Enhanced Antitumor Effects of CD20 over CD19 Monoclonal Antibodies in a Nude Mouse Xenograft Model

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ABSTRACT

We used a nude mouse xenograft tumor model to compare the efficacy of unconjugated CD19 and CD20 mAbs (IgG2a subclass) in mediating antilymphoma effects. Treatment with the CD20 mAbs BCA-B20 and CLB-CD19 resulted in a drastic decrease in tumor take rate (P < 0.0001) in comparison to controls, whereas the CD19 mAb CLB-CD19 was ineffective. Tumor growth rates were reduced by both CD19 and CD20 (P < 0.0001). The decrease in growth rate induced by NKI-B20 or BCA-B20 was larger than that induced by CLB-CD19 (P = 0.0022). In vitro experiments showed that NKI-B20 or BCA-B20 are more powerful than CLB-CD19 in mediating lysis by interleukin 2-activated natural killer cells. No difference was observed between different isotypes (IgG1, IgG2a, IgG2b) of the switch variants of NKI-B20 or CLB-CD19. A positive correlation between antigen density and the sensitivity to antibody-dependent cellular cytotoxicity was demonstrated with human lymphoblastoid B cells, JY, transfected with cDNA encoding the human CD19 antigen that expressed high levels of this antigen. These cells are more efficiently killed by natural killer cells when coated with CLB-CD19 mAbs than JY wild-type cells that express 1 log lower levels of the CD19 antigen. Antibody-dependent cell-mediated cytotoxicity experiments with thiolate-activated macrophages show a more complex relationship between antigen density, isotype of the mAb, and cytotoxicity. BCA-B20 (IgG2a) and CLB-CD19 (IgG2a) and all isotypes of NKI-B20 mediated strong cytotoxicity, whereas CLB-CD19 isotypes IgG1 and IgG2b were associated with limited cytotoxicity. Proliferation of Daudi cells was inhibited with high concentrations of all isotypes of CLB-CD19, but not with any of the CD20 mAbs. To our knowledge this is the first report showing that the antitumor effects in vivo of unconjugated CD20 mAbs are far superior to those of CD19 mAbs.

INTRODUCTION

Over the past decade attempts have been undertaken to treat lymphoid cancers with unconjugated antibodies, radiolabeled antibodies, or immunotoxins (1–3). Although the potency of antibodies can be enhanced by coupling to radioisotopes or toxins, these conjugates also have enhanced toxicity, and in the case of immunotoxins also induce therapy resistance due to enhanced immunogenicity. In our opinion the full potential of unconjugated antibodies has not yet been fully explored. The best results thus far with unconjugated antibodies in B cell cancers have been obtained with mAbs against the idiotype of the surface immunoglobulins on the patients’ tumor cells (4) and with CDw52 antibodies (CAMPATH-1G and CAMPATH-1H) (5). Unfortunately the applicability of tumor-specific anti-idiotype mAbs is restricted by the need to make individual mAbs. On the other hand CAMPATH mAbs are directed against a widely expressed leukocyte antigen, and may therefore deplete not only the tumor cells but also T cells, resulting in severe side effects.

In the past we have treated four lymphoma patients with anti-idiotype mAbs (6), resulting in two partial remissions. To avoid the laborious production of custom-made anti-idiotype mAbs, we have continued our studies using anti-pan-B cell mAbs. On the basis of results obtained in a xenograft tumor model in nude mice (7, 8), we performed two clinical trials in patients with B cell lymphoma using mouse CD19 mAbs alone (9) or in combination with recombinant IL-2 (10). The results of these trials are encouraging, but treatment needs further improvement.

To investigate the effect of the target antigen on the therapeutic effect of the mAbs, we now report a comparison of the antitumor effects of unconjugated CD19 and CD20 mAbs in a preclinical study, including killing of mAb-sensitized human lymphoma cells in vitro and in vivo in a xenograft model. The CD19 antigen is expressed on normal as well as malignant B cells as a 90-kDa glycoprotein (11, 12). The CD19 antigen is expressed from the pre-B cell stage but disappears on plasma cell differentiation. The CD19 molecule plays an important regulatory role in B cell activation and differentiation (13–15), and can be found in a complex with complement receptor-2 (CD21), TAPA-1 (CD81), and Leu-13 molecules (16). Facilitated by foreign antigen and complement, this CD19-CD21-CD81 complex might even multimerize with the B cell receptor complex (17), enabling a B cell response at low concentrations of foreign antigen (18).

