In Vivo Antitumor Effects of Unconjugated CD30 Monoclonal Antibodies on Human Anaplastic Large-Cell Lymphoma Xenografts

Zhi-Gang Tian, Dan L. Longo, Satoshi Funakoshi, Osamu Asai, Douglas K. Ferris, Michael Widmer, and William J. Murphy

ABSTRACT

CD30 is a Mr 120,000 surface antigen originally identified by the Ki-1 monoclonal antibody (moAb) against primary and cultured Reed-Sternberg cells present in Hodgkin's disease and anaplastic large-cell lymphomas (ALCLs). Examination of two ALCL cell lines (Karpas 299 and Michel) demonstrated cell surface expression of CD30. Incubation of these lymphomas with two anti-CD30 moAbs that recognize the ligand-binding site (M44 or HeFl-1) resulted in significant growth inhibition in vitro, with significant decreases in cell viability. Another anti-CD30 moAb, Ber-H2, which recognizes a determinant not involved in ligand binding, had no effect on ALCL growth in vitro. When these human ALCL lines were transferred i.v. into mice with severe combined immune deficiency, the ALCL cell lines in vivo, and unconjugated anti-CD30 moAbs may be of potential clinical use.

INTRODUCTION

CD30 is a M, 120,000 surface antigen originally identified by the Ki-1 moAb against Reed-Sternberg cells of HD (1). CD30 was found to be expressed in various non-Hodgkin's lymphomas, particularly ALCLs (2) and some nonhematopoietic tumors (3, 4). The association of the CD30 antigen with lymphoid malignancies has proven to be a useful marker for the identification of malignant cells within lymphoid tissues, particularly lymph nodes (5). An anti-CD30 moAb (Ber-H2) has been evaluated clinically, although none of the patients with refractory HD demonstrated responses to the native Ber-H2 antibody (6). Recently, Ber-H2 was conjugated with a plant ribosome-inactivating protein, saporin, to construct an anti-CD30 immunotoxin (Ber-H2/saporin immunotoxin), and it was demonstrated that this immunotoxin exerted antitumor activity in vitro and in SCID mice xenografted with human CD30+ ALCLs (7, 8). Interestingly, unmodified Ber-H2 did not demonstrate significant antitumor effects (7, 8). Gruss et al. (9) and Hecht et al. (10) developed other anti-CD30 moAbs (M44 and HeFl-1), and it was demonstrated recently that M44 and HeFl-1 recognize the region of the extracellular part of CD30 that contains the binding site for the CD30L, different from Ber-H2, which recognizes another nonoverlapping region of CD30 not involved in ligand binding (11). We and others (12–16) have observed that signals (ligand to receptor or antibody to receptor antigen) inducing activation of normal cells can result in antiproliferative effects in transformed cells. In this report, we have determined that, in contrast to Ber-H2, anti-CD30 moAbs that bind to the ligand-binding site of CD30 (i.e., M44 or HeFl-1) can inhibit the growth of CD30+ ALCL cell lines directly both in vitro and in vivo.

MATERIALS AND METHODS

Mice. CB-17 SCID mice were obtained from the Animal Production Area (National Cancer Institute-Frederick Cancer Research and Development Center) and were not used until 6–8 weeks of age. SCID mice were housed in microisolator cages, and all food, water, and bedding were autoclaved before use. SCID mice received trimethoprim and sulfamethoxazole (40 mg trimethoprim and 200 mg sulfamethoxazole/320 ml drinking water) in suspension in their drinking water.

Antibodies. Three anti-CD30 antibodies were studied: M44, which was provided by Immunex (9), HeFl-1, which was provided by the Biological Response Modifiers Program, National Cancer Institute-Frederick Cancer Research Center (10); and Ber-H2, which was purchased from DAKO (Glostrup, Denmark; Ref. 6). These three moAbs are murine IgG1; msIgGl, as a control antibody, was purchased from Cappel (West Chester, PA).

