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PROTECTION OF BALB/C MICE AGAINST *BRUCELLA ABORTUS* 544 CHALLENGE BY VACCINATION WITH COMBINATION OF RECOMBINANT HUMAN SERUM ALBUMIN-L7/L12 (*BRUCELLA ABORTUS* RIBOSOMAL PROTEIN) AND LIPOPOLYSACCHARIDE

Iraj Pakzad[1], Abbas Rezaee[2], Mohammad J. Rasaee[2], Ahmad Zavaran Hossieni[2], Bahman Tabbaraee[3], Anoshirvan Kazemnejad[2]

[1]*Department of Microbiology, Ilam University of Medical Sciences, Ilam / Iran;* [2]*Faculty of Medical Sciences, Tarbiat Modaress University, Tehran / Iran;* [3]*Department of Bacterial vaccines, Pasteur Institute of Iran, Tehran / Iran*

ABSTRACT

Background: The immunogenic B*rucella abortus* ribosomal protein L7/L12 and Lipopolysaccharide (LPS) are promising candidate antigens for the development of subunit vaccines against brucellosis. ***Objective****:* This study was aimed to evaluate the protection of combination of recombinant HSA-L7/L12 fusion protein with LPS in *Balb/c mouse*. ***Materials and Methods****:* The recombinant HSA-L7/L12 fusion protein in *Saccharomyces cerevisiae* was expressed and purified by affinity chromatography column. LPS was extracted by *n*-butanol, purified by ultracentrifugation. BALB/c mouses were immunized in 9 groups with PBS, HSA, tHSA-L7/L12, L7/L12, LPS, LPS+ HSA, LPS+ tHSA-L7/L12, LPS+ L7/L12, *B. abortus S19*. ELISA, LTT tests and challenging two weeks after last injection were carried out. Bacterial count of spleen of immunized BALB/c mouse was done four weeks after challenging with virulent strain *B.abortus* 544. ***Results****:* In ELISA test the specific antibodies of tHSA-L7/L12 exhibited a dominance of immunoglobulin IgG1 over IgG2a. LPS-HSA and tHSA-L7/L12+ LPS produced a significantly higher antibody titer than LPS alone and L7/L12+LPS ($P<0.05$). The predominant IgG subtype for LPS and L7/L12+LPS were IgG3. However, tHSA-L7/L12+ LPS and LPS+HAS elicited predominantly IgG1 and IgG3 subtypes.

In addition, the tHSA-L7/L12 fusion protein and L7/L12 elicited a strong T-cell proliferative response upon restimulation *in vitro* with recombinant tHSA-L7/L12 and L7/L12, suggesting the induction of a cellular immunity response in vivo. However, there was no significant difference proliferative response in L7/L12 and tHSA-L7/L12 fusion protein ($P > 0.05$). The combination of tHSA-L7/L12 fusion protein with LPS and *B. abortus S19* induce higher level of protection against challenge with the virulent strain *B. abortus* 544 in BALB/c mice than other groups ($P = 0.005$). ***Conclusions:*** The combination of tHSA-L7/L12 fusion protein with LPS had higher protective ability than LPS and fusion protein distinctly.

Key words: Lipopolysaccharide, *Brucella abortus*, fusion protein, L7/L12, subunit vaccine

INTRODUCTION

At present, there is no vaccine licensed for use against brucellosis in humans. Several live attenuated Brucella vaccines have been tried in humans, but none was found to be satisfactory [1-3]. However, no safe and effective vaccine is available for human use. Several strategies such as development of subunit vaccines [4], utilization of bacterial vectors [5] and over expression of protective homologous antigen [6] have already been introduced to develop *Brucella* vaccine. A number of genetically defined mutants that are attenuated for growth in macrophages or in animal models have been developed recently, but their suitability for human use has not been evaluated [7-11]. The recombinant L7/L12 protein and plasmid encoding the l7/l12 gene can elicit strong CMI and engender protection from *Brucella* infection in mice; however, the protective effect was much lower than what the live attenuated *B. abortus* vaccine S19 provides [4, 11, 12]. The protective role of other types of L7/L12 based-vaccines utilizing different vectors such as vaccinia virus and *Lactococcus lactis* have recently

* *Corresponding author:* Dr. Iraj Pakzad (PhD); Department of Microbiology, Ilam University of Medical Sciences; Ilam, Iran I.R., Tel: 988412231326 Fax: 988412227136; Email: pakzad_i2006@yahoo.com

been reported [13-15]. There have been a number of studies to show the protective effect of LPS Brucella subunit vaccines in animal models. Winter *et al*. showed that a single vaccination with a complex consisting of porin and smooth lipopolysaccharide from *B. abortus* strain 2308 provided significant protection against challenge with the same strain, equivalent to the protection achieved by vaccination with live attenuated strain 19 [16]. Jacques *et al*. showed that mice immunized with a *Brucella* O-polysaccharide-bovine serum albumin conjugate were protected against challenge with *B. melitensis* strain H38 [17]. Brucella O-polysaccharide-specific monoclonal antibodies were shown to provide protection against challenge with *B. melitensis* and *B. abortus* smooth strains [18,19]. Bhattacharjee *et al*. showed that mice subcutaneous and intranasal immunized with a *Brucella melitensis* Lipopolysaccharide Subunit Vaccine were protected against challenge with *B. melitensis* strain 16 M [20].

However, there is evidence that polyvalent vaccines, including protein and DNA vaccines, can engender more effective protection than univalent vaccines [21, 22]. Thus, polyvalent vaccines combining L7/L12 with other immunogenic antigen(s) such as Lipopolysaccharide (LPS) of *Brucella*, are important components in humoral immunity against brucellosis [23].

The anionic and amphiphilic nature of lipid A of LPS enables it to bind to numerous substances which are positively charged and also possess amphipathic character, such as Human Serum Albumin (HSA) (David, 1999). In order to prepare a natural complex of LPS with L7/L12, we have previously produced fusion protein tHSA-L7/L12 in *S. cerevisiae* and shown immunization of mice with tHSA-L7/L12 provided significant protection against disseminated infection of spleens [24]. In this study, protection of combination of this fusion protein with LPS was evaluated.

MATERIALS AND METHODS

Production and purification of tHSA-L7/L12 fusion protein

In order to construct yeast expressing vector for the tHSA-L7/L12 fusion protein, the *l7/l12* ribosomal gene was amplified by PCR. The expression plasmid pYtHSA-L7/L12 was constructed by inserting the *l7/l12* gene into the pYHSA5 shuttle vector (containing inulinase signal sequence, *HSA* gene and Gal10 promoter). The recombinant vector was transformed into *S. cerevisiae* and was then induced by galactose. The secreted recombinant fusion protein was detected in supernatant by SDS-PAGE and confirmed by western blot analysis using anti-HSA and anti-L7/L12 antibodies. The supernatant of induced cells were collected by centrifugation (1300 $\times$g, 3 min). Phenylmethylsulphonyl fluoride (PMSF, Merck) was added and the expressed protein from supernatant was precipitated by 60 % ammonium sulfate (Merck), dialyzed against water and purified by affinity chromatography column CN-Br activated sepharose CL-4B (Biogen) and anti-HSA antibody [24].

Preparation of purified LPS: The LPS was extracted from killed *B.abortus* 99 cells and purified by a method described previously [10, 25]. The protein content was estimated by Bradford's method of, with bovine serum albumin as a standard. Nucleic acid was estimated by measuring the A260 nm. The LPS content was determined by 1,9 dimethyl methylene blue with standard LPS in A510 nm [26]. SDS-PAGE was carried out by procedure [27].

Immunization and challenge of mice

Nine groups of female 6-8 weeks old BALB/c mice (15 mice in each group) were injected intraperitoneally with PBS, HSA (10 μg), tHSA-L7/L12 (10 μg), L7/L12 (10 μg) [a donation from Dr. Hamid Abtahi, Iran], LPS (10 μg), LPS (10 μg) + HSA (10 μg), LPS (10 μg)+ tHSA_L7/L12 (10 μg), LPS (10 μg) + L7/L12(10 μg), *B. abortus S19* (5×10^4 CFU). Injection volumes were 0.2 ml/mouse. A second dose was given 4 weeks after the first dose. Blood was collected from five killed mice in each group 2 weeks after the second dose of vaccine. Sera were collected and stored at -20°C until they were analyzed for antibody by an enzyme-linked immunosorbent assay (ELISA). Two weeks after the final vaccination, five mice from each group were challenged intraperitoneally according to published methods [28], but a relatively higher dose of *B. abortus* strain 544 (5×10^5 CFU) was used here. Four weeks postchallenge, the mice were killed by cervical dislocation, and their spleens were removed aseptically and weighed. Each spleen was homogenized in sterile PBS, serially diluted 10-fold, and plated in triplicate on trypticase-soy agar. *B. abortus* 544 colonies were counted after 3 days of incubation at 37°C with 10 % CO_2. The results were represented as the mean log CFU $\pm$ SD per group; this experiment was repeated three times. Statistical analyses were performed with a Student's *t* test. Log_{10} units of protection were cal-

culated as the mean $\log_{10}$ numbers of CFU of the negative control group (PBS) minus the mean $\log_{10}$ numbers of CFU of the experimental group.

ELISA

The presence of serum IgG specific to LPS, tHSA-L7/L12 and recombinant L7/L12 (a donation from Dr. Hamid Abtahi, Iran) was determined by indirect ELISA, two weeks after the final immunization. The purified LPS (10 μg/ml), tHSA-L7/L12 (5 μg/ml), L7/L12 (5 μg/ml) and HSA (5 μg/ml) were diluted in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene plate (100 μl/well; Nunc-Immuno plate with MaxiSorp surface). After overnight incubation at 4°C, the plates were washed, blocked, and then incubated with serially diluted sera for 3 h at room temperature. Following another washing, IgG specific rabbit anti-mouse horseradish peroxidase conjugates were added (100 μl/well) at the appropriate dilutions. After 30 minutes of incubation at room temperature, the plates were washed, and 100 μl of substrate solution (200 μmol of *o*-phenylenediamine and 0.04 % H_2O_2) was added to each well. The enzymatic reaction was allowed to proceed for 20 min at room temperature, after which the reaction was stopped with the addition of 50 μl of 2 M sulfuric acid/well. Absorbance at 450 nm was determined with an ELISA reader (Labsystems Multiskan MCC/340; Fisher Scientific, Pittsburg, PA). The titer, expressed in optical density (OD) units was obtained by multiplying the reciprocal dilution of the serum by the OD (*A*450nm) at that dilution [10]. All assays were performed in triplicate and repeated three times.

Splenocyte culture and lymphocyte proliferation

Two weeks after the last immunization, mice were sacrificed, and their spleens were removed under aseptic conditions. Single-cell suspensions were prepared from the spleens, and the red blood cells were lysed with ACK (150 mM NH4Cl, 1 mM $KHCO_3$, 0.1 mMNa2 · EDTA, pH 7.3) solution. Splenocytes were cultured at 37°C in 5 % CO_2 in a 96-well flat-bottom plate at a concentration of 4×10^5 cells/well in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10 % heat-inactivated fetal calf serum (Sigma), in the presence of 0.08 μg of purified tHSA-L7/L12 protein and 0.5 μg of L7/L12 protein or no additives (unstimulated control). The cells were cultured for 3 days and pulsed for 8 h with 0.4 μCi of [3H] thymidine (50 Ci/mmol; Amersham) per well. The radioactivity incorporated into the DNA was measured in a liquid scintillation counter. Cell proliferation was expressed as mean counts per minute (cpm) from five mice for each group. All assays were performed in triplicate and repeated three times.

Statistical analysis of the data

Antibody titers of groups of mice were expressed as means ± standard deviations. The intensities of bacterial infection in spleen were expressed as the mean log CFU ± standard deviation per infected organ. To compare the mean values in ELISA titers and log CFU per infected spleen was used ANOVA Analysis of Variance and then followed by multiple Tukey comparisons.

This study was approved by the Tarbiat Modarres University/Tehran Ethical Committee.

RESULTS

Extracellular production of fusion protein tHSA-L7/L12 in S. cerevisiae

The pYtHSA-L7/L12 shuttle vector has signal sequence, and the product was expected to secretion into extracellular. In induced culture, a band apparent molecular mass of ~ 52 KDa was present in the supernatant induced cell. Considering the length of two fused genes this weight was expected.

Western blotting of fusion protein and Extracellular protein purification

Anti-HSA and anti-L7/L12 antibody from rabbit was used for blotting. The result shows that fusion protein can be detected by both antibodies. After precipitation of supernatant protein by ammonium sulfate 60 % and overnight dialyzing against water that was purified in affinity chromatography, the amount of purified fusion protein was 0.5 mg/liter (Fig. 1).

Characterization of LPS of *B. abortus*. Purified LPS from *B. abortus* by butanol extraction was shown to have < 2% (wt/wt) contamination by protein and <1 % (wt/wt) contamination by nucleic acids. LPS was added to 14% polyacrylamide SDS-PAGE gels containing 4 M urea, stained with silver, resulting in patterns seen previously with LPS of *B. abortus* [25].

Humoral immune response elicited byL7/L12, tHSA-L7/L12 and LPS immunization

Sera collected 2 weeks after the last immunization were assayed for the presence of tHSA-L7/L12, LPS, specific antibodies by ELISA. The results showed that the total IgG titer of tHSA-L7/L12 from the hyperimmune sera of mice immunized with tHSA-L7/L12, L7/L12 , HSA, and live S19 strain reached 1:21,800,

1:14,800, 1:15,100, 1:54,200 respectively (Table 1). This results show immunization with tHSA-L7/L12 and live S19 strain elicited much higher humoral immune responses against tHSA-L7/L12 in mice .To analyze the potential roles of IgG subtypes in the mechanism of preventing *Brucella* infection, we examined the proportion of Th1-associated IgG2 and Th2-associated IgG1 in the total serum IgG of each group with ELISAs by diluting the sera at 1:100. The analysis of IgG subtypes showed a significant increase in IgG1 and IgG2a from L7/L12 group, tHSA-L7/L12 group, and live S19 group compared with HSA ($P < 0.01$) (Table 1). However, the ratio of the Th1-associated versus Th2-associated IgG subtype may reflect a substantial increase in certain IgG subtypes thus, the IgG2a/IgG1 value for each group was calculated. Results showed that the ratios were 0.63, 0.65 and 0.78 after L7/L12 , tHSA-L7/L12 and S19 immunization, although the tree types of antigen had higher IgG1 and/or IgG2a levels than the HSA, which suggested that the live S19 strain had a more dominant Th1 response than other groups ($P < 0.05$). This result shows L7/L12 and tHSA-L7/L12 induce Th2 response than Th1. The results showed that the total IgG titer of LPS from the hyper-immune sera of mice immunized with LPS, LPS + HSA, tHSA-L7/L12 + LPS, L7/L12 + LPS and live S19 strain reached, 1:14,853, 1:24,512, 1:23,710, 1:15,010 and 1:36,100 respectively (Table 2). Anti-LPS IgG antibody titers from S19 strain group was the highest IgG titer, followed by the groups that received LPS + HSA and tHSA-L7/L12 + LPS. There was no significant difference in the anti-LPS IgG antibody responses in mice immunized with LPS and L7/L12 + LPS. LPS + HSA and tHSA-L7/L12 + LPS produced a significantly higher antibody titer than LPS alone, and L7/L12 + LPS

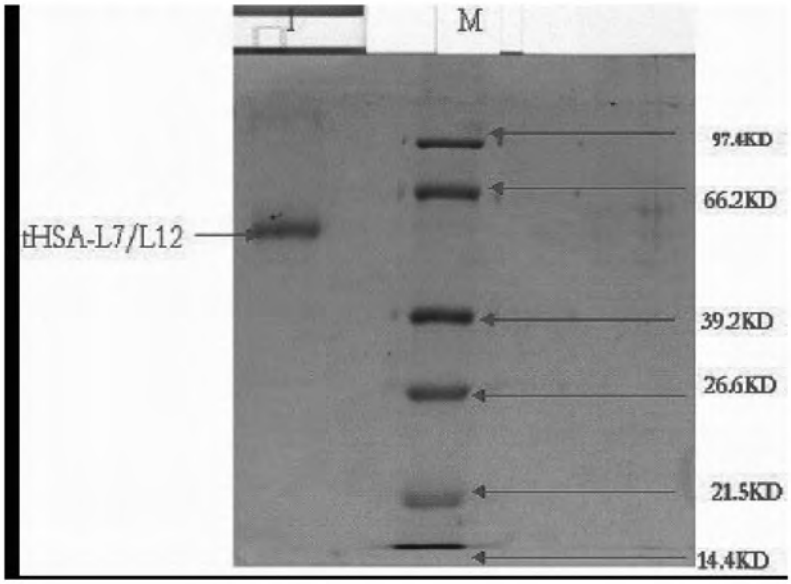

Figure 1 - Comassie blue-stained SDS-PAGE of purified protein by affinity chromatography, M (marker), lane 1: purified fusion protein tHSA-L7/L12

Table 1 - Anti-tHSA + L7/L12 IgG ELISA titers and IgG subtype profiles of mice two weeks after immunization[a]

Group	Vaccine	Anti -tHSA_L7/L12 IgG ELISA titer (mean ± SD)	Anti-tHSA_L7/L12 IgG subtype ELISA titer (mean ± SD)	
			IgG1	IgG2a
1	L7/L12	14,245 ± 238	8,875 ± 238	5,667 ± 214
2	tHSA_L7/L12	21,800 ± 715	12,800 ± 715	8,345 ± 567
3	HSA	15,178 ± 274	9,878 ± 274	4,034 ± 245
4	*B. abortus* S19	54,257 ± 3867	51,257 ± 3867	40,256 ± 3126
5	PBS	<2	<2	<2

[a]Mice were immunized i.m. Two doses of vaccine were given 4 weeks apart. Sera were collected from five of each group at 2 weeks after immunization. The data are expressed in OD units.

($P < 0.05$). The LPS IgG subtype titers are shown in Table 2. The predominant IgG subtype for LPS and L7/L12 + LPS were IgG3. However, tHSA-L7/L12 + LPS and LPS-HAS elicited predominatly IgG1 and IgG3 subtypes.

Cellular immune response induced by tHSA-L7/L12

To further investigate the CMI response induced by tHSA-L7/L12 fusion protein and L7/L12, live S19 strain we analyzed the proliferative T-cell response. As shown in Fig. 2, all three vaccines (tHSA-L7/L12, L7/L12 and live S19 strain) induced significant T-cell proliferation in immunized BALB/c mice in response to recombinant tHSA-L7/L12 and L7/L12, compared with PBS or HSA immunization ($P = 0.01$). Though in the tHSA-L7/L12 group and L7/L12 group, it could prime a specific T-cell proliferative response significantly; however, these effects were lower than that of the live S19 strain ($P = 0.05$). The T-cell proliferation in response to tHSA-L7/L12 compare with L7/L12 in three groups above mentioned was not significant ($P > 0.05$). It is worth mentioning, T-cell proliferation in immunized BALB/c mice with tHSA-L7/L12 and L7/L12 in response to two recombinant protein were not significant ($P > 0.05$). Among the three vaccines, the live S19 strain showed the strongest stimulant effect ($P < 0.05$). As a stimulus control, was used of RPMI 1640 medium.

Protection of mice after challenge. Two weeks after the last immunization, the vaccinated mice were challenged with intraperitoneal injection of Brucella virulent strain 544. Four weeks after the challenge, the infection level in each mouse was evaluated by determining the CFU in the spleen. Data from independent groups that immunization with tHSA-L7/L12 and L7/L12 resulted in a significantly higher degree of protection (1.4 and 1.3-log increase in protection respectively) than the controls that received PBS and HSA ($P < 0.05$), (Table 3). Protection of recombinant L7/L12 was already confirmed by other researcher [1]. Our result shows protection of tHSA-L7/L12 was not significantly different from protection of L7/L12 ($P > 0.05$). To compare the extent to which mice could be protected, we included the live *B. abortus* strain S19 group for immunization, and it induced 2.9-log protection compared with the PBS group ($P < 0.01$). The protection conferred by LPS, LPS + HSA, tHSA-L7/L12 + LPS and L7/L12 + LPS was statistically significant and different from that obtained with PBS ($P = 0.007$). The LPS + HSA and tHSA-L7/L12 + LPS vaccines provided significant protection compared to LPS ($P = 0.01$), the greatest protection was provided with *B. abortus* S19. The differences between the protections afforded by S19 compare LPS and LPS-HSA were statistically significant ($P = 0.01$). No reduction in the frequency of CFU was observed in animals injected with HSA compared to the PBS group (Table 3).

