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Depletion of B Cells In Vivo by a Chimeric Mouse Human Monoclonal Antibody to CD20

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Murine monoclonal antibody 2B8 specifically recognizes the CD20 phosphoprotein expressed on the surface of normal B lymphocytes and B-cell lymphomas. The light- and heavy-chain variable regions of 2B8 were cloned, after amplification by the polymerase chain reaction, into a cDNA expression vector that contained human IgG1 heavy chain and human κ-light chain constant regions. High-level expression of chimeric-2B8 antibody (C2B8) was obtained in Chinese hamster ovary cells. Purified C2B8 exhibited antigen binding affinity and human-tissue reactivity similar to the native murine antibody. In vitro studies showed the ability of C2B8 to bind human C1q, mediate complement-dependent cell lysis of human B-lymphoid cell lines, and lyse human target cells through antibody-dependent cellular cytotoxicity. Infusion of macaque cynomolgus monkeys with doses ranging from 1.6 mg/kg to 6.4 mg/kg resulted in greater than 98% depletion of peripheral blood (PB) B cells and 40% to 70% depletion of lymph node B cells. Recovery of PB B cells usually started at 2 weeks after treatment and required 60 to greater than 90 days to reach normal levels. As much as 95% depletion of B cells in peripheral lymph nodes and bone marrow was observed following weekly injections of 16.8 mg/kg antibody. No toxicity was observed in any of the animals. These results offer the possibility of using an "immunologically active" chimeric anti-CD20 antibody as an alternative approach in the treatment of B-cell lymphoma.

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B LYMPHOCYTES ARISE from pluripotent stem cells and differentiate to fully mature antibody-secreting plasma cells.[1] The human B-lymphocyte–restricted differentiation antigen Bp35 (CD20), is a cell surface nonglycosylated hydrophobic phosphoprotein of 35 kD, and is expressed during early pre-B–cell development just before the expression of cytoplasmic μ-heavy chains.[2] Uncommitted hematopoietic-precursor stem cells do not express CD20. CD20 is expressed consistently until the plasma cell stage and regulates a step in the activation process that is required for cell-cycle initiation and differentiation.[3-7]

The CD20 molecule expressed on neoplastic B cells provides a promising target for therapy of B-cell lymphomas and leukemia.[8] It is especially suitable as a target for antibody-mediated therapy because of accessibility and sensitivity of hematopoietic tumors to lysis by way of immune-effector mechanisms. The CD20 antigen is not normally shed from the cell surface, and there are no detectable serum levels of soluble CD20, which might block targeting of antibody to lymphomas.[9] CD20 does not internalize after binding to antibody.[10] Earlier studies have shown that partial clinical responses were achieved in lymphoma patients using high doses of murine anti-CD20 monoclonal (MoAb).[8]

We have identified a high-affinity murine anti-CD20 MoAb and constructed a mouse/human chimeric antibody that is expressed at high levels in mammalian cells. We have shown that the chimeric MoAb has specificity and affinity equivalent to the native-murine parent. However, unlike the murine antibody, the chimeric antibody binds human C1q, and mediates complement-dependent cell lysis (CDCC) in the presence of human complement, and antibody-dependent cellular cytotoxicity (ADCC) with human effector cells. Chimeric anti-CD20 antibody, when administered to cynomolgus monkeys, caused a marked B-cell depletion in peripheral blood (PB), bone marrow (BM), and lymphatic tissue.

MATERIALS AND METHODS

Production of murine anti-CD20 MoAb. Mice from the BALB/c strain were immunized weekly with the human lymphoblastoid cell line SB.[11] Spleens were taken from mice with high serum titers of anti-CD20 antibodies, and the splenocytes fused with the mouse myeloma SP2/0.[12] All assays for CD20 reactivity were accomplished by radio immunoassay. Briefly, purified anti-CD20 B1 (Coulter Corp, Hialeah, FL) was radiolabeled with ^{125}I by the iodobead method.[13] Hybridomas were screened by coincubation with ^{125}I-B1 (10 ng) in 1% bovine serum albumin (BSA), phosphate-buffered saline (PBS), and 100,000 SB cells. After incubation for 1 hour at room temperature, the cells were harvested by transferring to 96-well filter plates (V&P Scientific, San Diego, CA) and washed thoroughly. Duplicate wells containing unlabeled B1 and wells containing no inhibiting antibody were used as positive and negative controls, respectively. Wells containing greater than 50% inhibitions were expanded and cloned. The antibody showing the highest reactivity was derived from the cloned cell line designated 2B8.

Construction of the chimeric anti-CD20 Ig DNA expression vector. RNA was isolated from the 2B8 mouse hybridoma cell[14] from which single-stranded cDNA was prepared. Mouse Ig light-chain variable-region DNA was amplified from the cDNA by the polymerase chain reaction (PCR) using a set of DNA primers with homology to mouse light-chain signal sequences at the 5′ end and mouse light-chain J region at the 3′ end (see Fig 1 for primers). This DNA fragment was inserted directly into an expression plasmid in front of the human κ-light–chain constant domain and the entire construct sequenced. Two clones from two separate PCR reactions were sequenced to assure that no PCR errors occurred. The κ gene in the expression plasmid contained a synthetic signal sequence, unique restriction sites for the insertion of a light-chain variable domain, which maintain the reading frame and conserve the amino acids (Fig 2), and the human κ constant region (amino acid 108-214 Kabat numbering[15]). A schematic drawing of the expression plasmid can be found in Fig 3. The mouse light-chain variable region from 2B8 is in the mouse κ VI family.[15] Similarly, the mouse

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PCR Primers

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VKappa SENSE
                        -22                                        -13
5'   ATCACAGATCTCTCACCATG GAT TTT CAG GTG CAG ATT ATC AGC TTC 3'
         Bgl II      MET

VKappa ANTISENSE
               109  108  107 106  105  104 103
   5' TGCAGCATCCGT ACG TTT GAT TTC CAG CTT   3'
              Bsi WI

VHeavy SENSE
               -5   -4          -1  +1
   5'  GCGGCTCCCACG CGT GTC CTG TCC CAG 3'
               Mlu I
VHeavy ANTISENSE
               115  114  113        112    111      110
   5'  GG(G/C)TGTTGTGCT AGC TG(A/C) (A/G)GA GAC (G/A)GT GA 3'
               Nhe I
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Fig 1. DNA primers utilized in the PCR reactions to amplify the light-chain (VKappa) and heavy-chain (VHeavy) variable-region DNA.

heavy-chain variable region was isolated from the 2B8 hybridoma and inserted in front of human IgG1 constant domains and sequenced (see Fig 1 for primers). The γ-1 gene construct in the expression plasmid contained a synthetic-leader sequence, unique restriction sites for insertion of a heavy-chain variable domain (Fig 2), and the human γ-1 constant region (amino acids 114 through 478 Kabat numbering[15]). The mouse heavy-chain variable region from 2B8 is in the mouse VH 2B family.[15] The synthetic signal sequences were derived from consensus signal sequences of Ig genes.[15]