Tumor Cell Lines. Karpas 299 (DSM-ACC31) was provided by Immunex and established from peripheral blood blast cells of a 25-year-old white man with the diagnosis of CD30+ high-grade ALCL (17). The peripheral blood blasts with pleomorphic nuclei resembled primitive histiocytes, which bear the surface markers CD4, CD5, epithelial membrane antigen, HLA-DR, and CD30. The Karpas 299 cell line possesses the same cytochemical, immunological, morphological, and chromosomal profile, with a t(2;5) translocation, T-cell receptor B-chain gene rearrangement, and a typical anaplastic, morphological, CD4+, CD5+, epithelial membrane antigen+, HLA-DR, and CD30+ immunophenotype, the same as the original peripheral blood blast cells of the patient. The Michel cell line was also provided by Immunex and was established from a CD30+ ALCL tumor. The cell lines were maintained in suspension culture in RPMI 1640 at 37°C in a humified, 5% CO2 atmosphere and were subcultured every 3–4 days. Only cultures in the log phase of growth were used. The cell lines have been studied previously for receptor gene rearrangements, chromosomal alterations, cytochemical staining, cytokine secretion, and receptor expression (17).

In Vivo Experiments. All mice received 20 μl aASGM1 (Wako Pure Chemicals, Inc., Dallas, TX) by i.v. injection 1 day before tumor transfer to remove host natural killer cells and to facilitate engraftment of tumors (18). Karpas 299 and Michel ALCL cells (5 × 10⁵) were then injected either by i.v. or i.p. injection. SCID mice then received either 2 μg anti-CD30 antibody (either M44 or HeFl-1) or msIgG1 myeloma protein in 0.2 ml HBSS i.p. every other day for 20 days for a total 10 injections starting at day 5 or day 10. Tumor-bearing mice were then monitored for tumor development and progression. Moribund mice were euthanized and necropsied for evidence of tumors. Liver, kidney, and lymphoid organs were analyzed histologically for the presence of tumors. Both parametric (Student's t test) and nonparametric (Wilcoxon rank sum test) analyses were performed to determine whether the survival of the different groups differed significantly. All experiments had 6–10 mice/group and were performed three times.

Immunofluorescence Studies. Expression of the CD30 antigen on human ALCL cell lines was determined by flow cytometry or immunofluorescence microscopy. The procedures for flow cytometric analysis have been described...
IN VIVO ANTITUMOR EFFECTS OF UNCONJUGATED CD30 mAbs

and fixed in 1% paraformaldehyde. The cells were analyzed using an Electronically Programmed Individual Cell Sorter (EPICS) flow cytometer (Coulter). For the immunofluorescence studies, 1 million cells (Karpas 299 or Michel) were incubated with human AB serum-containing HBSS [5% (v/v)] for 20 min at 4°C. After being washed with HBSS, the cells were incubated with HeFi-1 (5 μg/ml) for 30 min at 4°C and washed again. The secondary antibodies (1:200; rhodamine-labeled goat antimouse IgG; Kirkegaard & Perry Laboratories, Geithersburg, MD) were then incubated for another 30 min at 4°C. After extensive washing, the cells were coverslipped and examined with a fluorescence microscope (Wild Leitz USA, Inc., Rockleigh, NJ). Photomicrographs were prepared with a Condonics NP600 Printer (Condonics, Middleburg Heights, OH).

