cell death and (b) further exploring the therapeutic efficacy of 1D09C3 in nonobese diabetic (NOD)/SCID mice. The chronic lymphocytic leukemia cell line JVM-2 and the mantle cell lymphoma cell line GRANTA-519 were used. Generation of reactive oxygen species (ROS) and mitochondrial membrane depolarization were measured by flow cytometry following cell incubation with dihydroethidium and TMRE, respectively. Western blot analysis was used to detect c-Jun-NH2-kinase (JNK) phosphorylation and apoptosis-inducing factor (AIF). NOD/SCID mice were used to investigate the activity of 1D09C3 in early- or advanced-stage tumor xenografts. In vitro, 1D09C3-induced cell death involves a cascade of events, including ROS increase, JNK activation, mitochondrial membrane depolarization, and AIF release from mitochondria. Inhibition of JNK activity significantly reduced 1D09C3-induced apoptosis, indicating that 1D09C3 activity involves activation of the kinase. In vivo, 1D09C3 induces long-term disease-free survival in a significant proportion of tumor-bearing mice treated at an early stage of disease. Treatment of mice bearing advanced-stage lymphoma results in a highly significant prolongation of survival. These data show that 1D09C3 (a) exerts a potent antitumor effect by activating ROS-dependent, JNK-driven cell death, (b) cures the great majority of mice treated at an early stage of disease, and (c) significantly prolongs survival of mice with advanced-stage disease. (Cancer Res 2006; 66(3): 1799-808)

Introduction

The development and approval of monoclonal antibodies (mAb) targeting tumor-specific antigens and inducing tumor cell death represents a major advance in the therapy of non-Hodgkin lymphomas (NHL; refs. 1–3). Used as single agent, the anti-CD20 mAb rituximab induces transient remissions in 40% to 60% of patients with indolent NHL and is also active although at a lower degree in other lymphoproliferative diseases, including chronic lymphocytic leukemia (CLL; ref. 4). However, only a minority of NHL patients achieve complete response and virtually all patients experience relapses (5). The anti-CD52 mAb alemtuzumab induces responses in 30% to 40% of patients with fludarabine-refractory CLL but the majority of patients experience infusion reactions and immunosuppression with opportunistic infections (6). The mechanisms of action of mAbs include triggering of apoptosis by caspase-dependent or caspase-independent pathways, antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity, and generation of reactive oxygen species (ROS; refs. 7–9). To elicit their antitumor activity, the majority of unconjugated mAbs require effector mechanisms to be intact in vivo in the patients. Thus, the development of new mAbs with an inherent tumoricidal activity that do not require complement activation and ADCC might represent a major advancement in mAb therapy.

The human leukocyte antigen (HLA)-DR is one of the three highly polymorphic genes of the class II MHC, which, under normal conditions, are selectively expressed on immune cells, including B lymphocytes, activated T lymphocytes, monocytes, and dendritic cells (10). The relatively limited expression of HLA-DR on normal cells facilitates the targeting of malignant lymphoma cells that express HLA-DR. Preclinical evidences suggest that both B-cell tumors (11, 12) and T-cell tumors (13) can be killed in vitro and in vivo by signals delivered through receptors involved in cell activation and growth, such as HLA-DR (14–16). The ability of signaling through MHC-II molecules to kill B-cell tumors has been shown in murine systems (17) and has been extended to human cells in vitro (18), although the selectivity of antitumor effects and the details of the signaling pathway leading to apoptosis are still controversial, with some studies suggesting caspase-8 activation (19) and others caspase independence (20, 21). Introduction and clinical application of the murine Lym-1 (18), although the selectivity of antitumor effects and the details of the signaling pathway leading to apoptosis are still controversial, with some studies suggesting caspase-8 activation (19) and others caspase independence (20, 21). Introduction and clinical application of the murine Lym-1 (18), although the selectivity of antitumor effects and the details of the signaling pathway leading to apoptosis are still controversial, with some studies suggesting caspase-8 activation (19) and others caspase independence (20, 21). Introduction and clinical application of the murine Lym-1 (18), although the selectivity of antitumor effects and the details of the signaling pathway leading to apoptosis are still controversial, with some studies suggesting caspase-8 activation (19) and others caspase independence (20, 21).
Recently, a fully human anti-HLA-DR antibody termed 1D09C3 has been generated by screening the human combinatorial antibody library (25). In vitro, 1D09C3 exerts a potent tumoricidal activity on several lymphoma and leukemia cell lines as well as primary cells from CLL patients (25). In vivo, injection of 1D09C3 in severe combined immunodeficient (SCID) mice inoculated with the mantle cell lymphoma (MCL) cell line GRANTA-519 resulted in a significant delay of tumor growth (25). However, the mechanism(s) of action of 1D09C3 as well as its curative potential in vivo are still to be elucidated.

