The Efficacy of CD3×CD19 Bispecific Monoclonal Antibody (BsAb) in a Clonogenic Assay: The Effect of Repeated Addition of BsAb and Interleukin-2

By Inez-Anne Haagen, Anette J.G. Geerars, Wim B.M. de Lau, Bert J.E.G. Bast, and Bert C. de Gast

To evaluate the potency by which human T cells are targeted and activated by bispecific monoclonal antibodies (BsAbs) to lyse tumor cells, a clonogenic assay was developed. The efficacy of a CD3×CD19 BsAb binding to both the CD3 T-cell antigen and the CD19 B-cell antigen was already proven in 51Cr-release assays and in 3-day activation cultures. To achieve more quantitative results, a 14-day clonogenic assay, based on limiting-dilution, was performed for the determination of the initial and residual number of clonogenic units obtained with a CD19+ pre-pre-B acute lymphoblastic leukemia (ALL-B) cell line. Elimination of up to 5 logs of ALL-B cells by freshly isolated peripheral blood mononuclear cells (PBMCs) cultured with BsAb plus interleukin-2 (IL-2) could be detected. The presence of human IgG did not abolish the effect. Repeated addition of each of the two agents was necessary, because a single treatment produced only a 1- to 2-log kill. CD3 monoclonal antibody and IL-2 stimulation ("lymphokine-activated killer cell" conditions) resulted in only a 2-log kill. The number of T cells proved critical in lysis of ALL-B cells, with a 5-log kill using a T-cell:B-cell ratio of 3:1 but with only a 1-log kill using a ratio of 1:1. PBMCs isolated from patients with non-Hodgkin's lymphoma, both in relapse or remission, proved to be as competent as those from healthy donors in removing ALL-B cells. This clonogenic assay shows the importance of repeated administration of CD3×CD19 BsAb and IL-2 and offers the possibility to compare it with other therapies in B-cell malignancy.

The treatment of leukemia, advanced lymphoma, and multiple myeloma includes multiple courses of polychemotherapy, but, despite aggressive treatment, 30% to 60% of the patients have resistant disease or relapse. To predict sensitivity of the tumor cells to chemotherapeutic drugs, chemosensitivity assays have been developed, including long-term clonogenic assays and short-term cytotoxicity assays relying on cell viability or DNA synthesis. Tumor cells resistant for chemotherapeutic agents may still be sensitive to immunologic agents that may be applied in drug-resistant tumors or in minimal residual disease to prevent relapse of the disease. These immunotherapeutic agents include monoclonal antibodies (MoAbs) acting with complement or in the form of immunotoxins. Alternatively, immune cells such as tumor infiltrating lymphocytes or lymphokine-activated killer (LAK) cells, activated by CD3 MoAb and IL-2 may be used. Recently, bispecific monoclonal antibodies (BsAbs) have been shown to combine both these aspects of specificity (provided by the antibody) and effectiveness (provided by, eg, T cells). BsAbs can bind both to antigens on the target cell and to trigger molecules on the effector cell such as T-cell receptors (TCRs). Therefore, the BsAbs can be used to focus normal cellular defense mechanisms to the tumor cells regardless of the specificity of the TCR.

The efficacy of BsAbs developed to trigger cytotoxic activity of the effector cells towards tumor target cells is generally measured in a standard 4-hour 51Cr-release assay. To predict the potential efficacy of treatment, the outcome of this assay may be rather deceptive because of constraints in time and maximal achievable effect. For instance, 60% lysis might indicate that 40% is not susceptible to this form of cytotoxicity, but the alternative explanation, ie, that the remaining cells are killed or inhibited in their growth after a longer incubation time, cannot be tested in this assay. To achieve a more sensitive measurement of both the cytostatic and cytotoxic capacity of this CD3×CD19 BsAb in vitro, we developed a clonogenic assay using peripheral blood (PB) lymphocyte-T cells and a clonogenic CD19+ pre-pre-B acute lymphoblastic leukemia (ALL) cell line (REH). In this system, the long-term efficacy of unprimed T cells of a PB mononuclear cell (PBMC) fraction from normal donors and non-Hodgkin's lymphoma (NHL) patients in the presence of CD3×CD19 BsAb could be tested. Conceptually, this would better reflect the efficacy after intravenous application of BsAb. Moreover, we could examine and show the value of repeated administration of BsAb and the supportive effect of interleukin-2 (IL-2).

