β-Glucan, a "Specific" Biologic Response Modifier That Uses Antibodies to Target Tumors for Cytotoxic Recognition by Leukocyte Complement Receptor Type 3 (CD11b/CD18)[1]

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β-Glucans were identified 36 years ago as a biologic response modifier that stimulated tumor rejection. In vitro studies have shown that β-glucans bind to a lectin domain within complement receptor type 3 (CR3; known also as Mac-1, CD11b/CD18, or $\alpha_M\beta_2$-integrin, that functions as an adhesion molecule and a receptor for factor I-cleaved C3b, i.e., iC3b) resulting in the priming of this iC3b receptor for cytotoxicity of iC3b-opsonized target cells. This investigation explored mechanisms of tumor therapy with soluble β-glucan in mice. Normal mouse sera were shown to contain low levels of Abs reactive with syngeneic or allogeneic tumor lines that activated complement, depositing C3 onto tumors. Implanted tumors became coated with IgM, IgG, and C3, and the absent C3 deposition on tumors in SCID mice was reconstituted with IgM or IgG isolated from normal sera. Therapy of mice with glucan- or mannan-rich soluble polysaccharides exhibiting high affinity for CR3 caused a 57–90% reduction in tumor weight. In young mice with lower levels of tumor-reactive Abs, the effectiveness of β-glucan was enhanced by administration of a tumor-specific mAb, and in SCID mice, an absent response to β-glucan was reconstituted with normal IgM or IgG. The requirement for C3 on tumors and CR3 on leukocytes was highlighted by therapy failures in C3- or CR3-deficient mice. Thus, the tumoricidal function of CR3-binding polysaccharides such as β-glucan in vivo is defined by natural and elicited Abs that direct iC3b deposition onto neoplastic cells, making them targets for circulating leukocytes bearing polysaccharide-primed CR3. Therapy fails when tumors lack iC3b, but can be restored by tumor-specific Abs that deposit iC3b onto the tumors. ***The Journal of Immunology*****, 1999, 163: 3045–3052.**

Biologic response modifiers (BRM)[4] derived from microbial products have represented important tools for defining mechanisms of host defense. However, most BRM are classified as nonspecific because their exact mode of action is unknown. β-Glucan BRM, first reported 36 years ago (1), have been extensively investigated for their tumoricidal activity. β-Glucan BRM are primarily derived from fungi and have backbone structures of β-1,3-linked D-glucose (β-1,3-D-glucan) with β-1,6-linked side chains of β-1,3-D-glucan of varying sizes and frequencies along the backbone (2). More than 500 publications have reported that β-glucans, either soluble or particulate, exhibit antitumor and antimicrobial activities in several animals, including mice (3–7). Some β-glucans have been used in patients for tumor immunotherapy with varying success, including lentinan, derived from mushrooms (8–10), and schizophyllan from culture filtrates of *Schizophyllum commune* (11–13).

Early reports showed that β-glucans functioned by stimulating host defense mechanisms and were not toxic for tumors. β-Glucans stimulated macrophages (4), neutrophils (14), and NK cells (15) to kill sensitive tumor cells. Some data also suggested that β-glucans could promote T cell-specific responses (16), perhaps through triggering the secretion of IFN-γ, IL-6, IL-8, and IL-12 from macrophages, neutrophils, and NK cells (17). A role for T cells in β-glucan function was also proposed because of absent tumoricidal activity in nude or T cell-depleted mice (16, 18).

A role for CR3 in mediating responses to β-glucan was first suggested by research into the mechanisms of neutrophil phagocytosis of iC3b-opsonized yeast. Stimulation of CR3-dependent phagocytosis or degranulation was found to require the simultaneous ligation of two distinct sites within CR3, one specific for iC3b and a second site specific for yeast cell wall β-glucans (19). Because they lack cell-surface CR3-binding polysaccharides, erythrocytes opsonized with iC3b were bound to neutrophils via CR3 but did not stimulate phagocytosis or degranulation. More recently, a lectin domain of CD11b (α-subunit of CR3) was mapped to a C-terminal region located distal to the I-domain binding sites for iC3b and ICAM-1 (20, 21). Soluble zymosan-derived polysaccharides (SZP) rich in mannans or β-glucans (SZP_m or SZP_g) were shown to bind to CR3 with high affinity, inducing a primed receptor state capable of triggering phagocyte or NK cell cytotoxicity of iC3b-opsonized tumor cells that otherwise did not trigger CR3-dependent responses (22).

The sugar specificity of CR3 was found to be broader than was first appreciated and included some polysaccharides containing

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[4] Abbreviations used in this paper: BRM, biologic response modifier; iC3b, factor I-cleaved C3b fragment of C3; MMTV, murine mammary tumor virus; SZP, soluble zymosan-derived polysaccharide made up primarily of either mannan (SZP_m) or β-glucan (SZP_g); S-180, sarcoma 180.

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mannose and *N*-acetyl-D-glucosamine as well as glucose (20). Although CR3 does not bind α-mannans, data reported with a β-oligomannan that mediated attachment of *Candida albicans* to phagocytes (23) via CR3 (24), as well as the finding of a mannose-rich SZP with high affinity for CR3 (20), suggest that a less common β-mannan in some yeast also binds to CR3. Murine CR3 was recently shown to have a sugar specificity and function similar to those of human CR3 in priming phagocytes and NK cells for cytotoxicity of iC3b-opsonized tumors (25).

This investigation focused on defining the in vivo mechanisms of tumoricidal activity induced by therapy with soluble CR3-binding polysaccharides such as β-glucan. Using mouse tumor models, C3 deposition onto tumors was shown to be primarily regulated by tumor-reactive Abs that directed complement activation. This targeting of tumor cells with opsonic iC3b was an essential factor in determining the specific cells to be eliminated in β-glucan therapy. The molecular requirements for β-glucan-mediated tumoricidal activity were defined in tests with C3- and CR3-deficient mice. It was concluded that the specificity of the cell-mediated cytotoxicity mediated by β-glucans is regulated by tumor-specific Abs that target only tumor cells with iC3b, and this spares normal tissues that are not opsonized with iC3b.

