Effects of gossypol acetic acid on cellular and humoral immune response in non-immunized and SRBC-immunized mice

DENG SIJUN[1], ALEKSANDRA PAWLAK[2], BŁAŻEJ POŹNIAK[2], AGNIESZKA SUSZKO[2], MARIANNA SZCZYPKA[2], YUAN HUI[1], BOŻENA OBMIŃSKA-MRUKOWICZ[2]

[1]Veterinary College, Hunan Agricultural University, Furong District Changsha City, Hunan, China
[2]Department of Biochemistry, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Wrocław, Poland

Abstract

Gossypol is a yellow polyphenolic compound from cotton plant. It has been shown to exert a potential for anti-cancer, anti-inflammatory and anti-fertility effects. Following effects of gossypol acetic acid (GA) in mice were determined in the present study: influence on the total number of lymphocytes, weight ratio and subsets of lymphocytes in the thymus, spleen and mesenteric lymph nodes, and humoral immune response to sheep erythrocytes (SRBC). Gossypol acetic acid was administered orally four times at 24 h intervals at three doses of 5.0, 25.0 and 125.0 mg/kg to SRBC-immunized mice (prior to or after priming), and non-immunized mice. It was found that GA decreased the total number of lymphocytes in thymus and mesenteric lymph nodes. Gossypol acetic acid dependent on the dosage decreased the percentage and absolute count of CD4+ thymocytes but increased the percentage and absolute count of CD8+ lymphocytes in spleen and lymph nodes. In SRBC-immunized mice, GA at the doses of 25.0 and 125.0 mg/kg administered prior to SRBC reduced the number of plaque forming cells (PFC) and the production of IgG. However, GA administered after priming decreased the production of IgM and IgG. In vitro, GA inhibited proliferation of J774.E murine macrophages in a dose- and time-dependent manner.

Key words: *gossypol acetic acid, cellular response, humoral response, lymphatic organs, mice.*

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Introduction

Gossypol is a yellow polyphenolic compound originally isolated from seeds and roots of the cotton plant (*Gossypium* sp.). It exists in a free and bound form. Free gossypol is the major toxic ingredient of cotton-seed meal [1]. Gossypol is generally known as a male anti-fertility and potential anti-cancer agent [2]. It also exhibits other biological actions like anti-parasitic [3], anti-bacterial [4], anti-inflammatory [5] and anti-viral activity [6]. Gossypol is also reported to be a small-molecule Bcl-2 inhibitor [7] and nonspecific protein kinase C (PKC) inhibitor [8]. B-cell lymphoma 2 (Bcl-2) was first identified at chromosomal breakpoint of t-bearing B-cell lymphomas [9] and was found to have the anti-apoptotic function in cancer cell. In haematopoietic cell system, the agents which inhibit PKC activity can suppress the growth of both normal and leukemic progenitors [10]. It suggests that, as PKC inhibitor, gossypol probably affects cell proliferation. Gossypol suppresses leukemic cell differentiation in response to tumor-promoting phorboids [11] and decreases the expression of interleukin 2 (IL-2) and interferon γ (IFN-γ) in activated human T lymphocytes [12]. The anti-inflammatory activity of the compound could be demonstrated on exhausting neutrophils and by preventing vasodilatation which induces inhibition of leukocyte extravasation [13]. Gossypol prolongs skin allograft survival in mice without affecting the bone marrow function [14]. Therefore, gossypol has been suggested to be a potential anti-inflammatory and immunosuppressive agent.

Correspondence: Prof. Bożena Obmińska-Mrukowicz DVM PhD, Department of Biochemistry, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wrocław, Poland, phone +48 71 320 54 03, fax +48 71 320 54 55, e-mail: b.mrukowicz@gmail.com

The purpose of the present study was to determine the immunomodulatory effects of gossypol acetic acid (GA) in mice, both *in vitro* and *in vivo*. The *in vitro* part included the effect of GA on the proliferation of mouse macrophages (cell line J774.E). The *in vivo* part included the assessment of the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes, calculation of the central and peripheral lymphatic organ weight ratio, evaluation of lymphocyte subsets in lymphatic organs and the assessment of humoral immune response with respect to the dosage and scheme of GA treatment in non-immunized and SRBC-immunized mice.

