The β-Glucan-Binding Lectin Site of Mouse CR3 (CD11b/CD18) and Its Function in Generating a Primed State of the Receptor That Mediates Cytotoxic Activation in Response to iC3b-Opsonized Target Cells[1]

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Mouse leukocyte CR3 (Mac-1, $\alpha_M\beta_2$ integrin) was shown to function as a receptor for β-glucans in the same way as human CR3. Soluble zymosan polysaccharide (SZP) or pure β-glucans labeled with FITC or ^{125}I bound in a saturable and reversible manner to neutrophils, macrophages, and NK cells. This lectin activity was blocked by anti-CD11b mAb M1/70 or 5C6 and did not occur with leukocytes from CR3$^{-/-}$ (CD11b-deficient) mice. SZP preparations containing primarily mannose or glucose bound to CR3, and the binding of ^{125}I-labeled β-glucan to CR3 was competitively inhibited by β-glucans from barley or seaweed, but not by yeast α-mannan. Also, as with human CR3, the lectin site of mouse CR3 was inhibited by α- or β-methylglucoside (but not D-glucose), α- or β-methylmannoside, and *N*-acetyl-D-glucosamine. Phagocytosis of zymosan and serum-opsonized zymosan was partially inhibited by anti-CR3 and was reduced to <40% of normal with leukocytes from CR3$^{-/-}$ mice. As with neutrophils from patients with CD18 deficiency, neutrophils from CR3$^{-/-}$ mice exhibited no phagocytosis of particulate β-glucan. SZP or β-glucans primed CR3 of neutrophils, macrophages, and NK cells for cytotoxicity of iC3b-opsonized tumor cells that otherwise did not trigger killing. β-Glucan priming for cytotoxicity was inhibited by anti-CR3 and did not occur with leukocytes from CR3$^{-/-}$ mice. The primed state of macrophage and NK cell CR3 remained detectable for 18 to 24 h after pulsing with β-glucans. The similarity of mouse and human CR3 in response to β-glucans highlights the utility of mouse tumor models for development of therapeutic β-glucans. ***The Journal of Immunology*, 1999, 162: 2281–2290.**

Biological response modifiers derived from microbial products have represented an important tool for defining mechanisms of host defense, but most biological response modifiers have remained classified as nonspecific because their exact mode of action was unknown and/or the chemical composition of their active component(s) was undefined. They have particularly represented an important area of research in tumor immunology because of the seminal work of William Coley more than 100 years ago who showed that certain bacteria culture extracts could occasionally induce complete remission in patients with cancer (1). β-(1,3)-D-Glucans have been studied for their ability to activate host defense mechanisms against tumors and microbial infections for the past 35 years. Numerous studies have demonstrated that β-glucans, either soluble or particulate, isolated from various natural sources and with variable molecular sizes and secondary structures, exhibit antitumor and antimicrobial activities in mouse model systems (2–11). Some of them have been applied clinically for tumor immunotherapy, such as the soluble fungal β-glucans lentinan and schizophyllan (12–21). In vitro studies to determine the mechanism of action of β-glucans have revealed that they activated macrophages, neutrophils, and NK cells to kill sensitive tumor cells (22–28) and that T cell responses to cellular Ags were potentiated (29–33). High m.w. soluble or particulate β-glucans have also been shown to stimulate neutrophil degranulation and respiratory bursts (34, 35), and the secretion of IL-1, TNF-α, and IL-6 from macrophages (36–38).

Analysis of the response of human leukocytes to β-glucans has shown that the $\alpha_M\beta_2$ integrin CR3 is primarily responsible for both the high affinity binding of particulate (34) or soluble (39) β-glucans and the cytotoxic and phagocytic responses mediated by β-glucans (35, 40, 41). By use of FITC- and ^{125}I-labeled polysaccharides, the β-glucan binding site (lectin site) of CR3 was mapped to a region of CD11b located C-terminal to the I-domain (39) and its distinct metal ion-dependent adhesion site ("MIDAS") for the many protein ligands of CR3 such as iC3b, ICAM-1, and fibrinogen (42, 43). Particulate or large soluble β-glucans were shown to cross-link membrane CR3 and trigger phagocytosis and a respiratory burst (34, 35), whereas small soluble β-glucans were shown to saturate individual cell surface CR3 molecules (39) and generate a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized tumor target cells that otherwise did not trigger this CR3-dependent response (35, 41). The lectin site of CR3 was shown to be divalent cation independent and to have a broad specificity for certain polysaccharides containing mannose

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and *N*-acetyl-D-glucosamine (NADG),[3] as well as glucose (39). Although initial studies that mapped the lectin site used recombinant chimeras between the α-chains of CR3 (CD11b) and CR4 (CD11c) expressed as heterodimers with the β-chain (CD18) (39), recent studies have been able to demonstrate lectin site activity with recombinant CD11b monomers from which the I-domain was deleted (44).

Few studies have attempted to identify the β-glucan receptor of mouse leukocytes. Because of the potential utility of β-glucans in tumor therapy, it is important to determine whether the murine receptor for β-glucans is the same CR3 molecule used by human leukocytes and whether the tumoricidal activity mediated by β-glucans also occurs through murine CR3. Previous studies of murine macrophage phagocytosis of unopsonized zymosan showed it to be inhibited by soluble β-glucan but not by soluble α-mannan (45). Small, soluble β-glucans released from baker's yeast with hot formic acid were shown to be particularly effective in blocking yeast phagocytosis by macrophages (46, 47). However, Giaimis et al. (48) later provided evidence that macrophage phagocytosis of unopsonized heat-killed yeast was mediated by both β-glucan and mannose receptors. Subsequently, Seljelid et al., who had produced a soluble β-glucan that activated murine macrophages for both antitumor and antibacterial activities (8, 24, 36, 49), showed that the activity of this soluble β-glucan could not be blocked by mannose or mannan, demonstrating that the murine receptor for soluble β-glucans was distinct from the mannose receptor (50). Okuda and Tachibana (51) were the first to suggest that β-glucan might function in macrophage tumoricidal activity through activation of CR3 to recognize C3 deposited on tumor cells by macrophages.

In this investigation, mouse leukocyte CR3 was examined for the presence and sugar specificity of a lectin site capable of recognizing soluble and particulate β-glucans. Leukocytes from normal and CD11b-deficient mice were used. Mouse CR3 was shown to contain a lectin site with sugar specificity and function in priming neutrophils, macrophages, and NK cells for cytotoxicity or phagocytosis that was the same as that in human CR3.

