BTR2003: Unified Science & Technology for
Reducing Biological Threats and Countering Terrorism
March 19-21, 2003

Oral WGP Beta Glucan Treatment Accelerates Myeloid Recovery after Radiation Exposure

Daniel J. Allendorf
James Graham Brown Cancer Center
Department of Microbiology/Immunology
529 South Jackson St., Room 3B
University of Louisville
Louisville, KY 40202
Tel: 502-852-5357; Fax: 502-852-2123; e-mail: djalle02@gwise.louisville.edu

Jarek T. Baran, Ph.D.
James Graham Brown Cancer Center
529 South Jackson St., Room 3B
University of Louisville
Louisville, KY 40202
Tel: 502-852-5357; Fax: 502-852-2123; e-mail: jtbara01@gwise.louisville.edu

Mariusz Z. Ratajczak, M.D., Ph.D., D.Sc.
Professor and Director, Stem Cell Biology Program
James Graham Brown Cancer Center
529 South Jackson St., Room 408
University of Louisville
Louisville, KY 40202
Tel: 502-852-1788; Fax: 502-852-3032; e-mail: mzrata01@gwise.louisville.edu

General Charles W. Dyke (retired)
International Trade and Technology Associates
1330 Connecticut Ave. N.W. Suite 210
Washington, DC 20036
Tel 202-828-2614; Fax 202-828-2617; e-mail: cdyke@itta.com

Gary Ostroff, Ph.D.
Chief Technology Officer
Biopolymer Engineering, Inc.
3388 Mike Collins Drive, Eagan, MN 55121
Tel: 651-675-0300; Fax: 651-675-0400; e-mail: gostroff@biopolymer.com

Gordon D. Ross, Ph.D.*
Professor and Acting Director
Chemoattractants and Cancer Program
James Graham Brown Cancer Center
529 South Jackson St., Room 429
University of Louisville
Louisville, KY 40202
Tel: 502-852-5523; FAX: 502-852-2123; Email gordon.ross@louisville.edu

* Corresponding Author

ABSTRACT

The threat of nuclear radiation terrorism has prompted a widespread search for defenses against this menace. Radiation destroys the bone marrow (BM) and depletes the body of white blood cells (WBC or leukocytes) that defend against infection and disease. In this immune-weakened state, the body is more susceptible to infection and may become overwhelmed before the immune system has time to recover. I.V. treatment with β-glucan, β(1,3) linked polymers of D-glucose isolated from the cell wall of baker's yeast, has previously been shown in mice to accelerate BM recovery and increase survival after lethal irradiation. Oral yeast β-glucan administration offers many practical and financial benefits as an alternative treatment, but has not been previously examined for a similar benefit in post-radiation myeloid recovery and survival. To test this hypothesis, mice were exposed to a sublethal dose of radiation (500 cGy) and treated daily with 80 μg of whole glucan particles (WGP Beta Glucan) by gastric gavage. This treatment with WGP Beta Glucan gave rise a to significantly faster recovery of leukocyte counts, as early as 7 days post-irradiation compared to control mice treated with PBS. A role for CR3 in mediating this hematopoietic recovery was established as oral WGP treatment in sublethally irradiated $CR3^{-/-}$ (i.e. $CD11b^{-/-}$) mice failed to enhance myeloid recovery. Complement receptor 3 (CR3, Mac-1, or CD11b/CD18) is a β_2-integrin found on monocytes, macrophages, and neutrophils that functions as a major leukocyte receptor for β-glucan, as well as for iC3b and ICAM-1. Subsequent investigation of mice that had been fed fluorescein-labeled WGP Beta Glucan revealed intestinal macrophages that had ingested WGP Beta Glucan and had migrated to the spleen and BM. Further experiments showed that wild-type, but not $CR3^{-/-}$ macrophages, stimulated by WGP Beta Glucan synthesized IL-12 and several other inflammatory cytokines including GM-CSF. IL-12 is known to stimulate T lymphocyte production of oncostatin-M, a potent hematopoietic cytokine. In addition, the damaged BM removed from irradiated mice showed evidence for deposition of the serum complement protein C3 (iC3b-fragment) that was shown *in vitro* to serve as a co-stimulator of hematopoietic stem cell CR3 when added in combination with fragments of the β-glucan that are probably released by macrophages that convey WGP Beta Glucan to the BM. A role for serum complement C3 was further suggested because both C3-deficient ($C3^{-/-}$) and $CR3^{-/-}$ mice exhibited a similar defect in recovery of blood leukocytes following sublethal radiation injury. While the exact mechanism of WGP Beta Glucan-enhanced myeloid recovery remains to be established, these data indicate that orally administered WGP Beta Glucan stimulates myeloid recovery following irradiation in a CR3-dependent manner. Consequently, oral treatment with WGP Beta Glucan may be a useful therapeutic intervention following radiation exposure to accelerate myeloid recovery after radiation exposure.

INTRODUCTION

The threat of terrorism has raised awareness that there are relatively few options to protect or treat the U.S. public from the effects of nuclear catastrophe or radiation terrorism. Exposure to radiation can cause a rapid depletion of immune cells and platelets derived from the bone marrow (BM) that are necessary for controlling life-threatening infections and bleeding episodes. It is the loss of these key immune cells that is responsible for the infectious morbidity and mortality following radiation exposure, due to the inability of radiation damaged BM to reconstitute the immune system.

