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Type of serum influences the rituximab dependent cytotoxicity and apoptosis of chronic lymphocytic leukemia cells *in vitro**

Rodzaj surowicy wpływa na zależną od rituximabu cytotoksyczność i apoptozę komórek przewlekłej białaczki limfocytowej w hodowli *in vitro*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Purpose: The aim of the study was to compare the influence of types of serum on the *in vitro* viability and on either spontaneous or rituximab (RIT)-induced apoptosis of chronic lymphocytic leukemia (CLL) cells.

Methods: The influence of fetal calf serum (FCS), patients' autologous serum (AS) and human AB-serum (ABS), used alone and in combinations consisting of two of them (v/v-1:1), on RIT-dependent cytotoxicity, apoptosis, detection of active forms of caspases-3,-9,-8 and disruption of mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by flow cytometry. RIT was used at the concentration of 10 μg/ml. The spontaneous apoptosis was assessed in culture without RIT.

Results: AS revealed the protective action on CLL cells, however this serum added *in vitro* to the culture either alone or in combination with FCS was the only one to allow RIT to exert its cytotoxic action against CLL cells. RIT-induced apoptosis involved changes in $\Delta\Psi_m$ and activation of caspases-3,-8,-9 when AS+FCS was applicated. Drug induced apoptosis (DIA) was 6.02 and 0.34, when FCS+AS and FCS alone were used, respectively ($p<0.01$). The RIT-dependent cytotoxic effect decreased when FCS+AS or FCS+ABS were used, as compared to effect of AS used separately. The cytotoxic effect of RIT did not depend on drug concentration, but on the type of serum added to the culture.

Conclusions: The strongest cytotoxic effect of RIT in the presence of AS suggests that this drug activity towards CLL cells is enhanced by known cytotoxic mechanisms, caspase-dependent apoptotic pathway and possible influence of other extracellular factors present in the patients' sera.

Key words: **chronic lymphocytic leukemia • Rituximab • Fetal Calf Serum • Autologous Serum • AB-Serum • apoptosis**

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Streszczenie

Cel pracy: Celem pracy było porównanie wpływu różnych rodzajów surowic na żywotność oraz na spontaniczną lub indukowaną rytuksymabem (RIT) apoptozę komórek przewlekłej białaczki limfocytowej (PBL) *in vitro*.

Metody: Metodą cytometrii przepływowej oceniono wpływ płodowej surowicy cielęcej (FCS), surowicy autologicznej (AS) oraz ludzkiej surowicy AB (ABS) na cytotoksyczność i apoptozę, aktywność kaspaz 3, 9, 8 oraz zmianę potencjału mitochondrialnego ($\Delta\Psi_m$) komórek PBL poddanych działaniu RIT w stężeniu 10 μg/ml w hodowli *in vitro*. Surowice stosowano pojedynczo lub w kombinacji dwóch z nich w stosunku 1:1. W hodowlach kontrolnych – bez RIT oceniano apoptozę spontaniczną.

Wyniki: Najwyższy odsetek komórek białaczkowych ulegających spontanicznej apoptozie stwierdzono po zastosowaniu wyłącznie FCS, podczas gdy najniższy odsetek tych komórek uzyskano stosując tyko AS. Zastosowanie FCS+AS w hodowli komórek PBL poddanych działaniu RIT zwiększało $\Delta\Psi_m$, jak i ekspresję kaspaz 3, 8 i 9, w porównaniu z hodowlami kontrolnymi. Apoptoza indukowana lekiem wynosiła 6,02 i 0,34, odpowiednio w hodowlach z FCS+AS i z FCS użytym pojedynczo ($p<0,01$). Zastosowanie FCS+AS lub FCS+ABS zmniejszało natomiast efekt cytotoksyczny RIT, w porównaniu z uzyskanym po samodzielnym zastosowaniu AS. Cytotoksyczne działanie RIT nie zależało od stężenia leku, lecz od rodzaju surowicy dodanej do hodowli.

Wnioski: Najsilniejszy efekt cytotoksyczny RIT w obecności surowicy AS sugeruje, że jego aktywność wobec komórek PBL zwiększa się na skutek znanych mechanizmów cytotoksyczności, apoptozy zależnej od kaspaz oraz możliwego wpływu innych pozakomórkowych czynników obecnych w surowicy chorych.

Słowa kluczowe: **przewlekła białaczka limfocytowa • rytuksymab • płodowa surowica cielęca • surowica autologiczna • surowica AB • apoptoza**

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Introduction

It has been well established that the accumulation of the chronic lymphocytic leukemia (CLL) cells in the bone marrow, peripheral blood, lymph nodes and eventually non-lymphoid organs results from both deregulated proliferation and the *in vitro* inhibition of apoptosis of leukemic cells. Currently, the association of purine analogues (fludarabine or cladribine) with an alkylating agent (cyclophosphamide) and anti-CD20 monoclonal antibody (rituximab, RIT) is considered as the most powerful regimen in front-line therapy [10,12,13,16,17].