MATERIALS AND METHODS

BsAb: SHR-1

The SHR-1 is a fusion product between the YTH12.5 and the MGI1CD19 cell lines. YTH12.5 is a rat IgG2b MoAb and has a specificity for the human CD3 α-antigen. MGI1CD19 is a mouse IgGl MoAb specific for human CD19. The production and purification of the SHR-1 has been described elsewhere. Briefly, SHR-1 was purified by ion-exchange chromatography. Fractions were analyzed using isotype-specific assays and, in addition, by (reducing) sodium dodecyl sulfate and native polyacrylamide gel electrophoresis, as well as isoelectric focusing. Production and purification was performed by the Department of Pathology (Cambridge, UK). Pure intact bifunctional BsAb was used in this study.

MoAbs

CD3-fluorescein isothiocyanate (FITC)/DR-phycocerythrin (PE; Leu4/HLA-DR), CD4-FITC/CD8-PE (Leu3a/Leu2a), CD3-FITC/CD16-CD56-PE (Leu3/Leu11c-Leu19), and CD45-FITC/CD14-PE (Hle1/LeuM3), CD5(Leu 1), CD10 (anti-Calla), and CD5/CD19 MoAbs were from Becton Dickinson (San Jose, CA). CD19 was obtained from the CBL (Central Laboratory of the Netherlands Red
When indicated, FITC-conjugated goat-antimouse subclass-specific Rat IgG2b CD3 MoAb (SHL45.6; derived from the rat hybridoma Cross Blood Transfusion Service, Amsterdam, The Netherlands). Rat IgG2b CD3 MoAb (SHL45.6; derived from the rat hybridoma Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Mononuclear Cells

Samples from patients were obtained after informed consent. PBMC samples from normal donors (n = 10) and patients diagnosed with NHL (n = 4) were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. PBMCs were diluted in RPMI 1640 supplemented with 10 mmol/L HEPES (GIBCO, Grand Island, NY), 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO), and 10% fetal calf serum (GIBCO).

Isolation of PBMC Subpopulations

PBMCs were first depleted for monocytes by adherence to plastic for 2 hours. Monocytes were recovered from plastic and kept at 4°C. Nonadherent cells were used for T-cell isolation by rosette formation with 2-aminooethylisothiouronium–treated sheep red blood cells followed by Ficoll-Paque density centrifugation.

Phenotype Determination

Cell fractions were determined for purity by immunofluorescence staining. A total of 0.5 × 10^9 to 1 × 10^9 pellet cells were incubated with the relevant MoAb. If nonconjugated antibodies were used, a second incubation step was introduced with goat-antimouse–FITC. One- and two-color immunofluorescence-stained cells were analyzed using a FACSscan (Becton Dickinson).

Cell Lines

The CD19+ pre-pre-B-ALL cell line REH (CD19+, CD11a−, CD54+, CD32+, B7+, MHC-I+), further referred to as ALL-B cell, with a doubling time of 48 hours, was used in the clonogenic assay. Cell lines were maintained in RPMI 1640 complete medium and used at a time of exponential growth. This cell line was found not to be sensitive for the biologic response modifiers with antitumor properties, ie, tumor necrosis factor-α (10 ng/mL) and interferon-γ (50 U/mL). Cells were regularly tested for mycoplasma infection (Gen-Probe, San Diego, CA).