Under 44 CFR 351.23(f), the Department of Health and Human Services is directed to provide guidance to state and local governments on the use of radioprotective substances and the prophylactic use of drugs to reduce the radiation dose to specific organs. Currently, the only drug that is FDA approved and considered appropriate for stockpiling to protect against radiation injury is potassium iodide (KI) (http://www.fda.gov/cder/guidance/4825fnl.htm). KI is useful to protect the thyroid from the long-term risk of thyroid cancer, but provides no protection against the acute BM-destroying (myelosuppressive) effect of radiation. Nonetheless, stockpiling of KI has begun for civilians living within 20-50 miles of the 103 active nuclear power plants in the U.S. Recently, Prussian Blue (ferric hexacyanoferrate) has been suggested as a sequesterant treatment for ingested thallium or cesium to reduce the absorption of these radioactive isotopes (http://www.fda.gov/OHRMS/DOCKETS/98fr/03-2597.htm) and the FDA has requested the submission of New Drug Applications for Prussian blue drug products (http://www. fda.gov/cder/ drug/infopage/ prussian_ blue/). However, there are currently no recommended treatment options to reconstitute radiation damaged BM to protect against both life-threatening infections and bleeding episodes.

β-Glucan is a well-known biological response modifier (BRM) that has been used as an immunoadjuvant therapy for cancer since 1980, mostly in Japan. It represents a class of fungal and yeast cell wall polysaccharides that is made up entirely of glucose β(1,3)-linked together in linear chains with variable frequency of β(1,6)-linked side chains [1]. Another activity demonstrated with β-glucan in the mid-1980's was its ability to stimulate hematopoiesis (blood cell formation), in an analogous manner as granulocyte monocyte–colony stimulating factor (GM-CSF) [2]. Research was carried out initially with particulate β-glucan and later with soluble β-glucans, all of which were administered intravenously to mice [3-6]. Mice exposed to 500-900 cGy of gamma radiation exhibited a significantly enhanced recovery of blood leukocyte, platelet and red blood cell counts when given i.v. β-glucans [7, 8]. Other reports showed that β-glucan could reverse the myelosuppression produced with chemotherapeutic drugs such as fluorouracil [9] or cyclophosphamide [10, 11]. Moreover, the anti-infective activity of β-glucan combined with its hematopoiesis-stimulating activity resulted in enhanced survival of mice receiving a lethal dose of 900-1200 cGy of radiation [7]. *In vitro* studies showed that β-glucan could enhance granulocyte and megakaryocyte colony formation by hematopoietic stem progenitor cells when used in combination with GM-CSF and interleukin-3 (IL-3), respectively [12]. Development of β-glucans for their hematopoietic activity was not considered worthwhile at that time because of advent of GM-CSF as a therapeutic agent. The Armed Forces Radiobiological Research Institute (AFRRI) that did much of the early research showing the radioprotective effects of β-glucan also considered β-glucan use to protect individuals exposed to radiation as a result of a nuclear power plant accident or nuclear war. However, the apparent need to administer β-glucans intravenously made it unfeasible to rapidly treat large numbers of people in such emergency situations.

Subsequently, the oral immunomodulatory activities of β-glucans have been recognized. It is believed that the oral uptake of certain β-glucans by M (microfold) cells in intestinal Peyer's patches leads to β-glucan presentation to macrophages in the underlying gut-associated lymphatic tissue (GALT). Orally delivered mushroom β-glucans have been shown to activate peritoneal and alveolar macrophages [13, 14]. Further, oral administration of the shitake mushroom-derived β-glucan, lentinan has been found to increase the number of T helper cells in blood of rats [15]. Oral β-glucan has also been shown to induce anti-infective [16, 17] and anti-tumor activities in both preclinical and clinical studies [18-20]. Summarizing available data, β-glucans function by stimulating host immune defense mechanisms, primarily macrophages,

neutrophils, NK cells, and dendritic cells, thereby enhancing microbial or tumor cell clearance and subsequently reducing mortality [21, 22].

This report details studies to evaluate the potential of the yeast-derived orally active β-glucan immune modulator WGP Beta Glucan to accelerate hematopoiesis following radiation in an analogous manner as intravenously administered β-glucans.

MATERIALS AND METHODS

Animals. A colony of CR3-deficient ($CD11b^{-/-}$) mice and their wild-type littermates on a C57BL/6 background was established at the University of Louisville from breeders provided by Dr. Tanya Mayadas (Harvard Medical School, Boston, MA) who had generated the founder mice [23]. Another colony of mice deficient in the serum complement protein C3 ($C3^{-/-}$) and their wild-type littermates on a C57BL/6 background was established from heterozygous breeders obtained from the Jackson Laboratory (Bar Harbor, ME) that came originally from founder mice generated by Dr. Michael Carroll (Center for Blood Research, Harvard Medical School, Boston, MA) [24]. Mice used for these experiments were all 10 weeks of age, and equal numbers of males and females were examined.

Immune modulators. WGP Beta Glucan (WGP, Imucell™ WGP Glucan, Biopolymer Engineering Inc., Eagan, MN, USA) is a component from the cell walls of Baker's yeast that is purified by extraction of cellular proteins, nucleic acids, lipids, and most non-glucose-based oligosaccharides (e.g., chitin and mannans) by a morphologically non-destructive proprietary process [25]. It is a highly purified, 3-5 micron, spherical β-glucan particle. WGP Beta Glucan was labeled with fluorescein using DTAF (Molecular Probes, Inc., Oregon) generating a green WGP-DTAF particle for fluorescence microscopy or flow cytometry.

