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Role of Soluble β-(1-3),(1-6)-D-Glucan from *Saccharomyces cerevisiae* in the Murine P388 Ascites Tumor Model

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Abstract. *Background: Therapeutic options for the treatment of malignant ascites are limited and could be broadened by immune-stimulatory drugs. Materials and Methods: Soluble β-(1-3),(1-6)-D-glucan from Saccharomyces cerevisiae was administered i.p. into DBA/2-mice bearing the P388 lymphoma either freshly inoculated or as an established ascites-tumor. Its effect on survival, ascites volume and production of cytokines was examined. Results: The early, but not the later, administration of β-glucan showed a tendency to induce interleukin (IL)-12 in the ascites, whereas both treatment schedules demonstrated a clear tendency to reduce production of interferon-γ in the abdominal fluid and had no notable impact on the level of tumor necrosis factor-α. Treatment with β-glucan at either time-point showed no effect on the ascites volume and mean survival time. Conclusion: β-(1-3), (1-6)-D-Glucan shows weak and differential modulation of immune-stimulatory and pro-inflammatory cytokines in tumor ascites dependent on the stage of tumor growth without affecting survival of the mice.*

Advanced stages of primary carcinomatosis and infiltration of solid and, rarely, hematological tumors into the peritoneal cavity are often accompanied by the production of malignant ascites due to inflammation-associated peritoneal vascular hyperpermeability (1). Malignant ascites contains detached tumor cells and elevated levels of white blood cells and, varying by type and progression of the tumor, inflammation-associated and immune-suppressive cytokines such as interleukin (IL)-6, IL-8, IL-10 and transforming-growth factor (TGF)-β1 (2, 3). Cancer patients abnormally accumulating abdominal fluid are commonly treated with paracentesis, diuretics or local or systemic chemotherapy, however, with overall limited clinical effects (4). Local treatment with immune-stimulatory agents such as β-(1-3),(1-6)-D-glucans, branched polysaccharide compounds derived from yeast or fungal cell wall, or picibanil (OK-432), a streptococcal preparation, has shown beneficial effects against malignant effusions in cancer patients, mostly when used combined or in combination with chemotherapy (5, 6). The therapeutic effect of these agents is mediated by the activation of peritoneal exudate cells (PEC) including macrophages, natural killer (NK) cells, neutrophils and dendritic cells (DC) indicated by increased serum or ascites levels of immune-activatory cytokines such as IFN-γ and IL-12(p70) (5-11). Furthermore, the inhibitory effect of *i.p.* injected β-glucans on murine ascites tumors has been shown to depend on the onset of treatment and the administered dose, whereas their effect on induction or suppression of cytokines in murine malignant ascites has not been determined yet (7, 8). In this study, the efficacy of a soluble, *i.p.* administered β-(1-3),(1-6)-D-glucan to activate PEC indicated by the production of immune-stimulatory and pro-inflammatory cytokines in the abdominal fluid was evaluated together with ascites production and the survival of mice bearing *i.p.* pre-B-cell lymphoma P388.

Materials and Methods

Reagents. The aqueous preparation of β-(1-3),(1-6)-D-glucan (20 mg/ml), derived from the inner cell wall of *Saccharomyces cerevisiae*, was obtained from Biotec Pharmacon (Tromsø, Norway) and dissolved in PBS. The endotoxin level was <0.05 EU/ml. Endotoxin-free PBS was purchased from Biochrom (Berlin, Germany).

Animals and cell line. Female DBA/2-mice were purchased from Charles River (Sulzfeld, Germany) and were used at 8-12 weeks of age. The mice were maintained in the pathogen-free animal facility of epo GmbH (Berlin-Buch, Berlin) following institutional guidelines and with approval from the appropriate authorities. The murine pre-B-cell lymphoma P388 was provided by epo GmbH and was twice *i.p.* passaged in female DBA/2 mice before being transplanted for treatment.

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Formation of malignant ascites and therapeutic treatment with β-glucan. To induce production of malignant ascites, the DBA/2-mice were *i.p.* inoculated with 2×10^5 P388 lymphoma cells. The animals received *i.p.* β-glucan (250 μg) once or the same volume (0.2 ml) of PBS either one hour after tumor injection or when formation of ascites was palpable, designated as early (each group n=8) or late treatment (each group n=12). The mice were observed daily for the development of ascites and leukemia-related symptoms and were sacrificed when they showed signs of moribundancy or the ascites volume exceeded approximately 3 ml.