Clonogenic Assay

A serial dilution assay for the determination of the initial and residual number of clonogenic units obtained with a B-cell line was performed as described by Bast et al, with some modifications. Cells from PBMC samples and from the ALL-B cell line were washed once and resuspended in RPMI 1640 complete medium plus 10% fetal calf serum. A series of nine serial fivefold dilutions were prepared from the B-cell line suspension (starting concentration 1 × 10^6 cells/mL) and also from the PBMC samples. The starting concentration of the PBMC fractions depended on the percentage of T cells (50% to 100%) and the chosen effector-to-target cell (E:T) ratio. Six aliquots, meaning six wells per dilution, of 100 μL of each PBMC dilution were plated in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark). Cells were incubated with 10 or 100 ng/mL BsAb, and 50 U/mL IL-2 (EuroCetus, Amsterdam, The Netherlands), and 10 ng/mL human IgG when indicated. Finally, the B-cell dilutions were added to wells (total volume, 200 μL/well). Plates were cultured for 14 days at 37°C in 5% CO2. On various days (mostly days 3, 6, and 9), fresh BsAb and IL-2 were added. Day 3 was chosen because, in previous experiments with 32P-release, addition of BsAb on day 3 resulted in restoration of optimal lysis.

Growth of ALL-B cell colonies at day 14 was scored visually using a microscope, and the nature of the clones was analyzed by immunofluorescence staining of cell surface markers. For immunofluorescence determination, 10 μL of a CD10/CD5 MoAb mixture was added to each well. After 10 minutes, cell suspensions from each well were transported into tubes, washed once, and analyzed on a FACScan using a lifetagte. Presence of ALL-B cells (CD10+) was scored in an all-or-nothing method: 10,000 cells/well were screened. If one CD10+ cell was found in one or more wells of the six wells of a certain combination, this was scored as positive for ALL-B cell growth. Statistical analysis of the number of clonogenic units was performed using a Spearman estimate as described by Johnson and Brown, in which the total number of wells in each separate plate showing ALL-B cell growth determines the number of clonogenic units. The initial number of clonogenic units obtained with ALL-B cells was greater than 10^7.

Reagents

Recombinant tumor necrosis factor-α and interferon-γ were purchased from Sigma Chemical Co (St Louis, MO) and Boehringer Ingelheim (Ingelheim, Germany), respectively. Human IgG was from the CLB (Amsterdam, The Netherlands).

RESULTS

Optimal Conditions for Killing of Tumor B Cells in the Clonogenic Assay

The effect of repeated administration of CD3×CD19 BsAb. To determine the optimal conditions by which freshly isolated PBMC become activated to eliminate tumor B cells from a 14-day culture, the CD19+ pre-pre-B-ALL cell line, REH, was used as target cell. We first determined whether this ALL-B cell line could grow in a limiting dilution assay supported by nonirradiated PBMCs when seeded in round-bottomed plates. Indeed, a correlation was found between the initial number of cells and the eventual measurement of ALL-B cell clonogenic units (data not shown). To determine optimal conditions for maximal B-cell elimination, ALL-B cells were mixed with PBMCs resulting in a final T:ALL-B cell ratio of 9:1, together with stimulating agents, ie, CD3×CD19 BsAb and IL-2 (Fig 1). When cultured with PBMCs alone, greater than 10^7 ALL-B cell clonogenic units were generated. A single treatment with BsAb or IL-2 resulted in a 1-log reduction of clonogenic units (92% inhibition). Repeated addition of BsAb resulted in minimal further growth reduction (Fig 1), whereas repeated addition of IL-2 showed no further growth reduction (data not shown). A single treatment with CD3×CD19 BsAb together with IL-2 on day 0 reduced the number of clonogenic units by 1 to 2 logs (Fig 1). Under suboptimal conditions (addition on day 0 or days 0 and 3), variation in killing was observed between donors. Repeated administration of BsAb at day 3 (and day 6), thereafter, gave an increase in reducing effect to 4 logs (>99% inhibition). Maximal killing of ALL-B cells (more than a 5-log kill, 100% inhibition) was observed when both BsAb and IL-2 were administered repeatedly. In control experiments, treating ALL-B cells with either CD3×CD19 BsAb, IL-2, or a combination in the absence of PBMCs had no effect on the number of clonogenic units as compared with that for culture medium alone (data not shown).

