Monoclonal Antibody Therapy of B Cell Lymphoma: Signaling Activity on Tumor Cells Appears More Important Than Recruitment of Effectors

Alison L. Tutt, Ruth R. French, Timothy M. Illidge, Jamie Honeychurch, Harry M. McBride, Christine A. Penfold, Douglas T. Fearon, R. Michael E. Parkhouse, Gerry G. B. Klaus and Martin J. Glennie

*J Immunol* 1998; 161:3176-3185; http://www.jimmunol.org/content/161/6/3176

References
This article cites 46 articles, 28 of which you can access for free at: http://www.jimmunol.org/content/161/6/3176.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Monoclonal Antibody Therapy of B Cell Lymphoma: Signaling Activity on Tumor Cells Appears More Important Than Recruitment of Effectors

Alison L. Tutt,* Ruth R. French,* Timothy M. Illidge,* Jamie Honeychurch,* Harry M. McBride,* Christine A. Penfold,* Douglas T. Fearon, † R. Michael E. Parkhouse, ‡ Gerry G. B. Klaus,§ and Martin J. Glennie²*  

Despite the recent success of mAb in the treatment of certain malignancies, there is still considerable uncertainty about the mechanism of action of anti-cancer Abs. Here, a panel of rat anti-mouse B cell mAb, including Ab directed at surface IgM Id, CD19, CD22, CD40, CD74, and MHC class II, has been investigated in the treatment of two syngeneic mouse B cell lymphomas, BCL1 and A31. Only three mAb were therapeutically active in vivo, anti-Id, anti-CD19, and anti-CD40. mAb to the other Ags showed little or no therapeutic activity in either model despite giving good levels of surface binding and activity in Ag-dependent cellular cytotoxicity and complement assays, and in some cases inhibiting cell growth in vitro. We conclude that the activity of mAb in vitro does not predict therapeutic performance in vivo. Furthermore, in vivo tracking experiments using fluorescently tagged cells showed that anti-Id and anti-CD40 mAb probably operate via different mechanisms: the anti-Id mAb cause growth arrest that is almost immediate and does not eliminate cells over a period of 5 or 6 days, and the anti-CD40 mAb have a delayed effect that allows tumor to grow normally for 3 days, but then abruptly eradicates lymphoma cells. This work supports the belief that mAb specificity is critical to therapeutic success in lymphoma and that, in addition to any effector-recruiting activity they may possess, in vivo mAb operate via mechanisms that involve cross-linking and signaling of key cellular receptors. The Journal of Immunology, 1998, 161: 3176–3185.

D
epite their specificity and ease of production, mAbs have failed to have the major impact on cancer treatment that had been hoped for in the 1980s. However, recent studies have been encouraging and suggest that, at least for certain hematologic malignancies, mAb may become a treatment of choice in the near future (1–4). For example, “naked” chimeric anti-CD20 mAb is now producing striking results in relapsed, low grade NHL. Maloney et al. (1, 4) have recently reported a 50% response rate (partial and complete) when chimeric human/mouse anti-CD20 Ab is given as a single agent. Similarly, over the last few years the anti-CD52 mAb, Campath-1H, has continued to generate impressive response rates in certain B and T cell malignancies, particularly when the neoplastic cells are concentrated in the blood and bone marrow (3). These accomplishments underline the potential of mAb in cancer treatment and also pose the important question of why some Abs eliminate tumor cells but many more do not (5, 6). The factors that have been considered as important in clinical investigations include the density of the Ag on the target cell and its tendency to modulate, the ability of the therapeutic mAb to activate effector systems, particularly Ag-dependent cellular cytotoxicity (ADCC), and the access of the mAb to the tumor, a problem that has been encountered with some solid tumors. Which of these factors is most critical is still not clear and probably varies from one tumor to another. However, there has been a general consensus, arising mainly from animal work and the improved performance of chimeric human/mouse mAb in patients, that ADCC is of critical importance in determining clinical outcome (7–9). ADCC-active mAb have usually performed better in animal immunotherapy, while mixed results have been obtained when investigating the role of complement in these systems (8–10). Because ADCC often works well in vitro using fresh PBMC effectors, it is an understandable assumption that it operates in vivo. However, this has never been formally proven, and certainly a number of mAb that have performed well in ADCC assays have failed in clinical trials (11), encouraging the search for methods to improve Ab potency, in particular by conjugation with toxic compounds, such as radioisotopes.

As an alternative explanation for the clinical success of some mAb, many researchers are now considering that mAb might have a direct regulatory effect on tumor growth. A signaling role for therapeutic mAb was shown by experiments in which anti-μ Ab was able to affect cytotoxicity by cross-linking the surface Ig complex (BCR) on certain B cell lines and on some fresh lymphoid tumors (12, 13). Recent clinical results from Vuist and colleagues (14) now indicate that the therapeutic activity of anti-Id mAb in B

---

3 Abbreviations used in this paper: ADCC, antigen-dependent cellular cytotoxicity; BCR, B cell receptor for antigen; CDC, complement-dependent cytotoxicity; PE, phycoerythrin; CFSE, 5- and 6-carboxyfluorescein diacetate succinimidyl ester.

4 Lymphoma Research Unit, Tenovus Laboratory, General Hospital, Southampton, United Kingdom; 5 Wellcome Trust Immunology Center, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom; 6 Institute for Animal Health, Pirbright, United Kingdom; and 7 National Institute for Medical Research, London, United Kingdom

Received for publication December 22, 1997. Accepted for publication May 12, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Tenovus of Cardiff, the Cancer Research Campaign, and the Leukemia Research Fund.