Materials and Methods

Cell lines and Abs

The BALB/c (H-2^d) mammary tumor line Ptas64 was provided by Dr. Wei-Zen Wei (Michigan Cancer Foundation, Wayne State University, Detroit, MI). The MMT (H-2^k) mammary tumor line and the sarcoma 180 (S-180) (MHC class I-negative) line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The C57BL/6-derived (H-2^b) EL-4 lymphoma line was from Dr. Nia-Kong Cheung (Sloan-Kettering Cancer Institute, New York, NY). These cell lines were maintained in RPMI 1640 containing 2 mM L-glutamine, antibiotics, and 10% FCS. The MC38 adenocarcinoma and MB49 bladder carcinoma lines (both H-2^b) were provided by Dr. Donald W. Kufe (Harvard University, Boston, MA) and maintained in DMEM containing 10% FCS, antibiotics, and 4.5 g/L glucose. The rat hybridomas secreting TIB211 IgM anti-mouse CD8 mAb and M1/70 IgG anti-mouse CD11b (CR3) were from the ATCC. Mouse 11C1 hybridoma secreting IgG2a mAb specific for murine mammary tumor virus (MMTV) (26) was generously provided by Dr. Hiroshi Fuji (Department of Molecular Immunology, Roswell Park Cancer Institute, Buffalo, NY). Each mAb was isolated from ascites fluid generated in BALB/c mice, and portions of each were coupled to biotin or FITC using standard methods. Biotinylated rat anti-mouse Ly-6G (Gr-1, anti-granulocyte) mAb, anti-mouse CD3-FITC, PE-labeled rat anti-mouse CD80 (B7-1), anti-mouse NK cell (DX5), both unlabeled IgG and IgG-PE, and streptavidin-PE were from PharMingen (San Diego, CA). Goat $F(ab')_2$ anti-mouse IgG-FITC and anti-mouse IgM-FITC were from Southern Biotechnology Associates (Birmingham, AL), and goat $F(ab')_2$ anti-mouse C3-FITC was from Cappel (Durham, NC).

SZP

Methods for isolation and characterization of SZP, a soluble low molecular mass (~10 kDa) polysaccharide consisting primarily of mannose or glucose, were previously reported (20). Briefly, polysaccharides are eluted from zymosan by heating to 95°C in 90% formic acid for 20 min. After discarding large polysaccharides precipitating with 70% ethanol, a small polysaccharide fraction is isolated by precipitation with 80% ethanol. After digestion with α-mannosidase and removal of anionic proteins by Mono-Q fast protein liquid chromatography, a uniform-sized soluble polysaccharide is isolated by chromatography with S-200HR. As reported recently (25), there has been some variation in the size and mannose/glucose composition of such SZP preparations that is dependent on the starting batch of yeast. SZP preparations used in this investigation either consisted almost entirely of mannose and are referred to as SZP_m (20), or consisted primarily of β-1,3-glucans and are referred to as SZP_g (25). The CR3-binding affinity of ^{125}I-labeled SZP_m preparations have been 5-fold higher than preparations of ^{125}I-SZP_g, and thus, whereas optimal priming of mouse or human CR3 for cytotoxic activation in response to iC3b-opsonized tumor cells in vitro required ~1 μg hexose per ml of SZP_m (22), comparable CR3 priming with SZP_g required ~5 μg/ml of hexose (25). Contaminating LPS was removed from SZP_m to levels below the limit measurable with the *Limulus* lysate test (E-Toxate, Sigma, St. Louis, MO) using Detoxi Gel (Pierce, Rockford, IL). With SZP_g, all detectable LPS was removed using Triton X-114 (27).

Mice

Female, 4–16-wk-old, normal and SCID $BALB/c_J$ mice, as well as normal C57BL/6 mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice deficient in C3 ($C3^{-/-}$ on a 129/J background) (28), CR1/2 ($Cr2^{-/-}$; 129/J) (29), or CR3 ($CD11b^{-/-}$; 129/J) were previously described (30). The C3- and CR3-deficient mice and their wild-type littermates were bred and maintained at Harvard Medical School until shipped to the University of Louisville for tumor therapy experiments. For some experiments as noted, the CR3-deficient trait was transferred onto a $BALB/c_H$ background (subscript "H" denoting Harvard Medical School origin of BALB/c mice) by eight or nine backcrosses to normal $BALB/c_H$ mice, so that age- and sex-matched $BALB/c_H$ wild-type and CR3-deficient littermates were available for tumor therapy experiments.

Purification of normal IgM and IgG reactive with the Ptas64 tumor cell line

Gammaglobulins from 50 ml of normal BALB/c mouse serum (Harlan Bioproducts for Science, Indianapolis, IN) were precipitated with 50% saturated ammonium sulfate, dialyzed into 10 mM Tris/HCl pH 7.5, and chromatographed on a 1.6 × 10-cm Mono-Q fast protein liquid chromatography column (Amersham Pharmacia Biotech, Piscataway, NJ) at 10 ml/min. Bound proteins were eluted with a linear salt gradient over 60 min, in which the limit buffer was 0.5 M NaCl in 10 mM Tris/HCl, pH 7.5. Fractions enriched for IgM and/or IgG Abs reactive with Ptas64 cells were identified by indirect immunofluorescence staining with anti-IgM-FITC or anti-IgG-FITC and flow cytometry. IgM and IgG Abs were isolated by S-300HR (Amersham Pharmacia Biotech) chromatography using a 1.6 × 50-cm column. The IgM fraction, 2.0 ml and 2.62 mg/ml of IgM (determined by ELISA; see below), stained 30% of Ptas64 cells by indirect immunofluorescence at a dilution of 1:20. Based on similar staining activity of the starting serum diluted 1:4, this represented a 40% yield of the IgM Abs. The IgG fraction of 4 ml had comparable staining activity of Ptas64 cells when diluted 1:50, representing a 20% yield of IgG Abs. Each Ig fraction was depleted of detectable LPS using Triton X-114 and sterilized by ultrafiltration with a 0.22 μm filter.

