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Mannan from *Aloe saponaria* inhibits tumoral cell activation and proliferation

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Abstract

In this study, we tested the antiproliferative effects of mannan from *Aloe saponaria* using normal murine (SpMC) and human cells (PBMC) and several tumoral cell lines. Employing flow cytometry, it could be determined that mannan inhibits the proliferative response in normal and tumoral cells. Mannan affects the expression of $CD3^+$ SpMC indicating that mannan inhibits mainly T lymphocyte proliferative response. Also in SpMC cultured with or without mitogen mannan produces an increase of an activation marker (CD25). On C1498 cell line, mannan reduces CD3 expression and abolishes the CD25 expression. In conclusion, mannan has a dual beneficial effect when applied to normal and tumoral cells at the same time by inhibiting the activation of cancer cells and improving that of normal ones.

Keywords: *Aloe saponaria*; Activation; Tumoral cell; Mannan

1. Introduction

The Aloe family is constituted by tropical or subtropical plants characterized by lance-shaped leaves with jagged edges and in some species sharply tapered as saponaria. Although the Aloe is an original species from South Africa, it also grows in tropical and tempered areas, and has successfully been introduced and cultivated in various regions of Argentina. Like most members of this group, *Aloe vera* L. (*Aloe barbadensis* Mill. or *Aloe vulgaris* Lam.) is a species that does not tolerate temperatures under 0 °C. *Aloe saponaria* (Ait.) Haw. (*Aloe latifolia* Haw.) seems to be an exception, since it can thrive in atmospheres with a minimum temperature of until −7 °C, which explains its wide distribution in the region of Córdoba, Argentina.

Two basic substances are extracted from the leaves of aloe. The so-called gel is derived from the pulp of the leaves and contains carbohydrate polymers such as glucomannanes or pectic acid [1]. One important

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feature that distinguishes *saponaria* mannan from *vera* mannan is the content of anthraquinone (Murature, personal communication). Saponaria gel is composed of approximately 98.5% water. The total solid is made up of over 60% polysaccharides, while organic acids and inorganic compounds account for the remainder of the solid. The mucilage, sugars, fiber, proteins, ash, fats, aloin and resin are the major constituents of the solid [2]. The carbohydrates found are galactose, glucose, mannose, rhamnose, xylose and uronic acids.

It has been reported that the polysaccharide has diverse immunomodulatory activities in vivo as well as in vitro (reviewed in Ref. [3]). It is a modulator of cellular and humoral immunity [4] and it also stimulates proliferation of murine pluripotent hematopoietic stem cells, granulocyte macrophage colony-forming cells, and cells forming myeloid and erythroid colonies [5–7]. As well as i.v. administration of polysaccharide induces protection of murine hematopoietic stem cells against X-ray exposure, thereby decreasing the mortality of the exposed animal.

Acemannan (ACM) extracted from the plant *A. vera* and *A. saponaria* is a polydispersed β (1–4)-linked acetylated mannan. Interest in ACM was focused initially for wound healing, but there have recently been several reports on its antiviral and antiproliferative properties in vivo. Parenteral administration of ACM has been followed by a great regression of tumors such as fibrosarcomas in rats, mice, dogs and cats [8–12]. Other aloe constituents, lectins and polysaccharides have been proposed to prevent carcinogenesis. Lectin-like substances from the leaves of *A. vera* and *A. saponaria* have been shown to have haemoagglutinating properties, and fresh preparations also promoted the growth of normal human cells in culture but inhibited tumor cell growth [13]. Most of these experiments were carried out using Acemannan® but little is known about the effects of the mannan extracted from *A. saponaria* on the immune system or its anti tumoral effects.

In this study, we tested the antiproliferative effects of *A. saponaria* gel using tumoral cell lines, C1498 (mouse acute myeloid leukemia), HT29 (human colorectal adenocarcinoma), PC-12 (rat pheochromocytoma), CHO (ovary of an adult Chinese hamster), L6 (rat myoblast), NIH 3T3 (fibroblast from mouse) and normal murine and human cells.

