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Cover photo: Thymic epithelial cell joining to neoplastic Hela cell in culture [courtesy of Bohdan Woźniewicz, Children's Memorial Health Institute]. Photo: Piotr Gawdziś

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Editorial correspondence should be directed to the Editor, Włodzimierz Maśliński, Department of Pathophysiology and Immunology, Institute of Rheumatology, Spartańska 1, 02-637 Warsaw, Poland
(tel./fax: +48 22 844 25 40), tel. +48 22 646 50 07, e-mail: zpatiir@warman.com.pl

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The analysis of L-selectin expression intensity (CD62L) on B-lymphocytes in B-cell chronic lymphocytic leukemia cells (B-CLL)

JOANNA KOPEĆ-SZLĘZAK[1], URSZULA PODSTAWKA[1], LECH KONOPKA[2], GRAŻYNA PAŁYNYCZKO[2]

[1]Department of Physiopathology, Institute of Haematology and Blood Transfusion, Warsaw, Poland; [2]Department of Internal Medicine and Haematology, Institute of Haematology and Blood Transfusion, Poland

Abstract

Decreased percentage of B cells expressing L-selectin in B-cell chronic lymphocytic leukemia (B-CLL) is known from literature. The purpose of our studies was to compare the intensity of L-selectin expression on subpopulation of L-selectin positive B-lymphocytes in untreated and treated patients with B-CLL.

The lymphocytes were obtained from peripheral blood of 70 patients suffering from B-CLL (40 untreated and 30 after the treatment) and 20 healthy blood donors. The percentage of positive cells and intensity of CD62 L-selectin expression (RFI) were measured on CD19+ B lymphocytes using tri-color fluorochrom labeled monoclonal antibodies and flow cytometric analysis.

The lower percentage of L-selectin positive B-cells paralleled with considerable decrease of this selectin intensity was observed in patients with B-CLL in comparison to healthy subjects. Both these changes may contribute to the impaired adhesion and migration of B-cells in B-CLL resulting in the accumulation of these cells in the peripheral blood. In a group of patients entirely responding to the treatment the tendency toward normalization of both measured parameters has been observed.

Key words: *L-selectin, B-cells, B-CLL.*

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Introduction

The migration of lymphocytes from blood, through peripheral vessel endothelium, to lymph node is regulated by the expression of adhesion molecules. The expression of L-selectin on lymphocyte surface plays a key role during the first phase of adhesion to the endothelium [1]. Published data document decreased percentage of B cells expressing CD62L (L-Selectin) in B-CLL in comparison to healthy donors [2-5]. A loss of L-selectin expression has also been observed on B-cells isolated from patients with lymphoma (e.g. MALT lymphoma [6, 7].

Using microarray technique, the decreased activity of genes responsible for a synthesis of surface adhesion molecules, including L-selectin, in B-CLL has been reported [8]. Therefore, it is possible that B-cells from B-CLL patients exert impaired expression of L-selectin. However, literature search revealed no data on the intensity of L-selectin expression (a direct indicator of density of surface expressed L-selectin) on the subpopulation of B-cells that have not lost CD62L molecules.

The purpose of our studies was to compare the intensity of L-selectin expression on subpopulation of L-selectin positive B-lymphocytes in untreated and treated patients with B-CLL.

Method

The object of research

We have investigated the lymphocytes obtained from peripheral blood of 70 patients suffering from B-cell chronic lymphocytic leukemia:

- 40 patients before treatment (BT), without validated contact with cytostatics; Rai 0 – IV; age 65.7±9.8; 22 men, 18 women,

Correspondence: Prof. M.S. Joanna Kopeć-Szlęzak, Department of Physiopathology, Institute of Haematology and Blood Transfusion, Chocimska 5, 00-957 Warsaw, Poland.

- 30 patients after treatment with cytostatics (AT):
  a) 20 patients with a partial response (AT-PR) after treatment with Chlorambucil or Chlorambucil and Cladribine (2-chlorodeoxyadenosine, 2CdA); Rai 0 – IV; age 60.1±13.1; 14 men, 6 women,
  b) 10 patients with a complete response to curing (AT-CR) after a Cladribine therapy – as a first – line therapy; age 58.9±5.3; 4 men, 6 women.

The diagnosis of B-CLL based on clinical morphological and immunological criteria (reactivity with monoclonal antibodies: CD5, CD19, CD20, CD23, κ or λ light chain). The clinical stage of disease was evaluated according to the Rai system [9]. The partial response (PR) and the complete response (CR) were defined in accordance with determined by an NCI-Sponsored Working Group criteria [10]. The control group comprised 20 healthy donors.

Procedure

Leukocytes were marked with fluorochroms labeled monoclonal antibodies directed against antigens CD62L, CD5, CD19 in tricolor fluorescention scheme. The adequate isotype controls were included in all series. After incubation with antibodies, erythrocytes were lysed using the reagent from Ortho. Cytometric analyses were performed directly after labeling (Cytoron Absolute Ortho flow cytometer). 10,000 cells were counted. The analysis of the material was carried out using the Immunocount II software (Ortho).

The expression of antigen was estimated as a percentage of positive cells (i.e. the percentage of CD62L-positive cells among the population of CD19+ cells). The intensity of CD62L-antigen expression was found by relative fluorescence intensity (RFI) using mean channels of fluorescence for the antigen and adequate value of isotype control according to Miwa equation [11]:

$$RFI = 10 \times [(3.5 \times \text{mean channel of the sample}) \div 255] - 10 \times [(3.5 \times \text{mean channel of the control}) \div 255]$$

(Mean channel of CD62L fluorescence intensity on B-cells in the analyzed sample and the control isotype sample, 255 – number of fluorescence channels in the Cytoron Absolute Ortho cytometer).

An example of B-lymphocytes stained with anti L-selectin antibody from patients and healthy donors is presented in Fig. 1. Obtained values are presented as means ±SD and ±SE. The statistical analysis was made by Mann Whitney U Test ($p<0.05$ were considered as statistically significant).

Results

- **Changes of percentage of B-cells with CD62L-expression**

In peripheral blood the percentage of B CD19+ lymphocytes with CD62L expression below the lower limit of control varied from 10% to 48% of tested patients respectively in CR and PR groups (Fig. 2).

The considerable differences in the number of B-cells with CD62L expression were observed among patients from BT and AT-PR groups. Therefore, wide range of values and consequently high values of standard deviations are reported (Tab. 1).

Table 1. The characteristic of BCD19+ cells from peripheral blood of patients suffering from B-CLL before treatment (BT), after treatment with a partial response (AT-PR) and a complete response (AT-CR) to the treatment. p – a p value (Mann Whitney U Test), a statistically significant difference compared to the control group

		Donors n=20	BT n=40	AT-PR n=20	AT-CR n=10
CD19 + cells as a % of lymphocytes	mean (±SD)	11.4 (±2.6)	87.8 (±8.6)	77.4 (±19.9)	13.8 (±13.1)
	min-max	7.5-16.5	56.0-98.7	19.9-32.9	2.2-46.0
	p		<0.0001	<0.0001	0.52
% CD5 cells in B CD19 + cells	mean (±SD)	4.9 (±2.5)	83.3 (±16.4)	88.8 (±15.3)	33.0 (±31.8)
	min-max	2.2-10.5	38.8-99.9	57.6-100	3.0-94.8
	p		<0.0001	<0.0001	0.0001
% CD5 + CD62L + cells in B CD19 + cells	mean (±SD)	4.7 (±2.5)	77.5 (±19.7)	71.5 (±18.7)	25.0 (±25.9)
	min-max	1.8-10.0	29.7-99.1	32.1-98.0	3.0-92.8
	p		<0.0001	<0.0001	0.0014
% CD62L + cells in B CD19 + cells	mean (±SD)	93.8 (±5.0)	87.4 (±17.7)	80.7 (±20.9)	86.2 (±15.6)
	min-max	85.0-98.6	29.7-99.9	31.1-99.7	50.8-97.0
	p		0.80	0.16	0.059

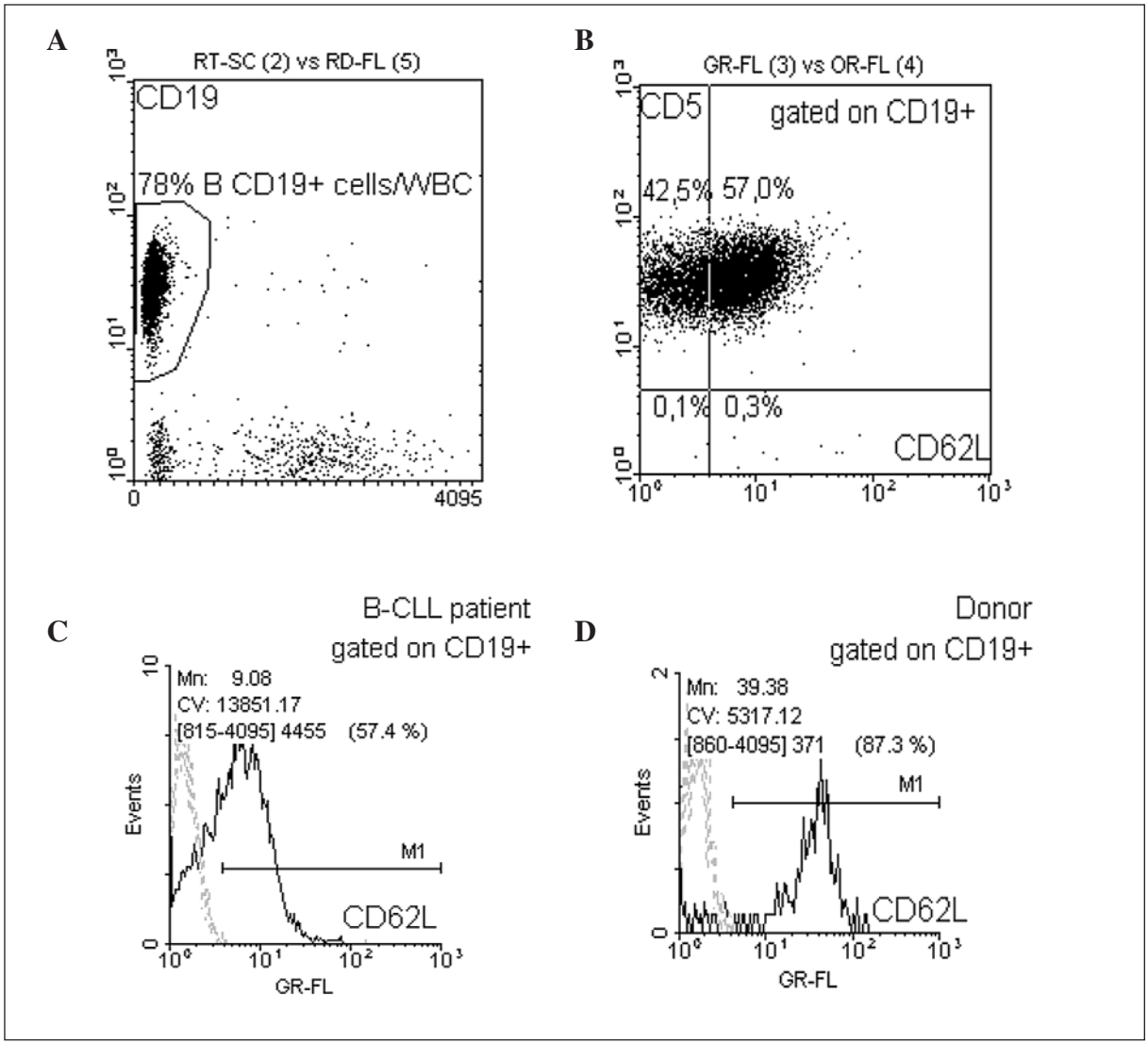

Fig. 1. An example of tricolor cytometric analysis of B CD19+cells in peripheral blood in patient suffering from B-CLL (cytograms A and B). Cells were labeled with antibody anti-CD19 RPE-Cy5 in a combination with FITC-anti-CD62L and with RPE-anti-CD5. CD19+ cells were gated in side scatter vs fluorescence RPE-Cy5 (A). Among CD19+ four populations of cells are visible (B). The histograms show examples of CD62L expression on B CD19+ cells in a patient suffering from B-CLL (C) and healthy donor's blood (D). The gray line – the negative control. The black line – cells after staining with anti-CD62L antibody

◗ The analysis of CD62L expression intensity on B CD19+ cells

The preliminary comparison of CD62 L-selectin expression intensity in BT and AT-PR groups of patients (n=10 in each group) revealed no significant differences between B CD19+CD5-positive (leukemic) cell and B CD19+CD5-negative cell subpopulations ($p>0.05$). In the BT group the value of CD62L RFI was 13.7±2.3 vs 15.3±2.1; in the AT-PR group – 15.3±1.9 vs 16.2±2.5. Thus, in the next analysis of CD62L expression intensity in these groups was done using entire B CD19+ cell population.

L-selectin expression intensity on B CD62L+ cells in B-CLL was considerably lower than L-selectin expression intensity on normal control B-lymphocytes in peripheral blood ($p<0.0001$, Fig. 3). It was noticed that in the majority of patients from BT and AT-PR groups (respectively: 65% and 76%) a subpopulation of B-lymphocytes exerted values of RFI CD62L below the minimal value of L-selectin expression intensity on B CD19+CD5+ lymphocytes in healthy donors (RFI=15.4).

In the AT-CR group the mean value of expression intensity of CD62L on B CD19+ lymphocytes (RFI=15.8±1.7) was closer to control values (RFI=17.6±0.8) than in untreated

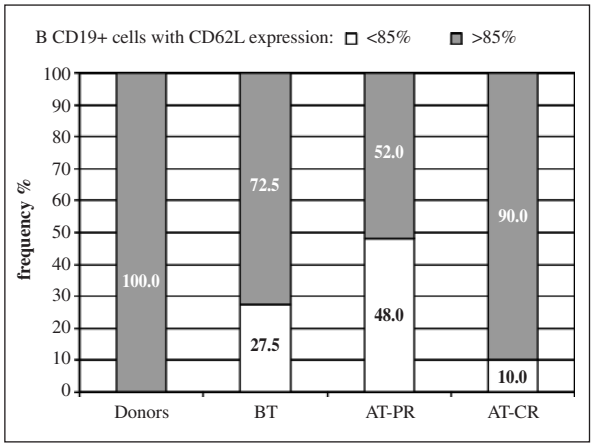

Fig. 2. The frequency of cases with a proper (>85%) and a diminished (<85%) percentage of B CD19+ with CD62L+ expression in peripheral blood of patients suffering from B CLL before treatment (BT), with a partial response (AT-PR) and a complete response (AT-CR) to the treatment

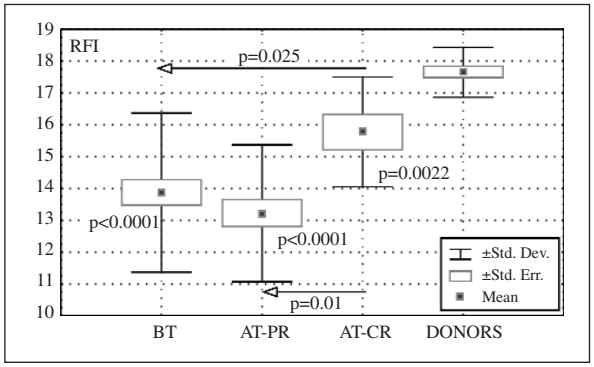

Fig. 3. RFI of CD62L on B CD19+ cells in peripheral blood of patients suffering from B CLL before treatment (BT), with a partial response (AT-PR) and a complete response (AT-CR) to the treatment.

p – a p value (Mann Whitney U Test), a statistically significant difference compared to the control group and between patients groups (arrows)

patients or those with a partial response to the treatment (Fig. 3). Because in some patients (7/10) from the AT-CR group the percentage of B CD5+ cells was lower than in other groups and became normalized, the analysis of CD62L expression intensity was carried out on separately gated B CD5-positive and B CD5-negative lymphocytes. In the healthy donors' peripheral blood RFI CD62L index was 16,2±0,7 for B CD5+ and 17.5±0.6 for B CD5-, and the difference was statistically significant (p=0.0013). In the AT-CR group the mean value of RFI CD62L on B CD5-positive cells was 14.9±2.4, on B CD5-negative cells (RFI =16.5±1.4, and did not differ considerably from control values (respectively: p=0.2, p=0.09).