ELISA for IgM

Immulon-2 plates (Dynatech, Chantilly, VA) were coated with affinity-purified anti-mouse IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN) for capture of mouse IgM test samples or MOPC-104E IgM (Sigma) tested in parallel to establish a standard curve. Bound IgM was detected with anti-mouse IgM-peroxidase (Boehringer Mannheim Biochemicals), followed by Sigma 104 phosphate substrate solution (Sigma). The OD_{490} of each well was measured with a Rainbow MicroELISA plate reader (SLT Instruments, Salzburg, Austria).

Mouse tumor models for SZP therapy

Mice were implanted s.c. in a mammary fat pad with 1–5 × 10^6 Ptas64, MMT, or MC38 cells freshly harvested from culture and suspended in 100–200 μl of PBS. Small, palpable tumors were allowed to form in all mice over 10–14 days before initiation of daily i.p. or i.v. (tail vein) injections of 200 μg SZP_m or SZP_g (100 μl, 2 mg hexose/ml of PBS), with a control group of mice receiving daily i.v. injections of 100 μl PBS. After 9–14 days of therapy (same time for all mice in an individual experiment), the mice were sacrificed, and tumors were removed, trimmed of surrounding tissue, and weighed.

Flow cytometry analysis of tumors

Single-cell suspensions of the freshly removed tumors were generated by gently teasing small tissue fragments between forceps in HBSS (Life Technologies, Grand Island, NY), followed by filtration through a cell strainer (Falcon Division, Becton Dickinson Labware, Franklin Lakes, NJ). Large debris was removed by layering cell suspensions over 3 ml of heat-activated FCS and allowing sedimentation at 1 × *g* for 10 min in an ice bath. After two washes with ice-cold RPMI 1640, the cells were suspended in RPMI 1640 and maintained on ice. Immunofluorescence staining of 5 × 10^5 cells was conducted on ice using standard techniques, including the addition of propidium iodide to exclude dead cells from analysis (31). An EPICS Profile II (Coulter Electronics, Miami Lakes, FL) was used for flow cytometry, and the listmode data were analyzed with WinList 3.0 (Verity Software House, Topsham, ME).

Table I. *Detection of naturally occurring IgM and IgG Abs reactive with various tumor cell lines in sera from BALB/c, C57BL/6, and 129/J mice*[a]

Mouse strain	Age (mo)	Tumor Cell Line	Linear Fluorescence Mean Channel IgM[b]	IgG[c]
BALB/c_J	1	Ptas64	3.90	10.1
	2	Ptas64	9.59	12.3
	3	Ptas64	20.0	28.3
	>3	Ptas64	20.1	30.2
	>3	S-180	16.8	27.4
BALB/c_H	3	Ptas64	7.79	10.2
BALB/c_H CD11b$^{-/-}$	>2	Ptas64	6.90	6.49
BALB/c_J SCID	>2	Ptas64	1.05	1.09
C57BL/6	>2	MC38	15.6	13.6
	>2	MB49	7.18	5.72
	>2	S-180	14.3	13.1
	>2	EL-4	5.1	2.1
129/J	>2	MMT	13.3	21.3
	>2	Ptas64	12.4	16.1
	>2	MC38	18.9	25.5
	>2	MB49	15.2	30.7
	>2	S-180	17.2	13.2
129/J C3$^{-/-}$	>2	MMT	14.8	12.0
	>2	Ptas64	11.3	11.3
	>2	S-180	12.3	12.4
129/J Cr2$^{-/-}$	>2	MMT	21.6	17.7
	>2	S-180	15.1	27.9

[a] Fresh-frozen sera were tested for levels of IgM and IgG Abs reactive with different cultured tumor cell lines using indirect immunofluorescence staining and flow cytometry. To assure equivalent fluorescence sensitivity, the flow cytometer was calibrated with Standard Brite beads (Coulter).
[b] All sera were diluted 1:4 for analysis of IgM Ab staining of tumor lines.
[c] All sera were diluted 1:16 for analysis of IgG Ab staining of tumor lines.

Results

Naturally occurring Abs coat syngeneic tumors with C3

Tests of normal sera from BALB/c, C57BL/6, and 129/J mice demonstrated naturally occurring IgM and IgG Abs reactive with syngeneic and allogeneic tumor cell lines (Table I). The presence of these Abs explained the ability of these tumor lines to activate complement with resultant C3 deposition, because no C3 was detectable on tumors cells incubated with fresh serum from SCID mice (not shown). The only exception among these tumors was the EL-4 lymphoma, in which levels of IgM and IgG tumor-reactive Abs in C57BL/6 sera (Table I) were too low to mediate detectable C3 deposition (not shown). Previous in vitro studies had shown that the density of C3 deposited onto Ptas64 cells by normal BALB/c_J serum was adequate to trigger the cytotoxic activation of the β-glucan-primed CR3 expressed by neutrophils, macrophages, or NK cells (25).

Naturally occurring Abs in normal sera also functioned to opsonize implanted syngeneic tumors with IgM, IgG, and C3 detectable by flow cytometry of cell suspensions prepared from the freshly excised tumors. Analysis of Ptas64 H-2^d BALB/c mammary tumors removed from BALB/c_J mice 3–4 wk after implantation is shown in Fig. 1. Young BALB/c (Table I) or 129/J mice (not shown) had lower levels of such tumor-reactive Abs than did older mice, and tumors removed from young mice exhibited less staining for Ig and C3 than did tumors removed from older mice (Fig. 1, *A* and *B*). Amounts of IgM and IgG tumor-reactive Abs increased with age in BALB/c_J (Table I) and 129/J (not shown) mice, reaching plateau levels at ≥3 mo of age. As expected, no tumor-reactive Abs were detected in SCID BALB/c_J mice (Table I), and tumors removed from SCID mice exhibited no staining for IgM, IgG, or C3 (Fig. 1*C*). Reconstitution of SCID mice with i.v. normal IgM or IgG generated in vivo opsonization of implanted tumors with C3 (Fig. 1*D*). Sera from 129/J mice deficient in either serum C3 (C3$^{-/-}$) or B cell CR1/2 (Cr2$^{-/-}$) exhibited levels of tumor-reactive IgM and IgG Abs that were similar to those of normal 129/J mice (Table I).