2 Address correspondence and reprint requests to Dr. Martin J. Glennie, Lymphoma Research Unit, Tenovus Laboratory, General Hospital, Southampton, United Kingdom SO16 6DY. E-mail address: m.j.glennie@sonoton.ac.uk

Copyright © 1998 by The American Association of Immunologists 0022-1767/98/$02.00
cell lymphoma correlates with the ability to induce Ig signal transduction. This work revealed a striking correlation between the tendency of anti-Id mAb to produce clinical responses in non-Hodgkin’s lymphoma patients and their ability to induce transmembrane signaling in a patient’s lymphoma cells, as measured by the phosphorylation of intracellular proteins.

While tailor-made anti-id mAb are not considered practical for general application in lymphoma treatment, they may still have much to teach us about the process of controlling cell growth in vivo. Although Ab-mediated signaling appears critical in anti-id mAb treatment, it may also play an important role in many other mAb treatments, involving both neoplastic and normal cells. A number of mAb that have been used, or at least considered, for treating lymphoma, such as anti-CD19 (15, 16), anti-CD20 (1, 4), anti-CD22 (17), anti-CD38 (11), and anti-CD40 (18, 19), are capable of generating transmembrane signals in normal and neoplastic B cells. In the current work we have investigated a panel of anti-mouse B cell mAb in two syngeneic B cell lymphoma models, BCL1 (20) and A31 (21), to try to establish whether it is direct growth regulation, ADCC, or complement that is of overriding importance in therapy. Our results clearly show that results from in vitro assays do not correlate with activity in vivo and that, at least for B cell lymphoma, therapeutically successful mAb appear to be those directed at key receptor molecules (Id, CD19, and CD40) involved in transmembrane signaling during B cell responses.

Materials and Methods

Animals and cell lines

BALB/c and CBA/H mice and Louvain (LOU/OlaHsd) rats were all supplied by Harlan UK (Blackthorn, U.K.), and maintained in local animal facilities.

BCL1 (20) and A31 (21) mouse B lymphoma lines were maintained in vivo, i.e., passage in BALB/c and CBA mice, respectively. Enlarged spleens were taken at the terminal stage of disease, and single cell suspensions were prepared as described previously (22). The BCL1-3B, cell line (23) is a variant of the BCL1 tumor that is maintained in culture using standard medium (see below).

All cell culture was performed in supplemented DMEM containing glucose (2 mM), pyruvate (1 mM), penicillin and streptomycin (100 IU/ml), fungizone (2 µg/ml), and 5 or 10% FCS (Mycobone; Life Technologies, Paisley, Scotland) or in supplemented RPMI (Life Technologies) containing the same supplements but with the addition of 50 µM 2-ME (BDH, Poole, U.K.).

Abs and fragments

All mAb used in this study are shown in Table I together with their sources. Three new mAb, Mc39-12, Mc39-16, and TI2-3, were generated during the project using standard somatic fusion technology as discussed previously (28). Mc39-12 and Mc39-16 mAb reacted with mouse µ-chain and A31 Id, respectively and were raised by immunizing LOU rats with mouse IgM that had been immunoprecipitated from the A31 lymphoma according to the method described by Hamblin et al. (29). Three days after the final booster, spleen cells from the rat were fused with NS-1 mouse myeloma cells, and supernatants from the resulting hybridomas were screened by ELISA, selecting mAb that bound all mouse IgM (Mc39-12) or only A31 idiotypic IgM (Mc39-16).

TI2–3 (anti-CD74) was raised by immunizing a LOU rat with whole A31 cells and then screening the resulting hybridomas for reactivity to both A31 and BCL1, lymphoma lines and normal mouse spleen cells. After cloning, the reactivity of the hybridomas was determined by flow cytometry and immunoprecipitation of normal and lymphoma mouse cells. The target Ag showed a major band of 33 kDa together with a range of minor bands that were typical of CD74 (30) (T. M. Illidge, H. M. McBride, and M. J. Glennie, manuscript in preparation).

Hybridoma cells were expanded in stationary culture using 5% supplemented DMEM. To purify the IgG mAb, the culture supernatants were concentrated 20 times by membrane filtration (Amicon, Beverly, MA), precipitated with saturated ammonium sulfate, and then dialyzed and fractionated on protein G (Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. Four of the rat mAb (Mc39-12, Mc39-16, Mc10-6A5, and M5-114) were prepared by ion-exchange chromatography on DEAE (Whatman, Clifton, NJ) as described by Elliott et al. (28). The purity of all IgG preparations was checked by electrophoresis (EP system, Beckman, Palo Alto, CA) and HPLC using a Zorbax GF250 Bio Series column (Du Pont, Wilmington, DE) (31).

Flow cytometry

Mouse lymphoma cells (splenic) were analyzed by direct immunofluorescence staining using a FACS vantage (Becton Dickinson, Mountain View, CA) as described previously (31).

Binding of [125I]mAb to the surface of lymphoma cells

mAb were trace radiolabeled for binding studies using carrier-free [125I] (Amersham International, Aylesbury, U.K.) and Iodo-Beads (Pierce, Rockford, IL) as the oxidizing reagent and were extensively dialyzed to remove unbound [125I].

The binding of radiolabeled mAb to cells was determined as described by Elliott et al. (28). Radiolabeled mAb were serially diluted before incubation with cells (0.5–1.25 × 10⁵/ml; final volume, 1 ml) in RPMI medium containing 10% FCS for 2 h at 37°C. Endocytosis of cell-bound mAb was prevented by inclusion of sodium azide (15 mM) and 2-deoxyglucose (50 mM). The cells were separated from the aqueous phase by rapid centrifugation through a 1.1/1 (v/v) mixture of dibutyl phthalate/dioctyl phthalate for 5 min at room temperature, incubated for 6 h at 37°C in a CO₂ incubator, and then centrifuged again, this time at 500 g for 5 min, before finally harvesting 100 l of the supernatant to estimate the release of radioactivity using a Packard Tri-Carb 2100CA liquid scintillation counter (Packard, Downers Grove, IL) as the oxidizing reagent and were extensively dialyzed to remove unbound [125I].