Therefore, it was the aim of the present study to investigate the mechanism(s) of 1D09C3-induced cell death and further explore its selective tumoricidal activity toward neoplastic cells. In addition, two tumor xenotransplant models were used to investigate in nonobese diabetic (NOD)/SCID mice the therapeutic efficacy of 1D09C3 in the treatment of mice with early- and advanced-stage NHLs.

Materials and Methods

Cell lines and primary cells. The CLL cell line JVM-2, the MCL cell line GRANTA-519, and the anaplastic large cell lymphoma cell line SU-DHL-1 were purchased from the DSMZ (Braunschweig, Germany). Normal CD34+ cells were enriched from the peripheral blood of consenting healthy donors of allogeneic stem cells undergoing peripheral blood stem cell mobilization (26). Normal CD14+, CD3+, CD19+, and CD16+CD56+ cells were enriched from the peripheral blood of consenting blood donors using the appropriate immunomagnetic mAbs according to the instructions of the manufacturer and the AutoMACS device (Miltenyi Biotec, Bergisch-Gladbach, Germany). Primary B-CLL mononuclear cells (MNC) were isolated at the time of diagnostic workup from the peripheral blood of consenting patients by centrifugation on a Ficoll/Hypaque density gradient and plastic adherence to deplete monocytes. As assessed by flow cytometry, the percentage of MNCs coexpressing CD19, CD5, and CD23 antigens was >95%.

Reagents. The pan-caspase inhibitor zVAD-fmk was purchased from PharMingen (San Jose, CA). The c-Jun-NH2-kinase (JNK) antibody inhibitor 1, 1-s-tosylamide-2-phenylethyl chloromethyl ketone (t-Tok) and the L-TAT control peptide were purchased from Alexis (Milan, Italy, EU). Tiron (4,5-di-hydroxy-1,3-benzenediolic acid) was obtained from Sigma-Aldrich (St. Louis, CA). Dihydroethidium and tetramethylrhodamine ethyl ester (TMRE) were purchased from Invitrogen Italia (Milan, Italy).

HLA-DR expression. Cells (1 x 10^6) were labeled with either FITC-labeled anti-HLA-DR mAb (clone L243; Becton Dickinson) or an appropriate FITC-labeled anti-IgG2a isotype control (Becton Dickinson) and then centrifuged (750 g, 10 minutes, 4°C) to pellet the nuclei. After centrifugation of the supernatant (10,000 g, 25 minutes), the cytosolic fractions was determined by Western blot analysis using anti-HSP60 antibody (Santa Cruz, San Diego, CA; ref. 34).

Cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli-
ium bromide assay. Tumor cells (0.5 x 10^4 to 10^5/mL) or normal cells (2 x 10^5 to 10^6/mL) were resuspended in culture medium supplemented with heat-inactivated serum were exposed for 4 to 48 hours to 1D09C3 (0.1-10 μg/mL). Controls were without antibody or with the murine anti-HLA-DR 10F12 antibody that fails to induce cell death (27). At the end of incubation, viable and dead cells were distinguished by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis. To obtain absolute cell counts by FACS, cell samples were supplemented with Flowcount beads. Cell counts were calculated by the following equation: viable cells x total beads / counted beads. Cell survival following was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake method. Briefly, cells (5,000-10,000 per well) were incubated in triplicate in a 96-well plate in the presence or absence of 1D09C3 in a final volume of 0.15 mL for 24 to 48 hours at 37°C. Thereafter, 0.015 mL MTT solution (5 mg/mL in PBS) were added to each well. After a 4-hour incubation at 37°C, plates were centrifuged (10 minutes, 1,000 rpm), the supernatant was removed, and 0.1 mL DMSO was added to each well. Then, the absorbance at 550 nm was measured by means of a 96-well multispecimen autoreader (Dynatech MR5000, Dynex Technologies, Chantilly, VA). The following formula was used to calculate cell viability: percentage cell viability = (absorbance of the experiment samples / absorbance of the control) x 100.

