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Eradication of Large Human B Cell Tumors in Nude Mice with Unconjugated CD20 Monoclonal Antibodies and Interleukin 2[1]

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ABSTRACT

Since antibody-dependent cellular cytotoxicity is considered an important mechanism by which mAbs may exert their antitumor effects, it seems likely that these antitumor effects can be enhanced by the activation of the appropriate effector cell populations. We have used nude mice xenografted with human Daudi tumor cells as a model to compare the antilymphoma effects of unconjugated CD19 (CLB-CD19) and CD20 (BCA-B20) mAbs (IgG2a subclass) alone or in combination with recombinant human interleukin 2 (rhIL-2) or recombinant mouse granulocyte-macrophage-colony-stimulating factor (rmGM-CSF). Treatment of established tumors with BCA-B20 or rhIL-2 or rmGM-CSF as a single agent, all resulted in highly significant decreases of tumor growth rates, but did not increase the number of complete regressions. The combination of CLB-CD19 or BCA-B20 mAbs with rhIL-2 or rmGM-CSF resulted in larger decreases of growth rates than either of the agents alone. Complete eradication of large Daudi tumors could be achieved when treatment with BCA-B20 mAbs was combined with rhIL-2, but not with the combination of CLB-CD19 mAbs and rhIL-2 nor with the combination of BCA-B20 mAbs and rmGM-CSF. Cured animals kept for 2–3 months after complete regression of the tumors were still tumor free. Regression of tumors was correlated with the infiltration of lymphocytes as well as macrophages into the tumor. This is the first report to show that unconjugated CD20 mAbs are to be preferred over unconjugated CD19 mAbs, and interleukin 2 over GM-CSF in the combinational treatment of large B cell tumors.

INTRODUCTION

Over the past decade attempts have been undertaken to treat lymphoid cancers with unconjugated antibodies, radiolabeled antibodies, or immunotoxins (1–4). For the treatment of B cell cancers, we have decided to focus on the B-cell-specific differentiation antigens recognized by CD19 and CD20 mAbs, since the applicability of tumor-specific anti-idiotype mAbs is restricted by the need to make individual mAbs (5). Also, the use of mAbs directed against more widely expressed leukocyte antigens, such as CD52, is less attractive, since it leads to a depletion of T cells as well as tumor cells (6) and may therefore result in severe side effects. A number of clinical trials have been performed with unconjugated murine CD19 (7, 8), murine CD20 (9), and chimeric CD20 (10, 11) mAbs. The results of these trials are encouraging, but treatment needs further improvement.

Recently, we have shown in a xenograft tumor model that the antitumor effects *in vivo* of unconjugated CD20 mAbs are superior to those of CD19 mAbs in preventing the outgrowth of human Daudi tumors (12). The full potential of unconjugated antibodies has not yet been fully explored. Notably, the combination of mAbs with cytokines that stimulate effector cells to lytic activity needs further investigation.

We now report on comparisons in a preclinical study of the treatment of established Daudi tumors with unconjugated CD19 and CD20 mAbs in combination with three different cytokines: IL-2,[4] G-CSF, and GM-CSF.

The CD19 antigen is expressed on normal as well as malignant B cells as a 90-kDa glycoprotein (13). The CD19 antigen is expressed from the pre-B cell stage onward, but disappears upon plasma cell differentiation. It plays an important role in B cell activation and differentiation and can be found in a multicomponent complex at the cell surface (14, 15). There is evidence that the malignant stem cell from which tumor cells originate also expresses the CD19 antigen (16). The CD20 antigen is expressed on normal and malignant B cells as a 33–35-kDa integral membrane phosphoprotein (17). It is expressed slightly later in B cell differentiation than the CD19 antigen and is lost at the plasma cell stage. Recently, CD20 was found to directly regulate transmembrane calcium conductance in B lymphocytes, probably by forming multimeric complexes in the plasma membrane (18). The CD20 antigen plays an important role in B cell proliferation and differentiation (19). On most B cells the expression of CD20 antigens is 2–10-fold higher than that of CD19 antigens. CD19 antigens are modulated to varying degrees on different cells, whereas CD20 antigens are not susceptible to antibody-induced modulation (20–22).

Of the cytokines explored in the present study, IL-2 is produced by T cells and has a pleiotropic activity (23). IL-2 can stimulate mature T cells both *in vitro* and *in vivo* to proliferate and to produce IFN-γ and tumor necrosis factor α. Of more relevance in nude mouse models is the fact that IL-2 can stimulate the proliferation and cytotoxicity of natural killer cells (24) and the tumoricidal activity of monocytes (25). Systemic administration of IL-2 to mice resulted in increased levels of ADCC with effector cells taken from various tissues (26). The cells mediating this ADCC were mainly NK/LAK cells. G-CSF and GM-CSF (and also monocyte-colony-stimulating factor and IL-3) are cytokines produced by multiple cell types, including fibroblasts, endothelial cells, stromal cells, and lymphocytes, that are widely distributed throughout the body. These cytokines support the production, maturation, and induction of function of granulocytes and monocytes/macrophages (27). G-CSF and GM-CSF have been used frequently as supportive agents to avoid neutropenia after chemotherapy, radiotherapy, or bone marrow transplantation (28). In the present study, we tested the capacity of these cytokines to improve the cytolytic capacity of possible effector cells, *e.g.*, granulocytes and monocytes/macrophages (29–31).