2. Materials and methods

2.1. Mannan purification

A. saponaria mannan purification was performed following the procedure described for *A. vera* mannan [1].

2.2. Purification of murine SpMC

Spleens from Balb-c mice (CNEA, Argentina) age 6–8 weeks were removed and cell suspensions were prepared by homogenization in a tissue grinder. The erythrocytes were lysed by brief incubation in 8.3 g/l NH_4Cl in 0.01 mol/l Tris–HCl pH 7.5 (Red Blood cell lysing buffer, Sigma-Aldrich, MO). The cells were washed three times with RPMI-1640 medium and their viability determined by trypan blue dye exclusion was always >99%.

2.3. Animal treatment

Male mice from a Balb-c strain (CNEA) were used. Animals were maintained under standard lighting and temperature conditions. All the mice were treated according to NIH regulations and the Institutional Care and Use of Animals Committee, Universidad National de Córdoba (exp. no. 15-99-40426) approved animal handling and experimental procedures. Mice under light ether anesthesia received intradermal injections in both hind feet with 0.25 ml of an emulsion constituted by 0.05 ml of phosphate-buffered saline (PBS) pH 7.4 and 0.20 ml of complete Freund's adjuvant (CFA, Sigma-Aldrich). After 10 days of inoculation, spleens were removed and cell suspensions were prepared as indicated above. The purified protein derivative (PPD) antigen from *Mycobacterium tuberculosis* was obtained from Instituto Nacional de Microbiología "Carlos Malbrán" (Buenos Aires, Argentina).

2.4. Purification of human PBMC

PBMC were isolated by Fycoll-Hypaque (Histopaque, Sigma-Aldrich) from freshly drawn heparinized blood or buffy coat of healthy donors. The layer of mononuclear cells was recovered and their viability determined by trypan blue dye

exclusion was always >99%. The investigation conforms to principles outlined in the declaration of Helsinki.

2.5. Cell lines

In this study, we used several cell lines: C1498 is an IL-4R$^+$ spontaneously occurring C57BL/6 myeloid leukemia; HT29 is a human colon cancer cell line; PC-12 is a cell line derived from a transplantable rat pheochromocytoma; CHO cell line derived from an ovary of an adult Chinese hamster; L6 is a rat myoblast; and NIH 3T3 fibroblast from *Mus musculus* (mouse). All the cells lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies Paisley, UK), 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin.

2.6. Cell proliferation assays

Cell cultures were set up in triplicate in 24-well microculture plates and incubated at 37 °C in RPMI-1640 medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere. SpMC (1×10^6 cells/ml) were cultured in the presence of concanavalin A (Con A) (3 μg/ml) with different concentrations of mannan, and as a control experiment the effects of vincristine and doxorubicin (10 ng/ml and 600 pg/ml, respectively) were tested simultaneously. SpMC (1×10^6 cells/ml) were cultured in the presence or absence of Con A (3 μg/ml) or mannan (0.6 mg/ml) in a total volume of 2 ml for 96 h. SpMC (1×10^6 cells/ml) from inoculated mice were culture in the presence or absence of Con A (3 μg/ml), PPD (15 μg/ml, antigen-specific stimulation) or mannan (0.6 mg/ml). Cultures were carried out for 96-h period since the mitogenic effect of Con A is fully displayed at this time, after longer culture times most cells die and the percentage of viable cells is very low to perform this kind of experiments [14]. Cultures containing 1×10^6 PBMC were cultured in the presence of phytohaemagglutinin (PHA) (5 mg/ml) with or without mannan (0.6 mg/ml) in a total volume of 2 ml for 96 h [15]. The tumoral cell lines were cultured without mitogen with or without mannan (0.6 mg/ml). Cell proliferation was evaluated by flow cytometry.