Annexin V/PI staining. The Annexin V-FITC assay (Bender MedSystems, San Bruno, CA) was used to quantitatively determine the percentage of cells undergoing apoptosis following exposure to 1D09C3 (28, 29). Controls were either without antibody, with the murine anti-HLA-DR 10F12 antibody (27), or the human IgG1 (41-764; Sigma-Aldrich) isotype control. Briefly, cells to be analyzed were washed twice with cold PBS and then resuspended in binding buffer [10 nmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L CaCl2 (pH 7.4)]. Following incubation, 0.1 mL of the cell suspension was transferred to a 5 mL culture tube and 5 μL of Annexin V-FITC was added. After vortexing, samples were incubated for 10 minutes at room temperature in the dark. At the end of the incubation, 0.4 mL of binding buffer and 10 μL of PI were added and the cells were analyzed immediately by flow cytometry. The Annexin V/PI double staining allowed to distinguish between apoptotic (Annexin V+/PI−) and nonapoptotic (i.e., dead cells; Annexin V+/PI+ plus Annexin V−/PI+).

Measurement of ROS. For analysis of ROS levels, 2 x 10^5 cells were plated in six-well plates (30). After exposure to 1D09C3, cells were incubated with dihydroethidium (5 minutes, 37°C) and analyzed by flow cytometry using FACS Calibur (Becton Dickinson). Data were processed by using the Cell Quest software (Becton Dickinson). The membrane-permeable fluorescent dye dihydroethidium is oxidized in the presence of ROS, intercalates within double-stranded DNA, and then fluorescently maximal at 600 nm. Increased cellular fluorescence, as measured by flow cytometry, indicates increased production of ROS.

Measurement of ΔΨm. Mitochondrial membrane depolarization was determined by using the fluorescent probe TMRE (31, 32). Briefly, cells (1 x 10^6/mL) were incubated with TMRE (37°C, 10 minutes) in the dark and analyzed by flow cytometry using FACS Calibur (Becton Dickinson). The fluorescent dye TMRE is accumulated by mitochondria and as a result of mitochondrial membrane depolarization, a shift in the left in the emission spectrum by apoptotic cells can be detected.

Subcellular fractionation. To analyze cytosolic and mitochondrial proteins, subcellular fractionation was done (33). Briefly, cells were resuspended in ice-cold buffer [250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 17 μg/mL phenylmethylsulfonyl fluoride (PMSF), 8 μg/mL aprotinin, and 2 μg/mL leupeptin], homogenized using a 22-gauge needle, and then centrifuged (750 x g, 10 minutes, 4°C) to pellet the nuclei. After centrifugation of the supernatant (10,000 x g, 25 minutes), the cytosolic fractions was harvested, while the pellet was solubilized in mitochondrion lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 0.2% Triton X-100, 0.3% NP40, 100 μmol/L PMSF, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). After centrifugation at 100,000 x g (30 minutes, 4°C), the supernatant representing the S-100 fraction was finally harvested. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). The purity of mitochondrial and cytosolic fractions was determined by Western blot analysis using anti-HSP60 antibody (Santa Cruz, San Diego, CA; ref. 34).

Western blot analysis. Proteins from whole cells or fraction lysates were run on 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked with 5% milk in TBS-Tween 20 (TBST) buffer (20 mmol/L Tris-HCl, 500 mmol/L NaCl, and 0.1% Tween 20) and incubated (overnight, 4°C) with the appropriate primary antibody in TBST containing 5% milk. Immunoreactivity was detected by sequential incubation with horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich) and enhanced chemiluminescence reagents (35). Antibodies used included anti-AIF, anti-HSP60, and anti-caspase-3 from Santa Cruz; anti-caspase-8, anti-caspase-9, and anti-poly(ADP-ribose)polymerase (PARP) from Becton Dickinson; anti-phospho-JNK and anti-phospho-c-Jun...
from Cell Signaling Technology (Beverly, MA) and anti-α-tubulin from Sigma-Aldrich.

In vivo tumoricidal activity in NOD/SCID mice. Six- to eight-week-old female NOD/SCID mice with body weight of 20 to 25 g were purchased from Charles River (Milan, Italy). Mice were housed under standard laboratory conditions according to our institutional guidelines. Experimental procedures done on animals were approved by the Ethical Committee for Animal Experimentation and were carried out in accordance with the guidelines of the United Kingdom Coordinating Committee on Cancer Research (36). Mice were inoculated i.p. with JVM-2 cells (0.25 x 10^6 or 1 x 10^6 per mouse), i.v. with GRANTA-519 cells (1 x 10^6 per mouse). 1D09C3 was injected s.c. Control mice received PBS or the human IgG4 isotype control (3 x 1 mg/mouse). Animals were checked twice weekly for tumor appearance, body weight measurements, and toxicity. Endpoint of in vivo experiments was death. Dose-titration experiments were done in mice (n = 10 per treatment group) xenografted with JVM-2 cells (1 x 10^6 per mouse) and treated with 1D09C3 at doses ranging from 1 x 0.01 mg/mouse to 1 x 1 mg/mouse. For treatment of early-stage disease, mice xenografted with JVM-2 cells (0.25 x 10^6 or 1 x 10^6 per mouse) received 1D09C3 at 3 x 1 mg/mouse (days 4, 7, and 9) or 1 x 1 mg/mouse (day 4), and mice xenografted with GRANTA-519 cells (1 x 10^6 per mouse) received 1D09C3 at 3 x 1 mg/mouse (days 1, 4, and 7). Treatment of advanced-stage disease consisted of 1D09C3 (6 x 1 mg/mouse) administered at 48-hour intervals starting on day 15 for JVM-2-bearing mice, and day 7 for GRANTA-519-bearing mice. Experiments evaluating the treatment of early- or advanced-stage disease were done at least on four separate occasions, using five mice per treatment group.