Discussion

The decrease in the percentage of B-cells with L-selectin expression in B-CLL has been demonstrated in literature [2-4], as well as in our patients. The observations carried out in this study indicate that these changes of L-selectin expression depend on many factors, including response to treatment.

Our key observation is that the decreasing of percentage of B-cells with L-selectin expression is accompanied with the lower CD62L intensity expression on the B-cells, that preserved this expression. Therefore, in patients with B-CLL, B CD62L+ cells possess less L-selectin than the control B-lymphocytes from healthy donors.

The reduction of L-selectin expression intensity in B-cells in CLL may be related to impaired glycosylation of this molecule, as described in literature [12]. However, it is most likely that alterations of expression and expression intensity of L-selectin in B-cells in B-CLL is linked with lower activity of genes responsible for CD62L-selectin expression [8].

Beside a lower susceptibility to apoptosis, the diminished percentage of B CD62L+ cells in B-CLL, may influence the accumulation of cancer B-cells in peripheral blood [13]. This notion is based on the impaired adhesion and migration of these cells through an endothelium related to a lack of L-selectin expression [5, 14].

Described in this study declined L-selectin expression intensity on B CD62L+ cells, may also contribute to the process of accumulation of leukemic B-lymphocytes in peripheral blood of patients suffering from chronic lymphocytic leukemia, untreated or with a partial response to the treatment.

The AT-CR group of patients was treated with 2CdA. The introduction of new purin analogues in the treatment of proliferation of low malignancy B-cells gives wider possibilities to achieve a complete clinical and hematological response, that is noticed in 29%-47% of patients suffering from B-CLL [15, 16].

The analysis of L-selectin expression and L-selectin expression intensity on B-cells in the AT-CR group of patients shows that both: the amount of B-cells with L-selectin expression and the CD62L expression intensity exert tendency to normalize. It is possible that a longer period of time is needed to normalize the selectin expression intensity.

To summarize: the decreased percentage of B-cells with L-selectin expression in B-CLL is associated with a significant decline of the expression intensity in B CD62L+ cells compared to control. Interestingly, the drop in the expression intensity in these cells is mostly pronounced in untreated patients or in patients with a partial response to the treatment. Both these changes could be related with an impaired adhesion and migration of B-cells in CLL, and as a consequence, their accumulation in peripheral blood. In the group of patients with a complete response to the treatment (CR), the percentage of B-cells

with L-selectin expression and CD62L expression intensity exert tendency to normalize.

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Assessment of IgA subclasses synthesis in children with selective and partial IgA deficiency

GRAŻYNA MAJKOWSKA-SKROBEK[1], DARIA AUGUSTYNIAK[1], ADAM JANKOWSKI[2]

[1]Immunology Unit, Department of General Microbiology, Institute of Genetics and Microbiology, University of Wroclaw, Poland;
[2]Department of Children's Immunology, Medical University of Wroclaw, Poland

Abstract

The aim of this study was to determine the failure degree of IgA_1 and IgA_2 synthesis in 21 children with selective IgA deficiency (IgAD) and in 8 partial IgA-deficient children (p-IgAD). Serum and secretory (salivary and fecal) levels of IgA subclasses were assessed by means of ELISA methods. Both IgA_1 and IgA_2 were detectable in all serum samples but only the concentration of IgA_2 increased with increasing age. The significantly lower values of serum IgA_1 and IgA_2 were shown in IgAD children as compared with p-IgAD children ($p < 0.0001$ for IgA_1 and $p < 0.005$ for IgA_2) as well as with the control group ($p < 0.0000001$). The level of serum IgA_1 was considerably decreased in p-IgAD group in comparison with the healthy controls ($p < 0.00005$), whereas no statistically significant differences in this parameter were detected between p-IgAD and IgAD groups. Moreover, the concentrations of IgA_2 were on the level of control group in 75% of sera from IgAD group and in almost 10% of sera from p-IgAD group. On the other hand, the observation of negative correlation between the percentage of the IgA_1 in the total serum IgA and the percentage of the IgA_2 in the total serum IgA in IgAD group may prove the compensative role of each other. Diminished IgA_1 synthesis indicated in part p-IgAD group, together with normal values of both S-IgA subclasses and serum IgA_2 may reflect the transient defect of IgA synthesis in such children followed by an increase and normalization of serum IgA level or a decrease and absence of immunoglobulin. Concluding, the systemic IgA_1 synthesis was more impaired than the IgA_2 production in the two tested groups of IgA-deficient children. An increase in serum IgA_2 concentration to the normal range may suggest that either among these children there are cases with selectively impaired synthesis of only one subclass or that there are factors, which independently regulate the synthesis of serum IgA_1 and IgA_2.

Key words: *selective IgA deficiency, partial IgA deficiency, IgA subclasses, serum, saliva, feces, ELISA.*

(Centr Eur J Immunol 2003; 28 (3): 110–118)

Introduction

The human IgA exists in two isotypic forms: IgA_1 and IgA_2, which differ both in their primary amino acid sequences and carbohydrate structures [1, 2]. IgA antibodies are found both in the blood and in the mucosal secretions. The IgA in human serum is primarily monomeric, with 90–95% being of the IgA_1 subclass. Most of the IgA in secretions is polymeric and the concentrations of IgA_2 is increased relative to serum. Serum IgA is derived primarily from the bone marrow, while IgA destined for external secretions is synthesized locally in mucosa-associated tissues and glands. The distribution of the two IgA subclasses in secretions is dependent on the mucosal site: IgA_1-secreting cells predominate in the respiratory tract, in

Correspondence: Grażyna Majkowska-Skrobek, PhD, Institute of Genetics and Microbiology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland. Phone: +48 71 375 62 96, fax: +48 71 325 21 51, e-mail: majkosia@microb.uni.wroc.pl

the upper gastrointestinal tract and in mammary glands (60–93%), whereas IgA_2-secreting cells predominate in the lower gastrointestinal and in the female reproductive tracts [3, 4, 5]. Interestingly, in humans predominantly monomeric IgA from the systemic pool contributes little to external secretions, and *vice-versa,* locally produced polymeric IgA is selectively transported into external secretions, and only small amounts of this IgA enter the circulation [6].

An inability to produce antibodies of the IgA subclasses is the most frequently recognized form of primary immunodeficiency, having an incidence of approximately 1 in 600 individuals of European ancestry [7, 8]. IgA deficiency (IgAD) has been associated with an increased frequency of sinopulmonary infections, gastrointestinal disorders, autoimmune diseases and allergies [9, 10]. However, approximately two-thirds of IgAD individuals remain healthy - apparently even when living under poor hygienic conditions [11]. The reason for this difference in susceptibility to infections, as well as to other diseases, is not well understood.

The aim of our study was to disclose possible differences between serum and secretory levels of the two IgA subclasses in children with selective IgA deficiency (IgAD) and with partial IgA deficiency (p-IgAD) and to determine the failure degree of these subclasses in both groups of patients.

Materials and methods

Patients and controls

Twenty-one children (10 females and 11 males, aged 4–17 years) with selective IgA deficiency (IgAD) and eight children (6 females and 2 males, aged 4–18 years) with partial IgA deficiency (p-IgAD) were included in the study. Selective IgA deficiency was defined according to criteria of WHO [7] such as: serum IgA level was less or equal to 0.05 g/l, associated with normal serum levels of both IgM and IgG. And then, partial IgA deficiency was diagnosed as a serum concentration of IgA more than 0.05 g/l, but less than two standard deviations below normal serum level of IgA, with IgM and IgG levels within the normal range. The IgAD and p-IgAD groups involved children who were suffering from at least 8 respiratory infections during last year including pneumonia and/or bronchitis. The control group (C) comprised 32 sex- and age-matched healthy volunteers (15 females and 17 males, aged 6-14 years) with no history of primary and secondary humoral immunodeficiency. The characteristic of studied children is shown in Table 1.

All children were apparently healthy at time of collection of samples. None of them have been receiving any antibiotics for at least 3 weeks before the sample collection. All subjects were Caucasians. The study was approved by the Bioethical Committee of the Medical University of Wroclaw.

Serum samples

Venous blood samples were drawn into vacuum blood collection tubes without additives. The samples were allowed to clot and centrifuged at 450 x g for 10 min, and sera were immediately frozen and stored at -70^{0}C in multiple aliquots until analysis. Serum samples were frozen and thawed only once.

Saliva samples

Whole unstimulated saliva samples were collected by spitting directly into sterilized plastic tubes and placed in melting ice at once. The samples were stored at -70^{0}C until assayed following clarification at 9 000 x g for 10 min at 4^{0}C.

Fecal extracts

Approximately 1 g of fecal sample from one bowel movement was suspended in 7 ml PBS (BIOMED-Lublin). Subsequent mixing was done with a vortex mixer for 10 min, and the mixing process was repeated after 15 min, achieving a well-mixed suspension, even for some hard stool samples. The sample suspension was then centrifuged at 20 000 x g for 10 min at 4^{0}C. Supernatant was pipetted into a new tube and the mixing process was repeated. The sample suspension was then centrifuged again 12 000 x g for 10 min at 4^{0}C. The resulting clear supernatant was pipetted into Eppendorf reaction vessel. The vessel was stored at -70^{0}C until measurement.

Table 1. Profile of studied children

	IgAD	p-IgAD	Control
No.	21	8	32
Age [years]	8.97 ± 4.1	8.37 ± 5.25	9.87 ± 2.53
Sex [M : F]	11 : 10	2 : 6	17 : 15
IgM [g/l]	0.794 ± 0.66	0.721 ± 0.66	0.954 ± 0.48
IgG [g/l]	15.66 ± 3.33	13 ± 5.72	17 ± 5.15

Age and concentrations of both IgG and IgM were expressed as geometric mean ± standard deviation

Purification of S-IgA from colostrum

The purification of S-IgA from colostrum was established using a modified procedure described by Gregory et al. [12]. Briefly, human colostrum obtained from several healthy women 48 hours *post partum* was diluted (1:1) with 0.15 M NaCl and clarified by ultracentrifugation at 100 000 x g for 60 min at 4^{0}C. Casein was precipitated at pH 4.5 by adding cold 2 M CH_3COOH and then removed by ultracentrifugation at 100 000 x g for 60 min at 4^{0}C. The pH was adjusted to 7.0 with 0.5 M NaOH and the sample dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and passed twice through a heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column (1.5 x 5 cm) to remove lactoferrin and lysozyme. The column was washed with sodium phosphate buffer (pH 7.4) and the void volume collected and dialyzed against sodium phosphate buffer (pH 7.4) overnight. The heparin-Sepharose column was rejuvenated by washing with 1.65 M NaCl. The sample was then passed twice through an anti-human γ chain (DAKO) conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) column (1.5 x 5 cm). The sodium phosphate buffer was used to wash the column and the void volume collected and dialyzed against sodium phosphate buffer (pH 7.4) overnight. Next, the sample was passed twice through an anti-human μ chain (DAKO) conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) column (1.5 x 5 cm). After washing the column, the void volume was collected and dialyzed against sodium phosphate buffer (pH 7.4) overnight and concentrated by liofilization. The presence of protein in isolated fractions was examined using Bradford method [13].

Separation of S-IgA$_1$ and S-IgA$_2$ from purified S-IgA

The method of separation of S-IgA$_1$ and S-IgA$_2$ from colostral S-IgA has been described in detail [12]. Briefly, purified S-IgA from colostrum was passed through a jacalin-agarose (Vector) column (1.5 x 5 cm) in a 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 0.1 mM $CaCl_2$, 20 mM galactose and 0.08% NaN_3. The column was washed with the buffer and S-IgA$_2$ was collected in the void volume. S-IgA$_1$ was eluted with the same buffer containing 0.8 M galactose. Isolated fractions were analysed using the Laemmli system of SDS polyacrylamide gel electrophoresis [14] and assessed for purity and thus for suitability as S-IgA$_1$ and S-IgA$_2$ standards by means of Western blotting. The concentration of S-IgA was established using ELISA method.

ELISA for measurement of serum IgA$_1$ and IgA$_2$

The concentrations of IgA subclasses in the sera were measured by ELISA. Briefly, 96-well microtitre plates (MaxiSorp, NUNC) were coated with either jacalin (Sigma) for IgA$_1$ or monoclonal anti-human IgA$_2$ antibodies (CHEMICON or Southern Biotechnology) for IgA$_2$ in appropriate dilution and kept for 2 h at 37^{0}C and overnight at 4^{0}C. After blocking the wells with Tris-HCl containing 0.05% Tween for IgA$_1$ or PBS containing 3% BSA (Sigma) for IgA$_2$, serially diluted test samples or standards were added to each well and cultured for 2 h at 37^{0}C. Purified human IgA$_1$ and IgA$_2$ standards (myeloma serum proteins) were purchased from CHEMICON. After washing, secondary antibodies were added and incubated for 2 h at 37^{0}C. For secondary antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA (DAKO) was used. After washing, citrate buffer (pH 5.0) containing *o*-phenylenediamine substrate (Sigma) was added to each well and reacted for 15 min at room temperature. The optical density was determined with an automated ELISA plate reader (Dynatech 500). Standard curves of absorbance at 490 nm vs concentration of IgA$_1$ or IgA$_2$ were prepared and were used to determine the IgA$_1$ or IgA$_2$ concentration of the serum test samples, allowing for the fact that they had been diluted.

ELISA for measurement of S-IgA and S-IgA$_1$

Immunoglobulins levels in saliva and fecal extracts were determined by ELISA methods, which were similar to the procedures measurement of serum IgA$_1$ or IgA$_2$ detailed above. Briefly, for measurement of S-IgA, 96-well microtitre plates were coated with rabbit anti-human SC antibodies (DAKO) and developed with (HRP)-conjugated rabbit anti-human IgA (DAKO). S-IgA$_1$ were measured by ELISA with monoclonal anti-human IgA$_1$ antibodies (ICN) - coated assay plates and developed with HRP-conjugated goat anti-rabbit Ig. Rabbit anti-human SC antibodies were utilized as the secondary antibody. The concentrations of S-IgA and S-IgA$_1$ were calculated using the linear ranges of the dilution colostral S-IgA (Sigma) and prepared in-house S-IgA$_1$ as standards, respectively.

Statistical analysis

The Mann-Whitney U test was used to compare IgA subclasses levels between the study groups. Coefficients of correlation (r) were calculated by the Spearman rank test. The p values less than 0.05 were considered as statistically significant.

Results

Standardization

Utilizing the commercially available IgA$_1$ and IgA$_2$ myeloma serum proteins, standard curves were generated for each subclass (Fig. 1). Graphs of protein concentration versus absorbance at 490 nm produced sigmoidal curves in the region between 0.0156 μg/ml and 2 μg/ml for IgA$_1$, and 0.0156 μg/ml and 16 μg/ml for IgA$_2$. These curves displayed linearity between 0.625 μg/ml and 0.5 μg/ml for IgA$_1$, and between 0.625 μg/ml and 1 μg/ml for IgA$_2$. Coefficients of variation values ranged from 0.52% to 20.65% for both standard curves.

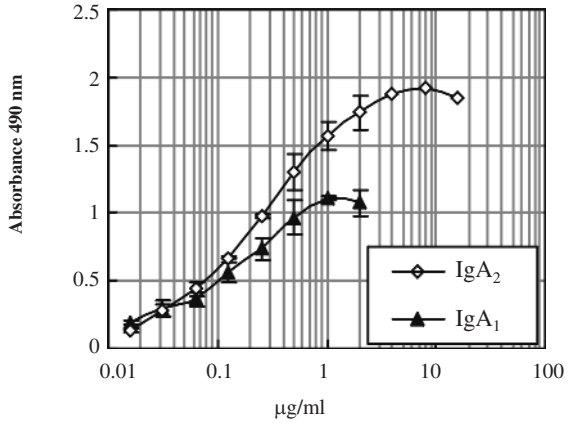

Fig. 1. Serum IgA_1 and IgA_2 standard curves. Concentration of myeloma IgA subclasses as standard versus absorbance at 490 nm. Each point represents a mean value ± standard deviation obtained from 6 consecutive assay runs, each with duplicate standards. The linear part of the curves was used for all measurement

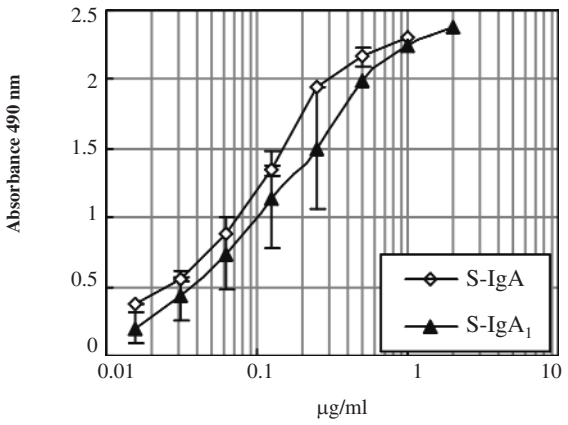

Fig. 2. Typical S-IgA and S-IgA_1 standard curves obtained by ELISA methods. Concentration of colostral S-IgA and S-IgA_1 as standards versus absorbance reading at 490 nm. Each point represents a mean value ± standard deviation obtained from 6 consecutive assay runs, each with duplicate standards. The linear part of the curves was used for all measurement

Fig. 2. illustrates the typical standard curves for S-IgA and S-IgA_1, using colostral S-IgA and prepared in-house S-IgA_1 as the standards. Both standards curves plotted as protein concentration versus absorbance reading at 490 nm were sigmoidal in the region between 0.0156 µg/ml and 1 µg/ml. The curves was linear and steep in the range of 0.031 µg/ml to 0.25 µg/ml for S-IgA and in the range of 0.031 µg/ml to 0.5 µg/ml for S-IgA_1. The S-IgA_2 concentration was calculated as the difference between S-IgA and S-IgA_1.