Therapy of mammary carcinoma with SZP

BALB/c_J mice with Ptas64 mammary tumors were tested for a therapeutic response to daily injections of SZP_m given either i.p. or i.v. for 2 wk (Fig. 2). This experiment with 18 mice was repeated twice with similar results and then was repeated three more times with LPS-free SZP_m. Because of variations in mean tumor weight in the PBS control groups (0.7–1.3 g), tumor weight reduction in each experiment was determined by comparison only with the PBS control group from the same experiment. Overall, in six experiments, mice given i.p. SZP_m exhibited less tumor reduction (45% ± 10%; $p < 0.001$) than did mice receiving i.v. SZP_m (90% ± 7.2%; $p < 0.001$). Similar results obtained with LPS-free SZP_m indicated that minor LPS contamination did not explain the tumoricidal response to SZP_m. In each experiment, one or two mice of six in the i.v. SZP_m group had no detectable tumor.

Abs to tumor Ags are required for SZP therapy

SZP_g therapy of young (5-wk-old) BALB/c_J mice implanted with Ptas64 tumors resulted in less tumor reduction (≤50%; not shown)

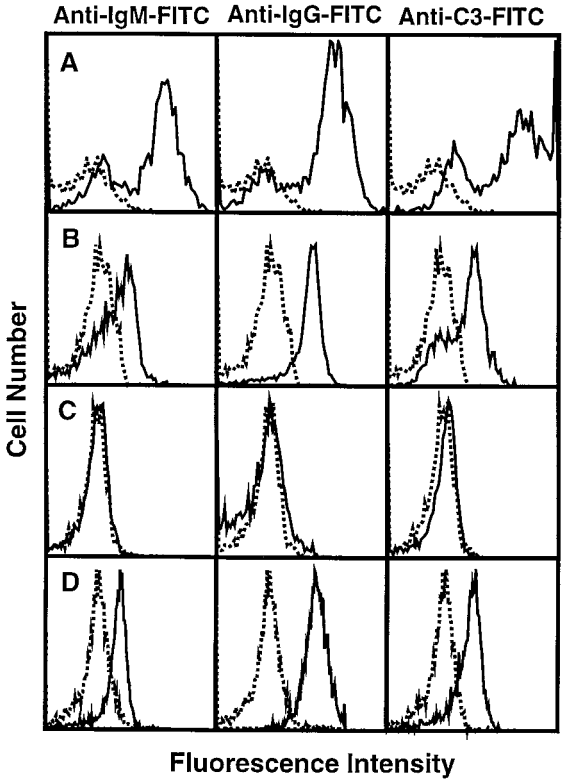

FIGURE 1. Detection by flow cytometry of Ab and C3 deposition on Ptas64 mammary tumors removed from BALB/c_J mice 3–4 wk after implantation. *A*, Staining of a tumor, with anti-IgM-FITC, IgG-FITC, or C3-FITC, removed from a 5-mo-old mouse. *Dotted lines* represent nonspecific staining with nonspecific IgG-FITC. *B*, Staining of a tumor from a 5-wk-old mouse. *C*, Staining of a tumor from a SCID BALB/c_J mouse. *D*, Staining of tumors from SCID BALB/c mice that had been reconstituted i.v. with either IgM or IgG purified from normal mouse sera. *D, left histogram*, Tumor from an IgM-reconstituted SCID mouse. *D, middle and right histograms*, Tumor removed from a SCID mouse reconstituted with normal IgG.

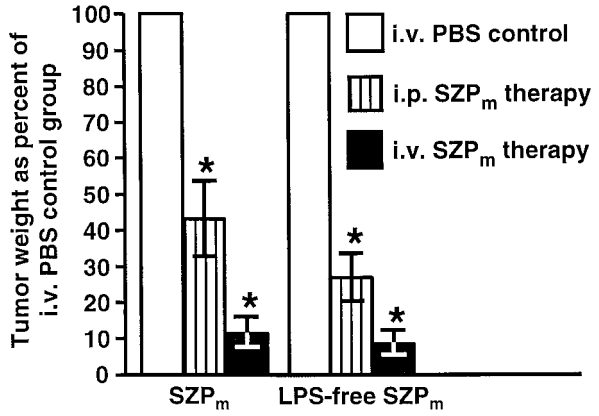

FIGURE 2. SZP$_m$ therapy of BALB/c$_J$ mice with Ptas64 mammary carcinoma. Data from six experiments are shown. For each experiment, three groups of six mice with small tumors were tested for a response to SZP$_m$ as indicated by the weight of tumors after 2 wk of therapy. For each experiment, two groups of six mice were given daily i.p. or i.v. injections of 200 μg of SZP$_m$. The third control group of six mice received daily i.v. PBS. For each experiment, the average weight for these two SZP therapy groups was determined and compared with the average weight of tumors removed from the PBS control group. Three experiments with 18 mice were conducted with SZP$_m$ using 54 mice, and then the same experiment was done three more times with another 54 mice using LPS-free SZP$_m$. Each bar represents the mean ± SD for each therapy group in three experiments. *, Tumor weight reductions were significant ($p < 0.001$) with either i.p. or i.v. SZP$_m$ therapy as compared with the PBS control groups.