Statistical analysis. Statistical analysis was done with the statistical package Prism 4.0 (GraphPad Software, San Diego, CA) run on a Macintosh G4 personal computer (Apple Computer). To test the probability of significant differences between untreated and treated samples, the Student’s t test for paired data (two tail) was used. Survival curves were created using the product limit method of Kaplan-Meier and survival differences were compared using the log-rank test. Differences were considered significant if P ≤ 0.05.

Results

In vitro antiproliferative activity of 1D09C3. The effect of 1D09C3 on in vitro tumor cell growth was investigated using the HLA-DR+ cell lines JVM-2 and GRANTA-519 and the HLA-DR- cell line SU-DHL-1 (Fig. 1A). Exposure of HLA-DR+, but not HLA-DR− cell lines, to 1D09C3 (2.5 μg/mL, 24 hours) significantly reduced both viable cell countings and cell survival (Fig. 1B–C). Treatment with 1D09C3 reduced the absolute number of viable cells by 3-fold (P < 0.001) and 48-fold (P < 0.0001), and the survival of tumor cells by 33-fold (P < 0.0001) and 25-fold (P < 0.0001) for JVM-2 and GRANTA-519, respectively (Fig. 1B–C).

Mechanism of 1D09C3-induced cell death. To confirm whether the antiproliferative activity exerted by 1D09C3 involved programmed cell death, the Annexin V/PI double staining was used to distinguish between apoptotic (Annexin V+/PI−) and nonapoptotic dead cells (Annexin V+/PI+ plus Annexin V−/PI+). Exposure to 1D09C3 (10 μg/mL) was associated with a potent time-dependent death of JVM-2 (Fig. 1D) and GRANTA-519 (Fig. 1E) cells. The levels of cell death triggered by 1D09C3 was unchanged if human serum was added in culture as a source of complement, suggesting that complement did not contribute to 1D09C3-induced cytotoxicity. Under our experimental conditions, GRANTA-519 cells were preferentially triggered to apoptosis,

![Figure 1](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-05-2714/FIG1.html)
whereas an increase of dead cells was the prominent finding with JVM-2 cells. For both cell lines, 1D09C3-induced cell death was dose-dependent and could be detected at antibody concentrations as low as 0.1 \( \mu \text{g/mL} \) (Fig. 1F-G).

Previous studies in NHL cell lines, primary CLL cells, and normal splenic B cells investigating the mechanism(s) of anti-MHC class II antibody-induced apoptosis showed different pathways of cell death, involving both caspase-dependent (19, 37) or caspase-independent mechanisms (20, 38), generation of ROS (39), and JNK activation (15, 21, 39, 40). Exposure of JVM-2 and GRANTA-519 to 1D09C3 induced apoptosis and cell death (Fig. 1), but was not associated with processing of the caspase-8 (mitochondria-independent/extrinsic pathway), caspase-9 (mitochondria-dependent/intrinsic pathway), caspase-3, or cleavage of PARP, which is typical of caspase-dependent apoptosis (Fig. 2A). 1D09C3-induced cell death was not reversed by the pan-caspase inhibitor Z-VAD-fmk, further supporting that 1D09C3 acts through a caspase-independent pathway (Fig. 2B).

To further examine the molecular mechanisms whereby 1D09C3 triggers cell death, we investigated whether (a) loss of mitochondrial potential was involved in 1D09C3-induced cell death and (b) ROS generation was required for triggering the mitochondrial pathway. Treatment with 1D09C3 induced a potent, time-dependent mitochondrial membrane depolarization that could be detected both in JVM-2 (Fig. 3A) and GRANTA-519 (Fig. 3B) cells, with loss of mitochondrial potential being detectable in 40% to 80% of 1D09C3-treated cells.