Creation of C2B8 producing transfectomas. Chinese hamster ovary (CHO) cells (DG44)[16] were grown in SSFM II minus hypoxanthine and thymidine (GIBCO). Approximately 25 μg of plasmid DNA was inserted into 4×10^6 CHO cells, by electroporation using a BTX 600 electroporation device (BTX, San Diego, CA). The plasmid DNA had been previously restricted with the enzyme *Not* 1, which separates the genes expressed in mammalian cells from the portion used to grow the plasmid in bacteria (see Fig 3). Conditions for electroporation were 210 V, 400 microfaradays, 13 ω. Each electroporation was plated into six 96-well dishes (about 7,000 cells/well). Dishes were fed with media containing G418 (Geneticin; GIBCO) at 400 μg/mL active compound 2 days after electroporation, and thereafter every second or third day until colonies arose. Supernatant from colonies was assayed for the presence of chimeric Ig by an enzyme-linked immunosorbent assay specific for human antibody. Colonies producing the highest amount of Ig were expanded and plated out into 96-well dishes containing media plus methotrexate (5 nmol/L) and fed every 2 or 3 days. Supernatant was assayed as above and colonies producing the highest amount of Ig were expanded. Chimeric antibody (C2B8) was purified from supernatant using protein-A affinity chromatography.

Immunoreactivity of C2B8. Purified antibodies produced by CHO cells were radiolabeled with ^{125}I or with fluorescein isothiocyanate (FITC). They were tested by direct binding to various CD20$^+$ target cells and detected either by gamma counter or by flow cytometry using a FACScan analyzer (Becton Dickinson). The B-lymphoblastoid SB cell line was used as a positive control to examine direct binding of C2B8. The CD20$^-$ T-cell leukemia line, HSB, was used as a negative control.[17] Competitive inhibition of binding of C2B8 to these cells was studied and compared with that observed for murine anti-CD20 antibodies 2B8 and B1.[4]

In vitro effector function of C2B8. A whole cell cytometry method for detection of human C1q binding was developed using the CD20$^+$ cell line SB. In this assay, chimeric-C2B8 and murine-2B8 antibodies are incubated for 30 minutes at room temperature followed by washing with PBS (pH 7.4), 1% BSA. After washing, the cells are incubated for an additional 30 minutes with 10 μg/mL C1q (Quidel, San Diego, CA), previously labeled with FITC. The cells were washed again and fixed with 0.15 mol/L NaCl, 1% paraformaldehyde. Negative-binding histograms were determined by SB cells incubated with irrelevant human IgG1 followed by addition of C1q-FITC and by incubation of C1q-FITC with SB cells in the absence of antibody.

Complement-dependent cytotoxicity and ADCC effector assays were accomplished by using ^{51}Cr-labeled SB cells that were exposed to antibodies and human serum, or activated PB effector cells (effector:target = 100:1), for a period of 4 hours at 37°C.[18] Control studies were performed simultaneously with the CD20$^-$ cell line HSB. The percentage of specific lysis was calculated according to the following formula:

$$\%\ \text{Specific Lysis} = \frac{\text{Experimental} - \text{Spontaneous Release}}{\text{Maximum} - \text{Spontaneous Release}} \times 100$$

Maximum release was determined by lysis in 0.1% Triton X-100. Assays used 10,000 SB (CD20$^+$) cells or 10,000 HSB (CD20$^-$) cells. Results were obtained in triplicate and averaged for both cell lines.

Fluorescent antibody staining of primate lymphoid cell population. After removal of plasma, primate blood cells were washed twice in Hanks' Balanced Salt Solution and resuspended in a plasma-equivalent volume of fetal bovine serum. A 0.2-mL volume of the blood preparation was distributed to each of four 15-mL conical centrifuge tubes. Human-specific fluorescent-labeled MoAbs, which corecognize monkey-lymphocyte surface markers CD2 (AMAC, Westbrook, ME) and CD20 (Becton Dickinson), were

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KAPPA INSERT SITE

                         LEADER
                   -20
AGA TCT  CTC ACC ATG AGG GTC CCC GCT CAG CTC CTG
                 MET ARG VAL  PRO ALA GLN LEU LEU
Bgl  II                       Ppu  MI

        -10                                -1  +1
GGG CTC CTG CTG CTC TGG CTC CCA GGT GCA CGA TGT GAT
GLY LEU LEU LEU LEU TRP LEU PRO GLY ALA ARG  CYS ASP
                                        Dra  III

101                    107
GGT ACC AAG GTG GAA ATC AAA
GLY THR  LYS VAL GLU ILE LYS
Kpn  I

          J  REGION

                        108  109
                        CGT  ACG
                        ARG THR
                        Bsi  WI

              HUMAN  KAPPA  CONSTANT


GAMMA 1 INSERT SITE

                         LEADER
           -19                                -10
GTC  GAC  ATG GGT TGG AGC CTC ATC TTG CTC TTC CTT
          MET GLY TRP SER LEU ILE LEU LEU PHE LEU
Sal  I

                    -5   -4   -3
GTC GCT GTT GCT ACG CGT GTC
VAL ALA VAL ALA THR ARG  VAL
                Mlu  I

                       114  115
                       GCT  AGC
                       ALA SER
                       NHE  I

              HUMAN  GAMMA  1  CONSTANT
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Fig 2. DNA sequence of the Ig vector indicating restriction endonuclease sites where variable-region DNA can be cloned. The PCR-DNA fragments of anti-CD20 were cloned into the *Bgl* II and *Bsi*WI (Kappa) and *Mlu* I and *Nhe* I (Gamma 1) sites, respectively.

added to two of the tubes for identifying T- and B-lymphocyte populations. C2B8 antibody bound to monkey B-cell surface CD20 was measured in the third tube using polyclonal goat-antihuman IgG coupled with phycoerythrin (AMAC). This reagent was preabsorbed on a monkey IgG-Sepharose column to eliminate background staining of monkey surface-IgG^+ B cells. A fourth sample was included for autofluorescence. Cells were incubated with antibodies for 30 minutes, washed and fixed with 0.5 mL of fixation buffer (0.15 mol/L NaCl, 1% paraformaldehyde), and analyzed on a Becton Dickinson FACScan instrument. Fluorescence measurements reflected only gated lymphocyte events.

Depletion of B cells from primate PB. Four cynomolgus monkeys ranging in weight from 4.5 to 7 kg received doses of C2B8 corresponding to 0.01 mg/kg, 0.1 mg/kg, 0.4 mg/kg, and 1.6 mg/kg each day for 4 consecutive days. Therefore, the dose range was 0.04 to 6.4 mg/kg body weight. The antibody was administered by intravenous infusion with blood samples drawn before each infusion. Additional blood samples beginning 24 hours after the last injection were taken at days 1, 2, 4, 8, 15, 29, and subsequently thereafter at biweekly intervals until completion of the study at day 90. Approximately 5 mL of whole blood from each animal was centrifuged at 2,000 rpm for 5 minutes. The pellet containing PB leukocytes and red blood cells was resuspended in fetal calf serum for staining with fluorescent antibodies.