DISCUSSION

Immunity against brucellosis may require induction of both cellular and humoral immunity. Most individual *Brucella* antigens do not possess the ability to induce a protective response by themselves [29]. Therefore, an effective subunit vaccine will likely require a combination of several antigens [30,31].

Outer membrane antigens, specifically LPS, are in direct contact with the host humoral immune system. For this reason, LPS may be one of the main components of a potential subunit vaccine [29,32]. Previously, L7/L12 has been reported as a most im-

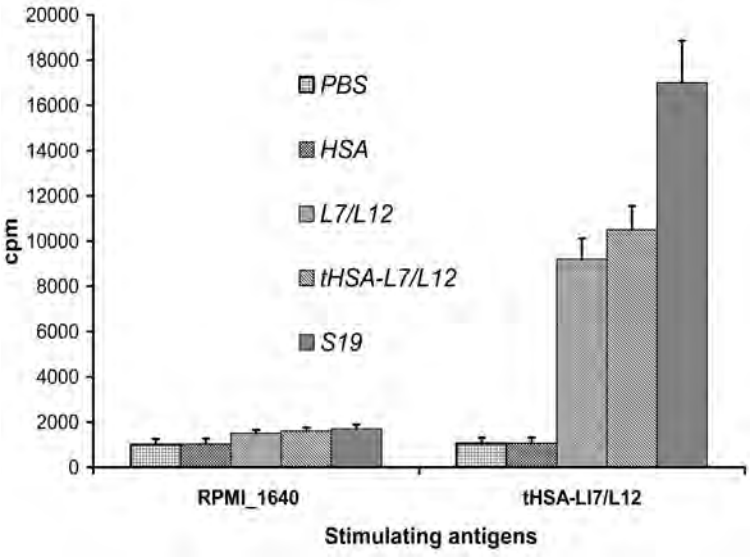

Figure 2 - Lymphocyte proliferation assay. BALB/c mice were immunized with tHSA-L7/L12 fusion protein, rL7/L12, strain S19, with PBS and HSA as negative immunization controls. The T-cell proliferation response was measured 2 weeks after the last immunization. Splenocytes from each group were prepared from 4×10^5 cells per well in a 96-well flat-bottom plate and stimulated in vitro with purified tHSA-L7/L12 (2 μg/ml) or the RPMI 1640 medium (control) as antigens. Each sample was assayed in quadruplicate wells. Data represent the mean cpm $\pm$ SD from five each group of five mice, $P < 0.01$

Table. 2 Anti-LPS IgG ELISA titers and IgG subtype profiles of mice two weeks after immunization[a]

Group	Vaccine	Anti-LPS IgG ELISA titer (mean ± SD)	Anti -LPS subtype ELISA titer (mean ± SD)			
			IgG1	IgG2a	IgG2b	IgG3
1	L7/L12+LPS	15,012 ± 6,263	1,745 ± 818	786 ± 487	1,229 ± 706	12,226 ± 4225
2	tHSA_L7/L12+LPS	23,710 ± 6,875	18,157 ± 6,266	2,018 ± 1473	1,448 ± 946	16,062 ± 5073
3	HSA+LPS	24,512 ± 7,346	19,157 ± 7,266	2,318 ± 1,673	2,448 ± 1346	17,862 ± 6,765
4	LPS	14,853 ± 5,996	1,045 ± 732	883 ± 786	1,329 ± 836	13,226 ± 3,025
5	*B.abortus S19*	36,100± 8,347	28,157 ± 6,465	2,018 ± 1,673	1,448 ± 1,146	26,062 ± 5,876
6	PBS	<2	<2	<3	<3	<3

Table 3 - ***Protection of mice against challenge with B. abortus 544 after immunization with various vaccines***

Vaccine	Mean ± SD	P value	
		Log CFU/spleen	Log protection
PBS	4.96 ± 0.23	0.00	>0.05
HSA	4.86 ± 0.26	0.1	>0.05
L7/L12	3.75 ± 0.27	1.2	<0.05
HSA+LPS	3.91 ± 0.16	1.0	<0.05
LPS	4.271 ± 0.02	0.7	<0.05
HSA-L7/L12	3.56 ± 0.16	1.4	<0.05
tHSA-L7/L12+ LPS	3.01 ± 0.14	1.9	<0.05
L7/L12+ LPS	3.45 ± 0.15	1.5	<0.05
S19	2.14 ± 0.18	2.82	<0.01

portant protein of this bacterium to stimulate cellular immunity. Immunity induced by L7/L12 recombinant protein has been previously demonstrated [11,33,34]. Recently, it has been recommended that a suitable subunit vaccine must contain a combination of LPS with L7/L12. Supporting this concept are reports that polyvalent vaccines can induce a more intensive immune response than a univalent vaccine [21,22].

We have previously shown immunization of mice with tHSA-L7/L12 provided significant protection against disseminated infection of spleens [24]. In the present work we compared the protective of combination of tHSA-L7/L12 +LPS with purified *B.abortus* LPS and tHSA-L7/L12 +LPS distinctly.

Immunization with the recombinant fusion protein tHSA-L7/L12 and L7/L12 could induce remarkable titres of total IgG (1:21,800, 1:14,245, respectively); however, the titres were less than those induced by live S19 strain (1:54,257). The IgG subtype assay demonstrated that the ratio of IgG2a/IgG1 in the fusion protein group and L7/L12 group (0.63 and 0.65, respectively) were much lower than that of the live S19 immunization group (0.78), suggesting that fusion protein vaccine similar to L7/L12 elicited a moderate Th1-type cellular immune response. The results showed that the total IgG titer of LPS from mice immunized with, LPS+HSA, tHSA-L7/L12+ LPS, (1:24,512, 1:23,710 ,respectively) higher than mice immunized with LPS, LPS+L7/L12(1:14,853, 1:15,010 , respectively) (Table 2), this indicates interaction between HSA and LPS, anti-LPS IgG antibody titers from S19 strain group was the highest IgG titer, followed by the groups that received LPS+HSA and tHSA-L7/L12+LPS. There was no significant difference in the anti-LPS IgG antibody responses in mice immunized with LPS and L7/L12+LPS. LPS+HSA and tHSA-L7/L12+LPS produced a significantly higher antibody titer than LPS alone, and L7/L12+LPS ($P<0.05$). These findings are consistent with reports that confirmed induce protection by O-polysaccharide-bovine serum albumin conjugate [17], LPS and LPS-GOMP in mouse against virulent strain [20, 35]. The mechanism of antibody-mediated protection against brucellosis has not been well described. The complement-mediated bacterial killing [36], antibody-dependent cytotoxicity by NK cells or macrophages, and phagocytosis and subsequent killing by activated macrophages [37,38] are potential mecha-

nisms of protection in which antibody might play a role.

The LPS IgG subtype titers are shown in Table 2. The predominant IgG subtype for LPS and L7/L12+ LPS were IgG3. However, tHSA-L7/L12+LPS and LPS+HAS elicited predominatly IgG1 and IgG3 subtypes.

The lymphocyte proliferation assays demonstrated that tHSA-L7/L12 fusion protein induced a significant T-cell response similar to L7/L12. However, the $CD4^+$ and $CD8^+$ subtypes of T cells that were primed were not identified. However, the difference in T-cell proliferation in immunized BALB/c mice with tHSA-L7/L12 and L7/L12 was not statistically significant ($P>0.05$), suggesting that the fusion protein could induce a similar T-cell response as L7/L12.

The S19 strain conferred the highest protection against *Brucella* infection of all groups in this study. It is possible that S19 can infect the host cells efficiently and produce endogenous antigens in antigen-presenting cells. These results are in agreement with other observations [39]. Although the tHSA-L7/L12+LPS fusion protein stimulated a lower immune response than the S19 strain especially in terms of cellular immunity, it still conferred 1.9-log protection. Protection of recombinant L7/L12 was already confirmed by others [4].Our result showed that the protection of tHSA-L7/L12 was not significantly different from that of L7/L12 ($P>0.05$). This indicates that the fusion of HSA with L7/L12 causes an increase in complexity of this protein without affecting protection. In another findings suggest protection conferred by tHSA-L7/L12 + LPS was higher than tHSA-L7/L12 and LPS distinctly.

In conclusion, the combination of tHSA-L7/L12 fusion protein with LPS had higher protective ability than LPS and fusion protein distinctly.

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CORRELATION OF XMAP AND ELISA CYTOKINE PROFILES; DEVELOPMENT AND VALIDATION FOR IMMUNOTOXICOLOGICAL STUDIES *IN VITRO*

Eleonora Codorean[1]*, Cornelia Nichita[2], Lucian Albulescu[1], Elena Răducan[1], Ionela Daniela Popescu[1], Alina Constantina Ioniță[1], Radu Albulescu[1,2]

[1]*„Victor Babeș" National Institute of Pathology, Bucharest, Telephone / fax: +4021 319 45 28; 0722 608 936;*
[2]*National Institute for Chemical Pharmaceutical R&D, Bucharest, Telephone:+4021 322 29 16; Fax:+4021 322 29 17*
**E-mail address: imunotox@vbabes.ro*

ABSTRACT

There is an emerging trend in immunotoxicological studies to use the multiplex technologies for testing the safety and the efficacy of new pharmaceuticals by using cytokines profiling as biomarker. The Luminex® 200™ xMAP (multi-analyte profiling) technology provides simultaneous measurement of multiple cytokines in small sample volumes, expressing rapidly the differences between various test compounds. The aim is to develop and validate the Luminex® 200™ multiplex immunoassays by correlation with ELISA (enzyme-linked immunosorbent assays) for implementation in evaluating cytokine profiling in immunotoxicological studies *in vitro*.

Methods. Human peripheral whole blood from healthy subject diluted 1+4 with RPMI 1640 was cultured 48 hours in 28 experimental variants: control, in presence of mitogens, bioflavonoid extracts (from *Crataegus monogyna* and *Echinacea purpurea*) as cytoprotectors and with a toxic compound [$Pb(NO_3)_2$]), separately or variously combined. IL-1β and IL-2 were comparatively performed by xMAP and ELISA immunoassays from the same sample to initialize validation of multiplex cytokine panel: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, usually performed by Luminex® 200™ system in our immunotoxicological studies.

The results indicate similarly typed trends of cytokine values obtained by both methods, with comparable relative changes in presence of mitogens, bioflavonoids and toxic, respectively. Although xMAP absolute cytokine values were higher than ELISA values, the correlation between multiplexed assay and ELISA was good for IL-1β and IL-2 with positive correlation coefficients near to 1.

Conclusions. Quantitative differences between absolute values for IL-1β and IL-2 obtained by xMAP and ELISA assays are found, but the relative values are comparable and the two methods keep similar trends in similar exposure conditions.

The performance parameters of the xMAP assay and the good correlation coefficients with the „gold standard" ELISA recommend to validate the multiplex assay for analyzing cytokine profiles in immunotoxicological studies *in vitro*.

Key words: *in vitro*, multiplex immunoassay, ELISA, cytokine profile, validation.

INTRODUCTION

Cytokines are important modulators of immune response pathways, secreted by cells of the immune system and interact with these cells modulating their following activities. In the presence of various stimuli, released cytokines can alter the behaviour and the properties of the immune or other cell types. *In vivo* or *in vitro* levels of multiple cytokines may reflect states of immune dysfunction and/or an immune-related disease and cytokine profiling has become an established method for the identification and characterization of the disease-associated immune responses [1], [2], [3].

The expression pattern of cytokine profiles frequently required for the characterization of the immune system status in immunotoxicological studies, provides rapid information about environmental exposure, food contamination or about safety and efficacy of new therapeuticals [4], [5], [6], [7].

Classically, analysis of cytokine expression patterns has been performed by enzyme-linked immunosorbent assays (ELISA) for each separate analyte, requiring large quantities of cells, more time and costs, to characterize a complete cytokine profile.

New xMAP array technology performed by Luminex® 200™ system, facilitates the simultaneous

evaluation of multiple immune mediators with advantages of higher throughput, smaller sample volume, and lower cost, cytokine profile and other biomarkers becoming powerfully requisite in detecting or monitoring various pathologies, including cancer [8-11].

In the last years, several papers have been focused on the development and validation of the multiplex assay by correlation with ELISA method on *in vitro* and *ex vivo* studies [4], [5], [12], [13], [14].

The paper presents the development of *in house* validation of Luminex® 200™ multiplex bead array for testing cytokine profile as biomarker of the immunomodulatory effects of bioflavonoids *in vitro*.

MATERIALS AND METHODS

Venous blood collected from a healthy subject directly into vacutainer containing sodium heparin were diluted 1+4 with RPMI 1640 culture media containing 1% Penicillin/Streptomycin. Samples of 200 µL were distributed in 96 wells microplate and cultured 48 h at 37°C in various exposure conditions (Table 1).

The experimental variants resulted from:

a) mitogen stimulation

50 ng/mL LPS (bacterial lypopolysaccharide-Difco, USA)

5 µg/mL PHA (phytohemagglutinin -Sigma, USA)

2,5 µg/mL ConA (concanavalin A -Sigma, USA)

b) exposure to bioflavonoid-rich extracts (obtained in National Institute of Pharmaceutical Researches, Bucharest)

15 µg/mL PA29, Crataegus monogyna extract, new test compound

15 µg/mL standardized extract (Echi) *Echinacea purpurea* as positive control

c) exposure to a cytotoxic compound used as a negative control

10 µg/mL $Pb(NO_3)_2$ (Merck, Germany)

Triplicates of exposure to these compounds, separately or in association resulted 72 samples, excluding blank variants (Table 1).

Cell-free supernatants were collected and stored at -80°C until cytokine analysis simultaneously performed from each sample (well) by xMAP and ELISA.

Quantitation of cytokine levels using xMAP technology

Human Fluorokine MAP Base Kit Panel A (R&D Systems, USA) was used with the following analyte-specific bead sets: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IFNα, GM - CSF and TNF-γ according to the manufacturer's protocol. Briefly, the beads provided within each kit were incubated with 50 µL buffer, kit standards or sample in a 96-well plate at room temperature, in the dark. Millipore multiscreen plates were used together with the Millipore filtration system. For measurement, Luminex® 200™ platform was used. Data were evaluated applying a 5-parameter logistic curve fit using the Software Luminex IS 2.3.

Quantitation of cytokine levels using ELISA

The assays were performed by using Quantikine HS Human Immunoassay kits contained precoated ELISA plates for IL-1β, IL-2, (R&D Systems, USA) according to each protocol of the kit. Standards or samples of cell culture supernates were added in the well and incubated 2 h at room temperature. The plates were washed and incubated for 30 min with horseradish peroxidase-conjugated streptavidin. After removal of nonbound horseradish peroxidase conjugate by washing, substrate reagent solutions were added and incubated 20 min in the dark. The reactions was stopped by the addition of 1.8 M H_2SO_4 to each well. The absorbency of all ELISAs was read within 30 min at 450 nm with a PR 3100 TSC (BIO-RAD, USA) reader.

Calibration curves for both xMAP and ELISA methods were prepared from human recombinant cytokine standards by dilution steps in RPMI 1640 medium. Samples were measured twice, and blank values were subtracted from all readings. Correlations between data sets obtained by the two methods were evaluated by using Pearson's correlation coefficient (r).

Experimental protocol was approved by the institutional ethical committee and the experiment was performed according to the Declaration of Helsinki (1964), amended by World Medical Assembly, Venetia (1983).

RESULTS

The experimental variants included *in vitro* exposure of human peripheral whole blood culture to a cytotoxic compound [$Pb(NO_3)_2$], separately or in association with the polyclonal mitogens - LPS, ConA or PHA, as reference compounds, a bioflavonoid-rich extract from Crataegus monogyna PA29 as test compound and a standardized bioflavonoid (Echi).

IL-1β and IL-2 from the same sample (well) were comparatively measured by xMAP Luminex® 200™ and by ELISA, previously validated in our laboratory.

Table 1 - Experimental design - exposure variants: RPMI 1640 diluted whole blood cultured in presence of the mitogens (LPS, PHA, ConA), bioflavonoid extracts (PA29, Echi) and toxic (Pb^{2+})

	1	2	3	4	5	6	7	8	9	10	11	12
A	RPMI 1640 (media)			LPS + RPMI 1640			PHA + RPMI 1640			ConA + RPMI1640		
B	Wb (Whole blood)			LPS + Wb			PHA + Wb			ConA + Wb		
C	Wb + PA29			LPS + PA29			PHA + PA29			ConA + PA29		
D	Wb + Echi			LPS + Echi			PHA + Echi			ConA + Echi		
E	Wb + Pb^{2+}			LPS + Pb^{2+}			PHA + Pb^{2+}			ConA + Pb^{2+}		
F	Wb + PA29 + Pb^{2+}			LPS + PA29 + Pb^{2+}			PA29 + Pb^{2+}			ConA + PA29 + Pb^{2+}		
G	Wb + Echi + Pb^{2+}			LPS + Echi+ Pb^{2+}			PHA +Echi+ Pb^{2+}			ConA + Echi + Pb^{2+}		
H												

A 1- A12: Blank samples
Column 1-3: diluted whole blood cultured with the test compounds
Column 4-6: LPS stimulated whole blood cultured in association with test compounds
Column 7-9: PHA stimulated whole blood cultured in association with test compounds
Column 10-12: ConA stimulated whole blood cultured in association with test compounds

Table 2 - Correlations between Luminex and ELISA performances for measuring IL-1β and IL-2 whole blood *in vitro*

Cytokine	Recovery (%)		CV (%)		Detection range, pg/mL		Regression coefficients (R^2)		ICC (%)
	Luminex	ELISA	Luminex	ELISA	Luminex	ELISA	Luminex	ELISA	
IL-1β	73-126	67-105	3,84	6,74	0,1-1600	1-250	1,00	0,9861	0,9734
IL-2	92-109	87-100	4,89	7,40	0,1-2250	0-2000	1,00	0,9912	0,9175

The Comparison of recovery/accuracy, defined as ratio of the observed amount compared to the expected known amount of cytokine in a sample.

To determine the recovery of each cytokine, recombinant human cytokines in a buffered protein base with preservatives, lyophilized, obtained from R&D Systems were used for both methods. Known amounts of it were spiked in a culture medium containing 10% human AB serum. These samples were treated as unknown samples and measured by xMAP and ELISA as triplicates. The concentrations of the samples were determined from the standard curves generated and the accuracy was determined as the concentration recovered, expressed as a percentage of the actual spiked concentration.

The recovery expressed as a percentage, falls for IL-1β within the range of 78-122% by xMAP and of 67-105% by ELISA. Recovery values for IL-2, falls within the range of 92-109% for Luminex® 200™ and 87-100 % for ELISA. (Table 2)

Dynamic range, representing lower and upper quantitation limits

The Luminex® 200™ standard curve for IL-1β ranged between 0,1-1600 pg/mL build upon a five parameters logistic equation resulted r^2 = 1,000; the ELISA standard curve for IL-1β have r^2 = 0,9861 and the range of concentration 1-250 pg/mL. The optimal working domain indicated by linearity of standard curves: 1 - 1500 pg/mL for Luminex and 5-250 pg/mL for ELISA.

The Luminex® 200™ standard curve for IL-2 ranging between 0,10 - 2250 pg/mL build upon a five parameters logistic equation, resulted r^2 = 1,00 and for ELISA standard curve ranging between 0 - 2000 pg/mL of concentration, r^2 = 0,9912. The optimal working domain indicated by curve linearity was 5-1000 pg/mL for Luminex® 200™ and 10-2000 pg/mL for ELISA. (Table 2).

Intra-assay variability, expressed as coefficient of variation (CV)

Coefficient of variation (CV) = standard deviation divided by the mean was obtained by measuring serial concentration of standard in triplicates.

The CVs for IL-1β were 3,84 % by xMAP and 6,74% by ELISA, respectively, for IL-2, CVs were 4,89 % for xMAP and 7.40% for ELISA.

The values obtained by Luminex® 200™ assay were about 2,3 -4 fold higher than ELISA values average. In any individual, very high levels of two cytokines (5 fold or more) were excluded as outliers.

