Monoclonal Antibodies to Idiotype Inhibit In Vitro Growth of Human B-Cell Lymphomas

By Peter M. van Endert, Bernd Heilig, Günter J. Hämmerling, and Gerhard Moldenhauer

REGULATION of B cell growth and activation is one function of membrane-bound Ig that serves as the antigen receptor for mature as well as immature B cells. In murine and human resting mature B cells, crosslinking of surface IgM and IgD induces activation, as indicated by RNA synthesis, size enlargement, and induction of major histocompatibility complex (MHC) class II antigen expression. In addition, membrane Ig ligation alone or in combination with soluble factors may induce proliferation.

In contrary, splenic and liver B cells from neonatal or very young mice are rendered unresponsive to antigen by pretreatment with anti-Ig antibodies or antigen. The inhibition of antigen-induced growth and Ig secretion has been considered an in vitro equivalent to B-cell tolerance. Only membrane IgM, but not IgD, seems to deliver tolerizing signals to immature B cells. Similar to immature normal B cells, the growth of some murine lymphomas is inhibited by surface Ig crosslinking so that these tumors have been considered as models of B-cell tolerance.

In malignant human B-cell populations, both proliferative and inhibitory signaling via the antigen receptor have been reported. Among the chronic lymphocytic leukemia (CLL) tumors that have been studied extensively, most were described to synthesize DNA upon stimulation either with anti-IgM alone or in combination with a variety of lymphokines or growth factors. However, in one case of CLL and two of hairy cell leukemia, growth inhibition by surface Ig crosslinking was observed. Such tumors, exhibiting the phenotype of immature B cells, may serve as models of B-cell tolerance in humans.

Besides their signaling function, Ig receptors determine the specificity of B-cell antibody secretion by displaying an enormous degree of diversity in the antigen-combining site. B-cell tumors, usually exhibiting a monoclonal idiotype, thus possess a highly individual surface marker that can be used for targeting therapeutic reagents. Monoclonal antibodies (MoAbs) recognizing idiotypic determinants on human B-cell lymphomas have been used for therapeutic purposes in more than 28 patients. Prolonged infusion of high doses of anti-idiotype (anti-Id) antibodies alone or in combination with α-interferon (α-IFN) was reported to result in partial or complete responses in 60% of the patients. The mechanism of these responses, however, could not yet be elucidated.

We report here that MoAbs recognizing idiotypic determinants drastically inhibit in vitro spontaneous DNA synthesis in three low- or intermediate-malignancy human B-cell lymphomas of different histologic subtypes. In two of these tumors, suppression of spontaneous tumor cell DNA synthesis persisted when MoAbs were removed after incubation for 48 hours or more. These results indicate that, in anti-Id therapy for B-cell lymphoma, inhibitory signaling via surface Ig may contribute to tumor regression.

MATERIALS AND METHODS

Patients and tumor cells. Patients were selected in the Medical Clinic and Policlinic, University of Heidelberg, Germany. All had been diagnosed by histologic and immunohistochemical investigation of lymph node or bone marrow specimen as having low- or intermediate-malignancy non-Hodgkin lymphoma (NHL). Using the Kiel classification, histologic diagnoses were centrocytic lymphoma in patient PH, immunocytoma in patients AS, TR, and LH, and CLL in patient MZ. The corresponding classification according to the Working Formulation is malignant lymphoma, follicular mixed small and large cell (PH), small lymphocytic plasmocytoid (AS, TR, and LH), and small lymphocytic consistent with CLL (MZ). All patients exhibited leukemia with a white blood cell count above 10,000/mm3.

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Tumor cells were obtained by leukapheresis and by Ficoll density cytometric analysis (see below) of pan-B-cell antigen expression. Tumor cells were obtained from PBMC using a modification of the direct or stored in liquid nitrogen until use. PBMC were either used or obtained. Tumor PB mononuclear cells (PBMCs) were used for proliferation assays. When cells were analyzed for contaminating non-B cells by flow cell (WBC) count of 25,000/μL in patient AS and between 80,000 and 400,000/μL in the other patients. With the exception of patient AS, peripheral blood lymphocytes (PBL) from all patients contained more than 97% lymphoma cells as determined by a flow cytometric analysis (see below) of pan-B-cell antigen expression. Tumor cells were obtained by leukapheresis and by Ficoll density gradient purification of mononuclear cells from heparinized blood samples. Cell material was obtained in a clinical trial of antidiotype therapy for which an informed written consent had been obtained. Tumor PB mononuclear cells (PBMCs) were either used directly or stored in liquid nitrogen until use.

