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Treatment of low-grade non-Hodgkin's lymphoma with continuous infusion of low-dose recombinant interleukin-2 in combination with the B-cell-specific monoclonal antibody CLB-CD19

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Abstract Seven patients with low-grade non-Hodgkin's lymphoma were treated with a combination of a murine monoclonal antibody directed against the B-cell-specific antigen CD19 (CLB-CD19), given twice weekly, and continuous infusion of low-dose recombinant interleukin-2 (rIL-2). We demonstrated stable serum CLB-CD19 levels throughout the 12 weeks of treatment, and homing of the antibody into the tumour sites. A variable degree of antigenic modulation was noted. Prolonged treatment resulted in a sustained increase in the number of natural killer cells in the circulation with enhanced cytotoxic capacity, including antibody-dependent cellular cytotoxicity. During the first weeks of treatment, T cell activation occurred in the majority of patients. Toxicity was related to the rIL-2 treatment and consisted of transient constitutional symptoms and a flu-like syndrome without organ dysfunction. A partial remission occurred in one patient, and in another patient who was primarily leukaemic a greater than 50% reduction of circulating B cells was noted. An antitumour effect occurred early during treatment and could not be related to rIL-2-induced modulation of natural killer cell or T lymphocyte activation.

Key words Interleukin-2 · Anti-CD19 antibody
Monoclonal therapy

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Introduction

Monoclonal antibody therapy in B cell malignancies can be directed against either the tumour-specific immunoglobulin idiotype of the malignant B cell clone [anti-idiotype monoclonal antibodies (mAb)] or against lineage-specific cell-surface antigens present on B lymphocytes. Treatment with anti-idiotype mAb has resulted in long-lasting tumour regressions in a substantial percentage of small groups of patients [7, 21, 24]. In recent years, clinical experience has been gained with murine mAb directed against several B-cell-specific antigens such as CD19 [16], CD20 [30] or polymorphic HLA-DR [17]. The modest therapeutic efficacy of this type of treatment may be enhanced by the conjugation of murine mAb to toxic agents [14] or radio-isotopes [31, 37] at the cost of increased toxicity. Effective treatment with unconjugated mAb depends on the ability to recruit the endogenous host effector system. Antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells and monocytes is one of the mechanisms involved [11, 15, 28, 36, 42]. Cytokines such as recombinant interleukin-2 (rIL-2), interferon γ (IFNγ), and macrophage-colony-stimulating factor are able to enhance ADCC mediated by NK cells, monocytes and even granulocytes [6, 26, 28]. It is therefore logical to use these cytokines to enhance the effect of mAb-based therapies.

In murine B cell lymphoma models, our group and others have demonstrated the superiority of treatment with the combination of rIL-2 and mAb to either agent

alone [3, 4, 45]. The mouse IgG2a mAb CLB-CD19 (murine antibody directed to CD19 antigen) [10] is directed against the B-lymphocyte-specific antigen CD19, which is present on the cell surface of normal and malignant B cells. The CD19 antigen is expressed during B cell differentiation from a very early stage, but is absent from plasma cells [40, 49]. Previously we have demonstrated that CLB-CD19 can be safely administered to B cell lymphoma patients [16]. From this study we concluded that a total dose of at least 1000 mg mAB would be necessary to saturate all tumour sites. Recently we have shown that rIL-2 given by continuous infusion for a period of more than 6 weeks will lead to a sustained and time-dependent increase in the number of NK cells and ADCC capacity without significant organ toxicity [43, 44].

On the basis of the pharmacokinetic characteristics of CLB-CD19 mAB and the immunomodulating and toxic effects of continuous infusion of rIL-2, for the present study we used a 12-week treatment schedule of 50 mg/m^2 CLB-CD19 twice a week in combination with continuous infusion of 3×10^6 IU m^{-2} (24 h)$^{-1}$ rIL-2. In this paper we describe the results in seven patients with low-grade non-Hodgkin's lymphoma. The treatment was designed to give sufficient mAb to allow adequate penetration to extravascular tumour sites and a prolonged increase in the number of NK cells and ADCC capacity.

Materials and methods

Patient selection

Eligible patients (age 16–70 years) had measurable or evaluable, histologically proven CD19$^+$ B cell non-Hodgkin's lymphoma of low or intermediate grade malignancy, as defined by the Working Formulation for clinical usage [27]. Patients had to be in good clinical condition [Eastern Cooperative Oncology Group (ECOG) performance status ≤ 1], with a life-expectancy of at least 3 months and normal bone marrow (leucocytes $\geq 3 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l), renal (calculated creatinine clearance ≥ 50 ml/min) and hepatic (bilirubin ≤ 20 μmol/l) function. Patients with rapidly progressive disease likely to require chemotherapy or radiotherapy within 12 weeks or with clinical central nervous system involvement were excluded. Radiotherapy, treatment with corticosteroids or chemotherapy within 4 weeks (6 weeks for nitrosoureas or mitomycin C) of study entry were not allowed and patients had to have fully recovered from previous therapy. Written informed consent was obtained in accordance with The Netherlands Cancer Institute guidelines. The protocol was approved by the Institute's Ethical Committee.

Treatment schedule

EuroCetus rIL-2 (Amsterdam, The Netherlands), diluted in 10 ml sterile water and 2% albumin, was continuously infused on an outpatient basis at a dose of 3×10^6 IU/m^{-2} (24 h)$^{-1}$ for a period of 12 weeks by a portable pump (Perfusor M, Braun, Uden, The Netherlands) via a central venous catheter as previously described [43]. The CLB-CD19 was given by peripheral vein at a dose of 50 mg/m^2 twice a week as a 2-h infusion for a total of 24 doses. The CLB-CD19 was stored at a concentration of 9.2 mg/ml in phosphate-buffered saline with 1 mg human serum albumin mg mAb at −70° C until use [16]. The required dose was diluted in 100 ml saline just prior to administration. Before the first dose of CLB-CD19, the patients were skin-tested with 2 μg mAb in 0.1 ml phosphate-buffered saline. The dose of rIL-2 was reduced to 1.8×10^6 IU m^{-2} (24 h)$^{-1}$ when necessitated by rIL-2-related toxicity. The dose of CLB-CD19 was adjusted according to serum CLB-CD19 levels to achieve a trough serum level of at least 1 μg/ml. Paracetamol was given at a maximum dose of 6×500 mg to relieve constitutional symptoms. Nausea and vomiting were treated with 10–20 mg metaclopramide. During the entire period of rIL-2 infusion, oral prophylactic antibiotic therapy was given with the quinolone derivative pefloxacin at a dose of 400 mg once a day.