Depletion of B cells from primate lymph nodes. Four cynomolgus monkeys ranging in weight from 4 to 6 kg were divided into two groups of two monkeys. One group was injected intravenously with a dose of 1.6 mg/kg (0.4 mg/kg each day for 4 consecutive days). The animals in the other group were injected with a single dose of 6.4 mg/kg. Animals were bled after the last injection at days 1, 2, 4, 8, 15 and 22, and blood was processed the same as before for fluorescent antibody staining. Lymph node biopsies from the inguinal area of the leg were taken at days 15 and 29 with cell preparations stained for quantitation of lymphocyte populations by flow cytometry. A lymph node biopsy was also taken from a normal animal injected with saline. The percent of B lymphocytes in the total lymphocyte fraction, detected by flow cytometry in the normal animal, was used as a reference for comparing the percentage of B lymphocytes in the lymph nodes of the treated animals. Depletion of lymph nodes was quantitated by assuming the fraction of B lymphocytes in normal saline-treated animals to be approximately 40%. Reduced amounts of B cells found in treated lymph nodes were calculated as percent depleted B cells by the following formula:

$$\% \text{ Depletion} = 100 - \frac{\% \text{ B Cells in Treated Lymph Node}}{\% \text{ B Cells in Saline Control}}$$

High-dose in vivo pharmacology study. Four monkeys were infused with 16.8 mg/kg C2B8 at weekly intervals over a period of 4 consecutive weeks. At 22 days beyond the end of the dosing period, two animals were anesthetized for removal of BM and lymph node biopsies. At 36 days beyond the end of the dosing period, the two remaining animals were anesthetized for BM and lymph node biopsies. Both sets of tissues were stained for the presence of B lymphocytes using the CD20 marker Leu-16 by flow cytometry.[19]

Evaluation of animal systemic and tissue toxicity. All animals used in the in vivo studies were routinely examined for toxic side effects during the course of the studies by routine blood chemistry, urinalysis, and physical examination including weight loss and loss of appetite. Two of the four animals in the multiple high-dose study were killed at the end of the study at 22 days after final dosing. Body tissues were thoroughly examined for evidences of toxicity and tissue damage.

RESULTS

Construction and expression of chimeric 2B8 antibody. The light and heavy chain variable regions from the murine 2B8 anti-CD20 antibody were cloned using PCR amplification and inserted directly into a cDNA mammalian chimeric antibody expression vector. The expression vector (TCAE see Fig 3) contains four separate eukaryotic cDNA genes arranged in a tandem head to tail fashion, each containing its own promoter and polyadenylation region. The four genes are the human κ gene (allotype Km3),[15] the human γ-1 constant gene (allotype z, a),[15] a neomycin phosphotransferase gene (NEO),[20] and a mouse dihydrofolate reductase (DHFR) gene.[21] The NEO gene is used for selecting mammalian cell clones that have integrated the gene and are resistant to the antibiotic G418.[19] The DHFR gene is used for gene amplification in the presence of the competitive substrate inhibitor methotrexate.[22]

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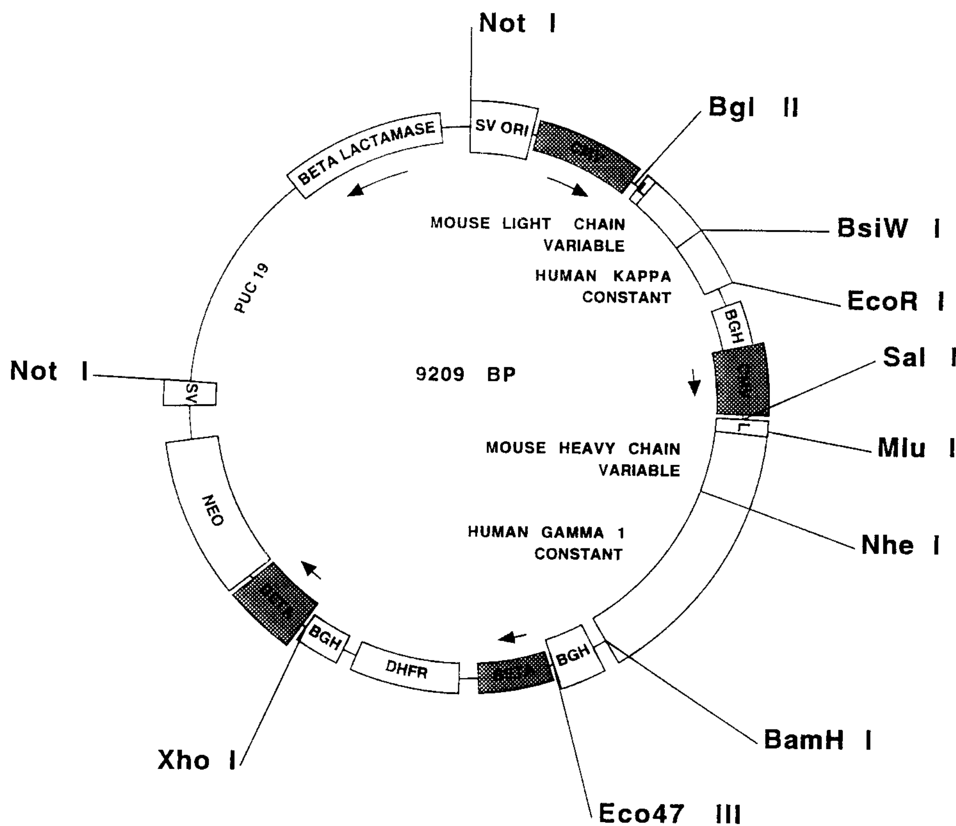

Fig 3. Schematic representation of the DNA expression vector tandem chimeric-antibody expression used to express anti-CD20 in CHO cells. L, leader; CMV, cytomegalovirus promoter; BETA, mouse β-globin major promoter; BGH, bovine growth hormone polyadenylation; SV, SV40 early polyadenylation; SV ORI, SV40 origin.

After electroporation into CHO cells and selection in G418, drug-resistant clones were isolated from cells that secreted 2 μg/10^6 cells/d. Cell lines have been derived in 5 nmol/L methotrexate in a single amplification step that secrete stable Ig levels at 30 μg/10^6 cells/d.