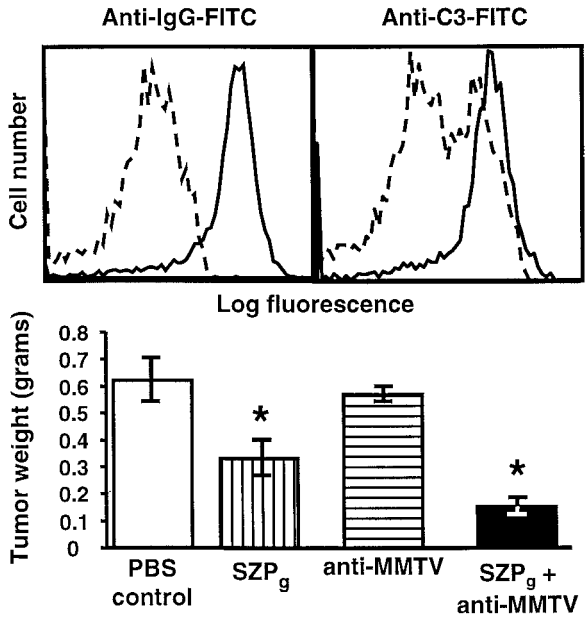

FIGURE 3. Therapy of young (5-wk-old) BALB/c$_J$ mice implanted with Ptas64 mammary carcinoma by passive immunization with 11C1 IgG2a anti-MMTV mAb, with or without simultaneous SZP$_g$. The *upper histograms* show flow cytometry analysis of tumors removed from these mice, and the *lower bar graph* shows the weight of tumors after 9 days of therapy. The *upper left histogram* compares IgG staining of a representative tumor removed from the PBS control group (dashed line) vs a tumor removed from one of the anti-MMTV-treated mice (solid line). The *upper right histogram* compares C3 staining with the same two tumors and shows that tumors from mice not receiving the anti-MMTV (dashed line) contained a subset of cells that were not coated with C3, whereas the solid line shows that the majority of tumor cells from the mice receiving anti-MMTV therapy were coated with C3. There was a 47% reduction in tumor weight in the mice receiving SZP$_g$ alone as compared with the PBS control group (*, $p < 0.001$), whereas treatment with anti-MMTV alone did not produce a significant reduction in tumor weight. Treatment with i.v. SZP$_g$ plus anti-MMTV resulted in a 70% reduction in tumor weight that was significant when compared with either the PBS control group ($p < 0.001$) or the group receiving only SZP$_g$ ($p < 0.05$). These results are from an experiment with 24 mice divided into four groups of six mice. Each bar represents the mean ± SD for six mice.

than with older (≥2-mo-old) BALB/c$_J$ mice (90%; Fig. 2). To determine whether this was due to a reduced amount of tumor-reactive Abs in the younger mice that resulted in less C3 deposition on tumor cells, the amount of IgG and C3 on tumors in the young BALB/c$_J$ mice was augmented by passive immunization with an IgG2a mAb to MMTV, a tumor Ag expressed on Ptas64 cells. Initial experiments to determine the optimal dose of the anti-MMTV mAb indicated that 200 μg of the IgG2a mAb given on alternate days for 2 wk resulted in maximum deposition of C3 onto tumor cells. When this mAb therapy was combined with daily i.v. SZP$_g$ in young BALB/c$_J$ mice implanted with Ptas64 tumors, the increased amount of C3 on tumors was associated with a significantly greater tumor reduction in response to SZP$_g$ (Fig. 3). Administration of the anti-MMTV mAb resulted in increased deposition of IgG and C3 on breast tumors (Fig. 3, *top*). However, despite this greatly increased complement activation by tumors, treatment with i.v. anti-MMTV alone had no significant effect on tumor growth. A therapeutic benefit with the i.v. anti-MMTV mAb was only observed when it was combined with i.v. SZP$_g$ (Fig. 3, *bottom*).

Normal IgM or IgG reconstitution of the therapeutic response to SZP$_g$ in SCID mice

Previous reports on the tumoricidal activity of β-glucans in mice had suggested a requirement for T cells because of therapy failures in nude (18, 32), thymectomized (6), or normal mice depleted of T cells with anti-CD4 and anti-CD8 (16). As expected, T cell-deficient nude BALB/c (not shown) and SCID BALB/c (Table I) mice lacked detectable Abs reactive with Ptas64 tumor cells, and Ptas64 tumors removed from nude (not shown) or SCID BALB/c (Fig. 1*C*) mice did not exhibit staining for Ig or C3. Reconstitution of SCID mice i.v. with normal IgM or IgG resulted both in C3 opsonization of tumors (Fig. 1*D*) and in a significant tumor reduction response to SZP$_g$ therapy (Fig. 4). Although tumor reduction with combined therapy was less than had been observed with normal BALB/c mice (Fig. 2), the proportions of tumor cells bearing C3 were also comparably less. With reconstitution of IgM tumor-reactive Abs to serum levels comparable with normal BALB/c mice, only 10% of tumor cells exhibited C3 staining, whereas with IgG reconstitution to normal serum IgG Ab levels, 25% of tumor cells were stained for C3 (Fig. 4). However, with IgG-reconstituted SCID mice, a tumor reduction of 50% was considerably less than the 90% tumor reduction observed in normal BALB/c mice (Fig. 2), in which 50–75% of tumor cells exhibited staining for C3 (Fig. 1).

SZP therapy requires both C3 on tumor cells and CR3 on leukocytes

Previous in vitro experiments had shown that SZP-mediated cytotoxicity required SZP priming of both leukocyte CR3 and iC3b on the tumor cells (25, 33). A correlation of tumor C3 density with resultant SZP-mediated tumor reduction was also suggested by current experiments with mice given Abs that enhanced the density of C3 on tumors (Figs. 3 and 4). The requirement for C3 in SZP therapy was confirmed in experiments with C3-deficient 129/J mice implanted with the MMT mammary carcinoma. SZP$_m$ therapy of normal 129/J mice resulted in tumor reduction (Fig. 5; $p <$