Serum IgA subclasses levels

The levels of IgA subclasses were determined by ELISA assays in the serum samples obtained from 21 IgAD children, 8 p-IgAD children and 32 healthy controls, with an age from 4 to 18 years. The results of serum levels of IgA_1 and IgA_2 in all studied groups of children are presented as scatter graphs in Fig. 3A and Fig. 3B, respectively. The mean concentration of serum IgA_1 (0.0085±0.012 g/l for IgAD; 0.16±0.1 g/l for p-IgAD) was decreased above 167-fold in IgAD group and around 9-fold in p-IgAD group comparing to the healthy control (1.41±0.47 g/l), (Fig. 3A). On the other hand, the mean concentration of serum IgA_2 was 17.6-fold lower in IgA-deficient patients (0.0026±0.0043 g/l) and 1.67-fold lower in p-IgAD children (0.027±0.026 g/l) in comparison with the control group (0.046±0.47 g/l), (Fig. 3B). Interestingly, the IgA_2 level was within the normal range in 2 of the 21 IgA-deficient patients and in 6 of the 8 children with partial IgA deficiency (Fig. 3B). The normal range was defined as the mean plus two standard deviations and included about 95% observations on a normalized frequency distribution. As expected, when serum levels of IgA_1 were determined, differences between healthy controls and both groups of patients were seen ($p < 0.0000001$ for IgAD; $p < 0.00005$ for p-IgAD).

Although there was a great deal of variability in the levels of IgA subclasses in all individuals participating in the study, the concentration of IgA_2 increased with increasing age ($r=0.3$; $p < 0.05$). Furthermore, the mean concentration of serum IgA_1 was slightly higher in healthy girls (1.63±0.52 g/l) than in healthy boys (1.22±0.33 g/l) and the difference was statistically significant ($p < 0.005$). By comparison, no significant dependence was found between the serum levels of IgA subclasses and sex in both group of patients.

The percentage of IgA_2 in the total IgA in serum was negative correlated with the percentage of IgA_1 in the total IgA in the group with selective IgA deficiency ($r=-0.92$; $p < 0.0000001$). In his group, the percentage of serum IgA_2 in the total IgA ranged from 0.94% to 62%. As shown in Fig. 4B, IgA_2 constituted more than 50% of the IgA in 3 patients and less than 10% of the IgA in 5 patients. The percentage of serum IgA_2 in the total IgA less than 10% stated also in 4 patients with p-IgAD.

Secretory S-IgA subclasses levels

The concentrations of secretory S-IgA subclasses were measured in saliva samples obtained from 15 IgAD children, 5 p-IgAD children and 23 healthy controls, whereas fecal S-IgA subclasses were detectable in 17 IgAD children, 5 p-IgAD children and 19 healthy controls. Both

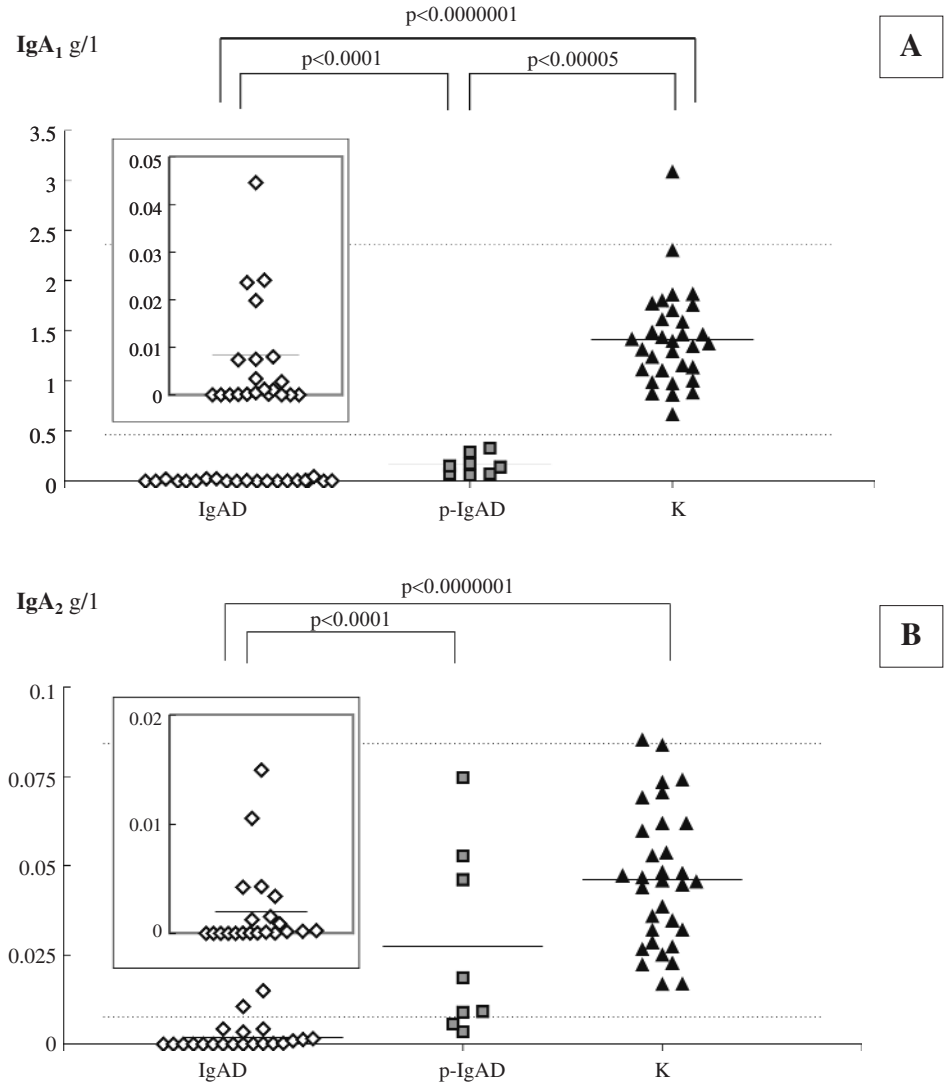

Fig. 3. Serum IgA_1 (A) and IgA_2 (B) levels (g/l) in IgAD (n=21) and p-IgAD (n=8) children as compared with healthy controls (n=32). The concentrations of IgA_1 and IgA_2 were quantified by isotype-specific ELISA. Each point represents an individual. Solid lines and dashed lines indicate geometric mean values and 95% confidence intervals, respectively, determined for normal control. Statistics were performed with Mann-Whitney U test

$S\text{-}IgA_1$ and $S\text{-}IgA_2$ were detectable in 35% of secretions (saliva and feces) from IgAD group and in 80% of secretions from p-IgAD.

The mean level of salivary $S\text{-}IgA_1$ in children with selective IgA deficiency (0.87±2.09 g/l) was significantly decreased as compared with partial IgA-deficient children (54.27±46.51 g/l; $p < 0.05$) as well as with healthy controls (137±78.4 g/l; $p < 0.0005$). On the other hand, the mean concentration of salivary $S\text{-}IgA_2$ (21.12±51.6 g/l) in such children was about 2.5-fold lower than that of both p-IgAD children (53.9±61.83 g/l; difference not significant) and control group (50.1±22.64 g/l; $p < 0.05$). By comparison, the differences between the mean concentrations of fecal S-IgA subclasses in IgA-deficient patients and control group were statistically significant (24.15±28.14 mg/g *vs* 129.92±128.45 mg/g; $p < 0.05$ for $S\text{-}IgA_1$, 18.62±11.34 mg/g *vs* 147.07±267 mg/g; $p < 0.05$ for $S\text{-}IgA_2$). There were not observed any statistically considerable differences in both $S\text{-}IgA_1$ and $S\text{-}IgA_2$ levels between p-IgAD children and healthy individuals (154.82±174.04 mg/g *vs* 129.92±128.45 mg/g for $S\text{-}IgA_1$, 112.21±121.1 mg/g *vs* 147.07±267 mg/g for $S\text{-}IgA_2$).

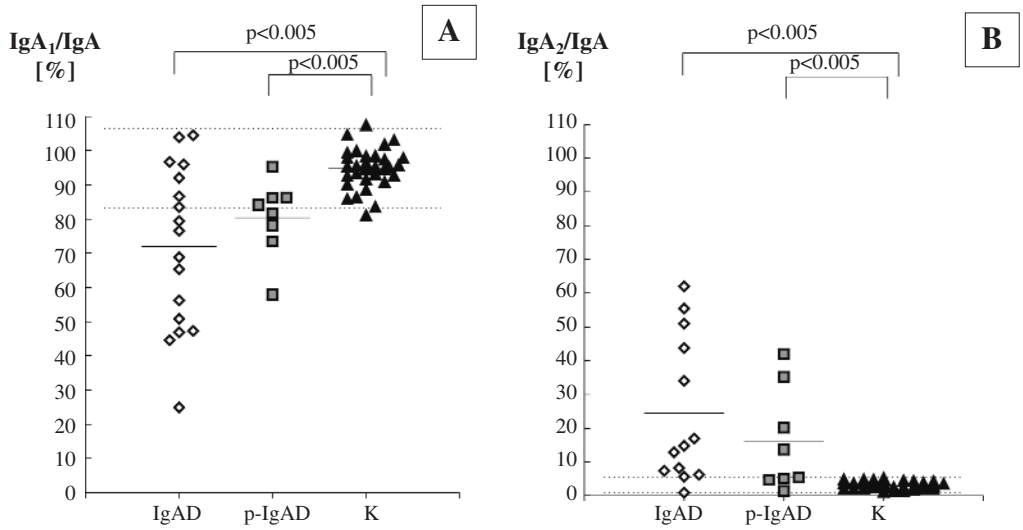

Fig. 4. Derived values for percentage serum IgA_1/total IgA (A) and serum IgA_2/total IgA (B) for selective (n=21) and partial (n=8) IgA-deficient children and healthy controls (n=32). Each point represents an individual. Solid lines and dashed lines indicate geometric mean values and 95% confidence intervals, respectively, determined for normal control. Statistics were performed with Mann-Whitney U test

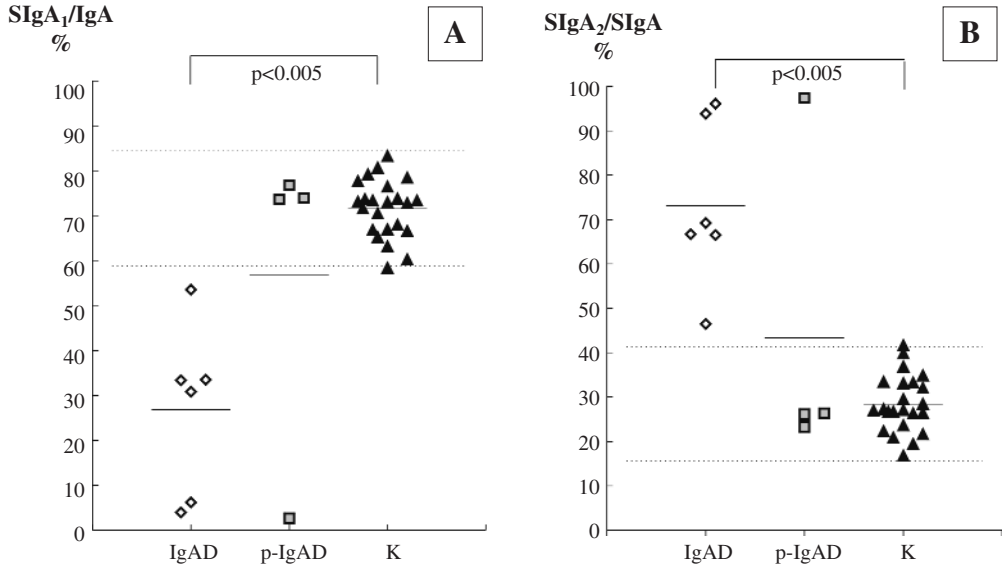

Fig. 5. Derived values for percentage salivary S-IgA_1/total S-IgA (A) and salivary S-IgA_2/total S-IgA (B) for selective (n=6) and partial (n=4) IgA-deficient children and healthy controls (n=23). Each point represents an individual. Solid lines and dashed lines indicate geometric mean values and 95% confidence intervals, respectively, determined for normal control. Statistics were performed with Mann-Whitney U test

Considering the mean percentage of the S-IgA_1 in the total salivary S-IgA and the mean percentage of the S-IgA_2 in the total salivary S-IgA in all studied groups, there were significant differences between IgAD children and healthy controls ($p < 0.005$), (Fig. 5A and 5B). In contrast to above results, the differences in the percentage values of S-IgA_1 or S-IgA_2 in feces, were not statistically significant in both IgAD and p-IgAD children comparing to the group of healthy controls (Fig. 6A and 6B). There were, also, no statistically significant differences in this parameter in saliva

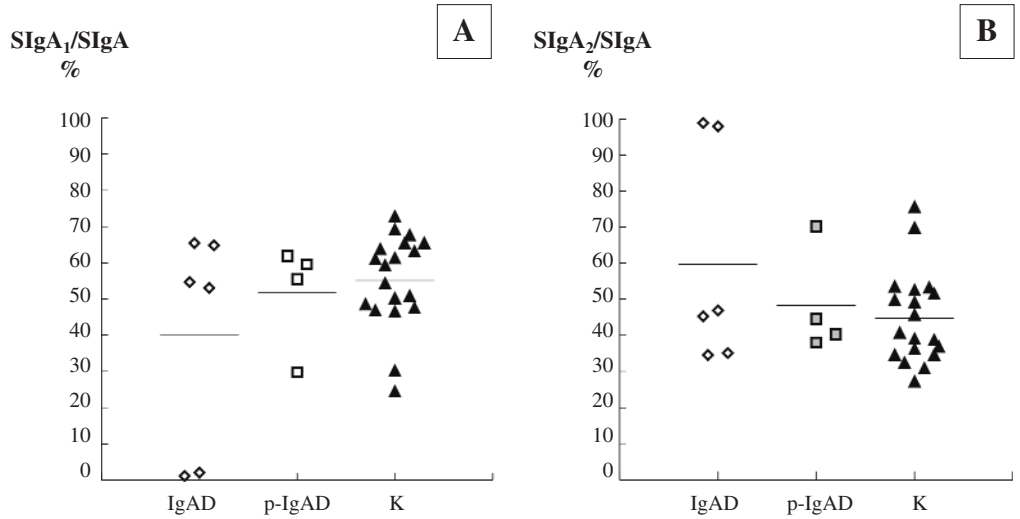

Fig. 6. Derived values for percentage fecal S-IgA$_1$/total S-IgA (A) and fecal S-IgA$_2$/total S-IgA (B) for selective (n=6) and partial (n=4) IgA-deficient children and healthy controls (n=19). Each point represents an individual. Bars indicate geometric mean of each group