Materials and Methods

Polysaccharides and monosaccharides

A soluble yeast β-glucan (~20 kDa), both labeled with FITC and unlabeled, was purchased from Molecular Probes (Eugene, OR) and is termed herein "MP β-glucan." Zymosan A particles for phagocytosis assays and soluble β-glucans from seaweed (laminarin, ~8 kDa), barley (~500 kDa), and soluble yeast α-mannan were purchased from Sigma Chemical Company (St. Louis, MO). NADG, α-methylglucoside, β-methylglucoside, α-methylmannoside, β-methylmannoside, and D-glucose were also from Sigma. A soluble low m.w. polysaccharide fraction was isolated from baker's yeast by generation of zymosan (52) followed by solubilization of the zymosan with hot formic acid, ethanol precipitation, and S-200HR chromatography as previously described (39). There was some variation in the size and sugar composition of such soluble zymosan polysaccharide (SZP) preparations that was dependent on the batch of yeast. Monosaccharide analysis (39) of various SZP preparations indicated a homogenous-sized polysaccharide of 5–10 kDa made up of variable proportions of glucose and/or mannose. Recently generated preparations of SZP were found to have no detectable mannose and to be made up entirely of β-(1,3)-glucan, whereas previously reported preparations were found to consist primarily of mannose and ~5% glucose (39). As reported previously, such preparations of pure β-glucan exhibited comparable CR3-binding (39) and priming (35) activity to the other SZP preparations containing primarily mannose. The SZP preparations used in this investigation either consisted almost entirely of β-glucan, and are referred to as "SZP β-glucan", or consisted primarily of mannose and are referred to as simply "SZP" as previously reported (39). Compositional and linkage analysis of the SZP β-glucans used in the current investigation were conducted as a research service by the Complex Carbohydrate Research Center, University of Georgia, Athens, GA, using a combination of gas chromatography-mass spectrometry and nuclear magnetic resonance spectrometry. SZP or SZP β-glucan was labeled with FITC or ^{125}I as previously described (39).

mAbs and cell lines

The rat M1/70 hybridoma line secreting anti-mouse CD11b (CR3) mAb was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used to generate ascites fluid in BALB/c mice previously given 400 rad of γ radiation. IgG was isolated from ascites fluid by precipitation with 50% saturated ammonium sulfate followed by Mono-Q (Amersham Pharmacia Biotech, Piscataway, NJ) anion exchange chromatography (53). Some of the M1/70 IgG was coupled either to FITC (54) or to ^{125}I (55). Preparations of M1/70-FITC were also purchased from PharMingen (San Diego, CA). Another rat IgG anti-mouse CD11b mAb, 5C6 (56), was purchased from BioSource, Camarillo, CA. Rat 2D7 anti-mouse CD11a (LFA-1) mAb, both unlabeled IgG and IgG-FITC, were purchased from PharMingen. Biotinylated rat anti-mouse Ly-6G (Gr-1, anti-granulocyte) mAb, phycoerythrin (PE)-labeled rat anti-mouse CD80 (B7-1), anti-NK1.1, both unlabeled IgG and IgG-PE, and streptavidin-Cy-Chrome were also from PharMingen. Anti-F4/80-PE was purchased from Accurate Chemical and Scientific, (Westbury, NY). Goat $F(ab')_2$ anti-mouse IgG-FITC was from Southern Biotechnology Associates (Birmingham, AL), and goat $F(ab')_2$ anti-mouse C3-FITC was from Cappel (Durham, NC).

The mouse monocytoid cell line $P388D_1$ was obtained from the ATCC and maintained in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS. Before use in assays for uptake of ^{125}I-SZP β-glucan, the cells were differentiated by cultivation for 96 h in culture medium supplemented with 12 nM 1,25-dihydroxyvitamin D_3 (Aldrich Chemical, Milwaukee, WI) and 1 μM retinoic acid (Sigma) (46). The BALB/c mouse-derived mammary tumor cell line Ptas64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI. This line was maintained in monolayer culture at 37°C in RPMI 1640 medium containing 10% FBS and antibiotics, with passage every 2 days following release of the adherent cells with 1 × trypsin-EDTA solution (Mediatech, Herndon, VA).

Mice

Female, 6–8 wk old, BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Generation of mice deficient in CD11b ($CR3^{-/-}$) and their heterozygous-deficient ($CR3^{+/-}$) littermates were previously described (57). For the current investigation, CR3-deficient mice of a mixed BALB/c/129JV strain was used, and sex-matched wild-type ($CR3^{+/+}$), heterozygotes, and homozygous-deficient littermates were available for parallel assays of isolated leukocytes. Mice were bred and maintained in a virus Ab-free animal facility at the Longwood Medical Research Center (Boston, MA).

Preparation of unstimulated and thioglycolate-elicited peritoneal neutrophils and macrophages

Mice were given a 3-ml i.p. injection of BBL fluid thioglycolate medium (Becton Dickinson, Cockeysville, MD), and peritoneal exudates rich in neutrophils or macrophages were harvested into PBS after 18 or 96 h, respectively. The exudate cells were washed twice and resuspended in ice-cold RPMI 1640/5% FBS and maintained on ice until tested. Peritoneal cells were double-stained with a combination of biotinylated anti-Ly-6G with second step streptavidin Cy-Chrome and anti-mouse CD80-PE for analysis of the relative proportions of neutrophils and macrophages using flow cytometry. These tests demonstrated that the 18-h-induced peritoneal exudates contained 85–90% neutrophils, whereas the 96-h exudates contained 75–80% of macrophages. Unstimulated peritoneal macrophages were also obtained for certain experiments as noted by peritoneal lavage with PBS using mice that had not been given thioglycolate.

Preparations of splenic NK cells and macrophages

Splenic NK cells and macrophages were isolated as described (58) with minor modifications. Briefly, a cell suspension generated by pressing minced spleen against the bottom of a petri dish containing RPMI 1640/10% FBS was passed through nylon mesh and centrifuged at 200 × *g* for

[3] Abbreviations used in this paper: CR3, complement receptor type three (Mac-1, CD11b/CD18, $\alpha_M\beta_2$ integrin); EAC1–3bi, sheep E opsonized with rabbit IgM antibody and mouse C1 through C3bi; MP β-glucan, soluble yeast-derived β-glucan from Molecular Probes; NADG, *N*-acetyl-D-glucosamine; PI the number of ingested particles per 100 neutrophils or macrophages; SZP, soluble zymosan polysaccharide; PE, phycoerythrin; LAD, leukocyte adhesion deficiency.

10 min. Erythrocytes were removed by suspending the cells in an ammonium chloride buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2) and incubating for 2 min followed by dilution with an equal volume of FBS and centrifugation at 200 × *g* for 10 min. Macrophages were isolated by adherence and release from plastic petri dishes (58), and NK cells were isolated from the nonadherent cells by positive selection with NK1.1 followed by anti-mouse IgG-coated paramagnetic beads according to the directions from the manufacturer (Dynal, Great Neck, NY). The cells were washed three times and suspended in RPMI 1640/10% FBS. NK cells and macrophages were distinguished in flow cytometry assays by staining with NK1.1-PE or F4/80-PE, respectively. NK cell preparations contained 75–85% $NK1.1^+$ and macrophage preparations contained 80–85% $F4/80^+$ cells, and each preparation contained <1% contamination with the other effector cell type.