Material and methods

Animals and cells

The studies were carried out on male and female Balb/c mice (8-10 weeks of age), each weighing 20-22 g. The experimental animals were obtained from the Breeding Centre of Laboratory Animals at the Institute of Occupational Medicine, Łódź, Poland. The studies were performed on non-immunized and SRBC-immunized mice. The mice were immunized intraperitoneally with 0.2 ml of 10% sheep red blood cells (SRBC) suspension (4×10^8 cells per mouse). The sheep blood was collected into Alsever's solution in a sterile manner and kept there at 4°C for at least 3 days. The SRBC suspension was prepared *ex tempore* in phosphate buffered saline (PBS, Institute of Immunology and Experimental Therapy, Wrocław, Poland). Principles of laboratory animal care (NIH publication No 86-23, revised 1985), as well as the specific national laws on the protection of animals were followed. The study protocol was approved by the II Local Ethics Commission in Wrocław, Poland (No 182/2010). For *in vitro* test the murine macrophage cell line J774.E, originally isolated from BALB/cN mice ascites, was used. The cell line was obtained from the collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

Drugs and treatment

Gossypol acetic acid was obtained from College of Light Industry, Zhejiang, China. The agent is water insoluble therefore for *in vivo* experiment, it was first dissolved in DMSO (Sigma, France) and then diluted in PBS (each dose contained 10% DMSO). The non-immunized mice were administered with GA orally (via stomach-tube) four times at 24 h intervals at three different doses of 5.0, 25.0 and 125.0 mg/kg. The SRBC-immunized mice were administered with GA orally at the same doses (5.0, 25.0 and 125 mg/kg) four times at 24 h intervals either prior to, or after priming. The volume of the administered drug was 0.2 ml/mouse. Animals in the control group received an equivalent amount of 10% DMSO in PBS. Each experimental group consisted of eight mice.

Measurements

The measurements included: 1) the total number of thymocytes, splenocytes and lymphocytes of mesenteric lymph nodes; 2) the weight ratio of the thymus, spleen and mesenteric lymph nodes calculated according to the following formula: weight of organ (mg)/body weight of mouse (mg) × 100; 3) CD subsets (CD4+CD8+, CD8+CD4–, CD4+CD8+ and CD8–CD4–) in the thymus and (CD3+, CD4+, CD8+ and CD19+) in the spleen and mesenteric lymph nodes were determined by flow cytometry assay using monoclonal antibodies (mAb) coupled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE); 4) the number of plaque forming cells (PFC); 5) anti-SRBC haemagglutinin titre in the serum; 6) the proliferation inhibition of J774.E.

The total number of thymocytes, splenocytes, lymphocytes of mesenteric lymph nodes, the weight ratio of the thymus, spleen and mesenteric lymph nodes, CD subsets in the thymus, spleen and mesenteric lymph nodes were determined three times: 24, 72 and 120 hours after the last GA administration. The numbers of PFC and anti-SRBC haemagglutinin titre were determined on day 4 after SRBC injection.

Assays of thymocyte, splenocyte and lymphocyte from mesenteric lymph node subsets

The mice were anaesthetized with halothane (Narcotan, Zentiva, Czech Republic) 24, 72 and 120 hours after the final dose of GA administration. The thymus, spleen and mesenteric lymph nodes were removed and placed in disposable Petri dishes containing a sterile, ice-cold PBS. The suspended cells were released from the lymphatic organs by passing them through a nylon mesh and centrifuging them on a layer of Ficoll 400 (Pharmacia, Fine Chemicals AB, Sweden)/Urografin 76% (diatrizoate sodium and meglumine diatrizoate, Bayer Schering Pharma, Poland) in 1 : 3 ratio, density of 1.071. After centrifugation at 4°C, the cells were collected from the interphase and washed twice with PBS supplemented with 1% bovine serum albumin (BSA, Sigma, Germany) at 4°C. After the second wash, the cells were suspended in PBS with 1% BSA at 1×10^7 cells/ml. The viability of each cell suspension was 90-98% according to a trypan blue dye exclusion assay. The cells were resuspended in 100 µl PBS solution containing 1% BSA. The cells in suspension were stained with monoclonal rat anti-mouse CD4: FITC/CD8: RPE dual colour reagent (Serotec, UK) or monoclonal rat anti-mouse CD19: FITC/CD3:RPE dual colour reagent (Serotec, UK) according to the manufacturer's instructions. The cells were incubated at 4°C for 30 min and washed three times with an ice-cold PBS buffer. The fluorescence was analyzed using a flow cytometer (FACS Calibur; Becton Dickinson Biosciences, Germany). The distribution of the thymocyte, splenocyte and lymphocyte of mesenteric lymph node markers was analyzed using the CellQuest 3.1f software.

Determination of plaque forming cells

The mice were anaesthetized with halothane and next killed by cervical dislocation 24 h after the final GA administration. The spleens were removed and placed in the Hank's saline (Institute of Immunology and Experimental Therapy, Wrocław, Poland). They were teased apart with forceps by gently tearing the capsule and releasing the cell. The suspended cells were centrifuged on a layer of Ficoll 400/Urografin 76% (density 1.071). After centrifugation at 4°C, the splenocytes were collected from the interface and washed twice in the Hank's saline. After the second wash, the cells were suspended in the Hank's saline at 1×10^6 cells/ml. The viability of the splenocyte suspension was 96-100% according to a trypan blue dye exclusion assay. The number of splenocytes producing haemolytic anti-SRBC antibodies (plaque forming cells, PFC) was determined by a local haemolysis technique in agar gel as described by Mishell and Dutton [15].