The reliability of xMAP for the immunotoxicological studies

The two methods were applied to multiple exposure variants: in the presence of mitogens as refe-

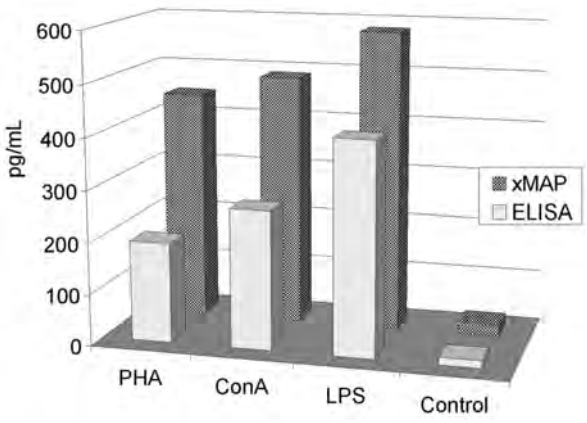

Fig. 1. IL-1β assayed comparatively by xMAP Luminex® 200™ and ELISA from the same sample/well of the human peripheral whole blood cultures stimulated with mitogens (5 μg/mL PHA, 2,5 μg/mL ConA, 50 ng/mL LPS). The modulation of IL-1β release, results in an increasing behaviour with the highest levels in LPS stimulated samples: LPS > Con A > PHA. The picture of both methods follows similarly typed trend with lower values obtained by ELISA in all samples

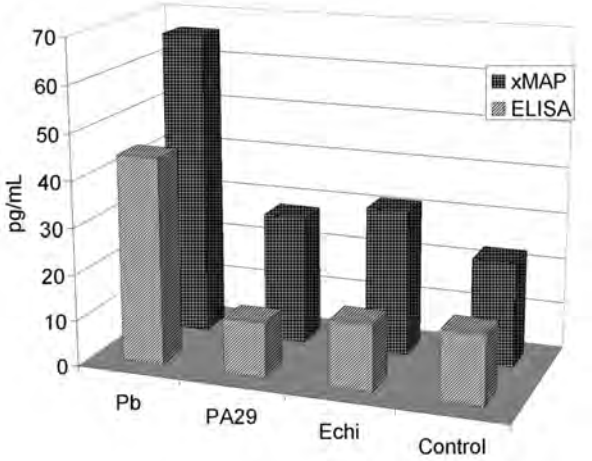

Fig. 2. Bioflavonoid effects on IL-1β level obtained by xMAP Luminex® 200™ and ELISA assays. The basal (unstimulated) response to toxic (Pb) exposure indicates an inflammatory effects by high level of IL-1β released compared to control. The IL-1β levels induced by bioflavonoids (PA29, Echinacea) were closely to control values.

rence substances, in the presence of toxic compound, respectively of standardized bioflavonoid (Echi) with expected (previously investigated) effects, administered separately or in association.

The IL-1β levels in the supernatants of cultures running in the presence of the mitogens (Fig. 1) or of the bioflavonoids (Fig. 2) assayed comparatively from the same sample (well) by Luminex® 200™ system and ELISA have expressed similar relative changes.

A similar typed behaviour showed the IL-2 in the samples stimulated with mitogens separately or in association with bioflavonoids and Pb^{2+} (Fig 3).

IL-1β and IL-2 release exhibits similar aspect of the levels, in mitogen stimulated cells and were mo-

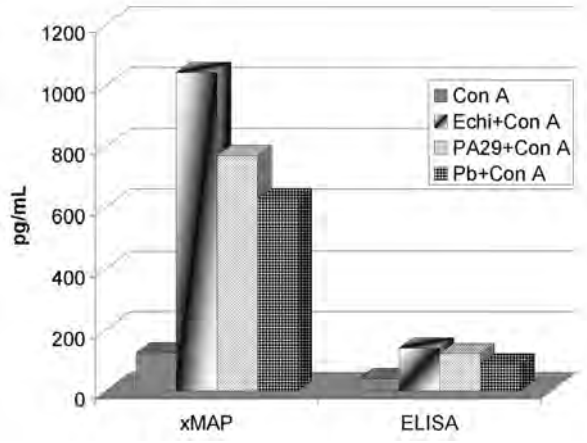

Fig. 3. Bioflavonoid effects in modulating IL-2 level with similar behavior by xMAP Luminex® 200™ and ELISA assays. Peripheral whole blood cultures from healthy control, stimulated with 2,5 μg/mL ConA, exhibits diferential response to bioflavonoids (PA29, Echi) and toxic (Pb^{2+}), indicating the higher effects/ interference of ConA in T cell response, and IL-2 secretion. The differences of the absolute values of IL-2 obtained by the two methods keep the similar comparative aspects.

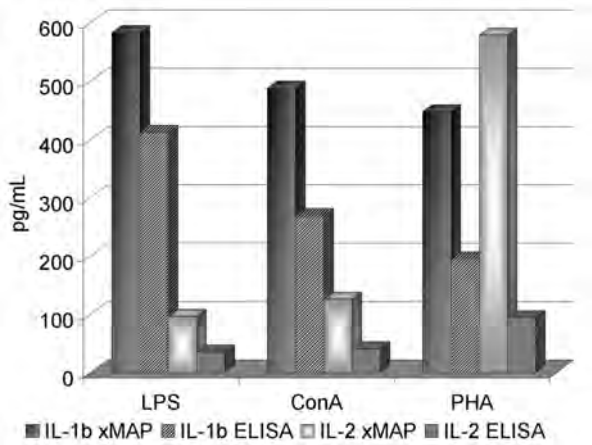

Fig. 4. Comparison of the Luminex® 200™ multiplex and ELISA assays for IL-1β and IL-2 in supernatants of peripheral whole blood cultures stimulated by mitogens. The behaviour of the cytokines indicates the highest sensitivity of IL-1β to LPS and high sensitivity of IL-2 to PHA. The absolute values keep a close rate xMAP/ELISA, excepting the unexpected high value of this rate resulting from xMAP IL-2 level in presence of PHA

dulated in similar trends in presence of bioflavonoid extracts or, respectively, toxic compound indicated the reliability of both methods (Fig. 4).

Correlation coefficient as a measure of the extent to which two variables tend to "vary together".

The degree of agreement between the two methods was good for IL-1β with the ratio Luminex/ELISA near to 1 in 50% of samples and near to 2 in the rest of the experimental variants (n = 28). The Correlation and Covariance coefficients between Luminex® 200™ system and ELISA assays were good: Pearson's correlation coefficient Luminex® 200™ / ELISA was 0.9734 for IL-1β and 0.9175 for IL-2 and covariance values were positive for these experimental conditions: large values of one variable tend to be associated with large values of the other.

DISCUSSION

To determine cytokine profiles in serum or plasma, one possible multiplex platform is the Luminex xMAP, a bead array coupled with fluorescent molecules to detect multiple soluble analytes. Luminex assays have the potential to measure up to 100 cytokines simultaneously in volumes of 25 to 50 μL of sample, in contrast with conventional individual measurement by ELISA that requires 50 to 200 μL of serum per analyte. In addition, Luminex assays may have a greater dynamic range (~1-10000 pg/mL) than ELISAs [11], [14].

Reproducibility data regarding Luminex cytokine assays are primarily limited for higher (supraphysiologic) levels, having distinct advantages for the evaluation of the majority of cytokines tested and representing a valid alternative method to ELISA for characterizing the diseases associated with inflammation or with other immune perturbation events [2],[3], [4].

We have applied their performance characteristics for measurement of soluble cytokines, growth factors, signaling molecules and other analytes in cancer researches performed in our laboratory; reproductibility and other quality parameters were proved by correlation of multiplex and ELISA parallel testing of the serum angiogenic growth factors (VEGF and bFG) [8-11].

The quantitative measurement of the cytokine release using immunoassay methods having a simple and easily quantifiable endpoint, recommended it also as a sensitive and relevant test for the *in vitro* evaluation of immunomodulatory effects of xenobiotics, becoming a powerful method in our laboratory [13], [18].

The quantification of cytokines in supernatants from whole peripheral blood cells stimulated with mitogens, with or without bioflavonoids or toxic compound may be simultaneously performed in a single sample using the Luminex200 xMAP array reader and it is usually performed in our laboratory in screening safety and efficacy of natural extracts or new drug candidate [6], [7], [20], [22].

However, prior to the replacement of ELISA assays with multiplex bead array assays, there is a need to know how these two methods for quantitative analyses are comparable. Without any regulatory guidelines for validating such complex biological assays, and with no reference standards and controls, the validation of xMAP assays has to attempt using existing guidelines for "gold standard" methods ELISA or by comparing the results obtained by two multiplex platform [10], [17-19], [23].

The study presents some performance parameters of xMAP Luminex® 200™ related to ELISA, validated in our laboratory for quantification of interleukins IL-1β and IL-2 released in supernatants of human peripheral whole blood cell cultures.

From each standard curve the lower limits will be the limiting factor of the assay, indicated by the lowest concentration that can be detected (in the linear part of the curve) that is different for Luminex® 200™ and for ELISA for IL-1β and for IL-2.

Intra-assay variability, expressed as a coefficient of variation, was analyzed by comparing the levels of cytokines secreted by samples that were either not stimulated or were treated with different stimuli (bioflavonoids - PA29 and Echi, toxic, LPS, ConA, or PHA), separately or in association.

These results obtained comparatively by the two methods in the quantification of the interleukins release in peripheral blood *in vitro*, in the presence of toxic compounds or bioflavonoids administered separately or in association with the mitogens, as reference substances, are in accord with our previous studies and with the results of other authors, and confirm that xMAP profile reflects sensitively the immunodulation events and recommend it as reliable for this purpose [4], [13], [15], [20], [22].

It appeared that, considered separately, the evaluations by ELISA were better than those by the xMAP assay in range of lower values. Reliability studies at high cytokine levels, which coincided with the linear ranges of the standard curves, which maximizes reliability, but it is less than optimal at low pg/mL concentrations, where the standard curves are sigmoidal.

Future evaluation of the validity of multiplex cytokine measurements needs comparative parallel assays

between ELISA and multiplex assays for the other cytokine from the panel: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, usually performed by Luminex® 200™ system in our immunotoxicological studies.

Given the importance of standardized reagents, platforms, and laboratory procedures for accurate measurements, the study design avoided other sources of variation such as differences in kit production lots, manufacturers, antibodies, and reference cytokines. Comparisons using simultaneously ELISA and multiplex assays of IL-1β and IL-2 in whole blood culture based on identical antibody pairs would aid the future evaluation of the validity of multiplex cytokine measurements *in vitro*.

CONCLUSIONS

Quantitative differences between absolutes values for IL-1β and IL-2 obtained by xMAP and ELISA assays are found, but the relative values are comparable and the two methods keep similar trends in similar exposure conditions.

The performance parameters of the xMAP assay and the good correlation coefficients with the „gold standard" ELISA recommend to validate the multiplex assay for analyzing cytokine profiles in immunotoxicological studies *in vitro*.

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INTRANASAL PUVA PHOTOTHERAPY IN NASAL POLYPOSIS - A PILOT STUDY

Andrea Koreck[1,4#*], Zsolt Bella[2#], Edit Kadocsa[2], Adam Perenyi[1], Laszlo Tiszlavicz[3], Istvan Nemeth[3], Maria Kiss[1], Tudor Rares Olariu[5,6], Jozsef Jori[2], Lajos Kemeny[1,7]

[1]Department of Dermatology and Allergology, University of Szeged, Hungary; [2]Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University of Szeged, Hungary; [3]Department of Pathology, University of Szeged, Hungary; [4]Department of Immunology, Victor Babes University of Medicine and Pharmacy Timisoara, Romania; [5]Clinical Laboratory, Municipal Clinical Hospital Timisoara, Romania; [6]Department of Parasitology, Victor Babes University of Medicine and Pharmacy Timisoara, Romania; [7]Dermatological Research Group of the Hungarian Academy of Sciences and the University of Szeged

[#]Both authors contributed equally

ABSTRACT

Nasal polyposis (NP) affects 4 % of the general population, representing a major health problem. In spite of complex (surgical and medical) treatment, the relapse rate is high and it has a negative impact on the quality of life. Recently we found that intranasal photochemotherapy with ultraviolet A light (PUVA) is effective in allergic rhinitis. In the present study PUVA was administered for 6 weeks in 7 patients with NP. Nasal lavages were performed in all patients before and at the end of the treatment; from four patients a biopsy specimen was also collected. Eosinophils significantly decreased in patients with NP and slightly in a patient who had associated aspirin sensitivity. IL-5 and eosinophil cationic protein (ECP) levels showed a decreasing trend in patients with NP and an increasing trend in patients with associated aspirin sensitivity. Our results suggest that intranasal PUVA might represent a future therapeutic method in a subset of patients with NP.

Key words: Nasal polyposis, intranasal PUVA phototherapy, Interleukin-5 (IL-5), Eosinophil Cationic Protein (ECP), eosinophil cell count

INTRODUCTION

Nasal polyposis (NP) is a chronic inflammatory disease of the upper airways (nose and paranasal sinuses) with a prevalence which has been estimated to be 4% [1]. Clinically, nasal polyps (NPs) are characterized by edematous inflammatory masses which prolapse into the nose, leading to nasal obstruction, loss of smell, secretion and headache. Quality of life of NP patients is comparable or even worst than that of patients with chronic obstructive pulmonary disease, coronary artery disease and asthma [2]. A significant percentage of patients with NPs are also diagnosed with asthma, allergic rhinitis and aspirin sensitivity and it has been shown that these diseases have an additional negative impact on quality of life [3].

The inflammatory infiltrate in NPs comprises a variety of cells, including eosinophils, mast cells, neutrophils, lymphocytes and plasma cells. The majority of NPs belong to the eosinophilic type [4]. The presence of high levels of proinflammatory mediators such as IL-5 and eosinophil cationic protein (ECP) and of epithelial damage are also characteristic of NPs.

Therapeutic strategies for NPs include medical treatment especially with corticosteroids (topical and oral), surgical treatment or a combination of both [5]. Independent of the applied treatment, NPs are characterized by a high recurrence rate [6]. NPs associated with asthma and aspirin sensitivity (ASA syndrome) have a worse therapeutic response, probably due to a more complex pathogenetic mechanism, involving also dysfunctions of the arachidonic acid metabolism [7].

Photochemotherapy with 8 methoxypsoralen (8-MOP) and ultraviolet A (UVA) light (PUVA) has been widely used for decades in the management of various inflammatory skin diseases. PUVA treatment has been successfully applied for the management of immune-mediated diseases of the oral mucosa such as oral lichen planus and chronic graft-versus-host disease [8]. Recently, we showed that intranasal

* *Corresponding author:* Andrea Koreck; Department of Immunology, Victor Babes University of Medicine and Pharmacy Timisoara; Splaiul Tudor Vladimirescu nr. 14, Timisoara, Romania; Tel.: +40 723 371 551; E-mail: akoreck@yahoo.com

PUVA therapy is also effective in allergic rhinitis [9]. In the present study we have investigated the effect of intranasal PUVA therapy on the inflammatory cell infiltrate and the level of proinflammatory mediators of patients with nasal polyposis.

MATERIALS AND METHODS

Patients and treatment schedule

We conducted an open-label pilot study in 7 patients with grade II-III bilateral nasal polyposis (3 females, 4 males; mean age: 46 years; 3 patients with ASA syndrome, 4 patients without ASA syndrome). The Ethical Committee of Szeged University has approved the protocol. All patients gave their written informed consent. We excluded potential subjects from the study who had a diagnosis of cystic fibrosis, oral steroid-dependent asthma, or had upper or lower respiratory infection within 4 weeks prior to the beginning of the study or had any significant nasal structural abnormalities which can interfere with the delivery of intranasal phototherapy. We also excluded patients with known photo-sensitivities or photoallergies to natural or artificial sunlight and those who were receiving any form of light therapy, or had used any of the following drugs: systemic corticosteroids within 4 weeks, topical corticosteroids within 2 weeks, membrane stabilizers within 2 weeks, antihistamines within 10 days, prior to the beginning of the study.

Patients received 8-MOP orally (0.6 mg/kg) one hour prior to intranasal irradiation. Each intranasal cavity was irradiated 3 times a week for 2 weeks and 2 times a week for the following 4 weeks, with increasing doses of UVA. Treatment was performed with a special instrument for targeted intranasal phototherapy (Rhinolight-PUVA, Hungary) with a starting dose of 0.5 J/cm^2 (highest dose: 0.92 J/cm^2; total cumulative dose: 8.5 J/cm^2). Patients were instructed to use photoprotection 24 hours after ingestion of the photosensitizer.

Nasal biopsies

Biopsy samples (3-4mm) were collected before starting PUVA therapy from all patients and in 4 cases also after the 6-week treatment regimen. Specimens were fixed immediately in 10% formaline. All samples were embedded in paraffin blocks. For detection of eosinophil cells hematoxylin-eosin staining was performed [10]. In order to identify CD3, CD4 and CD8 positive T cells immunohistochemistry was performed. Antibodies against CD3 (LabVision Corporation, USA; epitop specific rabbit antibody against an epitop of CD3, at a dilution of 1:150), against CD4 (Novocastra Laboratories, UK; clone 1F6, at a dilution of 1:40) and against CD8 (DAKO Laboratories, Denmark; clone C8/144B, 1:100) were used according to the criteria listed in the manufacturers datasheets. For visualisation LSAB2 (DAKO, Denmark) and diaminobenzidine (DAKO) were applied.

Eosinophils, T cells, CD4+ and CD8+ T cells were evaluated by calculating the average number of stained cells from 10 randomly selected high-power fields per polyp.

Nasal lavage

Before starting the treatment protocol and after the last treatment nasal lavage was performed in all patients. Nasal lavage was carried out by instilling 3 ml of pre-warmed (37^{o}C) normal saline solution into each nasal cavity. The samples were placed immediately on ice and were processed within 2 hours. The nasal lavage fluid was passed through a 40 μm nylon mesh filter (BD Biosciences, Bedford, MA, USA) and the filtrate was centrifuged at 420 g for 10 minutes at 4^{o}C. The supernatant was separated from the pellet. The samples were stored at -70^{o}C.

Cytokine assays

IL-5 levels in nasal fluids were quantified with a commercial ELISA kit (Quantikine, R&D System, Minneapolis), according to the manufacturer protocol. We also measured ECP levels in nasal lavage samples using the ELISA procedure (MBL Nagoya, Japan).

RESULTS

All patients had eosinophilic type NPs verified by histopathology. Three patients had associated asthma and aspirin sensitivity (NP and ASA syndrome group), 2 of these had also allergic rhinitis. Four patients had only nasal polyposis without associated asthma, allergic rhinitis or aspirin sensitivity (NP group).

All patients from the NP group completed the 6-week treatment protocol. In contrast only one of the 3 patients with NP and associated ASA syndrome was able to complete the treatment protocol. The other two patients dropped-out because of worsening of nasal symptoms and need of corticosteroid therapy, one after 23 days of treatment and the other after 37 days of treatment. From both drop-out patients a nasal lavage sample was obtained after the last PUVA treatment.

Nasal biopsy samples were collected after completing the 6-week phototherapy regimen from 3 patients from the NP group and the one patient with

NP and ASA syndrome who completed the whole treatment regimen.

Effect of intranasal PUVA treatment on infiltrating eosinophils and T cells

In all patients from the NP group eosinophils decreased with more than 50% (Figure 1), in one patient a 75% decrease of eosinophils was detected. In the patient with NP and ASA syndrome a slight decrease of eosinophils (14%) was seen after 6 weeks of phototherapy. After PUVA therapy no changes in CD3+, CD4+ and CD8+ T cell counts were detected in any of these patients. No changes in the CD4/CD8 cell ratio were present after intranasal phototherapy.

Effect of intranasal PUVA treatment on IL-5 and ECP levels

After PUVA treatment, IL-5 levels decreased in nasal lavage samples of patients from the NP group (Table 1). In contrast, in 2 out of 3 patients with NP and associated ASA syndrome a trend towards increase of IL-5 levels was detected (Table 1).

ECP levels decreased in 3 out of 4 patients with NP and increased in 2 out of 3 patients with NP and ASA syndrome (Table 2).