Preparation of sheep EAC1–3bi and iC3b-opsonized Ptas64 cells

Sheep E were opsonized with rabbit IgM Ab and fresh serum from C5-deficient AKR/J mice as previously described to generate EAC1–3bi (59). Freshly harvested Ptas64 cells with viability ≥95% were washed twice and resuspended in warm RPMI 1640 at 1×10^7 cells/ml. The cell suspension was mixed with an equal volume of a 1:2 dilution of fresh-frozen normal mouse serum complement (Harlan Bioproducts for Science, Indianapolis, IN) and incubated for 30 min at 37°C in a shaking water bath. The iC3b-opsonized Ptas64 cells were washed three times with ice-cold RPMI 1640/10%FBS and maintained on ice until used as targets for cytotoxicity assays. C3 deposition onto Ptas64 cells was confirmed by flow cytometry analysis with anti-C3-FITC.

Fluorescence staining and flow cytometry assay

Fluorescence staining techniques with FITC-labeled polysaccharides or mAbs and flow cytometry assay for the analysis of the receptor specificity of β-glucan-FITC staining were performed as with human leukocytes (39, 60). Listmode data files were analyzed, and histograms were generated using WinList 3.0 from Verity Software House (Topsham, ME).

Radioactive binding assay and Scatchard analysis of SZP β-glucan

Thioglycolate-elicited peritoneal macrophages or $P388D_1$ cells (viability, ≥95%) were harvested, washed twice, and resuspended in RPMI 1640/5% FBS. These mouse cells were analyzed for receptor-dependent (saturable/reversible) binding of ^{125}I-SZP β-glucan in the same way as described previously with human neutrophils (39, 61) with minor modifications. Briefly, 2.5×10^6 cells in 12- × 75-mm plastic tubes immersed in an ice bath were incubated with ^{125}I-SZP β-glucan or ^{125}I-M1/70 IgG mAb in the presence or absence of an unlabeled (competing) soluble polysaccharide, monosaccharide, or mAb in a final reaction volume of 100 μl. After 20 min of incubation on ice, 80 μl of the cell suspension (containing 2×10^6 cells) were overlaid onto 200 μl of a 6:4 mixture of *n*-butyl phthalate (Fisher Chemical, Fair Lawn, NJ) and dioctylphthalate (Aldrich) in 300-μl polypropylene microprecipitation tubes (Sardstadt, Newton, NC) and centrifuged at 14,000 rpm for 2 min in a microcentrifuge to separate the cells with bound ligand from unbound ligand. The radioactivity associated with the cells with bound ligand vs the supernatant with unbound ligand was determined in a gamma scintillation counter as previously described (39). Analysis of the binding affinity of the SZP β-glucan used in the current investigation was conducted by Scatchard plot analysis as previously described (39).

Assay for phagocytosis of zymosan and particulate β-glucan

Unopsonized zymosan and mouse serum-opsonized zymosan were prepared and used for assays of mouse neutrophil or macrophage phagocytosis in the same manner as described previously with human neutrophils (40) except that zymosan was opsonized with fresh-frozen mouse serum complement instead of human serum complement and thioglycolate elicited peritoneal neutrophils or macrophages were examined instead of peripheral blood neutrophils. Sterile suspensions of particulate yeast β-glucan were a generous gift from Dr. David L. Williams (East Tennessee State University, Johnson City, TN). Particle aggregates were dispersed by repeated and forceful expression through a 27-gauge needle and diluted into 1% BSA/HBSS (H-BSA) just before use at a final concentration of 1 mg/ml. For assays of phagocytosis, 50 μl of this suspension were mixed in a 10- × 75-mm plastic tube with 50 μl of neutrophils or macrophages (4×10^6/ml in H-BSA) and incubated on a tube rotator at 37°C for 10 min before analysis of phagocytosis by phase contrast microscopy (34). For tests of the receptor specificity of phagocytosis, the neutrophil or macrophage test suspension was incubated with 5 μg of IgG mAb or 250 μg/ml α-mannan or β-glucan for 15 min on ice before addition of zymosan, opsonized zymosan, or particulate β-glucan.

Cell-mediated hemolysis and cytotoxicity assays

EAC1–3bi or cells of the murine mammary tumor line Ptas64 and iC3b-Ptas64 cells were labeled with ^{51}Cr (Amersham Life Science, Arlington Heights, IL) and used as targets for neutrophil, macrophage, and NK cell-mediated hemolysis or cytotoxicity in the same manner as described previously with EC3bi bearing human C3, human mammary tumor cell lines, and human leukocytes (35, 41). As an alternative to the ^{51}Cr release assay, the nonradioactive CytoTox96 assay (Promega, Madison, WI) was used according to the instructions from the manufacturer for tests of peritoneal neutrophil cytotoxicity. Briefly, peritoneal neutrophils (5×10^6/ml in RPMI 1640/10% FBS; 50 μl/well) in V-bottom 96-well microplates (Dynatech Laboratories, Chantilly, VA) were incubated with 5.0 μg/ml of various activating or blocking agents (i.e., β-glucan, mAb) for 30 min at 37°C. IgG preparations were centrifuged (14,000 × *g* for 20 min at 4°C) just before addition to neutrophils to remove small soluble aggregates. Next, 50 μl of target cells (1×10^5/ml in RPMI 1640/10% FBS; giving a 50:1 E:T cell ratio) were added to each well, and the plate was incubated at 37°C for 4 h. The plate was centrifuged, and the absorbance at 490 nm was evaluated using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC). In preliminary studies, the optimum target cell number was determined to be 5×10^3, and E:T cell ratios of 5:1 to 100:1 were explored that indicated optimal CR3-dependent killing could be obtained with a 50:1 ratio. Therefore, all experiments shown herein used this 50:1 E:T cell ratio. Specific cell-mediated cytotoxicity was calculated using the formula: percent specific killing (% cytotoxicity) = $100 \times [(OD_{490}$ experimental − OD_{490} spontaneous) ÷ (OD_{490} maximum − OD_{490} spontaneous)] as described in the manufacturer's instruction, where spontaneous release was that obtained from target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

Results

CR3 functions as the mouse leukocyte receptor for SZP and β-glucan

Neutrophils, macrophages, and NK cells were examined for staining by FITC-labeled yeast MP β-glucan or SZP using flow cytometry (Figs. 1 and 2). Receptor-specific staining was confirmed with 1 μg/ml of each FITC-labeled polysaccharide by demonstration that staining was blocked in the presence of a 100-fold molar excess (100 μg/ml) of homologous unlabeled polysaccharide (Fig. 2). The fluorescence intensity of receptor-specific staining did not increase significantly when either of these FITC-labeled polysaccharides were used at higher concentrations. Neutrophils from both normal and CR3-deficient mice were stained equivalently with anti-LFA-1-FITC (Fig. 1). The profile of staining with MP β-glucan-FITC was similar to that obtained with anti-CR3-FITC, and neither occurred with neutrophils from homozygous CR3-deficient mice. MP β-glucan-FITC staining of neutrophils from normal or heterozygous CR3-deficient mice (that expressed similar amounts of CR3) was blocked by unlabeled anti-CR3 but not by unlabeled anti-LFA-1 (Fig. 1). Anti-CR3 also blocked almost all macrophage and NK cell staining by SZP-FITC (Fig. 2) or MP β-glucan-FITC (not shown).