Study design

Before entry a history was taken and a full physical examination, and the following investigations were carried out: a complete blood count with differential counting; an extensive chemistry profile including thyroid functions, coagulation tests, serum complement and immunoglobulin levels and urine analysis; an electrocardiogram, chest radiograph and appropriate radiological studies to delineate the disease. In addition, bone marrow aspiration and trephine, and fine-needle aspiration or excision biopsy of an involved lymph node were carried out for immunopathology and surface marker determination.

During treatment haematological and chemistry profiles, serum complement, immunoglobulin, and CLB-CD19 levels, and blood cultures from the central venous access, were monitored on a weekly basis and repeated 2–4 weeks after treatment. Before and within 2–4 weeks after treatment blood was taken to determine human anti-(mouse Ig) antibodies (HAMA).

Before, at weeks 1, 2, 3, 6, 9 and 12, and 2–4 weeks after treatment, 30 ml peripheral blood was taken, the lymphocytes were isolated by centrifugation over Ficoll/Hypaque (Nyegaard) gradients, cryopreserved by controlled-rate freezing in 10% dimethylsulphoxide and stored in liquid nitrogen until testing. When feasible, fine-needle lymph node aspiration was performed at regular intervals for surface marker determination and to evaluate localisation of CLB-CD19.

Response was evaluated every 4 weeks and toxicity was scored weekly according to the standard World Health Organization (WHO) criteria [25]. For those side-effects for which no standard WHO toxicity criteria are available, a scoring system was used as previously described [43]. Easily accessible tumour lesions were monitored every week for antitumour effect.

Immunofluorescence

Mononuclear cells were isolated from peripheral blood lymphocytes (PBL), bone marrow or from cell suspensions of metastatic lymph node by centrifugation over Ficoll/Hypaque. The immunological phenotype was determined by means of direct immunofluorescence, using either fluorescein isothiocyanate (FITC) or phycoerythrin-(PE)-conjugated mAb, or indirect immunofluorescence using fluorescein-conjugated goat F(ab)$_2$ anti-(mouse IgG) (absorbed with human Ig, Tago, Burlingame, Calif., USA) as second antibody. The following antibodies were used: fluorescein-conjugated rabbit anti-(human Ig) [IgM, IgD, IgG, κ or γ (Dako)] or mAb against these immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam) and mAb against CD19 (CLB; Leu12 Fitc Becton Dickinson), CD20 (B1 Coulter; Leu16 Fitc, Becton Dickinson), CD16 (CLB-FcRgran1, CLB; Leu11 Fitc; Leu 11 PE, Becton Dickinson) CD56 (Leu19; Leu19 PE, Becton Dickinson), CD25 (CLB-IL2R/1, CLB; CD25

Fitc, Dako), p75 (p75 Fitc, Endogen), CD27 (CLB-CD27, CLB), CD3 (SPV-T3b, Netherlands Cancer Institute, Amsterdam; OKT3 PE, Ortho), CD4 (OKT4, Ortho; Leu3[a] PE, Becton Dickinson), CD8 (OKT8, Ortho; Leu2[a] PE, Becton Dickinson), CD14 (LeuM3, Becton Dickinson), CD45 (CLB-T200, CLB), and HLA-DR (DK22, Dako, HLA-DR Fitc, Becton Dickinson). Double staining to determine the various surface antigens on $CD3^+$, $CD16^+$ or $CD56^+$ cells was carried out using the appropriate direct FITC- and PE-labelled mAb. Flow cytometry was carried out on a Becton Dickinson Facscan apparatus.

Binding of the infused mAb to malignant and normal B cells in vivo was detected by incubating cell suspensions with FITC-labelled goat anti- (mouse Ig) (GAM-FITC). To assess the degree of saturation, the mean fluorescence intensity (MFI) obtained with GAM-FITC alone was compared to the MFI of cells incubated with excess CLB-CD19 in vitro followed by GAM-FITC. Changes in antigen density during mAb administration were determined by comparing the MFI of cells taken during treatment after incubation with anti-CD19 plus GAM-FITC with the MFI of similarly incubated pre-treatment cells, using cryopreserved cells in the same experiment.

Concentration of free CLB-CD19 and HAMA in the serum

Before, weekly during treatment, and 2–4 weeks after treatment, patients' sera were collected in heparinized tubes, separated and stored at −20° C until use. Unbound CLB-CD19 concentrations in serum were determined using a double-determinant enzyme-linked immunosorbent assay (ELISA) [16]. Briefly, serum samples were incubated in microtitre plates coated with rat anti-(mouse IgG2a) and developed with peroxidase-conjugated rat anti-(mouse κ) followed by 3,3′5,5′-tetramethyl benzoic acid. Concentrations of mAb were calculated from the absorbance at 450 nm (Titertek Multiscan, Flow Laboratories) and a reference curve of known concentrations of CLB-CD19.

To detect HAMA, serum samples were incubated in microtitre plates coated with CLB-CD19 (10 μg/ml in PBS) followed by peroxidase-conjugated sheep anti-(human immunoglobulins) absorbed with mouse immunoglobulin (Amersham) and tetramethylbenzoic acid as described above.

Serum anti-IL-2 antibodies and soluble IL-2 receptor

The presence of rIL-2-binding antibodies in the sera obtained before and after treatment was studied by ELISA as previously described [44]. Patients' sera were incubated in flat-bottom microtitre plates (Nunc) coated with rIL-2 (100 μg/well), followed by rabbit anti-(human Ig), conjugated to horseradish peroxidase and *o*-phenylenediamine substrate. Absorbances were read at 492 nm. Before, at 3-week intervals during, and after treatment with rIL-2, serum levels of soluble IL-2 receptor were detected by ELISA (kindly provided by EuroGenetics, Amsterdam, The Netherlands), according to the manufacturer's instructions.