**Tumor cell purification.** For selected assays, highly purified B cells were obtained from PBMC using a modification of the methods described by Thiele and Lipsky. Before this purification, PBMC from patients PH, LH, TR, and MZ contained 1% to 1.5% cells expressing the T-cell antigens CD2 and CD3 and less than 1% staining for the myelomonocytic antigen CD14, as assessed by a flow cytometric analysis (see below). PBMC from patient AS contained 10% T cells and 7% cells belonging to the myelomonocytic lineage. For depletion of contaminating cells, PBMC were first treated with L-leucine methyl ester, a lysosomotropic agent known to kill human monocytes, natural killer (NK) cells, and, in part, CD8 T cells. Briefly, PBMC were suspended in serum-free RPMI 1640 medium (GIBCO, Karlsruhe, Germany) at 5 × 10^6/ml in the presence of 5 × 10^-3 mol/L L-leucine methyl ester (Sigma, Munich, Germany). Cells were incubated for 45 minutes at room temperature, washed in complete medium, and resuspended twice with sheep red blood cells (Behring, Marburg, Germany) treated with amino ethylisothiouroniumbromide hydrobromide (AET, Sigma). Cells not forming rosettes in the second set were considered as virtually pure B cells and were used for proliferation assays. When these cells were analyzed for contaminating non-B cells by flow cytometry, no T cells, myelomonocytic cells, or NK cells were detectable. In addition, highly purified cells did not synthesize DNA upon stimulation with 1 μg/ml phytohemagglutinin (PHA; Wellcome, Hannover, Germany) so that T cells seemed completely eliminated.

**Proliferation assays.** Proliferation assays were performed in RPMI 1640 medium supplemented with 5 × 10^-5 mol/L mercaptoethanol, nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine (all supplements from GIBCO), and fetal calf serum (FCS; Seromed, Berlin, Germany) or pooled heat-inactivated normal human serum. Serum concentration and sources were adjusted to the requirements of the individual tumor clones (see Results section). Tumor PBMCs were suspended at 2 × 10^6/ml in complete medium and seeded into 96-well flat-bottom polystyrene tissue culture plates (Costar, Cambridge, MA). Unless indicated otherwise, tumor cells were cultured for a total period of 5 to 7 days. All experiments were set up in triplicates. Sixteen hours before the scheduled end point of the experiment, 1 μCi of [³H]-thymidine (5 Ci/mmol; Amersham, Braunschweig, Germany) was added per well. Plates were harvested onto glass fiber filters using a Skatron cell harvester (LKB, Freiburg, Germany). BALB/c mice were injected IP into pristane primed BALB/c mice. MoAbs were purified from ascitic fluid by chromatography using diethyl aminomethyl (DEAE)-Affi Gel Blue (Biorad, Munich, Germany). BALB/c mice were treated with 5 × 10^6 viable tumor cells in FCS-medium with 0.1% NaN₃, were infected with 1 μg/ml anti-IgM MoAb, and incorporated radioactivity was measured with a liquid scintillation counter.

**Production of MoAb fragments.** F(ab')₂ and Fab' fragments of MoAbs with IgG1 isotype were produced using the methods described by Parham. MoAbs were adjusted to 2 mg/ml in 0.1 mol/L sodium citrate buffer (pH 3.5) and digested with 35 μg/ml pepsin (Sigma) at 37°C for 1 to 3 hours. The reaction was stopped by the addition of 1/10 volume of 1 mol/L Tris buffer (pH 8.5). Undigested Ig was removed by ion exchange fast protein liquid chromatography (FPLC) over a MonoQ column (Pharmacia). Resulting F(ab')₂ fragments were further processed by reduction with 10 mmol/L L-cysteine for 30 minutes at 37°C. After acetylation with 20 mmol/L iodoacetamide, bivalent and monovalent fragments were separated by gel filtration over a Superose 12 column (Pharmacia). In silver-stained 8.75% polyacrylamide SDS gels, the molecular weight of unreduced complete MoAbs was about 150 Kd, while F(ab')₂ fragments were found at about 120 Kd and monovalent fragments at about 50 Kd. The purity of the MoAb preparations was greater than 96%.