Thioglycolate-elicited peritoneal macrophages from normal and CR3-deficient mice were also examined for receptor-specific uptake of ^{125}I-labeled SZP β-glucan (Fig. 3). Receptor-specific binding of the ^{125}I-labeled β-glucan occurred only with normal macrophages, not with CR3-deficient macrophages, and was blocked by anti-CR3 (Fig. 3). These "macrophage" data obtained with an exudate containing 20–25% neutrophils, as well as similar data obtained with 18-h exudates that were rich in neutrophils (not shown), indicated that neutrophil binding of ^{125}I-labeled β-glucan also required CR3.

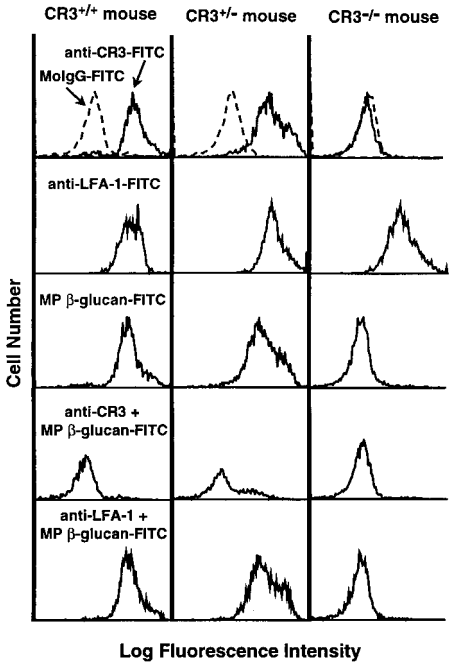

FIGURE 1. CR3-dependent staining of neutrophils with β-glucan-FITC. Flow cytometry was conducted with peritoneal neutrophils from normal ($CR3^{+/+}$) mice vs mice that were either homozygous ($CR3^{-/-}$) or heterozygous ($CR3^{+/-}$) for deficiency of CR3 (CD11b). Neutrophils from normal and heterozygous CR3-deficient mice were stained equivalently with M1/70 anti-CR3-FITC (*top*) or MP β-glucan-FITC (*middle*), but no staining with either of these markers was observed with neutrophils from the homozygous CR3-deficient mice. Equivalent expression of LFA-1 (CD11a) was noted on neutrophils from all three types of mice. *Bottom*, β-glucan-FITC staining of neutrophils from normal and heterozygous CR3-deficient mice was blocked by 10 μg/ml unlabeled M1/70 anti-CR3 but not by 2D7 anti-LFA-1.

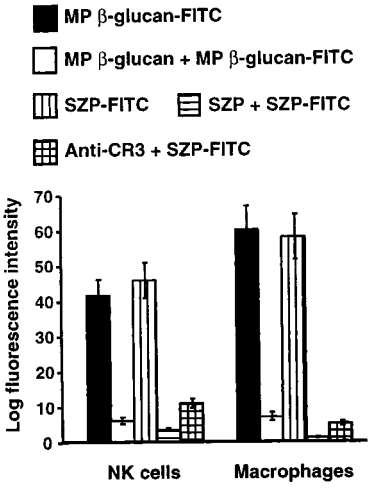

FIGURE 2. CR3-dependent staining of murine NK cells and macrophages with β-glucan-FITC or SZP-FITC. Murine splenocytes were examined for double staining by MP β-glucan-FITC and anti-NK1.1-biotin-streptavidin-PE to identify NK cells or F4/80-PE to identify macrophages. With both leukocyte types, β-glucan-FITC staining was blocked by a 100-fold molar excess of unlabeled β-glucan (MP β-glucan + MP β-glucan-FITC). NK cells and macrophages were also stained by SZP-FITC, and this staining was also inhibited by a 100-fold molar excess of unlabeled SZP (SZP + SZP-FITC). The staining produced by either SZP-FITC or MP β-glucan-FITC (not shown) was also inhibited by prior treatment of NK cells or macrophages with 10 μg/ml unlabeled M1/70 anti-CR3. Mean values ± SD for ≥3 assays of each type are shown.

Sugar specificity of the murine CR3 lectin site

The sugar specificity of CR3 was explored using the $P388D_1$ macrophage cell line and an assay for inhibition of ^{125}I-labeled SZP β-glucan uptake by competing unlabeled polysaccharides (Fig. 4) or monosaccharides (Fig. 5). As found previously with human CR3, the binding of yeast SZP β-glucan to mouse CR3 was blocked by competing unlabeled β-glucans from seaweed (laminarin) or barley. Laminarin and barley β-glucan exhibited less blocking activity (relative affinity) than did yeast β-glucan. No inhibition of ^{125}I-labeled β-glucan binding to $P388D_1$ cells was observed with competing unlabeled yeast α-mannan. Additional tests that compared the ^{125}I-labeled β-glucan-blocking activity of unlabeled SZP β-glucan to anti-CR3 mAbs showed ≥90% inhibition of specific binding with either M1/70 anti-CR3 or 5C6 anti-CR3 (not shown). Murine CR3 staining with M1/70-FITC was blocked only by unlabeled M1/70 and not by unlabeled 5C6, indicating that 5C6 recognizes a CD11b epitope that does not overlap with the I-domain epitope recognized by M1/70 (42) (not shown).

Unlabeled monosaccharides exhibited a pattern of inhibition of the murine CR3 lectin site that resembled previous observations with the human CR3 lectin site (39). Binding of ^{125}I-labeled SZP-β-glucan was inhibited competitively by α- or β-methylmannoside and α- or β-methylglucoside, but not by D-glucose (Fig. 5). Also, as with human CR3, the lectin site of murine CR3 could be blocked with competing NADG. The 10-fold lower binding affinity of the ^{125}I-labeled SZP β-glucan used in the current study (6.7×10^{-7} M with human neutrophils) as compared with the ^{125}I-SZP reported previously (6.7×10^{-8} M), probably explained the greater inhibition observed with the same monosaccharides in this investigation. Parallel tests of the currently used ^{125}I-SZP β-glucan with human neutrophils showed the same levels of blockade with NADG as observed with the mouse $P388D_1$ cells (not shown). Monosaccharide concentrations >25 mM increased the viscosity of cell suspensions such that some of the sugars produced less inhibition at concentrations >25 mM by promoting the nonspecific association of ^{125}I-labeled SZP β-glucan with cells during centrifugation through mineral oil.