Cytotoxicity assays

One patient's PBL taken before, during and after treatment were tested for their cytotoxic capacity in the same experiment, as previously described [44]. NK activity was defined by the ability of the lymphocytes to lyse K562 target cells, ADCC activity was defined as the ability of lymphocytes to lyse Jiyoye (Burkitt lymphoma) target cells in the presence of R24.3, a rat IgG2b mAb directed against a non-polymorphic epitope on HLA class II, and lymphokine-activated killer (LAK) activity was defined by the ability to lyse Jiyoye target cells without mAb. The murine mAb CLB-CD19 is not able to mediate ADCC in vitro using human effector cells. The cytotoxic capacities of lymphocytes in the three assays were also determined after additional in vitro stimulation with 600 IU rIL-2/ml for 18 h and were defined as NK_a, LAK_a, $ADCC_a$ respectively. The cytotoxic capacity was determined in triplicate at six effector/target (E/T) ratios between 80:1 and 2.5:1.

The cytotoxic capacity of lymphocytes from lymph nodes could be tested for one patient. Lymph node biopsy specimens were brought into suspension mechanically, and lymphocytes were isolated by centrifugation over Ficoll/Hypaque. Viability was 80%–90%.

The cytotoxic capacity of the patients' lymphocytes was expressed in lytic units (30%)/10^6 effector cells, calculated by the method described by Pross [33], where 1 LU is defined as the number of effector cells that produce 30% lysis of 1000 target cells.

Mouse immunoglobulin-induced proliferation and isolation of T cell clones

Patient peripheral blood lymphocytes and monocytes were incubated with or without CLB-CD19 (100 μg/ml) at 10^5 cells/well in round-bottom microtitre plates (Costar) in Iscove's medium supplemented with 5% pooled, inactivated human serum (CLB). Where available, Epstein-Barr-virus(EBV)-transformed autologous B cells (10^4 cells/well, irradiated with 40 Gy) were added to the culture. In control cultures, PBL were incubated in wells coated for 48 h with anti-CD3 (CLB, 1/2000 diluted ascites). After 7 days, cultures were pulsed for 4 h with 14.8 kBq [3H]dT well and harvested for liquid scintillation counting as previously described [46]. Data are presented from triplicate cultures as the stimulation index, indicating mean radioactivities (cpm) from cultures with CLB-CD19/mean radioactivities (cpm) from medium cultures. Standard deviation values were less than 10%.

PBL from patients 1 and 7 were incubated at 10^6/ml in the presence of the respective autologous EBV-transformed B cells (2×10^5/ml, irradiated with 40 Gy) and CLB-CD19 IgG2a-(1 μg/ml). Every 4 days, cultures were restimulated with rIL-2 (600 IU/ml) and 10% conditioned medium before being were cloned at day 7 (at 1 cell/well for patient 1) or at day 21 (at 0.3 cell/well for patient 7) after the start of the cultures. Clones were fed weekly with a mixture of allogeneic PBL (irradiated 40 Gy), autologous EBV-transformed B cells (irradiated 40 Gy), CLB-CD19 (100 ng/ml), rIL-2 (600 IU/ml) and phytohaemagglutinin (0.2 μg/ml). Clones were tested for specificity for mouse immunoglobulin by incubating $(2–4) \times 10^4$ cells/well in the presence of autologous EBV-transformed B cells (10^4/well) and CLB-CD19 (100 ng/ml). After 4 days cultures were pulsed with [^{3}H]dT and harvested as described above.

Results

Patient characteristics

Seven patients with non-Hodgkin's lymphoma of low-grade malignancy were treated in this single-centre study (Table 1). All patients had slow tumour progression prior to treatment. All were in good clinical condition (performance status 0: five patients; 1: two patients).

Treatment duration

Five of the patients completed the course of 11–12 weeks of continuous infusion of rIL-2 with a total of 24 infusions of CLB-CD19. In patient 3 rIL-2

Table 1 Patient characteristics (*C* cyclophosphamide, *V(O)* vincristine, *P* prednisone, *H* doxorubicin, *Vm* Vm26, *B* bleomycin; *abd.* abdomen)

Patient no. age/sex	Previous treatment	Involved tumor sites	Interval since last treatment (months)
1(54/F)	CVP radiotherapy	Lymph nodes (non-bulky)	7
2(61/M)	CVP	Lymph nodes (bulky) bone marrow, lung liver, spleen	8
3(43/F)	Chlorambucil radiotherapy (whole abd.)	Lymph nodes (bulky)	14
4(47/F)	CHVmP, CVP chlorambucil radiotherapy (whole abd.)	Bone marrow leukaemic lymph nodes (non bulky)	25
5(65/M)	CVP, CHOP chlorambucil radiotherapy	Lymph nodes (bulky) intestines bone marrow	24
6(50/F)	CVP, CHVmP V/B pentostatin radiotherapy	Lymph nodes (bulky) bone marrow	9
7(58/M)	Splenectomy chlorambucil+ prednisone radiotherapy (whole abd.)	Lymph nodes (non-bulky) bone marrow	13

treatment was interrupted from week 4 to 5 because of cellulitis at the entry site of the central venous access. The rIL-2 treatment was discontinued after 9 weeks in patient 1 because of persistent and symptomatic infection of the central venous access, but she completed the CLB-CD19 treatment. In patient 7 rIL-2 and CLB-CD19 treatment was discontinued after a total of 4 weeks because of subjective toxicity despite dose reduction. In patients 1, 5 and 7 the dose of rIL-2 was reduced to 1.8×10^6 IU/m^{-2}(24 h)$^{-1}$ because of subjective toxicity after 2–5 weeks. In patient 2 the dose of CLB-CD19 was increased from 50 mg/m^2 to 100 mg/m^2 because of low serum CLB-CD19 trough levels.