Determination of anti-SRBC antibodies in the serum

The blood samples were taken from a retro-ocular artery of halothane anaesthetized mice. The sera were obtained by blood centrifugation and inactivated at 56°C for 30 min. The total and 2-mercaptoethanol (2-ME, Sigma, Germany) resistant serum agglutination titres were defined by active haemagglutination test carried out on microplates [16]. The titre of 2-mercaptoethanol resistant haemagglutinin (2-MrH) is roughly equivalent to that of IgG in the serum, so the greater titre obtained without 2-mercaptoethanol is due to the IgM. The results were expressed as a value of $\log_2$. It was found that serum of non-immunized mice did not contain spontaneous anti-SRBC antibodies.

Determination of the proliferation inhibition of mouse macrophages (J774.E)

For the *in vitro* experiment, J774.E cells were seeded in a 96-well-plate (NUNC, Denmark) in a concentration of 1×10^5 cells per well. The cell medium consisted of RPMI-1640 (Institute of Immunology and Experimental Therapy, Wrocław, Poland) supplemented with 10% foetal bovine serum (FBS, Sigma, USA), L-glutamine (Sigma, UK) and antibiotics (penicillin and streptomycin, Sigma, Germany). Gossypol acetic acid was diluted in the culture medium at several concentrations (less than 1% DMSO in each dilution) and added to the plates with cells. The plates were incubated (5% CO_2, 37°C) for 24 h, 48 h and 72 h. After the respective incubation time, the MTT assay was carried out. The test is based on the enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] in living, metabolically active cells. The metabolite, purple-coloured formazan is measured colorimetrically, using a multiwell plate read-

Table 1. The effects of gossypol acetic acid on the total number of thymocytes, splenocytes and mesenteric lymph nodes cells and weight ratio of thymus, spleen and mesenteric nodes. The mean values (n = 8) and standard deviations are presented

Index	Hour	Control	Gossypol acetic acid treatment		
			4 × 5 mg/kg	4 × 25 mg/kg	4 × 125 mg/kg
Total number of thymocytes ($\times 10^7$)	24	38.1 ±4.1	30.0 ±11.7	24.1 ±4.6*	26.7 ±8.0*
	72	42.6 ±9.0	37.3 ±5.2	31.3 ±6.3*	27.3 ±2.0*
	120	39.0 ±9.3	36.8 ±5.8	35.3 ±5.8	44.1 ±5.6
Weight ratio of thymus	24	0.21 ±0.04	0.19 ±0.08	0.19 ±0.09	0.18 ±0.07
	72	0.21 ±0.09	0.24 ±0.13	0.20 ±0.04	0.20 ±0.10
	120	0.16 ±0.04	0.17 ±0.09	0.17 ±0.05	0.14 ±0.07
Total number of splenocytes ($\times 10^7$)	24	78.5 ±9.4	79.0 ±7.7	74.8 ±11.2	80.6 ±7.7
	72	80.4 ±4.8	78.9 ±7.9	79.6 ±3.8	81.6 ±9.4
	120	82.2 ±5.1	74.1 ±9.4	78.0 ±9.1	80.9 ±7.1
Weight ratio of spleen	24	0.61 ±0.14	0.52 ±0.08	0.61 ±0.08	0.65 ±0.11
	72	0.54 ±0.04	0.55 ±0.11	0.59 ±0.06	0.64 ±0.23
	120	0.51 ±0.06	0.56 ±0.12	0.56 ±0.03*	0.57 ±0.06
Total number of mesenteric lymph nodes cells ($\times 10^7$)	24	37.7 ±8.6	33.5 ±3.9	38.8 ±5.0	35.4 ±3.9
	72	39.6 ±6.4	38.6 ±4.5	36.8 ±4.3	31.6 ±5.8*
	120	38.6 ±4.7	34.1 ±8.8	33.5 ±5.6	24.7 ±6.0*
Weight ratio of mesenteric lymph nodes	24	0.48 ±0.08	0.40 ±0.05	0.37 ±0.10	0.42 ±0.09
	72	0.38 ±0.03	0.38 ±0.04	0.40 ±0.05	0.38 ±0.10
	120	0.35 ±0.09	0.36 ±0.06	0.42 ±0.08	0.38 ±0.14

**P < 0.05 as compared with the paired control*

Table 2. Percentage and absolute number of thymocyte subpopulations in non-immunized mice treated with gossypol acetic acid (5, 25 or 125 mg/kg) four times at 24 h intervals. The mean values (n = 8) and standard deviations are presented