Although immunological phenomena involving complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) play the most important role in the mechanism of RIT action, this drug has also pro-apoptotic properties [4,12]. RIT-dependent cytotoxicity *in vitro* requires the presence of appropriate serum in the culture medium. In numerous *in vitro* studies, the influence of RIT on the viability of CLL cells was tested in the presence of fetal calf serum (FCS) in the culture medium [2,5,18,19]. Some studies also checked the influence of some other sera on the RIT-dependent cytotoxicity such as autologous serum (AS) or human AB-serum (ABS) [1,2,7,9,14,20]. So far, only few studies have aimed at comparing the *in vitro* effect of different types of sera on the survival of malignant cells or RIT-dependent cytotoxicity towards CLL cells [2,3]. Given this complexity of RIT action on CLL cells *in vitro*, the investigations on this action have to be carried out in the presence of a serum or sera combination which ensure the optimal conditions for both drug-induced cytotoxicity and apoptosis. It is then justified to undertake a study aiming at comparing the effect of different sera and their combinations on the *in vitro* viability and apoptosis of CLL cells under the influence of RIT in order to optimize the experimental conditions for further *in vitro* studies.

The aim of our study was then to evaluate the *in vitro* influence of three types of serum: FCS, AS and ABS, used either alone or in different combinations, on the viability of CLL cells incubated with RIT. We also compared the influence of FCS used alone or together with AS on the apoptosis of CLL cells incubated with RIT.

Material and Methods

Patients

Peripheral blood samples were collected from 41 newly diagnosed CLL patients (19 females, 22 males) as a part of routine diagnostic procedures in the Department of Hematology, Medical University of Lodz. The mean age of patients was 62 years (range 21–83). The diagnosis was based on the IWCLL criteria [8]. The study was approved by the Local Ethics Committee. All specimens were collected after written consent of patients.

Therapeutics

Rituximab (RIT; Mabthera, Roche, Switzerland) is commercially available. The following final concentrations of the drugs were used: 5 μg/ml, 10 μg/ml and 20 μg/ml [5,7].

Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood by centrifugation in the Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) and centrifuging on a density gradient by 3600 rpm for 20 minutes. The buffy coat of PBMNCs was isolated and washed twice in phosphate buffered saline (PBS) (Lonza, Belgium). Afterwards, PBMCs at a concentration of 1.0×10^6 cells/ml were suspended in RPMI 1640 (PAA Laboratories GmbH, Austria) supplemented with antibiotics (streptomycin 50 mg/ml, penicillin 50 IU/ml; Life Technologies, Scotland) [18]. Mean B-cell (CD19+) purity was >95% as measured by flow cytometry (FACS).

Cell culture conditions

To assess the influence of serum type on the viability and apoptosis of CLL cells, all cultures were incubated for 24 and 48 hours, in RPMI 1640 medium containing 20% of appropriate type of serum, at 37°C, in the atmosphere of 5% CO_2 and full humidity. The following serum types were used:

1. 20% (v/v) heat inactivated fetal calf serum (HI-FCS) (Gibco, Life Technologies, Scotland).
2. 20% (v/v) autologous serum (AS).
3. 20% (v/v) human AB-serum (ABS)(PAA Laboratories GmbH, Austria).
4. 10% (v/v) HI-FCS + 10% (v/v) AS.
5. 10% (v/v) HI-FCS + 10% (v/v) ABS.
6. 10% (v/v) AS + 10% (v/v) ABS.

Assessment of the influence of serum type on the RIT-dependent cytotoxicity

The cytotoxic effect of RIT was tested using this drug in three concentrations listed above. The control cultures were incubated without RIT.

At the onset of cell cultures and after 24 and 48 hours, the absolute number of cells per culture was estimated using a hemocytometer. At the same time points the RIT-dependent cytotoxicity was assessed as a percentage of dead cells using propodium iodide (PI; Sigma Aldrich, Germany). Afterwards, PBMCs were washed twice with cold PBS and then resuspended in 100 μl of binding buffer containing 10 μg/ml of PI and incubated for 15 minutes at room temperature in the dark. The fluorescence was measured immediately after staining by flow cytometer (FACSCalibur; Becton Dickinson, San Diego, USA) at 490”20 nm using FL1, and at 530”20 nm using FL3 standard fluorescent filter [6,18].

The drug-induced cytotoxicity (DIC) was calculated as the difference between the percentage of dead cells after incubation with RIT and that in the control culture.