Treatment of cancers with combinations of mAbs and cytokines potentially has the benefit of both; sensitizing target cells, thereby making them suitable targets in ADCC, and stimulating the effector cells to lytic activity. It has been shown by others that this approach

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[4] The abbreviations used are: IL, interleukin; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; ADCC, antibody-dependent cellular cytotoxicity; FcγR, receptor for the Fc part of IgG; LAK, lymphokine-activated killer; NK, natural killer; PEC, peritoneal exudate cells; rhIL-2, recombinant human IL-2; rhG-CSF, recombinant human G-CSF; rmGM-CSF, recombinant mouse GM-CSF; CSF, colony-stimulating factor.

may result in enhanced antitumor effects in the treatment of (*a*) immunocompetent mice bearing syngeneic B cell lymphoma with anti-idiotype mAbs in combination with IFN-α (32) or IL-2 (33), (*b*) immunocompetent mice bearing syngeneic B16 melanoma with anti-B16 mAbs and IFN-α (34), (*c*) nude mice bearing human B cell xenografts with CD19 mAbs and IL-2 (35, 36), and (*d*) patients with colorectal carcinomas with 17–1A mAbs and GM-CSF (37).

The experiments presented in this article show that mAb-based immunotherapy for B cell lymphoma can be improved by the addition of cytokines. In comparing the antitumor effects of CD19 and CD20 mAbs, IL-2 and GM-CSF in the treatment schedules used, it is apparent that IL-2 strongly potentiates the therapeutic effect of CD20 mAbs and that of CD19 mAbs to a lesser extent. GM-CSF had much less activity than IL-2 in this regard.

MATERIALS AND METHODS

Mice. Athymic BALB/c *nu/nu* mice were bred and maintained at the animal department of The Netherlands Cancer Institute. The mice were kept in isolators under specific pathogen-free conditions and used when 8–12 weeks old. Mice used as a source of effector cells were up to 20 weeks old. Throughout all experiments male animals were used. 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 lines Daudi and Raji were obtained from the American Type Culture Collection (Rockville, MD). BJAB cells were obtained from Professor Dr. P. H. Krammer (Heidelberg, Germany). Daudi cells used in the *in vivo* experiments were taken from a 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) containing glutamine supplemented with 5–10% (v/v) FCS (GIBCO), 100 units/ml penicillin and 100 μg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany), and 20 μM 2-mercaptoethanol (Merck), at 37°C in humidified air with 6% CO_2. Cell lines were *Mycoplasma* free.

mAbs. The hybridomas NKI-B20 (CD20), BCA-B20 (CD20), CLB-CD19 (CD19), R24.3 (anti-HLA II), and K8 (anti-idiotype) have been described previously (12). All mAbs were dialyzed against PBS twice for 24 h before sterilization by filtration (Millipore; pore diameter, 0.22 μm) and stored at −20°C until use. The concentration of mAbs was determined by spectrometry; the purity (always 90–95%) was determined by means of SDS/PAGE. For phenotyping of murine effector cells, mAbs directed against the following murine leukocyte differentiation antigens were used *in vitro*: Thy-1.2 (59 AD2.2), FcγRII and FcγRIIIα (2.4G2) (according to the new nomenclature in Ref. 38), Mac-1 (M1/70), CD3 (145–2C11), CD4, and CD8.

Fluorescence-activated Cell Sorter Analysis. Immunofluorescence was performed as described previously (36). Fluorescent staining was analyzed with a FACScan (Becton Dickinson, Mountain View, CA).

Effector Cells 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 containing 500 Cetus Units rhIL-2/ml (Eurocetus, Amsterdam, the Netherlands) as described previously (12). PEC were obtained from rmGM-CSF (daily, for a period of 2 weeks 5×10^5 units) or PBS-treated animals and used as described previously (12).

ADCC with Stimulated Spleen Cells as Effectors. Standard 4-h ^{51}Cr release experiments were performed as previously described (35), 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. The concentrations of mAbs given in the figures are the final concentrations in the experimental wells. The spontaneous release of label never exceeded 20% of the maximum release and was usually approximately 10%.

ADCC Experiments with Macrophages as Effector Cells. This assay has previously been described (36) as a proliferation inhibition assay and has been used with a number of alterations (12).

***In Vivo* Experiments.** The previously described nude mouse xenograft tumor model (35, 36) has been used with a number of alterations (12). In brief, on day 0 the animals were whole-body irradiated with a 3 Gy dose from a Siemens stabilipan Röntgen radiation generator, adjusted to 250 kV, 15 mA, and using a Thoraeus I filter. Viable Daudi cells (5×10^6) 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, St. Louis, MO) or human serum albumin (Euroclone, Amsterdam, the Netherlands). Cytokines were given as follows: rhIL-2 (EuroCetus, Amsterdam, the Netherlands) was injected as a s.c. depot (200 μl) in incomplete Freund's adjuvant containing 2×10^5 Cetus Units rhIL-2 and 3% (w/v) BSA on the left flank; CSFs were given as i.p. injections of 200 μl PBS/BSA (0.5% w/v) containing either 1 μg rhG-CSF (Amgen, Thousand Oaks, CA) or 5×10^5 units rmGM-CSF (Sandoz, Austria, Vienna). Treatment with rhIL-2 was given three times with 1-week intervals, and CSFs were given daily for a period of 2 weeks. We started treatments on day 4 (model I) or day 18 (model II). In model I, after inoculation of tumor cells on day 0, rhIL-2 and mAbs were given on days 4, 11, and 18, and CSFs from day 1 onward daily for 2 weeks. In model II, animals bearing established tumors were treated on days 18, 25, and 32 with mAbs and/or rhIL-2, or with CSFs daily from day 18 onward for 2 weeks. Tumor sizes were measured every 7–10 days as the perpendicular diameters in two dimensions with precision callipers. Tumor take rates are given for day 40 (model I) because most control animals had grown very large tumors at that time and had to be sacrificed. The number of animals with tumors in complete regression are given for day 60 (model II). 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. For model I both tumor take rate at day 40, and tumor growth rates were compared between the treatment groups given in Table 1. For model II both the tumor growth rates, and the number of complete regressions were compared between the treatment groups given in Table 2. *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. The data were analyzed as described previously (12). Tumor take rates in model II were compared using Fisher's exact test. In comparisons of individual treatments, *P* values adjusted for multiple comparisons were calculated based on Hommel's procedure (39). Residual analyses gave evidence of a number of moderate deviations from the model assumption. However, these would not affect the main conclusions.