Antibody specificity and immunoreactivity. Chimeric anti-CD20 was purified by protein-A affinity chromatography and estimated to be greater than 95% pure by electrophoresis on polyacrylamide gels. It was first tested in direct and competitive binding assays and compared with murine 2B8, where it was shown to be of comparable affinity and specificity on $CD20^+$ SB cell line. The apparent-affinity constant (Kap) was determined by direct binding of radiolabeled C2B8 and compared with radiolabeled murine 2B8 by Scatchard analysis. The Kap of C2B8 was estimated as 5.2 $\times 10^{-9}$ mol/L (Fig 4), compared with a Kap of approximately 3.5×10^{-9} mol/L for the murine antibody. Direct competition by radioimmunoassay was used to confirm both the specificity and retention of immunoreactivity of the chimeric antibody by comparing its ability to effectively compete with binding of native murine antibody. Data illustrated in Fig 5 shows that virtually equivalent amounts of chimeric and native murine antibody were required to inhibit the binding of the parent murine-2B8 antibody to CD20 sites on SB cells.

In vitro functional characterization of C2B8 antibody. C2B8 was evaluated for human C1q binding by flow cytometry, as shown in Fig 6. In this assay, when C2B8 was incubated with SB cells followed by the addition of fluorescein-labeled C1q, a significant increase in fluorescent intensity was observed. In contrast, under the same conditions, murine-2B8 antibody, as well as an irrelevant human IgG1 (data not shown), failed to bind human C1q.

C2B8 was then tested for its ability to lyse B-lymphoid cell lines in the presence of human serum as a source of complement. Approximately 50% of the SB target cells (Fig 7) were lysed in the presence of a 2.2 μg concentration of C2B8 antibody and a 1:4 dilution of human complement (Pel Freeze, Rogers, AK). In contrast, no significant lysis was observed in experiments using $CD20^+$ SB cells incubated with murine-2B8 antibody, or $CD20^-$ HSB cells incubated with C2B8 antibody. A summary of data from an ADCC assay using C2B8 and activated human effector cells at a 100:1 effector:target ratio is presented in Fig 8. Approximately 50% of the $antigen^+$ SB cells were lysed during the 4-hour incubation period. In contrast, $CD20^-$ HSB cells were not lysed nor were $CD20^+$ cells incubated with murine-2B8 antibody.

In vivo depletion of PB B lymphocytes. Cynomolgus monkeys received doses of C2B8 corresponding to 0.01, 0.1, 0.4, and 1.6 mg/kg daily for a total of 4 consecutive days resulting in total doses ranging from 0.04 to 6.4 mg/kg. The data in Fig 9 shows that doses of 0.4 to 6.4 mg/kg depleted greater than 95% of PB B cells for as long as 8 days postinfusion. The lowest dose (0.04 mg/kg) effectively depleted greater than 50% of the peripheral B cells. It is also apparent

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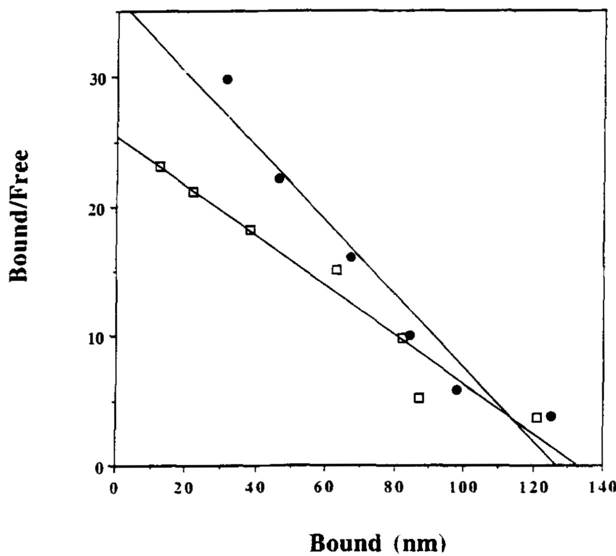
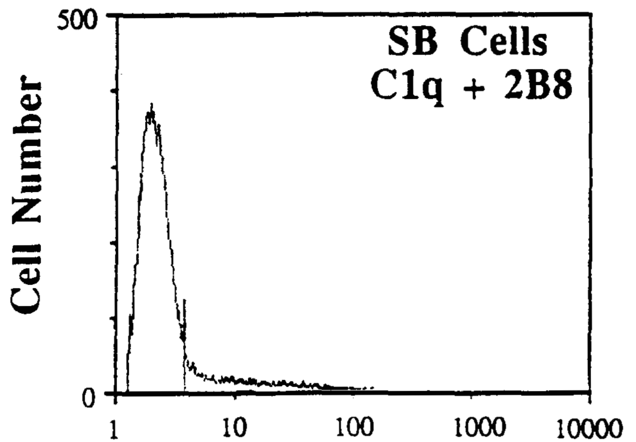


Fig 4. Determination of Kap for chimeric antibodies produced in CHO transfectoma. Antibodies produced in the CHO cell and the original murine cell line were purified, radiolabeled with I^{125}, and evaluated by direct binding to CD20 antigen expressed on 10,000 human SB cells. Apparent binding affinities were determined by Scatchard plots. (●) 2B8; (□) C2B8.

from the CD20 marker, Leu-16, that saturation of the CD20 antigen on the B-lymphocyte population in PB was not achieved with this dose of C2B8 antibody (Table 1). Table 1 compares the percentage of cells coated with C2B8 and the percentage of $CD20^+$ cells. The percentage of C2B8-coated cells at the lowest dose is basically identical to the $CD20^+$ cells at 24 hours. By day 2, the percent of C2B8-coated cells is only half that of the population of $CD20^+$ cells. Total saturation at 24 hours would have blocked binding of Leu-16 to these cells, as both reagents compete with each other for the same site on SB cells. At 0.04 and 0.4 mg/kg dose levels, B lymphocytes coated with C2B8 antibody were detected in the circulation during the initial 4 days after antibody infusion. By day 8, C2B8 antibody-coated cells at all doses were undetectable in PB. The levels of C2B8-coated cells were very low at the higher doses, presumably because of the absence of B cells by 24 hours. After day 8, the B cells began



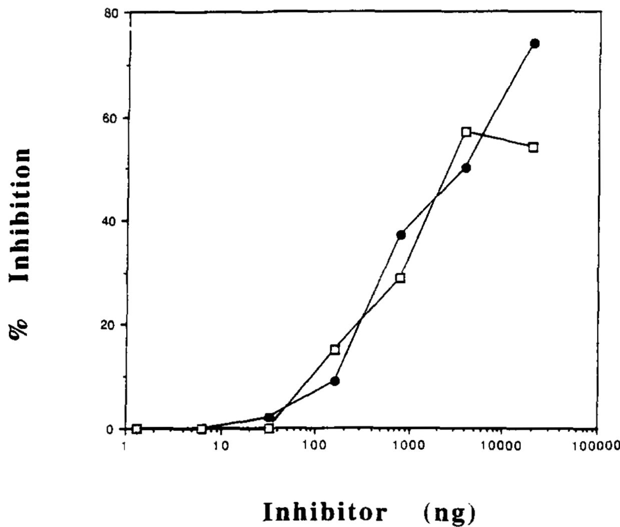


Fig 5. Immunoreactivity of purified antibodies produced by CHO transfectoma. Unlabeled chimeric 2B8 was incubated at various concentrations in the presence of 10,000 B-lymphoid cells and 10 ng of I^{125}-labeled native-murine 2B8. The amounts of chimeric antibody required to directly compete with the radiolabeled murine anti-CD20 antibody were compared with similar amounts of unlabeled murine antibody. Results indicate minimal loss of inhibiting activity due to chimerization. (●) 2B8; (□) C2B8.