Assessment of the influence of serum type on the RIT-dependent apoptosis

The RIT-dependent apoptosis was tested after 24 or 48 hours of the culture containing either 20% (v/v) of FCS or the combination of 10% FCS + 10% AS and in the presence of RIT in the concentration of 10 μg/ml.

The percentage of apoptotic cells was measured by flow cytometry using Annexin V (Ann-V; BD Pharmingen, USA). The cells were washed twice with cold PBS and then resuspended in 100 μl of binding buffer, containing 2 μl of FITC conjugated Ann-V. The fluorescence was measured as described above [6,18].

Apoptotic index (AI) was expressed as the percentage of Annexin V positive cells. Drug-induced apoptosis (DIA) was calculated as the difference between AI in the presence of RIT and the percentage of cells undergoing the spontaneous apoptosis in control cultures.

Detection of the active form of caspase-3, caspase-9 and caspase-8 by flow cytometry

The apoptosis after the 48 hour culture supplemented with 10% FCS + 10% AS was quantitatively assessed by flow cytometry measuring the caspase-3 activity on FACSCalibour (Becton Dickinson, San Diego, USA), using Becton Dickinson Pharmingen PE Active Caspase-3 Apoptosis Kit (San Diego, USA), according to the manufacturer’s protocol. Similarly, quantitive analyses of active forms of caspase-8 and caspase-9 were also performed on FACSCalibour using FAM-FLICA *in vitro* Caspase 8 Kit or FAM-FLICA *in vitro* Caspase 9 Kit (ImmunoChemistry Technologies, Bloomington, USA) respectively, according to the manufacturer’s protocol. Assays were done in duplicate.

Disruption of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) were evaluated using MitoTracker Red CMX Ros kit (Invitrogen, Australia). After the 48 hour culture supplemented with 10% FCS + 10% AS, PBMNCs were centrifuged, and the supernatant above the cell pellet was rejected. Afterwards, the cell pellet was gently resuspended in the staining solution containing the MitoTracker probe, prepared according to the manufacturer’s protocol, and incubated for 30 minutes at 37°C. The cells were then re-pelleted by centrifugation and resuspended in fresh warmed-up medium. Assays were done in duplicate and analysed by flow cytometry (FACSCalibour, Becton Dickinson, San Diego, USA).

Table 1. The influence of serum type on CLL cells viability *in vitro*, after 24 and 48 hours incubation

Incubation period		Type of serum						(p<0.05)
		FCS	ABS	AS	FCS + ABS	FCS + AS	ABS + AS	
		n=31	n=31	n=30	n=10	n=16	n=16	
		1	2	3	4	5	6	
24 hours [A]	Mean	16.3	14.0	12.5	12.5	11.8	12.3	1 *vs* 2; 1 *vs* 3 1 *vs* 6; 2 *vs* 5 2 *vs* 6; 4 *vs* 5
	SD	8.0	6.2	6.0	3.1	5.1	5.2	
	Range	6.2–32.5	5.6–30.5	1.7–28.6	8.9–18.2	5.7–22.4	5.0–22.9	
48 hours [B]	Mean	27.1	21.3	18.7	19.5	18.5	17.2	1 *vs* 2; 1 *vs* 3 1 *vs* 5; 1 *vs* 6 2 *vs* 6; 3 *vs* 5 4 *vs* 6;
	SD	13.9	9.3	10.9	8.0	9.6	7.8	
	Range	10.2–61.8	6.3–45.1	5.5–43.0	11.5–33.9	7.0–41.0	7.4–34.3	
(p<0.005)		A1 *vs* B1;	A2 *vs* B2;	A3 *vs* B3;	A4 *vs* B4;	A5 *vs* B5;	A6 *vs* B6	

FCS – fetal calf serum; ABS – serum devoid of anti-A and anti-B antibodies; AS – autologous serum; n – number of samples; Mean – mean percentage of dead cells (PI+); SD – standard deviation.

Statistical analysis

Statistical differences between experimental and control cultures or between the effects of different concentrations of the drug tested were evaluated by Wilcoxon signed-rank test, and the p value less than 0.05 was considered as statistically significant.

Results

Evaluation of the influence of serum type on the viability of CLL cells *in vitro*

The comparison of the influence of each of the sera tested on the viability of CLL cells in the absence of RIT revealed the highest percentage of dead cells in the culture supplemented with FCS, and the lowest in the presence of AS (p<0.03) (Table 1). The combinations of two types of serum yielded the results which were comparable with each other and lower than FCS, but only the combinations of AS with either FCS or ABS gave the percentage of dead cells significantly lower than the results obtained with FCS alone (Table 1). For each serum used alone or in combination the percentage of dead cells was significantly higher in the 48 hour culture than in corresponding the 24-hour one.