WGP absorption and distribution. Using WGP-DTAF, it was possible to measure phagocytosis by flow cytometry and to isolate cells with ingested WGP-DTAF by fluorescence-activated cell sorting (FACS). To monitor the uptake of orally administered WGP-DTAF, mice were fed 400 μg of the WGP-DTAF daily by intragastric administration and then examined on days 3, 7, and 12 for the presence of splenic, lymph node, and BM macrophages containing WGP-DTAF by FACS and fluorescence microscopy. Macrophages containing green WGP-DTAF were identified by red surface staining with the macrophage-specific antibody F4/80 coupled to the red fluorochrome, cychrome 5 (i.e., Cy5, BD Biosciences Pharmingen, San Diego, CA).

Radiation protection. To test the radiation protective effects of oral WGP Beta Glucan treatment, groups of 5 mice, either wild-type or $CR3^{-/-}$ mice, were given either an intragastric dose of 80 μg of WGP suspended in saline or saline only (control) one day prior to a sublethal radiation exposure (500 cGy). The mice received additional daily intragastric doses of WGP Beta Glucan or saline control for the entire 3-week period of observation. Other groups of wild-type or $CR3^{-/-}$ mice received a single i.v. injection of 400 μg of WGP Beta Glucan suspension in saline or saline control one day prior to radiation. A control wild-type group received no irradiation. White blood cell (WBC) counts were determined periodically over a 3-week observation period for each mouse and FACS was carried out to determine the proportions of granulocytes ($Gr\text{-}1^{high}$), monocytes ($Gr\text{-}1^{low}$), T cells ($CD3^{+}$) and B cells ($CD19^{+}$). Numerical count data were evaluated using Prism 3.0 (Graph Pad Software, San Diego, CA) that calculated the mean, standard deviation, and statistical significance (Student's T test) of values obtained from the individual groups of mice at each time point.

Cytokine synthesis. White blood cell cytokine synthesis was evaluated by intracytoplasmic staining of specific cytokines (IL-4, IL-6, IL-12, tumor necrosis factor (TNFα), IFNγ, and GM-CSF) using a test kit (BD Biosciences Phamingen) in which specific leukocyte types were labeled by surface staining and then the leukocytes were permeabilized for cytoplasmic staining using fluorochrome-labeled antibodies to specific cytokines. The stained leukocytes were examined by flow cytometry, and the specificity of cytoplasmic staining was evaluated by showing blockade of fluorochrome-labeled antibody staining with an excess of

homologous unlabeled antibody. This test was applied to mouse blood leukocytes or peritoneal leukocytes from wild-type or $CR3^{-/-}$ mice at various times after administration of WGP Beta Glucan by intragastric or intraperitoneal dosing. Cytokine secretion was evaluated in isolated human blood monocytes incubated *in vitro* with WGP Beta Glucan using an ELISA assay as previously described [26].

C3 deposition on bone marrow stroma. To determine if complement was activated by damage to bone marrow (BM) cells produced by radiation or cyclophosphamide, the BM of mice treated with these agents was removed and the isolated and washed BM cells were stained with rabbit anti-mouse C3c-FITC. The presence of bound C3 (iC3b fragment) on viable cells was evaluated by flow cytometry by gating on viable cells that were not stained with propidium iodide.

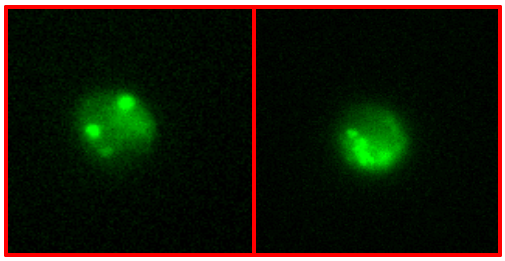
RESULTS

Parallel research on the ability of β-glucans to promote tumoricidal and anti-infective activities indicated that orally administered yeast-derived WGP Beta Glucan was equally effective as β-glucans given intravenously (i.v.) [17, 27]. Since, i.v. administered β-glucans have been shown to promote hematopoiesis and to protect mice from lethal gamma radiation injury, experiments were designed to determine if orally administered WGP Beta Glucan was equally effective as β-glucans given i.v.

Orally administered WGP are bioavailable. *In vitro* tests with resident peritoneal macrophages and the J774 murine macrophage cell line showed that the fluorescein label on WGP-DTAF did not alter the biological activities or CR3 specificity of macrophage phagocytosis (data not shown). Following 3-12 days of WGP-DTAF feeding to mice, cells containing WGP-DTAF were detected in spleen (Fig. 1), peritoneal lymph nodes (not shown), and BM (Fig. 2). FACS sorting of the fluorescein-positive cells allowed examination of cells by fluorescence microscopy. Microscopic visualization confirmed that the green fluorescence corresponded to ingested WGP-DTAF particles within cells, and that all cells containing green WGP-DTAF were also stained red with the macrophage specific antibody F4/80-Cy5 (Fig. 3). Although no WGP-DTAF were detected in macrophages from $CR3^{-/-}$ mice on day 3, approximately equivalent numbers of WGP-DTAF containing macrophages were detected in all lymphoid organs examined from $CR3^{-/-}$ versus wild-type mice on days 7 and 12 (data not shown).