Histological Sections. A number of animals with growing tumors or tumors in regression from different treatment groups were killed. Tumors were formalin fixed and paraffin embedded. Sections (3 μm) were periodic acid-Schiff-stained and examined "blind" (the person examining the sections did not know at that time from which treatment group the tumor was taken, or whether the tumor was regressing or growing). Photographs were taken with an Olympus camera mounted on a Zeiss microscope. In the legend to Fig. 2, the original magnifications are given.

RESULTS

Immunostimulating Effects on Treatment with CD19 or CD20 Antibodies of rhIL-2, rmGM-CSF, and rhG-CSF in Two Xenograft Tumor Models

Human Daudi tumor cells were inoculated s.c. on the flank of BALB/c nude mice. Daudi cells inoculated s.c. do not metastasize at all, as judged by histology (data not shown). Treatment with mAbs and/or cytokines followed shortly after inoculation of the tumor cells (model I) or was postponed until sizeable tumors had grown (model II). The presence of tumors and the size were determined regularly. On the basis of earlier experiments performed in our laboratory (35) and data from the literature (40), we decided to use the IgG2a isotypes of the CD19 (CLB-CD19) and CD20 (BCA-B20) mAbs. The doses and the treatment schedules of rhIL-2 and mAbs were based on earlier experiments performed in our laboratory (12, 35), and the doses and treatment schedules for rmGM-CSF and rhG-CSF were based on data from the literature (41–43).

Model I: Prevention of Tumor Outgrowth Using CD19 mAbs and/or rhIL-2, rmGM-CSF, or rhG-CSF. Previously we have shown that treatment with CD20 mAbs alone is sufficient to prevent the outgrowth of tumors in 85% of the mice (12). Treatment with CD19 mAbs is not, leaving possibilities for improvement of therapy. The effect of rhIL-2, rmGM-CSF, or rhG-CSF on the treatment with CLB-CD19 mAb was evaluated by two parameters: the tumor take rate on day 40 and the growth rate of tumors that did arise. CLB-CD19 and rhIL-2 were given on days 4, 11, and 18; rmGM-CSF and rhG-CSF were given daily for 2 weeks from day 1 onward. Table 1 summarizes, for four experiments, the number of mice, tumor take rate on day 40, and estimated growth rates (mm^2/day) for the different treatment groups.

The decrease in tumor take in comparison with controls was significant for the combination of (CLB-CD19 + rhIL-2; $P = 0.024$). All other P values were 0.05 or more.

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 when 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 (44). Differences in mean slopes between experiments were accounted for (12). Treatment with CLB-CD19 mAbs alone resulted in a significantly decreased growth rate as compared to controls ($P < 0.0001$) as did treatment with rhIL-2 alone ($P < 0.0001$). Treatment with rmGM-CSF or rhG-CSF did not result in significant decreases in growth rates. The addition to the treatment with CLB-CD19 mAbs of rhIL-2, rmGM-CSF, or rhG-CSF did not significantly influence growth rates further.

Model II: Treatment of Established Tumors with CD19 or CD20 mAbs and/or rhIL-2 or rmGM-CSF. Animals bearing large tumors (all animal average, 81 ± 46 mm^2) were treated with CLB-CD19 or BCA-B20 mAbs and/or cytokines. mAbs and rhIL-2 were given on days 18, 25, and 32; rmGM-CSF was given daily for 2 weeks from day 18 onward. Table 2 shows, for four experiments, the number of mice, average tumor size on the day the first treatment was given, estimated mean growth rate, and number of complete regressions for each treatment group. The data on growth rates were analyzed as described above. The data on tumor take rates were analyzed with the Fisher exact test. Table 3 shows the outcome of the statistical analysis of the comparison of a selected number of treatments. It is clear from Tables 2 and 3 that each of the treatments with a single agent (BCA-B20, rhIL-2, or rmGM-CSF) resulted in highly significant decreases of the growth rates as compared to controls (all P values <0.0040). The decrease in growth rate induced by rhIL-2 alone was improved by the addition of BCA-B20 mAbs as well as by the addition of CLB-CD19 mAbs. However, the combination of rhIL-2 and BCA-B20 mAbs was more effective than the combination of rhIL-2 and CLB-CD19 mAbs ($P = 0.0021$ single; $P = 0.0084$ Hommel corrected).

Both rhIL-2 (both P values <0.0001) and rmGM-CSF ($P = 0.0074$ single; $P = 0.015$ Hommel corrected) enhanced the effect of treatment with BCA-B20. The difference between growth rates between treatments with BCA-B20 + rhIL-2 *versus* BCA-B20 + rmGM-CSF was not significant (both P values = 0.34). However, a larger number of complete regressions was found in the group that had been treated with BCA-B20 + rhIL-2 (11/21) as compared to the BCA-B20 + rmGM-CSF treated group (1/10; $P = 0.0464$).