Index		Hour	Control	Gossypol acetic acid treatment		
				4 × 5 mg/kg	4 × 25 mg/kg	4 × 125 mg/kg
Thymocytes						
CD4–CD8–	(%)	24	3.0 ±1.1	2.4 ±0.4	2.4 ±0.3	3.0 ±0.4
	(× 10^7)		1.1 ±0.04	0.72 ±0.04	0.57 ±0.01	0.8 ±0.03
	(%)	72	2.5 ±0.6	2.6 ±0.6	2.6 ±0.4	2.9 ±1.0
	(× 10^7)		1.06 ±0.05	0.96 ±0.03	0.81 ±0.02	0.79 ±0.02
	(%)	120	3.6 ±0.8	3.0 ±0.4	3.9 ±1.0	4.4 ±1.0
	(× 10^7)		1.4 ±0.07	1.1 ±0.02	1.4 ±0.05	1.9 ±0.05
CD4+CD8+	(%)	24	81.0 ±2.8	83.3 ±1.4	82.6 ±3.1	79.0 ±2.0
	(× 10^7)		30.8 ±0.11	24.9 ±0.16	19.9 ±0.1*	21.0 ±0.16*
	(%)	72	82.4 ±3.7	83.1 ±1.2	82.3 ±1.9	78.9 ±3.9
	(× 10^7)		35.1 ±0.33	31.0 ±0.06	25.7 ±0.1*	21.5 ±0.07*
	(%)	120	80.7 ±0.9	82.4 ±2.6	81.7 ±1.6	77.7 ±5.6
	(× 10^7)		31.4 ±0.08	30.3 ±0.15	28.8 ±0.09	34.2 ±0.3
CD4+CD8+	(%)	24	12.9 ±1.5	11.7 ±1.1	11.9 ±1.9	14.0 ±1.0
	(× 10^7)		4.9 ±0.06	3.5 ±0.12*	2.9 ±0.08*	3.7 ±0.08*
	(%)	72	12.2 ±2.4	12.2 ±2.4	12.6 ±1.5	15.2 ±2.6*
	(× 10^7)		5.1 ±0.2	4.6 ±0.12	3.9 ±0.09*	4.1 ±0.05*
	(%)	120	14.4 ±1.4	12.6 ±1.0*	12.0 ±0.7*	14.3 ±3.5
	(× 10^7)		5.6 ±0.1	4.6 ±0.05*	4.2 ±0.04*	6.3 ±0.2
CD4–CD8+	(%)	24	3.1 ±0.7	2.7 ±0.5	3.0 ±0.8	4.0 ±1.1
	(× 10^7)		1.18 ±0.02	0.81 ±0.05	0.72 ±0.03	1.0 ±0.08
	(%)	72	2.9 ±0.8	2.7 ±0.4	2.5 ±0.3	3.0 ±0.7
	(× 10^7)		1.2 ±0.07	1.0 ±0.02	0.8 ±0.01	0.8 ±0.01
	(%)	120	2.9 ±0.8	2.7 ±0.2	2.4 ±0.4	3.7 ±1.2
	(× 10^7)		1.1 ±0.07	1.0 ±0.01	0.8 ±0.02	1.5 ±0.06

**P < 0.05 as compared with the paired control*

er. Due to the intense colour of GA, the drug-containing medium was replaced with fresh medium instantly before adding 20 µl of MTT (Sigma, USA) in concentration of 5 mg/ml. After 2 h of incubation at 37°C, 80 µl of lysis buffer was added. The buffer consisted of 225 ml dimethylformamide (DMF, Sigma, USA); 67.5 g sodium dodecyl sulphate (SDS, Sigma, Japan) and 275 ml distilled water. The optical density was measured after 4 hours using a spectrophotometric microplate reader (ELx800, BioTek, USA) at the reference wavelength of 570 nm. The optical density of formazan formed in control (untreated) cells was taken as 100%. Percent viability of test samples were determined as: % viability = (average OD for test group/average OD for control group) × 100. The IC_{50} values were obtained from more than 3 independent experiments. Data is presented as the mean ±SD of at least three separate experiments.

Statistical analysis

Statistical analysis was performed using student's *t*-test. A P value < 0.05 was considered to be statistically significant.

Results

Effects of gossypol acetic acid on the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes, weight ratio of central and peripheral lymphatic organs

It has been found that four exposures to GA at the three different doses of 5.0, 25.0 and 125.0 mg/kg did not change the weight ratio of thymus, spleen and mesenteric lymph nodes and total number of lymphocytes in spleen. But at doses of 25 and 125 mg/kg, GA significantly decreased the total number of thymocytes at 24 h and 72 h after last administration. The dose of 125 mg/kg significantly decreased the total number of lymph nodes cells at 24 h and 72 h after last administration. The suppressive effect of gossypol depended on the dosage (Table 1).

