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Original article

Influence of betulinic acid on lymphocyte subsets and humoral immune response in mice

Y. Jine[1], M. Lis[2], M. Szczypka[2], B. Obmińska-Mrukowicz[2]

[1] College of Veterinary Medicine, Hunan Agricultural University, Changsha 410128, PR China
[2] Department of Biochemistry, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Norwida 31, 50-375 Wroclaw, Poland

Abstract

Betulinic acid is a pentacyclic triterpene found in many plant species, among others, in the bark of white birch *Betula alba*. Betulinic acid was reported to display a wide range of biological effects, including antiviral, antiparasitic, antibacterial, anticancer and anti-inflammatory activities. The effects of betulinic acid (50, 5, 0.5 mg/kg) administered orally five times at 24 hours intervals to non-immunized and red blood cells (SRBC)-immunized mice were determined. The present study examined the total number of lymphocytes in the thymus, spleen and mesenteric lymph nodes, and the percentage of subsets of T cells ($CD4^+CD8^+$, $CD4^-CD8^-$, $CD4^+$, $CD8^+$) in thymus,T ($CD3^+$, $CD4^+$, $CD8^+$) and B ($CD19^+$) lymphocytes in the spleen and mesenteric lymph nodes, as well as white blood cell (WBC) and differential leukocyte counts in non-immunized mice, and humoral immune response in SRBC-immunized mice. SRBC was injected 24 hours after administration of the last dose of betulinic acid. It was found that betulinic acid administered orally five times at the dose of 0.5 mg/kg increased the total number of thymocytes, splenocytes, lymphocytes of mesenteric lymph node cells, and the weight ratio of the spleen and mesenteric lymph nodes in non-immunized mice. Betulinic acid also changed the percentage of T cell subsets in the thymus and T and B lymphocytes in peripheral lymphatic organs. The effects of betulinic acid on T and B cell subpopulations depended on the dose applied. The strongest stimulating effect of betulinic acid was observed when the drug was administered at the dose of 0.5 mg/kg. Five exposures to betulinic acid (0.5 mg/kg) decreased the percentage of immature $CD4^+CD8^+$ thymic cells with corresponding increases in the percentage and absolute count of mature, single-positive $CD4^+$ thymocytes and decreased the percentage and total count of $CD3^+$ splenocytes and mesenteric lymph node cells with corresponding decreases in the percentage and absolute count of $CD4^+$ and $CD8^+$ cells. Multiple administration of betulinic acid at the investigated doses augmented the percentage and absolute count of $CD19^+$ cells in the peripheral lymphatic organs. Moreover, betulinic acid at the dose of 5 mg/kg administered prior to SRBC immunization increased the number of plaque forming cells (PFC) but decreased the production of anti-SRBC antibodies on day 4 after priming. Thus, betulinic acid is a potential biological response modifier and may strengthen the immune response of its host.

Key words: betulinic acid, T and B lymphocyte subsets, humoral immune response, mice

Correspondence to: B. Obmińska-Mrukowicz e-mail: b.mrukowicz@gmail.com, tel.: +48 71 3205403

Introduction

The modulation of the immune system by various bioactive compounds derived from plants may have potential for the management of certain infections, autoaggression and neoplastic diseases. Generally, the mechanism by which bioactive compounds extracted from plants can enhance the immune responses in experimental as well as clinical situations are not clearly determined. The study of the influence and action of plant extracts as immunomodulators may be useful to elucidate some of the processes involved during the immune response. Betulinic acid, a pentacyclic lupane-type triterpene, is widely distributed in the bark of several plant species, including white birch (*Betula pubescens*), ber tree (*Zizyphus mauritiana)*, tropical carnivorous plants (*Tryphyllum peltaum, Ancistrocladus heyneaus, Diospyoros leucomelas),* the jambul (*Syzygium formosanum),* flowering quince (*Chaenomeles sinensis*) and *Pulsatilla chinensis* (Cichewicz and Kouzi 2004, Yogeeswari and Sriram 2005). Betulinic acid and its derivatives exhibit a broad range of biological activities, including inhibition of human immunodeficiency virus (Dang et al. 2009) and cytotoxic activity against various human cancer cell lines (Nakagawa-Goto et al. 2009, Eichenmuller et al. 2010, Kommera et al. 2011, Mullauer et al. 2011) as well as antibacterial (Schuhly et al. 1999, Chandramu et al. 2003), anti-inflammmatory (Recio et al. 1995, Yamashita et al. 2002, Viji et al. 2011) and immunomodulatory effects (Zdzisińska et al. 2003, Yun et al. 2003). It is believed that the mechanism of action of betulinic acid is associated with its inhibition of aminopeptidase, especially aminopeptidase N (APN) (Melzing and Borman 1998) which is an integral membrane metalloprotease, identical with surface molecule CD13 (Look et al. 1989). It is present in a wide variety of cells, e.g. hematopoietic cells of myeloid origin, epithelial-, endothelial- and fibroblast-cells. Deregulated expression of APN/CD13 has been found in many inflammatory and neoplastic diseases (Luan and Xu 2007). For this reason, administration of APN/CD13 inhibitors in the treatment of these illnesses has been studied extensively (Xu and Li 2005, Bauvois and Dauzone 2006).