Fig. 1 shows the tumor size *versus* time of individual mice in three groups (control, BCA-B20, and BCA-B20 + rhIL-2) in one experiment. Cured animals ($n = 12$) in this experiment did not develop tumors again by day 90 after initial inoculation of tumor cells. In another experiment we kept the cured animals ($n = 5$) until day 120, when all were still tumor free.

Table 1 *Model I: effect of CD19, rhIL-2, rmGM-CSF, rhG-CSF, and combinations on the prevention of tumor outgrowth*

BALB/c nude mice were inoculated s.c. with 5×10^6 viable Daudi cells on day 0 and were treated with mAbs and/or cytokines.

Treatment[a]	No.[b]	Tumor take rate[c] Total	Tumor take rate[c] %	Growth rate[d] (mm^2/day)
Control (PBS)	28	27/28	96	11.68 ± 0.60
CLB-CD19	29	28/29	97	4.66 ± 0.54
rhIL-2	30	30/30	100	7.93 ± 0.58
rmGM-CSF	10	10/10	100	10.89 ± 1.05
rhG-CSF	10	10/10	100	12.81 ± 1.13
CLB-CD19 + rhIL-2	29	20/29	69	3.69 ± 0.50
CLB-CD19 + rmGM-CSF	11	8/11	73	4.04 ± 1.10
CLB-CD19 + rhG-CSF	10	9/10	90	4.96 ± 0.96

[a] Treatment with PBS or CLB-CD19 (IgG2a) was given i.p., and rhIL-2 was given in a s.c. depot on days 4, 11, and 18; treatment with CSFs were given i.p. daily from day 1 onward for 2 weeks.
[b] Total number of mice in each group.
[c] Tumor take rate is given for day 40, when all animals were still alive.
[d] Estimated mean growth rates are given in mm^2/day ± SE.

Table 2 *Model II:* in vivo *effect of CD19 and CD20 mAbs and/or rhIL-2 and rmGM-CSF on established Daudi tumors*

Treatment[a]	No.[b]	Tumor Size[c] (mm^2)	Growth rate[d] (mm^2/day)	Complete regressions[e,f]
Control (PBS)	40	66 ± 45	14.72 ± 1.40	0/40
rhIL-2	18	54 ± 61	8.33 ± 2.03	0/18
rmGM-CSF	10	105 ± 35	8.11 ± 1.56	1/10
BCA-B20	21	88 ± 30	5.14 ± 1.24	3/21
CLB-CD19 + rhIL-2	21	86 ± 30	4.72 ± 1.94	1/21
BCA-B20 + rmGM-CSF	10	90 ± 44	0.37 ± 1.43	1/10
BCA-B20 + rhIL-2	21	98 ± 29	−1.34 ± 1.26	11/21

[a] BALB/c nude mice were inoculated s.c. with 5×10^6 viable Daudi cells on day 0 and treated with mAbs and/or cytokines from day 18 onward. Treatments with PBS, CLB-CD19 (IgG2a), and BCA-B20 (IgG2a) were given i.p., and rhIL-2 was given in a s.c. depot on days 18, 25, and 32; rmGM-CSF was given i.p. daily from day 18 onward for 2 weeks.
[b] Total number of mice in each group.
[c] Mean tumor size ± SD on day 18 in mm^2.
[d] Estimated mean growth rates are given in mm^2/day ± SE. Note that a negative growth rate is calculated for treatment group BCA-B20 + rhIL-2.
[e] Number of complete regressions *versus* total number of mice in each group achieved in a 60-day period after inoculation of tumor cells.
[f] Using Fisher's exact test P values on the number of complete regressions are as follows: BCA-B20 + rhIL-2 *versus* BCA-B20 alone ($P = 0.0203$) and BCA-B20 + rhIL-2 *versus* BCA-B20 + rmGM-CSF ($P = 0.0464$), treatments mentioned first being better than the latter.

Table 3 *Statistical analysis of biologically relevant comparisons of the effect of different treatments on growth rates of established tumors given in Table 2*

Comparison of treatment groups			P[a] Single	P[a] Corrected
BCA-B20 + rhIL-2	*vs.*	rhIL-2	<0.0001	<0.0001
		BCA-B20	<0.0001	<0.0001
		BCA-B20 + rmGM-CSF	0.3400	0.3400
		CLB-CD19 + rhIL-2	0.0021	0.0084
BCA-B20 + rmGM-CSF	*vs.*	BCA-B20	0.0074	0.0150
CLB-CD19 + rhIL-2	*vs.*	rhIL-2	0.0074	0.0150
BCA-B20	*vs.*	control	<0.0001	<0.0001
rhIL-2	*vs.*	control	<0.0001	<0.0001
rmGM-CSF	*vs.*	control	0.0008	0.0040

[a] 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 (single). In comparisons of individual treatments, P values adjusted for multiple comparisons were calculated based on Hommel's procedure (corrected). The treatment mentioned first being better than the latter.