The binding of radiolabeled mAb to cells was determined as described by Elliott et al. (28). Radiolabeled mAb were serially diluted before incubation with cells (0.5–1.25 × 10⁵/ml; final volume, 1 ml) in RPMI medium containing 10% FCS for 2 h at 37°C. Endocytosis of cell-bound mAb was prevented by inclusion of sodium azide (15 mM) and 2-deoxyglucose (50 mM). The cells were separated from the aqueous phase by rapid centrifugation through a 1.1/1 (v/v) mixture of dibutyl phthalate/dioctyl phthalate according to the method of Dower et al. (32). Radioactivity was measured in a Rackgamma spectrometer (LKB, Gaithersburg, MD).

Complement-dependent cytotoxicity (CDC) assay

The ability of the mAb to kill A31, BCL1, and BCL1-3B3 cells in a CDC assay was determined as previously described (24). Briefly, 10⁵ Cr-labeled target cells were exposed to various concentrations of each mAb and control reagents on ice for 30 min. Fresh rat serum (final dilution, 1/5 with supplemented DMEM) was then added as a source of complement, and the samples were warmed to 37°C. After 45 min the samples were centrifuged for 5 min to sediment cells, and specific ⁵¹Cr release was assessed by counting the supernatant. All samples were run in duplicate, and the maximum release was determined by measuring ⁵¹Cr release obtained after addition of 1% Nonidet P-40 to the cells.

ADCC assay

The ADCC assay was modified from previous work (33). ³¹⁵Cr-labeled A31, BCL1, and BCL1-3B3 target cells were prepared as described for the CDC assays and resuspended at a final concentration of 10⁵ cells/ml in supplemented DMEM. The effectors were peritoneal exudate cells washed from the peritoneal cavity of CBA mice 5 days after they had received an i.p. injection of thioglycolate broth (Becton Dickinson, Oxford, U.K.) (9). The exudate cells were washed once and resuspended in supplemented DMEM at the appropriate concentration.

For the assay, all samples were made in supplemented DMEM. First, 50–500 l aliquots of target cells (5 × 10⁵) and mAb were mixed in individual wells of 96-well U-bottomed culture plates (Life Technologies, Paisley, U.K.) and left on ice for 15 min. Aliquots of 100 µl of effector cells were then added at an E/T cell ratio of 50:1. The plates were centrifuged at 200 × g for 5 min at room temperature, incubated for 6 h at 37°C in a CO₂ incubator, and then centrifuged again, this time at 500 × g for 5 min, before finally harvesting 100 µl of the supernatant to estimate the released ³¹⁵Cr.

All determinations were performed in triplicate, and the maximum release of radioactivity was calculated using target cells to which 150 µl of 1% Nonidet P-40 had been added. The percentage of specific ³¹⁵Cr release

### Table I. Anti-mouse mAb used in this work

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Affinity (Kᵦ M⁻¹)</th>
<th>Source/Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc10-6A5</td>
<td>BCL1, Id</td>
<td>Rat</td>
<td>1 × 10⁸</td>
<td>24</td>
</tr>
<tr>
<td>Mc39-16</td>
<td>A31 Id</td>
<td>Rat</td>
<td>5 × 10⁸</td>
<td>Tenovus</td>
</tr>
<tr>
<td>Mc39-12</td>
<td>µ-chain</td>
<td>Rat</td>
<td>3 × 10⁴</td>
<td>Tenovus</td>
</tr>
<tr>
<td>1D3</td>
<td>CD19</td>
<td>Rat</td>
<td>2.7 × 10³</td>
<td>25</td>
</tr>
<tr>
<td>NMR6</td>
<td>CD22</td>
<td>Rat</td>
<td>1.2 × 10³</td>
<td>27</td>
</tr>
<tr>
<td>3/23</td>
<td>CD40</td>
<td>Rat</td>
<td>2.7 × 10³</td>
<td>27</td>
</tr>
<tr>
<td>TI2-3</td>
<td>CD74</td>
<td>Rat</td>
<td>5.3 × 10³</td>
<td>Tenovus</td>
</tr>
<tr>
<td>M5-114</td>
<td>MHC class II</td>
<td>Rat</td>
<td>2.1 × 10⁴</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

*a Estimates of the Kᵦ values were calculated from the curves shown in Figure 1 by determining the concentration of Ab required to achieve half-maximum binding.*
the plates were incubated for 24 h at 37°C before pulsing with 0.5 m
molecules of Ab bound per cell.

and the pellet was counted for cell-bound radioac-
centrifugation through a mixture of phthalate oils,
were separated from the aqueous phase by rapid
mAb was prevented by inclusion of sodium azide

(55x359) was calculated using the standard formula: % specific release = [(sample release − background release)/(maximum release − background release)] × 100.

Growth and clonogenic assays for Ab-treated cells

In the short term growth assay to assess direct effects of mAb on tumor cells in vitro, BCL1-3B3 cells were exposed to mAb while measuring DNA synthesis (uptake of [3H]thymidine). Culture plates (96-well, flat-bottom; Life Technologies) were first precoated for 2 h at 37°C with each test mAb in sterile PBS (50-μl aliquots/well), 5 × 10^6 BCL1-3B3 target cells were then added to each well in a volume of 150 μl of supplemented RPMI, and the plates were incubated for 24 h at 37°C before pulsing with 0.5 μCi/well of [3H]thymidine (Amersham) for an additional 16 h. The DNA-incorpo-
rated radioactivity was harvested onto glass filters (Whatman) and counted as described previously (33).