Effects of gossypol acetic acid on the subsets of lymphocytes in the thymus, spleen and mesenteric lymph nodes in non-immunized mice

It has been found that GA is able to change the percentage and absolute number of T cell subsets in the thy-

Table 3. Percentage and absolute number of splenocyte subpopulations in non-immunized mice treated with gossypol acetic acid (5, 25 or 125 mg/kg) four times at 24 h intervals. The mean values (n = 8) and standard deviations are presented

Index		Hour	Control	Gossypol acetic acid treatment		
				4 × 5 mg/kg	4 × 25 mg/kg	4 × 125 mg/kg
Splenocytes						
CD3+	(%)	24	34.9 ±2.3	34.1 ±1.3	33.3 ±7.0	36.0 ±7.6
	(× 10^7)		27.4 ±0.2	26.9 ±0.1	24.9 ±0.8	29.0 ±0.6
	(%)	72	32.5 ±2.3	36.3 ±5.1	30.7 ±4.3	36.1 ±4.3
	(× 10^7)		26.3 ±0.1	28.6 ±0.4	24.4 ±0.2	29.4 ±0.4
	(%)	120	36.1 ±4.3	34.4 ±4.0	34.4 ±4.0	38.4 ±2.7
	(× 10^7)		29.6 ±0.2	25.4 ±0.4*	26.6 ±0.3*	31.0 ±0.2
CD4+	(%)	24	23.1 ±2.0	23.7 ±1.8	24.0 ±3.8	23.2 ±5.6
	(× 10^7)		18.1 ±0.2	18.7 ±0.1	17.9 ±0.4	18.6 ±0.4
	(%)	72	24.0 ±5.9	24.7 ±2.5	20.1 ±3.0	23.4 ±2.2
	(× 10^7)		19.2 ±0.3	19.4 ±0.2	15.9 ±0.1	19.0 ±0.2
	(%)	120	24.3 ±4.9	20.1 ±2.0	19.6 ±2.5*	22.4 ±2.9
	(× 10^7)		19.9 ±0.2	14.8 ±0.2*	15.2 ±0.2*	18.1 ±0.2
CD8+	(%)	24	5.6 ±1.1	6.3 ±0.5	5.7 ±1.2	7.0 ±0.9*
	(× 10^7)		4.4 ±0.1	4.9 ±0.03	4.2 ±0.1	5.6 ±0.06*
	(%)	72	4.3 ±1.0	5.8 ±1.5*	3.5 ±0.6	5.5 ±0.8*
	(× 10^7)		3.5 ±0.04	4.6 ±0.1*	2.8 ±0.02*	4.4 ±0.07*
	(%)	120	5.6 ±0.7	5.0 ±1.4	4.3 ±0.6*	4.9 ±1.0
	(× 10^7)		4.6 ±0.03	3.7 ±0.1	3.3 ±0.05*	3.9 ±0.07
CD19+	(%)	24	58.9 ±2.6	58.9 ±1.8	60.1 ±7.4	57.4 ±7.8
	(× 10^7)		46.2 ±0.2	46.5 ±0.1	44.9 ±0.8	46.2 ±0.6
	(%)	72	61.5 ±5.8	58.4 ±5.2	65.0 ±4.3	58.8 ±4.2
	(× 10^7)		49.4 ±0.3	46.0 ±0.4	51.7 ±0.2	47.9 ±0.4
	(%)	120	60.3 ±6.0	61.9 ±4.1	62.7 ±2.2	57.5 ±2.8
	(× 10^7)		49.5 ±0.3	45.8 ±0.4	48.9 ±0.2	46.5 ±0.2

**P < 0.05 as compared with the paired control*

mus and T and B lymphocytes in the peripheral lymphatic organs. The effect of the agent is dependent on the dosage. As shown in Table 2, GA administered four times at the doses of 25 mg/kg and 125 mg/kg decreased the absolute count of immature CD4+CD8+ thymocytes (double-positive cells). Moreover GA, irrespectively of the dose applied, decreased the absolute count of single-positive, mature CD4+ thymic cells. But no effect of GA on the percentage of the immature double-positive (CD4+CD8+), double-negative (CD4–CD8–) and mature single positive CD8+ thymocytes was observed.