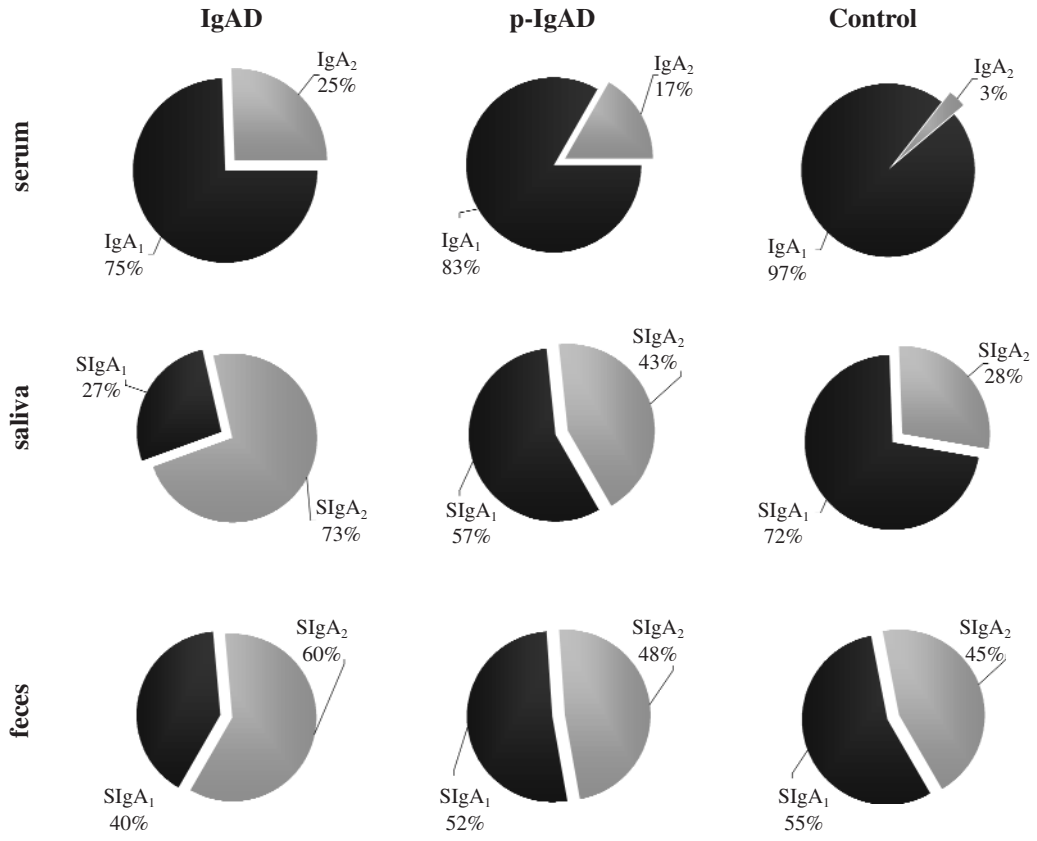

Fig. 7. Average percentage of IgA subclasses distribution in samples of sera, saliva and feces in selective and partial IgA-deficient patients and healthy controls

obtained from p-IgAD children and healthy children. As shown in Fig. 5A and 5B in all children from IgAD group and in one out four children with p-IgAD, the percentage either S-IgA_1 or S-IgA_2 in the total salivary S-IgA did not reach the levels of the healthy controls.

Fig. 8. illustrates comparison of the distribution of the two IgA subclasses both in sera and secretions in all studied groups. It was noteworthy that the quantities of S-IgA_2 exceeded the quantities of S-IgA_1 both in saliva and feces as well as in sera of IgA-deficient children comparing to healthy controls.

Discussion

The main finding of our study was demonstration that systemic IgA_1 synthesis was more impaired than systemic IgA_2 production in the two tested groups of IgA-deficient children. Our study revealed that the level of serum IgA_1 was significantly decreased in children with selective and partial IgA deficiency in comparison to the control group (Fig. 3A). On the contrary, the level of IgA_2 was significantly decreased only in IgAD group. Moreover, we demonstrated that the concentration of IgA_2 was on the level of control group in almost 10% of sera from IgAD group and in 75% of sera from p-IgAD (Fig. 3B). The reason for the differences in IgA subclass distribution found in this study is unclear. The IgA subclass shift found in this study may indicate that among these children there are cases with selectively impaired synthesis of only one subclass (defect of IgA_1, normal IgA_2). So far, such cases were present in closely related populations and as a general rule concerned the simultaneous deletion of heavy constant region genes different from $C\alpha1$ and $C\alpha2$ [15, 16, 17]. However, further studies are needed to confirm the presence of similar deletion in our patients. An increase in serum IgA_2 levels to the normal range may also suggest that there are factors, which independently regulate the production of serum IgA_1 and IgA_2. Molecules regulating the expression of immunoglobulins would thus be expected to be involved in the pathogenesis of IgA deficiency. Interleukins are obvious examples of that molecules. However, it has recently been shown that the production of IgA_1 by B cells from apparently healthy IgA-deficient patients may be efficiently up-regulated by IL-10 and IL-4 *in vitro*, whereas a corresponding up-regulation is not achieved in infection-prone IgAD subjects [18, 19].

Another important factor determining the different synthesis of various immunoglobulin class is fact that genes encoding a constant region of immunoglobulin are poliallelic. The reports by Litwin and Balaban [20] and Giessen et al [21] provided the suggestion that the total proportion of serum IgG that is IgG_2 or IgG_3 is related to the allotype of that IgG subclass. Except of γ chain, differentiation of allotypic variants referred to ε and $\alpha2$ chain, as well. Among three IgA_2 allotypes [A2m(1), Am2(2), A2(n)] over 90% of Caucasians have the allotype A2m(1) [2, 22]. Our studied groups included only Caucasians, therefore it is unlikely that the variation in the ratio of IgA_1 to IgA_2 seen in them can be attributed to allotype differences.

The differences in IgA subclass distribution found in this study could theoretically be the result of the slight differences in sex and age distribution in our study populations. Berth et al. [23] have recently shown that serum concentrations of both IgA_1 and IgA_2 were significantly higher in healthy men than in healthy women. In contrast to previous report, our present study revealed that the level of serum IgA_1 was significantly higher in girls than in boys from the control group. The discrepancies between our results and those of others can be explained by the sex and have no effects on synthesis of IgA subclass, although, as well known, the gender of parents determined transmitting the defect: affected mothers are more likely to pass the defect on to their offspring than affected fathers [24]. Furthermore, this dependence may reflect the stronger B cell responses in females than in males [25].

The results of previous study, concerning the development of IgA during ontogenesis of human being, showed that the levels of IgA subclasses reach adult concentration in secretions - faster than in serum [26, 27]. Significantly increased the proportion of IgA_2 in the total serum IgA both in IgAD and p-IgAD children comparing to the control group may suggest either more effective development of synthesis of intravascular IgA_2 in such children or that the secretory surfaces do contribute significantly to serum IgA_2 (Fig. 5B). According to Andre [28] if IgA production at mucosal surfaces with a relatively higher levels of IgA_2 contributed significantly to serum pool of IgA, one might expect that in infant a higher proportion of the serum IgA_2 would be derived from the secretory system. The results presented in this paper demonstrate a positive correlation between serum IgA_2 and the age of all children. It probably excludes the involvement of secretory IgA_2 in serum and confirms that serum and secretory pools of IgA derived from relatively independent sources. Similar association indicated both Conley et al. [29] who studied IgAD children and Berth et al. [23] who established reference values for serum concentration of the two IgA subclasses in Caucasians adults.

Observation of a negative correlation between the proportion of total serum IgA that was IgA_1 and the proportion of total serum IgA that was IgA_2 may suggest the compensative role of each other. This finding was accordant with previous study encompassing IgA subclass-deficient patients with deleted constant region genes [15]. The authors showed that the lack of IgA_1 can be partially compensated by IgA_2 antibodies, whereas the lack of IgA_2 can be fully compensated by IgA_1.

We also noted that the concentrations of both S-IgA_1 and S-IgA_2 in fecal extracts and S-IgA_2 in saliva were significantly decreased in IgAD children as compared with

the control group, whereas the salivary level of S-IgA_1 in such children was considerably lower in comparison to p-IgAD children as well as to control individuals. On the other hand, the normal values of both salivary and fecal S-IgA subclasses together with the diminished systemic IgA_1 synthesis observed in part children with partial IgA deficiency, may reflect the transient defect in such children followed by an increase and normalization of serum IgA level or a decrease and absence of the immunoglobulin. We can not also exclude that reduced IgA_1 symthesis in such cases is an effect of redundant or insufficient antigen esposure.

Summing up, the normal IgA_2 synthesis in mucosal system and "weaker failure" of its systemic synthesis in few children (in comparison to IgA_1 production) on one hand, and a serious impairment of both local and systemic IgA synthesis in the other ones, confirm the heterogeneity of IgA deficiency. The obtained results of the research can constitute a contribution to the knowledge of pathogenesis of IgA deficiency, requiring certainly their further widening by elements of the immunoregulatory mechanisms.

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The immunopotentiating effect of Listeriolysin O in the response against *Leishmania major* in BALB/c mice

KHALED S. TABBARA[1] and JAWAHER A. AL-SALEM[2]

[1]Department of Microbiology Immunology and Infectious Diseases, College of Medicine and Medical Sciences, and [2]Biotechnology Program, College of Higher Studies, Arabian Gulf University, Manama, Kingdom of Bahrain

Abstract

The development of a Leishmania major protective Th1 response is dependent on IL-12 production. Susceptible BALB/c mice develop a Th2 response. We have previously demonstrated that coinfection with Listeria monocytogenes resulting in type 1 cytokine production (IL-12 and IFN-γ), potentiated the response of BALB/c mice to L. major. We investigated here whether Listeriolysin O (LLO), essential for the development of a protective anti-listerial response, was also required to immunopotentiate the response against L. major. Coinfection with L. major and an LLO-producing (LLO^+), but not an LLO-nonproducing (LLO^-) L. monocytogenes strain, reduced lesion size and parasite load, and increased IFN-γ production. Semi-purified LLO also potentiated the response as shown by the containment of parasites in the spleens in well-defined focal granulomas in contrast to controls where more mature disorganized diffuse granulomas with higher parasite loads and necrosis were commonly seen.

Key words: *Leishmania, Listeria, coinfection, Listeriolysin O, IFN-γ, Th1 cells*

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Introduction

Listeria monocytogenes is a Gram-positive, facultatively intracellular bacterium, which invades and parasitizes macrophages, as well as a wide range of other cells, including mesenchymal and epithelial cells. Once internalized, *L. monocytogenes* escapes the phagosome, and multiplies in the cytosol of the parasitized cell. Escape from the phagosome is largely mediated by Listeriolysin O (LLO), a 58 Kd thiol-activated pore-forming cytolysin, which has been shown to be a major virulence factor in this organism. Immunization with LLO-producing, but not nonproducing strains induces protective T cell mediated immunity [1, 2]. The innate response to LLO-producing *L. monocytogenes* is associated with the production of several cytokines, including the type 1 cytokines IL-12 and IFN-γ [3–5], both of which are required for protective immunity [6, 7]. LLO itself appears to be a participant in this stimulatory activity, as shown by upregulation of expression mRNA of these cytokines following *in vitro* stimulation of spleen cells with purified LLO [8].

The protective immune response against *L. major* is dependent on the expansion of a population of IFN-γ--producing T lymphocytes, and the induction of Nitric Oxide (NO) – dependent killing within parasitized macrophages [9]. The cytokine milieu during the primary expansion of T lymphocytes is one of the major factors influencing the direction of Th cell polarization. Interleukin-12, a pro-inflammatory cytokine primarily produced by activated macrophages [10] and dendritic cells [11] has been shown to be central for the development of the protective Th1 response, while IL-4 is the major cytokine driving Th2 predominance and susceptibility [9].

The BALB/c mouse is highly susceptible to *L. major*, developing progressive non-healing lesions at the site of

Correspondence: Khaled S. Tabbara, Department of Microbiology, Immunology and Infectious Diseases, College of Medicine and Medical Sciences, Arabian Gulf University, P.O. Box 22979, Manama, Kingdom of Bahrain. Phone: + (973) 239-733, fax: + (973) 230-730, e-mail tabara@agu.edu.bh

infection, followed by visceralization and death. The inability of this mouse strain to resist *L. major* is associated with its failure to develop the appropriate Th1 response. Events in the early stages of infection, including an early burst of IL-4 production resulting in the down-regulation of expression of the β2 subunit of the IL-12 receptor [12], and a breakdown of IL-12 production [13], appear to contribute to the predominance of a Th2 response. Manipulations resulting in increased or sustained IL-12 production, particularly during primary encounter with the parasite or its antigens, reverse this response leading to Th1 predominance and protection [14–16].

Given the predominance of type 1 cytokines (IL-12 and IFN-γ) produced during the early immune response to *L. monocytogenes*, we have previously investigated the immunomodulatory effect of coinjection of an LLO-producing *L. monocytogenes* on the development of the immune response of BALB/c mice to *L. major*. In these studies (submitted for publication) [17], we showed the early polarization of the response against *L. major* towards a protective IFN-γ-dominated response resulting in a significant delay in lesion development, extended survival, control of parasite multiplication and dissemination. This response was associated with the upregulation of IFN-γ production and the downregulation of IL-4 protein and message expression in draining LN cells from coinjected mice. In this paper, and as a follow up to our earlier findings, we investigated whether the immunopotentiating role of *L. monocytogenes* in this model is related to LLO production.

Material and Methods

Mice

Female BALB/c mice raised and maintained at the animal facility of the Arabian Gulf University, from a stock originally obtained from Olac (U.K.), were used at 8–12 weeks of age. Mice were age-matched for each experiment.

Parasite preparation and footpad injections

A *Leishmania major* isolate (MOHM/SD/87ELD, isoenzyme LON1), originally obtained in 1987 from a cutaneous ulcer of a 50-year-old Sudanese male patient [18] was used throughout this study. Parasites were cultured at 22°C in Schneider's medium (Sigma), supplemented with 10% heat inactivated fetal bovine serum (FBS) (ICN), 5mM HEPES, 50IU penicillin/ml (ICN), 50 μg streptomycin/ml (ICN), and 2% human urine (complete Schneider's Medium). Cultures were refreshed every 2–3 months by re-isolating from infected footpads of BALAB/c mice to maintain virulence.

Stationary phase, four-day-old *L. major* promastigote cultures were used for injections. Promastigotes were collected by centrifugation, washed twice with Phosphate Buffered Saline (PBS), adjusted to $2x10^7$/ml and 50 μl ($1x10^6$) was injected subcutaneously in the footpad using a 25-gauge needle. Footpad thickness was measured weekly using a Vernier Caliper. The contralateral footpad was measured as a control.

Listeria monocytogenes culture and footpad injections

Two *Listeria monocytogenes* strains were used: 1) an LLO-producing (LLO^+) clinical isolate obtained from the pathology department at Salmaniya Medical Complex (Bahrain); and 2) an LLO-nonproducing (LLO^-) *L. monocytogenes* strain (NCTC 10357). *L. monocytogenes* stocks were maintained by monthly subculture on Brain Heart Infusion (BHI) agar plates.

Cultures for injection were prepared by inoculating 150 ml BHI broth with 1ml of an overnight culture. Growth at 37°C, on a rotary shaker, was monitored by hourly spectrophotometric readings at 600 nm. In preliminary experiments, the growth curves of both *L. monocytogenes* strains were determined and viable counts correlated to optical density (OD) at 600 nm. At the appropriate OD an aliquot of the culture was centrifuged and the pelleted organisms washed in cold PBS and adjusted to the desired concentration for injection. For coinjection experiments, organisms were mixed such that a 50 μl volume would simultaneously deliver the desired numbers of both organisms. Appropriate dilutions from the bacterial cultures used for injections were plated on BHI agar to ascertain actual viable counts.

Estimation of Parasite Load

Mice were sacrificed by cervical dislocation at various intervals following infection. The footpads, spleen and draining popliteal lymph nodes were aseptically removed. Footpads were de-boned and homogenized in cold HBBS using a glass homogenizer, while spleens and lymph nodes were mashed between the frosted ends of two glass slides to obtain single cell suspensions. Tissue suspensions were serially diluted in complete Schneider's medium in flat bottom 96-well microtiter plates. Plates were placed in a humidified box and incubated at 26°C for 7 days. Positive wells (showing growth) were scored and the number of viable parasites, per organ, was calculated from the reciprocal of the highest positive dilution.