In the clonogenic assay, BCL1-3B3 cells were plated in 96-well flat-bottom culture plates (Life Technologies) in supplemented RPMI (100 μl/well) at a frequency of two cells per well. mAb was included in the assay at 10 μg/ml throughout the culture period. For each assay, a complete 96-well plate of cells was set up per mAb. Colonies were grown for approxi-
ately 14 days and then counted and expressed as a percentage of those obtained in plates containing isotype-matched control mAb.

Ab immunotherapy

Groups of age-matched CBA and BALB/c mice were injected i.v. with compatible lymphoma cells. CBA mice received an i.v. inoculum of 10^5 A31 cells on day 0, followed by mAb treatments on days 3 to 6 (0.5 mg/day, 2 mg total). The first mAb treatment was given i.v.; the next three were given i.p. BALB/c mice received a similar protocol with 10^5 i.v. BCL1 cells on day 0 followed by four doses of mAb on days 9 to 12, again giving the first mAb treatment i.v. and the next three by the i.p. route. Both tumors develop primarily in the spleen, with a leukemic overspill toward the end of the disease (20, 21). Survival was monitored daily, and the results were analyzed using the χ² test of Peto (34).

Tracking tumor cells in vivo

To understand more fully the mechanism of action of anti-tumor mAb in vivo we developed two methods of tracking cells after therapy. In the first, fresh A31 cells were tagged in vitro with 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Leiden, The Nether-
lands). Stock CFSE (5 mM) was prepared by dissolving CFSE in DMSO and was stored frozen. A31 cells were resuspended in serum-free RPMI 1640 (Life Technologies) at a concentration of 2 × 10^6/ml and prewarmed to 37°C. Stock CFSE was then added to the cell suspension to give a final concentration of 5 μM and was mixed thoroughly by inversion. The sus-
pension was incubated for 10 min at 37°C, and the cells were washed twice with 4 vol of ice-chilled RPMI 1640 containing 10% FCS. Finally, the cells were suspended in PBS at a concentration of 2.5 × 10^9/ml for i.v. injection in mice.

In the second method, groups of mice were inoculated i.v. with 5 × 10^7 A31 or BCL1 cells in 200 μl of PBS on day 0 and then, when the tumor cells were growing in log phase (day 3 for A31 and day 4 for BCL1), were treated with the appropriate mAb (2 mg i.v.). The total yield of splenic tumor cells was estimated each day by staining spleen homogenates with a combination of PE-anti-Id and FITC-anti-CD19 (or FITC-anti-CD22), which allowed tumor cells to be identified by flow cytometry.

Results

Binding activities of various mAbs on BCL1, and A31 lymphoma lines

Using the phthalate oil method of Dower et al. (31), we have char-
acterized a range of rat anti-mouse B cell mAb (Table I) that bind to the two lymphomas, A31 and BCL1, and might be considered for treatment of these tumors. Only the anti-Id mAb are tumor specific, but, as discussed in the introduction, all the other speci-
ficities have at some time been considered for treatment of human lymphoma. Figure 1 shows typical binding curves performed at 37°C. Interestingly, the saturation levels of binding for this panel of mAb are essentially the same on both cell lines and BCL1-3B3 (data not shown), giving binding levels in the order anti-CD74 ≥ anti-MHC class II > anti-Id > anti-CD22 > anti-CD19 = anti-CD40. The BCL1-3B3 cell line is a variant of BCL1, which, unlike the parent line, can be maintained in long term culture (23). The invariant chain of class II, CD74, is very strongly expressed, par-
ticularly on A31 (~2.6 × 10^7 anti-CD74 molecules bound/cell). CD19 and CD40 are expressed at almost equal levels and at a relatively low density (<4 × 10^4 mAb molecules bound/cell) compared with the other surface molecules. The affinities of the mAb (Table I) are as expected, with apparent Kᵦ values falling in the range 10^5 to 10^9 M⁻¹.

Complement and Ab-dependent cellular cytotoxicity of A31, BCL1, and BCL1-3B3 cells

In an attempt to correlate in vitro assays with the performance of mAb in immunotherapy, we first performed CDC and ADCC as-
says. Figure 2 shows that the anti-MHC class II and anti-Id mAb produced marked cell lysis in CDC assays with all three cell lines. Likewise, anti-CD74 mAb was active on A31 cells. However, on
the BCL₁ and its variant, and despite the high levels of expression of CD74, the anti-CD74 mAb failed to induce appreciable levels of lysis. This is a somewhat surprising observation that remains to be explained. The other three mAb, CD19, CD22, and CD40, which were expressed at lower levels, were relatively inactive on all cell lines under the conditions investigated.

Next we conducted ADCC assays using mouse activated peritoneal exudate cells (9). The results in Figure 3 show that all the mAb mediated ADCC against A31, BCL₁, and BCL₁-3B3. In general, the A31 tumor was more sensitive to lysis than was BCL₁ and its variant. Again, Ag density appeared to be the over-riding feature determining the level of cytotoxic activity achieved, with anti-MHC class II and anti-CD74 mAb being consistently the most active. The main message from both CDC and ADCC assays is that Ag density appears to be the key factor in determining Ab potency within this group of reagents, there being no obvious preference in terms of rat isotype or Ab specificity.