Toxicity

Toxicity was due to rIL-2 and consisted mainly of the flu-like syndrome with reduction in performance status. As indicated in Table 2, these constitutional symptoms were most pronounced during the first weeks of treatment and lessened thereafter but prompted dose reduction in three of the seven patients. In addition, most patients had a runny nose and two patients had clinical and radiological signs of a (pan) sinusitis. All patients had a dry mouth with alteration in taste. Four patients had palmar erythaema with subsequent desquamation and one patient had a flare-up of psoriasis in both hands with onycholysis. Two patients experienced mental lability and one patient had grade I hair loss.

Table 2 Clinical toxicity. Malaise and fatigue: *grade 1* mild, no impairment of daily activity; *grade 2* moderate impairment of daily activity; *grade 3* severe, < 50% bedridden during waking hours; *grade 4* intolerable, > 50% bedridden during waking hours. Myalgia/arthralgia: *grade 1* mild, no use of analgesics; *grade 2* moderate, occasional use of analgesics; *grade 3* severe, constant use of analgesics; *grade 4* intolerable, bedridden and constant use of analgesics

	Grade			
Symptom	0	I	II	III
Performance status				
Before ($n = 7$)	5	2	–	–
Weeks 1–4 ($n = 7$)	–	3	3	1
Weeks 5–12 ($n = 6$)	–	1	5	–
Toxicity				
Fever				
Weeks 1–4 ($n = 7$)	1	2	3	1
Weeks 5–12 ($n = 6$)	5	1	–	–
Chills				
Weeks 1–4 ($n = 7$)	3	3	1	–
Weeks 5–12 ($n = 6$)	6	–	–	–
Fatigue/malaise				
Weeks 1–4 ($n = 7$)	–	2	4	1
Weeks 5–12 ($n = 6$)	1	3	1	1[a]
Myalgia				
Weeks 1–4 ($n = 7$)	1	5	1	–
Weeks 5–12 ($n = 6$)	5	1	–	–
Arthralgia				
Weeks 1–4 ($n = 7$)	3	4	–	–
Weeks 5–12 ($n = 6$)	4	2	–	–
Nausea				
Weeks 1–4 ($n = 7$)	2	5	–	–
Weeks 5–12 ($n = 6$)	5	1	–	–
Cheilitis				
Weeks 1–4 ($n = 7$)	4	3	–	–
Weeks 5–12 ($n = 6$)	4	2	–	–

[a] Related to persistent infection of the central venous access

No alterations in renal function or blood pressure were observed. Capillary leak syndrome did not occur. Transient elevations of the transaminases (grades II and III) were observed in two patients. No clinical features of thyroid dysfunction were observed, although three patients had transient and minimally elevated serum thyroxin levels (192, 169 and 159 nmol/l, N 70–145 nmol/l), with a transient decrease of the serum thyroid-stimulating hormone levels. Anti-microsomal and anti-thyroglobulin antibodies were not found in any of the patients.

Despite intravenous antibiotic prophylaxis during the insertion of the central venous access (CVA) and oral prophylaxis during the entire treatment period, positive blood cultures (*Staphylococcus epidermidis* in six patients, *Acinetobacter* in one) from the CVA were found in all patients. Three patients with a CVA infection had fever (above 38° C) and/or chills but negative peripheral blood cultures. In only one patient (patient 1) was removal of the CVA necessary because

of persistent symptomatic infection, which was associated with a thrombus at the tip of the catheter. Cellulitis of the thoracic wall at the entry site of the CVA with *Corynebacterium* bacteremia developed in patient 3 and was treated with temporary discontinuation of rIL-2 and appropriate antibiotics.

Haematological and immunological effects

All patients had a transient rise in the total leucocyte count with a peak at week 3 caused by a transient eosinophilia (see Fig. 1). Five patients developed anaemia requiring red blood cell transfusion. In three patients a transient grade I (two patients) or grade II (one patient) thrombocytopenia was observed.

During treatment, there were no consistent changes in the total lymphocyte and monocyte counts. Immunophenotyping was performed in six patients (the PBL of patient 5 were not viable after thawing). In patient 4 more than 90% of the lymphocytes were B cells, precluding reliable phenotyping of the other lymphocyte populations. In the remaining five patients substantial changes were observed within the lymphocyte subpopulations (Fig. 2). After 1 week of treatment the percentage and the absolute numbers of cells expressing CD3, CD4, CD25 (low-affinity IL-2 receptor) and HLA-DR changed substantially, showing considerable interpatient variation. During prolonged treatment there was a gradual and sustained increase in the percentage and absolute number of $CD8^+$ (especially $CD8^{dim}$) and $CD56^+$ cells, with a preferential increase of the $CD56^{bright}$ population, without changes in the absolute number of $CD3^+$ cells. The percentage of cells expressing p75 (intermediate-affinity IL-2 receptor) was closely correlated with the percentage of $CD56^+$ cells ($r = 0.964$).

To investigate the expression of the activation antigens p75, CD25, CD27, and HLA-DR on NK and T cells separately, double staining was performed on PBL taken before and after 1 week of treatment. The majority of the $CD56^+$ cells expressed p75 before treatment and the intensity of staining increased after 1 week of therapy. The NK cells had virtually no CD25. Approximately 15% of the $CD56^+$ cells expressed CD27. The changes within the $CD3^+$ subpopulation were more dramatic. The percentage of $CD3^+$ cells expressing CD25 increased to a variable extent after 1 week of treatment (Fig. 3), in three cases to at least 50%. The expression of HLA-DR on the T cells paralleled the CD25 expression. There was no change in the percentage or the level of expression of CD27.