Stimulation of *in vitro* IFN-γ Production

Two million popliteal lymph node cells were stimulated *in vitro* with $1x10^6$ live *L. major* promastigotes from a 4-day-old culture. Cells were cultured in a volume of 2 ml of RPMI 1640 (ICN) supplemented with 10% FBS, 25 mM HEPES, 50 IU penicillin/ml, 50 μg streptomycin/ml, 2 mM Glutamine, and 5×10^{-5} M 2-mercaptoethanol, in 24-well tissue culture plates. Supernatants were collected following 72hrs of culture, filtered and stored at -80°C until assayed.

Determination of IFN-γ Concentration

Interferon-γ concentrations in culture supernatants were determined by Enzyme Linked Immunosorbent Assay (ELISA) using a commercial kit (Pharmingen-U.S.A) following manufacturer's procedure.

Purification of Listeriolysin O

Purification of Listeriolysin O was carried out following the procedure of Tsukada, et al. [19]. Briefly, 3 liters of BHI broth were inoculated with the LLO^+ strain of *L. monocytogenes* and incubated for 18–22 hrs at 37°C. The supernatant was collected by centrifugation and filtered using a 0.45 µm Millipore filter unit. Supernatant proteins were precipitated in the cold with 60% saturation of ammonium sulfate. The precipitate was solubilized in a volume of PBS equal to 15% of the original supernatant volume, dialyzed at 4°C overnight against two changes PBS and then against 25 mM Tris-buffer, pH 8.0. The dialysate was applied to a DEAE-Sephacel column (LKB) and eluted with a gradient of 0–0.5 M NaCl while maintaining the column at 4°C. 1ml fractions were collected on ice, assayed for hemolytic activity, hemolytic fractions pooled and concentrated 15–20 times in a Centricon centrifugal filter devices (10 kDa).

Sodium Dodecyl Sulfate – Polyacrylamide Gel electrophoresis (SDS-PAGE)

Culture supernatant proteins and semi purified hemolytic fraction concentrates were analyzed using vertical SDS-PAGE (Mini-protean II Electrophoresis Cell, Bio-Rad). Samples were boiled for 4 min in an equal volume of sample buffer consisting of 3.8 ml deionized water, 0.1 ml of 0.5 M Tris-HCl buffer (pH 6.8), 0.8 ml glycerol, 1.6 ml of 10% SDS, 0.4 ml of 2-mercaptoethanol and 0.4 ml of 1% (w/v) bromophenol blue. Treated samples were applied to a 12% polyacrylamide gel and electrophoresed at 200 V and 60 mA for 40 min. Protein bands were visualized by staining with Coomassie blue and silver stain. Molecular weight standards were run in parallel. Gels were dried at 50°C for 45 min, laminated and photographed.

Tissue Processing and Staining for Light Microscopy

Spleens specimen were fixed in 10% buffered formalin (pH 7.2), processed routinely for light microscopy and 5µm paraffin sections were stained with Hematoxylin and Eosin.

Results

Coinjection with an LLO-Producing but not an LLO-Nonproducing *L. monocytogenes* strain attenuates *L. major* lesion development in BALB/c mice

To evaluate the role of LLO in protection of BALB/c mice against *L. major*, mice were coinjected with $1x10^6$ *L. major* plus an LLO^+ or an LLO^- *L. monocytogenes* strain. A dose of $1x10^3$ organisms, which was previously shown to be protective, was used for the pathogenic LLO^+ strain. Since the LLO^- strain lacks virulence, and are thus less replicative, a larger dose of $1x10^6$ was used to simulate a similar antigenic dose, based on peak numbers attained by the virulent strain when $1x10^3$ organisms are injected (results not shown).

The evolution of lesions, as measured by the increase in footpad thickness was monitored weekly. As shown in Figure 1, control group mice injected with $1x10^6$ *L. major*, as well as groups coinjected with the LLO^+ or LLO^- strains developed measurable lesions beginning week 4 following injection. Lesions size increased steadily over the following weeks in all three groups. The mean lesion size of mice coinjected with the LLO^+ *L. monocytogenes* strain was smaller than those of the two other groups and the difference was statistically significant different ($p<0.05$) between weeks 4 and 8 following injection when comparing the group coinjected with the LLO^+ strain to the two other groups. There was no difference in lesion size between mice coinjected with the LLO^- strain and those injected *L. major* only.

Differences among the three groups are further illustrated by the pattern of lesions developing. As shown in Figure 2, both the control group and the group coinjected with the LLO^- strain developed open, ulcerating lesions 8 weeks following injection. In contrast lesions of the group coinjected with the LLO^+ strain were nonulcerative.

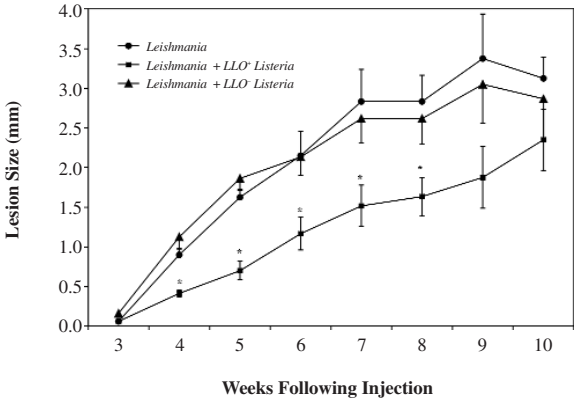

Fig. 1. Mean increases in footpad thickness (±SE) of BALB/c mice (10/group) injected subcutaneously in the footpad with $1x10^6$ *L. major* (●); $1x10^6$ *L. major* plus $1x10^3$ LLO^+ *L. monocytogenes* (■), or $1x10^6$ *L. major* plus $1x10^6$ LLO^- *L. monocytogenes* (▲). Asterisks indicate points of statistically significant differences ($p<0.05$) between the group injected with *L. major* plus LLO^+ *L. monocytogenes* and the groups injected with *L. major* alone and *L. major* plus LLO^- *L. monocytogenes*. Data shown are representative of three separate experiments

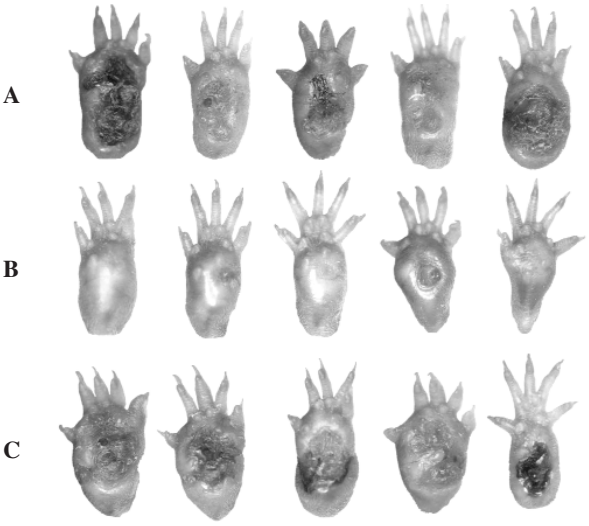

Fig. 2. Photographs of representative footpads of BALB/c mice (from a group of 8 mice) 8 weeks following subcutaneous footpad injection with $1x10^6$ *L. major* (A), $1x10^6$ *L. major* plus $1x10^3$ LLO^+ *L. monocytogenes* (B), or $1x10^6$ *L. major* plus $1x10^6$ LLO^- *L. monocytogenes* (C). Data shown are representative of two separate experiments

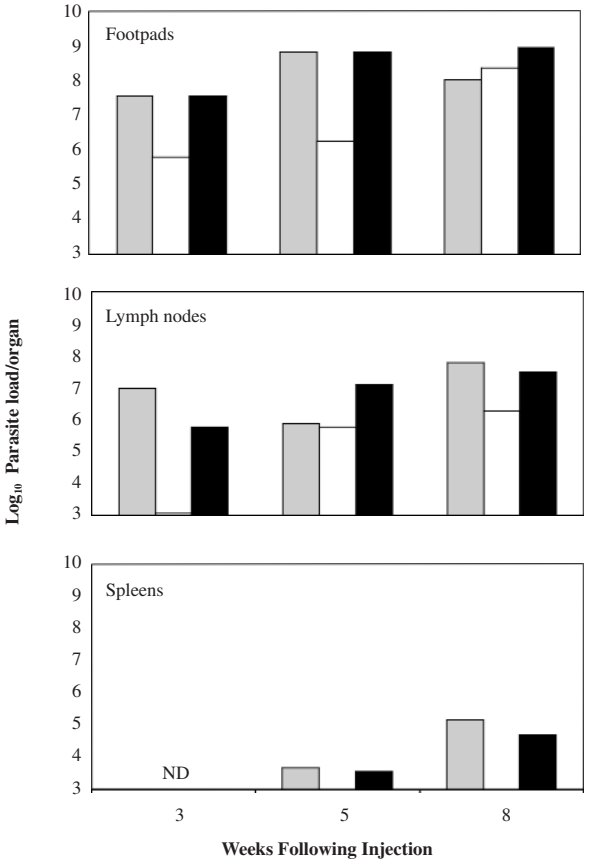

Fig. 3. Number of parasites per footpad, lymph node, and spleen of BALB/c mice injected subcutaneously in the footpad 3, 5 or 8 weeks previously with $1x10^6$ *L. major* (▒); $1x10^6$ *L. major* plus $1x10^3$ LLO^+ *L. monocytogenes* (□); or $1x10^6$ *L. major* plus $1x10^6$ LLO^- *L. monocytogenes* (■). Two mice were sacrificed at each time point. Data shown are representative of two separate experiments

Coinfection with the LLO-producing *L. monocytogenes* strain reduces the parasite load

In order to determine the effect of LLO production on *in vivo* parasite multiplication, footpads and popliteal lymph nodes were removed at 3, 5 and 8 weeks and spleens at 5 and 8 weeks following infection with *L. major* only, or together with LLO^+ or LLO^- *L. monocytogenes*. Two mice from each group were sacrificed at each time point. Footpads and spleens were assayed individually, while lymph nodes were pooled. The parasite burden of footpads from control mice and those coinjected with LLO^- *L. monocytogenes* followed a similar pattern. The averaged number of parasites per footpad of these two groups at the earliest point (3 weeks), when lesions were hardly measurable, was significantly increased ($4x10^7$) over the injection dose of $1x10^6$, further increased to about $9x10^8$ by week 5, then remained at a similar level by week 8. In comparison, the parasite load of footpads of mice coinjected with the LLO^+ *L. monocytogenes* strain was 60 fold lower at week 3, and 400 fold lower at week 5, but approached similar levels to the two other groups at week 8 (Figure 3).

Parasites numbers were lower in the lymph nodes than at the site of injection. At 3 weeks, the parasite loads of the control group and the group coinjected with the LLO^- strain were $8x10^6$ and $5x10^5$, respectively. In comparison, the group coinjected with the LLO^+ strain had a parasite load that was about 2.5 logs lower than the group injected with the LLO^- strain. However, by week 5, parasites increased in the LLO^+ coinjected group to a level similar to that of the control group, while the LLO^- coinjected group was about one log higher. At the peak of lesion development, at 8 weeks, the parasite loads of both control and LLO^- coinjected groups were further increased while the group coinjected with the LLO^+ strain remained essentially unchanged.

Differences in parasite loads of the three groups were most evident in the spleens. Based on earlier observations, which showed lack of visceralization before 4 weeks, the parasite load of spleens was only assayed at 5 and 8 weeks following injection. Parasites were detectable in spleens of the control and LLO^- coinjected mice 5 weeks following challenge and increased by about one log at 8 weeks. In contrast parasites were not detected at both points in spleens of mice coinjected with the LLO^+ strain ($<10^2$ per spleen).

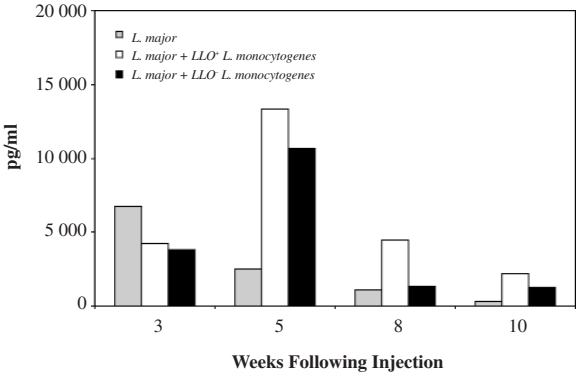

Fig. 4. Interferon-γ production by popliteal lymph node (LN) cells from BALAB/c mice injected subcutaneously in the footpad 3, 5, 8 or 10 weeks earlier with $1x10^6$ *L. major* (□), $1x10^6$ *L. major* plus $1x10^3$ LLO^+ *L. monocytogenes* (□), or $1x10^6$ *L. major* plus $1x10^6$ LLO^- *L. monocytogenes* (■). 2x106 LN cells were stimulated for 72hrs with $1x10^6$ live *L. major* promastigotes in a volume of 2 ml. Values shown are mean of antigen stimulated cultures minus values for medium control. Two mice were sacrificed at each time point. Data shown are representative of two separate experiments

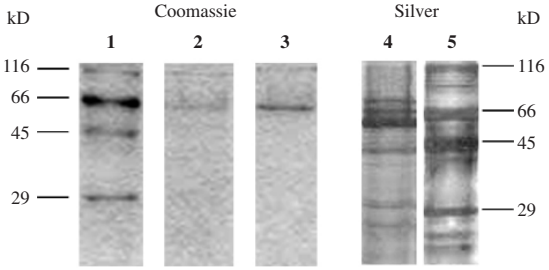

Fig. 5. SDS-PAGE gels of *L. monocytogenes* culture supernatant proteins and concentrated pooled hemolytic fractions. Lane 1 and 5 molecular weight markers, Lane 2, culture supernatant dialysate; lanes 3 and 4 concentrated DEAE-hemolytic fractions. Lanes 1-3 Coomassie blue stain, Lane 4 and 5 silver stain. Data shown are representative of three separate experiments

Lymph node cells from mice coinjected with the LLO-producing *L. monocytogenes* strain produce higher levels of Interferon-γ

The IFN-γ response of LN cells to *L. major* promastigotes was monitored following injection of *L. major* alone, or following coinjection with LLO^+ and LLO^- *L. monocytogenes* strains (Figure 4). Two mice were sacrificed at each time point, lymph nodes pooled and stimulated *in vitro*. Low levels of IFN-γ were detectable in all three groups as early at 3 weeks, peaked at 5 weeks, and then sharply decreased at the two following measurement points (weeks 8 and 10). The three groups of mice produced comparable levels of IFN-γ at 3 weeks. However, at 5 weeks, the group coinjected with LLO^+ strain produced the highest level (13,350 pg/ml) followed by mice coinjected with the LLO^- strain (10,700 pg/ml), while the control group produced significantly lower concentrations of IFN-γ (2,525 pg/ml). While all groups produced lower levels of IFN-γ at 8 and 10 weeks, mice coinjected with the LLO^+ strain were the dominant producers at both points (4,440 and 2,200 pg/ml Vs 1,075 and 320 for controls, and 1,330 and 1,275 for the group coinjected with LLO^- strain).

Effects of Coinjection of semi-purified Listeriolysin O

Listeriolysin O was semi-purified from *L. monocytogenes* culture supernatants to a single band on SDS gel as revealed by Coomassie blue staining (Figure 5). Silver staining, which detects lower concentrations of protein, however, revealed multiple bands. Coinjection of 80 μg of semi-purified LLO into footpads of mice together with $1x10^6$ *L. major* did not attenuate lesion development (data not shown). However, at peak lesion development, coinjected mice appeared less stressed than controls, which developed classical stress symptoms (ruffled fur, hunched back, sluggish movement). Nineteen weeks following injection, six mice were sacrificed from each of the two groups, and their spleens collected.

Spleens from LLO coinjected mice were significantly smaller than those of the control group (681±60 mg Vs 803±34 mg, $p<0.001$). Gross examination showed significant external granulomas on spleens from the control group, while only one of the six spleens from the coinjected group was grossly granulomatous. Histopathological changes in mice of the two groups (Figure 6) revealed significant differences. Spleens of mice injected with *L. major* alone developed extensive disorganized macrophage/monocyte granulomas with multinucleated giant cells, a very high parasite load, and occasional necrotic foci. In contrast, spleens from mice coinjected with LLO developed well-defined immature focal granulomas, in the red as well as the white pulp, with low parasite numbers and the absence of multinucleated giant cells and necrotic foci.