**In vitro growth of BCL₁-3B3 cells in the presence of mAb**

We next considered direct Ab toxicity on neoplastic B cells in the absence of any effector systems. This work was performed on the BCL₁ culture subline, BCL₁-3B3, since neither A31 nor the parental BCL₁ line can be sustained in culture for more than a few hours. In the initial assays the uptake of [³¹]Cr released by cultured cells was used as a measure of proliferation in the presence of the various mAb. Figure 4A shows a striking difference in the ability of mAb to influence the growth of BCL₁-3B3 cells. Two of the mAb, anti-Id and anti-CD74, had a direct antiproliferative effect. Anti-CD74 was always more active than the anti-Id mAb. In addition, the anti-CD19 mAb, 1D3, consistently gave a slight inhibition of proliferation. However, the most effective inhibitor of proliferation was the anti-μ mAb, Mc39-12, which appears to deliver a strong growth inhibitory signal, perhaps because of its ability to cross-link surface IgM (35). Because anti-Id mAb is less active in this assay, these results are consistent with it being relatively poor at cross-linking surface IgM. One explanation for such observations is that anti-Id mAb binds bivalently, but in what is sometimes called a monogamous union, in which each anti-Id mAb engages surface Ig symmetrically (IgM/D to IgG mAb), providing intra-
were conducted to determine an effective dosing regimen (data not shown). With the exception of anti-Id mAb, which was therapeutic at doses of <100 μg/animal, other mAb specificities required relatively high doses of around 2 mg/mouse to achieve near-maximum effectiveness. This high level ensured that all tumor cells were saturated and exposed to mAb for a sustained period, allowing weakly therapeutically mAb a chance to achieve a therapeutic effect. Figure 5 shows the results of a typical therapy in the A31 model in which mice received 10⁶ fresh cells i.v. on day 0 and then 2 mg of mAb between days 3 and 6 at a dose of 0.5 mg/day. Only two mAb, anti-CD40 and anti-Id, consistently prolonged the survival of A31-bearing mice and resulted in increases in survival of about 20 and 45 days, respectively. Surprisingly, as little as 5 μg/mouse of anti-Id mAb was enough to obtain 45 days of protection from the A31 lymphoma, far less than would be needed to saturate the tumor cells in vivo (data not shown). All the other mAb, despite binding to tumor cells in vivo, failed to show protection.

We next investigated the BCL₁ model using the same panel of mAb together with an appropriate anti-Id mAb and a treatment protocol similar to that used for A31. Figure 6 shows a typical immunotherapy experiment using 10⁵ BCL₁ cells and 2 mg of each mAb given over 4 days (0.5 mg/day). Treatment of the BCL₁ tumor was delayed until day 9 after tumor inoculation because this is a less aggressive malignancy than the A31. In the BCL₁ tumor, mAb to CD19, CD40, and Id were clearly therapeutic, with 80% of those mice receiving anti-CD40 mAb going on to become long term survivors (>100 days). Although the anti-Id and anti-CD19 mAb were less effective, there was nevertheless significant prolongation of survival in all treated animals (p < 0.01). Interestingly, the therapeutic effect of anti-CD19 mAb was highly dose dependent, and while treatment with 2 mg of mAb gave a clear survival benefit, a dose of 100 μg/animal did not (data not shown). The anti-MHC class II mAb had a small but statistically significant therapeutic effect (p < 0.05), and the other mAb were without detectable effect.

These results clearly show that within these models, none of the in vitro assays (cytotoxicity or growth arrest) had indicated which mAb would be effective in vivo; therefore, additional experiments...
were undertaken in vivo to investigate how therapeutic mAb control lymphoma growth.

Tracking A31 and BCL2 cells in vivo following mAb treatment

In our initial attempts to follow tumor cells in vivo we made use of the stable cell dye, CFSE, which tags cells by irreversibly fluorescing the cytoplasm. Once labeled with CFSE in vitro, the cells and their progeny remain detectable by flow cytometry for 6 to 7 days in vivo. The experimental protocol involved labeling freshly prepared tumor cells in vitro, injecting them i.v. into groups of mice (5 x 10^7 cells/mouse), and then either leaving the mice untreated or treating them with 2 mg of mAb (i.v.) 1 day later as indicated (anti-CD40, second column; anti-Id, third column). Each day after tumor inoculation, splenic lymphocytes were prepared from sample mice and stained with PE-anti-Id mAb for analysis by flow cytometry. A31 cells from mice given anti-Id mAb (third column) could not be stained with anti-Id mAb due to blocking or clearing of surface Ig. The numbers in each dot plot show the percentage of lymphocytes in the boxed region.

FIGURE 7. In vivo tracking of CFSE-labeled A31 cells with and without mAb treatment. CFSE-labeled A31 cells were given by i.v. injection to three groups of CBA mice (5 x 10^7/mouse) on day 0. The mice were then left untreated (first column) or were given 2 mg of mAb by i.v. injection 1 day later as indicated (anti-CD40, second column; anti-Id, third column). Each day after tumor inoculation, splenic lymphocytes were prepared from sample mice and stained with PE-anti-Id mAb for analysis by flow cytometry. A31 cells from mice given anti-Id mAb (third column) could not be stained with anti-Id mAb due to blocking or clearing of surface Ig. The numbers in each dot plot show the percentage of lymphocytes in the boxed region.

CFSE label dropped progressively as the tumor population expanded. Thus, the CFSE marks cell division.