In Vitro Proliferation Assay. The effects of anti-CD30 on human ALCL tumor cell growth in vitro were determined by [3H]thymidine incorporation and trypan blue exclusion. The cell lines were split 24 h before assays were performed. Cells were resuspended in culture medium to a concentration of 1 x 10^6, and 0.1 ml was plated in 96-well flat-bottom plates (Corning Glass Works, Corning, NY) that already contained 0.1 ml appropriately diluted reagents of the antibodies or controls. Seventy-two h later, 1 pCi [3H]thymidine/well (specific activity, 6.7 Ci/mmol; New England Nuclear Research Products, Boston, MA) was added for the final 12 h of culture. Cultures were then harvested onto glass fiber filters with a PhD Cell Harvesting System (Cambridge Technology, Inc., Cambridge, MA), and [3H]thymidine uptake was assayed by liquid scintillation on an LKB beta counter (LKB Instruments, Inc., Turku, Finland). Each experiment was performed three times with a representative experiment being shown. Counts per minute were analyzed, untransformed by regression analysis and ANOVA. A linear relationship

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Fig. 2. Surface expression of CD30 on Michel and Karpas 299 ALCL cell lines by immunofluorescence analysis. Michel and Karpas 299 cells were incubated with HeFi-1 antibodies (murine antihuman CD30 mAb), washed, and then incubated with secondary antibodies (rhodamine-labeled goat antimouse IgG). After washing, the cells were examined under a fluorescence microscope. A, Karpas 299 cells under Hoffman configuration. B, staining of Karpas 299 cells (same field) with msIgGl control antibody. C, staining of Karpas 299 cells with anti-CD30. D, staining of Michel cells with anti-CD30. ×1000.
between the cpm level and the logarithm of the concentration was assumed. When no significant departure from linearity was detected, the regression slopes for the control and treated samples were compared by t test. Values were presented as percentage inhibition of proliferation compared with control.

**Analysis of Apoptotic Cells.** Michel cells were cultured in regular tissue culture media containing 1.5% DMSO for 4–6 days to synchronize the cell cycle into G0-G1 (20). After washing with HBSS, the cells were incubated with 1 μg/ml either mslgG1 or M44 in culture medium with 5% FBS, and 1 ml cold 70% ethanol (per sample of 10⁶ cells) was added to the pelleted cells and incubated for 30 min at 4°C. Samples were washed twice with HBSS containing 1% FBS and resuspended in HBSS without FBS, followed by an addition of 10 μl 400 U/ml RNase (Sigma Chemical Co., St. Louis, MO). Samples were incubated for 1 h at room temperature and then with 100 μl 0.5 mg/ml propidium iodide (Sigma) overnight and analyzed with an EPICS flow cytometer with the apoptotic cells appearing in the <2n DNA peak (21). They could be distinguished from necrotic cells by analyzing the scatter profile by an increase in side scatter and a small decline in forward scatter.

![Fig. 3. Effect of anti-CD30 antibody on the proliferation of ALCL in vitro.](image)

![Fig. 4. Effect of anti-CD30 antibody on the proliferation of ALCL cell lines in long-term culture.](image)

![Fig. 5. Flow cytometric analysis of anti-CD30-induced apoptosis of ALCL cell lines.](image)
RESULTS

Surface Expression of CD30 on Human ALCL Cell Lines. We first examined the expression of the CD30 surface antigen on two human ALCL cell lines: Karpas 299 and Michel. As shown in Fig. 1, both Karpas 299 and Michel cell lines expressed high levels of CD30 antigen with a standard mean fluorescence intensity of 255 channels for each cell line through flow cytometry, in agreement with previous reports (9), which were verified further in an immunofluorescence analysis as shown in Fig. 2. We then ascertained the effects of anti-CD30 antibodies on the proliferative potential of these ALCL cells using a \[^{3}H\] thymidine incorporation assay.