The aim of this study was to determine the effect of betulinic acid in different dosages on the subsets of lymphocytes T in the thymus and T, and B lymphocytes in the spleen, and mesenteric lymph nodes in non-immunized mice, and on primary humoral immune response in sheep red blood cells (SRBC)-immunized mice.

Materials and Methods

Animals

The experiments were carried out on male and female Balb/c mice, each weighing 18-20 g (7-8 weeks of age). The mice were kept under conventional conditions and had *ad libitum* access to water and granulated food. The animals were obtained from a Breeding Center of Laboratory Animals of the Nofer Institute of Occupational Medicine, Łódź, Poland. Some of the mice were immunized intraperitoneally (i.p.) with 0.2 ml of SRBC suspension (4×10^8 cells/mouse). The sheep blood was collected into Alsever's solution in 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. 14/2007).

Drugs and treatment

Betulinic acid (Sigma-Aldrich Chemie GmbH, Riedstr. 2,D-89555 Steiheim, Germany. Lot: S43559-498) at the dose of 0.5, 5 or 50 mg/kg was administered orally (by stomach-tube) with a 1% starch jelly five times, at 24 h intervals in non-immunized and SRBC-immunized mice. Mice were immunized with SRBC 24 h after the last administration of betulinic acid. The trials in the control group were conducted in parallel. Animals in the control group received an equivalent amount of pure starch jelly. The volume of drug was 0.2 ml/ mouse. Each control and experimental group consisted of seven mice.

Measurements

The following measurements were taken: (i) the total number of thymocytes, splenocytes and lymphocytes of mesenteric lymph nodes; (ii) the weight ratio of the thymus, spleen and mesenteric lymph nodes calculated according to the following formula: weight of an organ (mg)/body weight of a mouse (mg) $\times$ 100; (iii) the percentage and count of lymphocyte subpopulations in lymphatic organs; (iv) white blood cell count (WBC) and differential leukocyte count; (v) the number of plaque forming cells (PFC) in spleen; (vi) anti-SRBC haemagglutinin titre in the serum.

The total number of thymocytes, splenocytes, lymphocytes of mesenteric lymph nodes, the weight ratio of the thymus, spleen and mesenteric lymph nodes, the percentage of CD subsets ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$, $CD8^+$ in the thymus; $CD19^+$, $CD3^+$, $CD4^+$ and $CD8^+$ in spleen and mesenteric lymph nodes), as well as the number of leukocytes and the picture of leukocytes in the blood were determined on days 1 and 3 after the last dose of betulinic acid in non-immunized mice. The number of PFC and anti-SRBC haemagglutinin titres in SRBC-immunized mice treated five times with betulinic acid were determined on day 4 after priming.

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

The mice were anaesthetized with halothane (Narcotan, Zentiva, Prague, Czech Republic) 1 and 3 days after the final dose of betulinic acid was administered. Thymuses, spleens 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 passage through a nylon mesh and then centrifuged (2250 x g, 15 min, 4°C) on a layer of Ficoll 400 (Pharmacia, Fine Chemicals AB, Uppsala, Sweden)/ Urografin 76% (diatrizoate sodium and megluminediatrizoate, Bayer Schering Pharma, Poland) at a 1 : 3 ratio at density of 1.076. After centrifugation, the cells were collected from the interphase and washed twice (375 x g, 8 min, 4°C) with a sterile, ice-cold PBS supplemented with 1% bovine serum albumin (BSA, Sigma). 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 thymocytes, splenocytes and lymphocytes of the mesenteric lymph nodes were stained with Rat Anti-Mouse CD4: FITC/CD8: RPE dual color reagent (Serotec, Kidlington, United Kingdom) at a dilution recommended by the manufacturer. The splenocytes and lymphocytes of the mesenteric lymph nodes were also stained with Rat Anti-Mouse CD19:FITC/CD3:RPE dual color reagent (Serotec, Kidlington, United Kingdom) at a dilution recommended by the producer.