Discussion

The ability to produce LLO is unique to virulent *L. monocytogenes*, and only LLO-producing strains are capable of inducing the critical T cell-dependent response required for protective immunity against this pathogen. Although LLO is in itself an important target antigen for the stimulation of the CD4+ T cell response [20] its main role in the induction of the protective response appears to be as a bacterial modulin in the cytokine response rather than as

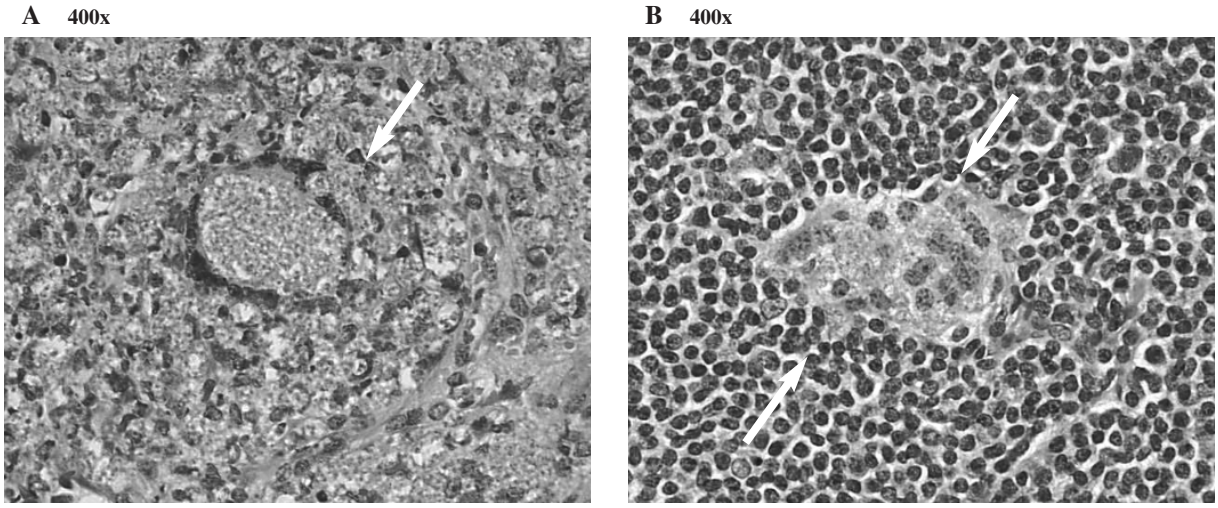

Fig. 6. Histopathologic changes in spleens from mice injected 19 weeks earlier with *L. major* or *L. major* plus 80 μg semi-purified LLO from culture supernatant proteins. Representative hematoxylin and eosin-stained sections are shown. Disruption of normal spleen structure is evident in the control group (*L. major*) with diffuse granuloma, high parasite load, and multinucleated giant cells (arrow) (A). Spleens of the experimental group injected with *L. major* plus semi purified LLO show better preservation of normal spleen architecture with well-defined focal granuloma (arrows) and a low parasite load (B). Original magnifications 400X. Sections shown are representative of six individual mice from each group

a target antigen [21]. Through its action on macrophages, LLO stimulates the production of IL-12, which in turn drives NK cells to produce the macrophage-activating cytokine IFN-γ [8]. Together those two cytokines, produced by the innate response, drive the developing acquired T cell response towards an IFN-γ- dominated protective Th1 response.

Our results suggest that Listeriolysin O production in our system is also closely linked to protection against *L. major* in that lesion evolution in mice coinjected with the LLO^+ strain was significantly delayed and reduced and that the lesion displayed a less aggressive pathology, while coinjection with the LLO^- strain offered no such advantage. Coinfection with the LLO^+ strain also clearly potentiated the *L. major*-specific IFN-γ response resulting in the control of parasites at the site of injection, the draining lymph nodes, and spleens. Of particular note was the significant delay of spread of the parasite to the spleen, in spite of significant parasite load at the site of injection and the lymph nodes at that time. It appears possible that a potentiated response limits visceralization though not able to entirely eliminate parasites at the injection site or to prevent spread to the local lymph node. The results suggest LLO produced by the virulent strain was essential for the generation of protective immunity, but the possibility that other cell products may be involved cannot be ruled out, particularly since the two *L. monocytogenes* strains utilized were not isogeneic.

A transient increase of *in vitro* IFN-γ response of mice coinjected with the LLO^- strain was noted at 5 weeks but returned later to levels comparable to those of mice challenged with *L. major* alone. It is possible that bacterial components, such as LPS, at the higher dose of injected LLO^- strain, may have lead to this observation, although one would have expected this at a time point closer to injection.

The injection of semi-purified LLO was not as effective as the injection of LLO^+ organisms. Although histological changes observed in the spleen including the containment of the parasite in focal areas with lower parasite loads, the preservation of normal histological features of the spleen and the absence of necrosis are consistent with protection, yet footpad lesion sizes were not affected. It is possible that various indicators of protection may not all be similarly affected. Soussi et al. [22] reported a similar observation where no reduction in lesion development was detected in BALB/c mice immunized with an LLO-producing attenuated (*ActA* mutant) *L. monocytogenes* expressing the LACK antigen (a major antigen) of *L. major*, although IFN-γ was specifically produced by spleen cells stimulated *in vitro* with *L. major* or the LACK antigen. Several possibilities may account for the observed lack of effect of LLO on lesion development. First, the amount of LLO injected may not have been sufficient to induce adequate macrophage stimulatory effects, particularly as our product was not pure, which would reduce the specific activity of the LLO preparation. In studies on the role of LLO in stimulating an anti *L. monocytogenes* response [23], immunization with double the quantity of crude LLO (of the one we used) achieved a reduction in bacterial load, which was 1000 times

lower than immunization with live, virulent, LLO^+ organisms and induced only one third of the amount of IFN-γ. Second, the "bolus" injection of LLO would be expected to persist for a shorter period than the situation following the introduction of live, LLO-producing organisms that survive and continue to replicate for at least 7 days. The breakdown of the injected LLO would lose its effects on the innate response and thus on the immunomodulatory of the response. Third, LLO is cytolytic, and thus it is possible that directly injected LLO may induce the lysis of initially encountered monocytic cells recruited to the site of infection and thus interfere with the early response. It is possible to speculate that the observed potentiation may have resulted from the lowering of the infecting *L. major* dose through the lytic effects of LLO however our observations showed no such effect on *L. major* when exposed to LLO *in vitro* (data not shown).

In conclusion, our results demonstrate that LLO plays a major role in the observed immunopotentiation of the response of BALB/c mice against *L. major*. This effect, shown by the reduction in lesion size and parasite load, is mediated by the upregulation of IFN-γ production, and the subsequent control of parasite replication and dissemination.

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The *in vivo* effect of *Echinacea purpurea* succus on various functions of human blood leukocytes

EWA SKOPIŃSKA-RÓŻEWSKA, IRENA SOKOLNICKA, DOROTA RADOMSKA-LEŚNIEWSKA, BEATA KAPAŁKA, EWA SOMMER, BEATA BIAŁAS-CHROMIEC, MAŁGORZATA FILEWSKA, URSZULA DEMKOW

Department of Laboratory Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

Abstract

17 healthy human volunteers were treated for 7 days with 6 ml of Echinacea purpurea succus daily (IMMUNAL drops, LEK Slovenia) or placebo. Blood from cubital vein was collected twice- before and after treatment. The following parameters were evaluated: (1) release of growth factors by mononuclear leukocytes (human leukocyte-induced angiogenesis, H LIA test); (2) in vitro mononuclear leukocytes response to mitogen PHA, (3H thymidine); (3) chemiluminescent activity of granulocytes, (4) CD4 and CD8 lymphocyte markers.

Echinacea administration resulted in stimulation of proliferative and angiogenic activity of blood mononuclear leukocytes, stimulation of blood granulocytes activity and increase of CD4/CD8 ratio.

Key words: *Echinacea purpurea, in vivo treatment, human volunteers, blood leukocytes.*

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Introduction

Echinacea purpurea and *Echinacea angustifolia* (Asteraceae family) belong to the most important herbal remedies with immunostimulatory properties. Commonly called the purple coneflower, Echinacea is also a favorite garden plant.

These medicinal plants originate from the North American continent, where they have been used by the Indians for centuries in folk medicine for curing burns, snake bites, severe colds, and all kinds of pains and infections. Later, Echinacea was adopted by white settlers. Between 1887-1895 Echinacea was introduced to Americans by pharmacists John King and John Uri Lloyd and at beginning of the 20-th century, it had become the best-selling tincture in the United States [1, 2]. By 1895 Echinacea products had become available in Germany. In the late 1930s, Gerhard Madaus started commercial cultivation of Echinacea purpurea in Germany. The majority of pharmacological and clinical studies performed since 1939 have involved fresh juice of the flowering Echinacea purpurea [3].

The name Echinacea is derived from the Greek word „echinos" (hedgehog) and refers to the prickly, conical receptacle of the plants. Many compounds of Echinacea extracts (polysaccharides, alkamides, polyphenols, glycoproteins) posses immunomodulatory, anti-oxidative and anti-inflammatory activity [4, 5]. Echinacea is one of the most powerful and effective remedies against many kinds of bacterial and viral infections. A lot of papers describe, on experimental models, stimulation by Echinacea extracts of various parameters of cellular and humoral immunity [6-13].

Different species of Echinacea, the part of the plant processed and the processing procedure are variables influencing its effectiveness and mode of action. However, many experts consider the fresh-pressed juice of Echinacea purpurea to be the best preparation, having the greatest level of clinical support.

Despite this statement and widespread use, the clinical value of Echinacea products is still questioned. *Placebo*-controlled randomized studies of the effect of high doses given for long time gave negative results [14-17]. On the other hand, clinical studies of short-term therapeutic administration in respiratory tract infections, or of topical application in skin diseases, were promising [18-25]. In our previous studies performed in mice we have found that *Echinacea purpurea* preparations may vary in their

Correspondence: prof. Ewa Skopińska-Różewska, Department of Laboratory Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, Płocka 26, 01-138 Warsaw, Poland.

immunomodulatory effect, and some of them suppressed lymphocyte reactivity to mitogen PHA in doses recommended by producers, being stimulatory in lower doses [26, 27]. It was in agreement with the results of Coeugniet et al [23] performed in humans. So, we believe that it is very important to determine proper dose of each new Echinacea product before introducing it to human therapy.

The aim of our present study was to evaluate the effect of succus *Echinacea purpurea* originated from Slovenia (IMMUNAL drops), given for 7 days to healthy human volunteers in dose recommended by producer, on various parameters of cellular immunity mediated by blood leukocytes.

Material and methods

Study was performed in 17 healthy male volunteers, 22-35 years old. Blood from cubital vein was obtained twice, before and after 7-days treatment.

The following materials were studied:

1. IMMUNAL drops, (LEK), 6 ml daily (Lot. 000172501C, caffeic acid deriv. 5.47 mg%, 9 persons).
2. *Placebo*, 1 tablet daily (sample 58/01, 8 persons).

The following immunological parameters were studied:

- Proliferative activity of blood mononuclear cells (MNC) stimulated by mitogen PHA [28, 29].
  Briefly: MNC were isolated from heparinized blood on Lymphoprep gradient, washed thrice with PBS, resuspended in culture medium (RPMI 1640 (Difco) enriched with L-glutamine, FCS and antibiotics) at a concentration of $2x10^6$/ml and cultured in microplates with PHA at concentration 0, 1, 2 or 5 mcg/ml. Following 48 h incubation at 37°C in a humidified 5% CO_2 atmosphere, 0.2 uCi of tritiated thymidine was added for the next 18 hours. Afterwards, the cultures were harvested using multiple sample harvester (Scatron, Norway) and the incorporated radiolabel was counted using liquid scintillation counter (Rack-Beta, LKB, Sweden). Mean of quadriplicate count was calculated and expressed as counts per minute (cpm).
- Angiogenic activity of blood MNC (human leukocyte-induced angiogenesis, HLIA assay) according to [30, 31]. Briefly: MNC isolated as above were resuspended in Parker medium and injected intradermally into 6-7 weeks old female inbred Balb/c mice ($5x10^5$ cells in 0.05 ml per inoculum). Before performing injections the mice were anaesthetized with 3,6% chloralhydrate (0,1 ml per 10 g of body weigh). Both flanks of each mouse were finely shaved with razor blade, on each flank we localized 2-3 injections. Cell suspensions were supplemented with 0.05 ml/ml 0,01% trypan blue in order to facilitate recognition of injection sites later on. After 72 hours mice were killed (Morbital) their skin was separated from underlying tissues, injection sites were localized on the inner side of skin, and newly-formed blood vessels were counted in dissecting microscope at 6x magnification. Identification was done according to the criteria proposed by Sidky and Auerbach (tortuosity and divarications).
- Chemiluminescent (CL) activity of blood granulocytes, according to [32].
  Briefly: 0.05 ml of heparinized blood was diluted 1: 4 with PBS supplemented with 0.1% of glucose and 0.1% of BSA. 0.05 ml of such diluted blood was added to 0.2 ml of luminol solution (10^{-5} M) in PBS and placed in the scintillation counter (Rackbeta 1218, LKB Wallac) in the „out of coincidence" mode for spontaneous CL measurement. Then, cells were activated by addition of 0.02 ml of fMLP (Sigma) in final concentration 10^{-7} M. Chemiluminescence of stimulated cells was then measured for 15 min. The results were calculated as maximal CL value (in cpm) for 1000 granulocytes.
- Analysis of blood mononuclear cells subpopulations was done by monoclonal antibody staining of $CD4^+$ (T helper/inducer lymphocytes), $CD8^+$ (T suppressor/cytotoxic lymphocytes), and $CD19^+$ (B lymphocytes), using DAKO APAP KIT System 40, USA, in Lymphoprep- isolated blood mononuclear cell suspensions, according to producer instructions.

Statistical analysis of results was performed by Student t test.

Results

Table 1 presents the effect of IMMUNAL drops on proliferative activity of blood MNC in mitogen- stimulated cell cultures. In this test system, we observed statistically significant increase of stimulatory index, calculated by dividing values of cultures with mitogen by values of mitogen-free cultures. *Placebo* had no effect (Table 2).

Table 3 presents the results of experiments performed for estimating angiogenic activity of blood MNC, chemiluminescent activity of granulocytes and CD4/CD8 lymphocyte ratio. Significant (granulocytes activity) and highly significant stimulation (blood MNC) was observed in comparison to the values obtained before treatment. In *placebo* group, no stimulation was observed. MNC of persons treated with IMMUNAL drops presented significantly higher CD4/CD8 lymphocyte ratio after treatment, in comparison to the values of *placebo* as well as to the values obtained before treatment. Also T/B lymphocytes ratio was significantly higher after treatment (10.7 se: 1.2) than before treatment (6.1 se: 0.56), with no difference in *placebo* group.

Discussion

In our present study we focused on the effect of IMMUNAL drops on 4 important parameters of cellular

Table 1. The effect of **IMMUNAL** (succus, 6ml daily) 7-days administration to 9 human volunteers on the reactivity of their blood lymphocyt to mitogen PHA in *in vitro* culture (3H thymidine, scintillation counter); n=number of cultures; I.S. = stimulation index calculated in comparison to corresponding mitogen-free cultures

	PHA concentration					
	1 μg/ml		**2 μg/ml**		**5 μg/ml**	
	Before IMMUNAL	**After IMMUNAL**	**Before IMMUNAL**	**After IMMUNAL**	**Before IMMUNAL**	**After IMMUNAL**
n	53	54	54	54	53	54
mean I.S. ±SE	39±1.6	53.1±2.7	42.5±2.1	62.8±51	43.4±2.6	61.4±5.2
Statistical significance of difference		p<0.001 ↑		p<0.001 ↑		p<0.001 ↑

Table 2. The effect of ***PLACEBO*** 7-days administration to 8 human volunteers on the reactivity of their blood lymphocytes to mitogen PHA in *in vitro* culture (3H thymidine, scintillation counter); n=number of cultures; I.S. = stimulation index calculated in comparison to corresponding mitogen-free cultures

	PHA concentration					
	1 mcg/ml		**2 mcg/ml**		**5 mcg/ml**	
	Before *PLACEBO*	**After *PLACEBO***	**Before *PLACEBO***	**After *PLACEBO***	**Before *PLACEBO***	**After *PLACEBO***
n	48	47	48	48	48	48
I.S. ±SE	45.5±2.7	41.6±2.6	46.1±3.4	46.7±3.8	43.4±3.6	45.6±4.1
Statistical significance of difference		n. s.		n. s.		n. s.