The CD20 antigen is expressed on normal and malignant B cells as a 33–35-kDa integral membrane phosphoprotein (19, 20). It is expressed slightly later in B cell differentiation than the CD19 antigen and is also lost at the plasma cell stage. A recent report states that the CD20 antigen is also expressed at low levels on a subpopulation of human T cells (21). The CD20 antigen plays an important role in B cell proliferation and differentiation (22, 23). CD20 mAbs can also induce down-regulation of surface expression of IgM (24) and rescue cells from anti-IgM-induced apoptosis (25). On most B cells the expression of CD20 antigens is higher than that of CD19 antigens. Press et al. (26) reported a ratio of the number of CD20 versus CD19 antigens on Daudi cells of about 3 (306,000/91,000). For the Daudi cells used in this article, Vervoordeldonk et al. (27) also found a ratio of 3, although the actual numbers of sites calculated were lower (111,000/33,000). For the cell lines Raji and Ros and cells from four patients, the CD20/CD19 antigen ratio was found to be 6–10, with one exception where the expression of CD19 was as high as that of CD20 antigens (27). CD20 antigen is not susceptible to antibody-induced modulation, whereas CD19 antigen is modulated to varying degrees on different cells (26, 27). Internalization rates of CD20 antigens were twice as high with IgG1 as with IgG2a isotype switch variants of CLB-CD19 mAbs on Daudi cells (28).

The experiments presented in this article revealed that two CD20 mAbs (BCA-B20 and NKI-B20) are very potent in mediating antitumor effects against Daudi target cells in a nude mouse xenograft tumor model. The tumor take rate was impressively decreased by treatment with both CD20 mAbs, but was hardly
affected by CD19 mAbs. In contrast the growth rate of tumors that did arise was reduced by both CD19 and CD20 mAbs. In vitro experiments showed that the nature of the target antigen, its density on the cell surface, and the isotype of the mAb all contribute to the cytotoxic effect on tumor cells.

MATERIALS AND METHODS

Mice. BALB/c and athymic BALB/c-nu/nu mice were bred and maintained at the animal department of The Netherlands Cancer Institute. The nude mice were kept in sterile isolators under specific pathogen-free conditions and used when they were 8–12 weeks old. Mice used as a source of effector cells or serum were up to 20 weeks old. Throughout all experiments we used male animals. All experiments were approved by the Animal Experimental Advisory Board of The Netherlands Cancer Institute.

Cell Lines and Cell Culture Conditions. The human Burkitt cell line Daudi was obtained from the American Type Culture Collection (Rockville, MD). Lymphoblastoid JY cells (29) were routinely cultured in our laboratory. Daudi cells used in the in vivo experiments were taken from our cell bank and grown in vitro for 7–10 days prior to inoculation. Cells were grown in complete medium, consisting of DMEM or Iscove’s medium (GIBCO, Paisley, Scotland) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany), and 20 μM 2-mercaptoethanol (Merck), at 37°C in humidified air with 6% CO₂. Cell lines were Mycoplasma free.

mAbs. The NKI-B20 hybridomas producing CD20 mAbs (IgG1 or IgG2b or IgG2a) have been described elsewhere and were submitted to the Fifth Leukocyte Differentiation Antigen Workshop in 1993 under the code D2—1H4 (30). NKI-B20 (IgG2a) has an association constant (Kₐ) of 0.8 × 10⁹ M⁻¹. The NKI-B20 hybridomas were grown in complete medium. The BCA-B20 hybridoma producing CD20 mAbs (IgG2a) has been described previously (31) and was obtained by us from Bioprobe bv (Amsterdam, The Netherlands). BCA-B20 mAb has been submitted to the Fifth Leukocyte Differentiation Antigen Workshop in 1993 under the code B9E9 and has been clustered as a CD20 mAb (30). BCA-B20 has an association constant (Kₐ) of 1.1 × 10⁹ M⁻¹. The BCA-B20 hybridoma was grown in complete medium with 15% (v/v) normal horse serum (GIBCO) instead of FCS. NKI-B20 and BCA-B20 mAbs were purified from ascites fluid using protein A-Sepharose CL-4B columns (Pharmacia, Uppsala, Sweden). The CLB-CD19 hybridomas producing CD19 mAbs (IgG1, IgG2b, or IgG2a) have been described previously (13). CLB-CD19 (IgG2a) has an association constant (Kₐ) of 2.0 × 10⁹ M⁻¹. CLB-CD19 mAbs, purified from culture medium, were obtained from Euroclon. The K8 mAb (isotype IgG2a) is directed against the idiotype of the surface immunoglobulin of the B acute lymphoblastic leukemia cell line (7). The P.24.3 mAb, a rat IgG2b directed against the receptor for the Fc part of IgG, was a gift from Becton Dickinson.

