Binding to CD20 by Anti-B1 Antibody or F(ab’)2 is sufficient for induction of apoptosis in B-cell lines

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Abstract CD20 is a B-cell-specific cell surface protein expressed on mature B lymphocytes and is a target for monoclonal antibody therapy for non-Hodgkin’s lymphoma (NHL). Though clear clinical efficacy has been demonstrated with several anti-CD20 antibodies, the mechanisms by which the antibodies activate CD20 and kill cells remain unclear. Proposed mechanisms of action include complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and induction of apoptosis. In this report we compared the activity of two anti-CD20 antibodies, Anti-B1 Antibody (tositumomab) and rituximab (C2B8), in a variety of cellular assays using a panel of B-cell lines. Anti-B1 Antibody showed a low level of activity in a CDC assay against complement-sensitive B-cell lines, Ramos and Daudi. We found that there is an inverse correlation between the expression of CD55 and CD59 and CDC mediated by either Anti-B1 Antibody or rituximab. Rituximab was more potent at inducing CDC when compared to Anti-B1 Antibody. Using Raji cells as target cells and human peripheral blood leukocytes as effector cells, Anti-B1 Antibody was a potent inducer of ADCC. The activities of Anti-B1 Antibody and rituximab were nearly identical in the ADCC assay. In addition, Anti-B1 Antibody showed direct induction of apoptosis in all B-cell lines tested. In general, cross-linking Anti-B1 Antibody with a goat anti-mouse Ig did not further enhance the percentage of cells undergoing apoptosis. Importantly, a F(ab’)2 fragment of Anti-B1 Antibody induced apoptosis, while the Fab fragment did not, indicating that the Fc region was not required and dimerization of CD20 may be sufficient for induction of apoptosis. In contrast, rituximab, which binds to an overlapping epitope on CD20 with a three-fold lower affinity than Anti-B1 Antibody, did not efficiently induce apoptosis in the cell lines tested in the absence of crosslinking. In conclusion, these two anti-CD20 antibodies have overlapping, but distinct mechanisms of action on B-cell lines.

Keywords CD20 · Anti-B1 Antibody · Rituximab · Apoptosis · Antibody therapy

Introduction

CD20 is a cell surface protein expressed on B-cell precursors and mature B cells, but is lost following differentiation to plasma cells [3]. Greater than 90% of B-lymphocytic lymphomas express CD20, while T-lymphocytic lymphomas are CD20-negative [27].

Many recent advances have been made in antibody-targeted therapies in non-Hodgkin’s B-cell lymphoma (NHL) [4]. Rituxan (C2B8, rituximab) is a mouse:human chimeric IgG1 anti-CD20 antibody and was the first monoclonal antibody approved by the FDA for the treatment of relapsed, low-grade and follicular lymphoma (for review see [20]). Another anti-CD20 antibody, Anti-B1 Antibody (tositumomab), is a mouse IgG2a currently under clinical investigation as one component of Bexxar therapy for the treatment of low grade NHL [16, 31, 17]. The efficacy of Bexxar, a novel radioimmuno therapy consisting of Anti-B1 Antibody followed by131I-labeled antibody, has been demonstrated in multiple studies in patients with relapsed/refractory disease, previously untreated low-grade NHL and in Rituxan failures [16, 31, 13, 7, 15]. Furthermore, clinical studies with unlabeled Anti-B1 Antibody have demonstrated significant anti-tumor activity and have shown efficacy similar to that described for rituximab [18, and Knox et al., submitted manuscript].

Monoclonal antibodies can exert their anti-tumor effects by various mechanisms, including: activation of
antibody-dependent cell-mediated cytotoxicity (ADCC),
induction by the antibody of complement-dependent
cytotoxicity (CDC), enhancement of complement release,
such as TNF and IL-1, and direct induction of apoptosis.
Several anti-CD20 antibodies have been shown to induce
apoptosis [28, 25, 11, 26]. One anti-CD20 antibody, 1F5,
has been shown to require the addition of crosslinking
group anti-mouse IgG to induce apoptosis [25, 26].
Numerous studies have been published regarding the
induction of apoptosis by another anti-CD20 antibody,
rituximab. However, the requirement for crosslinking of
rituximab to induce apoptosis has been debated in the
literature [7, 26, 8, 22, 11]. Anti-B1 Antibody has shown
clinical efficacy, but is less well characterized with respect
to induction of apoptosis [26, 25]. A variety of published
techniques are available for measuring apoptosis. They
include Annexin V, induction of ParP cleavage, DNA
fragmentation and caspase activation [23, 24]. The
Annexin V assay was chosen for measuring apoptosis in
these studies, as it is an early marker of this event.
Annexin V-FITC binds with high affinity to phosphotidylserine that has been translocated from the inner
membrane to the outer plasma membrane and this
binding can be monitored by flow cytometry [19].

Anti-B1 Antibody and rituximab can cross-compete
for binding to CD20 and likely recognize overlapping
epitopes. The purpose of this study was to further
characterize and compare the efficacy of these highly
potent anti-tumor agents; therefore, we examined their
biological activity in a range of in vitro assays, including
CDC, ADCC and the induction of apoptosis. In par-
cular, we evaluated the degree of crosslinking of CD20
required for activity. Fab and F(ab')2 fragments were
prepared and tested such that the effect of valency of the
antibody could be further understood. The data indicate
that the degree of crosslinking required for induction of
apoptosis varies with different antibodies. Anti-B1 IgG
or F(ab')2 fragment directly induced apoptosis in a panel
of B-cell lines. In contrast, rituximab induced apoptosis
only weakly in the absence of crosslinking and apoptosis
was significantly enhanced by adding goat anti-human
IgG or goat anti-human F(ab')2.