**Quantification of surface Ig molecules.** Cell surface Ig density was quantified by Scatchard plot analysis. Briefly, 100 μg of anti-IgM MoAb NMZ88 was labeled with 1 mCi of [¹²⁵I]iodide (Amersham) by the chloramine T method. Triplicate samples of 1 × 10^6 viable tumor cells in FCS-medium with 0.1% NaN₃, were incubated for 60 minutes at room temperature with increasing amounts of [¹²⁵I]-labeled MoAb (10³ to 3 × 10⁶ cpm). The number of cell surface Ig molecules and the avidity of MoAb was calculated assuming a one-site binding model.

**Immunofluorescent staining and flow cytometry.** For all dilutions and washings in the staining for flow cytometric analysis, RPMI medium containing 2% FCS and 0.1% NaN₃ was used. First step reagents included the MoAbs to Ig heavy chains and to tumor Ig idiotypes described above. The following MoAbs recognizing lymphocyte surface antigens, all derived from the International Workshop and Conference on Human Leucocyte Differentiation Antigens: were used: J5 (CD10), VIM13 (CD14), HD237 (CD19), B1 (CD20), B2 (CD21), HD50 (CD23), VIB-CS (CD24), anti-Tac
(interleukin-2 [IL-2] receptor, CD25), HD28 (CD37), and PA-1 (transferrin receptor, CD71). C156 (CD5) was kindly provided by Dr C. Jägle (Heidelberg, Germany); ISCR3 (HLA-DR) originated from Dr J. Johnson (Munich, Germany); and JA11 (IgD) was obtained from Dianova (Hamburg, Germany). OKT11 (CD2) was purchased from Ortho (Ortho Diagnostics Systems, Neckargemünd, Germany). OKT3 (CD3) hybridoma was obtained from American Type Culture Collection (Rockville, MD). The NK cell marker Leu7a was purchased from Becton Dickinson (Heidelberg, Germany). Fluorescent labeling of cells was performed by a standard indirect staining, using fluorescein isothiocyanate (FITC)-conjugated F(ab')2 goat antirabbit antibodies (Dianova) as second-stage reagents. Green fluorescence of viable cells was analyzed in the presence of propidium iodide (1 µg/mL) for exclusion of dead cells on a FACScan cytometer (Becton Dickinson).

Indirect cytotoxicity assay and determination of cell viability. Fresh lymphoma cells isolated from PB by density gradient centrifugation were suspended at 6 × 10^6/mL in RPMI 1640 medium with 0.5% FCS and 0.1% NaN3. Fifty microliters of cell suspension was mixed with 50 µL of appropriate dilutions of MoAb (10 to 100 µg/mL) and incubated for 4 hours at 4°C. After centrifugation and removal of the supernatant, cells were resuspended in serial dilutions of Low tox baby rabbit complement (Cedarlane, Hornby, Canada) or autologous serum and incubated 45 minutes at 37°C. Viability was determined by staining with acridine orange/ethidium bromide.

RESULTS

Requirements for lymphoma cell DNA synthesis in vitro. Five low- or intermediate-malignancy B-cell lymphomas were tested for cell density and serum conditions required for in vitro DNA synthesis. Two of these, plasmocytoid lymphomas AS and TR, exhibited a very low rate of unstimulated DNA synthesis even when cultured with 5% human or fetal serum and at cell densities of 2 × 10^6 cells/mL or higher. DNA synthesis of centrocytic lymphoma PH was best supported by incubation in 10% human serum. DNA synthesis of MZ CLL cells was critically dependent on cell densities of 2 × 10^6 cells/mL or higher. DNA synthesis of centrocytic lymphoma PH was best supported by incubation in 10% human serum. DNA synthesis of MZ CLL cells was dependent on mitogenic effects of 15% FCS. LH tumor cells showed the highest rate of spontaneous DNA synthesis even when cultured with 5% human or fetal serum and at 5 × 10^6 cells/mL. In all lymphomas, the rate of DNA synthesis varied with the serum batch used and between different time points at which cells had been obtained, and fresh tumor cells showed a higher proliferation than thawed cells.