Deposition Unit (Becton Dickinson, Mountain View, CA).

Fluorescence-activated Cell Sorter Analysis. Immunofluorescence has been performed as described previously (8). Fluorescent staining was analyzed with a FACScan and cells were sorted on a FACStar using an Automated Cell Deposition Unit (Becton Dickinson, Mountain View, CA).

**Effectors in Cytotoxicity Assays**. Spleen cells from male BALB/c nude mice were incubated in flasks (Falcon) for a period of 5–6 days in complete medium (2–3 × 10⁶ cells/ml) containing 500 Cetus Units rhIL-2 (Eurocetus, Amsterdam, the Netherlands)/ml. After this period dead cells and erythrocytes were removed by centrifugation over a gradient of Lymphoprep (Nycomed Pharma, Oslo, Norway). The resulting cell fraction mainly consisted of NK cells as determined by fluorescence-activated cell sorting analysis (see “Results”). PECs were obtained from thioglycolate-treated (Brewers; Difco, Detroit, MI; 1 ml injected i.p. 5–7 days before harvest) male BALB/c mice by washing their peritoneal cavities with ice-cold PBS after killing them in a CO₂ chamber. The harvested PECs were transferred to ice-cold FCS, washed twice in complete medium, and reconstituted at the desired cell density in complete medium. PECs were cultured in flat-bottomed 96-well microtiter plates (Costar).

**ADCC with Stimulated Spleen Cells as Effectors**. Standard 4-h ⁵¹Cr release experiments were performed as previously described (7), except that after labeling and washing the target cells were incubated for an additional 20–30 min in 1 ml complete medium to allow loosely bound label to leak out of the cells. After this the cells were washed once more and resuspended at the appropriate concentration. Effector cells were mixed with 1000 labeled target cells at varying E:T ratios in 96-well round-bottomed microtiter plates (Costar). Test or control mAbs were added to a final volume of 200 μl/well (the concentrations of mAbs given in Figs. 1, 2, 4, and 5 are the final concentrations in the experimental wells). Subsequently the plates were centrifuged for 1–2 min at 1000 rpm and incubated for 4 h at 37°C in humidified air with 5% CO₂. After this period 100 μl supernatant were harvested and the ⁵¹Cr content determined in a gamma counter (Packard). The percentage of specific chromium release was calculated according to the following formula:

% specific release = (T — SIM — S) x 100%,

where T = cpm in the test sample, SIM = cpm in medium control spontaneously released from the target cells alone, and M = cpm in maximum releasable label in 2% Triton X-100 solution. The spontaneous release of label never exceeded 20% of the maximum release and was usually approximately 10%.

**ADCC Experiments with Macrophages as Effectors**. With a number of alterations this assay has previously been described (8) as a proliferation inhibition assay. PECs were seeded in 96-well plates, the nonadherent cells were washed away 1 day later, and target cells (1000/well) were added to the wells at varying E:T ratios. mAbs were added (the concentrations given in Figs. 1, 2, 4, and 5 are the final concentrations in the experimental wells) and the macrophages were allowed to kill the target cells for 3 days. After this period the remaining target cells were fed [³H]thymidine (0.4 μCi/well in 20 μl medium; specific activity, 6.7 Ci/mmol; DuPont, Den Bosch, the Netherlands) and allowed to proliferate for a period of 4 h. Subsequently the cells were harvested with a Tittertek cell harvester (Flow Laboratories, Inc., Mclean, VA) and the incorporated [³H]thymidine was determined in a beta plate liquid scintillation counter (LKB, Uppsala, Sweden). The percentage of specific inhibition of [³H]thymidine incorporation was calculated according to the following formula: % specific inhibition = (C — T/C — S) x 100, where C = cpm incorporated in targets incubated with PECs and control mAbs (K8), T = cpm incorporated in target cells incubated with PECs and test mAbs, and S = cpm incorporated in PECs alone.

**Complement-mediated Cytotoxicity.** In microtiter plates 50 μl antibody dilution in complete medium and 50 μl ⁵¹Cr-labeled target cells (10⁵ cells/ml) were incubated on ice for 15 min. One hundred μl of the serum used as the source of complement were added, incubation was continued for 45 min at 37°C, the plates were centrifuged for 1 min at 1200 rpm, and 100 μl supernatant were collected for counting in a gamma counter (Packard). Specific ⁵¹Cr release was calculated using the formula given above. Freshly frozen serum from BALB/c nude mice, stored at —70°C, was used as a source of complement. Rabbit complement (low tox H) was purchased from Cedarlane (Ontario, Canada). Pooled serum from healthy donors was used as a source of human complement.