2.7. Phenotypic analysis of lymphocyte population and the murine cell line C1498

Cultures of SpMC (1×10^6 cells/ml) were incubated in RPMI-1640 medium (or DMEM for C1498 cell line) (24 wells/plates) at 37 °C during 96 h in the presence or absence of Con A (3 μg/ml) with or without mannan (0.6 mg/ml) in a total volume of 2 ml. The cells were washed with Hank's balanced salt solution (HBSS) containing 1% BSA and 0.1% NaN_3 and preincubated with anti-mouse CD32/CD16 for 1 h at 4 °C in order to block immunoglobulin non-specific binding through Fc receptor (constant fragment of immunoglobulin receptor). Following Fc blocking, cells were incubated with FITC-labeled anti-mouse CD3 monoclonal antibody (T lymphocyte) and PE-labeled anti-mouse IL-2R (CD25) monoclonal antibody (activated lymphocyte). Antibodies (1 μg/10^6 cells) were incubated for 30 min at 4 °C. The cells were washed three times with HBSS, fixed in 2% formaldehyde and stored at 4 °C in the dark until analysis. All antibodies were purchased from Pharmingen, San Diego, CA.

Platelets, erythrocytes (if any) and non-lymphoid cell were excluded from analysis by setting an appropriate gate on the forward light scatter (FW-SC) vs. 90° light scatter parameters (RT-SC).

FW-SC is primarily related to cell size and thus a high value is an index of blastogenic response. RT-SC (measured at 90°) is related to a variety of factors including refractive index, granularity and cell shape. We collected data from 10,000 cells in each sample using a flow cytometer (ORTHO Immuno Count Flow Cytometry System) and results were analyzed with WinMDI software version 2.7.

3. Results and Discussion

The effects of mannan on normal human and murine mononuclear cells were examined by flow cytometry. When SpMC were exposed for 96 h to different concentrations of mannan, it was found an inhibition of the blastogenic effect of Con A, a non-specific mitogen (Fig. 1). The experiment showed that with a dose of approximately 0.6 mg/ml the response

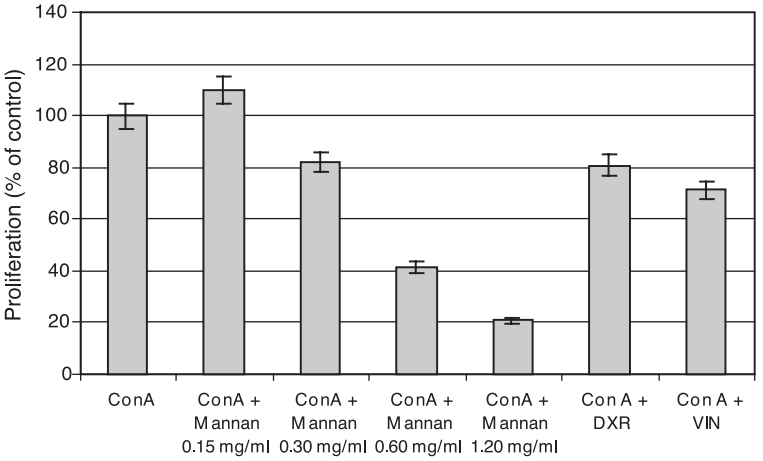

Fig. 1. Effects of different concentrations of mannan from *A. saponaria* on cell proliferation. SpMC were cultured for 96 h in the presence of the mitogen Con A and with different concentrations of mannan from *A. saponaria*. Concentrations are indicated in the figure. As control, cells were treated with doxorubicin (DXR) or vincristine (VCR) at 10 ng/ml and 600 pg/ml, respectively. Results represent the mean ± S.E. of triplicate experiments.

decayed to the half; thus, this concentration was selected to be employed in the next experiments. As control experiments, the effects of vincristine an doxorubicin were tested simultaneously, both these drugs inhibited the proliferative response elicited by Con A as previously described [16] (Fig. 1).

Stimulated SpMC and PBMC were cultured during 96 h in the presence or absence of purified mannan and the mitogens (Con A or PHA, respectively). Fig. 2A and B shows that mannan inhibits the lymphoproliferative response induced by the mitogen in both cases, since the population of cells with higher size (blast cells) was specifically reduced. The population of cells with the size of blastic ones comprised 80% of the total cell number in cultures of SpMC plus Con A and this population was reduced to 66% of the total cell number when mannan was added to the cultures (60% and 31% for PBMC plus PHA and PBMC plus PHA plus mannan, respectively). It is also interesting that the total number of cells bearing the volume of resting cells was not affected by the addition of mannan (Fig. 2A and B).