Role of CR3 in phagocytosis of zymosan and particulate β-glucan

Peritoneal neutrophils from normal and CR3-deficient mice were examined for phagocytosis of zymosan, serum-opsonized zymosan, and particulate β-glucan (Fig. 6). The low level of normal neutrophil ingestion of unopsonized zymosan was inhibited by anti-CR3 but not by anti-LFA-1. A lower level of zymosan ingestion was noted consistently with CR3-deficient neutrophils, but this was not affected significantly by either anti-CR3 or anti-LFA-1. This low level of zymosan phagocytosis by the CR3-deficient neutrophil preparation was shown to result from the 10–15% of macrophages in the 18-h peritoneal exudate used as a source of neutrophils. Macrophages differ from neutrophils in that they express mannose receptors in addition to CR3 (62). When 96-h peritoneal exudates (containing 75–85% macrophages) were examined instead for phagocytosis, nearly equivalent phagocytosis of zymosan was observed with cells derived from wild-type or CR3-deficient mice. However, with macrophages from CR3-deficient mice, zymosan phagocytosis was inhibited 64% by soluble α-mannan but not by SZP β-glucan, whereas with normal macrophages, zymosan phagocytosis was inhibited only by SZP β-glucan (45%) and not by α-mannan.

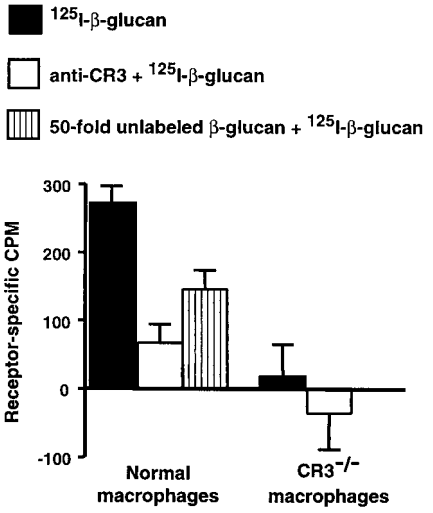

FIGURE 3. Peritoneal macrophages from normal, but not CR3-deficient mice exhibit receptor-specific uptake of ^{125}I-labeled SZP β-glucan. Peritoneal exudate cells (75–80% macrophages; 20–25% neutrophils) were examined. Saturation binding with ^{125}I-labeld β-glucan occurred at a hexose concentration of ~2 μg/ml with normal macrophages. Receptor-specific binding of ^{125}I-labeled β glucan was defined as the uptake of ^{125}I-labeled β-glucan inhibitable with a 100-fold molar excess of unlabeled β-glucan. Approximately one-half of the receptor-specific counts per minute of ^{125}I-labeled β-glucan was blocked in the presence of a 50-fold excess of unlabeled SZP β-glucan (100 μg/ml), and ~75% was blocked with 10 μg/ml unlabeled M1/70 anti-CR3. Macrophages from CR3-deficient (CR3$^{-/-}$) mice failed to demonstrate any receptor-specific binding of ^{125}I-labeled β-glucan.

With serum-opsonized zymosan, neutrophils exhibited a higher level of CR3-independent phagocytosis than was observed with unopsonized zymosan (Fig. 6). Anti-CR3 reduced phagocytosis by only 46%, and neutrophils from CR3-deficient mice ingested opsonized zymosan at levels that were about one-half the levels observed with normal neutrophils. By contrast, the phagocytosis of β-glucan particles demonstrated a requirement for CR3. Although anti-CR3 only reduced normal neutrophil ingestion by 49%, no ingestion of particulate β-glucan was noted with neutrophils from CR3-deficient mice.

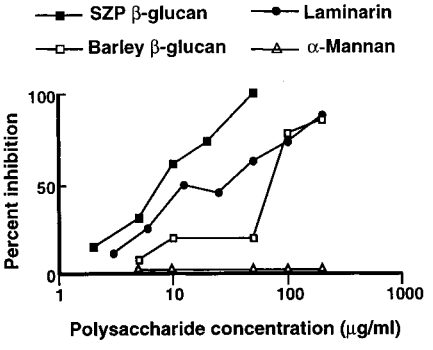

FIGURE 4. Inhibition of ^{125}I-labeled SZP β-glucan binding to CR3 of P388D$_1$ cells by competing unlabeled polysaccharides. P388D$_1$ cells were incubated in a 100-μl reaction mixture containing ~1 μg/ml of ^{125}I-labeled SZP β-glucan and 2.0 to 200 μg/ml unlabeled polysaccharides. Cell-bound ^{125}I-labeled SZP β-glucan was measured after centrifugation of the cell suspension through mineral oil. Maximum inhibition of cell-associated ^{125}I-labeled SZP β-glucan occurred in the presence of 50 μg/ml unlabeled SZP β-glucan, and accordingly, the net counts per minute value obtained with 50 μg/ml of unlabeled SZP was defined as 100% inhibition of receptor-specific binding. The percent inhibition of this value obtained with other unlabeled polysaccharides was determined and plotted vs polysaccharide concentration. The data shown represent the mean values of triplicates obtained from one experiment. Three similar experiments were conducted, and the results showed the same rank order of inhibition for the four competing unlabeled polysaccharides.

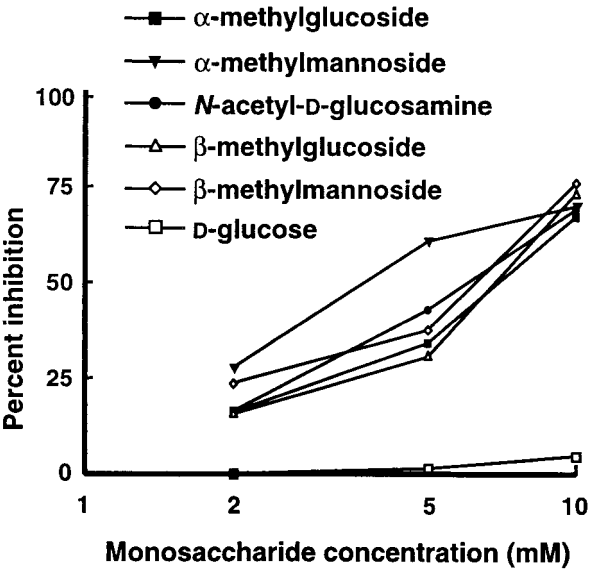

FIGURE 5. Inhibition of ^{125}I-labeled SZP β-glucan binding to CR3 of P388D$_1$ cells by competing unlabeled monosaccharides. Monosaccharides were tested for inhibition of the receptor-specific binding of ~1 μg/ml of ^{125}I-labeled SZP β-glucan as in Fig. 4. Data represent mean values of triplicates obtained from one experiment. Three similar experiments were conducted, and the results showed the same inhibition pattern for each monosaccharide.