Because ROS generation plays an important role in caspase-independent apoptosis (41) and ROS can be detected following HLA-DR ligation of activated B cells (42), we hypothesized that 1D09C3 treatment would lead to generation of ROS. A substantial increase in the production of ROS could indeed be detected in both JVM-2 (Fig. 3C) and GRANTA-519 (Fig. 3D) cells following 1D09C3 exposure. Subsequently, we verified whether ROS generation was a downstream effect of mitochondria perturbation or lied upstream of mitochondrial depolarization and cell apoptosis. To this purpose, we analyzed the effect of the ROS scavenger Tiron in cells incubated with the antibody. The combined treatment of the cells with 1D09C3 and the ROS scavenger Tiron prevented the generation of ROS (Fig. 4A) and mitochondrial membrane depolarization (Fig. 4B) while substantially reducing cell death (Fig. 4C). These findings allowed to assign to ROS increase a causative role in mitochondrial membrane depolarization and cell death induced by 1D09C3.

Increase in ROS intracellular levels can lead to activation of JNK that induces apoptosis in some cell systems (43–45). We therefore analyzed the activation of JNK and its translocation from the cytosol to mitochondria, where JNK is known to trigger mitochondrial membrane depolarization, thereby facilitating the release of mitochondrial apoptogenic proteins to the cytosol.

### Figure 2.

1D09C3 does not induce processing of the caspases or PARP. A, JVM-2 and GRANTA-519 cells were treated with 10F12 (10 \( \mu \text{g/mL} \)) or 1D09C3 (10 \( \mu \text{g/mL} \)) for 4 to 24 hours. Cytosolic proteins were then separated by SDS-PAGE and analyzed by immunoblotting with anti-caspase-8, anti-caspase-9, anti-caspase-3, and anti-PARP. No processing of the caspases or PARP was observed. CF, cleaved fragments. As positive control for caspase activation, the KMS-11 cell line exposed to soluble tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was used. B, JVM-2 and GRANTA-519 cells were treated with 10F12 (10 \( \mu \text{g/mL} \), 4 hours), zVAD-fmk (100 \( \mu \text{mol/L} \)), 1D09C3 (10 \( \mu \text{g/mL} \), 4 hours), or 1D09C3 plus zVAD-fmk. Treatment with the caspase inhibitor zVAD-fmk was started 1 hour before adding 1D09C3. Following treatments, cell death was assayed by flow cytometry using the Annexin V/PI double staining. Each cell line was tested on three independent experiments. Columns, mean; bars, SE.
Incubation of GRANTA-519 cells with 1D09C3 induced an evident activation of JNK, which was localized to mitochondria (Fig. 5A). In addition, we could appreciate the cytosolic release of the intramitochondrial AIF (Fig. 5B; refs. 49, 50). Because JNK was apparently activated in response to 1D09C3, we verified whether its activity was required for the proapoptotic effect of the antibody. To this purpose, we used the JNK peptide inhibitor 1-L-stereoisomer (L-JNKI1; ref. 51). Addition of the peptide to 1D09C3-exposed cells inhibited both JNK activity (Fig. 5C) and apoptosis (Fig. 5D), indicating that cell death induced by 1D09C3 involves activation of the kinase.

**Effect of 1D09C3 on primary B-CLL cells.** The capacity of 1D09C3 of triggering cell death was further evaluated by analyzing the effect of the antibody on primary leukemic cells purified from the peripheral blood of B-CLL patients at diagnosis. Typically, >95% of these cells coexpressed CD19, CD5, and CD23 antigens. As shown in Table 1, a 4-hour exposure to 1D09C3 (2.5 µg/mL) induced a marked B-CLL cell killing in all analyzed samples, with an average 7-fold increase of cell death compared with untreated cells (P ≤ 0.0001). Under the same culture conditions, the human IgG4 isotype controls had no effect on leukemic cells.

**Selectivity of 1D09C3-induced cell death.** Because HLA-DR is expressed by several hematopoietic cell types, 1D09C3 treatment might be expected to result in a broad myelolymphoid toxicity.

To investigate the selectivity of the tumoricidal activity of 1D09C3, the cell death–inducing effects of 1D09C3 were analyzed in vitro on normal CD34+ cells, CD14+ cells, resting and activated CD19+ and CD3+ lymphocytes, as well as CD16+/CD56+ cells (Table 2). HLA-DR expression on primary cells showed a median MFI ratio of 121 (range, 4-275). Following a 48-hour exposure to a high dose of 1D09C3 (10 µg/mL), no cell death-inducing effect could be detected for CD34+ cells, which were also analyzed by means of long- and short-term culture assays with no evidence of damage being detected at the level of primitive and committed hematopoietic progenitors (data not shown). No evidence of reduced cell counts or cell death induction was detected for CD14+ cells, resting CD3+ lymphocytes, and natural killer cells, whereas viable cell counts were reduced by 20% for activated T cells and by 50% for resting and activated B cells (Table 2). Incubation of primary cells with 1D09C3 in medium supplemented with human serum as a source of complement failed to increase the toxicity of the antibody, thus ruling out the occurrence of complement-mediated killing mechanisms.