Tumor Regression and the Presence of Infiltrating Lymphocytes and Macrophages

In order to investigate whether tumor regression was mediated by infiltrating cells or by necrosis/apoptosis, we took tumors from ani-

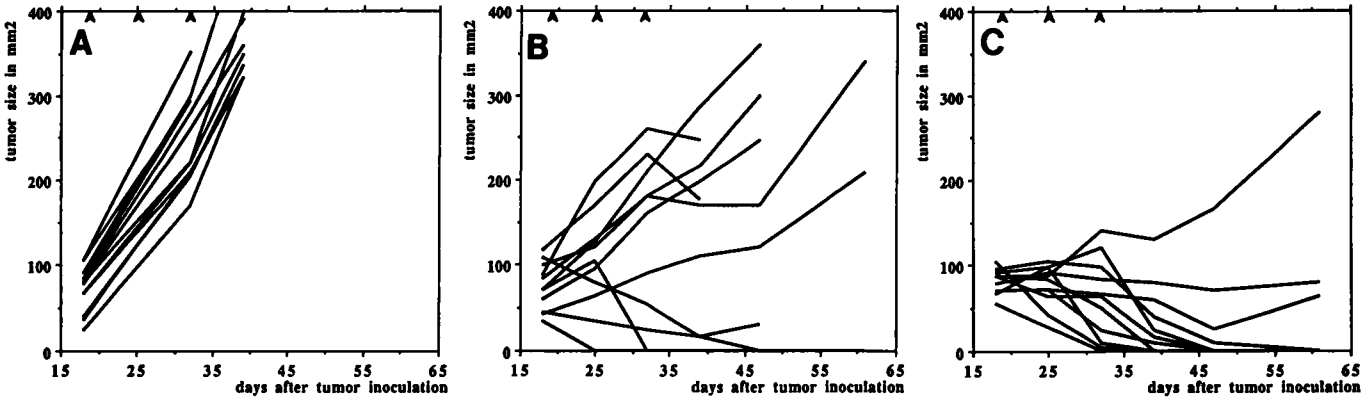

Fig. 1. Tumor size *versus* time. Time after inoculation of tumor cells is given in days, tumor sizes (measured as the perpendicular diameters in two dimensions) are given in mm^2. *Each line*, individual mouse, $n = 11$ in each group. *A*, control group treated with PBS; *B*, group treated with BCA-B20 (CD20) mAbs; and *C*, animals treated with BCA-B20 mAbs in combination with rhIL-2. *Arrowheads*, days of treatment (days 18, 25, and 32). Cured animals did not develop tumors again by day 90 after initial inoculation of Daudi tumor cells.

mals with tumors in regression or with growing tumors that had been treated with PBS, BCA-B20 + rhIL-2, BCA-B20 + rmGM-CSF, or GM-CSF alone (three animals/treatment group). Histological sections were stained and examined "blind."

From all tumors we examined at least two sections for the presence or absence of necrosis and infiltrating cells. Sections taken from growing tumors taken from either treated or untreated animals showed a Daudi tumor mass without infiltrating cells. In sections of very large tumors from these animals, we found fields of necrosis, varying in size, in the central part and the anticutaneous side of the tumor, but never in the periphery. In contrast, we found no or hardly any signs of necrosis in sections from tumors in regression. In these sections infiltrating lymphocytes (mainly perivascular) as well as macrophages were only seen in the periphery of the tumors. No qualitative difference in infiltrating cells was seen between animals treated with BCA-B20 + rhIL-2 or BCA-B20 + rmGM-CSF. In BCA-B20 + rhIL-2-treated animals, lymphocytes (most probably NK cells) and macrophages were seen in about equal numbers, whereas in BCA-B20 + rmGM-CSF-treated animals macrophages predominated. Fig. 2 shows sections representative for the different findings described here.

In Vitro Experiments with Activated NK Cells and Macrophages

ADCC Mediated by rhIL-2 Activated NK Cells. Spleen cells from nude mice were activated for 5 to 6 days with rhIL-2 *in vitro*; the resulting cells consisted of 90–95% of NK cells (35). Fig. 3 shows the results of ADCC experiments with activated NK cells as effectors and three human B cell targets (Daudi, BJAB, and Raji). A titration curve was made of CLB-CD19 (Fig. 3*a*), BCA-B20 (Fig. 3*b*), and negative control mAbs (all of isotype IgG2a) at a fixed E:T ratio of 25. Indicated by the *dotted lines* in Fig. 3 are the percentages of nonspecific killing by activated NK cells. Raji cells are more sensitive to LAK activity of NK cells than Daudi cells. BJAB cells show an intermediate sensitivity to LAK activity. The difference between the maximum levels of cytotoxicity reached when target cells were incubated with BCA-B20 mAbs can be explained completely by these differences in LAK sensitivity. When coated with CLB-CD19 mAbs Daudi and BJAB were less efficiently killed than when coated with BCA-B20. Compared to Daudi and BJAB, Raji cells were more susceptible to lysis with CLB-CD19 mAbs. No differences were observed between BCA-B20 and another CD20 mAb (NKI-B20), or between CLB-CD19 and two other CD19 mAbs (BU12 and B43; data not shown). It is important to note that the minimum amount of mAb needed to sensitize a given target cell to reach the maximum level of cytotoxicity is about 50 ng/ml for both CLB-CD19 and BCA-B20 mAbs.