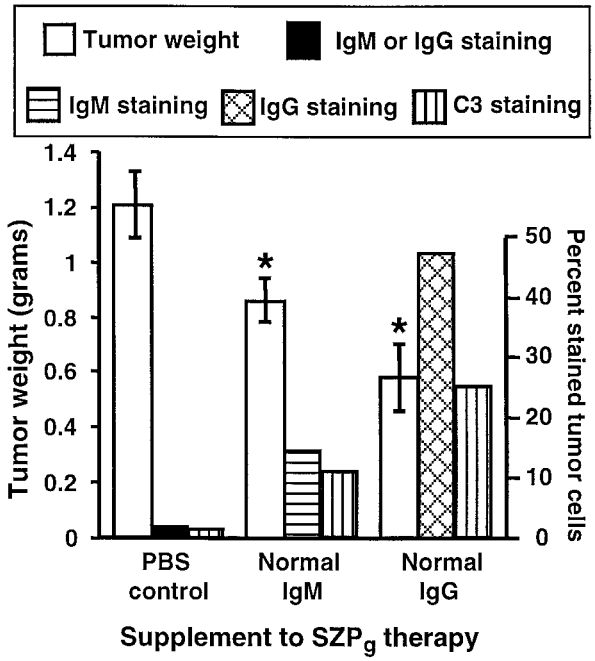

FIGURE 4. Therapy of SCID BALB/c$_J$ mice bearing Ptas64 mammary tumors with purified normal mouse IgM or IgG in combination with SZP$_g$. Groups of six mice each were implanted with Ptas64 mammary tumors as in Figs. 2 and 3 and then given daily i.v. therapy with SZP$_g$ supplemented with simultaneous i.v. IgM or IgG vs the control group given simultaneous i.v. PBS. The amounts of IgM or IgG given to mice throughout the therapy period were shown to be adequate to maintain the same serum levels of tumor-reactive IgM or IgG Abs as were detectable in normal adult BALB/c mouse serum (Table I). After 2 wk of therapy, the tumors were excised and weighed, and single-cell suspensions were analyzed for the presence of membrane IgM, IgG, and C3 by flow cytometry. Mean values ± SD for the tumor weights of each group are given. *, $p < 0.001$; significant tumor reduction occurred in mice given either normal IgM or IgG in combination with SZP$_g$.

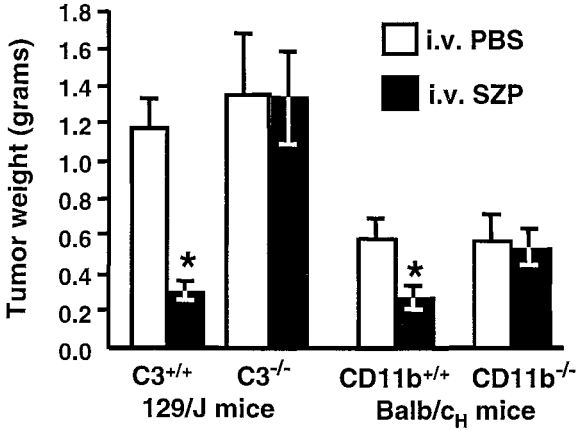

FIGURE 5. SZP therapy requires serum C3 and leukocyte CR3. Twelve normal (C3$^{+/+}$) and 12 C3-deficient (C3$^{-/-}$) 129/J mice were implanted with the MMT mammary carcinoma cell line, and palpable tumors were allowed to form before the start of daily i.v. therapy in groups of six mice each with PBS or SZP$_m$. A reduction in tumor weight of 79% ($p < 0.001$) occurred only with the normal 129/J mice receiving SZP$_m$ therapy. The same protocol was conducted with normal (CD11b$^{+/+}$) and CR3-deficient (CD11b$^{-/-}$) BALB/c$_H$ mice implanted with Ptas64 mammary tumors. A significant tumor weight reduction ($p < 0.001$) occurred only with i.v. SZP$_g$ therapy of normal and not CR3-deficient BALB/c$_H$ mice. Bars represent the mean tumor weight from groups of six mice ± SD.

0.01) similar to that of normal BALB/c mice (Fig. 2), and flow cytometry of tumors showed abundant deposition of C3 on >80% of cells (not shown). By contrast, in C3-deficient 129/J mice, there were no significant tumor reduction (Fig. 5) and no C3 on tumors (not shown), but the relative amount of IgG staining on tumors did not differ from that of the normal 129/J mice.

Initial experiments with CD11b$^{-/-}$ (CR3-deficient) mice examined 129Sv (H-2^b) × BALB/c$_H$ (H-2^d) F$_1$ mice implanted with MC38 (H-2^b) adenocarcinoma. In these F$_1$ mice, BALB/c H-2^d Ptas64 mammary tumors were rejected spontaneously and thus could not be used for experimental therapy. MC38 tumors removed from both the normal and CR3-deficient F$_1$ mice exhibited comparable staining for IgM, IgG, and C3, but significant tumor reduction (78 ± 14%, $p < 0.001$) was observed only in normal mice and not in CR3-deficient mice (not shown). After backcrossing these F$_1$ mice to BALB/c$_H$ for eight or nine generations, 99.6–99.8% of the unlinked loci should be derived from the BALB/c genome. Implanted Ptas64 tumors in these mice were not rejected spontaneously, allowing therapy with SZP$_g$ to be evaluated (Fig. 5). As expected, significant tumor reduction in response to SZP$_g$ therapy occurred only with normal (56.5 ± 10%, $p < 0.001$) and not with CR3-deficient mice (Fig. 5). However, unlike in BALB/c$_J$ mice, sera from both the normal and CR3-deficient BALB/c$_H$ mice exhibited lower levels of IgG Abs reactive with Ptas64 cells (Table I), and as a result, fewer cells from tumors were stained with anti-IgG-FITC or C3-FITC. There was no difference in serum IgM tumor-reactive Abs or staining of excised tumor cells with anti-IgM-FITC. However, with tumors from both normal and CR3-deficient BALB/c$_H$ mice, only 26% of cells stained for IgG, and only 29% of cells stained for C3. By contrast, with Ptas64 tumors removed from BALB/c$_J$ mice, a mean of 78% of cells stained for IgG and 61% stained for C3 (Fig. 1). Thus, the reduced therapeutic response in normal BALB/c$_H$ vs BALB/c$_J$ mice appears to have been due to lower levels of naturally occurring IgG tumor-reactive Abs that translated into a reduced surface density of C3 on tumor cells. No significant differences in IgG- or C3-specific staining were detectable on tumors removed from normal vs CR3-deficient mice.