The cells were incubated at 4°C for 30 min. and washed (375 x g, 8 min, 4°C) three times with an ice-cold PBS. The fluorescence was analyzed using a flow cytometer (FACS Calibur; Becton Dickinson Biosciences, San Jose, CA, USA). Data acquisition and analysis were done using a CellQuest 3.1f software. A two-color analysis was performed: fluorescence 1 (FL1) – FITC: emission peak 525 nm, fluorescence 2 (FL2) – PE: emission peak 575 nm. Instrument settings used in this study were as follows: FL1: log, 584V, FL2: log, 595V, the fluorescence compensation: FL1: -1.7 % FL2; FL2: -22.3 % FL1. A total of 10 000 events were collected.

Determination of the total number of thymocytes, splenocytes and mesenteric lymph node lymphocytes

The lymphatic organs after removing from anaesthetized with halothane mice were passed through a nylon mesh into 1 ml of a sterile, ice-cold PBS. Next, the suspended cells were diluted ten times in PBS. The number of mononucleated cells from central and peripheral lymphatic organs was counted in a Thoma hemocytometer using Türk solution.

Preparation of blood smears

The blood was smeared on microscopic glass and stained with Giemsa and May-Grunwald reagents. The blood smears were analyzed using 1000\ magnification in immersion oil. Up to 100 cells were counted on two blood smears for each mouse (seven mice per group). The results are presented as mean values (in total number) for each cell type (lymphocytes, band neutrophils, segmented neutrophils, basophils, eosinophils and monocytes).

Determination of plaque forming cells (PFC)

The mice were anaesthetized with halothane and then killed by cervical dislocation 24 h after the final dose of betulinic acid was administered. The spleens were removed and placed into sterile, ice-cold Hank's saline (Institute of Immunology and Experimental Therapy, Wrocław, Poland). The lymphocytes from the spleens were isolated as described above. After centrifugation at 4°C, the splenocytes were collected from the interface and washed twice (375 x g, 8 min, 4°C) in ice-cold Hank's saline. After the second wash, the cells were suspended in 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 hemolysis technique in agar gel, as described by Mishell and Dutton (1967).

Table 1. The effects of betulinic acid with respect to dosage on the total number of thymocytes, splenocytes and mesenteric lymph node cells, and weight ratio of the thymus, spleen and mesenteric lymph nodes. The mean values (n=7) and standard deviations are presented.

Index	Day	Control	Betulinic acid		
			5 x 0.5 mg/kg	5 x 5 mg/kg	5 x 50 mg/kg
The total number of thymocytes (x 10^6)	1	37.6 ± 5.4	42.0 ± 5.6	38.5 ± 7.1	38.3 ± 5.9
	3	31.9 ± 7.1	39.0 ± 5.3*	36.5 ± 9.8	31.6 ± 6.1
Weight ratio of thymus	1	0.164 ± 0.01	0.158 ± 0.01	0.136 ± 0.02*	0.145 ± 0.03
	3	0.1023 ± 0.02	0.092 ± 0.01	0.093 ± 0.01	0.090 ± 0.02
The total number of splenocytes (x10^6)	1	66.6 ± 11.0	70.4 ± 6.7	65.4 ± 9.5	68.6 ± 4.8
	3	52.1 ± 8.3	60.0 ± 3.0*	57.2 ± 4.3	54.8 ± 5.2
Weight ratio of spleen	1	0.558 ± 0.07	0.590 ± 0.04	0.531 ± 0.05	0.566 ± 0.08
	3	0.407 ± 0.04	0.473 ± 0.05*	0.408 ± 0.03	0.395 ± 0.04
The total number of mesenteric lymph node cells (x10^6)	1	31.6 ± 5.1	34.2 ± 5.3	32.8 ± 5.4	32.0 ± 7.4
	3	30.4 ± 7.7	43.3 ± 3.4*	39.3 ± 4.0*	33.0 ± 7.3
Weight ratio of mesenteric lymph nodes	1	0.334 ± 0.05	0.386 ± 0.03	0.309 ± 0.07	0.381 ± 0.06
	3	0.287 ± 0.06	0.362 ± 0.05*	0.252 ± 0.05	0.260 ± 0.02