**Antilymphoma activity of 1D09C3 in vivo.** The antitumor activity of 1D09C3 was investigated in NOD/SCID mice xenografted i.p. with JVM-2 or i.v. with GRANTA-519 cells. Both tumor models are characterized by extensive bone marrow infiltration and hind leg paralysis due to central nervous system involvement.

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**Figure 3.** 1D09C3 induces mitochondrial depolarization and generation of ROS. JVM-2 (A) and GRANTA-519 (B) cells were plated at 1 × 10⁶/mL and incubated with 10F12 (10 µg/mL) or 1D09C3 (10 µg/mL). At the indicated time points, loss of mitochondrial potential was measured using TMRE staining and flow cytometry. JVM-2 (C) and GRANTA-519 (D) cells were plated at 1 × 10⁶/mL and incubated with 10F12 (10 µg/mL) or 1D09C3 (10 µg/mL). At the indicated time points, generation of ROS was measured using dihydroethidium staining and flow cytometry.
Additionally, JVM-2-injected mice show enlargement of abdominal lymph nodes, and involvement of the lymphoid structures of the gastrointestinal tract, whereas GRANTA-519 show extensive liver and spleen infiltration. Disseminated disease could be detected 7 and 14 days after inoculation of GRANTA-519 and JVM-2 cells, respectively.

Preliminary dose-titration experiments using JVM-2 cells (1 x 10^6 per mouse) showed a dose-dependent antilymphoma activity of 1D09C3. The median survival time of untreated mice was 31 days, whereas the median survival times of mice treated with 1D09C3 at 1 x 0.01, 1 x 0.1, and 1 x 1 mg/mouse were 46 (P ≤ 0.0001), 58 (P ≤ 0.0001), and 67 (P ≤ 0.0001) days, respectively (Fig. 6A). No difference in median survival could be detected among untreated mice (31 days) and mice receiving either control vehicle (32 days) or the human IgG4 isotype control (32 days; Fig. 6A).

The therapeutic efficacy of 1D09C3 was then evaluated in mice xenografted with two different doses of JVM-2 cells (0.25 x 10^6 and 1 x 10^6 per mouse) and treated at an early stage of disease according to two treatment schedule (i.e., 3 x 1 mg/mouse; days 4, 7, and 9) or 1 x 1 mg/mouse (day 4). Mice xenografted with either 0.25 x 10^6 (Fig. 6B) or 1 x 10^6 (Fig. 6C) JVM-2 cells and treated with control vehicle died of disseminated disease (median survival times were 35 and 29 days, respectively). Treatment with a single injection of 1D09C3 resulted in a 70% overall survival for mice transplanted with 0.25 x 10^6 cells (Fig. 6B), and a 20% overall survival with a median survival of 64 days (P ≤ 0.0001) for mice xenografted with 1 x 10^6 cells (Fig. 6C). Increasing the dose of 1D09C3 up to 3 mg/mouse resulted in a 100% survival for NOD/SCID mice xenografted with 0.25 x 10^6 JVM-2 cells (Fig. 6B), and a 42% overall survival with a median survival of 99 days (P ≤ 0.0001) for animals xenografted with 1 x 10^6 cells (Fig. 6C). Mice receiving 3 mg of 1D09C3 and surviving up to 120 days were considered bona fide disease-free and indeed remained alive and well up to 360 days of observation.

A similar therapeutic efficacy was detected in mice xenografted with GRANTA-519 cells (1 x 10^6 per mouse) who received an early 1D09C3 treatment (3 x 1 mg/mouse, days 1, 4, and 7). All mice treated with control vehicle died with a median survival time of 36 days, whereas treatment with 1D09C3 resulted in a significant increase of median survival (108 days, P ≤ 0.0001), with 27% of NOD/SCID mice being alive and disease-free at the end of the 120-day observation period (Fig. 6D). Subsequently, all these mice developed signs of systemic disease and died within 240 days after tumor inoculation.