Humoral response to Ptas64 tumor cells in tumor-bearing mice

The sensitivity of Ptas64 tumors to SZP therapy appeared to correspond to serum levels of naturally occurring Abs to Ptas64 that regulated levels of C3 deposition onto tumors. The increase in tumor-reactive IgG Abs in 1–3-mo-old BALB/c$_J$ mice, as well as the variation between BALB/c mice at the same age but raised in different environments (i.e., BALB/c$_J$ vs BALB/c$_H$), further suggested that these Abs represented an elicited response rather than natural Abs that are primarily IgM. To determine whether Ptas64 cells might stimulate an immune response in BALB/c mice, sera from BALB/c$_J$ mice implanted with Ptas64 tumors for 6 wk were examined for levels of IgM and IgG tumor-reactive Abs (Fig. 6). Sera from mice given i.v. SZP$_g$ were compared with sera from mice in an i.v. PBS control group. The results indicated that 6 wk of tumor implantation stimulated a 3- to 4-fold increase in tumor-reactive IgG Abs, whereas there was no increase in IgM Ab levels. However, mice receiving SZP$_g$ did not exhibit a greater increase in Ab than mice in the PBS control group (Fig. 6).

Analysis of the effector cells functional in SZP therapy

Previous in vitro tests had shown that neutrophils, macrophages, and NK cells bearing SZP-primed CR3 were each capable of killing iC3b-opsonized Ptas64 cells (25). Flow cytometry of tumors from mice treated with SZP vs PBS found similar proportions of NK

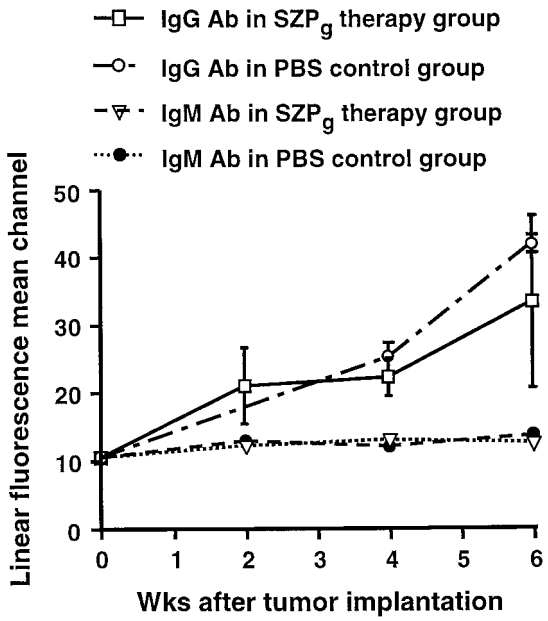

FIGURE 6. IgG immune response of BALB/c$_J$ mice to implanted syngeneic Ptas64 mammary tumors. Sera were collected from BALB/c$_J$ mice implanted with Ptas64 mammary carcinoma both before and during a typical i.v. therapy protocol with SZP$_g$ or PBS, as in Fig. 2. All serum samples were frozen for later analysis with a single culture of Ptas64 cells using the same flow cytometer settings. Sera were diluted 1:4 for analysis of IgM Ab with anti-IgM-FITC and 1:16 for analysis of IgG Ab with anti-IgG-FITC staining. Means ± SD for tests of five or six sera for each time point are given. There was so little variation in serum IgM Ab levels that SD values for IgM do not appear on the graph.

cells (15%), granulocytes (25%), macrophages (25%), $CD8^+CD3^+$ T cells (10%), and $CD8^-CD3^+$ T cells (15%). There was also no evidence for increased proportions of the minor subset of $CR3^+CD8^+$ T cells (≤2% of T cells) in tumors or spleens from these mice vs non-tumor-bearing mice. Moreover, the proportions of these major leukocyte subsets were the same in tumors removed from normal vs CR3-deficient mice. This shows that the reduced tumoricidal activity in CR3-deficient mice was not due to reduced leukocyte infiltration of tumors, suggesting that leukocyte diapedesis into such implanted s.c. tumors is normal in CR3-deficient mice.

Discussion

This investigation showed that the tumoricidal activity of soluble CR3-binding polysaccharides such as β-glucan was specific for neoplastic cells that had been opsonized with C3 through the action of naturally occurring tumor-reactive Abs. Tumors that bore a sufficient density of C3 for recognition by the CR3 of circulating leukocytes responded to therapy with β-glucans, whereas tumors that were not opsonized with C3 did not respond to therapy. Passive administration of Abs to tumor Ags caused tumors to be opsonized with C3 and allowed β-glucan therapy to be effective against tumors in which there was either a low or absent level of tumor-reactive Abs.

It is well known that β-glucan responses occur only in certain strains of mice bearing specific tumors. Our data suggest that reports of the sensitivity or resistance of specific tumors to β-glucan corresponds to the presence or absence of Abs capable of opsonizing the tumor with iC3b. Over the last 39 years, S-180 has been the most frequently used tumor to screen for β-glucan tumoricidal function (3, 34–36). Not only were Abs to S-180 found in sera from several mouse strains, but also S-180 was found to lack MHC class I that can suppress CR3-mediated NK cell cytotoxicity (37). Moreover, we confirmed (38) that C57BL/6 sera contain very low levels of Abs reactive with the syngeneic (H-2^b) EL-4 lymphoma, a finding consistent with the resistance of EL-4 tumors to β-glucan therapy (39). However, when a mAb to GD2 ganglioside was used in combination with C57BL/6 serum to opsonize the $GD2^+$ EL-4 cells, the cells exhibited abundant staining for C3 and were killed efficiently by β-glucan-primed neutrophils (J. Yan and G. D. Ross, unpublished observation). By contrast, with another H-2^b tumor, the MC38 adenocarcinoma, the presence of Abs in normal 129/J (H-2^b) sera produced in situ opsonization of tumors with iC3b and allowed β-glucan therapy to be successful.