Polysaccharide priming of CR3 for lysis or cytotoxicity of iC3b-opsonized target cells

Previous reports had shown that unstimulated peritoneal macrophages did not phagocytose EAC1–3bi, despite avid rosette formation (63). The current investigation showed that such unstimulated macrophages, as well as splenic NK cells, also did not lyse membrane-bound EAC1–3bi (Fig. 7). As with human CR3, addition of 1.0 μg/ml of MP β-glucan (Fig. 7) or SZP (not shown) primed CR3 for lysis of the bound EAC1–3bi. Lysis required target cell-bound iC3b, given that it did not occur with unopsonized E (not shown), and lysis was inhibited by anti-CR3 (Fig. 7).

Similar findings were obtained when the BALB/c mammary tumor cell line Ptas64 was examined as a target for cytotoxicity by murine neutrophils or NK cells (Fig. 8). Little cytotoxicity of unopsonized Ptas64 cells was noted with neutrophils ($\leq$8%) or NK cells ($\leq$15%), and there was no significant enhancement of cytotoxicity stimulated by addition of SZP (Fig. 8) or MP β-glucan (not shown). As noted previously with human breast cancer cell lines that were effectively opsonized with normal AB$^+$ human serum, this investigation showed that a BALB/c breast tumor cell line could be opsonized with iC3b using normal BALB/c mouse serum. Opsonization of Ptas64 cells with iC3b did not enhance cytotoxicity unless CR3 was primed with SZP (Fig. 8). Treatment of the neutrophils or NK cells with anti-CR3 blocked the cytotoxic activity generated by SZP.

The CR3 dependence of β-glucan-induced cytotoxicity of iC3b-Ptas64 cells was highlighted by tests of neutrophils from normal vs CR3-deficient mice (Fig. 9). A low level (~13%) of CR3-independent cytotoxicity was observed with both neutrophil types in the medium control. Addition of 5 μg/ml SZP β-glucan enhanced

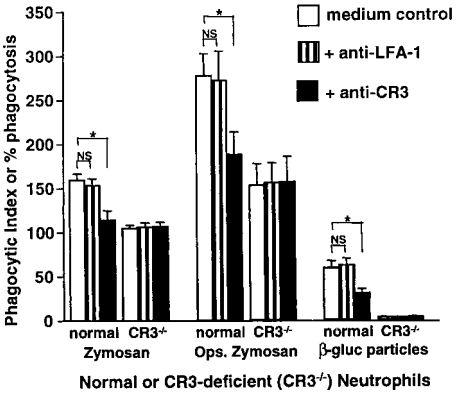

FIGURE 6. Phagocytosis of zymosan and particulate β-glucan by neutrophils from normal vs CR3-deficient ($CR3^{-/-}$) mice. Peritoneal neutrophils (85–90% neutrophils and 10–15% macrophages) were tested for phagocytosis of zymosan, mouse serum-opsonized zymosan (Ops. Zymosan), or particulate β-glucan (β-gluc particles). For zymosan and serum-opsonized zymosan, the phagocytic index (PI) was calculated (number of ingested particles per 100 neutrophils), whereas with particulate β-glucan the percent of phagocytosing cells (% phagocytosis) was determined. Mean values from ≥5 experiments are given, and in each of the individual assays in an experiment >300 neutrophils were observed microscopically for particle ingestion. For normal neutrophils, phagocytosis of zymosan, opsonized zymosan or β-glucan particles was significantly inhibited by anti-CR3 mAbs ($p \leq 0.05$), but not by anti-LFA1- mAbs. *, statistically significant differences; NS, not statistically significant differences.

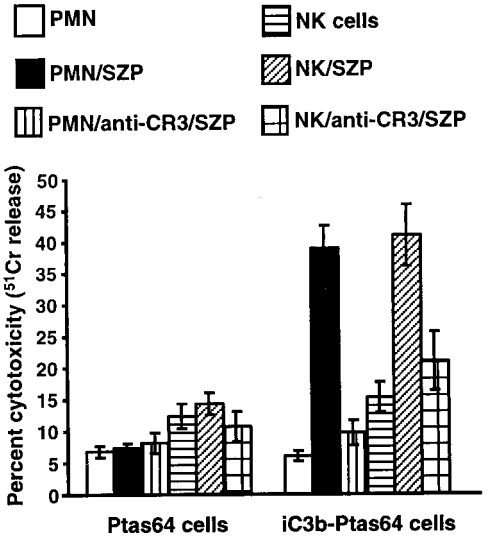

FIGURE 8. Soluble SZP primes CR3 of murine neutrophils or NK cells for cytotoxicity of iC3b-opsonized breast cancer cell line Ptas64. Ptas64 cells were resistant to cytotoxicity by peritoneal neutrophils (PMN) or splenic NK cells, even after opsonization with iC3b (iC3b-Ptas64 cells). Significant cytotoxicity was induced by 1 μg/ml SZP or MP β-glucan (not shown), occurred only with iC3b-opsonized cells, and was inhibited by 5 μg/ml M1/70 anti-CR3 but not nonspecific rat IgG (not shown). The mean ± SD of ≥3 experiments are shown.

the cytotoxicity of normal neutrophils from 13% to 73% in a CR3-dependent manner but had no effect on the cytotoxicity by CR3-deficient neutrophils.

Decay kinetics of the primed state of CR3

Splenic NK cells and unstimulated peritoneal macrophages were examined to determine how long CR3 remained primed for cytotoxicity of iC3b-Ptas64 cells following 30 min pulse priming with SZP and cultivation at 37°C (Fig. 10). A higher level of SZP-induced cytotoxicity was noted with NK cells as compared with macrophages, and the cytotoxicity produced by SZP-priming of NK cell CR3 was still greater than that of unprimed NK cells after 24 h. By contrast, SZP priming of macrophage CR3 became undetectable after 18 h of cultivation.

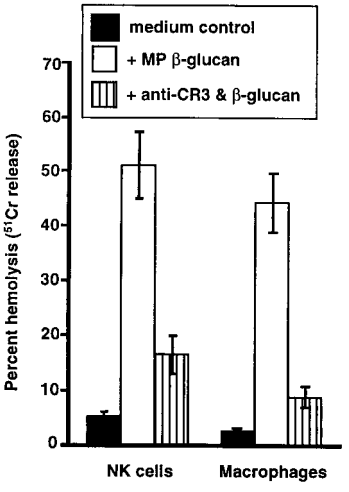

FIGURE 7. Soluble β-glucan primes CR3 of murine NK cells or macrophages for hemolysis of EAC1–3bi. Neither NK cells nor unstimulated macrophages (nonelicited, from peritoneal washes) lysed EC3bi despite avid CR3-dependent rosette formation. Hemolysis was induced by 1 μg/ml MP β-glucan or SZP (not shown) and was inhibited by 5 μg/ml M1/70 anti-CR3 but not by nonspecific rat IgG (not shown). Mean values ± SD from ≥3 experiments are shown.