Table 1. Levels of IL-5 in nasal lavage samples collected before first intranasal PUVA treatment and after the last treatment was delivered (# drop-out after 37 days; $ drop-out after 23 days)

Patients	IL-5 (pg/ml) before	IL-5 (pg/ml) after
P1 (NP)	3.72	0.98
P2 (NP)	3.57	0.06
P3 (NP)	1.6	0
P4 (NP)	0.64	0.64
P5 (NP + ASA)	1.87	24.43#
P6 (NP + ASA)	0.98	1.22
P7 (NP + ASA)	4.81	2.14$

Table 2. Levels of ECP in nasal lavage samples collected before first intranasal PUVA treatment and after the last treatment was delivered (# drop-out after 37 days; $ drop-out after 23 days)

Patients	ECP (ng/ml) Before	ECP (ng/ml) After
P1 (NP)	22.01	9.16
P2 (NP)	19.42	5.42
P3 (NP)	1.89	4.2
P4 (NP)	13.49	12.02
P5 (NP + ASA)	22.91	20.2#
P6 (NP + ASA)	21.09	21.27
P7 (NP + ASA)	13.05	20.15$

DISCUSSION

Nasal polyposis is a multifactorial disease, in which chronic inflammation is a major factor. Eosinophils represent the predominant inflammatory cell population in the majority of NPs and several data suggest that persistence of tissue eosinophilia is of major importance in the pathogenesis of NPs [4]. Eosinophils are terminally differentiated cells which migrate and accumulate in the tissues following release of various cytokines and chemokines, such as IL-5, IL-3, GM-CSF, RANTES [11]. Apoptosis is critical in the regulation of eosinophil removal and delayed apoptosis of eosinophils has been reported as an

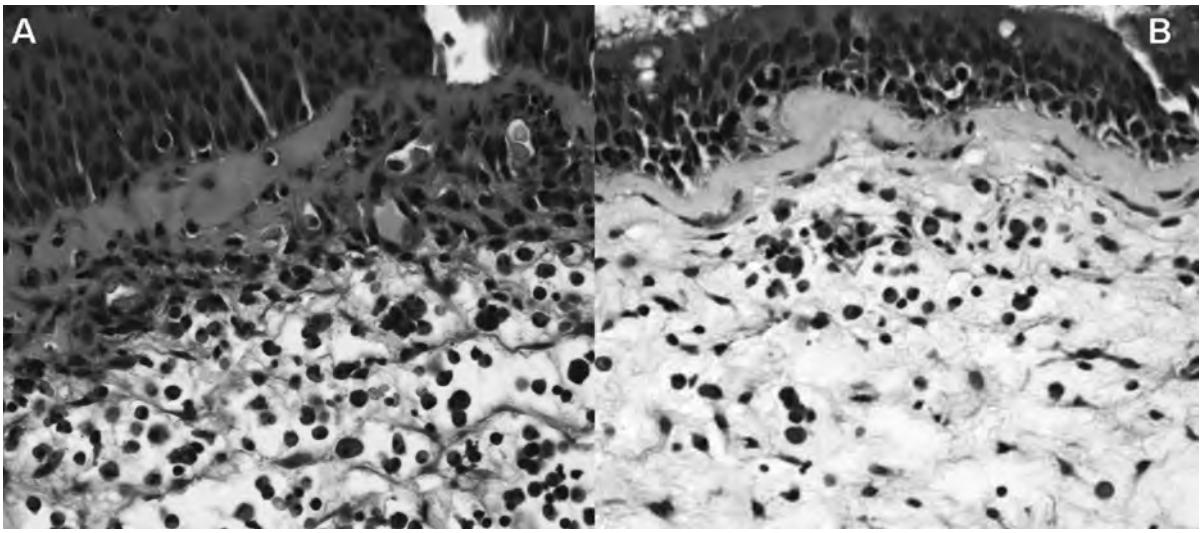

Figure 1 - Polyps were characterized at baseline by a dense eosinophilic infiltrate (A). Intranasal PUVA treatment for 6 weeks resulted in a significant decrease of eosinophils in patients with NP without ASA syndrome.

important mechanism for tissue eosinophilia in several diseases, including nasal polyps [12]. IL-5 is one of the major cytokines, which promote eosinophil maturation, activation and survival [13]. CD4+ T cells are considered to be the major source of IL-5, but other cell types including eosinophils also release this cytokine [14]. In this manner eosinophils themselves may promote cell survival in an autocrine way. Therefore, reduction of eosinophil cell number and of their major survival cytokine, IL-5 might represent a therapeutic target in NPs. It has been shown that long-term treatment of NPs with topical corticosteroids reduced the number of eosinophils *in vivo* [15]. Watanabe et al have shown that increased eosinophil apoptosis is present in NPs cultured in the presence of corticosteroids [16]. However, in a recent study Bloom et al have demonstrated that in the presence of high levels of IL-5, which are more closely related to those detected in clinical situations, glucocorticoids may actually decrease, rather than increase, the number of apoptotic eosinophils, suggesting that in *in-vivo* situations the local cytokine (or cytokine combination) levels can rescue eosinophils from glucocorticoid-induced apoptosis [17]. This may explain the relative efficacy or inefficacy of these drugs in NPs. Any new treatment modality which is able to decrease eosinophil cell number and IL-5 levels is of outstanding therapeutic importance in NP. In our study we have shown that both the number of eosinophils and the level of IL-5 decreased after intranasal PUVA phototherapy in NP but not in NP with associated ASA syndrome. In patients from the NP group the decrease of eosinophils was accompanied by a decreasing trend of the level of ECP, a cytotoxic mediator which participates in tissue damage. Thus intranasal PUVA phototherapy may have an anti-inflammatory effect in NP without ASA syndrome by stimulating the removal of eosinophils most probably by inducing apoptosis. The complex mechanism of inflammation in patients with ASA syndrome might be responsible for lack of response to PUVA treatment in this group.

Although our data are preliminary and the number of patients is low, the different trends in response of eosinophils and key eosinophil mediators between patients without and with ASA syndrome suggest that intranasal PUVA therapy may be considered for further evaluation for the treatment of a subset of patients with NP without associated ASA syndrome. A randomized placebo controlled study has to be conducted to evaluate the routine use of this new treatment option in patients with nasal polyposis.

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TH1/TH2 CYTOKINE LEVELS AS AN INDICATOR FOR DISEASE PROGRESSION IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION AND RESPONSE TO ANTIRETROVIRAL THERAPY

Chukwunomso E. Osakwe[1], Coralia Bleotu[3], Mariana Carmen Chifiriuc[1], Camelia Grancea[3], Dan Oţelea[2], Simona Paraschiv[2], Sorin Petrea[2], Magda Dinu[2], Cristian Băicuş[2], Adrian Streinu-Cercel[2], Veronica Lazăr[1]

[1]*University of Bucharest, Faculty of Biology, Microbiology and Immunology Department, 1-3 Aleea Portocalelor, 70709, Bucharest, Romania;* [2]*National Institute for Infectious Diseases, "Prof. Dr. Matei Bals", Bucharest, Romania;* [3]*Institute of Virology, S. Nicolau, Bucharest, Romania*

ABSTRACT

A recent theory stipulates that during the course of HIV infection, there is a shift in immune response from T-helper 1 to T-helper 2 responses, characterised by elevated secretions of relevant cytokines. Cytokine profiles of 15 asymptomatic (treatment naïve) and 26 symptomatic (undergoing treatment) HIV-1 patients was determined to investigate the validity of this theory. HIV-1 RNA was quantified using the COBAS® TaqMan® HIV-1 test, CD4 T-cell counts with the FACSCalibur flow cytometer and IL-1, IL-4, IL-6, IL-10 and IFN-gamma cytokine levels by ELISA method. The asymptomatic group had significantly higher RNA levels (p-value; 0.000006) and lower CD4 T-cell counts than the symptomatic group indicating ongoing disease progression in the absence of antiretroviral treatment and a positive response to HIV treatment by the symptomatic group. IL-1, IL-4 and IFN-gamma were undetectable in most study subjects. IL-10 and IL-6 levels was relatively lower in the asymptomatic group (mean value; 206.352 pg/ml, 10.516 pg/ml) than the symptomatic group (mean value; 417.539, 18.387 pg/ml). Lower levels of proinflammatory cytokines (IL-1, IFN-gamma) in both study groups and elevated levels of anti-inflammatory cytokine IL-10, confirms that there is a shift in immune response as HIV infection progress to AIDS. In addition, the presence of a progressive trend of anti-inflammatory cytokine, IL-10 and proinflammatory cytokine, IL-6 in 12 symptomatic patients tested 3 months after antiretroviral therapy indicates an attempt by antiretrovirals to restore immune function.

Key words: HIV TYPE 1, cytokines, disease progression

INTRODUCTION

In accordance with estimates by the Joint United Nations Programme on HIV/AIDS (UNAIDS), about 31.3 million adults and 2.1 million children were living with the Human Immunodeficiency Virus (HIV) as at the end of 2008.

Since the advent of HIV infection scientists have been able to shed more light on the mechanisms of transmission of the virus and its replication cycle. However, a lot remains to be understood on the pathogenesis of the disease [19] and how it affects the host immune system. It is commonly known that HIV pathogenesis first results in an acute infection stage, followed by an asymptomatic period which varies from individual to individual. In the absence of antiretroviral treatment, most HIV infected patients progress into the symptomatic period characterised by a generalized immune dysfunction that results in the manifestations of opportunistic infections and death [22].

Generalized immune system activation at the initial stage of infection, is one of the hallmarks of the HIV-1 virus, although the mechanism by which this is induced remains incompletely elucidated [1, 28]. In HIV-infected patients, increased immune system activation is typically characterized by increased turnover of $CD4^+$ and $CD8^+$ T cells [15, 25] and increased expression of activation markers, such as CD38 [11, 27] and HLA-DR [17], on immune system cells [39]. Therefore, the interactions of the virus with the host immune system have become increasingly clearer over the years.

* *Corresponding author:* Chukwunomso E. Osakwe - Microbiology and Immunology Department, Faculty of Biology, University of Bucharest, 1-3 Aleea Portocalelor, 70709, Bucharest, Romania. Phone: +40757913890; Email: ekeneosakwe@yahoo.com

It is very commonly stipulated that the control of the HIV epidemic would require the development of an effective vaccine that would either entirely prevent infection or significantly delay the rate of progression to Acquired Immune Deficiency Syndrome (AIDS) [8, 23,]. Therefore, gaining a better understanding of the interactions of the virus with host immune system and how host immunity attempts to control viral replication is necessary for the rational design of effective HIV vaccines [7, 33].

A simple theory which attempts to provide a scientific explanation for what triggers the progression of HIV infection to AIDS in most HIV-infected individuals has been gaining support. The theory holds that HIV-infected people switch from a T-helper type 1 (Th1) to a T-helper type 2 (Th2) state as the disease progresses [16]. However, the validity of this theory has been challenged with a number of experimental studies resulting in controversial results [2, 3, 24, 30, 32, 35]. For instance, *in vitro* infection with HIV of already established $CD4^+$ T cell clones indicated that Th2 and Th0 cells support HIV replication better than Th1 cells [19]. However, this does not provide any evidence for a shift in immune state. Also, in some HIV-infected individuals with low $CD4^+$ T cell counts, a prevalence of $CD8^+$ T cells producing type 2 cytokines was found in both peripheral blood and skin [19]. Consequently, evidence shows that enhanced production of type 2 cytokines in a proportion of HIV-infected individuals during the symptomatic stage may play a role in the pathogenesis of the disease [19]. Hence, cytokine levels at different stages of HIV-1 infection have been investigated by scientists to either confirm or disprove this theory [31].

Cytokines which are known for regulating cellular immune interactions are usually produced by lymphocytes, monocytes, macrophages, and, for some cytokines, also fibroblasts, neutrophils, endothelial cells, or mast cells [5]. In this study the cytokine profiles of HIV-1 infected patients at the asymptomatic and symptomatic stage of infection were determined in order to further investigate the validity of the Th1/Th2 theory and its role in HIV-1 disease progression.

MATERIALS AND METHODS

Study Group and Samples:

The study group consisted of 50 HIV-1 seronegative controls and 41 HIV-1 seropositive patients recruited from the Matei Bals Hospital. The HIV-1 seropositive individuals were further divided into two groups, 15 asymptomatic and 26 symptomatic patients. The classification into asymptomatic and symptomatic was done based on the WHO Disease Staging System for HIV Infection and Disease.

The 15 Highly Active Antiretroviral Therapy (HAART) naïve asymptomatic individuals were characterised as individuals in the Stage I phase of HIV infection with no manifestations of opportunistic infections despite increasing viral load levels and diminishing CD4 T cell counts, while the 26 symptomatic patients were characterised as individuals who had been diagnosed with one or more opportunistic infections at some point during HIV infection and are all presently undergoing HIV treatment. In 12 of the symptomatic patients, the viral load levels, CD4 T-cell counts and cytokine profile analysis was carried out twice within an interval of 3 months after treatment.

Whole blood was collected from all study individuals and centrifuged at 2000 - 2500 rpm for 5 minutes to obtain plasma which was stored at -80°C for further analysis. Informed consent was obtained from all study subjects.

Plasma Viral RNA Determination

The COBAS® TaqMan® HIV-1 test (Roche Molecular Systems, Inc., Branchburg, NJ, 08876 USA) was used according to manufacturer's recommendations to determine the viral load levels of all subjects. Plasma samples were thawed overnight and 5ml taken from each sample for viral load determination. HIV–1 virus particles were lysed by incubation at an elevated temperature with a protease and binding buffer that releases HIV-1 RNA and protects it from RNAses in plasma. A known number of HIV-1 Quantitation Standard Armored RNA molecules were introduced into each specimen along with the lysis reagent. Subsequently isopropanol was added to the lysis mixture for precipitation and the mixture was centrifuged through a column with a glass fibre insert. Impurities were removed during centrifugation and adsorbed nucleic acids were washed and eluted with aqueous solution. The HIV-1 target RNA and HIV-1 Quantitation Standard RNA was then reverse transcribed to generate complementary DNA which underwent PCR amplification [38] using HIV-1 specific complementary primers. Finally dual-labelled fluorescent probes which consisted of HIV-1 and HIV-1 Quantitation Standard specific oligonucleotide probes, with a reporter dye and a quencher dye provided for real-time [16, 14] detection and quantification of PCR product accumulation.

CD4 T-cell Counts

The CD4 T-cell counts of the subjects were estimated using the FACSCalibur flow cytometer

(Becton-Dickinson Biosciences, San Jose, CA, USA) in accordance with manufacturer's instructions. 50 µl of whole blood was distributed into tubes containing 10 µl of MultiTest CD3 FITC/CD8 PE/ CD45 PerCP/CD4 APC reagent (Multitest, San Jose, CA, USA), vortexed and incubated for 15 minutes at room temperature. 450 µl of FACS Lysing solution was added and further incubated for 15 minutes. Four colour analysis was performed to obtain the percentage of lymphocyte subsets. The absolute CD4 T-cell count was measured and expressed in cells/µl [21].

Quantification of Cytokine Concentration in Plasma Samples

Concentrations of IL-1, IL-4, IL-6, IL-10 and IFN-gamma cytokines in the plasma samples were determined using commercial sandwich enzyme-linked immunosorbent assay (ELISA) [9] kits (MABTECH AB, Sweden) in accordance with manufacturer's specifications. The concentrations of all cytokines used in this study were further obtained by using the ELISA reader (Apollo LB 911 Berthold Technologies) and results were expressed in pg/ml.

Statistical Analysis

Npar Tests was used for all statistical analysis and because the data set was not normally distributed, cytokine responses between the two independent groups, asymptomatic and symptomatic, were compared using the Mann-Whitney U test. P-values were also determined and p-values less than 0.05 were regarded as statistically significant. Ranges and mean values were also reported.

RESULTS

Brief Description of the Two Major Study Groups; Asymptomatic and Symptomatic

The asymptomatic group which had a mean age of 31 years, had a mean viral load level of 182 782 copies/ml with range from 1760 to 1 972759 copies/ml [Table 1] and mean CD4 T-cell count of 423.6 cells/µl with range from 94 to 960 cells/µl [Fig. 2]. While the symptomatic group with mean age of 21 years, had a mean viral load level of 18741.27 copies/ml with range from undetectable to 459 000 copies/ml [Fig. 3] and mean CD4 T-cell count of 518.69 cells/µl with range from 14 to 1201 cells/µl [Fig. 4]. From the asymptomatic group, two immunologically discordant individuals who had CD4 T-cell count of <100 cells/µl, with elevated viral load levels but no indication of opportunistic infections were identified. The difference in HIV-1 RNA levels between the asymptomatic and symptomatic groups was statistically significant with p-value of 0.000006.

IL-10 Cytokine Levels in All Study Groups

The study control group had IL-10 cytokine levels under the detection limits of the kit, while the asymptomatic group had mean IL-10 levels of 206.352 pg/ml (range; 13.55 to 983.554 pg/ml) and the symptomatic group had mean IL-10 levels of 417.539 pg/ml (range; 16.221 to 1298.861 pg/ml). The symptomatic group exhibited significantly higher levels of IL-10 than the asymptomatic group with a p-value of 0.046 [Fig. 5, 6].

Levels of IFN-gamma in The Control, Asymptomatic and Symptomatic groups

The control group showed undetectable levels of IFN-gamma. Most of the asymptomatic group members as well as the symptomatic group exhibited undetectable levels of IFN-gamma, except for just two asymptomatic individuals (the mean value was of 15.091 pg/ml) and one symptomatic (0.172 pg/ml) [Fig. 7, 8].

IL-6 cytokine levels in all study groups

For the control group, the mean value of IL-6 detected was 3.3 pg/ml with a range of 1.03 to 9.47 pg/ml. IL-6 cytokine was detected in almost every member of the asymptomatic group but in relatively low levels (mean value; 10.516 pg/ml and range; 0 to 40.87 pg/ml) as compared to the higher secretion levels observed in the symptomatic group (mean value; 18.387 pg/ml and range; 0 to 364.2) which had fewer members secreting IL-6 but in higher concentrations (p-value; 0.004) [Fig.9, 10].

Levels of IL-1 and IL-4 cytokines in the control, symptomatic and asymptomatic groups

IL-4 cytokine was undetectable in all study groups, while IL-1 which was also undetectable in the control and asymptomatic groups was detectable in only one member of the symptomatic group with cytokine level of 7.29 pg/ml [Table 1].

Comparison between the symptomatic groups tested twice

Out of the 26 patients included in the symptomatic study group, 12 individuals were tested twice for cytokine profiles within an interval period of three months after antiretroviral treatment. Our results have shown that for cytokines IL-4 and IFN-gamma, there were no significant changes in the expression

levels. However, there was an increase in IL-6 levels [Fig. 11] and also in IL-1 levels [Fig. 12]. IL-10 [Fig. 13] levels showed a large variation before and after the three months interval, however, its mean value increased after the three months of treatment from 387.962 pg/ml (range; 18.704 to 1133.171 pg/ml) to 527.357 pg/ml (range; 14.415 to 1171.199 pg/ml).

DISCUSSION

High-level HIV replication and the rapid breakdown of the mucosal immune system are the hallmarks of HIV infection of which cytokine dysregulation may be related to both phenomena [18]. In this study, cytokine profiles of asymptomatic and symptomatic HIV-1 infected patients were determined and very interesting observations were made.

In the selection of members of the asymptomatic and symptomatic groups [Fig. 1, 2], it was observed that the asymptomatic group who were also HAART naïve had relatively higher viral RNA levels and lower CD4 T-cell counts than the symptomatic group who were still undergoing HAART therapy. This indicates a generally favourable response to treatment displayed by the symptomatic group. The high viral replication rate in asymptomatic patients indicates the possible gradual progression of the asymptomatic to symptomatic condition, in the absence of antiretroviral treatment.

Th1 cells are generally known as the principal effectors of cell-mediated immunity against intracellular microbes, whereas Th2 cells are important in providing help to B cells in antibody production [29, 36, -34] and an imbalance between Th1 and Th2 responses has been suggested to be involved in the pathogenesis of HIV infection [4]. However while several studies have associated a shift in the cytokine pattern from Th1 to Th2 cytokine profile along with progression from the asymptomatic stage to the symptomatic stage with an increase in IL-10 levels, others are yet to confirm this [37].