Anti-idiotypic MoAbs inhibit spontaneous lymphoma cell DNA synthesis in vitro. MoAbs recognizing idiotypic determinants on individual tumors were raised by immunization with purified soluble tumor Ig. MoAbs were considered to be anti-idiotypic if they bound to tumor cells and soluble Ig derived from the autologous lymphoma, but not to control lymphoma cells and Ig of the same isotype and to a panel of tonsilary B cells from different healthy donors (data not shown). With the exception of MoAb AAS108 (isotype IgG2b), all anti-idiotypic MoAbs used in this study were of IgG1 isotype.

Patient PBMCs contained low amounts of contaminating T cells and monocytes (17% in patient AS, about 2% in other patients). To rule out indirect effects of anti-Id MoAb on contaminating cells, we used highly purified B cells for the proliferation assays. These cells contained no detectable contamination with T cells, monocytes, and NK cells and, in contrast to unpurified PBMCs, did not proliferate in response to the T-cell mitogen PHA.

Interestingly, proliferation of highly purified PH and LH lymphoma cells was significantly inhibited by PHA (Table 1). This effect could be due to an "abortive activation" of these tumor cells by the mitogen, which has been described in B cells treated with other mitogens.25 When highly purified tumor cells were cultured in the presence of 1 µg/mL anti-Id MoAb, the DNA synthesis of PH, LH, and MZ tumor cells was strongly inhibited, while AS and TR cells were not affected (Table 1). Control anti- HLA-DR MoAb ISCR3 had no effect on the 3H-thymidine incorporation of purified tumor cells.

The data in Table 1 represent maximum 3H-thymidine incorporation observed with lymphomas AS and TR. The absolute level of DNA synthesis by LH, PH, and MZ tumor cells exhibited considerable variation between individual experiments, depending on the batch of FCS used, the day of blood collection from the patients, and, especially, whether fresh or thawed cells were used. Spontaneous 3H-thymidine incorporation by thawed cells was significantly decreased after treatment with L-leucin methyl ester and rosetting. Data shown for lymphomas LH, PH, and MZ in Table 1 represent an average level of DNA synthesis observed with these tumor cells, as judged from at least five separate experiments. Due to the variation of background DNA synthesis, subsequent experiments were all performed at least three times.

Tumors susceptible and resistant to anti-Id MoAb-mediated inhibition do not differ in phenotype. Morphologic diagnosis and cell surface antigen expression of the lymphomas was

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Medium</th>
<th>PHA</th>
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<tr>
<td>PH</td>
<td>14,477 ± 539</td>
<td>3,906 ± 423</td>
</tr>
<tr>
<td>AS</td>
<td>1,322 ± 211</td>
<td>1,637 ± 312</td>
</tr>
<tr>
<td>LH</td>
<td>22,226 ± 593</td>
<td>2,100 ± 177</td>
</tr>
<tr>
<td>TR</td>
<td>2,184 ± 129</td>
<td>2,015 ± 282</td>
</tr>
<tr>
<td>MZ</td>
<td>5,291 ± 367</td>
<td>4,382 ± 345</td>
</tr>
</tbody>
</table>

**Table 1. Anti-Id MoAbs Inhibit Lymphoma Proliferation**

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>HLA-DR</th>
<th>Idiotype</th>
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<tbody>
<tr>
<td>PHA</td>
<td>13,988 ± 422</td>
<td>456 ± 29</td>
</tr>
<tr>
<td>AS</td>
<td>1,284 ± 49</td>
<td>1,391 ± 146</td>
</tr>
<tr>
<td>LH</td>
<td>24,173 ± 1,773</td>
<td>176 ± 21</td>
</tr>
<tr>
<td>TR</td>
<td>2,817 ± 323</td>
<td>2,234 ± 219</td>
</tr>
<tr>
<td>MZ</td>
<td>5,482 ± 514</td>
<td>211 ± 15</td>
</tr>
</tbody>
</table>

T-cell- and monocyte-depleted tumor cells from PB were cultured in medium only, with PHA (1 µg/mL) or with 1 µg/mL MoAb to idiotype or HLA-DR. Cells were cultured in 15% (MZ, AS, and TR) or 10% (LH) FCS or in 10% human serum (PH). After incubation for 120 hours, 3H-thymidine was added for an additional 16-hour period. Mean cpm of triplicates is given ± standard deviation.
compared to detect features correlating with sensitivity or resistance to anti-Id-mediated inhibition of DNA synthesis. Four of five tumors belonged to the subgroup of small lymphocytic lymphomas, with one CLL and three immunocytomas. Among these four, two were susceptible and two were resistant to inhibition (Table 2). In addition, a single centrocytic lymphoma in the study was sensitive.