Evaluation of RIT influence on CLL cells viability depending on serum type

The comparison of the influence of different types of serum used separately on the viability of CLL cells incubated with RIT at all three concentrations, showed the highest cytotoxic effect, as compared to the control culture, when AS was added to the medium. A similar effect was observed after both 24 and 48 hours (p<0.03) (Table 2). The 48 hour incubation of PBMNCs with RIT at the concentration of 10 μg/ml and in the presence of AS, yielded the DIC value 18.6, whereas it was only 2.6 when FCS was used (Table 2).

The application of 1:1 (v/v) mixture of the two types of serum revealed that the highest cytotoxic effect, as compared to the control culture, was demonstrated after 48 hours of incubation, and when FCS in combination with AS was added to the culture medium (p<0.0004) (Table 3). The incubation of leukemic cells with RIT at the concentrations of 10 μg/ml or 20 μg/ml in the presence of FCS+AS resulted in the similar DIC values for both RIT concentrations (7.0 and 7.2; respectively). However, a longer incubation period significantly increased the cytotoxic effect of the drug (p<0.002) (Table 3).

To evaluate the influence of serum type on the RIT-dependent cytotoxicity, the effects of sera combinations with the effect of serum used separately were compared. In case of FCS used together with ABS, no significant differences as compared to the results for each of serum used separately, were observed (Fig. 1A). The combinations of FCS either with AS or ABS decreased the cytotoxic effect of RIT as compared to the effect obtained with AS used separately (Fig. 1B, C).

Evaluation of RIT influence on percentage of apoptotic cells depending on serum type

The mean percentage of apoptotic CLL cells after 48 hours of incubation with FCS was significantly higher than that observed when FCS was used in combination with AS (17.28 and 11.09; respectively) (p=0.04) (Fig. 2). The incubation of CLL cells with RIT at the concentration of 10 μg/ml in the presence of FCS revealed differences in the percentage of apoptotic cells as compared to the control culture (17.6 and 11.09; respectively). Such difference was not observed when FCS alone was added to the culture. In case of FCS used in combination with AS, the DIA was 6.02 and it was significantly higher than that obtained after incubation with FCS only (DIA=0.34) (p<0.01) (Fig. 2).

Evaluation of RIT influence on caspases expression and mitochondrial membrane potential depending on serum type

The mean expressions of caspases -9, -3 and -8 as well as changes in $\Delta\Psi_m$ were significantly higher when CLL cells

Table 2. The influence of serum types on the viability of CLL cells incubated *in vitro* with rituximab in different concentrations

Incubation period		Type of serum												(p<0.05)
		FCS				ABS				AS				
		Control	Rituximab			Control	Rituximab			Control	Rituximab			
			5 µg/ml	10 µg/ml	20 µg/ml		5 µg/ml	10 µg/ml	20 µg/ml		5 µg/ml	10 µg/ml	20 µg/ml	
		1	2	3	4	5	6	7	8	9	10	11	12	
24 hours [A]	n	31	31	31	31	31	31	31	31	30	30	30	30	1 *vs* 5; 1 *vs* 9 2 *vs* 10; 3 *vs* 11 4 *vs* 12; 6 *vs* 10 7 *vs* 11; 8 *vs* 12
	Mean	16.3	16.2	16.8	16.5	14.0	18.2	18.0	18.2	12.5	26.5	27.9	30.0	
	SD	8.0	6.5	6.6	7.1	6.2	6.6	6.4	6.8	6.0	16.4	17.1	17.8	
	Range	6.2–32.5	5.6–33.1	6.6–30.9	5.6–33.6	5.6–30.5	8.5–38.8	6.4–30.9	4.7–34.0	1.7–28.6	5.0–81.2	4.9–83.2	4.8–83.9	
	DIC (%)		0.0	0.5	0.2		4.2	4.0	4.2		14.1	15.4	17.5	
48 hours [B]	n	31	31	31	31	31	31	30	31	30	30	30	29	1 *vs* 5; 1 *vs* 9 4 *vs* 8; 6 *vs* 10 7 *vs* 11; 8 *vs* 12
	Mean	27.1	29.1	29.7	30.2	21.3	26.1	26.1	25.8	18.7	34.2	37.3	37.0	
	SD	13.9	14.3	14.3	14.9	9.3	10.3	9.4	8.9	10.9	18.2	18.9	21.2	
	Range	10.2–61.8	12.4–60.4	9.8–61.3	7.4–72.6	6.3–45.1	11.3–44.3	8.5–45.4	12.3–43.6	5.5–42.9	6.6–76.8	8.4–80.8	8.6–82.4	
	DIC (%)		2.0	2.6	3.1		4.8	4.8	4.5		15.5	18.6	18.3	
(p<0.001)		A1 *vs* B1;	A2 *vs* B2;	A3 *vs* B3;	A4 *vs* B4;	A5 *vs* B5;	A6 *vs* B6;	A7 *vs* B7;	A8 *vs* B8;	A9 *vs* B9;	A10 *vs* B10;	A11 *vs* B11;	A12 *vs* B12	