In those animals given anti-CD40 mAb 1 day after receiving labeled cells (Fig. 7, second column), the tumor cells in the spleen continued to proliferate for 2 or 3 days following mAb treatment. This is shown by both the increase in the percentage of cells labeled with CFSE and PE-anti-Id, and the progressive reduction in the intensity of CFSE staining. The failure of the treatment to perturb cell growth was most clearly seen 2 days after the mAb was given, as at this time there appeared to be as many if not more tumor cells in the anti-CD40 mAb-treated animal (Fig. 7, day 3; 9.9% gated) than in the untreated control (Fig. 7, day 3; 6.2% gated). However, by day 4 differences started to emerge, with a slight reduction in the percentage of tumor cells after anti-CD40 mAb treatment. By day 6 this reduction was marked (Fig. 7, second column; 3.6% after anti-CD40 mAb compared with 54% in the untreated control), and by day 8 tumor cells were no longer detectable following anti-CD40 mAb treatment.

The profile of tumor cell-growth obtained after anti-Id mAb treatment was clearly different (Fig. 7, third column). First, as expected, the PE-anti-Id label was unable to detect the tumor cells due to blocking and internalization of their surface Ig by the treatment mAb. This
FIGURE 8. Yield of splenic tumor cells recovered in A31 and BCL₁ after mAb treatment. Groups of mice were given 5 × 10⁷ fresh A31 or BCL₁ cells on day 0 (T arrow). They were then treated with 2 mg of mAb (of the specificities indicated) on the days shown (Ab/arrow). Each day, spleen cells from sample mice were prepared, and the yield of tumor cells was calculated by flow cytometry with PE-anti-Id mAb and FITC-anti-CD19 or FITC-anti-CD22 mAb. Each point on the graph represents the mean yield of tumor cells recovered from two mice. This represents one of three similar experiments.

introduced some difficulties in determining a precise gate for the tumor cells in flow cytometry. Second, anti-Id mAb treatment appeared to have a quicker effect on tumor growth than anti-CD40 mAb and resulted in arrested tumor cell division between days 3 and 4, as shown by the comparative lack of increase in the percentage of tumor cells. Interestingly, the tumor cells that remained after treatment showed a relatively small reduction in their levels of CFSE staining between days 3 and 6, consistent with the belief that they are growth arrested. Comparison of the intensity of CFSE staining in tumor cells on day 6 showed a clear distinction between cells taken from untreated controls and anti-CD40 mAb-treated animals (Fig. 7, first and second columns) and those recovered from mice given anti-Id mAb (third column). This difference in mean fluorescence intensity (CFSE) for the two groups was about 10-fold. Finally, and despite impressive immunotherapy by anti-Id in A31 lymphoma (Fig. 5), this treatment did not eradicate the tumor cells, and they remained detectable as a CFSE-labeled population throughout the study period. In recent experiments we have been able to extend this period to 10 days, at which point small numbers of splenic CFSE-labeled tumor cells can still be detected after anti-Id mAb treatment.

Experiments with CFSE-labeled BCL₁ tumor also showed that anti-CD40 mAb treatment eradicated tumor (data not shown), and that the kinetics were very similar to those seen in the A31 lymphoma (Fig. 7), i.e., 2 to 3 days of proliferation followed by a rapid decline (data not shown). In addition, anti-Id and anti-CD19 mAb produced results similar to those seen with anti-Id in the A31 tumor (Fig. 7, third column). These two mAb prevented the growth of BCL₁ as shown by cell numbers and the rate of loss of CFSE stain. However, the growth-arresting effects of anti-Id and anti-CD19 mAb were less evident than those seen following anti-Id mAb in the A31 tumor, probably because these two mAb are comparatively poor in immunotherapy of BCL₁ (Fig. 6) and consequently less able to contend with the large tumor load that resulted from injecting 5 × 10⁷ cells. Finally, as expected, nontherapeutic mAb (Figs. 5 and 6), such as anti-CD22 mAb, failed to have a significant impact on CFSE-labeled tumor growth in either lymphoma model (see below).

To confirm and extend these observations we next investigated the development of A31 and BCL₁ lymphoma cells without pre-labeling in vitro by calculating the total yield of splenic tumor cells recovered following mAb treatment. In these studies mice were given 5 × 10⁷ fresh A31 or BCL₁ cells on day 0 and then treated with 2 mg of mAb on day 3 (A31) or day 4 (BCL₁) at a time when the lymphoma cells were growing in log phase. Tumor cells were detected using flow cytometry after double staining spleen cells with PE-anti-Id mAb and FITC-anti-CD19 or FITC-anti-CD22 mAb. The results in Figure 8 confirm the rapid growth of both tumors and show that mAb, such as anti-CD22 and anti-MHC class II, that were not effective in standard therapy experiments (Figs. 5 and 6) had no impact on the yield of tumor cells recovered from the spleens of tumor-bearing animals. They also show that anti-CD40 and anti-Id mAb in A31 and anti-CD40 mAb in BCL₁ had a profound impact on tumor growth, which reflected the effects seen in CFSE labeling experiments. In neither model did anti-CD40 mAb cause any inhibition of tumor cell expansion for the 3 days following treatment, but then tumor cell number dropped markedly as the mAb had its therapeutic effect. This reduction in tumor cell number was particularly dramatic in the BCL₁ model, where tumor cells were almost completely eradicated between days 7 and 8. In contrast to this delayed therapeutic effect, anti-Id mAb treatment of A31 lymphoma caused an immediate halt to tumor cell proliferation, but did not eradicate tumor over a 5-day period, a result that confirms the growth arrest seen in the CFSE experiments (Fig. 7). Measuring tumor load in the A31 tumor following anti-Id mAb treatment was achieved due to an unusual level of CD22 Ag on the tumor cells compared with that on the normal B cells in CBA mice. Thus, by staining with FITC-anti-CD22 mAb we were able to distinguish the tumor cells despite their surface Ig being blocked or internalized by the treatment mAb. Unfortunately, a similar distinction could not be achieved with the BCL₁ tumor in BALB/c mice, and consequently, this experiment could not be performed with anti-Id mAb in the BCL₁ model. Interestingly, when anti-CD19 mAb was used for treatment of BCL₁ it failed to control tumor growth and, as shown in the example in Figure 8, sometimes appeared to cause modest growth promotion of tumor for a short period after its administration.