The effect of E:T ratio. In the 4-hour cytotoxicity assay,
the E:T ratio greatly influences the release of 51Cr. To determine the optimal E:T ratio in the clonogenic assay, ALL-B cells were mixed with different concentrations of PBMCs (Fig 2). The total amount of PBMCs added was dependent on (1) the percentage CD3+ cells (the effector cells) and (2) the T-cell:ALL-B cell ratio, variably chosen from 1:1 to 9:1. Under optimal conditions, with repeated addition of both CD3×CD19 BsAb and IL-2 and a T-cell:ALL-B cell ratio of 9:1, the number of clonogenic units reduced more than 4 logs (100% inhibition for each donor tested). A T cell:ALL-B cell ratio of 3:1 was almost as effective, but a T-cell:ALL-B cell ratio of 1:1 appeared to be less efficient and resulted in only a 1-log reduction (90% inhibition).

Reduction of ALL-B Number of Clonogenic Units: Comparison of CD3×CD19 BsAb and CD3 MoAb

PB lymphocytes that are activated in vitro with IL-2 and CD3 acquire LAK activity and have been applied for adoptive immunotherapy of cancer. Therefore, we compared the lytic activity of PBMCs induced by CD3 rat IgG2b MoAb plus IL-2 and CD3×CD19 BsAb plus IL-2 in the clonogenic assay (Table 1). Under optimal conditions with a ratio of 9:1 and addition of stimuli on days 0, 3 and 6, experiments with BSAb plus IL-2 resulted in a 5-log ALL-B cell kill; in contrast, only 2 logs of reduction were found with CD3 MoAb plus IL-2 (“LAK-conditions”). Repeated addition of CD3 MoAb plus IL-2 at days 0, 3 and 6, hardly improved killing as compared with addition at day 0 and 3. IL-2 alone had no effect on the number of clonogenic units.

PBMCs isolated from patients with NHL efficiently eliminate ALL-B cells. To determine whether T cells in PBMCs isolated from NHL patients in remission (patients no. I and II) or relapse (patients no. III and IV) are as efficient as donor PBMCs, four patient samples were tested in the clonogenic assay. For patients no. I, II, and IV, the percentage of CD3+ cells was below the 30%. Therefore, before cells were used in the clonogenic assay, T cells were first isolated by treatment with 2-aminoethylisothiouronium-coated sheep red blood cells and subsequently mixed with the autologous non−T-cell fraction to approach percentages obtained in donor PBMCs. The T-cell:ALL-B cell ratio chosen was dependent on the amount of T cells that could be isolated, indicating that from patients no. I and IV only a 1:1 ratio could be determined. Treatment of patient-PBMCs with CD3×CD19 BsAb plus IL-2 reduced clonogenic units up to 5 logs (5:1

Table 1. Reduced ALL-B Cell Number of Clonogenic Units After Stimulation With CD3 × CD19 BsAb or CD3 MoAb, Both in the Presence of IL-2

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>Day1</th>
<th>0+3</th>
<th>0+3+6</th>
</tr>
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<tbody>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
<td>2 × 10⁶</td>
</tr>
<tr>
<td>BsAb and IL-2</td>
<td>1 × 10⁶</td>
<td>7 × 10⁵</td>
<td>6 × 10⁴</td>
</tr>
<tr>
<td>CD3 MoAb and IL-2</td>
<td>1 × 10⁶</td>
<td>4 × 10⁴</td>
<td>2 × 10⁴</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* PBMCs from normal donors (no. VI and VII) were mixed with ALL-B cells in an E:T ratio of 9:1 and were stimulated with BsAb/MoAb (100 ng/mL) and IL-2 (50 U/mL).
† Day of treatments.
‡ Data represent the number of clonogenic units after 14 days of culture.
CELL DEATH BY BsAb MEASURED IN A CLONOCYGENIC ASSAY