At the same time, some changes in the percentage and the absolute number of T and B cells in the peripheral lymphatic organs were found (Tables 3 and 4). Gossypol acetic acid did not change the percentage of CD3+ (Pan-T lymphocytes) and the percentage and absolute count of CD19+ (B cells) in spleen, but independently of dosage, temporarily increased the percentage and absolute count of CD8+ lymphocytes in spleen. At the dose of 25 mg/kg, it decreased the absolute count of CD3+ (Pan-T lymphocytes) and the percentage and absolute count of CD4+ and CD8+ in the spleen 120 h after last dose. At the dose of 125 mg/kg, it increased the percentage and absolute count of CD8+ 24 h and 72 h after the last dose (Table 3). Four exposure to the GA doses under investigation did not change the percentage and absolute number of B lymphocytes (CD19+ cells) in the spleen. It has been found that four exposures to GA at the dose of 5 mg/kg initially increased the percentage and absolute count of CD3+ (Pan-T lymphocytes), which corresponded with an increased percentage and absolute count of CD4+ lymphocytes in lymph nodes. This effect was short-lasting because 120 h after last administration of GA at the doses of 5 mg/kg and 25 mg/kg decreased of the percentage and absolute count of CD3+ and CD4+ cells in lymph nodes was observed. It has been also found that gossypol at the doses of 25 mg/kg and 125 mg/kg temporarily increased the percentage and absolute count of CD8+ lymphocytes in lymph nodes. However, 72 h after the last administration of GA, irrespective of the dose applied, a reduction in the percentage and count of B cells in lymph nodes was found. This effect also was observed 120 h following the exposure to four higher GA doses (125 mg/kg).

Table 4. Percentage and absolute number of lymphocyte from the mesenteric lymph nodes subpopulations in non-immunized mice treated with gossypol acetic acid (5, 25 or 125 mg/kg) four times at 24 h intervals. The mean values (n = 8) and standard deviations are presented

Index		Hour	Control	Gossypol acetic acid treatment		
				4 × 5 mg/kg	4 × 25 mg/kg	4 × 125 mg/kg
Mesenteric lymph node cells						
CD3+	(%)	24	48.9 ±3.6	49.7 ±5.1	50.9 ±6.7	52.3 ±5.4
	(× 10^7)		18.4 ±0.3	16.6 ±0.2	19.7 ±0.3	18.5 ±0.2
	(%)	72	41.8 ±5.4	49.4 ±3.8*	48.0 ±6.9	50.4 ±8.7*
	(× 10^7)		16.6 ±0.3	19.0 ±0.1*	17.6 ±0.3	15.9 ±0.5
	(%)	120	45.9 ±4.2	37.0 ±5.3*	35.5 ±5.3*	46.5 ±5.8
	(× 10^7)		17.7 ±0.2	12.6 ±0.5*	11.8 ±0.3*	11.4 ±0.3
CD4+	(%)	24	40.0 ±3.7	40.8 ±4.0	40.1 ±5.2	44.6 ±4.4*
	(× 10^7)		15.0 ±0.3	13.6 ±0.2	15.5 ±0.2	15.7 ±0.2
	(%)	72	33.6 ±3.9	39.6 ±3.7*	38.7 ±4.2*	41.0 ±7.4*
	(× 10^7)		13.3 ±0.2	15.2 ±0.1*	14.2 ±0.2	12.9 ±0.4
	(%)	120	42.9 ±5.3	34.5 ±5.2*	32.7 ±4.2*	42.3 ±5.3
	(× 10^7)		16.5 ±0.2	11.7 ±0.4*	10.9 ±0.2*	10.4 ±0.3
CD8+	(%)	24	6.1 ±1.3	6.7 ±1.1	6.7 ±1.1	9.0 ±1.0*
	(× 10^7)		2.3 ±0.1	2.2 ±0.04	2.6 ±0.05	3.1 ±0.03
	(%)	72	4.4 ±2.3	6.9 ±2.2	8.4 ±2.0*	8.0 ±3.5*
	(× 10^7)		1.7 ±0.1	1.5 ±0.04	3.0 ±0.08*	2.5 ±0.2*
	(%)	120	4.3 ±0.7	4.1 ±0.9	4.3 ±1.2	6.0 ±0.9*
	(× 10^7)		1.6 ±0.03	1.4 ±0.07	1.4 ±0.06	1.5 ±0.05
CD19+	(%)	24	48.2 ±3.6	47.6 ±5.5	47.7 ±4.1	45.0 ±5.7
	(× 10^7)		18.2 ±0.3	15.9 ±0.2	18.5 ±0.2	15.9 ±0.2
	(%)	72	55.6 ±5.5	47.6 ±4.0*	49.9 ±5.3	47.0 ±9.0*
	(× 10^7)		22.0 ±0.4	18.3 ±0.2*	18.3 ±0.2*	14.8 ±0.5*
	(%)	120	52.5 ±3.9	61.4 ±5.6*	62.5 ±5.3*	51.3 ±6.4
	(× 10^7)		20.2 ±0.2	20.9 ±0.5	20.9 ±0.3	12.6 ±0.4*

**P < 0.05 as compared with the paired control*

The effect of gossypol acetic acid on humoral immune response to SRBC

Plaque forming cells assay (Fig. 1) showed that GA at the doses of 25.0 mg/kg and 125.0 mg/kg prior to SRBC immunization reduced the number of splenocytes producing anti-SRBC antibodies (plaque-forming cells). However, four administrations of GA after priming did not change the number of splenocytes producing anti-SRBC antibodies. As further reported in Fig. 1, GA could depress the production of total (IgM + IgG) and 2-MrH (IgG) titres in SRBC-immunized mice. At a dose of 5.0 mg/kg, the decrease in total and 2-MrH titers was significantly different ($P < 0.05$) on day 4 after priming. At a dose of 125.0 mg/kg, the production of IgG differed significantly as compared to control in both, prior to and after SRBC GA-treated groups.