**Proliferation Assays.** Cells (10,000/well) were incubated with antibody dilutions in a volume of 100 μl medium for 24 h at 37°C and pulsed with [³H]thymidine for an additional 4 h. Cells were harvested and the incorporated [³H]thymidine was counted in a beta plate liquid scintillation counter (LKB).

**In Vivo Experiments.** The previously described nude mouse xenograft tumor model (7, 8) has been used with a number of alterations. In brief, on day 0 the animals were whole-body irradiated with a 3-Gy dose from a Siemens 841
stabilised Röntgen radiation generator, adjusted to 250 kV, 15 mA, and using a Thorex filter. Viable Daudi cells, 5 x 10^6, in 200 μl PBS were inoculated s.c. on the right flank 1–2 h after irradiation. Injections of 1 mg mAb were given i.p. in 1 ml PBS with 0.5% (w/v) BSA (Sigma Chemical Co., St. Louis, MO) or human serum albumin (Euroclone). The treatments were given on days 4, 11, and 18 after inoculation of the tumor cells. Tumor sizes were measured every 7–10 days as the perpendicular diameters in two dimensions with precision calipers. Tumor take rates are given for day 40 because most control animals had grown very large tumors by that time and had to be sacrificed. The animals were 8–12 weeks of age at the onset of the experiments and were evenly distributed among the different groups according to their age.

**Statistical Analysis of in Vivo Data.** Both tumor take at day 40 and tumor growth rates were compared between the treatment groups given in Table 1. Exact logistic regression (34) was used for statistical analysis with the aid of the computer program LogXact-Turbo 1.1 (CYTEL Software Corporation). P values were adjusted for differences between experiments. Two-sided P values are reported; these are calculated by doubling the smallest one-sided P value.

In the analyses of growth rates only those animals were used for which a tumor was observed at least once (75 animals and 259 measured points). Starting point for the calculation of the growth rate is the time at which the tumor is first detected. Therefore, negative growth rates are possible and indeed observed. The data were analyzed using a linear growth model with coefficients randomly varying between animals within the same experiment and with the same treatment (35). Differences in mean slopes between experiments as well as overall effects of age on tumor size were accounted for. Restricted maximum likelihood was used for estimation; P values were calculated from the Wald statistic. Program 5V of the statistical package BMDP was used for the calculations.

**Vector Construction.** The EcoRI fragment of pSP65-B4—19 containing the cDNA encoding human CD19 antigen (12) was inserted in pJFE14-SRα-Hyg (36, 37), placing both the cDNA encoding human CD19 antigen and the hygromycin resistance gene under the transcriptional control of strong SRα promoters. This plasmid is called pHZM-B4—19.

**Electroporation.** For electroporation experiments we used a Bio-Rad Electroporation Apparatus and 0.4-cm cuvettes. Viable log-phase JY cells (6 x 10^6) were taken up in 400 μl cold PBS and mixed with 10 μg of the plasmid encoding human CD19 antigen (pHDM-B4—19). The electroporation apparatus was set at 290 V and 960 @F (pulseduration, 30 ms), resulting in about 30% cell survival as measured with trypan blue exclusion 12 h later. The cells were put back on ice for 10 min, then taken up in 8 ml complete medium. Selection medium containing 250 μg/ml hygromycine (Calbiochem, La Jolla, CA) was added 24 h after electroporation. Selection pressure was maintained for 3 weeks, after which the cells were sorted using a FACStar for high CD19 antigen expression. One day after the sort cells were transferred to medium containing 500 μg/ml hygromycin.

**RESULTS**

**Comparison of the Efficacy of CD19 and CD20 Antibodies in the Xenograft Tumor Model**

The effect of one CD19 mAb (CLB-CD19) on the outgrowth of human Daudi tumor cells was compared to the effect of two CD20 antibodies (BCA-B20 and NKI-B20) in the nude mouse xenograft tumor model. On the basis of earlier experiments performed in our laboratory (7) and data from the literature (38), we decided to use the IgG2a isotypes of the available isotype switch variants of CLB-CD19 and NKI-B20 and BCA-B20. Daudi tumor cells were inoculated s.c. on the flank of BALB/c nude mice. The animals were treated with PBS (control) or antibodies (CLB-CD19, BCA-B20, or NKI-B20 or the combination of CLB-CD19 plus BCA-B20). The presence of tumors and the sizes (in two dimensions) were determined regularly.