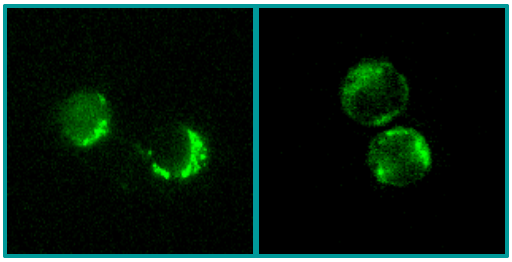
Macrophages that have ingested yeast cell walls or large soluble β-glucan molecules are known to degrade these materials and release small soluble fragments of β-glucan [28, 29]. Microscopic evaluation of WGP-DTAF in macrophages isolated from BM or spleen showed the presence of intact and spherical

Figure 1



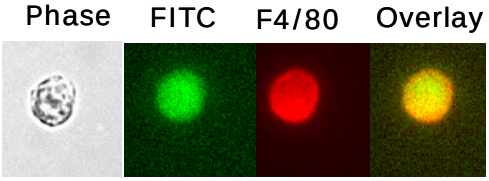
Splenic macrophages isolated from mice that had been given daily intragastric doses of WGP-DTAF for 3 days were sorted by FACS for evaluation by fluorescence microscopy. The macrophage in the left panel appears to contain intact spherical WGP, whereas the WGP within the macrophage in the right panel appears somewhat degraded into smaller green particles.

Figure 2



Bone marrow macrophages isolated from mice that had been given daily intragastric doses of WGP-DTAF for 12 days were sorted by FACS for evaluation by fluorescence microscopy. Only small green particles are seen within the macrophages.

Figure 3

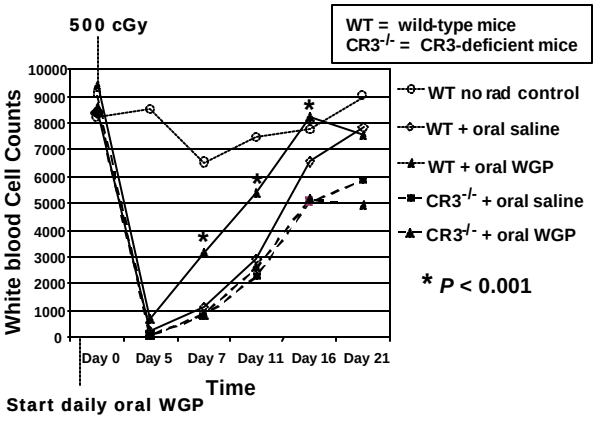


Bone marrow macrophages isolated from mice that had been given daily intragastric doses of WGP-DTAF for 7 days were surface stained with the red Cy5-F4/80 macrophage-specific antibody and sorted for green-stained cells by FACS for evaluation by fluorescence microscopy. All of the FACS-sorted green-stained cells contained particles of WGP-DTAF and also exhibited red surface staining, indicating that they were all macrophages (and not neutrophils or dendritic cells)

WGP-DTAF particles early after feeding (Fig. 1 shows splenic macrophages isolated 3 days after feeding WGP- DTAF), whereas the particles appeared more fragmented and diffuse in BM macrophages isolated 7-12 days after feeding DTAF-WGP (Fig. 2). These results show that WGP Beta Glucan is orally bioavailable and that the WGP Beta Glucan -containing intestinal macrophages migrate to BM, spleen, and lymph nodes where the WGP are degraded *in situ* into smaller fragments of β-glucan, which may in part be responsible for the hematopoeitic activity of oral WGP.

Oral WGP Beta Glucan protects against radiation damage. In irradiated mice, WBC counts reached a nadir of <1,000 per mm^3 on day 5, and WBC counts returned to normal on day 21 in irradiated wild-type mice treated with i.v. or oral saline (Fig. 4). In wild-type mice receiving i.v. WGP, WBC counts were higher than i.v. saline controls on day 7 only (not shown). In comparison, wild-type mice given WGP Beta Glucan orally exhibited significantly higher WBC counts than did mice given oral saline on days 7, 11, and 16 after irradiation and returned to normal 5 days sooner than mice receiving oral or i.v. saline (i.e., day 16 vs. 21) and 3 days sooner than mice receiving a single dose of i.v. WGP Beta Glucan (not shown). It is particularly noteworthy that the saline-treated $CR3^{-/-}$ mice exhibited a significantly slower hematopoietic reconstitution than did the saline-treated wild-type mice, and that there was no enhancement of recovery induced by oral (Fig. 4) or i.v. WGP Beta Glucan (not shown). Flow cytometry analysis showed equivalent recovery of each of the major leukocyte types examined in the WGP Beta Glucan -treated mice as compared to the saline-treated mice (not shown).

Figure 4



Groups of 5 mice, either WT or $CR3^{-/-}$ were given either an intragastric doses of 80 μg of WGP or saline control one day prior to a sublethal radiation exposure (500 cGy). The mice received additional daily intragastric doses of WGP or saline control for the entire 3-week period of observation.

Figure 5

FACS Figure to be inserted

Flow cytometry analysis of mouse bone marrow cell suspensions for the presence of deposited iC3b following exposure of the mice to gamma radiation (750 cGy) or treatment with cyclophosphamide. Deposited iC3b was detected by staining with rabbit anti-mouse C3c-FITC. Net specific C3-staining was calculated by subtracting background non-specific staining obtained by carrying out a parallel analysis of bone marrow cells from mice genetically deficient in C3, i.e., $C3^{-/-}$ mice. The dashed lines show staining of marrow cells from untreated mice, whereas the solid lines show the staining of BM cells from mice exposed to radiation or treated with cyclophosphamide.