Quantification of ascites volume and concentration of cytokines. The ascites volume, which consisted of a fluid and a viscous fraction, was determined by aspiration with a 16 mm needle after the animals were sacrificed. The ascites was centrifuged at 500 ×*g* for 10 minutes and the fluid fraction was stored at –20˚C until quantification of the content of IFN-γ, IL-12(p70) and TNF-α using commercially available sandwich-ELISA kits (eBioscience, Frankfurt, Germany) following the instructions of the manufacturer. Analysis of the ascites was performed as applicable when a sufficient liquid volume from individual mice was available.

Statistical analysis. Data of the cytokine measurements are representative of single or duplicate determinations of individual mice and the mean±SEM of all the evaluable mice of a treatment group. Data from the analysis of ascites volume and survival represent the mean±SEM of all the mice from a treatment group of the respective experiment. Student's *t*-test served for statistical analysis of the data. A *p*-value <0.05 was considered to indicate a statistically significant difference.

Results

Effect of β-glucan on intraperitoneal cytokine production. The ascites of five to seven mice of each group provided a sufficient liquid fraction to allow parallel determination of the concentration of three cytokines. Approximately half of the evaluated tumor-bearing control mice showed either a high level of more than 1,000 pg/ml IFN-γ or a concentration mostly below 400 pg/ml in their abdominal fluid (Figure 1). Treatment with β-glucan on the early or later stage of tumor growth strongly reduced the percentage of mice producing a high amount of IFN-γ in the ascites without being significant, due to the portion of mice producing low levels of IFN-γ in both the control and the drug-treated groups.

IL-12 was rarely detected in the ascites of the control mice and was absent from the peritoneal cavity of the mice administered with β-glucan at the stage of advanced tumor (Figure 1). In contrast, the majority of mice which received β-glucan early after tumor inoculation produced IL-12, although this increase was not significant compared to control mice (p=0.065). TNF-α was present in the ascites of all the tested mice in both treatment schedules ranging from 300 pg/ml to 1,000 pg/ml. Treatment with β-glucan administered early after tumor injection slightly, but non-significantly, increased the average concentration of TNF-α in ascites, whereas mice treated with PBS or the carbohydrate at the stage of established ascites showed no difference in TNF-α levels. Furthermore, high levels of IFN-γ were associated with a higher concentration of TNF-α and *vice versa* in the ascites of control mice (Figure 1). The two mice exhibiting the highest *ascites* concentration of IFN-γ in ascites after early treatment with β-glucan showed the highest level of TNF-α.

Effect of β-glucan on ascites production. All the DBA/2 mice showed the formation of ascites within three to four days after *i.p.* inoculation of P388 leukemia cells (not shown). The single treatment with *i.p.* β-glucan at the early or the later time-point had no impact on the terminal ascites volume (Figure 2).

Effect of β-glucan on survival of mice. The single administration of β-glucan did not prolong the mean survival time in the mice with early-stage ascites tumor or in the mice with advanced tumor (Figure 3). The survival of PBS- and β-glucan-treated mice was in the range described by others for mice bearing the ascites-form of this tumor (12, 13).

Discussion

Inhibition of ascites tumors by β-glucans in mice has previously been shown to depend on the dosage and time-point of administration (7, 8). The administered dose of β-glucan in this study was within a range described to potently activate innate immune cells including macrophages, DC, granulocytes and NK cells *in vivo* which also underlie the therapeutic activity of β-(1,3)-(1,6)-D-glucans on murine ascites tumors (7, 8, 10, 14, 15). Since activation of macrophages and maturation of DC is indicated by the induction or enhanced production of the TH1 priming and NK cell and $CD8^+$ T-cell stimulatory IL-12 (6, 10, 16-18), the tendency of the β-glucan to induce production of IL-12 in the mice with freshly inoculated tumor suggested a weak immune-stimulatory activity of the agent. However, antitumor immune responses potentially induced by the β-glucan may not have been effective at prolinging survival due to the rapid progression of the tumor. Furthermore, tumors were shown to suppress the activation of macrophages and maturation of DC (19, 20), which could have inhibited or limited the induction of IL-12 by the β-glucan in mice with advanced ascites or freshly inoculated tumor, respectively. In addition, the lack of tumor-inhibitory effect could have been due to the use of a potentially ineffective therapeutically dose since the administration of less than 2 mg β-glucan even prior to or early after tumor injection reportedly induced only a weak or no prolongation of the survival of ascites tumor-bearing mice (7, 8, 21).