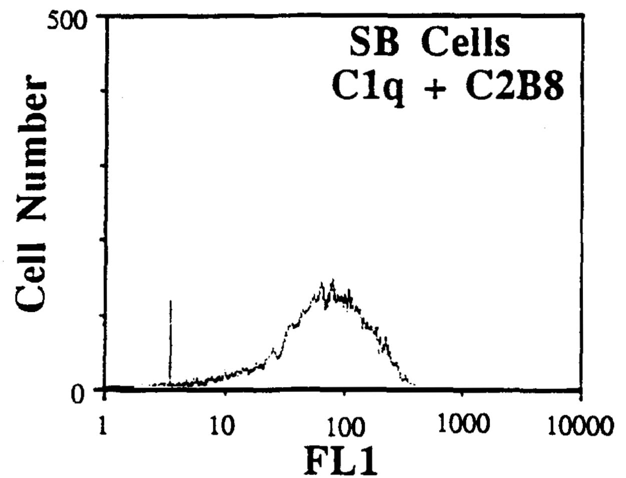


Fig 6. Binding of fluorescent-C1q to Chimeric MoAb C2B8 associated with antigen on the surface of human B-lymphoid cells detected by flow cytometry. Purified-C2B8 and murine-2B8 antibodies were incubated with human SB cells, washed to remove unattached antibody and incubated with fluoresceinated C1q. Cells were washed again, fixed and C1q fluorescence measured by flow cytometry. Controls were C1q-FITC and C1q-FITC plus irrelevant human IgG1,κ.

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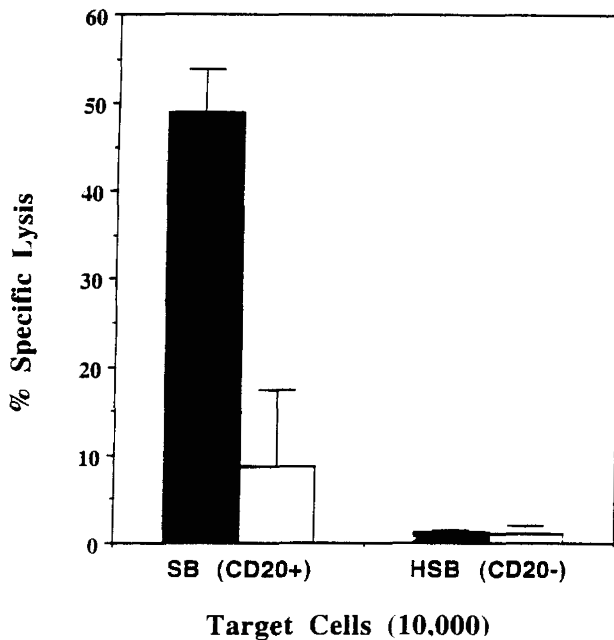

Fig 7. Chimeric C2B8 (■) was compared with murine 2B8 (□) for complement-related lysis in a concentration of 2.2 μg/mL. Ten thousand SB cells ($CD20^+$) were labeled with Cr^{51} followed by incubation in the presence of anti-CD20 antibodies and human complement was used at a 1:4 dilution (Pel Freeze). $CD20^-$ control cell line was 10,000 HSB cells.

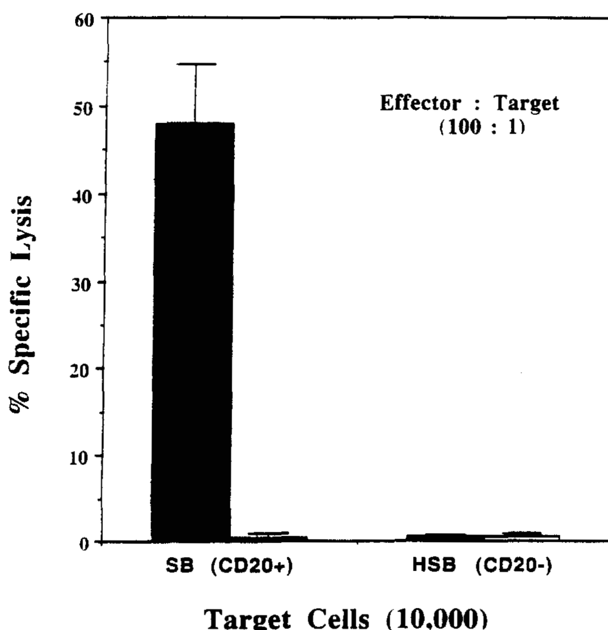

Fig 8. ADCC with in vitro human effector cells comparing chimeric-C2B8 and murine-2B8 lysis of human lymphoid target cells. C2B8 (■) and 2B8 (□) concentration with target cells was 3.9 μg/mL. Human peripheral lymphocytes were used as effector cells in a 100:1 ratio to target cells previously loaded with Cr^{51}. $CD20^-$ control cells were HSB at the same effector to target ratio.

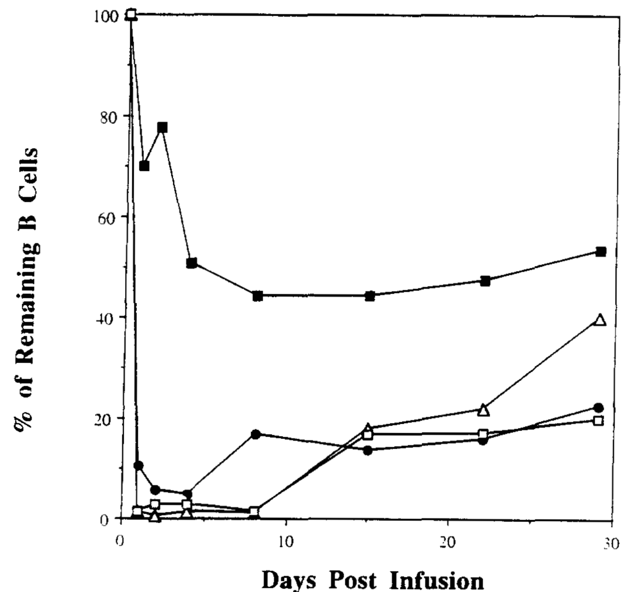

Fig 9. Depletion of PB B lymphocytes in cynomolgus primates infused with four daily injections of C2B8 antibody purified from CHO transfectoma. Fluorescein-labeled MoAb with specificity for human B lymphocytes (Leu-16, anti-CD20; Becton Dickinson) was used to stain and detect the B-lymphocyte population. Dose: (■) 0.04 mg/kg; (●) 0.4 mg/kg; (△) 1.6 mg/kg; (□) 6.4 mg/kg.

a slow recovery. This occurred more rapidly in some monkeys than others and was seemingly dose independent.