The PBL of patient 2, and to a much lesser extent of patient 7, contained a substantial proportion of $CD3^+$ $CD56^+$ lymphocytes. These cells expressed CD25 as well as p75 (Fig. 4), suggesting the presence of the high-affinity IL-2 receptor on these lymphocytes. The other patients had no p75 on their $CD3^+$ cells. These

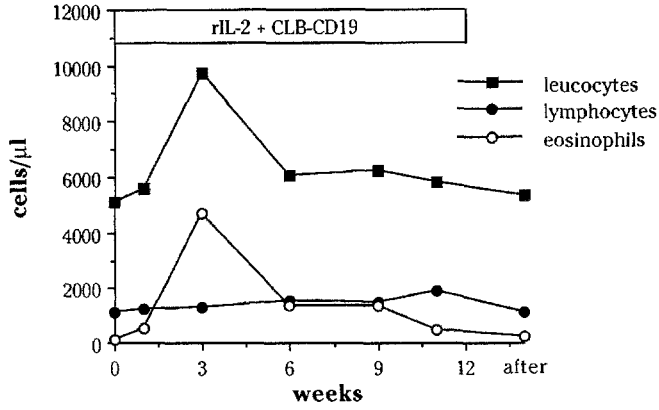

Fig. 1 Mean values of the leucocyte, lymphocyte and eosinophil count of patients 2, 3, 5 and 6 treated with CLB-CD19 and recombinant interleukin-2 (rIL-2). Patients 1 and 7 are excluded because they did not complete the total course of rIL-2 treatment and patient 4 is excluded because she was leukaemic

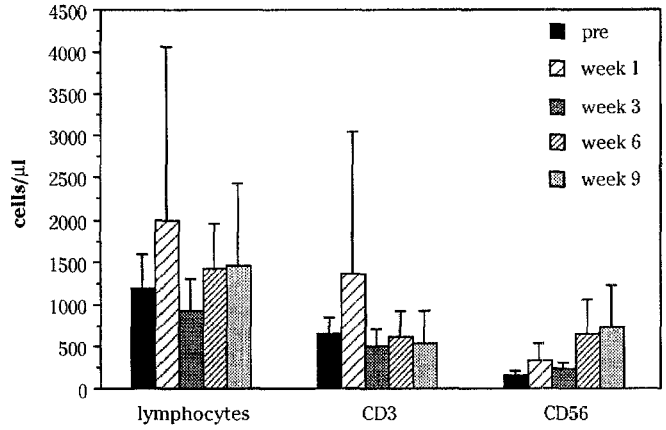

Fig. 2 Total lymphocyte count and the numbers of ($CD3^+$) T lymphocytes and ($CD56^+$) natural killer cells during treatment with CLB-CD19 and rIL-2. The results are expressed as mean values of the number of cells (μl^{-1}) ± standard deviation. *Bars* the data from patients 1, 2, 3, 6 and 7 sampled before and after 1 week of treatment and from patients 1, 2, 3 and 6 sampled at week 3, 6, and 9

data are evidence for T cell activation during the first weeks of treatment in three of the five patients tested.

CD20 was used as an independent B cell marker to follow the number of B cells in the blood. Patients 2 and 4 had an increased percentage of circulating B cells, 44% and more than 90%, respectively, expressing a single light chain. In both patients the number of circulating $CD20^+$ B cells decreased significantly during treatment (Fig. 5). The number of $CD20^+$ cells in the other four patients did not change.

Binding of mAb to cells in blood and extravascular sites

Figure 6 shows the binding of CLB-CD19 to the B cells in the blood of the leukaemic patient 4. After 1 week of treatment, all CD19-binding sites were saturated with CLB-CD19 (Fig. 6A, B). Partial modulation of

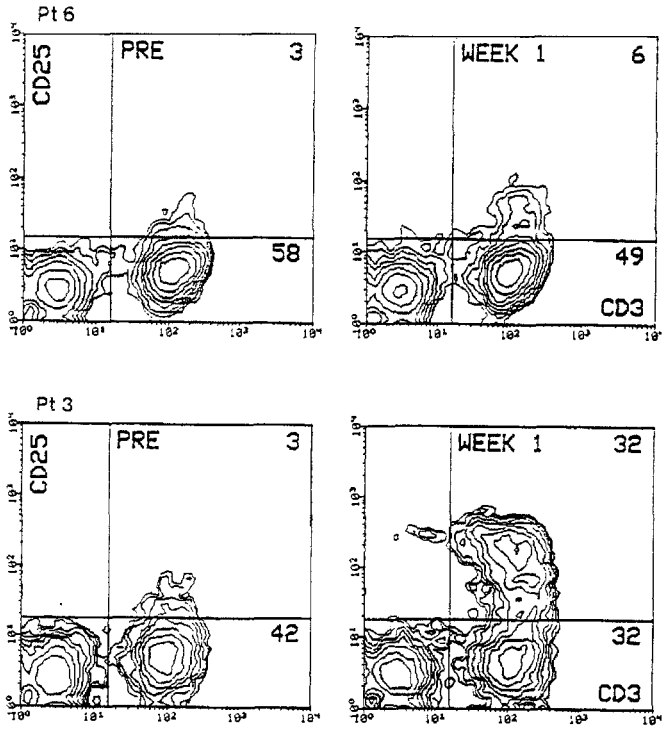

Fig. 3 Double staining of the peripheral blood lymphocytes (PBL) of patients 6 (above) and 3 (below) before (*left*) and after 1 week (*right*) of treatment. The cells were stained with phycoerythrin-labelled CD3 and fluorescein-isothiocyanate (FITC)-labelled CD25

the CD19 antigen was indicated by the modest reduction of the antigen density (Fig. 6C). The complete coating of the peripheral B cells as well as the partial modulation of the CD19 antigen persisted throughout treatment. On the PBL of patient 2 the reduction in CD19 antigen density at week 1 was much more pronounced. All remaining antigen sites were saturated with mAb. Because of the reduction in the number of circulating B cells there were too few B cells left to draw reliable conclusions regarding the pattern of coating and modulation during the later stages of treatment. In the other patients tested (patients 1, 3, 6 and 7), who had a normal number of circulating B cells, the B cells were (nearly) completely coated with CLB-CD19 after 1 week of treatment.