Discussion

This investigation showed that mouse CR3 functioned as a receptor for soluble and particulate polysaccharides in the same way as human CR3. With normal mice, β-glucan receptor activity with all leukocyte types was blocked by M1/70 or 5C6 mAbs to CD11b but not by a mAb to CD11a. Moreover, β-glucan receptor function was undetectable with leukocytes from CR3-deficient mice, suggesting that CR3 may be responsible for all previously reported functions of β-glucans with mouse leukocytes. Murine and human CR3 exhibited the same specificity for certain polysaccharides containing mannose and NADG, as well as glucose. Phagocytosis of zymosan and serum-opsonized zymosan was partially dependent on CR3, whereas phagocytosis of particulate β-glucan required CR3. Soluble polysaccharides that bound to CR3 primed the receptor for cytotoxic activation by tumor cells bearing iC3b that otherwise did not stimulate killing. The decay kinetics of the primed state of CR3 showed that NK cells or macrophages exposed briefly to a soluble CR3-binding polysaccharide were still capable of killing iC3b-targeted tumor cells up to 24 h later.

The absence of β-glucan receptor function with all leukocytes from CD11b-deficient mice, as well as the inhibition of β-glucan receptor function with normal leukocytes by two mAbs to CD11b, confirmed that mouse CR3 shares the same type of β-glucan-binding lectin site as human CR3. Without the recombinant molecular chimeras between CD11b and CD11c that were available for human CR3, it was not possible to map the murine lectin site as had been done with human CR3 (39). As had been noted with human CR3, a mAb to the I-domain, M1/70, blocked lectin site activity, despite the conclusion from other data that the lectin site mapped to a C-terminal region. A similar finding has also been made with

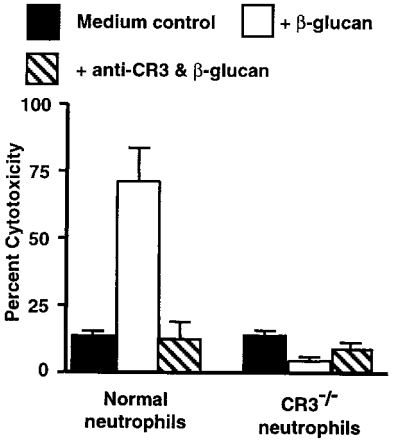

FIGURE 9. Neutrophils from CR3-deficient mice do not mediate cytotoxicity of iC3b-opsonized murine breast tumor cell line Ptas64 in response to soluble SZP β-glucan. M1/70 anti-CR3 (5 μg/ml) blocked β-glucan-induced killing by normal neutrophils but had no effect on the cytotoxicity mediated by the CR3-deficient neutrophils. Mean $\pm$ SD from three experiments are shown.

recombinant human CD11b, i.e., mAbs to the I-domain blocked lectin site function (44). However, lectin site function was also preserved in recombinant fragments of CD11b from which the I-domain had been deleted (manuscript in preparation).

Characterization of the CR3 lectin site was facilitated by FITC- and ^{125}I-labeled β-glucans. Use of small soluble β-glucans to block baker's yeast phagocytosis was first described in an investigation of the mouse macrophage line P388D$_1$ (46, 47). These studies showed that the murine receptor for baker's yeast could be blocked by soluble β-glucan but not by soluble α-mannan. Similar findings had been reported earlier with rat macrophages (64) and human monocytes (65). Recent reports about the cell wall architecture of baker's yeast have shown that it consists of β-glucan linked to a mannoprotein (66, 67). A similar polysaccharide structure apparently also exists in other yeast including *Candida albicans*. As with baker's yeast, the phagocytosis of unopsonized *C. albicans* has been shown to occur via a receptor now identified as CR3 (68) but first identified as a β-glucan receptor that could be

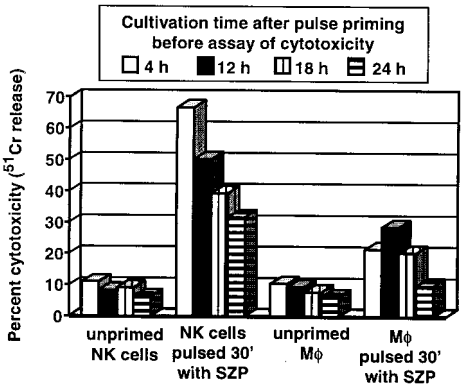

FIGURE 10. Decay kinetics of CR3 priming by SZP. Splenic NK cells or unstimulated (nonelicited) peritoneal macrophages (Mϕ) were incubated with 2 μg/ml SZP at 37°C for 30 min, washed three times, and cultured in RPMI 1640/10% FBS at 37°C for different time intervals before being tested for CR3-dependent cytotoxicity of iC3b-opsonized Ptas64 cells. Unprimed NK cells and macrophages were cultured in parallel and tested for their cytotoxic function at the same time intervals as the pulse primed cells. Results shown are mean values from three experiments.

blocked by soluble β-glucan but not by soluble α-mannan (69). However, another investigation showed that a soluble mannoprotein extracted from *C. albicans* was also able to block phagocytosis in the same way as soluble β-glucan, even though soluble α-mannan had no effect (70). More recent reports have suggested that this second polysaccharide component that blocks phagocytosis of *C. albicans* is a β-oligomannan and not α-mannan (71). Based on these observations about the CR3 lectin site specificity for adhesion to *C. albicans*, it is reasonable to propose that the original SZP mannan isolated from baker's yeast that bound to both human and mouse CR3 contained a similar β-oligomannan structure.

As with human CR3, mouse CR3 was found to have equivalent binding and priming activity with SZP preparations containing either $>$90% mannose and $\sim$5% glucose (original SZP) or $>$90% glucose with no detectable mannose (MP β-glucan). With either of these polysaccharides, saturation of CR3 could be demonstrated with 1.0 to 2.0 μg/ml hexose by flow cytometry (39), and the priming function for CR3-dependent NK cell-mediated cytotoxicity also did not increase with higher concentrations of these two polysaccharides (35). As shown earlier with human CR3, the binding of pure β-glucan to mouse CR3 could be blocked with α- or β-methylmannoside, as well as with α- or β-methylglucoside, providing further evidence for an unusual lectin site with dual specificity for either mannose- and glucose-containing polysaccharides.