In vivo depletion of B lymphocytes in peripheral lymph nodes. The objective of this study was to determine the effects of the two highest C2B8 doses that previously depleted greater than 98% of the B cells in the PB on the lymph node B-cell populations. The results summarized in Table 2 are obtained from lymph node biopsies of four animals infused with 1.6 mg/kg or 6.4 mg/kg of C2B8 and a single control animal injected with saline. Examination of lymphatic tissue removed on days 15 and 29 after administration of 1.6 and 6.4 mg/kg shows effective depletion of B lymphocytes (Table 2). B-cell depletion varied widely in individuals from both dose groups ranging from 34% to 78%, based on calculations using 39.5% B cells present in the inguinal node of a saline-treated animal. In the most highly depleted individuals, a major increase in the percentage of T cells was observed, confirming the depletion of B cells. In each of the antibody doses used (Table 2), complete depletion of the B cells in the lymphatic tissue with C2B8 still was not achieved. Furthermore, day 15 and day 29 B-cell populations were quite similar in each animal indicating most of the depletion occurred before day 15, with very little regeneration within the first 30 days.

Using the data derived from these studies, a high-dose pharmacology/toxicology study of C2B8 in cynomolgus monkeys was conducted. This protocol was designed to evaluate the toxicity (if any) associated with the administration of C2B8, as well as the efficacy of B-cell depletion from lymph nodes and BM. Also, because the majority of lymph node B cells were depleted within 15 days after antibody

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Table 1. Coating of CD20 Antigen Positive B Lymphocytes in PB of Cynomolgus Monkeys Treated With Various Doses of C2B8 Antibody

Monkey	Dose (mg/kg)	Day	Percent of Total Lymphocytes: Leu-16⁺ B Cells*	C2B8⁺ Cells†	CD2⁺ T Cells‡
672	0.04	Pre	16.7	0	74.7
		1	11.7	11	74.7
		2	13.0	6.3	80.6
		4	8.5	2.6	80.6
		8	7.4	0.2	82.7
848	0.4	Pre	12.4	0	80.1
		1	0.1	1.3	85.7
		2	0.1	0.7	87.8
		4	0.1	0.6	90.7
		8	1.6	0.5	88.5
660	1.6	Pre	28.3	0	72.9
		1	0.4	0.4	94.0
		2	0.1	0.2	95.6
		4	0.1	0.4	96.2
		8	0.0	0.3	96.0
427	6.4	Pre	7.1	0	84.7
		1	0.0	0.1	92.1
		2	0.0	0.2	92.0
		4	0.0	0.2	94.1
		8	0.0	0.1	91.3

* Determined as a percentage of the total gated lymphocyte population by flow cytometry using the CD20 marker antibody, Leu-16–FITC.

† Determined as a percentage of the total gated lymphocyte population by detecting the percentage of C2B8-coated cells using monkey IgG absorbed goat antihuman-phycoerythrin (RPE).

‡ Determined as a percentage of the total gated lymphocytes using the CD2 specific antibody T-cell marker (antihuman CD2-RPE, AMAC).

treatment, a weekly dosing regimen was adopted. In this study, four animals were given four weekly doses of approximately 16.8 mg/kg C2B8. At the completion of the dosing schedule, lymph node and BM specimens were removed and analyzed by flow cytometry for the presence of CD20⁺ B cells. Two animals were examined at 22 days after the last dose and the other two examined at 36 days. Figure 10 illustrates the results of a typical flow cytometry experiment with one of the monkeys used in the study. Two normal animals treated with saline were examined for comparible lymph node and BM tissues. Figure 11 histograms compare T-cell populations in normal animals and depleted animals from both lymph node and BM tissue specimens. Table 3 summarizes the results of all the animals in the study. It can be seen that lymph nodes from both animals evaluated at 22 days after cessation of treatment contained from 5.3% to 6.3% B cells as compared with 39.5% in control lymph nodes (Table 3). Similarly, in the BM of these animals, the levels of CD20⁺ cells ranged from 3% to 4.3%, as compared with 16.6% in the normal animal. In animals evaluated at 36 days after cessation of treatment, studies showed that one of two animals had approximately 12% B cells in the lymph node and 4.4% B cells in BM. The other animal had approximately 5% and 0.8% B cells in the lymph node and BM, respectively. T-cell populations generally increased dramatically with the absence of B cells in lymph nodes, ranging from 85% to 90%. T-cell populations in the BM of depleted animals ranged from slightly below normal at 25.6% to slightly over 46%. The normal control animal 908 BM sample had 29.8% T cells.

All animals used in the in vivo studies were routinely examined for symptoms of toxicity during the course of the studies by routine blood chemistry, urinalysis, and physical examination. Two of the four animals in the multiple high-dose study were killed at the end of the study at 22 days after final dosing. Body tissues were thoroughly examined for evidences of toxicity and tissue damage. No weight loss or other visible effects of antibody treatment other than loss of B cells were noted.

DISCUSSION

MoAbs have been used for therapy of hematologic malignancies. Radiolabeled, toxin-conjugated, and unmodified MoAbs have all been tested clinically.[8,23-25] Some of the problems associated with MoAb therapy of neoplasms include specificity of the antibody for tumor cells as opposed to normal tissues, heterogeneity of tumor-specific antigen expression, low distribution of antibody in solid neoplasms, human antimouse antibody responses, which often limit the usefulness of murine antibodies for repeated therapy, and toxicity of radiolabeled or toxin-conjugated antibodies.[23]

We have expressed at high levels a chimeric mouse/human MoAb to CD20, a cell-surface phosphoprotein expressed on cells of the B-cell lineage. We have shown that the chimeric antibody, C2B8, has specificity and affinity equivalent to the mouse MoAb from which it was derived. The chimeric antibody has shown human effector functions using in vitro assays for C1q binding, complement-mediated cell lysis and antibody-dependent cellular-mediated cytotoxicity. Unlike its mouse counterpart (data not shown),

Table 2. T- and B-Lymphocyte Populations in Lymph Node Biopsies Taken From C2B8-Treated Cynomolgus Monkeys, 15 and 29 Days After Treatment

Monkey	Dose (mg/kg)	Day	% CD2⁺ *	% CD20⁺ †	% Depletion‡
425	Saline	ND	ND	ND	0
684	1.6	15	76.9	22.6	42.8
688	1.6	15	83.2	14.5	63.3
692	6.4	15	74.1	23.9	39.5
696	6.4	15	74.1	8.7	77.9
425	Saline	29	52.1	39.5	0
684	1.6	29	61.6	26.0	34.2
688	1.6	29	84.1	14.6	63.0
692	6.4	29	66.9	21.4	45.8
696	6.4	29	84.1	12.9	67.3

Abbreviation: ND, not determined.