Damage to marrow caused by radiation, cyclophosphamide, or G-CSF activates complement and deposits iC3b on viable marrow stromal cells. Following BM injury, it is known that hematopoietic stem progenitor cells (HSPC) home to the site of marrow injury and attach to the injured cells prior to initiating the repair process. Attachment of HSPC to the site of marrow injury may involve CR3 that is expressed on the membranes of HSPC and can function as an adhesion molecule to allow HSPC attachment to stromal cells [30]. HSPC that lack CR3 fail to attach to the BM and initiate hematopoiesis [31]. A major ligand for CR3 that could mediate such attachment is the iC3b fragment of C3 that can become attached to injured tissues through activation of the complement system. The current studies showed that BM cells from normal but not $C3^{-/-}$ mice exposed to gamma radiation or

cyclophosphamide had surface deposits of the iC3b on injured (but still viable cells) that was detectable by immunofluorescence staining and flow cytometry (Fig. 5). The attachment of HSPC via CR3 to this iC3b deposited on BM cells has been suggested by the finding of enhanced release of HSPC from the BM into the blood of either normal mice treated with a blocking antibody to CR3 [32] or mice genetically deficient in CR3 or C3 (M. Z. Ratajczak and G. D. Ross, unpublished observation). Attachment of HSPC to this iC3b on injured BM cells in the presence of soluble β-glucan (released from macrophages that have ingested WGP) could provide a signal that activates the HSPC for enhanced hematopoiesis. For example, the activation of mature myeloid cells by soluble β-glucan is known to require the co-stimulation of CR3 by membrane-bound (deposited) iC3b [33].

Stimulation of cytokine synthesis. Analysis of murine peritoneal macrophages by intracellular staining and flow cytometry demonstrated that macrophages stimulated by WGP Beta Glucan phagocytosis *in vivo* synthesized TNFα, IL-6, IL-12, and GM-CSF (data not shown). Notably, these cytokines were detected only in macrophages from wild-type but not from $CR3^{-/-}$ mice that were isolated 24 h after intraperitoneal injection of WGP Beta Glucan. The only change in cytokines observed among blood leukocytes from mice given daily intragastric doses of WGP Beta Glucan for 6 days was that there was a significant increase in the proportion of $CD4^+$ helper T cells containing the Th1 cytokine IFNγ (increased from 0.5% to 3.5%). This finding may be due to the presence of WGP Beta Glucan -stimulated macrophages in lymph nodes that secreted IL-12, a cytokine known to stimulate the formation of Th1 cells. ELISA assays of human blood monocytes similarly demonstrated that ingestion of WGP Beta Glucan stimulated the secretion of TNFα, IL-6, and IL-12. Thus, it is likely that the WGP Beta Glucan -containing intestinal macrophages that migrate to BM secrete these cytokines and that these cytokines may contribute to action of oral WGP Beta Glucan in stimulating hematopoiesis.

DISCUSSION

The threat of nuclear radiation terrorism in America has become a real and frightening possibility and has prompted a widespread search for defenses against this danger. One of the deleterious effects of gamma radiation exposure is damage to the bone marrow (BM), depleting the body of white blood cells that defend against infection and disease. The current investigation demonstrated that immune modulation by oral WGP Beta Glucan treatment may offer a practical treatment option to protect the military and the public from these BM-injuring (myelosuppressive) effects of radiation exposure.

Yeast-derived β1,3-glucans work, in part, by stimulating innate anti-fungal immune mechanisms to fight a range of pathogenic challenges from bacteria, fungi, parasites, viruses, and cancer. Research to define the mechanism of action of β-glucans has shown that they function through the priming of macrophages, neutrophils, monocytes, and NK cells, giving these cells an enhanced activity to kill microbial pathogens or tumor cells. Two β-glucan-binding receptors on leukocytes have been characterized that function to promote the phagocytosis of yeast cells walls via binding to β-glucan. First, the iC3b-receptor CR3 (also known as Mac-1, CD11b/CD18, or $\alpha_M\beta_2$-integrin) was shown to have a β-glucan-binding lectin site that functioned in the phagocytosis of yeast cell walls by neutrophils, monocytes, and macrophages [34, 35]. CR3 binds soluble fungal β-glucan with high affinity (5×10^{-8} M) and this primes the receptor of phagocytes or NK cells for cytotoxic degranulation in response to iC3b-coated tumor cells [35-37]. The tumoricidal response promoted by soluble β-glucan in mice was shown to be absent in mice deficient in either serum C3 (complement 3) or leukocyte CR3, highlighting the requirement for iC3b on tumors and CR3 on leukocytes in the tumoricidal function of β-glucans [38].

Dectin-1 represents the second membrane receptor for β-glucan involved with glucan particle phagocytosis [39]. Dectin-1 is expressed at high levels on thioglycolate-elicited peritoneal macrophages and its activity predominates over that of CR3 in the phagocytosis of yeast via β-glucan binding by these activated cells [40, 41]. However, yeast phagocytosis by neutrophils and resident peritoneal macrophages is

blocked by anti-CR3 and does not occur with CR3-deficient ($CD11b^{-/-}$) neutrophils or resident macrophages [35]. Moreover, dectin-1 is not expressed by NK cells [41] that use CR3 to mediate tumoricidal activity against iC3b-opsonized mammary carcinoma cells following priming with β-glucan [35, 42]. Thus the role of dectin-1 in mediating β-glucan activities appears to be limited to activated peritoneal macrophages and perhaps also the intestinal $CR3^{-/-}$ macrophages observed to contain WGP-DTAF in this investigation.