FCS – fetal calf serum; ABS – serum devoid of anti-A and anti-B antibodies; AS – autologous serum; n – number of samples; Mean – mean percentage of dead cells (PI+); SD – standard deviation; DIC – drug induced cytotoxicity

Table 3. The influence of serum combinations on the viability of CLL cells incubated *in vitro* with rituximab in different concentration

Incubation period		Type of serum												(p<0.05)
		FCS + ABS				FCS + AS				ABS + AS				
		Control	Rituximab			Control	Rituximab			Control	Rituximab			
			5 µg/ml	10 µg/ml	20 µg/ml		5 µg/ml	10 µg/ml	20 µg/ml		5 µg/ml	10 µg/ml	20 µg/ml	
		1	2	3	4	5	6	7	8	9	10	11	12	
24 hours [A]	n	10	10	10	10	16	16	16	16	16	16	16	16	1 *vs* 2; 1 *vs* 3 1 *vs* 4; 1 *vs* 5 2 *vs* 10; 5 *vs* 6 5 *vs* 7; 5 *vs* 8 6 *vs* 10; 7 *vs* 11 8 *vs* 12; 9 *vs* 10 9 *vs* 11; 9 *vs* 12
	Mean	12.5	15.0	14.2	14.8	12.3	17.3	18.1	19.2	11.8	14.8	15.8	17.1	
	SD	3.1	2.0	3.0	3.0	5.2	8.0	8.3	7.7	5.1	6.2	7.1	7.8	
	Range	8.9–18.2	12.5–18.0	9.9–19.2	11.0–20.3	5.0–22.9	8.1–34.4	8.0–35.0	9.0–33.2	5.7–22.4	8.0–26.7	7.1–27.0	8.4–30.2	
	DIC (%)		2.5	1.7	2.3		5.0	5.8	6.9		3.0	4.0	5.3	
48 hours [B]	n	10	10	10	10	16	16	16	16	16	16	16	16	1 *vs* 2; 1 *vs* 3 1 *vs* 4; 1 *vs* 9 2 *vs* 10; 5 *vs* 6 5 *vs* 7; 5 *vs* 8 6 *vs* 10; 7 *vs* 11 9 *vs* 10; 9 *vs* 11 9 *vs* 12
	Mean	19.5	22.1	23.2	24.6	18.5	23.1	25.5	25.7	17.2	20.4	21.8	24.0	
	SD	8.0	8.4	9.2	9.2	9.7	10.5	10.7	10.4	7.8	9.1	9.9	11.2	
	Range	11.5–33.9	12.3–36.3	12.1–39.0	12.8–39.8	7.0–41.0	9.3–41.8	11.1–43.9	11.2–45.8	7.4–34.3	10.0–40.4	9.8–44.8	11.6–48.0	
	DIC (%)		2.6	3.7	5.1		4.6	7.0	7.2		3.2	4.6	6.8	
(p<0.01)		A1 *vs* B1;	A2 *vs* B2;	A3 *vs* B3;	A4 *vs* B4;	A5 *vs* B5;	A6 *vs* B6;	A7 *vs* B7;	A8 *vs* B8;	A9 *vs* B9;	A10 *vs* B10;	A11 *vs* B11;	A12 *vs* B12	

FCS – fetal calf serum; ABS – serum devoid of anti-A and anti-B antibodies; AS – autologous serum; n – number of samples; Mean – mean percentage of dead cells (PI+); SD – standard deviation; DIC – drug induced cytotoxicity