* $p<0.05$ as compared to the control group

Determination of anti-SRBC antibodies in the serum

The blood samples were taken from retro-ocular arteria of halothane anaesthetized mice. The sera were obtained by blood centrifugation and inactived at 56°C for 30 min. The total and 2-mercaptoethanol-(2-ME) resistant serum agglutination titres were defined by active haemagglutination test carried out on microplates. The titre of 2-mercaptoethanol resistant antibody is roughly equivalent to that of the 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 the serum of non-immunized mice did not contain spontaneous anti-SRBC antibodies.

Statistical analysis

The data obtained in this study were analyzed statistically using a t-Student test. The differences were considered significant at $p<0.05$.

Results

The effects of betulinic acid on the total number of thymocytes, splenocytes, mesenteric lymph node lymphocytes, and the weight ratio of the thymus, spleen and mesenteric lymph nodes in mice

As reported in Table 1, the total number of thymocytes, splenocytes and mesenteric lymph node cells, and the weight ratio of the spleen and mesenteric lymph nodes in mice treated with betulinic acid at the dose of 0.5 mg/kg markedly increased as early as 3 days following the exposure to drug. At the same time, the total number of mesenteric lymph node cells was increased in the mice treated with betulinic acid at the dose of 5 mg/kg. Administration of betulinic acid at the dose of 5 mg/kg did not change the total number of thymocytes and splenocytes or the weight ratio of lymphatic organs. Also the total number of thymocytes, splenocytes and mesenteric lymph node cells as well as the weight ratio of lymphatic organs remained unchanged after administration of betulinic acid five times at 24 h intervals at the dose of 50 mg/kg.

The effects of betulinic acid on thymocyte, splenocyte and mesenteric lymph node cell subpopulations in mice

As reported in Tables 2, 3 and 4, the trials conducted on mice confirmed the modulating effect of betulinic acid on T cells in the central lymphatic organ, and T and B cells in the peripheral lymphatic organs. The results of the study show that there is a relationship between the effect induced by betulinic acid and the dose of the drug applied. The strongest effect was found on day 3 after administration of betulinic acid at the dose of 0.5 mg/kg. Five exposures to betulinic acid (0.5 mg/kg) decreased the percentage, but increased the absolute number of the immature $CD4^+CD8^+$ thymic cells (double-positive cells) with corresponding increases in the percentage and abso-

Table 2. Percentage and absolute count of thymocyte subpopulations in mice treated five times with betulinic acid. The mean values (n=7) and standard deviations are presented.

Index		Day	Control	Betulinic acid		
				5 x 0.5 mg/kg	5 x 5 mg/kg	5 x 50 mg/kg
Thymocytes						
$CD4^+CD8^+$	(%)	1	76.7 ± 3.2	76.7 ± 1.5	76.9 ± 0.9	76.3 ± 1.7
	(x 10^6)		28.8 ± 0.17	32.2 ± 0.08	29.6 ± 0.06	29.2 ± 0.1
	(%)	3	78.1 ± 2.1	74.6 ± 2.3*	78.8 ± 1.4	78.6 ± 3.2
	(x 10^6)		24.9 ± 0.15	29.0 ± 0.1*	28.7 ± 0.1	24.5 ± 0.2
$CD4^-CD8^-$	(%)	1	5.8 ± 2.0	5.3 ± 1.6	5.3 ± 0.9	5.7 ± 1.4
	(x 10^6)		2.1 ± 0.1	2.2 ± 0.08	2.0 ± 0.06	2.1 ± 0.08
	(%)	3	4.1 ± 1.1	5.2 ± 0.7	4.2 ± 0.8	4.8 ± 3.2
	(x 10^6)		1.3 ± 0.07	2.0 ± 0.03	1.5 ± 0.07	1.5 ± 0.2
$CD4^+CD8^-$	(%)	1	13.8 ± 1.2	14.6 ± 0.5	14.4 ± 0.6	14.3 ± 1.4
	(x 10^6)		5.2 ± 0.06	6.1 ± 0.02	5.5 ± 0.04	5.5 ± 0.08
	(%)	3	14.0 ± 1.3	16.9 ± 1.8*	14.0 ± 1.6	14.0 ± 1.4
	(x $c10^6$)		4.5 ± 0.09	6.6 ± 0.09*	5.1 ± 0.1	4.4 ± 0.08
$CD4^-CD8^+$	(%)	1	3.6 ± 0.7	3.1 ± 0.4	3.2 ± 0.4	3.6 ± 0.7
	(x 10^6)		1.4 ± 0.03	1.3 ± 0.02	1.2 ± 0.02	1.4 ± 0.04
	(%)	3	2.6 ± 0.5	3.1 ± 0.7	2.9 ± 0.4	2.4 ± 0.3
	(x 10^6)		0.8 ± 0.03	1.2 ± 0.03	1.0 ± 0.03	0.8 ± 0.01