**Tumor Take at Day 40 after Treatment with CD19 and CD20 mAbs.** Table 1 shows that CLB-CD19 treatment did not result in a significant antitumor effect compared to the control group if the take rate at day 40 is scored (P = 1.00). The antitumor effects of CD20 antibodies (BCA-B20 and NKI-B20), on the other hand, are clear: only 10–20% of the animals treated with CD20 mAbs had a tumor at day 40 (P < 0.0001 taking all CD20 groups together). The antitumor effects were long-lasting since only 3 of 28 of the animals treated with BCA-B20 not bearing a tumor at day 40 had grown a tumor by day 90. There is no evidence for a difference between treatment with BCA-B20 alone versus the combination of CLB-CD19 plus BCA-B20 mAbs (statistical data not shown).

**Growth Rates of Tumors in Different Treatment Groups.** To compare growth rates of tumors, only those animals were used in which a tumor was observed at least once. The starting point for the growth rate determination was the time at which the tumor was first detected. The data were analyzed using a linear growth model with coefficients randomly varying between animals within the same experiment and with the same treatment (39). Differences in mean slopes between experiments were accounted for (for details, see "Materials and Methods"). Data from treatment groups BCA-B20 and NKI-B20 were pooled together for the statistical analysis. The estimated mean growth rates for the different treatment groups are given in Table 2. All three treatments resulted in decreased growth rates as compared to controls (P < 0.0001 in all cases). The decrease induced by CD20 (BCA-B20 or NKI-B20) is larger than that by CLB-CD19 (P = 0.0022). There is no difference between treatment with BCA-B20 alone versus the combination of CLB-CD19 plus BCA-B20 mAbs (statistical data not shown).

**Comparison of the Effects of CD19 and CD20 Antibodies in In Vitro Assays**

**ADCC Experiments with IL-2-stimulated Spleen Cells as Effectors.** As effectors in ADCC experiments nude mouse spleen cells activated for 5–6 days with rHL-2 were used. These cells were mostly

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**Table 1** Effect of CD19 and/or CD20 mAb treatment on the take rate of Daudi tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
<th>Total</th>
<th>ln %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>9/9</td>
<td>8/10</td>
<td>12/12</td>
<td>9/9</td>
<td>38/40</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>CLB-CD19</td>
<td>10/10</td>
<td>7/10</td>
<td>9/9</td>
<td>26/29</td>
<td>90</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>BCA-B20</td>
<td>2/10</td>
<td>0/10</td>
<td>2/12</td>
<td>4/32</td>
<td>13</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>NKI-B20</td>
<td>2/10</td>
<td>2/12</td>
<td>2/12</td>
<td>2/10</td>
<td>17</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>CLB-CD19 + BCA-B20</td>
<td>2/10</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

* Shown is the take rate (no. of mice bearing a tumor/total no. of mice) in each experiment, the sum and the percentage of four experiments and the P values as compared to the control (PBS-treated animals) on day 40. The BCA-B20 and NKI-B20 groups were taken together for the analysis. Exact logistic regression was used for statistical analysis. On day 0 BALB/c nude mice were irradiated prior to s.c. inoculation of Daudi cells. mAbs (CLB-CD19, BCA-B20, NKI-B20) were injected i.p. on days 4, 11, and 18.