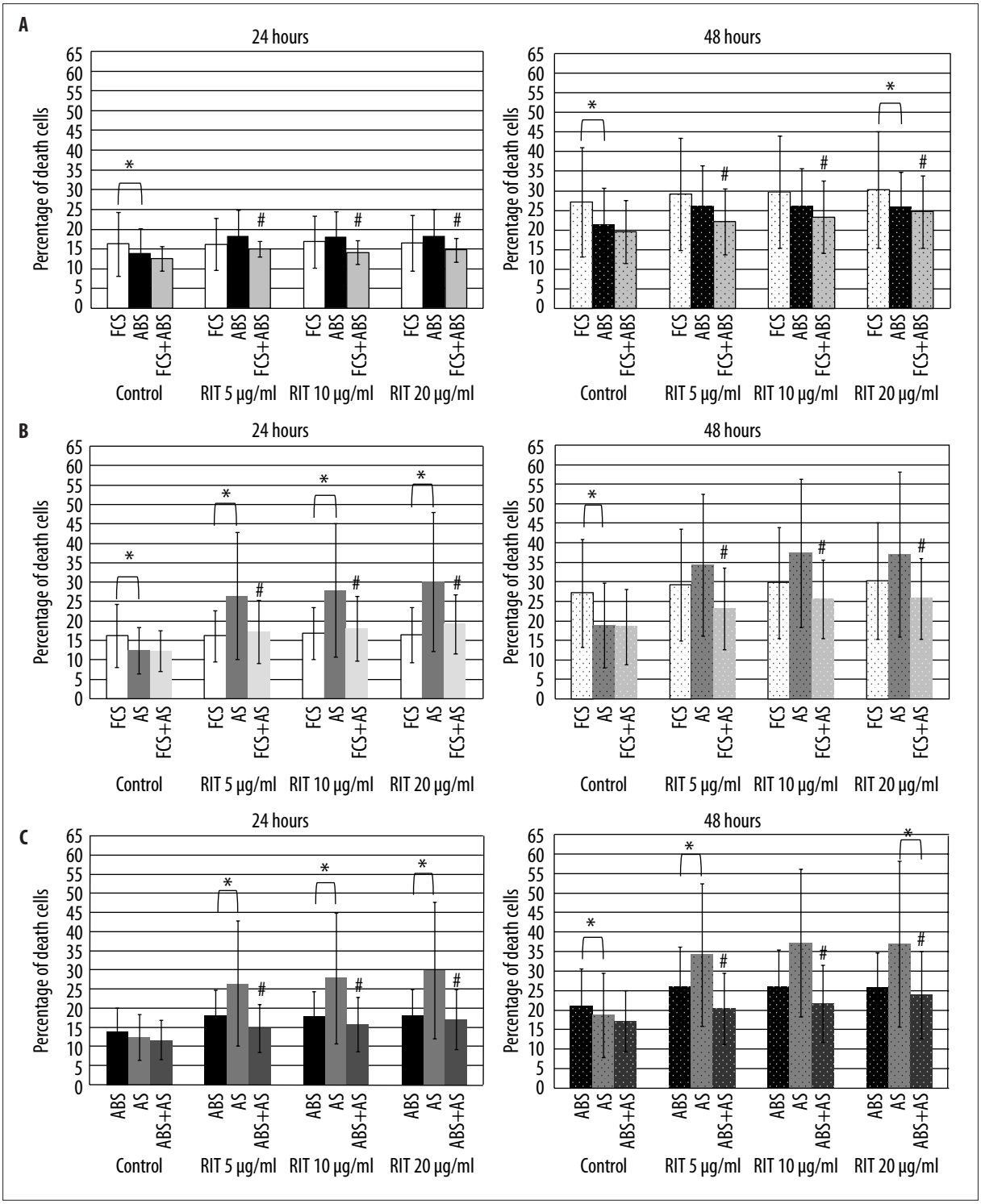

Fig. 1. The comparison of two different types of serum used separately or in combination on the viability of CLL cells incubated with different concentrations of rituximab, after 24 and 48 hours *in vitro* cultures. FCS – fetal calf serum; ABS – serum devoid of anti-A and anti B antibodies; AS – autologous serum; serum obtained from patient studied; plain columns – 24 hours incubation; columns with dots – 48 hours incubation; RIT – rituximab; * – difference statistically significant ($p<0.01$), # – difference statistically significant *vs* control ($p<0.01$)

were incubated with 10 µg/ml RIT and in the presence of FCS+AS, as compared to the control cultures (23.1 and 17.6; 22.1 and 17.3; 24.0 and 17.7; 34.5 and 28.5; respectively for caspase-9, caspase-3, caspase-8 and $\Delta\Psi_m$) (Fig. 3).

Discussion

RIT, a monoclonal antibody against the surface antigen CD20 which is currently widely used in the management of B-cell lymphoid malignancies, has a complex mechanism of action involving both antibody- and complement-dependent

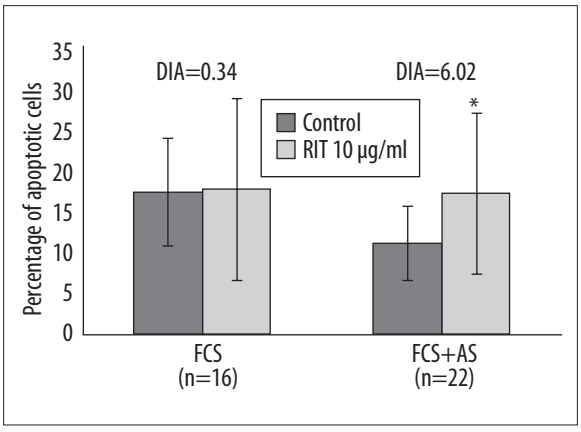

Fig. 2. The influence of fetal calf serum or combination of fetal calf serum and autologous serum on apoptosis of CLL cells incubated *in vitro* for 48 hours with 10 µg/ml rituximab. FCS- fetal calf serum; AS – autologous serum; RIT – rituximab; * – difference statistically significant vs control ($p<0.01$); DIA – drug induced apoptosis; n – number of samples