Lymph node cells were obtained by fine-needle aspiration in five patients during treatment. The presence of mAb bound in vivo, the degree of saturation and possible changes in antigen density were tested by flow cytometry. To reduce interassay variation the cells taken during treatment were tested simultaneously with pretreatment cells. There was evidence of extravascular penetration of CLB-CD19 in all patients. After 4–7 weeks of treatment the CD19 antigen density on the lymph node cells diminished to a variable degree. In all but one (patient 2) of the patients, the CD19 binding sites were saturated with CLB-CD19, indicating adequate tissue penetration and dosing with the

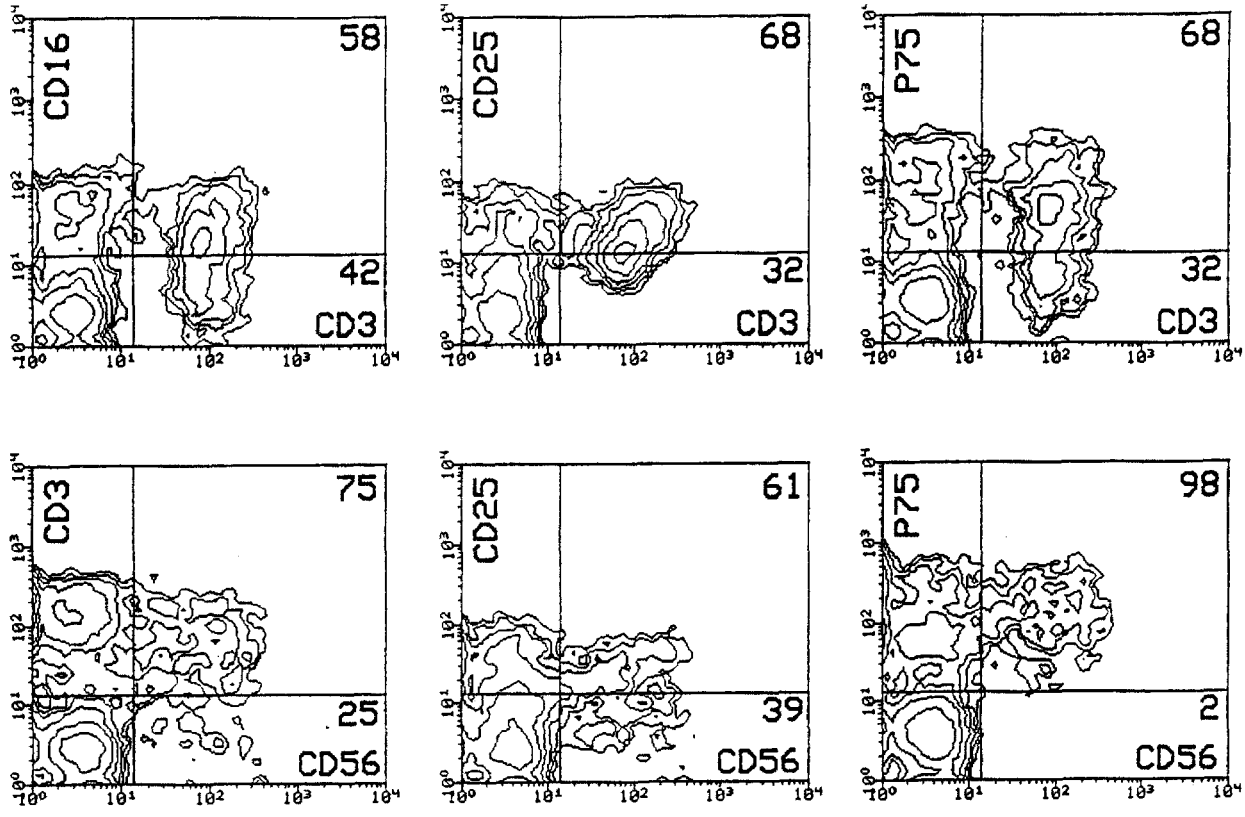

Fig. 4 Double staining of the PBL of patient 2 after 1 week of treatment. A high percentage of $CD3^+$ $CD16^+$ and $CD3^+CD56^+$ cells is present with a high percentage of CD25 and p75 on CD3+ (*above*) as well as CD56+ (*below*) cells

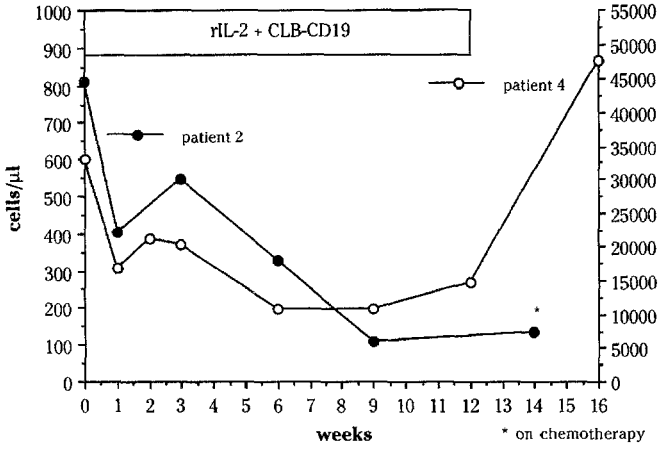

Fig. 5 Changes in the numbers of B cells during treatment in two patients with an increased number of circulating monoclonal B cells. The number of cells (μl^{-1}) of patient 2 are depicted on the *left axis*, and the cells of patient 4 on the *right axis*

antibody. During treatment the percentage of ($CD3^+$) T cells as well as the CD4/CD8 ratio in the lymph nodes were unchanged. Little if any (up to 3%) infiltration of ($CD56^+$) NK cells could be detected in any of the samples.