* Determined by flow cytometry with antihuman CD2-RPE (AMAC).

† Determined by flow cytometry with Leu-16 antihuman CD20-FITC (Becton Dickinson).

‡ Values calculated assuming 39.5% B cells from a normal animal treated with saline and biopsied at day 29 after last saline injection. Day 15 and day 29 treated animals all calculated based on 39.5% B cells in normal lymph node.

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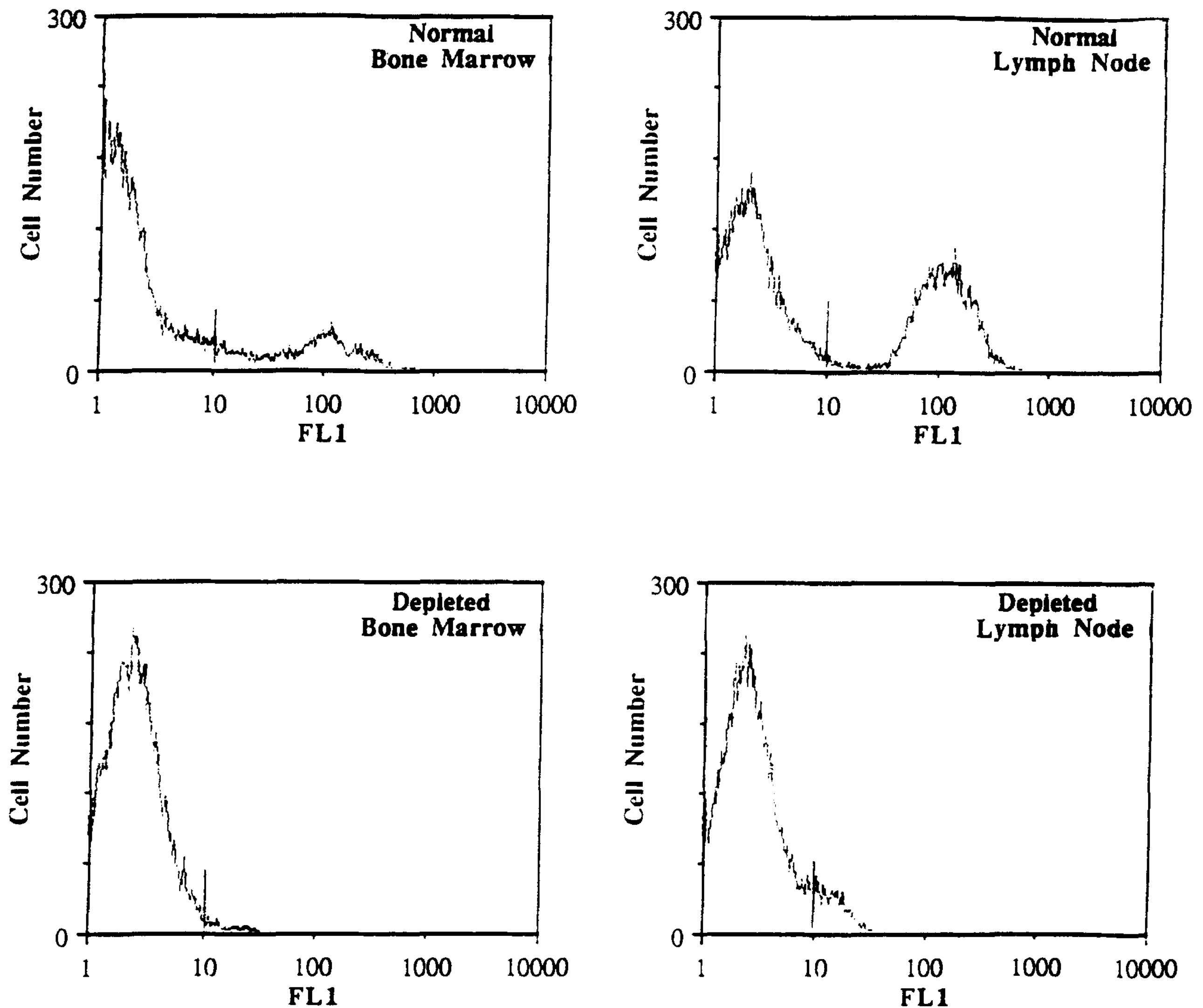

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Fig 10. Depletion of monkey lymph node and BM B lymphocytes after infusion of four weekly 16.8 mg/kg doses of C2B8. Chimeric C2B8 was injected into four monkeys: 677, 892, 681, and 884. Inguinal lymph nodes were surgically removed for flow cytometry cell analysis along with BM aspirates. Monkey-681 B-lymphocyte BM and lymph node histograms compared with nondepleted animals 425 (lymph node) and 908 (BM), injected with saline. C2B8-treated monkey 681 was examined at 36 days after last injection.

a low dose of chimeric anti-CD20 leads to long-term peripheral B-cell depletion in primates. Furthermore, significant depletion of B-cell populations was achieved in peripheral lymph nodes and BM when repetitive high doses were administered.

We have chosen CHO transfectomas to produce antibody for future clinical trials because of the previous use in humans of other CHO-derived recombinant proteins, and because we are able to derive CHO cells that secrete 80 to 100 $\mu g/10^6$ cells/d. These transfectomas are capable of producing greater than 1 g/L in short fermentation-tank studies.

C2B8 antibody has shown in vivo characteristics that support its use in treatment of B-cell malignancies. Tumors of this type are particularly accessible and readily susceptible to immunotherapy.[26] The CD20 target is strongly expressed on most B-lymphocyte tumors and is not shed into the circulation nor expressed in nonhematopoietic tissues, where it could interfere with antibody therapy. The concern for immunoreactivity against mouse/human chimeric antibodies is reduced because of the immunocompromised nature of lymphoma patients[27,28] and perhaps aided further by the human constant region.[29] We have not yet compared di-