Antitumor Activity of Macrophages Activated *in Vivo* with rmGM-CSF. To examine the effect of treatment with rmGM-CSF on the activation of macrophages *in vivo*, peritoneal macrophages were isolated from mice that had been treated daily with rmGM-CSF for a period of 2 weeks or with PBS as a control. The animals had been inoculated with tumor cells on day 0. Treatment with rmGM-CSF started on day 18 and was repeated daily for 2 weeks. One day after the last injection of rmGM-CSF, PEC were harvested. All of these animals had grown very large tumors (>300 mm^2), so treatment had failed. We were able to harvest four to six times as many PEC ($8–12 \times 10^6$/animal) from rmGM-CSF-treated animals as from controls (about 2×10^6). The harvested PEC from GM-CSF-treated animals consisted mainly of activated macrophages since they expressed high levels of Mac-1 and FcγRII and/or FcγRIIIα (according to the new nomenclature in Ref. 38), but were negative for CD3, CD4, CD8, Thy-1, and surface immunoglobulin (data not shown). The PEC from animals treated with PBS showed 1–2 log lower levels of Mac-1 and FcγRII/IIIα expression. The adherent cells were tested in ADCC experiments as described previously (12, 35). In this assay the macrophages are allowed to kill or phagocytose antibody-coated Daudi target cells for 3 days. The remaining fraction of the target cells is measured by incorporation of [^{3}H]thymidine. The reduction in incorporation of [^{3}H]thymidine is used as a measure of ADCC mediated by the antibodies. The macrophages taken from control animals showed hardly any cytotoxicity toward the antibody-coated Daudi target cells (Fig. 4, *dotted lines*). Whereas macrophages taken from rmGM-CSF-treated animals gave high levels of cytotoxicity with R24.3 and BCA-B20 mAbs and intermediate levels of cytotoxicity with CLB-CD19 at different E:T ratios (Fig. 4).

DISCUSSION

Treatment of cancers with combinations of mAbs and cytokines does not amount to a mere addition of the benefits of each treatment modality alone, but clearly can have synergistic value as demonstrated by this study. Synergism probably results from local cytokine activation of immune effector cells (*e.g.*, NK cells and macrophages) that have bound to mAb-sensitized tumor cells via the Fc receptors. The efficacy of treatment of nude mice bearing human B cell tumors (Daudi) with mAbs, cytokines, and combinations of these agents was

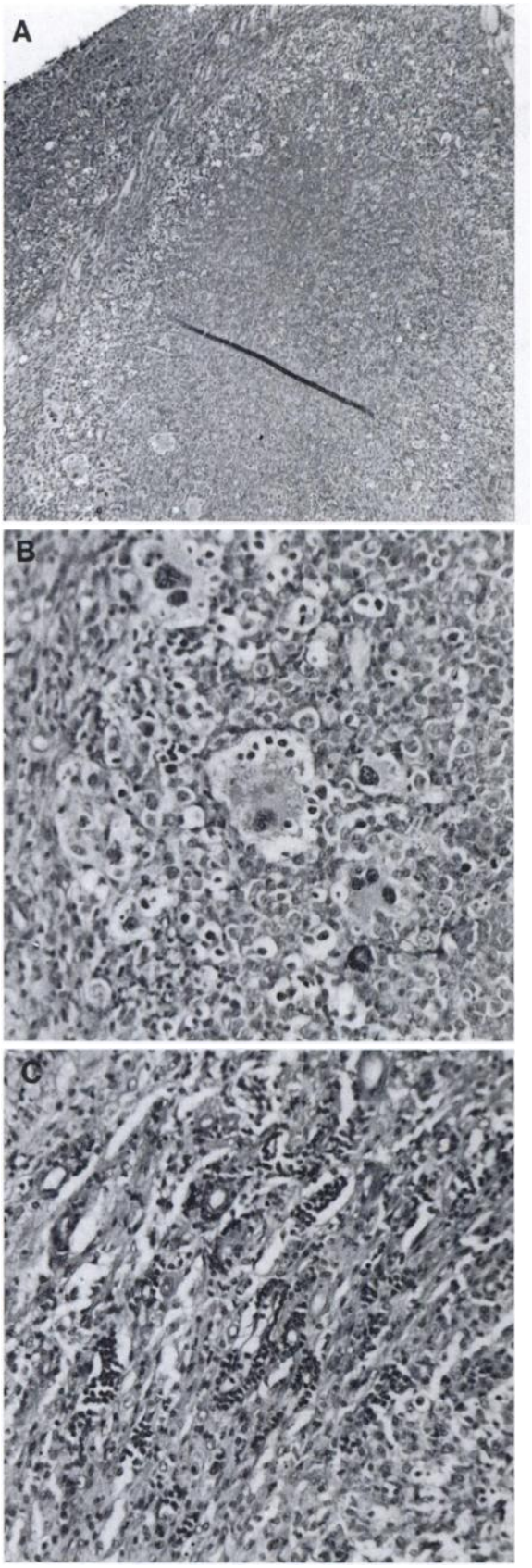

Fig. 2. Histological sections from tumors in regression. *A*, overview of a tumor in regression after treatment with BCA-B20 in combination with rhIL-2 (× 10). A horseshoe-shaped ring of infiltrating cells can be seen in the periphery of the tumor. No signs of necrosis can be seen. At higher magnification (× 40) infiltrating cells can be identified as macrophages (*B*), showing their typical foamy aspect or lymphocytes; most probably NK cells (*C*). The findings described here were seen in all tumors in regression that were examined.