Effect of Anti-CD30 Antibodies on Human CD30\(^{+}\) ALCL Cell Proliferation in Vitro. Incubation with anti-CD30 antibodies (M44 or HeFi-1) inhibited the proliferation of Karpas 299 and Michel cells significantly, with an optimal inhibition of thymidine incorporation (of 45–68\%) occurring at 10–100 ng/ml soluble anti-CD30 antibody when compared with an isotype-matched control antibody. Another anti-CD30 antibody (Ber-H2) did not inhibit proliferation of either of the ALCL cell lines significantly (Fig. 3). The two antibodies (M44 and HeFi-1) exerted similar inhibitory effects on the growth of Karpas 299 and Michel cell lines (Fig. 3). We then counted the viable cell numbers of the Karpas 299 and Michel lines in culture with either anti-CD30 antibodies (M44, Hefi-1, or Ber-H2; 100 ng/ml) or with an isotype-matched control antibody. Every 24 h, the absolute cell number and viability were determined by trypan blue exclusion. As shown in Fig. 4, both M44 and HeFi-1 lowered the Karpas 299 and Michel viability and cell number significantly after 96-h cultures, whereas Ber-H2 did not affect cell viability or number. These data demon-
stated that the anti-CD30 moAbs M44 and HeFi-1 inhibit ALCL lymphoma growth directly in vitro. To determine the possibility that the anti-CD30 antibodies had rendered the ALCL cells more susceptible to apoptosis, the treated ALCL cells were analyzed by flow cytometric analysis using propidium iodide. As shown in Fig. 5, 60.41% (peak A, representing the <2n DNA peak) of Michel cells underwent apoptosis when cocultured with anti-CD30 antibodies (HeFi-1) compared with the control (peak a, 32.55%). Peaks B and b represent cells in G0 and G1, respectively. This demonstrates that anti-CD30 was directly capable of inducing activation-induced cell death of the ALCL cells in vitro.

**Antitumor Effects of Anti-CD30 moAbs M44 and HeFi-1 in SCID Mice Bearing Human ALCL.** Before the lymphomas were injected into SCID mice, all mice received antisera to ASGM1, a marker present on murine natural killer cells, to remove host resistance to the tumor (18). Recipient mice then received the Karpas 299 and Michel cells by i.v. injection, which resulted in similar patterns of metastatic growth. The mice developed s.c. tumors around the neck and head, as well as brain and eye metastases (Fig. 6). When $5 \times 10^6$ Karpas 299 or Michel cells were injected i.v., the mice began to die at days 30–35, and all mice succumbed to tumors by day 45. After the human ALCL xenograft model was established, we then examined the effect of the anti-CD30 antibody on the survival of tumor-bearing mice. As shown in Fig. 7, the SCID mice bearing Karpas 299 cells were treated with different doses of M44. Although 0.2-μg M44 group exerted significant antitumor effects ($P = 0.009$ compared with control), the 2.0-μg and 20-μg M44 groups demonstrated significantly greater antitumor effects ($P = 0.039$ and 0.007 compared with the 0.2-μg M44 group, respectively). There were no significant differences between the 2.0-μg M44 group and the 20-μg M44 group ($P = 0.10$). As shown in Fig. 8, treatment with anti-CD30 increased significantly the survival in Karpas 299- and Michel-bearing SCID mice receiving 2 μg M44 every other day for a total of 10 injections starting at day 5 after tumor inoculation. The mean days of death for Karpas 299- and Michel-bearing SCID mice were 35.9 and 42.8 days, respectively, but after treatment with M44, the mean days of death increased to >78.6 ($P < 0.01$) and >100 ($P < 0.01$), respectively. Interestingly, 10 of 10 Michel-bearing and 6 of 10 Karpas 299-bearing SCID mice survived more than 100 days (Table 1; experiment 3) and remained disease-free after anti-CD30 treatment. We then delayed the time of treatment from day 5 to day 10 and noted that even after 10 days of tumor growth before initiating treatment, M44 showed significant antitumor effects with SCID mice bearing Karpas 299 and Michel ALCL lymphoma (Fig. 9). These data indicate that M44 is efficacious in the treatment of established tumor burdens of CD30+ ALCL lymphomas in vivo in mice. To examine another route of tumor inoculation, we injected Karpas 299 cells ($5 \times 10^6$) i.p. into SCID mice and found that all mice developed peritoneal tumors with extensive metastases in the lymph nodes. After treatment with anti-CD30, significant increases in survival were again noted (Fig. 10), indicating that unconjugated anti-CD30 is efficacious against CD30+ ALCL cell lines in vivo.
We demonstrate here that certain unconjugated anti-CD30 moAbs are efficacious against CD30⁺ ALCL cell lines both in vitro and in vivo. The precise mechanisms underlying this inhibition in vivo are not known. The recent cloning of the CD30 gene established CD30 as the newest member of the nerve growth factor receptor superfamily, the members of which (CD27, CD40, tumor necrosis factor receptor, and Fas) are involved in cellular activation or apoptosis (22). We have reported previously that CD40 stimulation inhibits the proliferation of various CD40⁺ B-cell lymphomas directly in vitro and is efficacious against a wide variety of CD40⁺ B-cell lymphomas in a similar xenograft model (16). The antitumor effects of CD40 stimulation were mediated presumably by activation-induced cell death of the neoplastic lymphocytes. CD30 belongs to the nerve growth factor/tumor necrosis factor receptor family and also serves as a transmembrane growth factor receptor, a possibility supported by identification and characterization of the CD30L (23). Biochemical studies of the CD30 molecule provided strong evidence to indicate a signal-transducing role. All the forms of CD30 are phosphorylated at serine and/or tyrosine residues and its intracellular component possesses kinase activity (24). The nature of the intracellular signals involved in the induction of cell death in the ALCL by anti-CD30 remains to be elucidated although our data demonstrating the induction apoptosis in the treated cells by anti-CD30 is in agreement with previous reports (9).