The most rigorous preclinical evaluation of an antineoplastic agent is to determine its ability to induce responses in well-established tumors. To test the therapeutic efficacy of 1D09C3 in animals with advanced disease, NOD/SCID mice were injected with either JVM-2 (1 x 10^6 per mouse) or GRANTA-519 (1 x 10^6 per mouse) cells and randomly assigned to receive 1D09C3 or control vehicle. Treatment with 1D09C3 (6 x 1 mg/mouse) was started on day 15 or day 7 for mice xenografted with JVM-2 or GRANTA-519, respectively. Each mouse received a total of six doses of 1D09C3 at 48-hour intervals. As compared with controls, 1D09C3 injection

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**Figure 4.** ROS scavenging inhibits 1D09C3-induced mitochondrial membrane depolarization and cell apoptosis. GRANTA-519 cells were plated at 1 x 10^6/mL and treated without or with the ROS scavenger Tiron (10 mmol/L). After 1 hour, the appropriate samples were stimulated with 1D09C3 (4 μg/mL, 30 minutes). A, generation of ROS was measured using dihydroethidium staining. B, mitochondrial membrane depolarization was measured using TMRE staining. C, apoptosis was measured by the Annexin V/PI double staining.
resulted in a significant prolongation of the median survival times both for mice xenografted with JVM-2 (29 versus 52 days, \( P < 0.0001 \); Fig. 6E) and GRANTA-519 (34 versus 54 days, \( P < 0.003 \); Fig. 6F) cells. Immunophenotypical analysis of GRANTA-519 cells growing in mice relapsing following an initial therapeutic response to 1D09C3 revealed a persistent expression of HLA-DR as well as a maintained sensitivity to 1D09C3-induced cell death, thus ruling out that failure of antibody therapy was due to the regrowth of tumor cells who had lost the target antigen expression or function (data not shown).

**Discussion**

Effective curative therapy without significant toxicity is the ultimate goal of cancer treatment. The development and approval of antitumor mAbs, such as rituximab, alemtuzumab, and trastuzumab, were major steps in this direction, although response rates and durations remained limited using antibodies as single agents (5, 6, 52). The efficacy of these antitumor antibodies usually depends on intact immunologic effector mechanisms. Therefore, the development of antibodies with a tumoricidal activity independent of complement and ADCC and with superior selectivity toward neoplastic cells represents a major advancement of cancer treatment.

The relatively limited expression of HLA-DR on normal cells facilitates the targeting of malignant lymphoma cells expressing HLA-DR. Hu1D10 (apolizumab; ref. 22) and Lym-1 (18) recognize posttranslational modifications on HLA-DR molecules that occur preferentially in B cell–derived tumors, thus providing a margin of selectivity, although some expression was also noted on normal B cells and monocytes. Neither of these antibodies has inherent tumoricidal activity, and, thus, Lym-1 is developed in a \(^{131}I\)-labeled form (Oncolym), whereas the efficacy of Hu1D10 relies on intact immunologic effector mechanisms of the patient. Furthermore, Lym-1 is a murine antibody with substantial immunogenicity for humans and Hu1D10 is a humanized antibody.

Recently, a new class of fully human, HLA-DR-specific mAbs capable of inducing tumor cell death has been described (25).
Among these antibodies, 1D09C3 has been shown to exert the most potent antitumor activity. In vitro data reported herein confirm that 1D09C3 has an intrinsic tumoricidal activity, as shown by the potent cell death and growth suppression effects detected in vitro in on primary B-CLL cells as well as two HLA-DR+ tumor cell lines used in our study (i.e., the CLL cell line JVM-2 and the MCL cell line GRANTA-519).

Our data in NOD/SCID mice xenografted with JVM-2 or GRANTA-519 cell lines significantly extends previous findings showing that 1D09C3 delays the growth of lymphoma cells in SCID mice (25). Total doses of 1D09C3 used in our in vivo experiments (i.e., 1, 3, or 6 mg/mouse), are similar to total doses of rituximab that were used by others (53, 54). Additionally, a 3 mg dose in mice is equivalent to 450 mg/m2 in humans and compares well with the conventional dose of rituximab (375 mg/m2), which is repeatedly used by others (17, 22, 24), and B-CLL.

Cancer Res 2006; 66: (3). February 1, 2006 1806 www.aacrjournals.org

1D09C3 might have a major clinical effect on the patients with NHL not due to loss of target antigen expression. Our observations in JVM-2 or GRANTA-519 cell lines significantly extend previous findings showing that 1D09C3 as single agent exerted a potent therapeutic effect in animals with a minimal tumor burden who achieved up to 100% tumor growth inhibition (25). Total doses of 1D09C3 used in our study (i.e., the CLL cell line JVM-2 and the MCL cell line GRANTA-519).

Another controversial point is the mechanism of HLA-DR-mediated cell death. Studies demonstrating either direct (15) or indirect CD95-mediated (55) apoptotic effects have been reported, along with studies describing a nonapoptotic morphology (56, 57) as well as caspase dependence (19, 37) or caspase independence (20) of HLA-DR-mediated cell death. The controversial role of these events in MHC class II-induced cell death might be due to the use in different studies of murine and humanized antibodies targeting several different components of the HLA-DR α or β chain in different cell models.