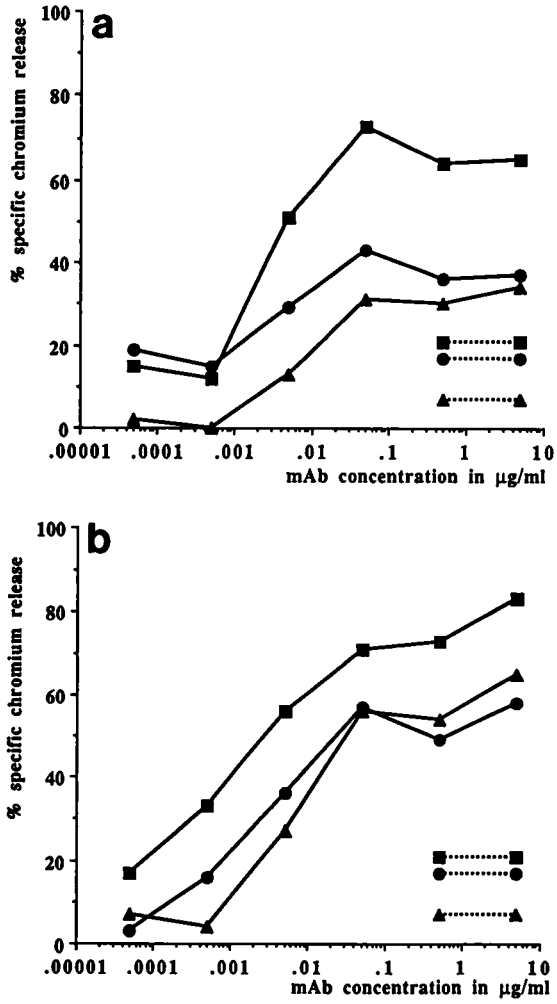

Fig. 3. ADCC with rhIL-2-activated NK cells. NK cells were incubated with different B cell targets (Raji, ■; Daudi, ▲; BJAB, ●) at an E:T ratio of 25. CLB-CD19 (*a*) and BCA-B20 (*b*), both IgG2a, were titrated from 5 μg/ml down to 50 pg/ml (*solid lines*). The level of cytotoxicity after incubation with isotype-matched irrelevant control mAb (K8) is indicated by the *dotted lines* using the same symbols for the different cell lines. Indicated is the mean percentage of specific ^{51}Cr release of triplicate determinations. SD never exceeded 5% of the mean. Data are representative of three independent experiments.

tested in two tumor models: prevention of tumor outgrowth (model I) and treatment of visible lesions (model II).

The results obtained with the latter model in which treatment of established tumors started on day 18 are summarized in Tables 2 and 3. It is clear that both rhIL-2 and rmGM-CSF resulted in a significant reduction of tumor growth rates in comparison to controls. Probably these effects are exerted by activated effector cells, but we cannot exclude the possibility of a direct negative effect on Daudi tumor cells. It has been reported previously by several groups, in different tumor models, that cytokines by themselves can have marked antitumor effects (23, 31, 42). It seems likely that the reduction in growth rate induced by BCA-B20 is due to sensitization of the tumor cells with mAb, thereby making them excellent targets for NK cells and macrophages mediating ADCC, since a direct negative effect on the proliferation of Daudi cells could not be shown *in vitro* (12). Additive antitumor effects were seen when treatment with BCA-B20 was combined with rmGM-CSF, whereas synergistic antitumor effects were seen when treatment with BCA-B20 was combined with rhIL-2.

Activation of effector cells *in vitro* with rhIL-2 (Fig. 3) or *in vivo* with rmGM-CSF (Fig. 4) resulted in high levels of cytotoxicity when

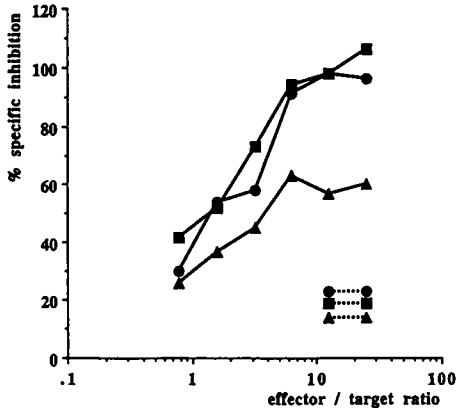

Fig. 4. ADCC with rmGM-CSF-activated macrophages. Macrophages were obtained from rmGM-CSF- or PBS-treated, tumor-bearing animals. Daudi target cells were incubated with macrophages and mAbs (2.5 μg/ml) directed against HLA class II (R24.3-rat-2b, ■); CD19 (CLB-CD19–2a, ▲); or CD20 (BCA-B20–2a, ●). E:T ratios ranged from 25:1 to 0.6:1. *Solid lines*, inhibition obtained after incubation with *in vivo* rmGM-CSF-activated macrophages. *Dotted lines*, inhibition obtained after incubation with macrophages taken from PBS-treated animals. Effector cells, target cells, and mAbs were incubated for 3 days, and proliferation of the remaining target cells was measured by incorporation of [^{3}H]thymidine. Indicated is the mean percentage of specific proliferation inhibition (n = 6). SD never exceeded 7% of the mean.

target cells were incubated with specific mAbs (CLB-CD19, BCA-B20, or R24.3). Nonactivated effector cells were not capable of killing coated target cells (Ref. 35 and Fig. 4). This is also reflected *in vivo*, in the sense that treatment with a combination of a given mAb and a given cytokine induced larger reductions in growth rates than either agent alone. The growth rates in the group treated with BCA-B20 + rmGM-CSF did not differ significantly from those in the group treated with BCA-B20 + rhIL-2. However, a significant difference in favor of BCA-B20 + rhIL-2 was found in the number of complete regressions (P = 0.0464). The treatment schedule for rhIL-2 has been optimized by Vuist *et al.* (35, 36). The treatment schedule for rmGM-CSF has been adopted from the literature (41–43) and might be optimized further. In the schedules used in the experiments in this study, rhIL-2 was found to be more active than rmGM-CSF. Regression of tumors was found to correlate with the presence of lymphocytes and macrophages in these tumors (Fig. 2 and data not shown). There is no correlation between the initial size of a tumor and the outcome of treatment with BCA-B20 + rhIL-2; the largest tumor that went in complete regression was 140 mm^2, while the smallest tumor that did not respond to this treatment was 70 mm^2. Therefore, immune status of the treated animal and vascularization and accessibility of the tumor to mAbs, cytokines, and infiltrating cells may play a more important role in the outcome of a given treatment than the initial tumor size. On the day the first treatment was given (day 18), those tumors that went into complete regression (n = 17) were on average 80 mm^2 (SD = 29), which is equal to the average of 81 mm^2 (SD = 46) of all animals.