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**Table 2** Effect of CD19 and/or CD20 mAb treatment on the growth rate of Daudi tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Estimated growth rate (mm²/day)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>38</td>
<td>12.55 ± 0.50</td>
<td></td>
</tr>
<tr>
<td>CLB-CD19</td>
<td>26</td>
<td>5.25 ± 0.57</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BCA-B20 or NKI-B20</td>
<td>6</td>
<td>1.21 ± 1.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CLB-CD19 + BCA-B20</td>
<td>2</td>
<td>1.20 ± 2.12</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Shown is the number of mice in each group, the estimated mean growth rate (± SE) in mm²/day for the different treatment groups and the P values as compared to the control group (PBS-treated animals). The decrease induced by CD20 (BCA-B20 or NKI-B20) is larger than that by CLB-CD19 (P = 0.0022). Data were analyzed using a linear growth model with coefficients randomly varying between animals within the same experiment and with the same treatment. Differences in mean slopes between experiments as well as overall effects of age on tumor size were accounted for.
NK cells; 80–90% positive for Thy-1 and FcyRIIa (33) and negative for Mac-1, CD3, CD4, CD8, and surface immunoglobulin (data not shown). As shown in Fig. 1 all CD19 and CD20 mAbs mediated ADCC with these effector cells at saturating concentrations of the mAbs (2.5 μg/ml). The CLB-CD19 antibodies (isotypes IgG1 and IgG2a) mediated killing of 40% of the target cells at the highest E:T ratio used (100:1), as does IgG2b (data not shown). Two other CD19 mAbs (BU12 and B43) of isotype IgG1-mediated levels of cytotoxicity comparable to CLB-CD19-IgG1 (data not shown). The binding of a fourth CD19 mAb (4G7, FITC labeled) was completely inhibited by CLB-CD19, BU12, and B43, indicating that these CD19 mAbs bind to the same or a closely adjacent epitope (data not shown). All isotypes (IgG1, IgG2a, and IgG2b) of NKI-B20 were capable of mediating killing of 80% of the target cells at an E:T ratio of 100:1 (Fig. 1), as is BCA-B20 (IgG2a) (data not shown). It is noteworthy that no real difference existed in cytotoxic capacity between different isotypes of mAbs of the same specificity. At the concentrations used the mAbs by themselves inhibited nor stimulated proliferation of the target cells (Daudi) in these assays.

ADCC Experiments with Activated Macrophages as Effector Cells. The same mAbs and a positive control mAb R24.3 were tested in ADCC experiments using thioglycolate-activated mouse PECs as effectors (7). We have used the adherent cells, consisting mainly of macrophages. These cells expressed Mac-1 and FcyRII and/or FcyRIIIa, but were negative for CD3, CD4, CD8, Thy-1, and surface immunoglobulin (data not shown). In this assay the macrophages are allowed to kill or phagocytose the antibody-coated target cells (Daudi) for 3 days. The remaining fraction of the target cells is measured by incorporation of [3H]thymidine. The percentage of proliferation inhibition as a measure of ADCC mediated by the antibodies is calculated using the formula given in "Materials and Methods." Fig. 2A shows that at saturating concentrations (2.5 μg/ml) of the CLB-CD19 mAbs the IgG2a isotype showed considerably higher antibody-dependent cytotoxicity compared to CLB-CD19-IgG1 (data not shown). The binding of a fourth CD19 mAb (4G7, FITC labeled) was completely inhibited by CLB-CD19, BU12, and B43, indicating that these CD19 mAbs bind to the same or a closely adjacent epitope (data not shown). All isotypes (IgG1, IgG2a, and IgG2b) of NKI-B20 were capable of mediating killing of 80% of the target cells at the highest E:T ratio used (100:1), as is BCA-B20 (IgG2a) (data not shown). It is noteworthy that no real difference existed in cytotoxic capacity between different isotypes of mAbs of the same specificity. At the concentrations used the mAbs by themselves inhibited nor stimulated proliferation of the target cells (Daudi) in these assays.

Effect of Antigen Density on ADCC. To study the influence of antigen density on the target cells on the sensitivity to lysis by means of ADCC by NK cells or macrophages, we transfected the human B cell line JY with a vector carrying the cDNA encoding the human CD19 antigen (pHJM-B4–19). Fig. 3 shows the membrane expression of the CD19 antigen on JY wild-type cells and JY*CD19 transfectants. The expression of the CD19 antigen on the JY*CD19 transfectants is about 1 log higher than on JY wild-type cells and is comparable to the expression of the CD20 antigen (which is not altered by the transfection).

Fig. 1. ADCC with rIL-2-stimulated NK cells as effectors. Daudi target cells were sensitized with CD19 or CD20 mAbs of different isotypes or with negative control mAb K8. Final mAb concentration was 2.5 μg/ml. E:T ratios ranged from 100:1 to 3:1. Indicated is the mean percentage of specific [3H]Cr release of triplicate determinations. SD never exceeded 5% of the mean. Data are representative of nine independent experiments.
We have used both cell types in ADCC experiments using rhIL-2 stimulated-nude mouse spleen cells as effectors and mAbs of the IgG2a isotype (K8, CLB-CD19, and NKI-B20) and the rat mAb R24.3 (IgG2b) as a positive control. As shown in Fig. 4 the sensitivity to lysis mediated by CLB-CD19 mAbs of the JY*hCD19 transfectants was considerably higher than that of the JY wild-type cells and reached the level of the CD20 mAbs. Although the transfectants are more sensitive than the wild-type cells to lysis by NK cells without specific mAb (K8), the conclusion remains valid because the specific lysis of the transfectants with R24.3 and NKI-B20 is also higher, and the lysis with CLB-CD19 reaches the level of R24.3 and NKI-B20.