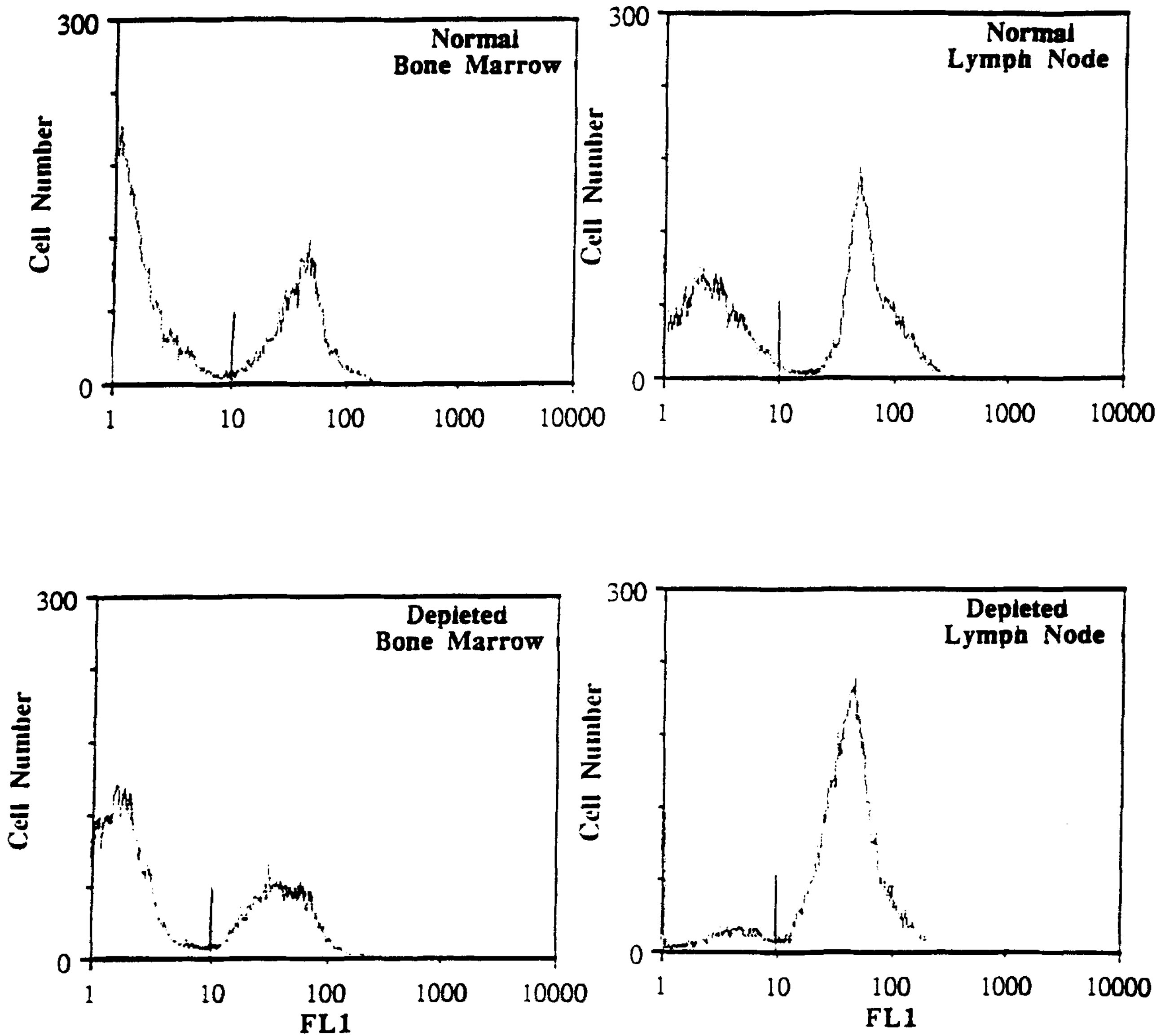

Fig 11. T-cell populations in monkey lymph node and BM tissues after infusion of four weekly 16.8 mg/kg doses of C2B8. Chimeric C2B8 was injected into four monkeys: 677, 681, 884, and 892. Inguinal lymph nodes were surgically removed from anesthetized animals for immunohistochemical staining and flow cytometry cell analysis along with BM aspirates. Monkey-681 T-lymphocyte BM and lymph node histograms compared with nondepleted animals 425 (lymph node) and 908 (BM), injected with saline. C2B8-treated monkey 681 was examined at 36 days after last injection.

rectly in monkeys the immunogenicity of the murine and chimeric antibodies. We have analyzed all antibodies produced in these animals against C2B8 and found them to be exclusively anti-idiotypic and antijoining region specific. None of these antibodies cross-react to other human or murine antibodies; however, immunogenicity still remains an issue for further investigation.

Despite the fact that all animals were dramatically depleted of peripheral B lymphocytes during the first week after treatment, there have been no adverse effects observed in any of the treated animals. In our studies, all antibody-treated monkeys have shown no apparent depletion of other cell types; however, the lack of suitable marker reagents for monkey hematopoietic tissues other than B- or T-lymphocytes, have made it difficult to establish this claim with certainty. Lymph nodes in B-cell–depleted animals become al-

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Table 3. T- and B-Lymphocyte Populations in Lymph Node and BM Biopsies Taken From Cynomolgus Monkeys Treated with Four Weekly Doses (16.8 mg/kg) of C2B8

Monkey	% CD2⁺ *	% CD20⁺ †	Day‡	Dose (mg/kg)	% Depletion§
Inguinal lymph node					
425	52.1	39.5	29	Saline	0
677	90	5.3	22	16.8	86.6
892	91	6.3	22	16.8	84.1
681	89.9	5.0	36	16.8	87.4
884	85.4	12.3	36	16.8	68.9
Bone marrow					
677	46.7	4.3	22	16.8	74.1
892	41.8	3.0	22	16.8	81.9
681	35.3	0.8	36	16.8	95.2
884	25.6	4.4	36	16.8	73.5
908	29.8	16.6	36	Saline	0

* Determined by flow cytometry with antihuman CD2-RPE (AMAC). Values are percentages of total lymphocytes.

† Determined by flow cytometry with Leu-16 antihuman CD20-FITC (Becton Dickinson). Values are percentages of total lymphocytes.

‡ Days after final weekly injection.

§ Values are calculated assuming 39.5% of total lymphocytes in animal 425 treated with saline as B-cell fraction in a normal lymph mode. Percent depletion represents the decreased fraction of B cells compared with the normal lymph node, found in the treated animals. The percent depletion of BM calculated in treated animals was based on fraction of B cells in normal BM sample from saline animal 908 as 16.6% B cells.

most entirely populated with T cells. All nonsacrificed animals in the studies eventually recovered their B-cell populations, indicating that monkey pluripotent stem cells are not affected. This is further supported by our in vitro studies on human BM stem cells (data not shown), which demonstrated that the CD20 antigen is not expressed on stem cells. In addition, other studies in humans that have used anti-CD20 antibodies to purge BM have seen no adverse effects on BM reconstitution.[30]

The application of C2B8 to human lymphoma therapy will provide a much greater challenge than the primate model described here. Human disease will likely present excessive tumor burdens within lymphatic tissues involving bulky fibroidal lesions with markedly decreased accessibility.[23] Our present plans are to determine how effective C2B8 is in reducing tumor bulk or in erradicating micrometastases in patients suffering from non-Hodgkin's B-cell lymphoma.

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