The apparent need to administer β-glucans intravenously made it unfeasible to consider their use as a treatment for large numbers of people in emergency situations. However, with the current demonstration that orally administered WGP Beta Glucan functions to accelerate hematopoiesis following irradiation in an analogous manner as i.v. administered β-glucan, there is renewed interest in determining the mechanism and potential usefulness of β-glucan as a radioprotective drug for these types of nuclear emergencies.

The oral anti-infective and radiochemoprotective activities of a wide range of mushroom and yeast-derived β-glucans have been widely reported, and the oral uptake of these high molecular weight β-glucans has been proposed via M cells in intestinal Peyer's patches. The results presented in this communication extend these observations to demonstrate that the oral uptake of yeast WGP Beta Glucan particles leads to β-glucan presentation to macrophages in the underlying GALT. These WGP Beta Glucan-containing cells then transport the WGP Beta Glucan into the organs of the reticuloendothelial system (lymph nodes, spleen and BM). This oral uptake and systemic distribution of WGP appears to be independent of the CR3-mediated mechanism of yeast particle phagocytosis, as there was the same uptake and distribution of WGP-DTAF in both wild-type and $CR3^{-/-}$ animals. The dectin-1 receptor, or other unknown receptor maybe responsible for this oral uptake of WGP Beta Glucan into the GALT.

Of importance to explain the hematopoietic properties of oral WGP Beta Glucan treatment is the observation that daily feeding of WGP-DTAF leads to the appearance of WGP Beta Glucan-containing BM macrophages. Two explanations for oral WGP Beta Glucan hematopoietic activity were considered: 1) WGP Beta Glucan breakdown and secretion of stimulatory soluble β-glucans that combine with deposited iC3b to stimulate stem cells via CR3, and 2) WGP Beta Glucan activation of macrophages to produce hematopoietic stimulatory cytokines such as GM-CSF.

Macrophages that have ingested yeast cell walls or large soluble β-glucan molecules have been shown to degrade these materials and release small soluble fragments of β-glucan [28, 29]. Examination by fluorescence microscopy of BM macrophages from animals fed WGP-DTAF for 7-12 days clearly showed evidence of WGP degradation (Fig. 2). Macrophage culture supernatants and lysates are currently being tested for biologically active β-glucan fragments using an assay that incorporates the limulus G-factor that agglutinates in response to picogram concentrations of soluble β(1-3)glucans. The current study also demonstrated that BM cells injured by gamma radiation or a cytotoxic drug (cyclophosphamide) stimulated the activation of complement with deposition of iC3b on injured but still viable BM cells. Such cell-bound iC3b in combination with soluble β-glucan has been shown to activate the CR3 of mature myeloid cells, and in the BM may activate the CR3 of immature myeloid stem cells, causing accelerated hematopoiesis. Hematopoietic stem progenitor cells have been shown to express CR3 [30, 31, 43] and to respond to soluble β-glucan *in vitro* (M. Z. Ratajczak and G. D. Ross, unpublished observation). Also, supporting a putative role for soluble β-glucans released from macrophages are the previous reports showing that soluble β-glucans given i.v. could promote hematopoiesis in the same way as shown here with orally administered WGP Beta Glucan [5, 7].

An absolute requirement for CR3 in mediating the enhanced hematopoietic affect of orally administered β-glucan is clearly evidenced by the failure of oral WGP Beta Glucan treatment to stimulate the recovery of WBC counts in $CR3^{-/-}$ animals following irradiation (Fig. 4). A direct role of CR3 in promoting hematopoiesis is supported by the observation that oral WGP-DTAF are efficiently taken up and transported to the BM in $CR3^{-/-}$ mice and yet these mice do not respond with an accelerated hematopoietic recovery in the same way as wild type mice. As outlined above, the role of CR3 could be mediated either through

macrophages that are stimulated by WGP Beta Glucan to secrete hematopoietic cytokines only in wild-type and not in $CR3^{-/-}$ mice, or by the direct stimulation of $CR3^{+}$ hematopoietic cells through the co-stimulation by both the iC3b deposited on BM stromal cells and the soluble β-glucan released by macrophages that have ingested WGP Beta Glucan.

In conclusion, this investigation has demonstrated the feasibility of using orally administered WGP Beta Glucan as a therapeutic agent to protect individuals from the BM injury produced by exposure to gamma radiation. Orally administered WGP Beta Glucan functions through accelerating the normal process of hematopoiesis, making disease-fighting white blood cells available to the body several days sooner than would occur spontaneously. Orally administered WGP Beta Glucan is bioavailable through its uptake by intestinal macrophages that transport it to the bone marrow and spleen. Based on available data, two CR3-dependent mechanisms that could allow the macrophage-ingested WGP Beta Glucan to promote hematopoiesis have been proposed. Because no other similar oral therapeutic is available to protect individuals from harmful radiation exposure, the current terrorist threat to detonate a nuclear weapon, or so-called dirty bomb within the USA dictates that further research on the potential use of oral WGP Beta Glucan should be pursued vigorously.