To test whether the number of cell surface Ig molecules correlated with inhibition by anti-Id, a Scatchard analysis was performed using a $^{125}$I-labeled MoAb recognizing $\lambda$ heavy chain. All tumors expressed surface Ig of IgM isotype (Table 3). Both tumors with high amounts of surface Ig and one of three lymphomas with low Ig density were sensitive to inhibition (Table 2). Therefore, neither morphology nor membrane IgM density correlated with inhibitory effects of anti-Id.

In a flow cytometric analysis, all tumors stained with MoAb to pan-B–cell antigens CD19, CD20 and HLA-DR (Table 3). Tumors with low surface Ig density expressed a number of further B-restricted antigens (CD21, CD23, and CD24) and, in the case of AS and MZ, the CD5 antigen associated with CLL.26 In addition, both in susceptible and resistant tumors, IgD expression was found equivalent to (AS, LH, and MZ) or below (PH and TR) IgM surface expression.

MoAbs to IgM but not to IgD inhibit lymphoma DNA synthesis. Because IgM and IgD molecules on B-cell lymphomas express the same idiotype,27 anti-Id MoAbs may exert their inhibitory effect via surface Ig of one or both isotypes. We therefore investigated the effects of two MoAbs recognizing distinct epitopes (data not shown) on $\mu$ heavy chain and two MoAbs binding to $\delta$ heavy chain. Before the assays, these MoAbs were shown to bind to surface Ig on lymphomas PH, LH, and MZ in a flow cytometric analysis (Table 3). Among the MoAbs recognizing $\mu$ chain, MoAb NMZ88 bound more strongly than NMZ8. Figure 1 shows that in all inhibitable lymphomas, MoAb to IgM but not to IgD could substitute for anti-Id MoAb. The inhibitory potency of anti-IgM MoAb was slightly (LH and MZ) to considerably (PH) lower than that of anti-Id MoAb. Corresponding to a weaker staining in FACS analysis, inhibition by NMZ8 was weaker than by NMZ88.

Because anti-IgM MoAbs have been reported to elicit mitogenic effects at higher concentrations,28 a dose-response curve of anti-Id and anti-IgM MoAbs was performed. Optimal inhibition by anti-Id MoAb was achieved at a concentration of 0.5 to 2 $\mu$g/mL. Both anti-Id and anti-IgM MoAbs were still inhibitory at concentrations as high as 250 $\mu$g/mL (Fig 1).

Anti-IgD MoAbs HD9 and JA11 did not affect the DNA synthesis by lymphomas at concentrations less than 50 $\mu$g/mL (Fig 1). The lack of inhibition by anti-IgD MoAb could not be due to a lack of binding to the lymphoma cells because clear surface staining was achieved with both MoAbs used for the proliferation assays (Table 3). At higher concentrations of anti-IgD MoAb HD9, DNA synthesis in tumors PH and MZ was affected to a maximum of 40% inhibition at 250 $\mu$g/mL. When a threefold excess of an isotype-matched control anti-IgD MoAb (AAS108, IgG2b) was added to the cultures to block Fc receptors on tumor cells, the effect of high concentrations of HD9 was abolished (data not shown). Therefore, the observed inhibition may have been caused by a physical crosslinking of surface IgD with receptors for Fc, a well-known phenomenon that requires ligation of both proteins by the same antibody molecule.