In this study, IL-10 levels were observed to be quite high in both the asymptomatic and symptomatic groups however, relatively higher levels was seen in the symptomatic patients than in the asymptomatic individuals. Also in majority of the 12 symptomatic individuals tested twice at an interval period of three months after HAART, IL-10 levels exhibited a progressive trend with more elevated levels being detected during the second ELISA test. Considering that majority of symptomatic patients already had undetectable levels of viral RNA and increasing CD4 T-cell count after HAART therapy, increasing IL-10 levels could be an indication for immune repair and reconstitution.

Furthermore, IFN-gamma levels were observed to be undetectable in majority of members of both the asymptomatic and symptomatic group. This cytokine which is known to play an important role in effective host immune responses against bacterial and viral infections [26] is also critical for inducing cell-mediated immunity, especially cytotoxic-T-cell (CTL) responses [12] and studies have shown that IFN-gamma knockout mice are highly susceptible to viral infections [6]. In addition, CTL responses have been observed to be preserved in asymptomatic HIV-1-infected individuals but not in HIV-1-infected patients who progress to AIDS [10]. Therefore, the absence of IFN-gamma secretion in both groups could also be regarded as an indicator for disease progression in the asymptomatic group as well as the presence of an already declined immune state in the symptomatic group.

Pro-inflammatory cytokine IL-6, showed increasing levels of secretion in both asymptomatic and symptomatic groups but much higher elevation in the symptomatic group. Also in the 12 symptomatic patients tested twice, IL-6 cytokine levels remained elevated and on the increase after three months of HAART.

In this study, sparse secretion levels of pro-inflammatory cytokines IFN-gamma, IL-1 and IL-6 in the asymptomatic group indicates the presence of ongoing disease progression in the absence of HAART. While elevated secretion levels of anti-inflammatory cytokine IL-10 in the symptomatic group, also shows disease progression. This therefore confirms the existence of a possible relationship between the cytokine pattern and a shift from asymptomatic stage to symptomatic stage. However, the mechanism by which this cytokine shift is achieved is yet to be fully elucidated. In addition, in the 12 asymptomatic patients whose cytokine profiles were determined twice, observable increases in IL-1, IL-6 and IL-10 cytokines after three months of HAART, indicates an ongoing though incomplete process of immune reconstitution and repair as confirmed by restoration of CD4 T-cell counts and decline in viral load levels.

More in depth research is still required using a larger and widely distributed sample population, with the inclusion of HIV-1 infected individuals in the acute stage of disease, in order to thoroughly establish the validity of the Th1/Th2 theory as well as to further elucidate the mechanisms involved in the pathogenesis of disease.

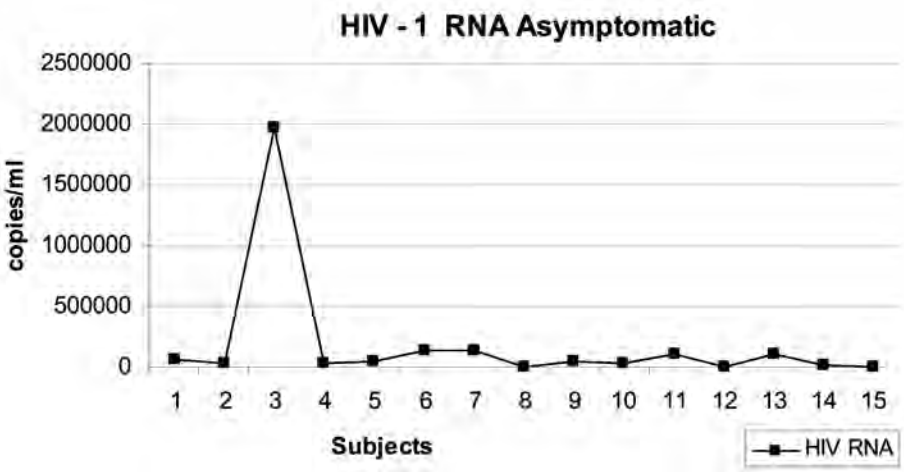

Figure 1: HIV-1 RNA levels asymptomatic subjects with RNA levels expressed in copies/ml; most subjects indicate presence of viremia.

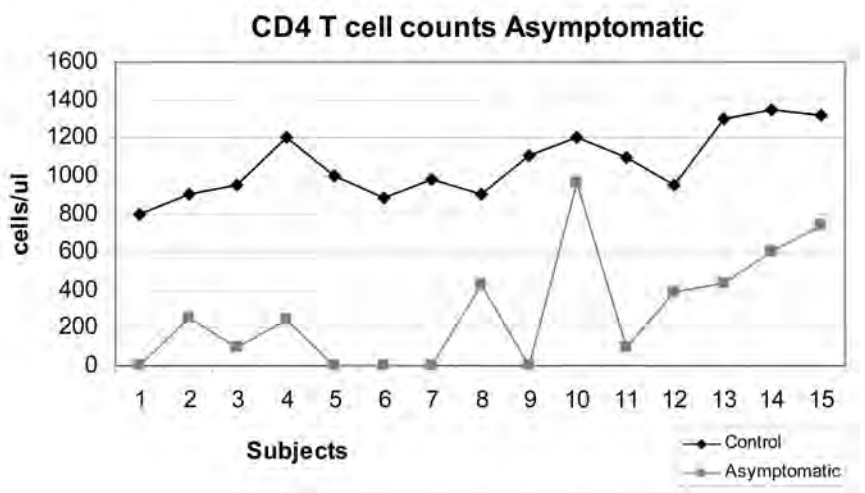

Figure 2 : CD4 T cell counts of both the control group and the asymptomatic group, expressed in cells/μl ; CD4 T cell counts of the control group are much higher than that of the HIV infected asymptomatic group.

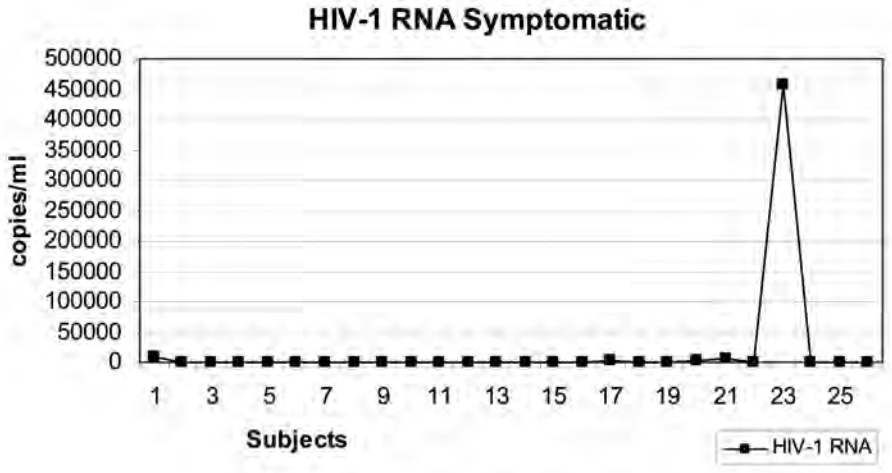

Figure 3: HIV-1 RNA levels of the symptomatic group with RNA levels expressed in copies/ml; most members of this group have undetectable viral load levels.

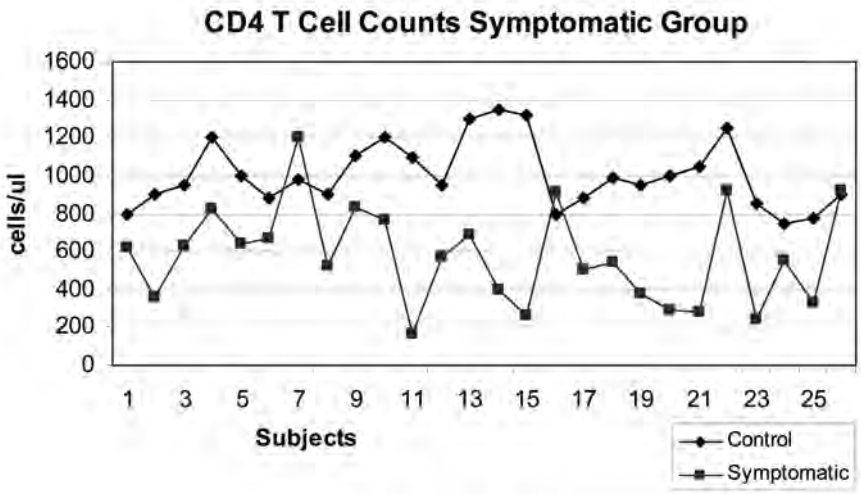

Figure 4: CD4 T cell count of the control group and the symptomatic group expressed in cells/μl; most patients had elevated CD4 T cell counts, an indicator for good response to HAART.

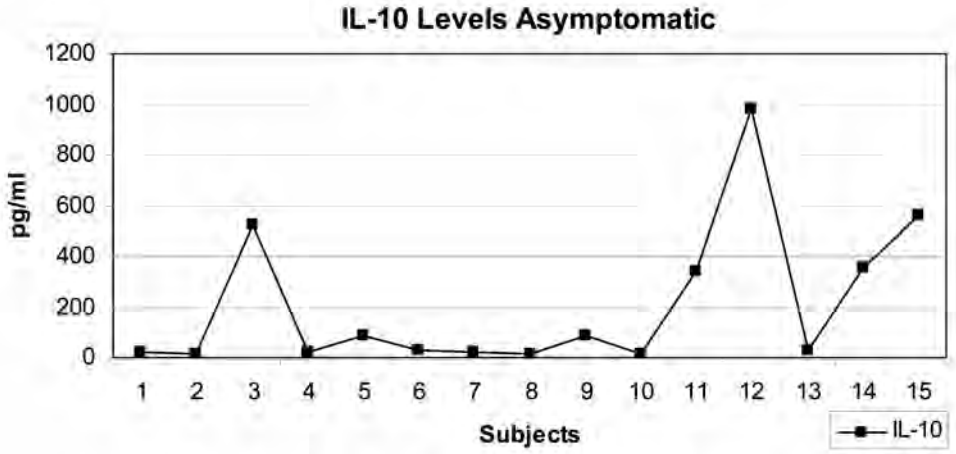

Figure 5: IL-10 cytokine levels determined in asymptomatic subjects by ELISA method as described in the materials and methods section and expressed in pg/ml.

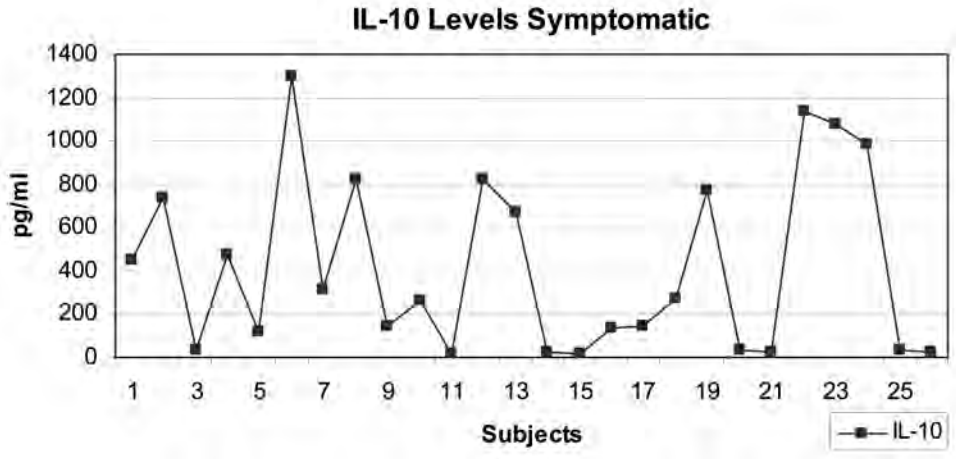

Figure 6: IL-10 cytokine levels determined in the symptomatic group and expressed in pg/ml; IL-10 cytokine levels appear higher in the symptomatic group than in the asymptomatic group.

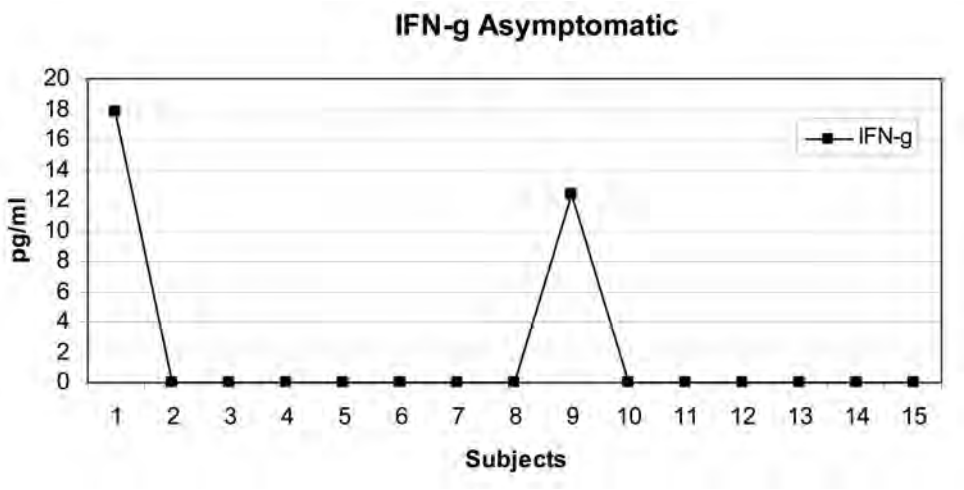

Figure 7: IFN-gamma cytokine levels determined in the asymptomatic group by ELISA method and expressed in pg/ml; as clearly observed only two asymptomatic individuals secreted the cytokine.

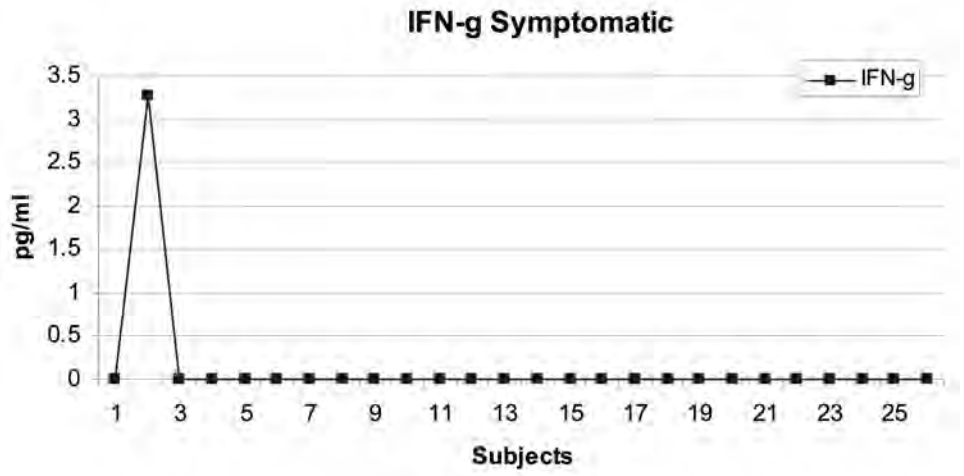

Figure 8: IFN-gamma levels in symptomatic group expressed in pg/ml; only one symptomatic individual showed detectable levels of the cytokine.

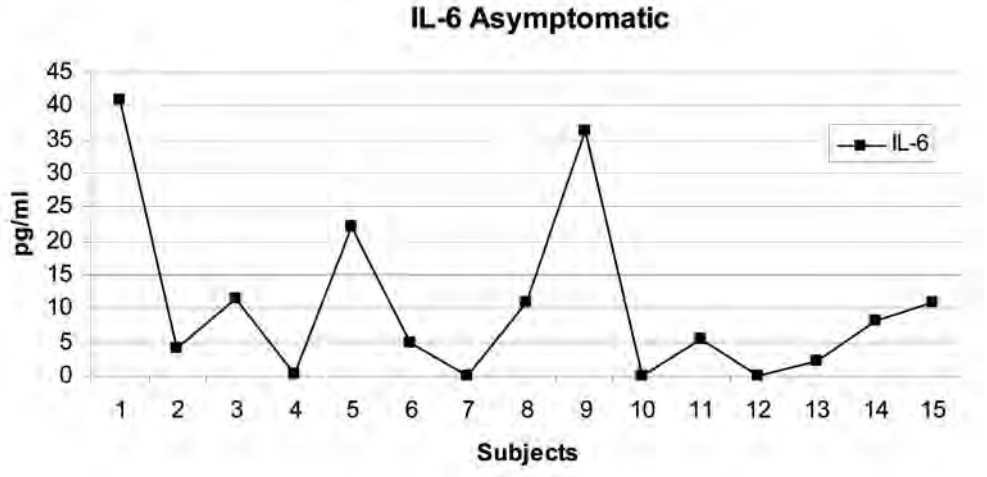

Figure 9: IL-6 cytokine levels determined in the asymptomatic group by ELISA and expressed in pg/ml; more members of the asymptomatic group than the symptomatic group secreted this cytokine but at lower levels.

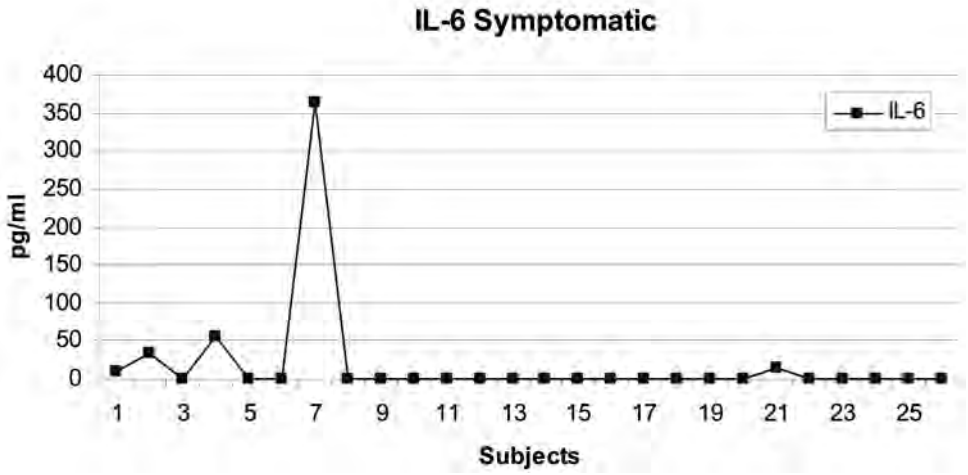

Figure 10: IL-6 cytokine levels in the symptomatic group as expressed in pg/ml; fewer members of the symptomatic group secreted this cytokine but at much higher levels than the asymptomatic group.