Cortenbach et al. and Bast et al. developed to measure the clonogenic assay is based on previous reports (Slaper-Cortenbach et al. and Bast et al.) of immunotoxins, MoAb-complement- and chemotherapy. Thus, these results may now be compared with those of immunotoxins, MoAb-complement- and chemotherapy. Human IgG Has No Effect on the Reduction of Clonogenic Units

MoAbs directed against the CD3 antigen induce T-cell activation on cross-linking of the antibody by Fcγ receptors (FcγRs) expressed on monocytes. Cross-linking of the CD3/TCR complex is an essential step for T-cell activation. In vivo, human IgG, the natural ligand for FcγRs, may prevent binding of the antibody to the different FcγRs. Addition of human IgG can block CD3 MoAb-mediated T-cell mitogenesis completely (data not shown). Therefore, we assessed the effect of human IgG in the clonogenic assay. Addition of 10 mg/mL human IgG at day 0 did not inhibit tumor cell lysis by PBMCs when cultures were treated with BsAb plus IL-2 on days 0, 3, and 6 (E:T ratio of 9:1; see Table 3).

DISCUSSION

An important prerequisite of direct intravenous administration of BsAb is their capacity to activate resting T cells to become cytotoxic. In previous studies, we showed the efficacy of CD3×CD19 BsAb to activate PB T cells to proliferate and become cytotoxic within 1 day. Addition to the effector phase, ie, BsAb-mediated lysis by activated T cells of tumor cells, proved to be independent of HLA class I expression. In this study, a clonogenic assay, based on a limiting dilution assay, was performed to evaluate the duration and efficacy of single and multiple CD3×CD19 BsAb administration to PBMCs to eliminate CD19+ ALL-B cells. A maximum elimination of 5 logs (100% inhibition) of ALL-B cell clonogenic units was achieved after repeated addition at days 0, 3, and 6 of 100 ng/mL CD3×CD19 BsAb plus 50 U/mL IL-2 to PBMC cultures. A T cell:ALL-B cell ratio of 3:1 was found sufficient to obtain maximal killing.

To our knowledge, this is the first report on a clonogenic assay to measure the efficacy of BsAb treatment quantitatively; thus, these results may now be compared with those of immunotoxins, MoAb-complement- and chemotherapy. The clonogenic assay is based on previous reports (Slaper-Cortenbach et al. and Bast et al.) developed to measure the effect of either immunorosette depletion and complement or MoAb and complement on malignant clonogenic B cells. These investigators used the presence of irradiated bone marrow feeder cells and flat-bottomed culture plates. In preliminary experiments, we found that feeders (irradiated PBMCs), when added to ALL-B plus CD3×CD19 BsAb, already caused a reduction in clonogenic units of 2 logs. Without the addition of BsAb, freshly isolated PBMCs provided optimal feeder function as well. Therefore, in our assay where a cellular effector system is examined, freshly isolated PBMCs were used to fulfill the feeder function and, at the same time, to serve as the effector (cytotoxic) cells. Cell-cell contact is required for T-cell activation as well as for targeted cytotoxicity; therefore, we used round-bottomed plates instead of flat-bottomed microtiter plates. Furthermore, scoring wells visually for ALL-B cell growth (clonogenic units) appeared to be impossible because of T-cell expansion through BsAb plus IL-2 stimulation. Consequently, in the assay, CD10 and CD5 conjugates were added to the wells to identify, by immunofluorescence, the presence of ALL-B cells or T cells, respectively.