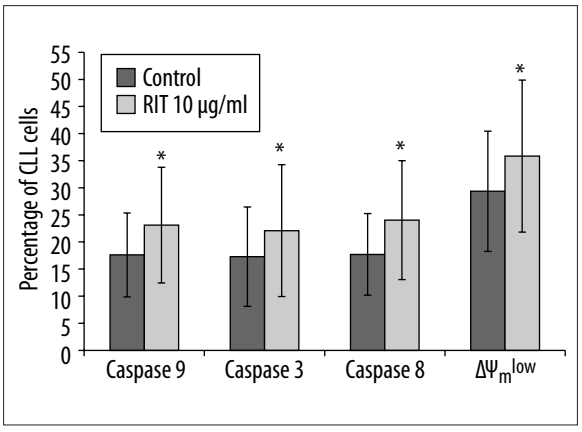

Fig. 3. The *in vitro* influence of rituximab on the percentage of CLL cells expressing caspases activity, and on mitochondrial potential ($\Delta\Psi_m$) after 48 hours incubation in the presence of FCS in combination with AS. n – (number of samples) = 27; * – differences statistically significant *vs* control ($p<0.001$)

cytotoxicity, and a direct induction of apoptosis. The importance of soluble extracellular factors for RIT action *in vivo* renders it necessary to devise appropriate cellular culture conditions for *in vitro* studies of the mechanism of its action. The type of serum added to the culture medium is crucial for providing the experimental system with all factors necessary for the drug to be able to exert its action towards target cells.

In the first part of our study we checked the influence of sera added to short-term culture on the viability of CLL cells. The cultures were supplemented with FCS, ABS or AS separately or with their 1:1 mixtures: FCS+ABS, FCS+AS or ABS+AS. We observed the highest percentage of dead cells when FCS was added to the culture separately. The viability of CLL cells was the highest when AS alone or the combinations of the FCS+AS or ABS+AS were used. Those observations favor the hypothesis that autologous serum used either separately or in combination with FCS or ABS exerts a protective action on CLL cells *in vitro*, probably due to antiapoptotic cytokines present in patients' sera *in vivo*. This finding may account, at least partially, for the generally known phenomenon that the CLL cells quickly undergo apoptosis when cultured *in vitro*, but their lifespan *in vivo* is very long. It is also possible, that the high rate of cell death in FCS-supplemented culture is attributable, at least partially, to a low concentration of albumins in such culture medium. Pettitt et al. [15] noted that the medium supplemented with FCS, in which CLL cells are usually cultured, contained a concentration of albumin twofold lower than required for its maximal antiapoptotic effect. These authors underlined that the concentration of albumin in the culture medium is important for the survival of CLL cells, since the viability of these cells was found to be consistently higher after a 4 day culture supplemented with 10 mg/ml of bovine serum albumin (BSA) than in the culture with 10% FCS.

As the type of serum itself modifies the CLL lymphocytes viability in the culture *in vitro*, this property of the culture medium must be taken into consideration when the cytotoxicity of RIT is tested in such culture. Previous publications on this issue were based on different experimental conditions; in a study of Smolewski et al. [18] 20% (v/v) of HI-FCS was used, Zent et al. [20] added to the culture human serum complement, Alonso et al. [1] incubated cells with RIT in the presence of 10% of human AB serum. In other studies, Moran et al. [11] used BSA in the concentration of 10 mg/ml, Bellosillo et al. [2] used either FCS or ABS, Patz et al. [14], Furlan et al. [7] and Johnson et al. [9] used autologous serum, and Bomstein et al. [3] compared the effect of FCS and AS.

To the best of our knowledge, the present study is the first one to compare the cytotoxic effect of RIT *in vitro* in the presence of such spectrum of sera and their combinations, on CLL cells viability and apoptosis.