The potential use of the CD30 antibody as a tool for immunotherapy, based on the data that span over more than 10 years, has been reviewed recently (22). Because of restricted expression of CD30 in normal human tissue (primarily appearing in activated or proliferating lymphoid cells; Ref. 22), CD30 seems to be a potential target for therapeutic attack in tumor-expressing CD30. Recently, Falini et al. (6) provided immunological evidence that the restricted in vitro reactivity of an unmodified anti-CD3O moAb (Ber-H2) with normal tissue is maintained in vivo, and that optimal in vivo targeting of HD cells can be achieved by injecting low doses of Ber-H2. Despite optimal in vivo targeting of tumor cells, none of their patients with refractory HD showed tumor regression to Ber-H2, and from this finding they suggested that, for therapeutic goals, anti-CD30 antibodies should be conjugated to other cytotoxic agents (either isotopes or toxins; Ref. 22). However, our data suggest that unconjugated anti-CD30 moAbs can be used, if the antibodies are directed at the ligand-binding site (11). Unconjugated moAbs are attractive, because they avoid toxicity associated with toxins and isotopes. The results also imply that the sCD30L may be effective against CD30⁺ tumors. It has been reported that CD30 stimulation by a soluble recombinant CD30L inhibited the growth of human ALCL cell lines in vitro, at least in part through the induction of apoptosis (9), in agreement with our results (Fig. 5). This suggests that sCD30L, M44, or HeFi-1 may be promising agents in the treatment of human CD30⁺ ALCL. A potential explanation concerning the differences between Ber-H2 failure in clinical Hodgkin’s disease treatment and our treatment in SCID mice may be that the Ber-H2 antibody recognizes a different epitope on the CD30 molecule.