Inhibition of lymphoma cell DNA synthesis is dependent on crosslinking but not on Fc-mediated effects. To rule out the possibility that crosslinking of IgM surface molecules with Fc receptors caused the observed inhibition, MoAb fragments were produced by pepsin digestion and subsequent reduction. As shown in Fig 2, undigested antibodies and F(ab')$_2$, fragments of anti-Id and anti-IgM MoAb were equally efficient in mediating inhibition of DNA synthesis in LH lymphoma cells, while equal concentrations of monovalent Fab' fragments of both reagents had no effect. Equivalent results were obtained when MZ and PH tumor cells were cultured in the presence of MoAb fragments (data not shown). Therefore, growth inhibition required crosslinking of surface IgM molecules but not Fc receptor interaction.

Differential survival of tumor cells under long-term anti-Id incubation. The observed inhibition of DNA synthesis after prolonged incubation with anti-Id MoAb could be a

### Table 2. Surface Ig Density on Lymphomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Histologic Diagnosis</th>
<th>IgM Molecules/Cell</th>
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<tbody>
<tr>
<td>PH</td>
<td>Centrocytic lymphoma</td>
<td>223,000</td>
</tr>
<tr>
<td>AS</td>
<td>Immunocytoma</td>
<td>5,800</td>
</tr>
<tr>
<td>LH</td>
<td>Immunocytoma</td>
<td>42,700</td>
</tr>
<tr>
<td>TR</td>
<td>Immunocytoma</td>
<td>3,800</td>
</tr>
<tr>
<td>MZ</td>
<td>CLL</td>
<td>3,300</td>
</tr>
</tbody>
</table>

The lymphomas were classified according to the Kiel nomenclature, based on the histochemical studies of lymph node biopsies. IgM molecules on the surface of lymphoma cells were quantified by Scatchard analysis of $^{3}H$-labeled MoAb NMZ88 (anti-IgM) binding.
Fig 1. Effect of anti-Id, anti-IgM, and anti-IgD MoAbs on the in vitro growth of lymphoma cells. Purified B cells from the PB of indicated patients were cultured in medium alone (○) or with increasing amounts of MoAb to idiotype (■), to IgM (+→+ and +→→+), or to IgD (□→□). 3H-thymidine incorporation was measured after 6 days of incubation. Standard deviations were less than 15% of the data.

Consequence of direct tumor cell killing. In a short-term cytotoxicity assay with rabbit or autologous human serum as a source of complement, no decrease of tumor cell viability was detected (data not shown). This result was not unexpected because all of the anti-Id MoAbs were of IgG1 isotype, which does not bind complement. Therefore, tumor cells were incubated for 10 days in the presence or absence of anti-Id MoAb and cell number and viability were determined in 3-day intervals during this period. Data shown in Fig 3 are representative for three independent experiments. Consistently, the pattern of cell survival was unique to each tumor. When incubated in medium alone, PH, LH, and MZ tumor cells showed a viability of 80% or better at all time points. In the presence of anti-Id MoAb, however, the viability of PH and LH tumor cells was markedly decreased. Impairment of tumor cell viability was first detectable on day 4 (PH) or 6 (LH) and resulted in a 35% rate of viable cells on day 10. In contrast, the viability of MZ cells was not affected by the addition of anti-Id MoAb.

Prolonged incubation with anti-Id MoAb may cause suppression or stimulation of DNA synthesis after MoAb removal. Because most of the tumor cell (in tumor MZ) or at least subpopulations (PH and LH) survived long-term anti-Id incubation, we next asked whether suppression of DNA synthesis would be reversible or persistent after the withdrawal of MoAb. Given the observed absence of direct tumor cell killing, only a persistent suppression would suggest a clinical usefulness of the reported inhibitory effects of anti-Id MoAb. Tumor cells were incubated with anti-Id for 4 hours to 6 days, washed extensively, adjusted to a density of 1 × 10^6 (LH) or 2 × 10^6 (PH and MZ) viable cells per milliliter and recultured in medium alone or with anti-Id MoAb for a further period of 5 days. At this time point, no MoAb could be detected on the surface of lymphoma cells in a flow cytometric analysis (not shown) if cells had been cultured in the absence of anti-Id MoAb after washing. When the MoAb was removed by washing, tumors PH and LH did not resume spontaneous DNA synthesis after 2 days of incubation with anti-Id (Fig 4). For tumor PH, 4 hours of incubation was sufficient to completely inhibit further spontaneous 3H-thymidine incorporation. In contrast, MZ cells showed an equal or even considerably higher rate of DNA synthesis after anti-Id MoAb was withdrawn. Thus, depending on individual lymphomas, either enhancement or suppression of spontaneous tumor cell DNA synthesis occurred after prolonged preincubation with anti-Id MoAb.