In ADCC experiments using PECs as effectors (mostly macrophages) we saw the same effect, although the difference was not as large because the reactivity on the JY wild-type cells was already considerable (data not shown).

Lack of Complement-mediated Lysis of Target Cells. As expected we were unable to detect any effect of CLB-CD19 antibodies, of NKI-B20 antibodies, or of BCA-B20 in complement-dependent cytotoxicity assays using serum taken from nude BALB/c mice as a source of complement (data not shown). Using rabbit or human serum as a source of complement we were able to detect different levels of cytotoxicity.

Inhibition of Proliferation Induced by CLB-CD19 but Not by BCA-B20 nor by NKI-B20 mAbs. We tested the direct effects of the mAbs on proliferation of the target cells in vitro as measured by [3H]thymidine incorporation. Proliferation of Daudi cells was inhibited by CLB-CD19 mAbs. Half-maximum inhibition was achieved with 30 μg/ml, and at concentrations above 60 μg/ml proliferation was blocked completely. The CD20 mAb BCA-B20 was not able to inhibit proliferation at any of these concentrations (Fig. 5), nor were NKI-B20, R24.3, and K8 mAbs (data not shown). There was no difference between the different isotypes of the mAbs used (data not shown).

The JY*hCD19 transfectants are not more sensitive than JY wild-type or Daudi cells to inhibition of proliferation. About the same concentrations of CLB-CD19 were needed to achieve half-maximum and complete inhibition (data not shown). CD20 mAbs had no effect on the proliferation of these cells either.

DISCUSSION

We have used a nude mouse xenograft tumor model to explore the efficacy of treatment with unconjugated mAbs directed against differentiation antigens on B cell lymphoid cancers. Because the effect of the choice of target antigen on the outcome of immunotherapy has not been studied systematically, we compared therapy with mAbs directed against two B cell-specific antigens, CD19 and CD20. On the basis of the outcome of earlier experiments performed in our laboratory (7, 8) and data from the literature (38), we decided to use the IgG2a isotypes of the available isoform switch variants of CLB-CD19 and NKI-B20. Antibody BCA-B20 is also of this isotype. There is no real difference in affinity between these mAbs. As is apparent from the data summarized in Table 1, NKI-B20 and BCA-B20 are much more effective in mediating antitumor activity than CLB-CD19. Only about 15% of the animals treated with CD20 mAbs (BCA-B20 and NKI-B20) had grown a tumor by day 40. Antitumor effects were long-lasting, since only about 10% of the animals treated with BCA-B20 that were tumor free on day 40 had grown a tumor by day 90. The observation that CLB-CD19 had no effect on tumor take when treatment was started...
or BCA-B2OmAim (all of IgG2aisotype), final concentrations ranged from 250 g@gJml to

Targetcells were incubated for 24h with various concentrations of CLB-CD19, NKI-B20, or BCA-B20 mAbs (all of IgG2a isotype), final concentrations ranged from 250 µg/ml to 125 ng/ml, and pulsed for 4 h with [3H]thymidine. cpm are indicated as the mean (n = 6) ± SD. Data are representative of four independent experiments.

Fig. 5. Inhibition of proliferation of Daudi cells by CD19 but not by CD20 mAbs. Target cells were incubated for 24 h with various concentrations of CLB-CD19, NKI-B20, or BCA-B20 mAbs (all of IgG2a isotype), final concentrations ranged from 250 µg/ml to 125 ng/ml, and pulsed for 4 h with [3H]thymidine. cpm are indicated as the mean (n = 6) ± SD. Data are representative of four independent experiments.

on day 4 is in agreement with our previously published results (7, 8), where CLB-CD19 prevented tumor take when treatment was started on day 0, but not on day 7. Although we do not know the mechanisms by which tumors are eliminated in vivo, mAbs against different target antigens might operate by different mechanisms. In the case of BCA-B20 and NKI-B20, tumor cells seem to be eradicated by effector cells, while in the case of CLB-CD19 there seems to be an inhibition of growth rather than an elimination of the tumor cells (Tables 1 and 2). This notion is largely supported by the in vitro data obtained with these mAbs.