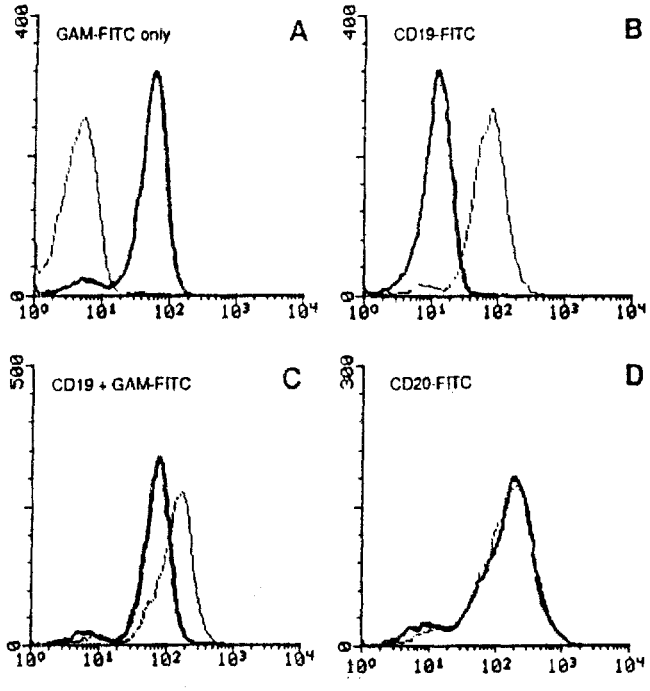

Fig. 6A–D Immunofluorescence test with peripheral blood lymphocytes of patient 4, containing more than 90% $CD19^+$ B cells, obtained before ( ) and at 1 week ( ). Cells were incubated in vitro with FITC-labelled goat anti-(mouse Ig) (*GAM-FITC*) to determine the presence of murine immunoglobulin on the cell surface (**A**), anti-CD19-FITC to determine the uncoated CD19 antigen (**B**), anti-CD19 followed by GAM-FITC to determine the total (coated and uncoated) amount of CD19 (**C**) and anti-CD20-FITC (**D**)

Table 3 Serum CLB-CD19 levels

Patient	Dose (mg/m^2)	CLB-CD19 levels (µg/ml)		
		Day 10		Last day of treatment
		Trough	Peak	Trough
1	50	8.2	23.4	6.2
2	50	0.7	2.7	
	100	1.3[a]	9.7[a]	6
3	50	12.1	37	2.9
4	50	6.1	29.2	3
5	50	11	27.6	11.3
6	50	4.3	12.4	3.8
7	50	3.3	8.8	3.4

[a] Ten days after CLB-CD19 dose escalation from 50 mg/m^2 to 100 mg/m^2

Serum levels of CLB-CD19

Sera were sampled at weekly intervals just before infusion of CLB-CD19 to determine the serum trough concentration of CLB-CD19. At the end of the fourth infusion (day 10), the peak level was determined (Table 3). The serum trough levels remained nearly constant throughout the entire treatment period without accumulation. Patient 2 had low trough and peak levels at day 10 (0.7 µg/ml and 2.68 µg/ml) and only a partial coating of lymph node B cells. The dose of CLB-CD19 was doubled to 100 mg/m^2 resulting in satisfactory trough and peak levels.

Cytotoxic capacity

The cytotoxic capacity of the PBL could be determined in patients 1, 2, 3 and 6. The degree of the cytotoxic capacity varied among the patients. During the course

Table 4 Cytotoxic capacity of peripheral blood lymphocytes of four patients and of lymph node cells of one patient before and during treatment. Cells were cultured overnight in medium with 600 IU/ml rIL-2. One lytic unit (LU) is defined as the number of effector cells that produce 30% lysis of target cells. As targets K562 (NK_a) and Jiyoye (LAK_a) were used. The killing of Jiyoye cells in the presence of the rat mAb R42.3 was defined as $ADCC_a$. –, not tested

Effector	Cytotoxic capacity (LU 10^6 effector cells)		
	K562	Jiyoye	Jiyoye+R24.3
Peripheral blood lymphocytes			
1 Pre	–	7	45
Max	1840	387	543
2 Pre	–	0	0
Max	1205	94	399
3 Pre	79	0	1
Max	350	0	23
6 Pre	9	0	6
Max	193	0	69
Lymph node lymphocytes			
2 Pre	0	–	0
Week 6	99	–	7

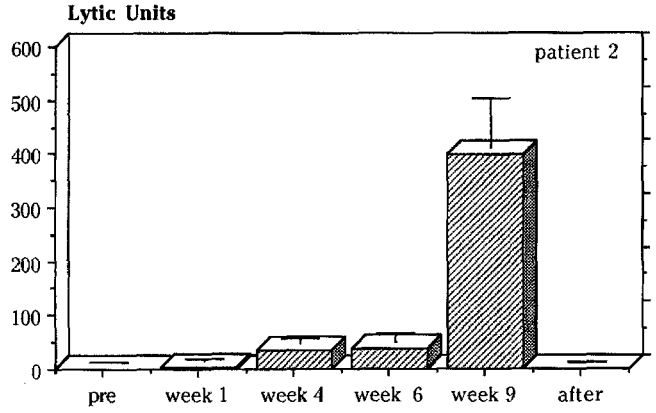

Fig. 7 The antibody-dependent cellular cytotoxicity capacity of the peripheral blood lymphocytes of patient 2 before and during treatment. The PBL were cultured overnight in medium with 600 IU/ml rIL-2 and the capacity of the PBL to lyse Jiyoye cells in presence of the rat IgG2b mAb R24.3 was determined in a standard ^{51}Cr-release assay. Cytotoxicity is expressed as lytic units (30%)/10^6 effector cells (*LU*)

of the treatment, NK activity increased. Additional stimulation of the PBL with rIL-2 in vitro revealed a pronounced increase in NK activity (see Table 4). Only after at least 6 weeks of treatment was an increase in the ADCC capacity (measured as the cytotoxic activity against Jiyoye cells in the presence of the rat mAb R 24.3) observed, which was further enhanced after in vitro stimulation of the PBL with rIL-2. A typical time course of the $ADCC_a$ capacity is shown in Fig. 7.

In patient 2 lymphocytes were isolated from lymph node biopsy specimens before and at 6 weeks of treatment. The cytotoxic capacity of thawed cryopreserved lymphocytes isolated before treatment and freshly isolated lymphocytes at week 6 were tested in the same experiment. As indicated in Table 4, week 6 lymph node cells showed significant NK and modest, but clearly detectable, ADCC activity after in vitro stimulation with rIL-2.

Proliferative response and generation of T cell clones against mouse immunoglobulin

In patients treated with murine mAb, a T cell response against the mouse immunoglobulin, processed and presented by the tumor cells, has been reported [19, 20]. In order to test for a proliferative T cell response to mouse Ig the PBL of patients 1, 2, 3, 4, 6 and 7 were cultured in the presence of additional CLB-CD19. Autologous B cells and monocytes present in the bulk population functioned as antigen-presenting cells. In addition, autologous EBV-transformed B cells were used as antigen-presenting cells in three cases. No specific proliferative response to CLB-CD19 was seen despite significant reactivity to anti-CD3 antibody. Using PBL from patients 1 and 7, secondary in vitro stimulation with CLB-CD19 was used to generate T cell clones that were tested for mouse Ig specificity. There was no indication that even minimal numbers of such T cell clones were generated (data not shown).