To determine whether the antiblastogenic effect of mannan on normal cells could be extended to blastogenic tumoral cells, the same analysis was carried out employing several murine and human tumoral cell

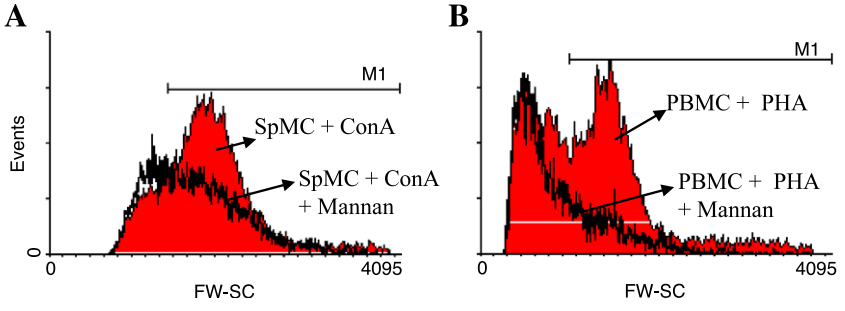

Fig. 2. Effects of mannan from *A. saponaria* on cell size. SpMC and PBMC were cultured in the presence of a mitogen with (open, black histograms) or without mannan (filled histograms) (panels A and B, respectively). Forward scatter light values (cell size) were determined by flow cytometry and represented as forward scattered versus cell number histograms. The curves display the mean of data from three separate experiments.

Table 1
Effect of mannan on tumoral cell proliferation

Cell lines	Number of cells		
		plus mannan	% of inhibition
PC12	2700 ± 100	1360 ± 70	50
CHO	4200 ± 200	580 ± 30	86
L6	7500 ± 400	1590 ± 80	79
NIH 3T3	7600 ± 400	720 ± 40	91
C1498	4050 ± 400	1800 ± 80	45
HT29	6500 ± 300	2275 ± 90	35

Tumoral cell lines were cultured for 96 h with and without mannan. The proliferation was evaluated by flow cytometry. Data are reported as mean of four experiments carried out in triplicate done with different cells and mannan preparations and expressed in number of cells ± S.D.

lines. Table 1 shows that mannan inhibits spontaneous proliferation of all the lines tested.

In order to discern whether the inhibitory effect of mannan on the balstogenic response elicited by Con A affected or not the relative proportion of lymphocytes (T cell vs. B cell) in cultures of spleen cells, the percentage of $CD3^+$ SpMC (T cells) after 96 h of Con A stimulation and cultured with and without mannan was evaluated by flow cytometric analysis. Fig. 3A shows that mannan does not change the proportion of T cells in cultures of SpMC after 96 h of culture. Fig. 3C shows that, when cells were cultured with Con A, mannan affects only $CD3^+$ SpMC, while no effect could be observed on $CD3^-$ SpMC, thus indicating that mannan inhibited mainly T lymphocyte proliferation.

The multisubunit receptor of interleukin-2 cytokine (IL-2R or CD25) is both essential in mediating T-cell growth/clonal expansion following antigen (or mitogen) stimulation, and a marker of T-cell activation since it is expressed only upon activation with the stimuli [17–20]. When cells were cultured without mitogen, mannan produced an increase in the expression of CD25 (Fig. 3B). The percentages of SpMC $CD25^+$ were 18% in absence of mannan and increased up to 74% when cells were cultured with mannan. This fact could explain some of the immunostimulant effects of mannan observed in vivo [8–12]. Cultures that contain simultaneously Con A and mannan had a higher proportion of the $CD25^+$ cells (Fig. 3D) than cells cultured with mitogen alone, to an extent similar to the one observed on resting cells, indicating that mannan has an immunomodulatory effect controlling the extent of the cell expansion without interfering with interleukin receptor expression. The mean fluorescence intensity (MFCh) of positive cells, which depends on the number of CD25 molecules/cell surface unit, follows the same trend as the population of CD25 positive