Target cells coated with CLB-CD19 mAbs are inhibited in their proliferation, whereas target cells coated with BCA-B20 or NKI-B20 mAbs are not (Fig. 5). It has been shown by Ghetie et al. (40) that the CD19 mAbs BU12, 4G7, and HD37 inhibit proliferation of Daudi cells by inducing cell cycle arrest. Most likely the inhibitory activity of CLB-CD19 is due to the same mechanism. In vitro 30 µg/ml CLB-CD19 are needed to achieve 50% inhibition of proliferation of Daudi cells. Since a nude mouse weighs about 30 g, this concentration could be reached after every i.p. injection (1 mg/treatment) even if the mAb would be distributed evenly among all tissues.

Various factors appear to play a role in the efficacy of ADCC, depending on the effector cells used. With IL-2-activated NK cells as effectors, antigen density appears to have a major effect, whereas the isotype of the antibody is irrelevant. The effect of antigen density, rather than other properties of the target antigen, is formally shown by the higher specific cell kill of JY*CD19 cells overexpressing CD19 antigen.

With macrophages as effector cells, it seems that a combination of target antigen and antibody isotype determines the level of cytotoxicity. In the case of CLB-CD19 mAbs, only the IgG2a isotype mediated high levels of cytotoxicity, which is in agreement with data from the literature (33). With NKI-B20 mAbs the IgG2a again gave the highest levels of cytotoxicity, but the other isotypes IgG1 and IgG2b were also effective. The most likely explanation for the different isotype effects of CLB-CD19 and NKI-B20 would be the difference in antigen density. Compensation for lesser binding of isotypes IgG1 and IgG2b to FcγRs could be found in a higher antigen density on the target cells and this would give rise to appreciable levels of cytotoxicity with these isotypes.

Our in vivo findings are in agreement with those of Buchsbaum et al. (41) who showed that established Raji tumors can be treated efficiently with unlabeled anti-B1 (CD20) mAbs. However that study does not provide any in vitro data that might explain the results. In a recent article of Ghetie et al. (40) it has been shown that several, but not all, CD19 mAbs induce cell cycle arrest, but no apoptosis, in Daudi cells in vitro with intact (IgG1) immunoglobulins. It has been suggested that cell cycle arrest would also be the mechanism by which CD19 treatment of SCID/Daudi mice induces a prolonged survival. This is in agreement with our findings, showing reduced growth rates of developing Daudi tumors in mice treated with CLB-CD19 mAbs as compared to control mice and with findings of Flavell et al. using BU12 mAbs in SCID/NALM-6 and SCID/RAMOS tumor models. However since our in vivo experiments were carried out with intact immunoglobulins of isotype IgG2a it cannot be excluded that, besides the induction of cell cycle arrest, other (Fc-dependent), effector mechanisms play a role in the antitumor effects of CD19 mAbs. This might also explain why intact HD37 IgG1 has a significantly better antitumor effect in the SCID/Daudi model than HD37 (Fab')2, as reported by Ghetie et al. (42).

Our data support the hypothesis that with antibodies BCA-B20 and NKI-B20 ADCC mediated by lymphocytes (NK cells) and/or macrophages may be important for the observed antitumor effects in vivo, while these mechanisms might be less effective for CLB-CD19 due to the lower CD19 antigen density in combination with a higher degree of antibody-induced antigenic modulation. On the other hand, most, but not all, CD19 mAbs may have an effect on tumor growth by means of an entirely different mechanism: inhibition of proliferation by cell cycle arrest (40). A correlation of a direct effect on tumor cells in vitro and the ability to induce tumor regression in patients was shown recently for anti-idiotypic mAbs (43). Nonetheless, Vuis et al. (7, 8) have shown that the in vivo efficacy of the IgG2a isotype of CLB-CD19 is much higher than that of other isotypes. A possible way to distinguish between these two mechanisms would be treatment of xenografted mice with either CLB-CD19-IgG1 or B43-IgG1, since the latter does not induce cell cycle arrest (40).

In the treatment schedule we have used here, we did not see an additive or synergistic effect between CD19 and CD20 mAbs; the effect of CD20 alone is already very strong. If the mechanisms by which CD19 and CD20 mAbs mediate antitumor effects are indeed different, a possible additive or synergistic effect might be observed with other treatment schedules, e.g., starting treatment much later after tumor inoculation.

The present study indicates that for the treatment of B cell cancers in patients using unconjugated mAbs, CD20 mAbs may be superior to CD19 mAbs. Press et al. (44) have reported on four patients treated with murine CD20 mAbs without adverse side effects and encouraging results. Since chimeric CD19 (45) and CD20 (46, 47) mAbs are now available, these are preferred in clinical trials because of expected lower immunogenicity and a potentially better interaction with human effector mechanisms.

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