**Material and methods**

**Cells**

The Daudi, WIL-2, Raji and Ramos B lymphoma cell lines were obtained from the ATCC (Bethesda, Md.). The BALL-1 B cell
lymphoma cell line was obtained from Coulter Corporation (Mi-
ami, Fla.). The DU-DHL-4 cells were obtained from Dr. Alan
Epstein (USC Medical Center, Calif.). All cells were maintained in RPMI-1640, L-glutamine (4 mM), 10% FBS, penicillin (100 U/ml),
streptomycin (100 U/ml) at 37 °C and 5% CO₂.

**Antibodies and reagents**

Clinical grade Anti-B1 Antibody, (Coulter Pharmaceutical) and
rituximab (IDEC/Genentech) were shown to be greater than 97%
pure monomeric IgG by SDS-PAGE and gel filtration HPLC.
Antibodies used were: CD19-FITC, CD55-FITC, and CD59-FITC
(PharMingen, San Diego, Calif.). Anti-CD20-FITC (clone B1) was
purchased from Jackson Labs (West Grove, Pa.). Where indicated, goat anti-mouse F(ab')2 or goat anti-human F(ab')2 was used for crosslinking
(Pierce, Rockford, Ill.). Human complement was purchased from
Quidel Corp (Los Angeles, Calif.). AlamarBlue reagent was purchased from Trek diagnostics (Chicago, Ill.). Annexin V-FITC
apoptosis detection kit was purchased from BD Pharmingen (BD
Biosciences, Calif.).

**Preparation of Fab and F(ab')2**

Anti-B1 Fab was prepared by digestion with immobilized papain
(Pierce). IgG was buffer exchanged into 20 mM phosphate buffer
(pH 7) containing 10 mM EDTA. Immobilized papain was washed
twice in fresh digest buffer (20 mM phosphate, 10 mM EDTA,
20 mM cysteine HCl, pH 7.0). IgG was then diluted 1:1 in digest
buffer and incubated with the immobilized papain at 0.5 ml per
10 mg IgG for 4 h at 37 °C. The digest was continually monitored
by gel-permeation HPLC to determine when digestion was com-
plete. HPLC was carried out using a Zorbax GF-250 column run at
1 ml/min in 0.2 M phosphate buffer (pH 7.0). After digestion,
papain was removed by centrifugation followed by filtration, and
the resulting Fab was purified using ion-exchange chromatography
on a mono-Q column (Amersham-Pharmacia) run at pH 8 with
eletion by a linear gradient of 0–500 mM NaCl. Fractions cont-
taining pure Fab were pooled, concentrated and dialyzed into
phosphate buffered saline.

Anti-B1 F(ab')2 was prepared by digestion of IgG with Ach-
romobacter lysis endopeptidase (Wako Chemicals USA, Rich-
mond, Va.). IgG at 10 mg/ml in 50 mM Tris-HCl (pH 8.5) was
incubated with lysis endopeptidase at a ratio of 500:1 (ab:enzyme)
for 1 h at 37 °C. Digestion was monitored by gel-permeation
HPLC, as described for papain digestion. The reaction was stopped
by addition of 30 mM TLCK, and F(ab')2 purified by chroma-
tography on protein A-Sepharose to remove residual IgG and Fc
fragments, followed by ion-exchange, as described for the papain
digest. Purified F(ab')2 was concentrated and dialyzed into
phosphate buffered saline.

**Iodination of antibodies**

The anti-CD20 IgGs were labeled with Na¹²⁵I using Iodo-Gen
(Pierce Chemical, Rockford, Ill.) as described [5]. Approximately
140 µg of IgG in PBS was added to a glass vial coated with 4 µg
of Iodo-Gen. The IgG was adjusted to approximately 0.1 M sodium
phosphate buffer (pH 7.2) and 1 mCi of Na¹²⁵I (NEN, Life Science
Products Boston, Mass.) was added. After a 5-min incubation,
while shaking at room temperature, the labeled IgG was separated
from free ¹²⁵I by ion-exchange chromatography. The iodination
protocol yielded specific activities of approximately 3–6 mCi/mg
and radiocytotoxicity of routinely greater than 97%.

**Scatchard analysis**

BALL-1 cells, grown in RPMI containing 10% FCS, were washed
twice with Hanks Balanced Salt Solution (HBSS) at room tem-
perature. The cells were adjusted to 4x10⁶ cells/ml in TBS binding
buffer (24 mM Tris, 1.7 mM NaCl, 2.7 mM KCl, 0.1% HSA,
2 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂ pH 7.4). Millipore
plates (MAFB NOB) were coated with 1% nonfat dry milk in water
and stored at 4 °C overnight. The plates were washed with binding
buffer and 25 µl of unlabeled antibody (100-fold excess) in TBS
binding buffer was added to control wells in a Millipore 96-well
glass-fiber filter plate (non-specific binding wells, NSB). Twenty-five
microliters of buffer alone was added to the maximum binding