The failure of therapeutic anti-CD19 mAb (and probably anti-Id mAb if it could have been tested) to control tumor growth in the BCL₁ model may simply reflect the large tumor loads present during treatment in these experiments (Fig. 8). In the standard immunotherapy systems shown in Figure 6, anti-Id and anti-CD19 mAb were therapeutically active, but the BCL₁ tumor load at the time of
treatment was less than \( \frac{1}{30} \)th of that present in these tracking experiments (data not shown). Together, these in vivo tracking experiments clearly show that two therapeutic mAb, anti-Id and anti-CD40, appear to be working in very different ways: anti-Id mAb cause a growth arrest that is almost immediate and does not eliminate cells over a period of 5 or 6 days, and anti-CD40 mAb have a delayed effect after 2 to 3 days of uninterrupted growth, but then eradicate lymphoma cells very effectively.

**Discussion**

In this paper we have attempted to correlate the in vitro anti-lymphoma activity of mAb with their therapeutic effectiveness in vivo and to show that therapeutically effective mAb probably work through multiple mechanisms. Two mouse models have been employed, BCL₁ (20) and A31 (21), together with a panel of six mAb specificities (Table I; anti-Id, -CD19, -CD22, -CD40, -CD74, and -MHC class II). In vivo the results were very clear, with only three mAb, anti-Id and anti-CD40 in both tumor models and anti-CD19 in BCL₁, showing good therapeutic activity. All the other mAb showed little or no therapeutic activity despite being given at doses of 2 mg/mouse at a time when tumor burden was low. Interestingly, these in vivo results could not have been predicted from the performance of the same mAb in standard in vitro cytotoxicity assays. In both CDC and ADCC, activity appeared to correlate with the level of mAb binding. Thus, mAb such as anti-MHC class II (M5-114), anti-CD74 (TI2-3), and anti-Id, that bound at high levels tended to give good cytotoxicity, while those that bound at lower levels (anti-CD19, anti-CD22, and anti-CD40) were usually less active. One exception to this general observation was anti-CD74 mAb on BCL₁. This bound at very high levels in both models (>10⁵ molecules/cell) and was fully active on the A31 tumor, but failed to induce marked complement cytotoxicity of BCL₁, or its in vitro variant BCL₁-3B3. We are currently unable to explain this discrepancy.

In growth and clonogenic assays using the in vitro cell line, BCL₁-3B3, although showing the profound effect of “naked” mAb on cell growth, we were again unable to see a correlation between mAb behavior in vitro with the treatment results achieved in mice. In both assays the mAb directed at the BCR (anti-Id and anti-\( \mu \))-caused growth inhibition, particularly the anti-Id mAb in the clonogenic assay, which stopped all cell growth. In addition, the few colonies seen in the clonogenic assay with anti-\( \mu \) were very small and fragile compared with those in controls. Other mAb, such as anti-CD74 and anti-CD40, did not produce results that were consistent between the two assays. Thus, while anti-CD74 caused a marked growth arrest in the short term assay, it had no effect in the clonogenic assay, and the slight growth promotion seen with anti-CD40 mAb in the short term assay did not result in increased cloning efficiency in the second assay. Therefore, it is our belief that the current work casts serious doubt on the ability of conventional cytotoxicity or growth assays to identify useful mAb for the treatment of many types of cancer (5–9) and leaves open the question of what are the main mechanisms operating during mAb treatment of neoplastic cells in vivo.

The most important results obtained in the current work to help explain the mechanisms of action of mAb come from in vivo experiments in which lymphoma cells were either tagged with CFSE dye or monitored by flow cytometry following mAb treatment. This work clearly showed that two therapeutically useful mAb, anti-Id and anti-CD40, control tumor growth in completely different ways: anti-Id mAb caused immediate growth arrest without tumor eradication, and anti-CD40 mAb had no effect on lymphoma growth for 2 or 3 days and then caused a dramatic loss of dividing cells. Both these patterns of tumor control are consistent with metabolic changes in the target cells that could have resulted from transmembrane signaling.

A number of recent investigations have suggested that certain therapeutic mAb, particularly anti-Id, might operate at least in part via a direct effect on cell proliferation, and that such activity probably requires cross-linking activity by the mAb to generate intracellular signals (14, 15, 18, 37). Anti-Id mAb in animals and patients appears to deliver signals that inhibit the growth of B cell tumors and, provided the BCR becomes sufficiently cross-linked by Ab, might even drive cells into apoptosis, as has been shown following treatment of lymphoma-bearing SCID mice treated with polyclonal anti-\( \mu \) Ab (35, 38). Racila et al. (38), working in the BCL₁ model, have presented convincing data showing that anti-Id and anti-\( \mu \) Ab, given passively or produced following active immunization (39), provoke intracellular signals that regulate tumor growth and leave cells in a state of dormancy for extended periods (40). The current work also shows rapid growth arrest of lymphoma cells following anti-Id mAb, especially in the A31 model, and it is quite conceivable that these cells, having not been destroyed by the treatment, might remain dormant for an extended period. In addition, maximum protection in the A31 model requires very little anti-Id mAb (5 \( \mu g/mouse \)), far less than would be required to saturate the target cells for recruitment of effector systems, but perhaps enough to allow signaling for metabolic perturbation.