The effect of gossypol acetic acid on the proliferation of mouse macrophages (J774.E)

The MTT assay (Fig. 2) revealed that the concentrations of GA causing 50% inhibition of macrophage proliferation (IC_{50}) was 4.68, 3.63 and 2.91 µg/ml after 24, 48 and 72 h incubation, respectively. These results indicate that the cytotoxic effect of GA on murine macrophages was both dose- and time-dependent.

Discussion

The objective of this study was to investigate the *in vitro* and *in vivo* immunomodulatory effects of GA in mice. The results of the *in vitro* assay showed that GA inhibited proliferation of macrophage cell line J774.E in a time and dose-dependent manner. The obtained IC_{50} for GA was 4.68, 3.63 and 2.91 µg/ml after 24, 48 and 72 h incubation respectively. This is approximately 5 times less than IC_{50} established for human nasopharyngeal carcinoma cell line (CNE2) [17]. Similar IC_{50} was obtained for gossypol in prostate cancer cell lines PC-3 (18.56 µg/ml) and DU-145 (18.35 µg/ml) after 72 h incubation [18]. The presented data suggest a relatively high susceptibility of murine macrophages to GA. Many reports have postulated different mechanisms to explain the cytotoxic effects of GA, e.g. inhibition of several enzymes (protein kinase C, lactate dehydrogenase, adenylate cyclase, acrosin) [19], disruption of cell-to-cell

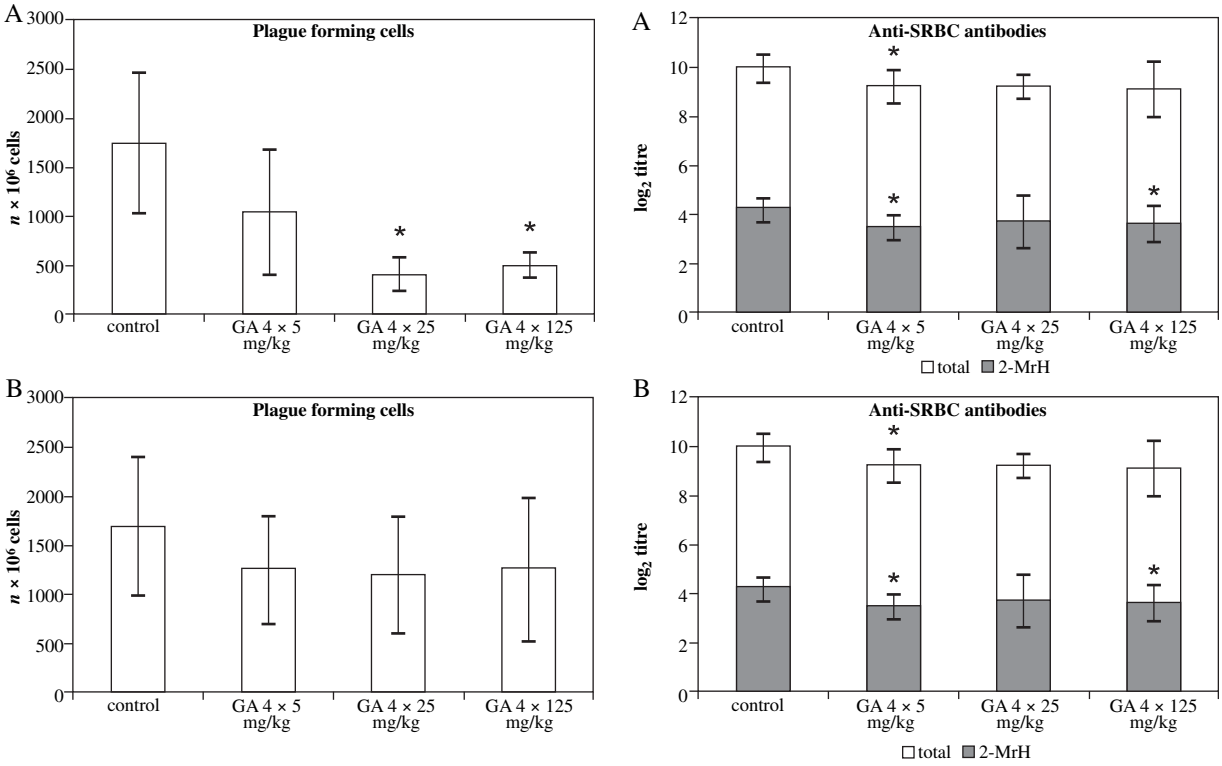

Fig. 1. The number of plaque forming cells and anti-SRBC haemagglutinin titres in SRBC immunized mice treated four times with GA at 24 hours intervals prior to (A), or after priming (B). The mean values ($n = 8$) and standard deviations are presented. $*P < 0.05$ as compared with the paired control