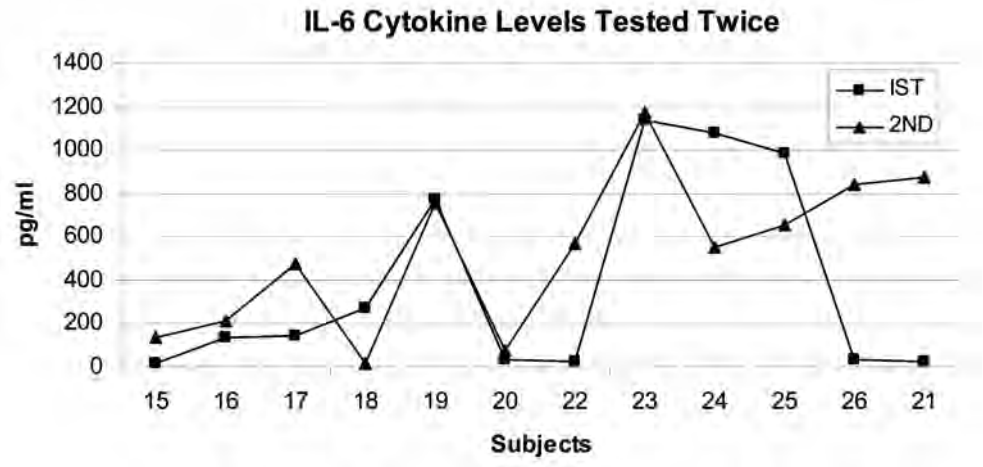

Figure 11: Comparison of first and second determinations of IL-6 cytokine in 12 symptomatic patients within an interval period of 3 months after treatment with antiretrovirals; there is a noticeable increase in IL-6 cytokine levels in most symptomatic patients after three months of HAART

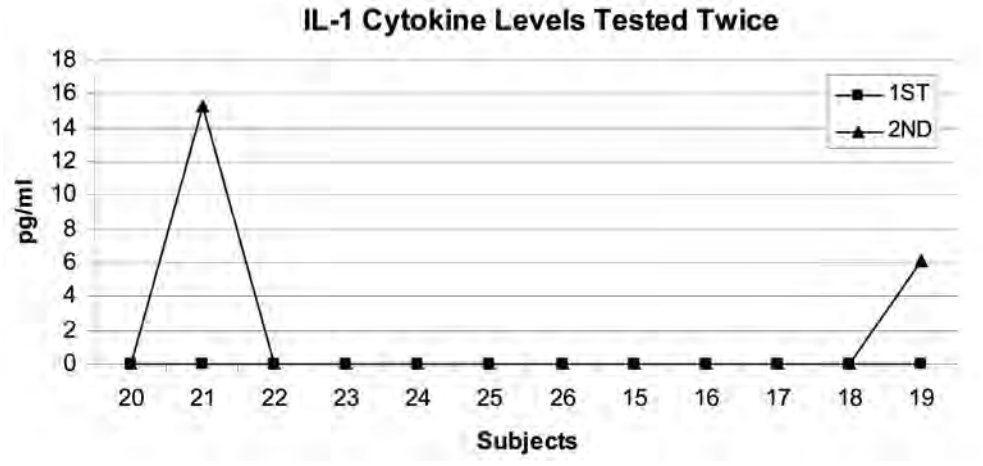

Figure 12: Comparison between first and second determinations of IL-1 cytokine in 12 symptomatic patients within an interval period of 3 months after antiretroviral treatment; IL-1 levels rose from undetectable levels to moderately high levels in two of the twelve symptomatic patients after three months of HAART

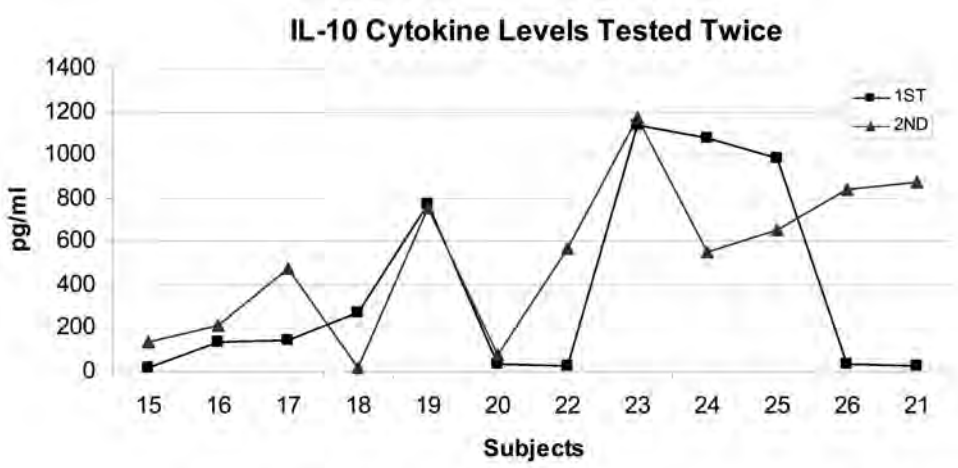

Figure 13: Comparison between first and second determinations of IL-10 cytokine in 12 symptomatic patients within an interval of 3 months after antiretroviral treatment; there were marked increases in IL-10 levels in 8 of the 12 symptomatic patients whose cytokine profiles were determined twice after HAART.

Table 1: Viral RNA, CD4 T cell Counts and Cytokine Levels of Control, Asymptomatic and Symptomatic groups

	Control	Asymptomatic	Symptomatic
RNA (Mean ± S.D)	0.0 ± 0.0	182781.7 ± 497540.5	18741.27 ± 89829.19
Range	0.0 – 0.0	1760 - 1972759	0.0 - 459000
CD4 (Mean ± S.D)	1044.29 ± 176.86	423.6 ± 278.56	578.69 ± 258.42
Range	800 - 1350	94 - 960	164 - 1201
IL-1 (pg/ml, Mean ± S.D)	0.0 ± 0.0	0.0 ± 0.0	7.29*
Range	0.0 – 0.0	0.0 – 0.0	7.29*
IL-4 (pg/ml, Mean ± S.D)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Range	0.0 – 0.0	0.0 – 0.0	0.0 – 0.0
IL-6 (pg/ml, Mean ± S.D)	3.3 ± 2.8	10.516 ± 12.896	18.397 ± 71.692
Range	1.03 – 9.47	0.0 – 40.87	0.0 – 364.2
IL-10 (pg/ml, Mean ± S.D)	0.0 ± 0.0	206.352 ± 289.032	417.539 ± 412.522
Range	0.0 – 0.0	13.55 – 983.554	16.221 – 1298.861
IFN-gamma (pg/ml, Mean ± S.D)	0.0 ± 0.0	2.012 ± 5.409	0.172 ± 0.751
Range	0.0 – 0.0	0.0 – 17.818	0.0 – 3.273

* IL-1 was detectable in only one member of the symptomatic group.

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INVESTIGATION OF THE CYTOTOXIC CAPACITY OF SOME ADHERENT OPPORTUNISTIC ENTEROBACTERIAL STRAINS BY THE MTT ASSAY AND TRANSMISSION ELECTRON MICROSCOPY

Veronica Lazăr[1], Mariana Carmen Chifiriuc[1], D.E.S. Steward-Tull[3], Coralia Bleotu[2], D. Candlich[3], A.C. Wardlaw[3]

[1]*Dept. Microbiology, Faculty of Biology, University of Bucharest, Aleea Portocalelor 1-3, 70709, Romania;* [2]*Institute of Virology St. S. Nicolau, Bucharest, 285 Mihai Bravu Ave., Romania;* [3]*Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G128QQ, Scotland.*

ABSTRACT

The purpose of this study was to determine the cytotoxic effect on CaCo-2 intestinal cells of dialysates obtained from bacterial cultures of some enterobacterial opportunistic strains with different sources of isolation (food, stool culture, acute diarrhoea, urine culture), previously tested and selected for their intensive adherence and invasion capacity to the cellular substratum and also for their cytotoxic effect on cell monolayers. In this study the level of cytotoxicity was measured quantitatively by means of the MTT assay and qualitatively by transmission electron microscopy (TEM). The MTT method uses a tetrazolium salt for the quantitative spectrophotometric assay of CaCo-2 cells survival and proliferation rates in the presence of bacterial dialysates. This test detects the viable cells, which are able to reduce the tetrazolium salt and offers the advantages of a very simple, rapid and precise method. For TEM examination the ultrathin sections were prepared following the standard protocols. The most cytotoxic strains proved to be *Citrobacter freundii 93* strain isolated from stool culture, and *Enterobacter cloacae* 43, isolated from food followed by *E. coli 115* strain isolated from acute diarrhoea. These results correlate well with TEM results pointing out the cytotoxic effect of *Enterobacter cloacae 43* strain and also its ability to induce attachment and to destroy the cell surface (A/E) of HEp-2 cells. Besides their great adherence and invasion capacity, the production and release of cytotoxic factors into the extracellular medium represent virulence factors in these strains. This could be responsible for the increase of the pathogenic potential of opportunistic bacteria and explain their implication in the etiology of severe infections and food-borne diseases. This study proved that the virulence of opportunistic pathogens is not correlated with the strain's origin, the most evident virulence features being exhibited by an *Enterobacter cloacae* strain isolated from food.

Key words: adherence, opportunistic enterobacterial strains, electronic microscopy, cytotoxicity

INTRODUCTION

Despite their occurrence as commensals in the intestine, most members of the *Enterobacteriaceae* family may cause opportunistic infections in hospitalized or debilitated patients [1-4]. Classical enterobacterial pathogens involved in intestinal and extraintestinal infections, predominantly *Escherichia coli*, are known to harbour virulence-associated features distinguishing them from random faecal isolates [5-8]. A number of studies have elucidated the epidemiology and significance of these virulence-associated properties, including somatic antigens, adhesins [9-12] and ability to develop biofilms on cellular and inert substrata, serum resistance and the production of enterotoxins [13], colicins, siderophores and hemolysins [14, 15). Moreover, penetration of the epithelial layer of the intestinal mucosa is a key virulence mechanism of several enteric pathogens, such as *Salmonella, Shigella, Escherichia*, and *Yersinia* species, which can be assayed *in vitro* with human epithelial cell lines, such as HEp-2, Vero and CaCo-2 cells [13, 16]. Some enterobacterial genera, for example *Citrobacter* sp., *Enterobacter* sp., are still considered as typical commensals. The fact that there has

been no evidence to suggest their clinical significance extends beyond their role in opportunistic infections, may explain why there are, with rare exceptions, few studies related to their ability to produce the virulence factors mentioned above. *Citrobacter* species are infrequent nosocomial pathogens, local or systemic breaches of host defenses can allow them to cause a range of infections, including urinary tract infections, neonatal sepsis, brain abscesses, meningitis, blood stream infections, intra-abdominal sepsis and pneumonia. Invasive *Citrobacter* infections are associated with a high mortality rate, with 33 to 48% of patients succumbing to *Citrobacter* bacteremia [17-21]. Awareness of the potential of *E. cloacae* strains to cause disease has been reflected in the increasing number of epidemiological studies of these microorganisms showing that they could be a serious cause of nosocomial Gram-negative bacteremia [26]. Bacterial toxins are currently detected according to their lytic action on erythrocytes and changes in morphology of mammalian cells [26]. These morphological changes are defined as cytopathic effects and can be useful to classify a cytotoxin. Most bacterial toxins cause initially cytopathic effects, but only those denominated as a cytotoxin lead to the death of cultured cells. In addition, viability of the cell monolayer treated with a cytotoxin can be determined by the tetrazolium salt reduction assay [28-30]. The purpose of the present study was to determine the cytotoxic effect on CaCo-2 intestinal cells of dialysates obtained from bacterial cultures of some selected enterobacterial opportunistic strains isolated from food, stool culture, acute diarrhoea and urine culture.

MATERIALS AND METHODS

Bacterial strains

Seven bacterial strains from different genera and sources, namely: *E. coli* 89 (Food-F), *E. coli* 115 (Urine culture-U), *E. coli* 115 (Stool culture from a patient with acute diarrhea -S), *Enterobacter cloacae* 39 (F), *E. cloacae* 41 (F), *E. cloacae* 43 (F), *Citrobacter freundii* 93 (S), *C. freundii* 112 (S) were carefully selected from a number of 120 *Enterobacteriaceae* strains (60 isolated from clinical and 60 from food sources) characterized and identified using API 20 E and tested for their adherence capacity to the cellular substratum represented by the CaCo-2 cells, using the qualitative Cravioto method (16). The strains exhibiting high adherence capacity (quantified by "++" and "+++") were further tested for adherence and invasion capacity of the CaCo-2 cells by quantitative modiefied Cravioto's method [31]. The most invasive 7 strains were selected for the present study.

Extraction of cytotoxin using polymyxin B

The method produced a preparation of intracellular cytotoxins from whole bacterial cells, released by polymyxin B, which was removed by exhaustive dialysis. Bacterial cultures were grown in Nutrient broth and incubated at 37°C for 48 h. The cells were harvested by centrifugation at 10000rpm for 10 min. The pellet in 10 ml sterile 0.85% w/v phosphate saline solution (PBS), mixed and centrifuged at 5000 rpm for 5min (Centrifuge Serval RC-5B). The pellet was resuspended in PBS containing polymyxin at 1.5 mg/ml to 2.0 at $O.D._{600nm}$ (corresponding to a bacterial density of 10^9 cells /ml). The suspension was incubated at 37°C for 2 h and centrifuged at 5000 rpm for 5min. 7. The supernates were dialysed at 4°C against 10.0L PBS, changed three times over a 24 h period. The dialysates were sterilized by passage through a 0.2 μm Millipore filter membrane and stored in 500 μl aliquots at -20°C.

Rapid colorimetric method for the quantitative assay for cytotoxicity

A tetrazolium salt - MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was used to develop this quantitative colorimetric assay for mammalian cell survival and proliferation. This assay detected CaCo-2 living cells after reaction with the cytotoxin (2, 15). CaCo-2 confluent monolayers, cultivated in 96 multiwell plates were infected with 200 μl of two- fold serial dilutions of cytotoxin and incubated for 18 -72 hrs at 37°C in CO_2 atmosphere; 200 μl of tetrazolium salt (MTT) were added and the plates were further incubated for 4 h at 37°C in the CO_2 atmosphere; 100 μl DMSO/0/0.4N HCl per well were added and the A540 nm was measured in an ELISA reader (Anthos). The results were calculated from the average $A_{540\ nm} \pm S.D>$ of 5 assays/specimen [32] and the percentage of viable cells was calculated with the formula:

$$\% \text{ viable cells} = \frac{\text{Mean } A_{540nm} \cdot \text{ of treated cells}}{\text{Mean } A_{540nm} \cdot \text{ of control cells}} \times 100$$

Transmission electron microscopy (TEM)

Bacterial cultures grown in 10 ml nutrient broth at 37°C for 24 h were centrifuged at 10000 rpm for 10 min and the pellet was used to obtain bacterial

suspensions in PBS, adjusted to a final density of 10^9 cells/ml (OD = 2.0); HEp-2 monolayers of 80% confluency, cultivated in Eagle MEM supplemented with 10% foetal calf serum, glutamine 1% and antibiotics were infected with bacterial suspensions at a final density of 10^7 /ml; and incubated for 2 h at 37°C; The cells were fixed with 2.5% (v/v) glutaraldehyde and prepared for examination by TEM in an Hitachi-HU-11 microscope [16, 28].

RESULTS AND DISCUSSION

The MTT quantitative cytotoxicity assay demonstrated that opportunistic enterobacterial strains from different sources, genera and species exhibited a high cytotoxic potential for CaCo-2 cells. The results obtained by MTT cytotoxicity assay revealed that *Citrobacter freundii* 93 isolated from stool culture exhibited the highest cytotoxic effect among the enterobacterial strains, followed by *Enterobacter cloacae* 43 strain isolated from food and *E. coli* 115 isolated from acute diarrhoea (Fig. 1-3). Previous studies have demontrated that *Citrobacter freundii* biotype 4280 produces attaching and effacing (AE) lesions in the large intestine of laboratory mice and is the causative agent of transmissible murine colonic hyperplasia. Southern analysis revealed that biotype 4280, but not 20 other strains of *C. freundii*, contained DNA homologous to the eae (*E. coli* attaching and effacing) gene which is necessary for AE activity by enteropathogenic *E. coli* in vitro [2]. Reports about the production of toxins by *E.cloacae* are very scarce. Recently, Paton and Paton (24) isolated a strain pro-

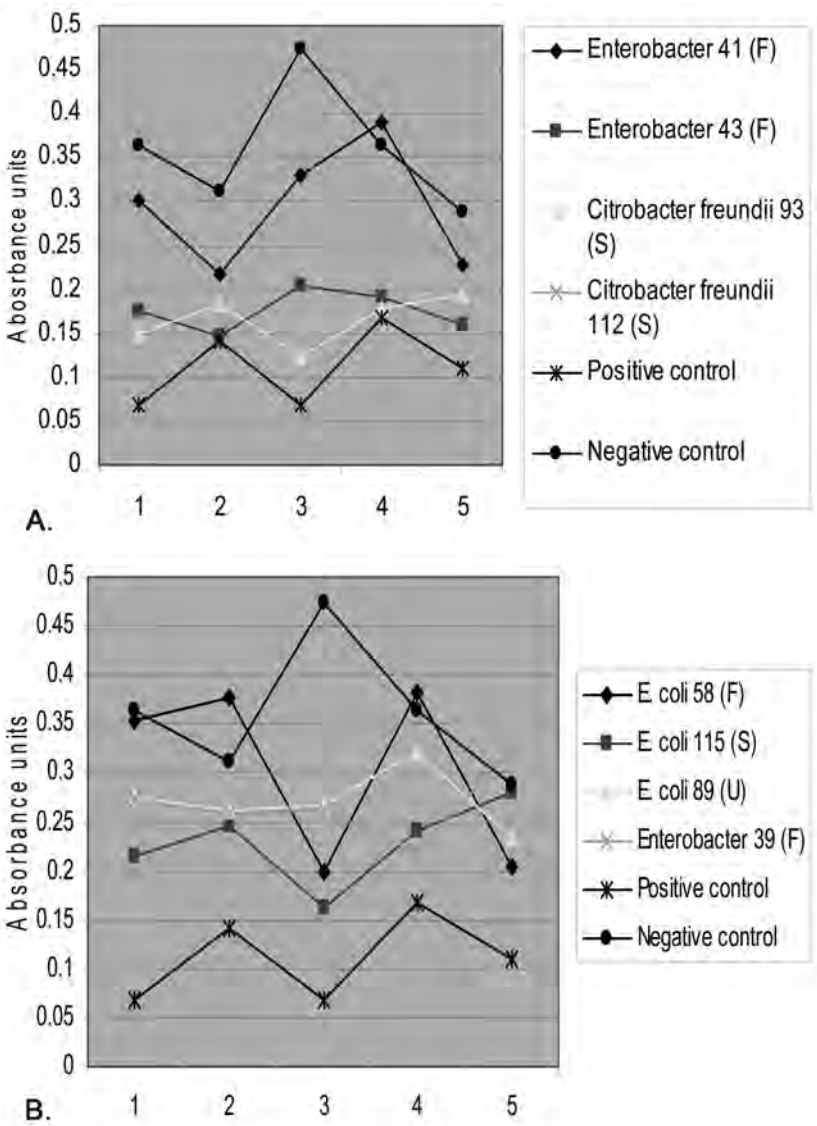


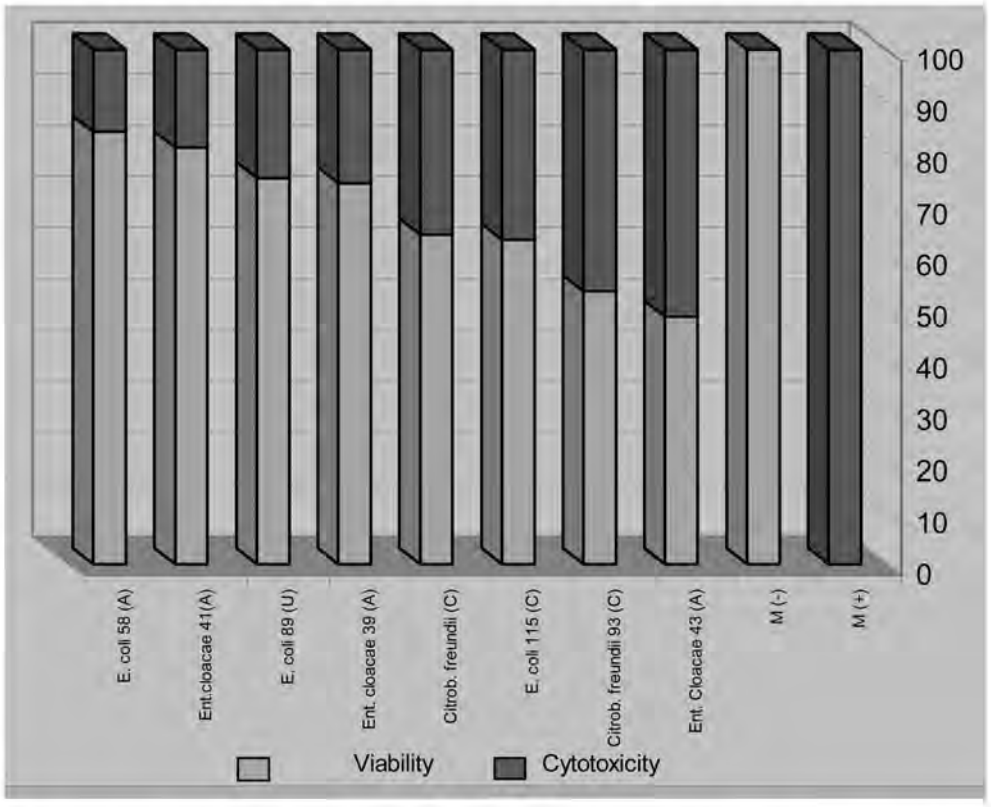
Figure 1.A,B - The degree of cytoxicity on CaCo-2 cells of enterobacterial culture dialysates (5 assays / specimen) expressed as absorbance units assayed by MTT colorimetric method.

ducing a Shiga-like toxin II-related cytotoxin from a patient with haemolytic-uremic syndrome. In our study, *E. cloacae* 43 strain with food origin exhibited a high level of cytotoxicity as evidenced by the MTT assay. These results also correlated with the cell monolayer detaching effect observed during previous adherence/invasion assays with CaCo-2 cells, and confirmed by electron microscopy showing the cytotoxic effect of *Enterobacter cloacae* 43 strain on the HEp-2 cells. TEM revealed a very intimate contact between the bacterial cell and the cell membrane of HEp-2 cells, as well as the capacity to induce attaching-effacing (A/E) lesions (Fig. 4 A, B). Schmidt et al. [15] have demonstrated the intergeneric occurrence of SLT-II-related toxins, which may well be a new marker of enteropathogenicity in *C. freundii*. The findings that the toxin genes belong to the *slt*-II family plus their evident instability in the majority of strains should help pave the way to a better understanding of their role in diarrhea or food poisoning, especially in the presence of host predisposing factors [33-35].