* $p<0.05$ as compared to the control group

Table 3. Percentage and absolute count of splenocyte subpopulations in mice treated five times with betulinic acid. The mean values (n=7) and standard deviations are presented.

Index		Day	Control	Betulinic acid		
				5 x 0.5 mg/kg	5 x 5 mg/kg	5 x 50 mg/kg
Splenocytes						
$CD3^+$	(%)	1	30.1 ± 6.0	27.3 ± 2.3	27.3 ± 2.3	27.6 ± 4.0
	(x 10^6)		20.0 ± 0.7	19.2 ± 0.1	17.8 ± 0.2	18.9 ± 0.2
	(%)	3	32.3 ± 5.6	18.9 ± 3.5*	31.3 ± 2.4	31.4 ± 4.4
	(x 10^6)		16.8 ± 0.5	11.3 ± 0.1*	17.9 ± 0.1	17.2 ± 0.2
$CD4^+$	(%)	1	24.6 ± 4.0	23.0 ± 1.0	26.1 ± 5.1	23.5 ± 3.6
	(x 10^6)		16.3 ± 0.4	16.1 ± 0.06	17.0 ± 0.5	16.1 ± 0.2
	(%)	3	23.6 ± 5.9	17.4 ± 3.7*	24.9 ± 7.9	24.0 ± 3.0
	(x 10^6)		12.3 ± 0.5	9.4 ± 0.1*	14.2 ± 0.3	13.1 ± 0.1
$CD8^+$	(%)	1	4.9 ± 1.1	3.1 ± 1.1*	3.7 ± 0.4*	4.0 ± 1.2
	(x 10^6)		3.3 ± 0.1	2.1 ± 0.07	2.4 ± 0.03	2.7 ± 0.05
	(%)	3	6.8 ± 1.7	2.9 ± 0.3*	6.7 ± 4.1	6.1 ± 1.8
	(x 10^6)		3.5 ± 0.1	1.7 ± 0.01*	3.8 ± 0.1	3.3 ± 0.09
$CD19^+$	(%)	1	62.7 ± 6.7	66.9 ± 2.0	64.6 ± 6.5	67.8 ± 4.5
	(x 10^6)		41.7 ± 0.7	47.0 ± 0.1*	42.2 ± 0.6	46.5 ± 0.2*
	(%)	3	60.5 ± 6.2	75.1 ± 4.4*	61.8 ± 3.8	61.4 ± 5.1
	(x 10^6)		31.5 ± 0.5	45.0 ± 0.1*	35.3 ± 0.1	33.6 ± 0.3

* $p<0.05$ as compared to the control group

lute count of mature, single-positive $CD4^+$ thymocytes (Table 2). At the same time, some changes in the percentage and absolute count of T and B cells in spleen and mesenteric lymph nodes were found. Administration of betulinic acid five times at 24 h intervals at the dose of 0.5 mg/kg led to a decrease in the percentage and absolute count of $CD3^+$ with corresponding decreases in the percentage and absolute number of $CD8^+$ and $CD4^+$ splenocytes and mesenteric lymph node cells. However, administration of betulinic acid at the investigated doses increased the percentage and absolute count of B cells

Table 4. Percentage and absolute count of mesenteric lymph node cell subpopulations in mice treated five times with betulinic acid. The mean values (n=7) and standard deviations are presented.