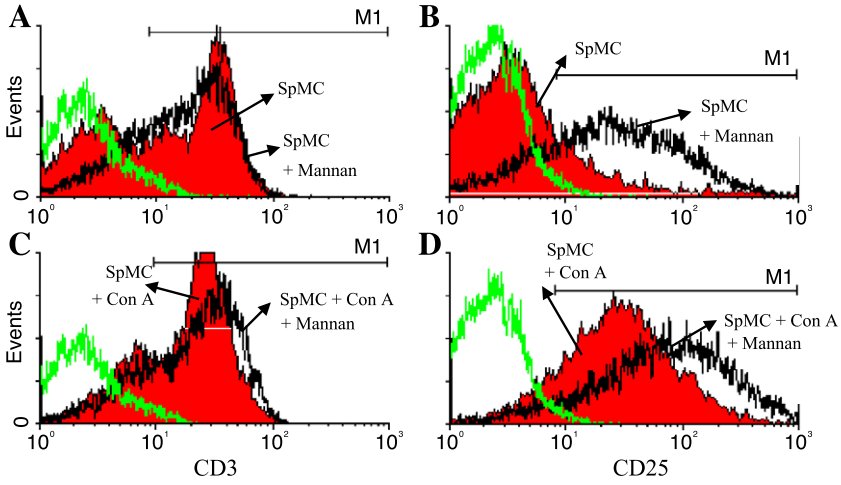

Fig. 3. Effect of mannan from *A. saponaria* on CD3 and CD25 surface expression on murine SpMC. SpMC were cultured in the absence (A–B) or presence (C–D) of Concanavalin A with (open, black histograms) or without mannan (filled histograms), and the cell-surface expression of CD3 and CD25 was analyzed by flow cytometry at 96 h of culture. The *x*- and *y*-axes represent fluorescence (three-decade log scale) and relative cell number, respectively. Histograms from cells stained with control isotype-matched mAb are indicated by an open gray histogram. The curves display the mean of data from three separate experiments.

cells (SpMC + Con A: 2133, SpMC + Con A + mannan: 2609); thus, mannan increases the surface density of IL-2 receptor on T cells.

To test the effect of mannan on tumoral cells, we observed the expression of the lymphocitic marker CD3 and the activation marker CD25 on C1498 cell line that has been reported to respond to IL-2 stimuli [20]. The effect of mannan on CD3 expression was similar to that observed for SpMC stimulated with mitogen (Figs. 3C and 4A). The most interesting result was that mannan abolished the expression of CD25 on this tumoral cells contrasting with the effect of mannan on normal murine cells cultured with or without mannan (Figs. 3B,D and 4B).

To discard the possibility that mannan inhibits the action of a non-specific stimuli like Con A and PHA but not the proliferative effect of a specific stimulus, a mouse model was employed. SpMC from mice immunized with CFA were stimulated in vitro with PPD for 96 h with and without mannan. The blastogenic responses to the stimulus and CD3 and CD25 expression were analyzed by flow cytometry. Table 2 shows that PPD increased 20% the cells in the blastogenic region and that the presence of mannan halved the number of blastic cells. More interestingly, mannan increased the percentage of CD3−CD25$^+$ cells. The control experiment carried out employing Con A instead of PPD as in vitro challenge followed a similar trend although the number of blastic cells was higher than in the experiments of cells challenged with PPD.

In conclusion, our results show that mannan inhibits its proliferation of many types of normal activated and tumoral cells. We focused the study on the question if this antiproliferative effect was the consequence of an antiactivation effect. To test this hypothesis, we employed lymphocytic cells since its activation markers are well known. It was found that, while mannan inhibits the expression of activation markers (CD3−CD25$^+$ cells) of tumoral cells of lymphocytic origin, it enhances its expression on normal lymphocytes. Thus, the beneficial effects of mannan on animals bearing tumors [8–12] could be due, at least in part, to the direct effects of mannan on tumoral and normal cells inhibiting the activation of cancer cells and improving that of normal cells. This action could be exerted through the mannose receptor (MR) since it has been reported its involvement in the effect of acemannan on a macrophage cell line by inhibiting its action after pre-incubation with Con A [21].