DISCUSSION

We have shown that in human B-cell lymphomas of different histologic types, anti-Id antibodies frequently...
suppress DNA synthesis and may even induce spontaneously irreversible arrest of DNA synthesis in vitro. While inhibitory signaling by surface Ig ligation in murine,5,7,30 as well as human31,32 normal and malignant B cells has been described previously, the effects of anti-Id antibodies on the proliferation of B-cell lymphomas are of special interest both in a physiologic and a clinical context. Firstly, different from commonly used heavy chain-specific reagents, anti-Id MoAbs bind to Ig receptors of both μ and δ isotype, thereby closely resembling the physiologic ligand of surface Ig, nominal antigen. Ligation of surface IgM and IgD has been reported to exert distinct effects,31 and simultaneous crosslinking of IgD molecules may protect B cells from negative signaling by IgM.31 Secondly, promising results have been achieved in a considerable proportion of patients treated with anti-Id MoAb,44 and a study of the direct effects of anti-Id MoAb may help to understand the mechanism of tumor regression as well as to determine which patients are promising candidates for this therapeutic approach.

In the lymphomas described here, the effects of anti-Id MoAb could not be distinguished from those of anti-μ heavy chain antibodies. In accordance with a previous report,51 ligation of surface IgD molecules did not inhibit tumor cell DNA synthesis. Furthermore, an IgD-mediated protection from IgM-mediated inhibitory signaling that was observed in another system35 was not detected. This lack of
inhibitory signaling by surface IgD molecules could not be explained by a lack of anti-Id MoAb binding to IgD or a low surface IgD density. Anti-Id MoAb precipitated both Ig isotypes from the surface of tumor cells. In addition, tumor LH, which was highly sensitive to the inhibitory effects of anti-Id but completely unresponsive to anti-8 MoAb, exhibited similar high surface staining with both reagents. However, the observation, that high concentrations of anti-IgD MoAb partially inhibited DNA synthesis in clones PH and MZ may indicate that, as reported previously, concomitant ligation of IgD molecules and Fc receptors was able to transmit inhibitory signals in the investigated clones. Our experiments nevertheless show that, in the absence of additional signaling by other surface receptors, only IgM receptors could transmit inhibitory signals.

Similar to models of tolerance induction, the inhibition of lymphoma cell DNA synthesis was a direct effect of surface IgM crosslinking and did not involve Fc receptor ligation, as evidenced by an equal inhibitory potential of complete MoAb to idiotype or Fc, heavy chain and their F(ab')2 fragments, respectively. Monovalent Fab' fragments had no effect.

In the study reported here, three of five randomly selected low malignancy B-cell lymphomas were sensitive to inhibitory signaling by surface IgM. In humans, only a few similar cases have been described. Mongini et al reported two cases of hairy cell leukemia, a rare lymphoma subtype, and one CLL that were inhibited by anti-μ MoAbs. In two of these tumors, only T-cell supernatant or B-cell growth factor (BCGF)-induced growth could be blocked by anti-λ antibody. In addition, Baeker and Rothstein found inhibition of mitogen-induced proliferation in three of 24 tested lymphomas. The tumors investigated by us belonged to different frequently diagnosed subtypes of NHL, and proliferation was investigated in the absence of growth factors besides human or bovine serum. Our results indicate that both lymphomas of small lymphoctic and follicular center cell morphology are frequently sensitive to anti-IgM-mediated growth inhibition in vitro so that the majority of low-malignancy NHLs may receive negative signals by surface IgM. Differing from our data, most published studies have not described inhibitory effects of anti-IgM on low-malignancy lymphoma cell DNA synthesis but have focussed on the requirements for stimulation by anti-Ig. In a variable proportion of the tumors, proliferation could be induced by stimulation with a-μ antibody predominantly in combination with phorbol esters. B-cell growth factor, or T-cell supernatant. Thus, depending on the experimental system, both positive and negative effects of surface Ig crosslinking in B-cell lymphomas have been described, suggesting that factors such as the quantity of the signal and the presence of additional signals determine the outcome of surface Ig ligation in lymphomas.