Table 3. The effect of **IMMUNAL** drops or *placebo* administration to human volunteers for 7 days on various parameters of their cellular immunity. The results are presented as stimulation indices (value after treatment/value before treatment) ±s.e.; n = number of tests

Chemiluminescent activity of blood granulocytes			**Angiogenic activity of blood lymphocytes**		**CD4/CD8 blood lymphocytes ratio**	
	n	**S.I. ±s.e.**	**n**	**S.I. ±s.e.**	**n**	**S.I. ±s.e.**
IMMUNAL	18	1.39±0.13	267	1.17±0.02	26	1.37±0.12
Placebo	16	0.77±0.11	238	1.04±0.01	22	0.83±0.09
Statistical significance of difference		p<0.05		p<0.001		p<0.01

immunity in human, mediated by blood granulocytes and mononuclear leukocytes (lymphocytes and monocytes). We obtained good stimulation of all parameters studied using daily dose recommended by producer (6 ml) given for 7 days.

The response of lymphocytes to the plant mitogen, PHA, represents an *in vitro* correlate of an *in vivo* immunological response and involves various subpopulations of mononuclear leukocytes. We suppose, that IMMUNAL drops may be used as a drug of choice in persons presenting lowered T-cell mediated immunity.

The human leukocyte-induced angiogenesis (HLIA) test is used in the laboratory diagnostics for evaluation of total reactivity of the subject's cellular immune system. Activated lymphocytes and monocytes release large spectrum of cytokines and growth factors, among them factors possessing angiogenic activity. These factors are important in various neovascular processes, including wound repair, fractures healing, healing of ischaemic heart and brain disease, e.t.c.

IMMUNAL drops increased angiogenic potential of mononuclear leukocytes of treated persons, what suggests, that IMMUNAL may be used as a complementary drug in these pathological conditions.

Very important are the results obtained in granulocytes chemiluminescence test. Polymorphonuclear leukocytes (PMNs) provide the first line of defense against microbial pathogens. The main bactericidal mechanism of these cells

is oxygen-dependent. The most important event in the killing process is the generation of the series of reactive oxygen species during the oxidative burst. This process leads to the emission of light proportional to free radical quantity – measured as chemiluminescence (CL). CL is widely accepted as a modality for the assessment of overall PMN metabolism.

The present study shows that treatment with IMMUNAL drops significantly increased this parameter of nonspecific cellular immunity.

The Comission E of the German Institute for Drugs and Medical Devices approves several Echinacea preparations for use in colds and other upper respiratory tract infections. We suppose, that *Echinacea purpurea* succus may have wider application. Stimulation by this drug of granulocytes activity may be important for fighting many bacterial and viral infections in various body organs, not only of respiratory tract ailments. Stimulation of angiogenic growth factors release by mononuclear leukocytes opens new promising fields of application of Echinacea in ischemic diseases, disturbed healing processes, chronic infections of bones, chronic ulcerations e.t.c. Our earlier studies revealed, that some antibiotics (for example clindamycin) combined with some herbal immunomodulators (aloe extract, peat preparation) exerted synergistic stimulatory effect in HLIA test [33]. As clindamycin is often used in patients with bones infections, it would be interesting to check whether such type of synergy and better clinical results might be obtained by adding *Echinacea purpurea* succus to the therapy regimen of these patients. Echinacea preparations, given for 7-10 days are safe drugs. Toxicity studies in rats gave negative results [34]. Contraindications are controversial. There are no medical reports of Echinacea administration worsening autoimmune diseases, and western medical herbalists use Echinacea in various autoimmune conditions. The contraindications in tuberculosis and AIDS are also speculative. There are even reports (although not controlled) of successful use of Echinacea in tuberculosis [35].

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Review paper

Application of neutrophils chemiluminescence test in medical diagnostics

ALEKSANDRA LEWANDOWICZ-USZYŃSKA and EDYTA JARGULINSKA-FORYSIAK

Department of Pediatric Immunology, University of Medicine, Wrocław, Poland

Abstract

Presented work contains basic informations describing „luminescence" of cells which due to the use of luminophores is called chemiluminescence (CL). In the first part of the paper the author reviews the literature on environment in which the luminescence of neutrophiles occurs and briefly describes redox reactions within neutrophils which are called „oxidative burst". The second part of the paper reviews the literature on neutrophils' CL in selected diseases. The article contains informations on the first researchers in the field of luminescence dating from 1961 – Tarusow et al. – through the group of Italians (Colli, Facchini) to contemporary scientists as R. Allen, P. Stevens and K. van Dyke. Their research on the cellular luminescence contributed to the practical use of chemiluminescence test in clinical diagnostics (mostly in chronic granulomatous disease). The author cites the observations of others and her own on the usefulness of neutrophils' CL test in the prognosis of duration and the prediction of complications in the course of severe infections: bacterial, parasitic and AIDS. The paper contains data on neutrophils' CL in selected neoplastic diseases and the diseases of the respiratory system (including bronchial asthma and sarcoidosis). Various reports on free oxygen radicals in the pathology of the urinary tract and circulatory system are cited, where neutrophiles CL test allows to observe the severity of the process and the effects of the treatment. The work contains also informations about the metabolic potency of neutrophils in the course of certain self-aggressive disorders of the liver and during some surgical procedures.

Key words: *Chemiluminescence, oxygen metabolism, neutrophils.*

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Introduction

The ability of lighting is a characteristic trait of alive organisms on the organ, tissue or cell level. It is often called as a ultra-weak luminescence or even "cold lighting" [1, 2]. This phenomenon could appear *in vivo* spontaneously. The described situation is in need of the energy coming from some biochemic reactions, which product very active compounds as oxygenic and lipids radicals. All these processes could be accompanied by the luminescence (the light quantum emission). This luminescence, intensifed by the substances increasing the intensity of lighting – luminophores, is called chemiluminescence (CL).

To make the exist of chemiluminescence the following conditions have to be done:

- one of the reaction's part has to give the energy, in the amount not fewer than one light quantum energy, which is emissed,
- an activated molecule must have the energetic levels with possibilities of moving from higher to the lower one with proper efficiency,
- the energy should be left in 10^{-9}-10^{-11} second time, not to go away as a thermic energy [1-3].

The total efficiency of chemiluminescence *in vivo* is about 10^{-14}-10^{-9} photones per second.

There are some factors light reducing: inhibitors of chemical reactions, cleaners of radicals, stoppers of reactions.

There are the following traits characterising chemiluminescence:

- the light intensity – depends on the quantum efficiency and the constant reaction speed,
- the light amount – the number of photones, which emissioned while the time unit, counting per field unit,

Correspondence: dr med. Aleksandra Lewandowicz-Uszyńska, Department of Pediatric Immunology, University of Medicine, Kasprowicza 64/66, 51-137 Wrocław, Poland. Phone: +48 71 323 64 50, e-mail: alusz@dilnet.wroc.pl

The NADPH-oxidase catalyzes the oxygen (O_2) reduction to the supraperoxide anion (O_2^-):

$$NADPH + 2O_2 \longrightarrow NADP^+ + O_2^- + H^+$$

The supraperoxide anion is subject to be dismutated into a hydrogen peroxide and into a singlet oxygen:

$$2O_2^- + 2H^+ \xrightarrow{\textit{peroxide dismutase}} H_2O_2 + {}^1O_2$$

The hydrogen peroxide, in the presence of ferrous ions, could be a substrate in Fenton reaction leading to hydroxylic radical' production:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + {}^{\cdot}OH^{\cdot}$$

In the presence of chloride anions and of myeloperoxidase (MPO), releasing from azurophil granules of neutrophiles, the hypochloric acid is produced:

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + OH^-$$

In the reaction with aminate groups of proteins, HOCL produces toxic and more constant chloramines, however with a hydrogen peroxide - singlet oxygen ($^1O_2$):

$$HOCl + H_2O_2 \longrightarrow {}^1O_2 + H_2O + Cl^- + H^+$$

Fig. 1. The basic reactions leading to the free oxygenic radicals production

- the emission spectrum – depending on the kind of emitter reaching from 200 nm to 700 nm.

Some of authors postulate even the wider light emission spectrum from 180 nm to 800 nm, or more wide, adding that the lighting borders are influenced by the detectors possibilities, not by the nature of phenomenon [1, 4-6].

The bases for researches on the spontaneous bioluminescence of tissues and cells in mammals were given by Tarusow et al in 1961 [2, 7]. He related some data of spontaneous luminescence on the surface of liver and brain. In the works of the luminescence of tissues and cells there were also distinguished Italian scientists: Colli, Facchini and Americans too, like Allen, Stevens, Van Dyke, Wilson [1, 5, 8, 9].

There were appeared the suspections of light possibilities of morphotic elements in blood and plasma. These observations in 70-th, became the basis for Allen to use chemiluminescence to test the neutrophils activity during phagocytosis [10]. There was showed the increased production of free oxygenic radicals because of a strange molecule adhesion to granulocyte or this molecule phagocytosis. This production is called „oxygenic burst" [11].

It is characterised by an increased uptake of oxygen, an increased oxygen utilization in pentose-monophosphoric cycle working with an enzymatic system described as NADPH oxidase, which is helped by (MPO) myeloperoxidase and at the end the free oxygenic radicals are produced (RT): suprahydroxylic and hydroxylic radicals, supraperoxide aniono-radical, an singlet oxygen [12, 13] (Fig. 1). RT have a very strong physical and chemical power.

It is possible thanks to the one out-of-pair electron present on the external orbit, which binds with other different electrons into a chemical bond and emites a light quantum. This phenomenon is one of main ways of pathogens destruction by neutrophils and is called an oxygen-dependent killing way.

The reactions lead to RT- producing with luminophores' help (luminol, lucygenina) [14] could be measured by different instruments for instance – scintillation counter β working on one photocopier, – Polon or a special constructed-luminometer. The RT are responsible for different destructions of cells, tissues or organs (Fig. 2) [15- 17].

The possibility of an intermediate estimation of the ability to RT-production using chemiluminescence, gave us many informations about granulocytes and their role, a role of RT in plenty diseases' pathology, allowed us to find them, to observe their dynamic changes and treatment results [18, 19].

Immunodeficiencies

The neutrophils luminescence measurement was a real breakthrough in diagnostics of a Chronic Granulomatous Disease (CGD). The defect of catalazo-positive patogens killing is the source of the disease and might deal with the lack of one of NADPH oxidase subparts in a cell membrane or in plasma (or its abnormal activation) [20]. There is no spontaneous or stimulated chemiluminescence (CL) in patients with chromosomeX-linked CGD. The decreased chemiluminescence (CL) is observed in mothers of CGD children, what could exists as its marker. The CGD diagnosis might be done in pregnancy. Huu et al [21] used prenatal diagnostics in children with CGD-trait inheritance, during twentieth week of pregnancy and also showed very high sensitivity and specificity of that method.

Stevens et al. [22] as first, have used the methods of the luminol-dependent chemiluminescence to discover MPO deficit. They showed that CL in patients with MPO deficit was about 50% decreased than in healthy people. The use of stimulators: f-MLP (formyl-methionyl-leucyl-phenylalanine) or Zymosan (polypeptide produced from yeast) showed a higher CL than in healthy people. The situation was controversial indeed, especially there were no changes in a luminol-dependent CL of granulocytes after the MPO-inhibitors use.

The induction of an acid arachidic metabolism-dependent CL, which is the possible way to produce some substracts for cells' luminescence, is a suppose cause of this situation.

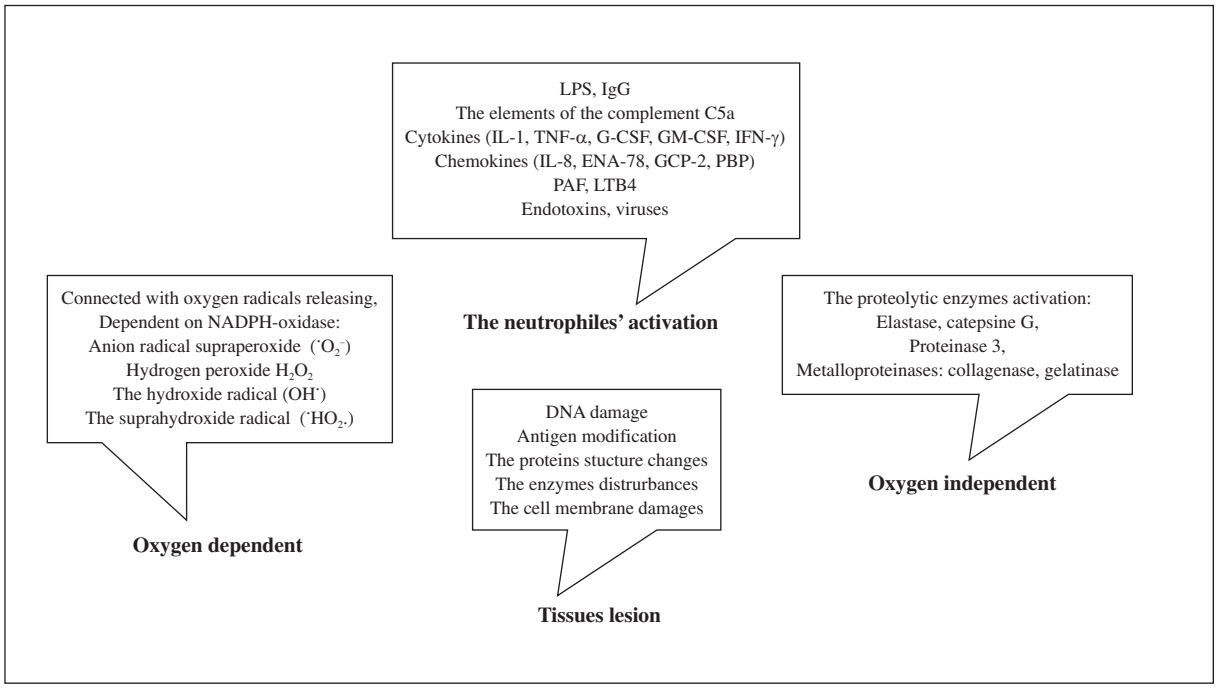

Fig. 2. The effects of the neutrophiles activation

Infections

There are many researches deal with CL use and role to predict some viral or bacterial infections, also help to expect the degree of its severity in definite patient [23-25].

The bacterial infections

Our own papers [26] showed an decreased CL of neutrophiles in children with frequent pneumonias, comparing to children who suffer from pneumonias from time to time. There was also observed a very slow recovery in children with a low activity of neutrophils at the onset. Zabuska-Jabłońska et al [27], also Zgliczyński et al [28] observed the same lower values of neutrophils' CL in adults with recurrent pneumonias. The decreased CL was described in children with recurrent respiratory tract infections [29]. The low values of neutrophils' CL in children with prolonged or chronic pneumonias were noticed what obliged to intensify the treatment. Werner et al [30] showed an exact increasing of CL in children from cities with bigger pollution.

Braun et al [31] showed the increased CL of neutrophils in patients during severe period of bacterial pneumonia, comparing to the patients with pulmonary oedema because of heart failure. Tekeuchi et al [31], also Adachi et al [32] described the higher CL of neutrophils, both: isolated and in full blood, in patients during a first period of pneumonia comparing to the values in healthy people. Through the treatment and the relieving of symptoms the CL collapsing was observed to the normal values in health. Very high values of CL were noticed during peritonitis.

AIDS

In 1994 there were many observations of decreased CL of neutrophils in patients with AIDS of different periods of disease [33]. The more severe phase the less active potential of neutrophils metabolism. The precised CL- decreasing was showed in patients with accessory *Pneumocystis carini* invasion [34]. To intensity oxygenic metabolism of neutrophils and to make the immunological deficiency of the patient stronger there were used *in vitro* and *in vivo* G-CSF or IFN-γ with good results [35, 36].

Parasites' invasions

The increased CL in patients with *Plasmodium falciparum* in severe phase of disease was observed [37, 38]. In some chronic cases the reduced CL was observed, what predisposed to prolonging the disease. There was no luminescence of neutrophils in trichinellosis [39].

Neoplasmal diseases

There were showed plenty of abnormalities dealing with the neutrophils metabolic activity in neoplasmal diseases. For instance the weak neutrophils response after LTB-4 and fMLP stimulation in patients with true polycythaemia comparing

to healthy people [40]. There were no changes after PMA-stimulation (phorbol-12-miristate-13-acelate). It is a great evidence to proof a specific defect of a stimulated-CL [1]. Itala et al [42] described an abnormal (decreased) RT-production during infections in patients with lymphoblastic leukaemia. Cheze et al. [43] noticed a great low CL in patients with aplasia (panmyelophthisis). Within the time of disease remission they observed a slow CL increasing. Teramoto et al [44] showed a CL-decreasing in patients with pulmonary cancers during a cisplatine treatment.