Index		Day	Control	Betulinic acid		
				5 x 0.5 mg/kg	5 x 5 mg/kg	5 x 50 mg/kg
Mesenteric lymph node cells						
$CD3^+$	(%)	1	53.6 ± 6.4	58.5 ± 4.5	55.1 ± 3.3	53.7 ± 5.0
	(x 10^6)		16.9 ± 0.3	20.0 ± 0.2*	18.0 ± 0.2	17.1 ± 0.37
	(%)	3	45.5 ± 7.7	31.5 ± 3.7*	40.3 ± 5.4	48.9 ± 5.1
	(x 10^6)		13.8 ± 0.6	13.6 ± 0.1	15.8 ± 0.2	16.1 ± 0.4
$CD4^+$	(%)	1	47.4 ± 3.1	45.4 ± 3.1	47.4 ± 3.2	48.0 ± 3.9
	(x 10^6)		14.9 ± 0.2	15.5 ± 0.2	15.5 ± 0.2	15.3 ± 0.3
	(%)	3	37.2 ± 5.0	27.3 ± 3.5*	31.9 ± 10.0	40.6 ± 1.8
	(x 10^6)		11.3 ± 0.4	11.8 ± 0.1	12.5 ± 0.4	13.3 ± 0.1
$CD8^+$	(%)	1	9.3 ± 1.6	14.1 ± 2.3*	10.2 ± 1.4	9.0 ± 1.5
	(x 10^6)		2.9 ± 0.08	4.8 ± 0.1*	3.3 ± 0.07	2.9 ± 0.1
	(%)	3	8.9 ± 1.4	3.2 ± 0.8*	7.0 ± 3.8	9.5 ± 1.9
	(x 10^6)		2.7 ± 0.1	1.3 ± 0.02*	2.7 ± 0.1	3.1 ± 0.1
$CD19^+$	(%)	1	43.0 ± 6.7	37.8 ± 4.6	39.8 ± 2.7	42.9 ± 5.5
	(x 10^6)		13.6 ± 0.3	12.9 ± 0.2	13.0 ± 0.1	13.7 ± 0.4
	(%)	3	50.7 ± 5.7	65.4 ± 3.7*	56.1 ± 5.9	47.5 ± 5.5
	(x 10^6)		15.4 ± 0.4	28.3 ± 0.1*	22.0 ± 0.2*	15.7 ± 0.4*

$p<0.05$ as compared to the control group

Table 5. Changes in the composition of blood cell types in mice after administration of betulinic acid five times at 24 h intervals. The mean values (n=7) and standard deviation are presented.

Number of cells (10^3/µl)	Day	Control	Betulinic acid		
			5 x 0.5 mg/kg	5 x 5 mg/kg	5 x 50 mg/kg
Leucocytes	1	6.6 ± 1.9	8.5 ± 1.1*	7.9 ± 1.0	7.6 ± 1.4
	3	5.1 ± 1.0	6.9 ± 0.8*	6.9 ± 1.1*	6.8 ± 1.2*
Lymphocytes	1	4.8 ± 1.4	7.2 ± 1.0*	6.9 ± 0.9*	6.3 ± 1.1*
	3	3.7 ± 0.6	5.9 ± 0.7*	5.8 ± 0.9*	5.5 ± 1.2*
Band neutrophils	1	0.2 ± 0.1	0.27 ± 0.1	0.2 ± 0.09	0.13 ± 0.1
	3	0.2 ± 0.09	0.19 ± 0.09	0.19 ± 0.1	0.24 ± 0.1
Segmented neutrophils	1	1.1 ± 0.6	0.8 ± 0.1	0.6 ± 0.1*	0.8 ± 0.2
	3	0.9 ± 0.3	0.7 ± 0.1	0.6 ± 0.1*	0.8 ± 0.2
Basophils	1	0.09 ± 0.06	0.01 ± 0.03	0	0.07 ± 0.01
	3	0.06 ± 0.07	0	0.05 ± 0.05	0.02 ± 0.04
Eosinophils	1	0.01 ± 0.07	0.02 ± 0.04	0.03 ± 0.04	0.1 ± 0.09
	3	0.08 ± 0.01	0.02 ± 0.05	0.07 ± 0.05	0.01 ± 0.03
Monocytes	1	0.1 ± 0.07	0.1 ± 0.05	0.1 ± 0.09	0.1 ± 0.1
	3	0.1 ± 0.07	0.07 ± 0.08	0.2 ± 0.09	0.2 ± 0.1