Interestingly, our results are in contrast to the report of Lowder et al, who investigated the in vitro proliferation of seven lymphomas, three of which had undergone a partial or complete clinical response to anti-Id treatment in vivo. Only one of these showed a reduction of 37% and 86% on days 5 and 7, respectively, when cultured in the presence of anti-Id MoAb. How does this fit in with our observation of inhibitory effect in the majority of the tumors?

Inhibition of DNA synthesis cannot be observed in the absence of a sufficient level of spontaneous [H]-thymidine incorporation, which was shown to be critically dependent on several variables in the cases reported. These variables comprised cell density and source and concentration of serum supplement. Thus, spontaneous DNA synthesis of most lymphomas in vitro is highly dependent on cell culture conditions, and under inappropriate conditions growth inhibition by anti-IgM reagents may well be overlooked. In the study by Lowder et al, tumor cells were cultured in 5% FCS at 1 × 10^6 cells/mL. Among the five tumors studied here, only lymphoma LH would have proliferated under these conditions. Similarly, among the seven lymphomas investigated by Lowder et al, only the tumor with a significant spontaneous DNA synthesis could be inhibited by anti-λ. This lymphoma underwent a major clinical response, suggesting that in vitro inhibition of DNA synthesis may correlate with in vivo tumor regression after anti-Id therapy.

None of the phenotypic variables tested in this study correlated significantly with tumor susceptibility to inhibition. These parameters included histologic subtype, surface IgM density, surface IgM to IgD ratio, and leukocyte antigen expression. First, both of the two resistant and two of the three susceptible tumors belonged to the small lymphocytic subtype. Second, the very low amounts of surface IgM that were found in both resistant lymphomas were detected in one of the susceptible tumors, too. Lowder et al found no correlation between surface Ig density and modulation on tumor cells and MoAb isotype or avidity and clinical responses. Third, a clearly predominant IgM expression as well as a roughly equal density of IgM and IgD (as assessed by a flow cytometric analysis) could be found in both groups. Finally, similar patterns of expression of differentiation antigens were displayed. While no phenotypic differences were detectable, resistant tumors constantly showed a lower rate of spontaneous thymidine incorporation in vitro. One might speculate that inhibition could not be observed in these lymphomas because the adequate conditions for significant spontaneous growth were not provided.

What is the significance of the observed inhibition of DNA synthesis for anti-Id MoAb treatment in vivo? Only therapeutic trials including both lymphomas that have been characterized as resistant and susceptible to inhibition of thymidine incorporation by MoAb in vitro can answer this question conclusively. Some issues, however, can be tested in vitro: can tumor cells recover from anti-Id-mediated growth arrest, and does incubation with anti-Id MoAb impair lymphoma cell viability? We performed long-term incubation assays for up to 1 week, thus mimicking in vivo therapies in which MoAb levels of more than 1 μg/mL were maintained for more than 2 weeks. Our data indicate that the biologic response of lymphomas to prolonged incubation with anti-Id MoAb may vary considerably. Tumor MZ may represent lymphomas in which prolonged anti-Id treatment does not affect or even enhances tumor cell
survival so that the lymphomas resume increased growth after cessation of anti-Id treatment, possibly resulting in clinical progression. This tumor thus seems to be different from CLL tumors that progressed to a prolymphocytic inhibition of DNA synthesis when incubated with anti-\( \mu \) MoAb.\(^7\) Other lymphomas, such as immunocytoma LH and, most significantly, centrocytic lymphoma PH, may not be able to recover spontaneously from negative signaling by anti-Id MoAb. As shown in tumor PH, even surface IgM-mediated signaling for a few hours can be sufficient for permanent arrest of DNA synthesis, and decreased tumor cell viability. It is tempting to speculate that the apoptosis that has been reported to occur in a high proportion of normal germinal center B cells\(^8\) could be induced by antigen or anti-Id in certain lymphomas such as PH that are related to germinal center cells in morphology. Taken together, our results indicate that anti-Id therapy could take advantage of the particular sensitivity of a majority of B-cell lymphomas to negative signaling by the antigen receptor. Further studies are needed to determine the frequency of sensitive lymphomas among tumors of different subtypes and to clarify the relationship between in vitro and in vivo responses to anti-Id treatment.

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