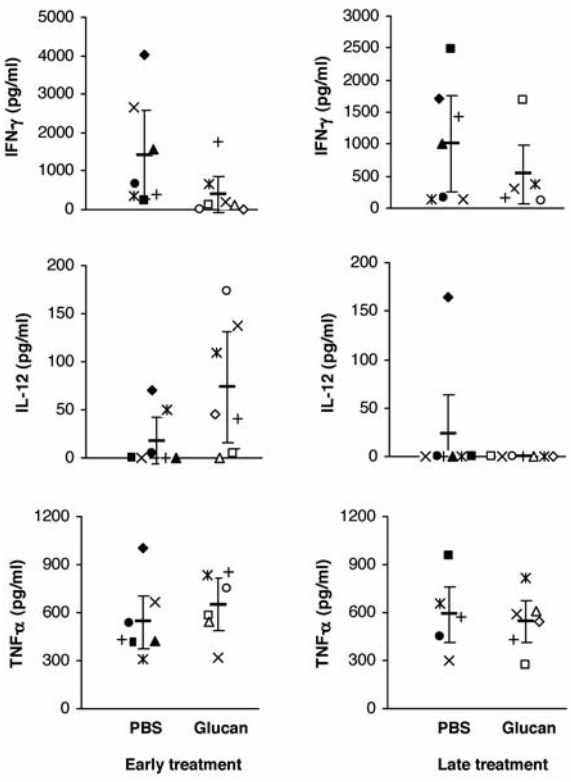

Figure 1. *Cytokine production of P388 lymphoma-bearing DBA/2 mice administered β-glucan or PBS, i.p. 1 hour after tumor cell injection (early treatment) or when ascites was palpable (late treatment). The data represent individual mice (different symbols) and the mean±SEM of all tested mice.*

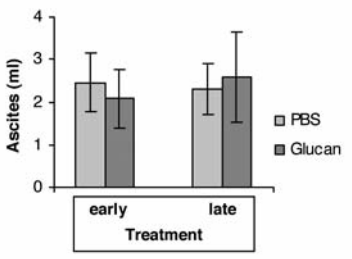

Figure 2. *Ascites volume of P388 lymphoma-bearing DBA/2 mice administered β-glucan or PBS, i.p. 1 hour after tumor cell injection (early treatment) or when ascites was palpable (late treatment). Ascites was aspirated when the mice were sacrificed. Mean±SEM of all mice.*

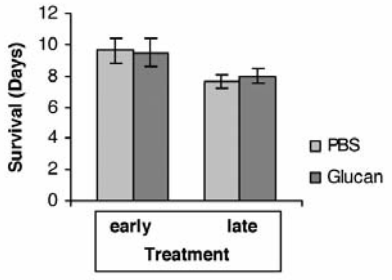

Figure 3. *Survival of DBA/2 mice i.p. inoculated with P388 lymphoma cells and treated with β-glucan or PBS, i.p. 1 hour after tumor cell injection (early treatment) or when ascites was palpable (late treatment). Mice were sacrificed when they became moribund and/or volume of ascites exceeded approximately 3 ml. Mean±SEM of all mice.*

The ascites of P388 tumor-bearing mice revealed a inflammatory cytokine profile shown by the presence of high levels of IFN-γ and TNF-α. The tendency of β-glucan to reduce ascites levels of IFN-γ attributed to increased production of the anti-inflammatory cytokines IL-10 or TGF-β or a shift to Th2-type T cell-priming has been reported in murine models of inflammatory diseases (22-24). The observed tendency of the β-glucan to reduce IFN-γ despite elevation of the levels of IL-12 could be explained by the recently demonstrated parallel induction of IL-12 and immune-suppressive cytokines by β-glucans in DC and macrophages (25-28). The presence of notable levels of TNF-α in the ascites of the PBS-treated mice indicated constitutive production of this cytokine enhanced by tumor-induced inflammation since PEC of tumor-free DBA/2-mice have been shown to produce significantly lower levels of TNF-α following repeated injections of PBS (29). The slight tendency towards enhanced production of TNF-α in the mice which received β-glucan early after tumor inoculation could indicate a weak pro-inflammatory activity of the agent and was similarly observed in sarcoma-180 ascitic tumor-bearing mice following *i.p.* treatment with β-glucan (10). However, levels of TNF-α detected in the ascites of this study were described to promote ascites production and progression of murine carcinomas (30-32). Furthermore, a therapeutic effect of the carbohydrate against the P388 ascites tumor could have been impeded by its tendency to reduce production of IFN-γ, which plays a central role in orchestrating antitumor-immune responses by macrophages and DC and priming of Th1 cells and has demonstrated antitumor effects in ascites xenograft models (14, 16, 33). Taken together, these data demonstrate β-(1,3)-(1,6)-D-glucan shows a tendency towards a differential induction or suppression of pro-inflammatory and immune-stimulatory cytokines in the ascites of P388-tumor-bearing mice dependent on the stage of tumor growth, whereas ascites production and survival of the mice is not affected.

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