Respiratory tract diseases

There were showed a much increased rest-CL in patients suffer from ARDS (acute respiratory distress syndrome), comparing to the risk group of this disease and also to healthy people [45]. There were also noticed significant higher results of a stimulated-CL by PMA in ARDS patients than in the risk group or in healthy people. As the authors wrote this test might be very useful to predict the risk of ARDS.

Barth et al. [46] showed the increased RT production and the increased CL in patients during second phase of pulmonar sarcoidosis comparing to the values in healthy people. That emission was also higher than of pulmonar macrophages. However, there was no correlation between CL and cells markers estimating the severity of the disease, although the authors recommended CL-test as one more examination to proof the sarcoidosis' activity.

Asman et al [47] showed the increased CL of separated granulocytes and of the full blood in patients with bronchial asthma. Teramoto et al. [48] showed the increased stimulated-CL in patients suffer from bronchial asthma and chronic obturative pulmonary (pulmonal) disease comparing to healthy people. The spontaneous CL was higher only in patients suffer from asthma. The same results were in our own researches in 1993-1994, which showed the increased CL in full blood, in children with asthma [49]. Li et al. [50] showed the increased CL in asthma, together with a collapse of an antioxidatic potential. That process was as more observed as more exact the decreasing of SOD was in the serum and as more higher the IgE-level was.

Urinary system

The researches of free oxygenic radicals in patients with renal problems deal with neutrophils and also monocytes [51]. The interesting observation came from Kuźniar et al. [52] about the increasing of CL in patients with different types of glomerulonephritis treated by methyloprednisolon. This observation is a real surprise – because there was observed *in vitro* that steroides decreased an oxygenic metabolism by NADPH-oxidase inhibition. The way corticosteroides influence on neutrophils is not known exactly, to the end.

Plenty of opposite theories and facts about chronic renal failure appeared. Eckhardt's et al researches [53] showed an increasing RT-generation and a higher CL of granulocytes in full blood. Okada et al. [54] had similar results of isolated neutrophils. Those informations are different than Pawlicki's [55] ones and Lucci's who noticed the increased rest CL of isolated neutrophils, while the stimulated CL was decreased. During haemodialysis a considerable decreasing of isolated neutrophils chemiluminescence was observed. Pawlicki et al [56] showed a great number of supraperoxide anions measured by a reduction of cytochrom C in the same time as CL, what seams to be controversed to CL results. The authors explained that the fact is probably caused by RT-relieving from neutrophils out of the cell, because of granulocytes degranulation what provide to leaving for example MPO-responsible for CL. The observed RT-increasing could be caused by the complement activation and a direct contact of cells with dialysing membrane.

In patients with chronic pyelonephriritis the decreased oxygenic metabolism of neutrophils and CL of these cells were observed [57].

Autoimmune diseases

The disturbed oxygenic metabolism of neutrophils leading to RT-over production plays a great role in rheumatoid arthritis (RA) [58]. There were observed the increasing of both spontaneous and stimulated luminol-dependent CL of neutrophils in full blood [59] and of neutrophils coming from the arthral liquid of patients with RA [60]. There were also higher spontaneous and stimulated CL of the granulocytes taken from healthy people and incubated with an arthral liquid from people suffer from RA. There was also higher CL of granulocytes in patients with progressive sclerodermia [61]. There was observed an intensification of basic CL together with a progression of skin change. The stimulated CL was similar to value in healthy people. The authors suggested that a neutrophils preactivation begins *in vivo* in organism.

There were showed an intensive oxygenic metabolism of neutrophils and of course an increased CL in children suffer from rheumatoid arthritis [62]. The intensification of CL was observed coming together with an aggravation of the disease. Miesel et al. [63] did not notice any increased values of CL in patients with RA during remission time, but in an aggravation of the disease they observed the increased CL coming with TNF-α elevation. These authors also observed, in another work, up to 8-times intensified CL of neutrophils which was caused by their phagocytic activity, comparing to healthy people.

Arnchold et al. [64] also showed the increased native CL in patients with RA comparing to healthy people.

Liver diseases

Lunel et al. [65] showed much increased basic CL in patients with an alcoholic hepatitis (liver failure). After latex, Zymosan and PMA stimulation there was observed

a significant weaker luminescence of neutrophils than observed in healthy people. Iushuk et al. [66] made a proof of the CL-test usefulness in a differential diagnostics between a viral hepatitis and bacterial one, leading on own observations. They showed the decreased oxygenic metabolism of granulocytes (luminol-dependent CL) in patients with an acute viral hepatitis comparing to CL in patients with *Yersinia enterocolitica* during its severe phase. Uehara M. et al. [67] noticed a significant decreased CL of neutrophils in patients suffer from a liver cancer and a liver fibrosis in confrontation with healthy people. The low values of CL were also observed during some chronic hepatitis, but the differences were not so clear as in liver cancer.

The surgical procedures

The meaningful collapsing of granulocytes oxygenic metabolism was also observed in some patients after splenectomy and after cholecystectomy too, but in a smaller degree [68]. Sakumoto et al [69] showed the increased CL of neutrophils in patients after the urologic operations through abdomen on the contrary to those through an endoscopy.

The others scientists showed a negative influence of lidokaine on CL of neutrophils both *in vivo* and *in vitro* [70]. The granulocytes of healthy people were treated by thiopental and had the significant decreased CL which was stimulated by *Staphylococcus aureus* and *Escherichia coli* [71]. Heberer et al. [72] noticed quite different results, showing no influence of analgesia and of severe abdominal operations on CL in full blood.

The ischaemic diseases

There were showed decreased values of CL in patients with an acute phase of myocardic ischaemia and CL increasing during a reperfussion period [73, 74]. Kowalski et al. [75] described the correlation between an increased CL of neutrophils and a decreasing of C3c, C5 of complement and also their haemolytic activity during an unstable ischaemic angina. Takeshita et al. [76] showed an intensified oxygenic metabolism of neutrophils tested by CL in patients with ischaemic angina. They investigated whether increased PMN activity in the peripheral blood is a marker for high-grade coronary artery stenosis in patients with angina pectoris [77]. Hansen et al. [78] showed on contrary, lower values of CL in patients with infarct, cured by streptokinase.

In spite of many observations and uses, chemiluminescence is not a specific method of diagnosis (except CGD) for any disease. We could observe its intensity and also its impairment in plenty diseases like we observe erythrocytes sedimentation and CRP-protein elevation or its reduction. Chemiluminescence is a very useful diagnostic test what helps in estimating the results of the treatment, the inflammatory progression and the degeneration, where there are any free-radicals reactions. CL is a fast, simple laboratory test used more and more often by many scientists and physicians.

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Antimicrobial peptides – their role in immunity and therapeutic potential

HANNA GAŁKOWSKA and WALDEMAR L. OLSZEWSKI

Department of Surgery and Transplantology, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

Abstract

Antimicrobial peptides (AMPs) have a broad antimicrobial spectrum (gram-positive and gram-negative bacteria, fungi, certain viruses) and lyse microbial cells by interaction with biomembranes. AMPs are produced by bacteria and by other species of all kingdoms. Recently, it has been recognized that their function is essential to the immune response. AMPs participate primarily in the innate immune system of many organisms, including plants, insects and vertebrates. They have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells, influencing diverse processes such as cell proliferation, wound healing, cytokine release, chemotaxis, immune induction. Recently, pharmaceutical companies have started research programmes related to therapeutical usefulness of AMPs. This review summarises the current knowledge about the basic and applied biology of AMPs.

Key words: *antimicrobial peptides, immunity.*

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Nomenclature and tissue distribution of AMPs

Antimicrobial peptides (AMPs) are an extremely diverse group of more than 700 small proteins and can be divided into several categories on the basis of their structures. Most AMPs are cationic (polar) molecules. There are two classes of AMPs based on the mechanism of their cellular synthesis: (1) non-ribosomally synthesised (largely produced by bacteria) and (2) ribosomally synthesised, gene- encoded peptides [1]. Non-ribosomally synthesised AMPs are produced by multifunctional peptide synthetases. These substances are already used in clinical applications: polymyxin B, polymyxin E, colimycin, cyclosporin, tripeptide ACV (precursor of penicillin and cephalosporins) [2, 3]. AMPs, in a more narrow sense, refer to gene-encoded peptides. These AMPs can be grouped according to their size, conformational structure or predominant amino acid structure. On the basis of their gross composition and 3D structure, AMPs can be divided in four main groups: I: linear peptides with an α-helical structure; II: β-sheet structures stabilised by disulphide bridges; III: peptides with predominance of one or more amino acids; IV: peptides with loop structures [4]. This review will concentrate on the biological relevance of two mammalian AMPs- families: defensins (from group II) and cathelicidins (from group I).

The defensins families of AMPs

Defensins are typically 28 to 44 amino acids long and contain 6 to 8 cysteine residues that form characteristic intramolecular disulfide bridges. Defensins are found in mammals, and distantly related peptides appear in insects and plants [5]. They are classified into 3 families- the α-defensins, the β-defensins and the θ-defensins.

• α-defensins

These defensins are 29 to 35 residues in lenght and contain 3 disulfide bridges. The first human α-defensin was isolated from neutrophils in 1985 [6]. At the present time, six α-defensins have been identified from humans. Human neutrophil peptides 1-4 (HNP1-4) are localised in azurophilic granules [7, 8]. The two other α-defensins, human defensins 5 and 6 (HD5-6) are located in Paneth's cells of the small intestine [8] and in epithelial cells of the female urogenital tract [9]. Unlike neutrophils, Paneth's cells do not store defensins as matured peptides; instead, they store them as

Correspondence: Hanna Gałkowska, PhD, Department of Surgery and Transplantation, Medical Research Center, Polish Academy of Sciences, 5 Pawinskiego, 02-106 Warsaw, Poland. Phone: +48 22 608 64 05, fax: +48 22 668 53 34, e-mail: hgalk@cmdik.pan.pl

propeptides [10]. Elevated plasma levels of α-defensins were observed in patients with active pulmonary tuberculosis [11] and children with severe sepsis [12].

• β-defensins

These peptides are 36-42 amino acids in lenght and contain 6 cysteine motifs connected by 3 disulfide bridges. The first β-defensin was isolated from cow airway epithelial cells [13]. In human beings, 4 types of β-defensins have been identified so far. The first human β-defensin, called hBD1 was originally isolated from large volumes of hemofiltrate [14] and is expressed constitutively in epithelial cells of the urinary and respiratory tracts [15, 16] and in human skin epidermis [17, 18]. Human β defensin-2 (hBD2) was isolated from psoriatic skin [19] and in contrast to hBD1 is upregulated in cultured keratinocytes by inflammatory stimuli like IL1 and live Pseudomonas aeruginosa [20] or Staphylococcus aureus, S. epidermidis, E. coli [21]. E. coli LPS is a very weak direct inducer of hBD2 mRNA and peptide, but the induction is amplified by IL1-mediated signaling [22]. Inducible human β defensin-3 was isolated from lesional psoriatic scales and investigations of different tissues revealed skin and tonsil to be major hBD3 mRNA- expressing tissues [23]. Human β defensin-4 was identified solely by searches of genomic database [24]. Recently, 28 new genes for human β defensins were identified by screening of human genome database [25]. Peripheral blood monocytes, monocyte-derived macrophages and monocyte-derived dendritic cells all express hBD1mRNA and hBD2mRNA. HBD2 peptide was demonstrated in blood monocytes and alveolar macrophages [26].

• θ-defensins

This family of defensins has been isolated from rhesus monkey neutrophils [27] and no data about the presence of these molecules in tissues are aviable at this time.

Cathelicidins

The term „cathelicidins" was coined as an attempt to unify a variety of peptides 12 to 80 or more amino acids in length, related by similar „cathelin" precursor domain (cathepsin L inhibitor) [28]. To date, cathelicidins have been found only in mammals [29]: mouse (CRAMP), rat (rCRAMP), pig (protegrin, PMAP-23, PR-39), monkey (rhLL37, RL37), rabbit (CAP-18), sheep (SMAP-29, SMAP-34) and human (hCAP-18/ LL37). Human LL37 was isolated from human bone marrow and is present in neutrophils [30], γδT cells, NK cells, B cells, monocytes/ macrophages from peripheral blood [31]. LL37 is also present in epithelial cells in the respiratory tract, urogenital tract, gastrointestinal tract [32, 33] and skin epidermis, mainly in response to infammatory stimuli [34-36] and cutaneous injury [37]. Human CAP-18/LL37 is also induced in keratinocytes by growth factors like IGF1 and TNFα, both important in wound healing process [38]. In addition, plasma has been reported to contain LL37 [39]. The mouse cathelin related antibicrobial peptide (CRAMP) expression resembles that of LL37 in human [40].

The role of AMPs in immune response

In higher vertebrates such as mammals, there are two types of immunity: innate (or natural) and adaptive (or acquired). The effector branch of innate immunity consists of two major aspects. One is the generation of humoral antibacterial mediators such as complement and AMPs (including defensins and cathelicidins), as well as large antimicrobial substances (lysozyme, cathepsin G, lactoferrin). The other is the recruitment and activation of phagocytic granulocytes, monocytes/ macrophages, and NK cells to sites of microbial (bacteria, fungi, enveloped viruses) invasion. HBD1 and LL37 are produced constitutively by keratinocytes and other epithelial cells and contribute to the barrier functions in the first line of antimicrobial defence. In mice, a deficiency in CRAMP production leads to the increased susceptibility to necrotic skin infections caused by Group A Streptococcus [41]. Neonatal skin in mice and humans expresses increased levels of AMPs compared to adult [42]. Furthermore, hBD1 expression was described in milk and breast tissue during lactation [43]. Recombinant hBD2 produced by baculovirus had a direct antimicrobial activity in vitro [20]. Human defensins, hBD2 and LL37 induce the activation and degranulation of mast cells resulting in the release of histamine and PDG2 [44, 45]. Because mast cell granule products increase neutrophil influx, AMPs can indirectly promote accumulation of these cells at inflammatory sites. Degranulation of neutrophils releases more defensins and upregulates innate host innflammatory defenses against microbial invasion. HNP1 and HNP2 are chemotactic for human monocytes [46] dendritic cells and T cells [47, 48]. Murine β defensin 2 acts directly on immature dendritic cells as an endogenous ligand for Toll-like receptor 4 (TLR4), inducing up-regulation of costimulatory molecules and, subsequently, dendritic cells maturation, acting as the natural adjuvant [49].

In addition to their role in host defense, defensins may also contribute to some pathophysiologic mechanisms. Neutrophil defensins can inhibit fibrinolysis and modulate tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells [50]. Cathelicidin LL37 can also play a part in wound closure and its reduction in chronic wounds can impair re-epithelialization [51].

AMPs as therapeutic agents

The broad spectrum of activity and the low incidence of bacterial resistance are attractive features of AMPs. Animal

studies provided first proof that AMPs can be used to modify the course of infection and inflammatory diseases. Small biotech companies in association with larger pharmaceutical companies carried out human studies using AMPs of animal origin. AMPs from the skin of frogs, called magainins, have been one of the first substances to go through a drug development process. Its derivative, pexiganan was investigated in phase III trials of 926 patients and topical use has been found to show equivalence to oral ofloxacin against polymicrobic diabetic foot ulcer [52]. The porcine neutrophil-derived protegrins are the first of the mammalian cathelicidin peptides used in clinical trials [53]. A phase II study showed that this peptide used orally by patients undergoing bone marrow transplantation significantly reduced mucositis and number of febrile days. A phase III study of protegrin is now underway [54]. In future, animal studies may resolve the role of AMPs in tissue protection, wound healing and interaction with the aquired immune system. Better understanding of AMPs relationship between the innate and the aquired immune systems is the critical step to give us insite into how we naturally prevent infections and how to design new drugs to fight diseases.

Concluding remarks

AMPs have emerged as effector substances of the innate immune system involving not only activities as endogenous antibiotics but also as mediators of inflammation. Several important topics will have to be addressed in the future: (I) AMPs might contribute to the development of diseases as pro- or anti- inflammatory substances; (II) clinical useful of AMPs as novel therapeutic agents promises to revolutionize treatment of many inflammatory and infectious diseases.

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