* $p<0.05$ as compared to the control group

in these peripheral lymphatic organs (Table 3 and 4). The strongest stimulating effect of betulinic acid on the B lymphocytes was observed when the drug was administered at the dose of 0.5 mg/kg. The effect of betulinic acid on T and B cells was found on day 3 following exposure to the agent at the dose of 0.5 mg/kg. On the other hand, no significant changes in the percentage and absolute count of T cells in the thymus and T cells in the peripheral lymphatic organs were observed when the daily dose of betulinic acid was increased ten (5 mg/kg) or hundred (50 mg/kg) times.

The effects of betulinic acid on WBC and differential leukocyte counts

As shown in Table 5, five times administration of betulinic acid, irrespective of the dose applied, increased the count of peripheral blood leukocytes.This resulted from an increase in the number of blood lymphocytes. However, the exposure to betulinic acid at the dose of 5 mg/kg administered five times at 24 hours intervals decreased the count of peripheral blood segmented neutrophils on days 1 and 3 after drug treatment.

The effects of betulinic acid on the primary humoral response in SRBC-immunized mice

Betulinic acid administered five times at 24 h intervals prior to antigen stimulation modulated the primary humoral response in SRBC-immunized mice, which resulted in an increased number of cells producing haemolytic anti-SRBC antibodies (PFC) and in decreased total and 2-mercaptoethanol resistant serum haemagglutinin titre (Fig. 1 and 2). Modulating effect of betulinc acid on the humoral response to SRBC depended on the dose-schedules of the treatment. Betulinic acid administered at the dose of 0.5 mg/kg five times prior to SRBC did not change the number of PFC, but discontinued the production of total and 2-mercaptoethanol resistant anti-SRBC haemagglutinins. However, five exposures to betulinic acid at the dose of 5 mg/kg prior to antigen stimulation increased the number of PFC, but decreased the total anti-SRBC antibody titers. No effect of betulinic acid administered at the dose of 50 mg/kg on the humoral immune response to SRBC was observed.

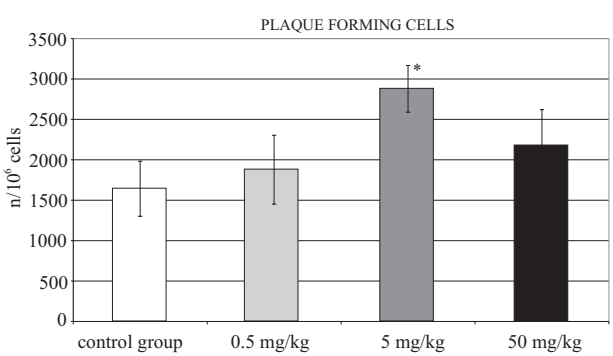

Fig. 1. The number of plaque forming cells (PFC) in SRBC-immunized mice treated with betulinic acid (0.5, 5 and 50 mg/kg) five times at 24 hours intervals prior to priming. The mean values (n=7) and standard deviations are presented. *p<0.05 as compared to the control group.

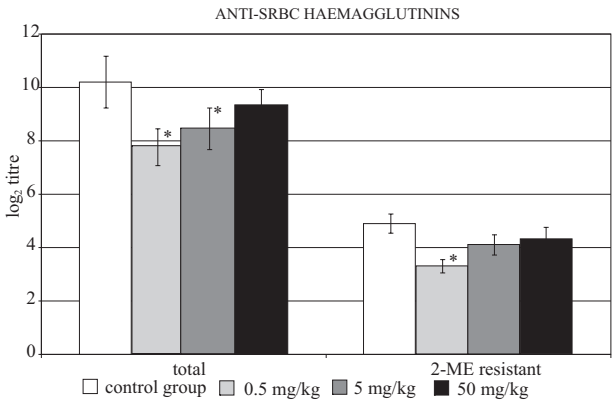

Fig. 2. The total and 2-mercaptoethanol-resistant (2-ME-resistant) anti-SRBC haemagglutinin titres in SRBC-immunized mice treated with betulinic acid (0.5, 5 and 50 mg/kg) five times at 24 hours intervals prior to priming. The mean values (n=7) and standard deviations are presented. *p<0.05 as compared to the control group.