IL-2 by itself was not effective in this assay, whereas the BsAb alone could reduce the number of clonogenic units up to 2 logs. Optimal conditions for eliminating ALL-B cells included multiple treatment with BsAb and IL-2. This positive effect of repeated addition of BsAb may relate to the inability of TCR/CD3 complexes, initially clustered by BsAb, to continuously transduce signals. Readdition of the BsAb to cytotoxic T lymphocytes instantly restored lytic activity of the cytotoxic T lymphocytes that had lost BsAb-targeted cytotoxicity, which was confirmed by us in release experiments previously. The finding that IL-2 was necessary together with BsAb to achieve optimal effect may be due to the fact that T cells need two signals to become optimally activated, the first signal through the TCR/CD3 complex and a costimulatory signal through CD28/CTLA-4, which activates and stabilizes IL-2 production in the activated T cell. In our assay system, the first signal is given by the BsAb through the CD3 complex, but a second signal through the CD28 antigen is lacking because the ALL-B cell line is B7−, comparable with most NHL- and leukemic-B cells. The addition of exogenous IL-2 may substitute for the absence of a second signal. The positive effect of the repeated addition of BsAb and IL-2 in vitro may indicate that a daily intravenous administration of both BsAb plus

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>BsAb and IL-2t</th>
<th>BsAb, IL-2, and Human IgGt</th>
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<td>Donor no. IV</td>
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</tr>
<tr>
<td>Donor no. V</td>
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<td>0</td>
</tr>
<tr>
<td>Patient no. III</td>
<td>4×10⁴</td>
<td>0</td>
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</tbody>
</table>

* PBMCs were mixed with tumor B cells and stimulated on days 0, 3, 6, and 9 with BsAb (100 ng/mL), IL-2 (50 U/mL), and human IgG (10 mg/mL) where indicated.

† Data represent the number of clonogenic units after 14 days of culture.
IL-2 in vivo may be successful in the elimination of tumor B cells.

IL-2, often in combination with CD3 MoAb, has frequently been used in vitro to stimulate killer cells (LAK cells) to be used for cancer therapy. In this clonogenic assay, we could show that BsAb- and IL-2–retargeted T cells were more effective (log-2 difference) than LAK cells in reducing the clonogenicity of ALL-B cells.

The E:T ratio appeared to be another important factor determining the effectiveness of BsAb plus IL-2 treatment. A ratio of 3:1 that was found to be effective in this study may not be observed in end-stage patients, indicating that this therapy may only be effective in minimal residual disease. T cells of patients with NHL proved equally effective as donor T cells when added in similar E:T ratios.

Monocytes are known to play an essential role (cross-linking of the CD3 MoAb by FcγRs and production of cytokines) in T-cell activation induced by CD3 MoAb. Addition of human IgG to PBMC cultures inhibited T-cell mitogenesis by the BsAb by blocking of the FcγRs. How-ever, addition of human IgG to the clonogenic assay cultures, stimulated with BsAb plus IL-2 on days 0, 3, and 6, did not influence the reduction in the number of clonogenic units and maximal elimination of tumor cells measured. These findings support the concept that better T-cell activation is achieved by administration of both BsAb plus IL-2.

Our results with the clonogenic assay are in concordance with in vivo mouse studies. These have shown that, under selected conditions, tumor growth can be prevented by CD3×anti-Id BsAb. However, when BsAb is administered to mice with a large tumor load, a low therapeutic effect is observed. Low-dose BsAb repeated 3 times resulted in a better long-term survival of those mice with a high tumor load. T cells were essential for the therapeutic effect of CD3×anti-Id BsAb was shown in these studies as well. The synergy of exogenous IL-2 in combination with BsAb was shown by Weiner et al. and resulted in the elimination of a larger tumor burden.

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The efficacy of CD3 x CD19 bispecific monoclonal antibody (BsAb) in a clonogenic assay: the effect of repeated addition of BsAb and interleukin-2

IA Haagen, AJ Geerars, WB de Lau, BJ Bast and BC de Gast

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