It has been known for some time that anti-CD19 mAb is therapeutic in xenografts (15) and patients (16). Furthermore, considerable evidence now points to an important signaling component within the therapeutic activity of this mAb specificity (37, 41). This signaling role of anti-CD19 mAb is supported by data showing that when given at very high levels it functions therapeutically as a F(ab')₂, a result that clearly rules out FcR-bearing effectors in this system (15). While our data show that anti-CD19 mAb is therapeutic in the BCL₁ model, we were unable to obtain a clear indication from the tracking experiments of how this reagent was working. Further investigations will be required to show whether tumor cells treated with anti-CD19 mAb follow a similar response pattern as those receiving anti-Id mAb. Interestingly, recent data from normal B cells suggest that the BCR and surface CD19 may be part of a common signaling unit and cooperate in optimizing responses to Ag (42).

While treatment with anti-CD19 and anti-Id mAb may have features in common, our data show that anti-CD40 is operating via a completely different pathway. CD40 is a major costimulatory molecule on normal B cells. It is normally engaged by CD40 ligand on helper T cells during Ab responses and appears to be necessary for driving B cell proliferation and rescuing them from the tolerogenic signals delivered via the BCR (43–45). However, work from Funakoshi et al. (18, 19) has recently shown that the situation is slightly different in many B cell malignancies in which anti-CD40 mAb, if cross-linked appropriately, can result in profound growth arrest of cultured cells. Furthermore, when used in SCID/xenograft models, the same anti-CD40 mAb is effective in protecting mice from tumor development. At least part of this in vivo activity appears to depend on signaling activity of the anti-CD40 mAb because it is partially effective even in the absence of FcR-expressing effector cells. Our current in vivo work confirms and extends these observations and shows that even in syngeneic lymphomas anti-CD40 is therapeutically active. Interestingly, in vitro we found no evidence of growth arrest, but, rather, the anti-CD40 tended to stimulate growth at least in short term culture. This result does not agree with those of Funakoshi et al. (18, 19), who reported only growth arrest in various lymphoma lines. It is quite possible that
the BCL-3/B3 line used here behaves differently from the human B cell lines used previously, or that the assay conditions, such as the level of cross-linking, are critically different in the two studies. Whatever the explanation, it is clear that anti-CD40 mAb can be therapeutically very active and that part of its mechanism of action may result from inhibitory signaling. It is important to note that in normal B cells, signaling via CD40 leads to profound phenotypic changes, including up-regulation of Fas, and that in certain circumstances such surface changes can result in B cell depletion (43, 46). It is possible that similar changes occur following anti-CD40 mAb treatment of neoplastic B cells and that these are also important in the removal of tumor. The current work shows that anti-CD40 mAb operates with very interesting kinetics, in which tumor cells continue to proliferate normally for 2 or 3 days after mAb treatment; only then do their numbers start to decline compared with those in untreated controls (Figs. 7 and 8). This picture is very different from the situation following anti-Id mAb treatment, where cells underwent rapid growth arrest. Investigations are underway to establish the mechanism of anti-CD40 mAb and whether in vivo it works indirectly, perhaps killing cells via the Fas system. The relatively slow kinetics of 2 to 3 days are consistent with up-regulation and activation of surface Fas; however, it is not clear how this Fas would be engaged, since Fas ligand is generally on activated T cells and, as yet, information is not available to show whether such activation occurs during mAb therapy (43, 47). As an alternative explanation we will also be considering whether in vivo the anti-CD40 mAb, rather than delivering a direct or indirect cytotoxic effect, operates by blocking critical growth signals that are dependent on the expression of surface CD40.

In conclusion, it is probably more than coincidence that those mAb, such as anti-Id, anti-19, and anti-CD40, that have been successful in the treatment of two mouse B cell lymphomas are all directed at key signaling molecules. Under normal circumstances these same receptors would be required to drive B cell activation and differentiation following interaction with Ag (BCR) and Th cells (CD40). It is now clear that such responses are highly coordinated and only occur when an array of signals is delivered following the appropriate receptor-ligand interactions. It appears that cells that are unable to receive a full complement of these signals are probably rendered anergic or may be deleted and thereby prevented from responding inappropriately to Ag. We speculate that a somewhat similar process may occur when these receptors are heavily cross-linked by individual mAb on lymphoma cells. Such treatment would be expected to deliver an intense, but inappropriate, signal via a single receptor. The target cell, “knowing” that it was not receiving all the signals required for a specific immune response, may be triggered into growth arrest and apoptosis. At present it appears that there are at least two pathways to such inappropriate signaling, one via the BCR and possibly CD19 (46) which is unlikely to involve Fas (48), and a second through CD40, where Fas up-regulation is a distinct possibility (43, 47). If correct, then the implications of such a mechanism on the design of future mAb and vaccine design would be profound. No longer should we think of anti-cancer Ab simply as glycoproteins that recruit cytotoxic effectors; special attention must be given to Ab specificity and their signaling activity on target cells. A major part of this new thinking should include reassessing the in vitro assays normally used for selecting potential therapeutic agents, with perhaps more emphasis being placed on systems that detect Ab-induced transmembrane signaling and triggering of metabolic changes in cell growth and gene expression.

Acknowledgments

We thank Prof. George Stevenson together with members of the Tenevos Research Laboratories for helpful discussion during this project. We are also indebted to Lisa Davies, Maureen Power, and Richard Reid for excellent technical assistance throughout, and to Prof. Ellen Vitetta for the BCL-3/tumor line.

References