The results of this study showed that among the *E. coli* tested strains, the *E. coli* 115 strain, isolated from stool culture of a patient with acute diarrhoea, exhibited the highest cytotoxic potential (Fig. 1-3).

CONCLUSIONS

Our results have shown that different genera of non-pathogenic enterobacteria isolated from different sources, especially *C. freundii* 93 isolated from stool culture and *E. cloacae* 43 strain isolated from food, possess some virulence properties recognized as important during the onset of intestinal and extra-intestinal infections. Since they have the ability to adhere and to invade eukaryotic cells, it appears clear that they have the capacity to cause systemic infections after intestinal translocation. The presence of specific virulence determinants as significant cytotoxic activity on CaCo-2 and HEp-2 cells of *E. cloacae* and *C. freundii* may play a definitive role in the genesis of opportunistic infections.



$$\% \text{ D.C. (dead cells)} = 1 - \frac{\text{Medium O.D. of the treated cells}}{\text{Medium O.D. of the negative control cells}} \times 100$$

Fig. 2. Cytotoxic activity of bacterial cultures dyalisates on CaCo-2 cells viability

It would seem that the virulence features harboured by the opportunistic pathogens is not correlated with the strain origin as demonstrated by the virulent strains *C. freundii* 93 and *E. cloacae* 43.

Our results confirm the fact that opportunistic infections triggered by the enterobacterial strains, may be the result of both host predisposing factors and the bacterial virulence determinants such as those detected in the present study.

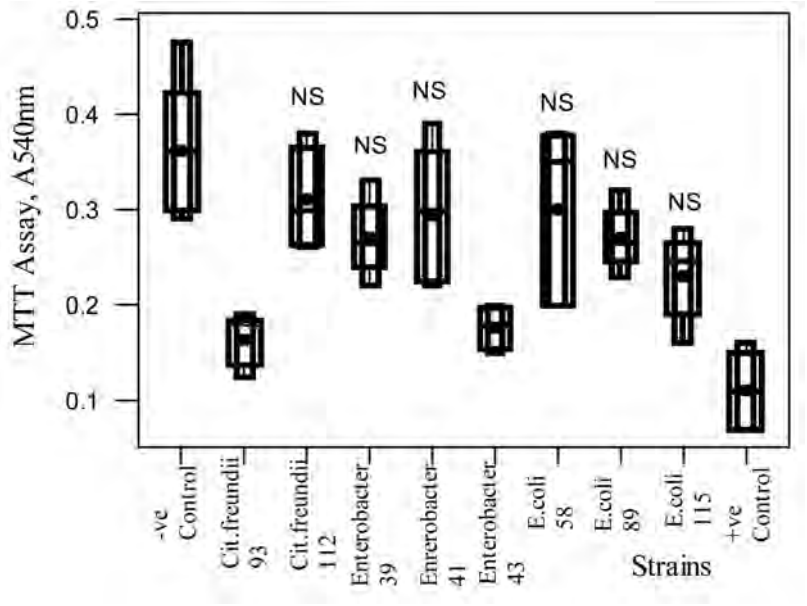


Fig. 3. Boxplot of the pooled values for five assays for each strain in the cytotoxicity assay with CaCo-2 cells.
Legend: NS. Not statistically significant difference from the positive controls by the Dunnett and Tukey analyses. The outer box shows the interquartile range with the median bar, the circle is the mean value and the inner box outlines the 945% confidence limits of the media (30).

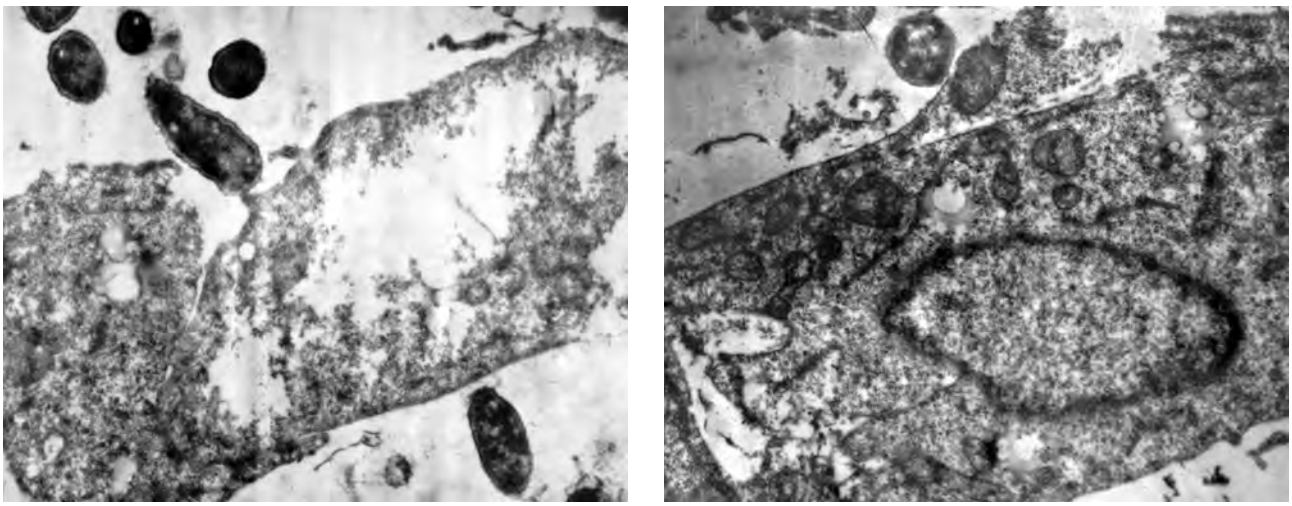


Fig. 4. A,B - *Enterobacter cloacae* 43 cytotoxic effect of adherent bacteria on HEp-2 cells (magnification x 23,900)

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IN VITRO ASSESSMENT OF THE ANTIMICROBIAL ACTIVITY OF NEW N-ACYL-THIOUREA DERIVATIVES

Lia Mara Diţu[1], Gr. Mihăescu[1], Carmen Chifiriuc[1], Coralia Bleotu[3], Laurenţiu Morusciag[2], George Mihai Nitulescu[2], Alexandru Missir[2]

[1]*Bucharest University, Faculty of Biology,* [2]*Pharmaceutical Chemistry Department, Faculty of Farmacy, "Carol Davila" University of Medicine and Pharmacy,* [3]*Institute of Virology Prof. C.C. Iliescu*

ABSTRACT

The qualitative screening of the susceptibility spectra of different microbial strains to the newly synthesized substances complexes was performed by adapted disk diffusion techniques, while the quantitative assay of the minimal inhibitory concentration (M.I.C., μg/cm^3) value was based on liquid medium serial microdilutions. The compounds were solubilized in dimethylsulfoxide (DMSO). The *in vitro* biological screening effects were tested against a microbial inoculum of ~1.5x10^8 UFC/cm^3, corresponding to 0.5 McFarland standard density, obtained from Gram positive (*Staphylococcus aureus, Bacillus subtilis*), Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and fungal strains (*Candida albicans*). In order to investigate the influence of the subinhibitory concentration of the tested substances on the expression of different virulence features, the strains were incubated overnight in the presence of the newly synthesized thiourea derivatives (vol:vol) and different virulence features were investigated, i.e: adherence capacity to the cellular substrate represented by HeLa cells and to inert substrata quantified by slime test and soluble enzymatic virulence factors (haemolysins and other pore-forming toxins, proteases activity, DN-ase and siderophores production). The cytotoxicity was assessed microscopically, by observing the effect of the tested compounds on the cellular substratum integrity.

Key words: thiourea derivatives, antimicrobial activity, new treatment strategies

INTRODUCTION

Bacterial resistance to existing drugs is a constantly growing problem that, combined with a decline in the development of new antibiotics, constitutes a significant threat to human health (Projan, 2003). The identification of new antimicrobial agents is therefore of considerable importance. The general necessity to find new antimicrobial compounds is determined by three major aspects: continuous emergence of new etiological agents, antibiotic resistance and bioterrorism. The dynamic of microorganisms include new bacterial strains with new clinical manifestations under the environmental influence including human intervention, antibiotics and biotechnology.

N-acyl-thiourea derivatives are associated with a wide spectrum of biological activities, especially antibacterial and antifungal, a large number of such compounds being already reported (Morusciag *et al.*, 2008). Taking into consideration the antibacterial potential of the thiourea scaffold we synthesized a series of 2-(2'-phenylethyl)-benzoyl-thioureides. In previous papers (Misir et al, 2009) synthesis methodology and the physico-chemical characterization of these new compounds were presented (Fig. 1).

The aim of the present study was the screening of the antimicrobial activity of new N-acyl-thiourea compounds towards some pathogenic bacterial and fungal strains.

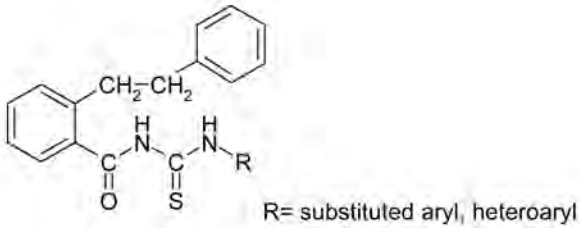

Fig. 1. The chemical structure of the new 2-(2'-phenylethyl)-benzoyl-thioureides

MATERIALS AND METHODS

Microbial strains

The antimicrobial activity of the investigated compounds was tested against bacterial and fungal strains recently isolated from clinical specimens, as

Table 1. Chemical structure of the new thioureides

Code	R=	Molecular formula	Chemical name
C11	2-C_2H_5	$C_{24}H_{24}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(2-ethyl-phenyl)-thiourea
C12	3-C_2H_5	$C_{24}H_{24}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(3-ethyl-phenyl)-thiourea
C13	4-C_2H_5	$C_{24}H_{24}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-4-methyl-phenyl)-thiourea
C14	2-n-C_3H_7	$C_{25}H_{26}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(2-propyl-phenyl)-thiourea
C15	4-n-C_3H_7	$C_{25}H_{26}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-4- propyl-phenyl)-thiourea
C16	2-i-C_3H_7	$C_{25}H_{26}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(2-isopropyl-phenyl)-thiourea
C17	4-i-C_3H_7	$C_{25}H_{26}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(4-isopropyl-phenyl)-thiourea
C18	4-n-C_4H_9	$C_{26}H_{28}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(4-butyl-phenyl)-thiourea
C19	2-s-C_4H_9	$C_{26}H_{28}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(2-secbutyl-phenyl)-thiourea
C20	4-s-C_4H_9	$C_{26}H_{28}N_2OS$	N-[2-Phenetyl)-benzoyl]-N'-(4-secbutyl-phenyl)-thiourea

well as reference strains belonging to the following genera and species: *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans*. Bacterial suspensions of 1.5 x 10^8 CFU/mL or 0.5 McFarland density obtained from 15-18 h bacterial cultures developed on solid media were used in the experiments. The antimicrobial activity was tested on Mueller-Hinton medium recommended for the bacterial strains and Yeast Peptone Glucose (YPG) medium for *Candida albicans*.

Qualitative screening of the antimicrobial properties of the tested compounds

The qualitative screening was performed by an adapted disk diffusion method. Petri dishes with Mueller Hinton (for bacterial strains)/YPG (for yeasts) media were seeded with bacterial inoculums as for the classical antibiotic susceptibility testing disk diffusion method; 5 mm diameter paper filter disks were placed on the seeded medium, at 30 mm distance. Subsequently, the disks were impregnated with 10 µL tested compound solution (1000 µg/mL). The plates were left at the room temperature for 20-30 minutes and then incubated at 37°C for 24 h. The positive results were read as the occurrence of an inhibition zone of microbial growth around the disk (Balotescu *et al.*, 2005).

Quantitative assay of the antimicrobial activity

MIC (Minimal Inhibitory Concentration) value for N-acyl-thiourea derivates was determined by the twofold microdilution technique, using 96 multi-well plates, starting from 1000 µg/mL to 1.953 µg/mL, for each tested strain. Simultaneously, there were achieved serial dilutions for DMSO in the same volume, in order to obtain the negative control. 20 µL of microbial suspension at the standard density of 0.5 Mc Farland was added in each well. The plates were incubated for 24 h at 37°C, and MICs were read as the lowest concentration of compound which inhibited the microbial growth.

Soluble enzymatic virulence factors

The microbial strains were cultivated in nutrient broth with and without addition of subinhibitory concentrations of N-acyl-thiourea derivatives (0.5 mg/mL) and the obtained overnight microbial cultures were used for the performance of the following virulence tests:

Plate haemolysis: the strains were streaked on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation at 37°C for 24 h the clear zone (total lysis of red blood cells) around the colonies was registered as positive reaction (Lazar et al., 2005).

Protease activity: it was determined using two different media as substrate: 15% soluble casein and 3% gelatine agar, respectively (Cantacuzino Institute Media Laboratory). The strains were spotted and after incubation at 37°C up to 24 h, precipitation surrounding the growth area indicated casein/gelatine proteolysis (caseinase/gelatinase presence) (Lazar et al., 2005).

DN-ase production: it was studied using DNA agar medium. The strains were spotted and after incubation at 37°C up to 24 h, a drop of a 1mol/L HCl solution was added upon the spotted cultures; a clearing zone around the culture was registered as positive reaction (Lazar et al., 2005).

Lipase production: the cultures were spotted on Tween 80 agar with a substrate at a final concentration of 1% and were incubated at 37°C up to 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction (Lazar *et al.*, 2005).

Mucinase production: it was determined using porcine stomach mucine (final concentration 1% in

Table 2. The quantitative results (MIC) of the antibacterial activity for the tested thioureide compounds

Microbial strains	MIC value (μg/mL)									
	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
S.aureus 1263	500	500	500	500	500	500	500	500	500	500
S.aureus IC 13204	500	500	250	500	250	250	250	500	500	500
K.pneumoniae 1204	500	500	500	500	500	500	500	500	500	500
K.pneumoniae IC 13420	500	500	500	500	500	500	500	500	500	500
E.coli IC 13529	500	500	500	500	500	500	500	500	500	500
E.coli IC 13147	500	500	500	500	500	500	500	500	500	500
B.subtilis ATCC 6633	500	500	500	500	500	500	500	500	500	500
C.albicans IC 249	125	125	125	125	125	125	500	500	500	500
C.albicans 101404	500	500	500	500	500	500	500	500	500	500
Ps.aeruginosa ATCC 27853	250	250	250	250	250	250	250	250	250	250
Ps.aeruginosa 1246	250	250	250	250	250	250	250	250	250	250

brain heart agar with 2% NaCl). The strains were spotted and incubated up to 7 days at 35°C; the enzyme activity was detected by the presence of precipitation zones around the growth area (Lazar *et al.*, 2005).

The adherence capacity o the cellular substrate represented by HeLa cells (Cravioto's adapted method) (Lazar *et al.*, 2005).

In this purpose, HeLa cells were routinely grown in MEM enriched with 10% heat-inactivated (30 min at 56°C), fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), supplemented 0.5 mL of gentamycin (50 μg/mL) (Gibco BRL) and incubated at 37°C for 24 h. HeLa cells monolayers grown in 6 multi-well plastic plates were used at 80-100% confluency. For the adherence assay, the HeLa cell monolayers were washed 3 times with phosphate buffered saline (PBS); 1 mL of bacterial suspensions of 10^7 CFU/mL prepared in PBS was used for the inoculation of each well with and without addition of subinhibitory concentrations of N-acyl-thiourea derivatives (0.5 mg/mL). The inoculated plates were incubated for 2 h at 37°C. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 min) and Giemsa stained (Merck, Darmstadt, Germany) for 30 min. The plates were washed, dried at room temperature overnight, examined microscopically (magnification, × 2500) with I.O. and photographed with a Contax camera adapted for Zeiss microscope.

Cell cycle analysis. The cell cultures used were represented by HeLa (ECACC # 93021013) cells. The cells were grown in Dulbecco's Modified Essential medium DMEM (Sigma) supplemented with 10% fetal calf serum (Sigma) at 37°C, 5% CO_2, in a humid atmosphere; after 24 h of incubation 100 μg/mL of tested compound were added. Cells from the supernatant and monolayer were harvested and 1×10^6 cells were fixed in 70% cold ethanol, stained with propidium iodide and analyzed to the Coulter EPICS XL flow cytometer (Beckman Coulter).

RESULTS

Aantimicrobial activity

The new compounds did not exhibit significant antimicrobial activity when using the qualitative adapted diffusion method.

The tested thioureides of the 2-phenethyl-benzoic acid generally presented a low level antimicrobial activity with high MIC values ranging from 125 to 500 μg/mL (table 2). However, the lowest MIC values were exihibited against one *C. albicans* (125 μg/mL) and also against the *Ps. aeruginosa* ATCC 27853 and *Ps. aeruginosa* 1246) strains (250 μg/mL).

The N-[2-Phenethyl)-benzoyl]-N'-(4-methyl-phenyl)-thiourea (C13) and N-[2-Phenetyl)-benzoyl]-N'-(4-propyl-phenyl)-thiourea (C15) compounds exhibited a broad spectrum of antimicrobial activity against a large number of tested microbial strains, i.e. *S. aureus* IC 13204, *Ps. aeruginosa* ATCC 27853, *Ps. aeruginosa* 1246 and *C. albicans* IC 249 (table 1).

Expression of the cell associated and soluble enzymatic virulence factors

Because the high MIC values were indicating a low antimicrobial activity and the impossibility to use these compounds as microbicidal agents, we further tested the influence of the subinhibitory concentrations of the new compounds on the expression of different virulence factors implicated in the pathogenicity of the tested strains, in order to detect a possible anti-pathogenic effect probably exhibited by interfering

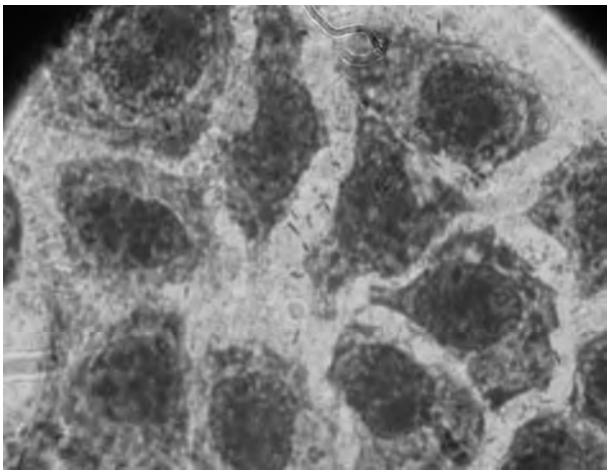

Fig. 2. The adherence pattern of *Ps. aeruginosa* ATCC 27853 - control (Giemsa staining, HeLa cells, x1500)

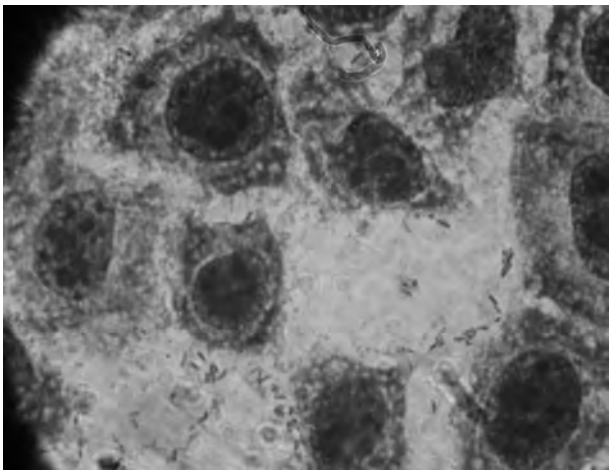

Fig. 3. The adherence pattern of *Ps. aeruginosa* ATCC 27853 in the presence of sub-inhibitory concentration of N-[2-Phenetyl)-benzoyl]-N'-4-propyl-phenyl)-thiourea (C15) (Giemsa staining, HeLa cells, x1500)

with the bacterial signaling molecules used in the coordinated regulation of the virulence and pathogenic factors.