REFERENCES

1. Stone,B.A. and Clark,A.E. 1993. Chemistry and Biology of (1-3)-beta-Glucans. Portland Press, Ltd., London.
2. Patchen, M. L. and T. J. MacVittie. 1983. Dose-dependent responses of murine pluripotent stem cells and myeloid and erythroid progenitor cells following administration of the immunomodulating agent glucan. *Immunopharmacology* 5:303-313.
3. Patchen, M. L., N. R. Di Luzio, P. Jacques, and T. J. MacVittie. 1984. Soluble polyglycans enhance recovery from cobalt-60--induced hemopoietic injury. *J. Biol. Response Mod.* 3:627-633.
4. Patchen, M. L., T. J. MacVittie, and L. M. Wathen. 1984. Effects of pre- and post-irradiation glucan treatment on pluripotent stem cells, granulocyte, macrophage and erythroid progenitor cells, and hemopoietic stromal cells. *Experientia* 40:1240-1244.
5. Petruczenko, A. 1984. Glucan effect on the survival of mice after radiation exposure. *Acta. Physiol. Pol.* 35:231-236.
6. Patchen, M. L. and T. J. MacVittie. 1985. Stimulated hemopoiesis and enhanced survival following glucan treatment in sublethally and lethally irradiated mice. *Int. J. Immunopharmacol.* 7:923-932.
7. Patchen, M. L. and T. J. MacVittie. 1986. Comparative effects of soluble and particulate glucans on survival in irradiated mice. *J. Biol. Response Mod.* 5:45-60.
8. Patchen, M. L., T. J. MacVittie, and I. Brook. 1986. Glucan-induced hemopoietic and immune stimulation: therapeutic effects in sublethally and lethally irradiated mice. *Methods Find. Exp. Clin. Pharmacol.* 8:151-155.
9. Matsuo, T., Y. Kurahashi, S. Nishida, K. Kumada, T. Hayami, and T. Takagi. 1987. Granulopoietic effects of lentinan in mice: Effects on GM-CFC and 5-FU-induced leukopenia. *Jpn. J. Cancer Chemother.* 14:1310-1314.
10. Wagnerová, J., A. Lísková, J. Navarová, A. Kristofová, T. Trnovec, and M. Ferencík. 1993. The effect of two glucan carboxymethyl derivatives with various substitution degrees on cyclophosphamide immunosuppression in mice. *Immunopharmacol. Immunotoxicol.* 15:227-242.
11. Patchen, M. L., T. Vaudrain, H. Correira, T. Martin, and D. Reese. 1998. In vitro and in vivo hematopoietic activities of Betafectin PGG-glucan. *Exp. Hematol.* 26:1247-1254.
12. Turnbull, J. L., M. L. Patchen, and D. T. Scadden. 1999. The polysaccharide, PGG-glucan, enhances human myelopoiesis by direct action independent of and additive to early-acting cytokines. *Acta Haematol.* 102:66-71.
13. Suzuki, I., H. Tanaka, A. Kinoshita, S. Oikawa, M. Osawa, and T. Yadomae. 1990. Effect of orally administered β-glucan on macrophage functions in mice. *Int. J. Immunopharmacol.* 12:675-684.