communication at the level of gap junctions [20] and also damage of cellular ultrastructure, with mitochondria being the main target [21]. Studies performed on rat and human lymphocytes revealed that GA increases DNA strand breaks only when cell viability is affected. Therefore, the genotoxic effect of GA is likely to be secondary to cytotoxicity [22, 23]. The thymus is the central lymphoid organ that provides site for lymphocyte differentiation, maturation and antigen commitment. The peripheral lymphoid organs (spleen and lymph nodes) are involved in antigen detection and provide site for lymphocyte activation [24]. The present trials conducted on non-immunized mice showed that thymus and mesenteric lymph nodes were the organs most significantly affected by GA. High dose (125 mg/kg) of GA reduced the total number of thymocytes and nodal lymphocytes. A similar effect was also shown in a study by Xu *et al*. [25], in which daily administration of GA (25 mg/kg for 7 days *i.p.*) markedly alleviated the DTH (delayed type hypersensitivity) in mice, decreased the relative weight ratio of thymus and proliferative capacity of the nodal lymphocytes. In the present study, it was found that GA suppressed the primary humoral response in SRBC-immunized mice. A decrease in total and 2-mercaptoethanol resistant haemagglutinin titres was found in mice exposed even to the low dose of GA (5 mg/kg). Immunoglobulin G is the most abundant class of antibodies in serum, and IgM is the first immunoglobulin class produced in a primary response to an antigen [24]. The adaptive immune response is based on antigen recognition and presentation, specific T- and B-cells' activation and proliferation, and antibody synthesis by B-cells. Gossypol acetic acid can significantly inhibit the proliferation of murine lymphocytes induced by phorbol dibutyrate (PDB) and ionomycin [25]. The decreased pro-

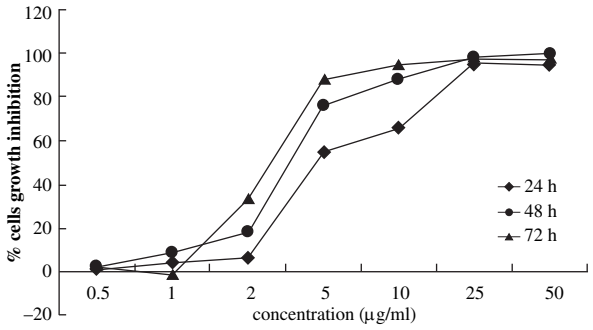

Fig. 2. Effect of gossypol acetic acid on the proliferation of mouse macrophages (J774.E) measured by MTT assay

duction of total and 2-mercaptoethanol resistant haemagglutinin titres found in the present study is in agreement with the findings of Xu *et al*. [25] and confirms the depressive effect of GA on humoral response in mice. The immunomodulatory effect of GA on lymphocyte subsets was expressed by increasing the percentage and count of CD8+ lymphocytes in spleen and mesenteric lymph nodes. Out of all investigated lymphatic organs, the subsets of lymphocytes in mesenteric lymph nodes seem to be most affected by GA. At a high dose (125 mg/kg), GA transiently decreased the percentage and absolute count of CD19+ and, apart from increasing the CD8+ subset, it transiently increased also the percentage and absolute count of CD3+ and CD4+ lymphocytes. Later, a reverse shift was observed in the mice administered with the lower (5 mg/kg) and middle (25 mg/kg) dose of GA. At the time point of 120 h, these mice presented an increase in CD19+ subset accompanied by a decrease in the CD3+ subset (mainly due to decreased CD4+ subpopulation). This suggests that GA may have a complex and ambiguous effect on different lymphocyte subpopulations. No specificity however, is visible in the PDB-induced lymphoblastic transformation, when the proliferation of T and B-cells is suppressed by GA equally [25]. The shifts in lymphocyte subpopulations observed in the present study may contribute to the anti-inflammatory activity described by several authors [26-28]. Gossypol acetic acid, as an old oral nonsteroidal male contraceptive agent [5], was studied extensively for its reproductive toxicity in animals. The reproductive ability of male mice was affected after 20 days of exposure to GA in a daily dose of 120 mg/kg [29]. A daily dose of 100 mg/kg prevented germ cells of a rat from entering meiosis [30]. In the present study, the exposure time was shorter and much lower doses had an effect on the immune response in mice. It may indicate that the immune system is even more sensitive to GA than the reproductive system. The experimental data on the immunomodulatory effects of GA is limited. The results obtained in the present study can be the basis for further investigations of the immunomodulatory effects of GA.

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