Although in the experiments with established tumors, presented in this article, we did not test the antitumor effects of CLB-CD19 alone to compare to treatment with BCA-B20 alone, it is clear from Tables 2 and 3 that the antitumor effect of BCA-B20 + rhIL-2 is superior to the effect of CLB-CD19 + rhIL-2. After high-dose treatment with mAbs of different specificities but of the same affinity, Sung *et al.* (45) showed better accumulation at high antigen densities on the target cells in solid tumors. Since the expression of CD19 antigens is about one third of that of CD20 antigen on the Daudi cells used in our experiments (22), and the affinity of the mAbs used is about the same (12), this possibly results in better accumulation of BCA-B20 mAbs *versus* CLB-CD19 mAbs in the tumor. The difference in antigen density is increased further by the susceptibility of CD19 antigens to antibody-induced modulation. Along with the higher levels of cytotoxicity reached with BCA-B20 *versus* CLB-CD19 mAbs in ADCC with NK cells and macrophages, this might explain the better antitumor effects of CD20 mAbs.

Buchsbaum *et al.* (46) showed that nude mice bearing established Raji tumors can be treated with a small amount of unlabeled anti-B1 (CD20) mAbs (also of isotype IgG2a), leading to an average decrease in tumor size of 90% three weeks after treatment had started. With BCA-B20 alone we found an average increase in Daudi tumor size of 85% on day 18. A number of reasons can be given to explain these different findings. From Fig. 3 it is apparent that Raji target cells are more sensitive to lysis by activated NK cells with and without specific mAb than Daudi cells. The difference in NK sensitivity might also explain our unpublished observation that inoculation of Raji tumor cells on the flank of irradiated nude mice did not result in the outgrowth of tumors (n = 5), whereas 27 of 28 of the control animals developed Daudi tumors under the same conditions (Table 1).

Vascularization of the tumors and thereby accessibility for mAbs, cytokines, and effector cells may be completely different between tumors (47, 48) and between the same tumors in different animal strains (49). The first point was shown by Schmid *et al.* (50) with xenografted nude mice bearing BJAB, Nalm-1, OCI.LY1, or Namalwa tumors. We can speculate that Raji tumors resemble OCI.LY1 and Namalwa tumors which both show generalized leakiness of intratumoral vessels. Daudi tumors might resemble BJAB and Nalm-1, in which accumulation of mAbs occurred exclusively in peripheral layers of the lymphoma nodule, while central areas were not accessible irrespective of mAb dose (50). To achieve maximum levels of ADCC, only about 50 ng/ml of a specific mAb is needed to sensitize target cells for NK cells (Fig. 3) or for macrophages (data not shown). If the penetration and accumulation of mAbs into the tumors are indeed different for Raji and Daudi tumors, this may contribute to the different antitumor effects achieved. The notion that the accumulation of mAbs in Daudi tumors indeed resembles that seen in BJAB tumors is supported by our data showing infiltrating lymphocytes and macrophages only in the periphery of regressing tumors and not in the central parts of these tumors (Fig. 2 and data not shown). It would be very interesting to examine histological sections taken from Raji tumors in regression after treatment with CD20 mAbs and compare these with our findings.

Table 1 summarizes the data obtained in model I when treatment started shortly after inoculation of tumor cells. Previously, we have shown an overwelming effect of treatment with BCA-B20 and a marginal effect of treatment with CLB-CD19 in this model (12). Now we have combined treatment with CLB-CD19 mAbs with a number of cytokines in order to improve the antitumor effect. The effects of starting treatment with CLB-CD19 + rhIL-2 on day 4, although less pronounced, are in agreement with earlier findings starting treatment on day 7 after inoculation of tumor cells (36). Treatment with CLB-CD19 + rmGM-CSF or CLB-CD19 + rhG-CSF did not result in a significant difference in tumor take rate as compared to controls.

It has been shown by Ghetie *et al.* (51) that several (BU12, HD37, and 4G7), but not all (B43), CD19 mAbs induce cell cycle arrest, but no apoptosis, in Daudi cells *in vitro* with intact immunoglobulins (IgG1). In that article it was suggested that cell cycle arrest would be the mechanism by which CD19 treatment of SCID/Daudi mice induces a prolonged survival (51). In *in vitro* assays CLB-CD19 behaved like most CD19 mAbs in inhibiting proliferation of Daudi cells, most probably by the induction of cell cycle arrest (12). Therefore, it is possible that CLB-CD19 also exerts its *in vivo*

effect through the induction of cell cycle arrest, and this mechanism may perhaps be more important than ADCC by NK cells and macrophages.

Treatment of patients with B cell cancers with unconjugated murine CD19 (7, 8) or CD20 mAbs (9) has not led to high response rates. Recently, Phase I and Phase II trials have been performed with unconjugated chimeric CD20 mAbs (10, 11). The response rate in the Phase II trial was 42%. On the basis of the experiments presented in this article, we suggest that unconjugated chimeric CD20 could be combined with IL-2 for the treatment of B cell cancers in patients. Moreover, if the mechanisms by which CD19 mAbs exert their antitumor effects indeed differ from those of CD20 mAbs, it would be interesting to combine CD19 and CD20 mAbs with each other and with IL-2 for maximum antitumor effects.

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