14. Sakurai, T., K. Hashimoto, I. Suzuki, N. Ohno, S. Oikawa, A. Masuda, and T. Yadomae. 1992. Enhancement of murine alveolar macrophage functions by orally administered β-glucan. *Int. J. Immunopharmacol.* 14:821-830.
15. Hanaue, H., Y. Tokuda, T. Machimura, A. Kamijoh, Y. Kondo, K. Ogoshi, H. Makuuchi, H. Nakasaki, T. Tajima, and T. Mitomi. 1989. Effects of oral lentinan on T-cell subsets in peripheral venous blood. *Clin. Ther.* 11:614-622.
16. Hotta, H., K. Hagiwara, K. Tabata, W. Ito, and M. Homma. 1993. Augmentation of protective immune responses against Sendai virus infection by fungal polysaccharide schizophyllan. *Int. J. Immunopharmacol.* 15:55-60.
17. Vetvicka, V., K. Terayama, R. Mandeville, P. Brousseau, B. Kournikakis, and G. Ostroff. 2002. Orally administered yeast β1,3-glucan (Imucell™ WGP) prophylactically protects against anthrax infection and cancer in mice. *J. Amer. Nutrit. Assoc.* 5:1-5.
18. Nanba, H., K. Mori, T. Toyomasu, and H. Kuroda. 1987. Antitumor action of shiitake (*Lentinus edodes*) fruit bodies orally administered to mice. *Chem. Pharm. Bull. (Tokyo)* 35:2453-2458.
19. Suzuki, I., T. Sakurai, K. Hashimoto, S. Oikawa, A. Masuda, M. Ohsawa, and T. Yadomae. 1991. Inhibition of experimental pulmonary metastasis of Lewis lung carcinoma by orally administered β-glucan in mice. *Chem. Pharm. Bull. (Tokyo)* 39:1606-1608.
20. Toi, M., T. Hattori, M. Akagi, K. Inokuchi, K. Orita, K. Sugimachi, K. Dohi, Y. Nomura, Y. Monden, and Y. Hamada. 1992. Randomized adjuvant trial to evaluate the addition of tamoxifen and PSK to chemotherapy in patients with primary breast cancer. 5-Year results from the Nishi-Nippon Group of the Adjuvant Chemoendocrine Therapy for Breast Cancer Organization. *Cancer* 70:2475-2483.
21. Onderdonk, A. B., R. L. Cisneros, P. Hinkson, and G. Ostroff. 1992. Anti-infective effect of poly-β1-6-glucotriosyl-β1-3- glucopyranose glucan in vivo. *Infect. Immun.* 60:1642-1647.
22. Kaiser, A. B. and D. S. Kernodle. 1998. Synergism between poly-(1-6)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranose glucan and cefazolin in prophylaxis of staphylococcal wound infection in a guinea pig model. *Antimicrob. Agents Chemother.* 42:2449-2451.
23. Coxon, A., P. Rieu, F. J. Barkalow, S. Askari, A. H. Sharpe, U. H. Von Andrian, M. A. Arnaout, and T. N. Mayadas. 1996. A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. *Immunity* 5:653-666.
24. Wessels, M. R., P. Butko, M. H. Ma, H. B. Warren, A. L. Lage, and M. C. Carroll. 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc. Natl. Acad. Sci. USA* 92:11490-11494.
25. Jamas, S., D. D. Easson, Jr., and G. R. Ostroff. *U.S. Patent 5,504,079.* Method of immune system activation by administration of a β(1-3) glucan which is produced by *Saccharomyces cerevisiae* strain R4 (1996).
26. Ross, G. D., V. Vetvicka, J. Yan, Y. Xia, and J. Vetvicková. 1999. Therapeutic intervention with complement and β-glucan in cancer. *Immunopharmacology* 42:61-74.
27. Kournikakis, B., R. Mandeville, P. Brosseau, and G. Ostroff. 2003. Anthrax protective effects of yeast β1,3 glucans. Submitted for publication.
28. Nagi, N., N. Ohno, S. Tanaka, J. Aketagawa, Y. Shibata, and T. Yadomae. 1992. Solubilization of limulus test reactive material(s) from *Candida* cells by murine phagocytes. *Chem. Pharm. Bull. (Tokyo)* 40:1532-1536.
29. Suda, M., N. Ohno, T. Hashimoto, K. Koizumi, Y. Adachi, and T. Yadomae. 1996. Kupffer cells play important roles in the metabolic degradation of a soluble anti-tumor (1-->3)-β-D-glucan, SSG, in mice. *FEMS Immunol. Med. Microbiol.* 15:93-100.
30. Coombe, D. R., S. M. Watt, and C. R. Parish. 1994. Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. *Blood* 84:739-752.
31. Ishida, A., H. Zeng, and M. Ogawa. 2002. Expression of lineage markers by $CD34^+$ hematopoietic stem cells of adult mice. *Exp. Hematol.* 30:361-365.

32. Velders, G. A., J. F. Pruijt, P. Verzaal, R. van Os, Y. Van Kooyk, C. G. Figdor, E. J. de Kruijf, R. Willemze, and W. E. Fibbe. 2002. Enhancement of G-CSF-induced stem cell mobilization by antibodies against the β2 integrins LFA-1 and Mac-1. *Blood* 100:327-333.
33. Ross, G. D. 2000. Regulation of the adhesion versus cytotoxic functions of the Mac-1/CR3/$\alpha_M\beta_2$-integrin glycoprotein. *Crit. Rev. Immunol.* 20:197-222.
34. Ross, G. D., J. A. Cain, B. L. Myones, S. L. Newman, and P. J. Lachmann. 1987. Specificity of membrane complement receptor type three (CR_3) for β-glucans. *Complement Inflamm.* 4:61-74.
35. Xia, Y., V. Vetvicka, J. Yan, M. Hanikyrova, T. N. Mayadas, and G. D. Ross. 1999. The ∃-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. *J. Immunol.* 162:2281-2290.
36. Thornton, B. P., V. Vetvicka, M. Pitman, R. C. Goldman, and G. D. Ross. 1996. Analysis of the sugar specificity and molecular location of the β-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J. Immunol.* 156:1235-1246.
37. Vetvicka, V., B. P. Thornton, and G. D. Ross. 1996. Soluble β-glucan polysaccharide binding to the lectin site of neutrophil or NK cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. *J. Clin. Invest.* 98:50-61.
38. Yan, J., V. Vetvicka, Y. Xia, A. Coxon, M. C. Carroll, T. N. Mayadas, and G. D. Ross. 1999. ∃-Glucan, a "specific" biologic response modifier that uses antibodies to target tumors for recognition by complement receptor type 3 (CD11b/CD18). *J. Immunol.* 163:3045-3052.
39. Brown, G. D. and S. Gordon. 2001. Immune recognition. A new receptor for β-glucans. *Nature* 413:36-37.
40. Brown, G. D., P. R. Taylor, D. M. Reid, J. A. Willment, D. L. Williams, L. Martinez-Pomares, S. Y. Wong, and S. Gordon. 2002. Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* 196:407-412.
41. Taylor, P. R., G. D. Brown, D. M. Reid, J. A. Willment, L. Martinez-Pomares, S. Gordon, and S. Y. Wong. 2002. The β-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.* 169:3876-3882.
42. Vetvicka, V., B. P. Thornton, T. J. Wieman, and G. D. Ross. 1997. Targeting of NK cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and β-glucan-primed CR3 (CD11b/CD18). *J. Immunol.* 159:599-605.
43. Janowska-Wieczorek, A., M. Majka, J. Kijowski, M. Baj-Krzyworzeka, R. Reca, A. R. Turner, J. Ratajczak, S. G. Emerson, M. A. Kowalska, and M. Z. Ratajczak. 2001. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood* 98:3143-3149.