1D09C3 failed to induce any processing of caspase-8, caspase-9, and caspase-3, as well as PARP, which is typical of caspase-dependent apoptosis. In addition, we did not detect any effect of the pan-caspase inhibitor z-VAD-fmk on 1D09C3-induced cell death. Data reported herein show that ROS were generated early following treatment with 1D09C3 and ROS generation was a prerequisite for both mitochondrial depolarization and apoptosis. Therefore, intracellular ROS generation, by either a plasma membrane (NADPH oxidase) or a cytoplasmic source, seems to be a primary event induced by 1D09C3 ligation (58, 59). Mitochondrial depolarization, possibly through ROS, induced activation of JNK (43–45), cytosolic release of AIF, and possibly other proteins (endonucleases, etc.) from the mitochondria (38), and apoptosis then follow. Inhibition of JNK activity significantly delays the growth of lymphoma cells in SCID mice (25). Total doses of 1D09C3 used in our study (i.e., the CLL cell line JVM-2 and the MCL cell line GRANTA-519).

Table 2. Cell death–inducing effects of 1D09C3 on primary normal cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HLA-DR expression (MFI ratio)*</th>
<th>Viable cell counting (% control)</th>
<th>Cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ cells</td>
<td>60</td>
<td>108 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>CD14+ cells</td>
<td>68</td>
<td>92 ± 5</td>
<td>13</td>
</tr>
<tr>
<td>CD3+ cells (resting)</td>
<td>4</td>
<td>96 ± 5</td>
<td>12</td>
</tr>
<tr>
<td>CD3+ cells (activated)</td>
<td>200</td>
<td>82 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>CD19+ cells (resting)</td>
<td>188</td>
<td>48 ± 10</td>
<td>8</td>
</tr>
<tr>
<td>CD19+ cells (activated)</td>
<td>275</td>
<td>44 ± 9</td>
<td>15</td>
</tr>
<tr>
<td>CD16+/CD56+ cells (resting)</td>
<td>17</td>
<td>101 ± 4</td>
<td>14</td>
</tr>
<tr>
<td>CD16+/56+ cells (activated)</td>
<td>175</td>
<td>120 ± 5</td>
<td>13</td>
</tr>
</tbody>
</table>

*Freshly isolated peripheral blood cell subsets were resuspended (2 × 10^5·5 × 10^7/mL) in RPMI 1640 supplemented with heat-inactivated FBS (10%, v/v) and incubated (48 hours, 37°C, 5% CO2) with 1D09C3 (10 μg/mL). Controls were without antibody or with the murine anti-HLA-DR 10F12 antibody that fails to induce cell death (27). At the end of incubation, viable cell countings were done by PI staining and FACS analysis, and cell death (i.e., Annexin V+/PI−, Annexin V−/PI+, and Annexin V−/PI+ cells) was evaluated by Annexin V/PI double staining. Each value represents the mean of three separate experiments.
reduced 1D09C3-induced apoptosis, indicating that the activation of the kinase exerts a major role in the apoptogenic mechanism of 1D09C3.

Altogether, these results establish a sequence of cause-and-effect events in the apoptotic pathway triggered by 1D09C3 ligation. However, in addition to JNK activity, other factors could contribute to 1D09C3-induced cell death. In lymphoid cells, redox state–dependent levels of NAD can directly influence the survival/death balance. Indeed, NAD provides the substrate for ADP-ribosyltransferase-2 that catalyzes ADP ribosylation, activates the cytolytic P2X7 purinoceptor, and induces lymphoid cell death (60–62). Expression of NAD receptors and their role in lymphoid cell response to 1D09C3 deserve further investigation.

In conclusion, data presented herein show that 1D09C3 exerts a potent and selective toxicity on HLA-DR+ tumor cells by activating ROS-dependent, JNK-driven cell death, cures the great majority of lymphoma-bearing mice treated at an early stage of disease, and significantly prolongs survival of mice treated at an advanced stage of disease. These data suggest that patients may ultimately benefit from 1D09C3. A phase I clinical trial aimed at exploring the toxicity and tolerability of 1D09C3 injection in patients with refractory and relapsed NHL is currently ongoing.

Acknowledgments

Received 4/6/2005; revised 11/6/2005; accepted 12/6/2005.

Grant support: Ministero dell’Istruzione, dell’Università e della Ricerca (Rome, Italy), Ministero della Salute (Rome, Italy), and Michelangelo Foundation for Advances in Cancer Research and Treatment (Milan, Italy).

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