We evaluated the DIC after 24 and 48 hours of incubation and, as expected, we found that a longer incubation period significantly increased cytotoxic effect of RIT. The appearance of this action of RIT was strongly dependent on the serum added to the culture medium. RIT led to a significant increase of cell death as compared to control culture only in the presence of AS. When ABS was used for the culture, RIT brought about some increase of cell death rate as compared to the controls, but this increase did not reach the threshold of statistical significance. We could not observe any cytotoxic effect of RIT in the cultures supplemented with heat-inactivated FCS. Moreover, the rate of dead cells was similar for all RIT concentrations used. It is then plausible, that the autologous serum, i.e. the natural medium for circulating CLL lymphocytes in *in vivo* conditions, prevents leukemic lymphocytes from spontaneous death *in vitro*, but enhances the cytotoxic effect of RIT on those cells. Moreover it is noteworthy, that the cytotoxic effect of RIT was significantly higher in the presence of AS as compared to ABS. It therefore seems legitimate to hypothesize that the drug in question fails to exert its action in the presence of HI-FCS not only due to the lack of proteins of complement system in this medium, but also because of the absence of some other factors which are present in the patient's serum but not in healthy AB donors. In other terms, RIT action towards CLL cells probably requires the presence of some exogenous substances contained solely in the patient's serum.

Our results are somewhat discordant with observations published by Bellosillo et al. [2] who evaluated *in vitro* the cytotoxic effect of RIT on CLL cells in the presence of ABS. They demonstrated that no effect was observed when cells were incubated with RIT in the presence of 10% heat-inactivated FCS, however the addition of human AB serum as a source of complement produced a cytotoxic effect after 24 hours of incubation. Additionally, this effect was dose-dependent for both RIT and ABS and could be detected using doses as low as 10 µg/ml and 1% ABS. However, the most significant cytotoxic effect was observed when 50 µg/ml and 10% ABS were used. Our failure to confirm the dose-dependency of RIT action on CLL cells might result from lower drug concentration than that used by Bellosillo et al. [2], as well as a smaller size of our sample.

Our above-quoted findings suggest that the autologous serum provides the best experimental conditions for *in vitro* assays of RIT toxicity. However, when planning a set of experiments one might face an ethical difficulty to obtain a quantity of patients' sera which would be sufficient for performing the experiments in the best conditions. We then wished to test the different combinations of two out of three sera in order to find out which one would be suitable for this purpose and might be used instead of AS as a single agent.

The combination of FCS+AS allowed higher DIC than FCS used alone or together with ABS. Therefore we chose to carry on further experiments on the mechanism of RIT-dependent apoptosis in the culture supplemented with FCS mixed with AS. We checked the total percentage of apoptotic cells and then the different stages of apoptotic cascade (changes in mitochondrial transmembrane potential and the expression of active forms of caspases-9, -3 and -8) were assessed.

Firstly, we compared the occurrence of spontaneous apoptosis of CLL cells after the 48-hour culture in the presence of FCS alone and FCS combined with AS. The mean percentage of cells undergoing apoptosis, i.e. displaying the affinity to Annexin V, was 17.28 and 11.09 respectively. This difference was statistically significant (p=0.04) which means that the presence of AS in the culture medium decreases not only the overall percentage of cell death, but also the occurrence of apoptosis.

Therefore, we checked the influence of RIT on apoptosis of CLL cells cultured in the presence of FCS combined with AS and found that both the expression of all caspases and the changes in $\Delta\Psi_m$ were significantly higher when the cells were incubated with RIT than in control cultures. This suggests that both extrinsic and intrinsic apoptotic pathway are involved in RIT-dependent apoptosis. RIT added to the cultures supplemented with FCS in association with AS allowed to obtain on average 17% of apoptotic cells, which is significantly higher than the control culture (11%; DIA=6.02). Our results are not very different from those obtained by Patz et al. [14] who used AS separately at the concentration of 20%, and incubated the CLL cells in the presence of 10 mg/ml of RIT. Those authors obtained 13% and 20% of apoptotic cells, respectively for the whole peripheral blood and freshly isolated CLL cells. It corroborates our observation that the use of AS in association with FCS provides appropriate conditions for *in vitro* studies on RIT-induced CLL cells apoptosis.

Conclusions

In conclusion, we found that in short-term CLL culture, the viability of cells was the lowest when FCS was used alone, whereas the highest *in vitro* viability of these cells was stated in the presence of AS. In contrast to the spontaneous cell death decreased by the use of AS, we found that this serum used alone or in combination with FCS was the only one to significantly enhance the cytotoxic effect of RIT on CLL cells *in vitro*. Additionally we stated, that the RIT action on CLL cells triggers an apoptosis cascade including the activation of some caspases and changes in mitochondrial transmembrane potential. Finally, we suggest that the antitumor activity of RIT against CLL cells is mediated by complex cytotoxic mechanisms, caspase-dependent apoptotic pathway and a possible action of some other extracellular factors present in the patients' sera. Therefore, the use of appropriate serum as a supplementation of culture medium is essential for achieving the antitumor effect of RIT *in vitro*.

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