Serum immunoglobulin and complement levels

During and up to 4 weeks after treatment no significant changes occurred in the serum levels of IgA, IgG, IgM, C_{1q}, C_3, C_4, or CH50. No HAMA or antibodies against IL-2 were detected.

Serum soluble IL-2 receptor levels

Serum soluble IL-2 receptor levels varied greatly before treatment but were not correlated with the clinical tumour burden. In most patients, the serum level reached a peak within the first 2 weeks of treatment and gradually decreased thereafter. There was no relation with the number of $CD25^+$ cells ($r = 0.256$) or any other immunological parameter.

Antitumour effect

After 1 week of treatment patients 1 and 7, who had non-bulky disease, experienced more than 50% reduction in the easily assessable tumour sites. At week 4 of treatment, the first formal evaluation point, a partial remission at all tumour sites was still present in patient 1, while progression had occurred in patient 7. Patients 2, 3 and 6 had up to 50% reduction in the tumour size during the first weeks of treatment, while patient 5 had a mixed response. In patients 2 and 4 a significant decrease in the number of circulating monoclonal B cells occurred during treatment (Fig. 5). The more than 50% reduction in circulating B cells in patient 4 persisted throughout treatment, but there were no changes in the minimally enlarged lymph nodes.

At the end of the treatment the partial remission in patient 1 was still present, one patient had no significant change in tumour size compared to the week-evaluation, while three others had evidence of progression. Two months after discontinuation of treatment all patients had tumour progression.

The clinical course of patient 2 was of special interest. According to the κ/λ ratio two monoclonal populations were present at different sites in this patient. During treatment the lymph node in the left groin (κ/λ ratio 1/4) and the number of circulating B cells (κ/λ ratio 1/5) substantially decreased, while the lymph nodes with an inverted ratio (κ/λ ratio 9/1) progressed in size.

Discussion

This study describes the immunological effects, toxicity and therapeutic potential of combined treatment

with the anti-(B cell) monoclonal antibody CLB-CD19 and prolonged continuous infusion of rIL-2 for a period of 12 weeks in seven patients with low-grade non-Hodgkin's lymphoma. The immunomodulating objectives of the combination therapy were fulfilled. The treatment schedule of CLB-CD19 resulted in a constant excess of mAb in the serum of all patients, and serial examination of involved lymph nodes showed the presence of CLB-CD19 on B cells as well as modulation of the CD19 antigen on the lymphocytes. These data are indicative of homing into and continued presence of the mAb in the tumour sites. This study confirmed that prolonged rIL-2 treatment results in a time-dependent and sustained increase in the number of circulating NK cells [8, 9, 38, 44]. Our functional studies indicated that continued treatment with rIL-2 results in an enhanced cytotoxic capacity, including ADCC, of lymphocytes isolated not only from peripheral blood but also from involved lymph nodes.

The therapeutic effect included more than 50% reduction in tumour size in two patients and more than 50% reduction in circulating B cells in another patient, who was primarily leukaemic. The two patients had non-bulky disease and the tumour reduction occurred in the first week of treatment, but was of very short duration (less than 2 weeks) in one of them. This result is not very different from what one would expect from treatment with unconjugated mAb alone [15, 16] or rIL-2 with or without LAK cells [1, 5, 12, 23, 39, 47] but seems to compare unfavourably with results of treatment with anti-idiotype antibodies [21] or immunotoxins [14, 15]. Unexpectedly, the antitumour effect in the present study occurred within the first weeks of treatment although NK cell activation occurred only after prolonged treatment.

The mechanisms and the effector cells involved in tumour reduction during mAb treatment have not been fully elucidated [15]. Despite extensive studies we could define no single clinical or laboratory parameter that correlated with the achievement of even a short-lived antitumour effect.

Binding of the antibody to the CD19 antigen induces internalization of the complex both in vitro [32] and in vivo [16]. The consequences of this antigenic modulation are not clear. We found tumour cell reduction in involved lymph nodes and in peripheral blood despite nearly complete modulation of the CD19 antigen in this and our previous studies [16], although modulation has been reported to interfere with the antitumour effect [34]. Internalization may also have beneficial effects. Since B cells are able to present internalized proteins as antigen in the context of MHC molecules, internalization of the mAb may evoke a specific T cell response. In fact specific T cells, primed to murine immunoglobulin, have been demonstrated in patients treated with murine mAb [19, 20]. Although we demonstrated signs of T cell activation in the majority of the patients during the first weeks of treatment, we found no evidence for the presence of T cells specifically reacting against mouse Ig and we noted no correlation between T cell activation and clinical response.

The toxicity of the combination of rIL-2 and CLB-CD19 is moderate and easily manageable. The side-effects consisting of constitutional symptoms and the flu-like syndrome without significant organ dysfunction are related to the rIL-2 treatment and no additional toxicity due CLB-CD19 was noted. Nevertheless, the severity of subjective toxicity prompted reduction of the rIL-2 dose in three of the patients. In most patients the toxicity was transient reaching a peak during the first month of treatment. The rIL-2 treatment-associated eosinophilia and the serum soluble IL-2 receptor level followed a similar transient pattern.

The high infection rate of the central venous access is a problem also noted by others [5, 22]. The infection may be associated with rIL-2 induced altered neutrophilic function [18] or abrogated humoral response [13], or a deleterious effect of CLB-CD19 mAb treatment on B cell-function [29]. In accordance with our previous results, we found no decrease in the serum immunoglobulin levels during treatment [16]. The present study confirms that, in contrast to patients with solid tumors, patients with B cell malignancies do not easily develop HAMA during or after treatment with murine mAb [11].

This study confirms the feasibility of immunotherapy using mAb in combination with rIL-2 [2, 48]. The desired characteristics of the mAb need to be defined and the effector mechanisms involved in man need to be delineated if the therapeutic efficacy of mAb treatment in human cancer is to be improved.

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