Naturally occurring Abs present in both BALB/c and 129/J sera produced in situ deposition of C3 on tumors, making them sensitive to SZP therapy. The presence of naturally occurring IgG Abs suggests that these are not "natural Abs" per se that are usually IgM, but are probably elicited Abs to environmental Ags that cross-react with tumor Ags. Similar levels of tumor-reactive Abs were found in sera from C3- and CR1/2-deficient mice, even though CR1/2-deficient mice have a limited repertoire of natural Abs, and both types of complement deficiency lead to a reduced response to T cell-dependent Ags (29, 40). Moreover, mammary tumors implanted into BALB/c mice elicited an increased level of tumor reactive IgG. These findings have a human counterpart, because normal human sera contain IgM and IgG Abs reactive with human mammary tumor lines, and likewise, levels of tumor-reactive IgG are elevated in patients with breast cancer (33). Similar findings of natural and elicited Abs to murine (38, 41) or human (42, 43) mammary carcinoma have been reported. Our studies suggest that although such humoral responses by themselves are inadequate to mediate tumor rejection, the resulting C3 deposition targets the tumor cells for cytotoxic recognition by leukocytes bearing β-glucan-primed CR3.

This investigation showed an important role for tumor-reactive Abs in facilitating a response to β-glucan. Passively administered normal IgG was more effective than normal IgM in opsonizing tumors in SCID mice, and the best C3 opsonization of tumors was achieved in mice given a mAb to the MMTV tumor Ag. However, it is noteworthy that the greatly increased complement activation mediated by this mAb had virtually no effect on tumor growth. This emphasizes the inherent resistance of tumors to complement-mediated cytotoxicity and shows why passively administered mAbs to tumor Ags or vaccines that generate a good humoral response are frequently ineffective therapeutically. Nevertheless, the enhanced response to β-glucan in mice given Abs to tumor Ags, particularly SCID mice, shows the potential efficacy of passive mAb therapy in combination with β-glucan in cancer patients who may be immunosuppressed.

Although the tumors used in this study required surface C3 for recognition by CR3-bearing leukocytes, other tumors may express natural ligands for β-glucan-primed CR3. For example, SZP-primed human NK cells mediate CR3-dependent cytotoxicity of unopsonized K562 cells (22), and SZP-primed murine NK cells mediate CR3-dependent cytotoxicity of unopsonized YAC-1 cells (V. Větvička, unpublished observation). Moreover, there have been several reports showing a tumoricidal activity of β-glucan-treated macrophages with unopsonized tumor cell line targets in vitro (44–46).

Previous investigators had proposed that CTL were essential for β-glucan tumoricidal activity because therapy was unsuccessful in either nude (18, 32) or T cell-depleted mice (16). However, the tumor reduction induced in SCID mice reconstituted only with IgG argues against a necessary role for CTL in β-glucan therapy. Moreover, the absence of MHC class I on S-180 sarcoma that has

been used frequently as a tumor model for β-glucan therapy argues further against an essential role for CTL. Nevertheless, with other tumors that express tumor-specific peptides on class I, a role for β-glucan in augmenting Ag presentation cannot be excluded. In particular, the enhanced CR3-dependent functions of macrophages, and possibly also dendritic cells, may enhance tumor Ag uptake and presentation to T cells. Furthermore, polysaccharide-dependent activation of macrophage CR3 stimulates the secretion of several cytokines, particularly IL-12 (17, 47). By contrast, in the absence of β-glucan therapy, IL-12 secretion might even be suppressed within iC3b-opsonized tumors that mediated CR3-dependent monocyte adhesion without stimulating toxicity. For example, monocyte IL-12 secretion is suppressed by ligation of CR3 to iC3b-opsonized erythrocytes that lack CR3-priming polysaccharides (48).

Similar proportions of $CR3^+$ granulocytes, macrophages, and NK cells were noted in tumors from mice treated with SZP vs PBS, suggesting that SZP therapy does not lead to a specific recruitment of leukocytes into tumors. The inflammatory response resulting from Ab-dependent activation of complement with release of C3a and C5a should occur equally in tumors from SZP-treated mice vs PBS control mice, and this may serve to recruit $CR3^+$ granulocytes and macrophages. Each of these leukocytes, as well as NK cells, are capable of CR3-dependent tumoricidal activity in vitro (25).

The unpredictable outcome of adoptive therapy with BRMs has limited their use in cancer. Although β-glucans have been viewed as adjuvants that could enhance immune responses, the current data indicate that β-glucans function through naturally occurring or elicited Abs by providing a cell-mediated effector mechanism that is normally functional only against microbial pathogens. Furthermore, in BALB/c mice with Ptas64 tumors, β-glucan did not enhance levels of tumor-reactive Abs above those observed in mice receiving PBS. With this recognition that tumors that lack opsonizing C3 will not respond to β-glucan therapy, it would be preferable to restrict the use of β-glucans to patients known to have tumor-reactive Abs through active or passive immunization.

Although SZP therapy of BALB/c mice bearing Ptas64 tumors resulted in an average tumor weight reduction of 90%, some therapy failures also occurred. These were particularly noted in several mice in one experiment in which i.v. SZP therapy was continued for up to 6 wk (data not shown). Examination of the tumors from these mice showed that ≥80% of the tumor cells were not stained by anti-IgG- or anti-C3-FITC, despite an increase in serum levels of tumor-reactive IgG. These data suggest that SZP therapy can function to select mutant cells that are tumor Ag negative and thus resistant to SZP therapy because they are no longer opsonized with Ab and C3. Tumor escape through immune selection for tumor Ag-negative cells is a well-known confounding factor in immunotherapy.

β-Glucans can potentially be used to generate a novel cell-mediated effector mechanism for tumor vaccines and mAbs to tumor Ags that otherwise rely mostly on the direct cytotoxic action of C. This therapy appears to have the greatest applicability to metastatic tumors that have lost MHC class I and thus have escaped recognition by CTL (49, 50). Such metastatic tumors frequently express polysaccharide or ganglioside tumor Ags for which there is an array of available vaccines and mAbs. Although the complement activation stimulated by such mAbs is inefficient when it relies solely on the complement membrane attack complex, it might be adequate to mediate the iC3b-opsonization of tumors required for cytotoxicity by leukocytes bearing β-glucan-primed CR3. Finally, if β-glucan therapy were combined with vaccines that stimulated both Abs and immune CTL, then the likelihood for immunologic escape would be further reduced, because it would require tumors to lose both MHC class I and all class II-dependent tumor Ags.

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