### Table 1 Effect of anti-CD30 treatment on survival in tumor bearing SCID mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor (route)</th>
<th>Treatment (initiation)</th>
<th>No. mice</th>
<th>Mean day of death</th>
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<tbody>
<tr>
<td>1</td>
<td>Karpas(i.p.)</td>
<td>mslgG1 (day 5)</td>
<td>5</td>
<td>75.9 ± 30.2</td>
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<td>2</td>
<td>Karpas(i.p.)</td>
<td>M44 (day 5)</td>
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<td>&gt;128.1 ± 31.4</td>
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<td>mslgG1 (day 5)</td>
<td>10</td>
<td>38.8 ± 5.4</td>
</tr>
<tr>
<td>4</td>
<td>Karpas(i.v.)</td>
<td>M44 (day 5)</td>
<td>10</td>
<td>&gt;66²</td>
</tr>
<tr>
<td>5</td>
<td>Karpas(i.v.)</td>
<td>HeFi-1 (day 5)</td>
<td>10</td>
<td>35.9 ± 4.5</td>
</tr>
<tr>
<td>6</td>
<td>Karpas(i.v.)</td>
<td>M44 (day 5)</td>
<td>10</td>
<td>278.6 ± 26.1</td>
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<tr>
<td>7</td>
<td>Michel(i.v.)</td>
<td>mslgG1 (day 5)</td>
<td>10</td>
<td>42.8 ± 4.0</td>
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<tr>
<td>8</td>
<td>Michel(i.v.)</td>
<td>M44 (day 5)</td>
<td>10</td>
<td>&gt;10²</td>
</tr>
<tr>
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<td>Michel(i.v.)</td>
<td>M44 (day 5)</td>
<td>10</td>
<td>53.0 ± 9.2</td>
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<td>Michel(i.v.)</td>
<td>M44 (day 10)</td>
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<td>&gt;50.0 ± 12.1²</td>
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<td>M44 (day 5)</td>
<td>10</td>
<td>&gt;66²</td>
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<td>Michel(i.v.)</td>
<td>M44 (day 10)</td>
<td>10</td>
<td>&gt;58.2 ± 4.6²</td>
</tr>
</tbody>
</table>

*SCID mice were treated as described in "Materials and Methods." All mice received anti-ASGM1 i.v. 1 day before tumor transfer. On the day the antibody was initiated, the mice received either mslgG1 antibody or anti-CD30 antibody (M44 and HeFi-1; 2 μg i.p.) administration every other day for a total of 10 injections.

¹Six of seven mice showed no evidence of tumor at day 140.

²Ten of 10 mice showed no evidence of tumor at day 60.

³Ten of 10 mice showed no evidence of tumor at day 60.

⁴Six of 10 mice showed no evidence of tumor at day 60.

⁵Ten of 10 mice showed no evidence of tumor at day 100.

⁶Six of 10 mice showed no evidence of tumor at day 60.

⁷Five of 10 mice showed no evidence of tumor at day 60.

⁸Ten of 10 mice showed no evidence of tumor at day 60.

⁹Eight of 10 mice showed no evidence of tumor at day 60.

Fig. 9. Effect of anti-CD30 treatment on survival of ALCL (Karpas 299 and Michel)-bearing SCID mice. SCID mice received Michel (A) or Karpas 299 (B) i.v. as described in "Materials and Methods." Five days later (M44; day 5) or 10 days later (M44; day 10), the mice were treated i.p. with M44 or mslgG (2 μg/injection) every other day for 20 days, for a total of 10 injections. The mice were monitored for tumor growth and survival. Moribund mice were euthanized and showed evidence of extensive tumor burden. The representative experiment was with 10 mice/group. Anti-CD30 treatment improved the survival of tumor-bearing SCID mice significantly (for Michel: M44 day 5 versus mslgG1, P = 0.001; M44 day 10 versus mslgG1, P = 0.006; M44 day 5 versus M44 day 10, P = 0.146; for Karpas 299: M44 day 5 versus mslgG1, P = 0.0005; M44 day 10 versus mslgG1, P = 0.0007; M44 day 5 versus M44 day 10, P = 0.752).
and may fail to initiate signal transduction through the receptor. It was noted that Ki-1 (another anti-CD30 moAb) did not affect function on normal or neoplastic CD30+ cells when compared with M44 (22), suggesting that the different CD30 moAbs may have markedly different results concerning clinical efficacy in ALCL. Thus, unconjugated M44 and HeFi-1 may be of potential use in the treatment of CD30+ ALCL.

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