Discussion

The part of this study which was carried out on non-immunized mice shows that betulinic acid can change the percentage and absolute count of T lymphocytes in the thymus, and T, and B lymphocytes in peripheral lymphatic organs. The findings prove that there is a relationship between the immunological effect induced by betulinic acid and the dose of the drug as well as the duration of the exposure. The strongest modulating effect was observed on day 3 following exposure to betulinic acid administered five times at 24 h intervals at the dose of 0.5 mg/kg. Moreover, betulinic acid at a ten times higher dose (5 mg/kg) can also modulate the primary humoral response to SRBC resulting in an increased number of splenocytes producing hemolytic anti-SRBC antibodies (PFC) and decrease in the total and 2-mercaptoethanol resistant serum haemagglutinin titre. It is likely that the stimulating effect of betulinic acid on the number and activity of B lymphocytes is due to the activation of T lymphocytes and macrophages through the cytokine cascade (IL-1) that is enhanced by this agent (Yun et al. 2003). IL-1 can induce the maturation of pre-B-cells (Giri et al. 1985). This cytokine acts synergistically with various B cell growth factors, which results in augmented proliferation, increased level of immunoglobulin production. Moreover, it induces or augments T cells to produce IL-2, IL-4, IL-5 and IL-6, all of which have immunomodulatory effects on B cells. The previous study conducted in vitro on human whole blood cell cultures (Zdzisińska et al. 2003) has indicated that betulinic acid is a modulator of cytokine production by Th1/Th2 cell subpopulations which slightly enhances IL-10 formation (anti-inflammatory cytokine) and inhibits IFN-γ pro-

duction (pro-inflammatory cytokine), thus reducing IFN-γ/IL-10 ratio. However, in this experiment betulinic acid did not change TNF-h production by human leucocytes. In contrast, the studies of Yun et al. (2003) have shown that betulinic acid stimulates RAW 264.7 cell, a murine macrophage cell line, to release TNF-α and IL-1β regardless of LPS co-treatment. The authors also have revealed that the surface $CD40^+$ molecule, which belongs to the TNF receptor family, was expressed on RAW 264.7 macrophages treated with betulinic acid regardless of LPS co-treatment. However, betulinic acid suppresses the production of nitric oxide (NO) and inhibits the gene expression of COX-2 in LPS-stimulated RAW 264.7 cells. These results demonstrate the ability of betulinic acid to both directly activate macrophages to produce pro-inflammatory cytokines, and indirectly, to inhibit NO and COX-2 mediated functions (inflammatory response). Our previous study carried out *in vivo* on Balb/c mice revealed that administration of betulinic acid at the daily dose of 0.5 mg/kg for 2 weeks significantly increased the production of TNF-α by peritoneal macrophages stimulated *in vitro* with LPS from E. coli (2.5 μg/ml). In contrast, the serum concentration of IL-2 (Th1) and IL-6 (Th2) was decreased in mice treated with betulinic acid (Yi et al. 2010). This observation may suggest that multiple administration of betulinic acid at the low dose of 0.5 mg/kg has a mixed, Th1 and Th2 adjuvant activity. As the present study demonstrated, betulinic acid is able to modulate the primary humoral response in SRBC-immunized mice, which results in an increased number of cells producing haemolytic anti-SRBC antibodies (PFC) and decreased total (IgM + IgG) and 2-mercaptoethanol resistant (IgG) haemagglutinin titres. Modulating effect of betulinc acid on the humoral response to SRBC depended on the dose-schedules of the treatment. A decrease in 2-mercaptoethanol resistant (IgG) haemagglutinin titres was found in mice exposed even to the lowest investigated dose of betulinic acid (0.5 mg/kg). IgG is the most abundant class of antibodies in serum, and IgM is the first immunoglobulin class produced in a primary response to an antigen (Kindt et al. 2006). 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.

It is concluded that betulinic acid, an inhibitor of APN/CD13, is a potential biological response modifier and may strengthen the immune response of a host. The effects of betulinic acid on the cellular and humoral immunity depended on the dose applied. The strongest immunomodulatory effect of betulinic acid was found after multiple administration of this drug at the lowest investigated dose (0.5 mg/kg).

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