Presumably the secondary structure of MP β-glucan, such as the frequency of β-(1,6)-glucan branching points that are known to contribute to the macrophage-activating activity of soluble β-glucans (72–74), explained its higher affinity for CR3. Regrettably, we must note that Molecular Probes discontinued distribution of this specific probe in 1996 and that a different β-glucan-FITC now marketed by Molecular Probes no longer exhibits receptor-specific staining with any leukocyte type; i.e., the staining produced is not blocked by excess unlabeled β-glucan from any source. The experiments shown in Fig. 1 were completed with some of the last remaining MP β-glucan-FITC obtained before 1996. The lower affinity of the SZP β-glucan did allow receptor-specific radiobinding assays following labeling with ^{125}I (Figs. 3–5).

Ligation of neutrophil CR3 to surface β-glucan on zymosan or particles of pure β-glucan stimulated phagocytosis. The results obtained in the current investigation with murine neutrophils treated with anti-CR3 or with neutrophils from CR3-deficient mice were remarkably similar to earlier results obtained with anti-CR3-treated human neutrophils and neutrophils from patients with leukocyte adhesion deficiency (LAD) (34, 75). Previous studies of murine neutrophils and macrophages have shown that phagocytosis of zymosan was mostly dependent on β-glucan receptors, except with macrophages where there was some contribution by mannose receptors (47, 48). Mannose receptors, which are inhibited by soluble α-mannan, are not expressed by murine neutrophils (62). Even though anti-CR3 inhibited zymosan phagocytosis, a reduced level of phagocytosis was detectable with the neutrophil preparation from CR3-deficient mice. The low number of cells ingesting zymosan in the CR3-deficient peritoneal neutrophil preparation was probably derived from the 10–15% contaminating macrophages detected separately by flow cytometry. Indeed, specific tests of CR3-deficient macrophages demonstrated avid phagocytosis of unopsonized zymosan that was blocked only by soluble α-mannan and not by soluble β-glucan. The failure of soluble α-mannan to inhibit zymosan phagocytosis by normal macrophages is consistent with some previous reports (45, 47), and indicates that the glucan-receptor activity of CR3 is dominant over mannose receptor function on normal macrophages.

Serum-opsonized zymosan presents a variety of opsonins. In addition to iC3b, serum-opsonized zymosan also can bear C3b,

IgG, and fibronectin that mediate ingestion via Fc receptors and fibronectin receptors, as well as mannan that triggers mannose receptors. The limited inhibition of opsonized zymosan phagocytosis produced by anti-CR3, as well as only a 40% reduction in phagocytosis by CR3-deficient neutrophils as compared with normal neutrophils, is similar to findings reported previously with normal human neutrophils vs neutrophils from patients with LAD (34, 75). By contrast and also similar to neutrophils from LAD patients, phagocytosis of particles of pure β-glucan derived from zymosan did not occur with neutrophils from CR3-deficient mice.

CR3 appears to have evolved to recognize iC3b-opsonized microorganisms and to ignore iC3b-opsonized host cells. Soluble polysaccharides that bind to the lectin site of CR3 apparently substitute for the CR3-binding polysaccharides of bacteria and fungi that are missing from host cells, allowing the high affinity adhesion mediated by opsonizing iC3b to stimulate extracellular cytotoxicity of host cells lacking these microbial polysaccharides. Concentrations of 1 to 5 μg/ml soluble β-glucan-primed murine neutrophil, macrophage, or NK cell CR3 for lysis or cytotoxicity of iC3b-opsonized cells that otherwise did not stimulate cytotoxicity. In addition to cell-mediated cytotoxicity, the ingestion of particulate β-glucan indicates that the lectin site of CR3 can also stimulate phagocytosis. CR3-mediated phagocytosis may require that the priming polysaccharide be attached to the target cell surface such as with iC3b-opsonized zymosan. Moreover, rabbit E differ from sheep E in that they are ingested by monocytes and neutrophils by way of a rabbit cell surface glucan-like polysaccharide (76) and CR3 (77).

After exposure to SZP, macrophage and NK cell CR3 remained primed for cytotoxicity of iC3b-opsonized tumor cells for up to 24 h. This is important for the development of β-glucan therapeutics because it means that a single daily dose of β-glucan may be adequate for stimulation of a continuous process of leukocyte CR3-mediated tumor destruction.

For the past 35 years, numerous reports have described how soluble and particulate β-glucans from a wide variety of fungi and with highly variable structures and sizes promote tumoricidal activity in the mouse. The clinical use of β-glucans for patients with cancer, primarily in Japan, has had variable success. As with other biological response modifiers, the lack of a clear understanding of their mechanism of action has made it difficult to refine their use. The current investigation indicated that mouse leukocytes respond to β-glucan via CR3 in the same manner as human leukocytes and that β-glucan-mediated tumoricidal activity by neutrophils, macrophages, and NK cells requires tumor cell opsonization with the CR3 ligand iC3b. Thus, it is now possible to predict that β-glucan therapy may be successful when tumor cells generate a humoral response and are opsonized with Ab and C3 and is likely to fail when tumors are not opsonized with C3. Likewise, specific β-glucans shown to have the highest affinity for CR3 should be the most effective in priming CR3 of circulating leukocytes.

The complete range of functions stimulated by primed CR3 that has been triggered for cytotoxic activation by an iC3b-opsonized target cell has not been fully defined. Most studies of CR3 have focused on its role as an adhesion molecule and its mechanisms for inside-out signaling that result in development of its high affinity adhesion site for ICAM-1 and its function in mediating the diapedesis of neutrophils into sites of infection. The relationship of the CR3 activation state for high affinity adhesion vs the CR3 activation state for cell-mediated cytotoxicity is unknown, but available data suggest distinct differences. Even though both ICAM-1 and iC3b bind to overlapping sites within the I-domain of CD11b, neutrophils activated for high affinity adhesion to ICAM-1 do not degranulate with a respiratory burst when they attach to endothelium via ICAM-1. Furthermore, neutrophils bearing β-glucan-primed CR3 do not exhibit high affinity adhesion to substrates or display the mAb24 reporter epitope corresponding to the high affinity ICAM-1 binding state (35). A large number of reports have examined cytokine (e.g., IL-1, TNF-α, IL-6) secretion by murine macrophages stimulated by high m.w. soluble or particulate β-glucans (78–83). With human neutrophils and monocytes, such polysaccharides have been shown to be large enough to cross-link multiple cell surface CR3 molecules, thereby both priming and triggering CR3 activation.

β-Glucan appears to be one of the first microbial biological response modifiers for which the cellular mechanism of action has been defined at the specific receptor level. Because the cytotoxic host defense function of β-glucans is specific for target cells bearing iC3b, most of which results from Ab to target cell Ags and the classical pathway of C activation, its action in promoting host defense relies on the specificity of the Ab, thus differentiating it somewhat from other "nonspecific" biological response modifiers.

Acknowledgments

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