Table 2
Effect of mannan on CD3$^+$−CD25$^+$ SpMC proliferation

	Proliferation (number of cells)	Percentage of cells CD3$^+$−CD25$^+$
SpMC infected	4400 ± 200	7
SpMC infected + PPD	5200 ± 300	7
SpMC infected + PPD + mannan	2900 ± 100	29
SpMC infected + Con A	6500 ± 400	2
SpMC infected + Con A + mannan	2300 ± 100	40

SpMC, infected with CFA, were cultured for 96 h in the presence or absence of Con A, with and without mannan. The proliferation of the total cells and the cells that express CD3 and CD25 were evaluated by flow cytometry. Data are reported as mean of four experiments carried out in triplicate done with different cells and mannan preparations and expressed in number of cells ± S.D.

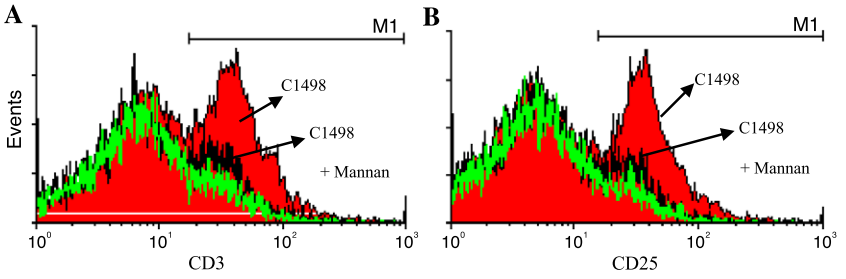

Fig. 4. Effect of mannan from *A. saponaria* on CD3 and CD25 surface expression on the tumoral cell line C1498. C1498 cells were cultured with (open, black histograms) or without mannan (filled histograms), and the cell-surface expression of CD3 and CD25 was analyzed by flow cytometry at 96 h of culture. The *x*- and *y*-axes represent fluorescence (three-decade log scale) and relative cell number respectively. Histograms from cells stained with control isotype-matched mAb are indicated by an open gray histogram. The curves display the mean of data from three separate experiments.

Sequence alignment identified the protein DEC-205 as a member of the mannose receptor family based on the overall structural similarity to the MR and phospholipase A2 receptor (PLA2R). DEC-205 was identified as the 200-kDa glycoprotein (gp200) recognized by mAb MR6. gp200-MR6 was of interest due to its expression by epithelial cells in the cortex of the thymus [21], dendritic cells and at low levels by T lymphocytes [22]. In addition, mAb MR6 was shown to have an anti-proliferative effect on cultures of T lymphocytes proliferating in response to interleukin-4 (IL-4) and to inhibit the IL-4-dependent production of IgE by B cells [23–26]. DEC-205 has recently been assigned the cluster of differentiation number CD205.

Correct control of the cell cycle results from the coordinated and sequential activation-inactivation of key regulators known as cyclin-dependent kinases (CDKs). The CDK activity is highly regulated by a number of different elements through the phases of the cycle. Since the cell cycle is a tightly regulated process, one might predict that the deregulation of some elements could cause abnormal proliferation contributing to cancer. It has been proposed that most, if not all tumors have altered the restriction checkpoint through an alteration of the p16/cyclinD1/pRb pathway [26]. This proposal is supported by the ratio of primary tumors and tumoral cell lines (like C1498) that display an alteration in this pathway.

This is the first report on the effects of mannan from *A. saponaria* on the proliferative response of normal and tumoral cells in vitro. Moreover, the results could explain the beneficial effects of aloe when employed as therapeutic agent against cancer, because it does not interfere with normal lymphocyte activation and inhibits tumoral cell proliferation and activation.

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