Among the tested microbial strains, some changes in the virulence factors expression was observed only for *Ps. aeruginosa* strains. The tested strains exhibited an increased ability to adhere to the cellular substratum in the presence of sub-inhibitory concentrations of the thioureide derivatives probably by inducing changes in adhesins synthesis or by modulating the adherence due to their hydrophobic character. Two of the tested compounds (C12, C15) changed the adherence pattern (from a diffuse to a localized one) in case of *Ps. aeruginosa* ATCC 27853 strain (fig. 2, 3) and three compounds (C15, C17, C18) exhibited the same effect on *Ps. aeruginosa* 1246 strain. The stimulation of adherence ability was also demonstrated by an increased adherence index (from 70% to 100%) for these two tested strains.

Concerning the soluble virulence factors expression, the results showed that all 10 tested compounds inhibited the expression of mucinase and DN-ase in *Ps. aeruginosa* ATCC 27853.

Influence of the tested compounds on eukaryotic cell cycle

Cell cycle analysis in the presence of 100 μg/mL tested compounds showed that C12, N-[2-Phenetyl)-benzoyl]-N'-(3-ethyl-phenyl)-thiourea and C14, N-[2-Phenetyl)-benzoyl]-N'-(2-propyl-phenyl)-thiourea induced an increase in G2/M phases (table 4) after 24 h (table 3).

The appearance of a peak in the left part of the histogram, associated with apoptosis was observed for C19, N-[2-Phenetyl)-benzoyl]-N'-(2-secbutyl-phenyl)-thiourea (43.99%) (fig. 4), demonstrating that this compound exhibited a cytotoxic effect on the eukaryotic cells.

Table 3. The effect of 100 μg/mL tested compounds on HeLa cell cycle

	G0/G1 %	S %	G2/M %
HeLa Control	76.15	11.85	5.88
C11	76.89	9.96	6.66
C12	69.27	14.22	7.33
C13	60.50	11.77	5.72
C14	74.41	15.89	6.73
C15	77.19	10.38	5.63
C16	63.95	11.25	5.00
C17	76.55	11.82	5.88
C18	78.22	12.10	4.93
C19	46.05	6.32	3.97
C20	78.48	11.56	6.07

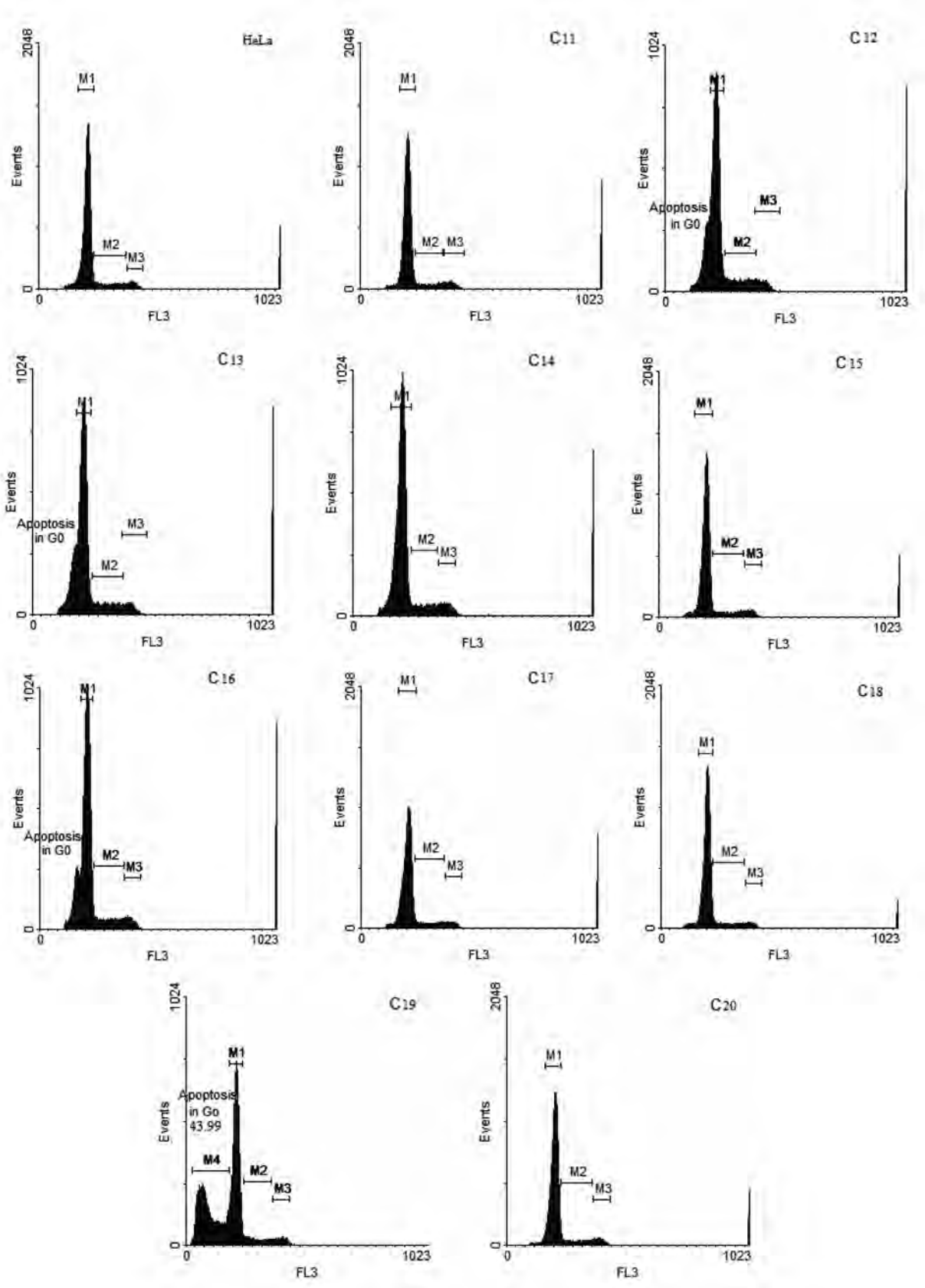

Fig. 4. HeLa cells cycle histograms in the presence of 100 μg/mL of tested compounds (HeLa cells)

DISCUSSION

The continuing emergence of multiple-drug-resistant bacterial strains has necessitated finding novel strategies for treating bacterial infections. The N-acyl-thiourea derivatives are associated with a wide spectrum of biological activities, especially antibacterial and antifungal (Morusciag et al, 2008, Limban et al, 2008). In the attempt to develop new alternative strategies for antibiotherapy, ten new N-acyl-thiourea derivatives were synthesized and tested against different microbial strains.

In our study, only two tested compound (N-[2-Phenetyl)-benzoyl]-N'-4-ethyl-phenyl)-thiourea (C13) and N-[2-Phenetyl)-benzoyl]-N'-4-propyl-phenyl)-thiourea (C15)) demonstrated a relatively antimicrobial activity with an MIC of 250 µg/mL towards the two tested *Ps. aeruginosa* strains. *Ps. aeruginosa* is an opportunistic human pathogen that tends to infect individuals with cystic fibrosis or immunocompromised patients, such as those suffering burns or undergoing cytotoxic chemotherapy (Lyczak *et al.*, 2000, Takaya *et al.*, 2008).

The results showed that the thioureides derivatives having a smaller substituent in *para* position on the aromatic nucleus ((N-[2-Phenetyl)-benzoyl]-N'-4-ethyl-phenyl)-thiourea (C13) and N-[2-Phenetyl)-benzoyl]-N'-4-propyl-phenyl)-thiourea) exhibited a wider antimicrobial spectrum, including *S. aureus, Ps. aeruginosa* and *C. albicans*. Growing of the molecular weight of the substitute radical on the phenyl group lead to a greater antimicrobial effect only towards *Ps. aeruginosa* tested strains (N-[2-Phenetyl)-benzoyl]-N'-(4-butyl-phenyl)-thiourea (C18), N-[2-Phenetyl)-benzoyl]-N'-(2-secbutyl-phenyl)-thiourea (C19) and N-[2-Phenetyl)-benzoyl]-N'-(4-secbutyl-phenyl)-thiourea (C20).

The discovery that a wide spectrum of organisms using quorum sensing to control virulence factors production makes it an attractive target for antimicrobial therapy. Through blocking this cell-to-cell signaling mechanism, pathogenic organisms that use quorum sensing to control virulence could potentially be rendered avirulent (de Kievit et al, 2000). The pathogenesis of *Ps. aeruginosa* infection is attributed to the production of both cell-associated and extracellular virulence factors (Rumbaugh *et al.*, 1999). The stimulatory effect exhibited by the sub-inhibitory concentration of thioureide derivatives on *Ps. aeruginosa* strains adherence ability can be the results of changes occurred in the adhesins synthesis or in the hydrophobicity character of the bacterial cell wall, influencing the adherence to the eukaryotic cellular substrate, taking into account that any increase in cell wall hydrophobicity increase the adherence to different substrata. The inhibition of mucinase and DN-ase activity in one of the two tested *Ps. aeruginosa* strain could account for the decrease of the invasive ability of the respective strain in the presence of sub-inhibitory concentrations some of the tested compounds.

CONCLUSIONS

Taking these findings together, our study is suggesting that the antimicrobial spectrum of tested thioureide derivatives was influenced by the size of the substitutent groups and the degree of ramification and also on their positioning on the benzene nucleus. For this reason, the ethyl and methyl substituents in *meta* position induced a better activity than n-butyl and sec-butyl substitutes. This observation is leading us to the tentative of synthesizing new similar compounds with other basic aromatic amines.

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BIOMARKERS DISCOVERY IN CANCER - UP-DATES IN METHODOLOGY

Cristina Tacu[1], Monica Neagu[2]*, Carolina Constantin[2], Maria Sajin[1]

[1]*Universitary Hospital, 169 Splaiul Independentei, sector 5, Bucharest, Romania*

[2]*„Victor Babes" National Institute of Pathology, 99-101 Splaiul Independentei, sector 5, 050096 Bucharest, Romania*

ABSTRACT

Biomarkers are biomolecules that can indicate normal/pathological processes, or physiological responses to therapy. Due to the serum abundance in proteins, such as albumin and lypo/glycoproteins, biomarkers are difficult to assess. Serum biomarkers identification can contribute to personalized medicine and improve cancer diagnostic and prognostic. The paper summarizes some of the proteomics techniques and the workflow used for protein signatures identification associated to cancer development. Thus, biomarkers validated for prostatic, breast, cervical or lung cancers are presented as examples for clinical application of serum markers. In spite of the continuous research efforts, there are only few validated biomarkers that have proved a good predictive power in cancer. Modern technology and the combination of various techniques used for proteins quantification represent important means for the identification and validation of new biomarkers.

Key words: cancer, serum biomarkers, proteomics

INTRODUCTION

Biomarkers research starts with the discovery of potential candidates and ends with their validation, stage in which they need to be robust enough to prove their clinical application [1]. International networks involved in biomarker discovery focus on the following areas of research:

- Developing general tests and establishing unitary discovery schemes;
- Developing multiplex technology adapted for proteomic and genomic biomarkers simultaneous discovery;
- Accurate quantification of body fluids components as future, possible biomarkers candidates;
- Efficient strategy implementation for the translation of research towards clinic.

Each possible biomarker is subjected to an operational flux that consists of discovery, confirming and validation stages (Figure 1). In the first stage of proteomic studies, a separation method is used, electrophoretic or chromatographic, followed by identification techniques, such as mass spectrometry. After finding out the molecular mass of the compounds, international data base can identify the actual compound. This identification is based on the availability of data regarding the compound, namely the properties of the molecule (isoelectric point, molecular mass, component peptides, and so on) in relation to the isolation source. The validation of an identified protein is done by immune-type techniques like ELISA, immunoblotting, N-terminal sequencing. The proteomic technology can be applied to any protein mix from any type of fluid. Thus, in the last years proteomics contributed to the discovery of protein-biomarkers, used for developing new diagnostic, prognostic and therapy monitorization [2].

In clinical investigation, proteomic technology [3] focuses on the comprehensive analysis of proteins expressed by the genome. In the work-flow of biomarker discovery, this evaluation, needs to be correlated with a pathology and/or a therapy [2] as the commonly accepted definition of biomarker is "an indicator measurable in biological systems as a result of normal and pathological processes or as a result of therapy" [4].

It is known that the human serum contains 60-80 mg/mL total protein among various molecules such as aminoacids, lipids, salts, carbohydrates, etc. Analyzing the human serum "proteome" new biomarkers can be detected in relation with pathology. One of the major difficulties in biomarkers discovery is the low concentration of a secreted protein due to a pathological process that can be totally masked by abundant proteins. For example, albumin synthesized by the liver at 12 g/day has a 21 days half life [5], therefore it represents more than 50% of the serum proteins and it can camouflage all other less abundant protein components. Taking into account the abundance and the dynamics of several proteins

* *Corresponding author:* Monica Neagu, „Victor Babes" National Institute of Pathology and Biomedical Sciences, 99-101 Splaiul Independentei, 050096 Bucharest, Romania; Telephone: 40 21 319 45 28; Fax: 40 21 319 45 28; e-mail: neagu.monica@gmail.com

(e.g. albumin, immunoglobulin's), it is mandatory to fractionate somehow the sera prior to identifying possible biomarkers candidates [6].

After protein denaturation, separation and purification is performed using mainly their physical properties. Purification techniques are based on the isoelectric point (pI), hydrophobicity and molecular mass. Using pI, serum proteins can be isolated by ionic exchange chromatography and pH gradient polyacrialmide gel separation. Ionic exchange chromatography is a high resolution simple technique that can separate proteins from a complex mix using the difference in electric charge. Choosing the type of ionic exchanger and the working pH, proteins can be separated from complex mixtures [7]. In 2005 the first reports were published in which ionic exchange chromatography was used to detect proteins that can be candidate biomarkers [8].

Using the property of hydrophobicity, proteins can be separated by Reversed-Phased High Performance Liquid Chromatography (RP-HPLC). RP-HPLC is a high resolution technique that can separate proteins and peptides. Moreover, using this technique the very low concentrations compounds in body fluids can be subjected to further concentration. It is a technique that can be adapted for high number of proteins separation [9] and that can be compatible with ionic exchange chromatography and mass spectrometry.

Using the molecular mass, proteins can be separated with Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) or Size Exclusion Chromatography (SEC). Applying these techniques, the information regarding molecular mass is only qualitative. SEC, although with a low resolution, has been used in the study of high molecular mass plasmatic proteins that bind chemotactic peptides secreted by neutrophils [10]. These peptides later became serum markers in colon cancer when mass spectrometry has validated them [11].

PROTEIN IDENTIFICATION

2-Dimension Electrophoresis (2-DE)

2-DE separates thousands of proteins [12] and compares the protein profiles of various biological samples. Although is not highly accessible it is the general method for identifying proteins based on the differences in the pI and molecular masses. Using this method, from the sera of hepatocellular carcinoma patients, C3a complement component was identified as a possible biomarker [13]. Recently, using the same method in correlation with Western-blot, in the sera of patients with cervical lesions alteration of protein profiles was detected in comparison with normals. These alteration comprised haptoglobin, apolipoproteins, metalloproteinases and transthyretine [14].

2-DE is still a limited method because it does not detect very small or very big proteins, plasma membrane associated proteins, very hydrophobic proteins, very acid or very basic [15].

SELDI-TOF-MS (Surface-enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry)

Mass spectrometry when applied to serum proteins delivers good information regarding the molecular mass. It is a method highly used in proteomics because it allows a semi quantitative analysis of many proteins from biological samples. Using this method proteins are selectively retained [16] on a chip and then covered by a specific matrix. After applying the laser, each sample displays a protein profile displayed as a classical spectrogram. SELDI-TOF-MS was used for serum protein profiling in correlation with various pathologies and thus for biomarkers discovery [17].

Two-dimensional Liquid Chromatography Mass Spectrometry (2DLC-MS)

Protein profiles can be obtained using ionic exchange chromatography [9] or cromatofocussing [18]. 2-DLC was used for biomarkers discovery [19] and for proteomic analysis of the sera [20]. Multidimensional liquid chromatography (MDLC) combines reverse phase chromatofocussing separation and mass spectrometry [21]. In proteomic studies, MDLC by-passes 2-D electrophoresis limitations by having the possibility to analyze complex samples as human serum rich in protein components with moderate pI [22]. The obtained data are complex and can be analyzed only with SEQUEST, a data analysis program used for protein identification. Multidimensional protein identification (MudPIT) [23] is a complex methodology comprising the above mentioned technology that can be applied in the discovery and confirmation stage of biomarkers (see Figure 1).

Matrix-Assisted Laser Desorption- Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS is the most precise method for protein identification [24]. After samples are subjected to 2-DE, the obtained spots are excised and denaturated with trypsin and the resulted peptides are incorporated in a matrix and analyzed on a chip by mass spectrometry. The analysis gives data regarding molecular mass, pI and component peptides. Obtained data are matched in the data base for the final identification using *Swiss-Prot, TrEMBL* **(UniProt**

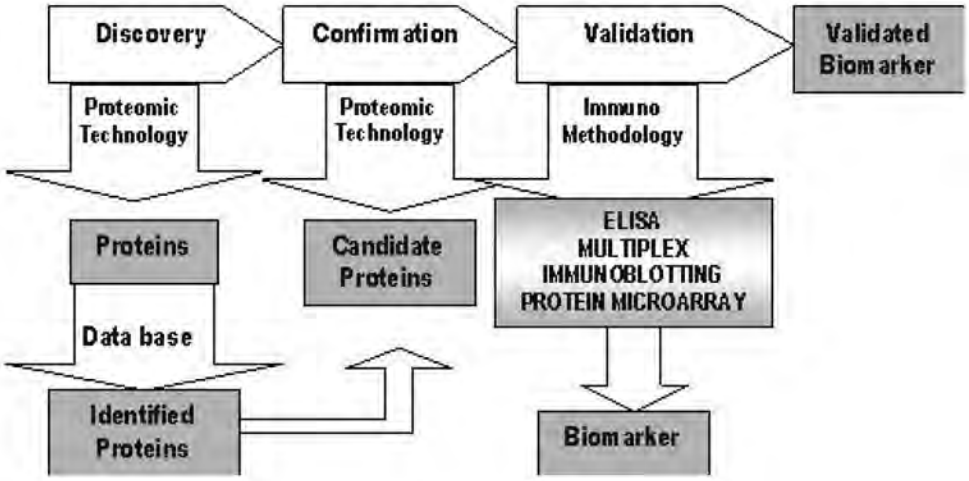

Figure 1. Work-flow for validating biomarkers

Knowledgebase) or other known data bases. MALDI-TOF-MS has been utilized for identification of transferine associated to ovarian cancer [25]. In other pathologies, like multiple sclerosis, this technique delivered a specific serum protein pattern [26].

Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS)

ESI-MS, in comparison with other mass spectrometry techniques, utilizes a more discrete ionization, therefore the protein fragmentation is less extended. For structural studies the molecule is further splited and analyzed in the tandem technology, namely ESI-MS coupled with MS [27]. Recently, using this technology in the sera of patients diagnosed with pancreatic cancer has been discovered various candidate proteins such as alpha-2 macroglobulin, ceruloplasmin and 3C component of the complement system [28].

PROTEIN VALIDATION

Immunoanalysis

ELISA (Enzyme-Linked ImmunoSorbent Assay) is the most exploited technique in the validation stage of any new discovered protein that has a chance to turn into a biomarker. It is a rapid and precise method that can detect low concentrations of proteins from complex biological samples. The techniques has its variants in the fluorescence detection mode, when it is called Fluorescent ImmunoAssay (FIA). The improved alternative of this procedure is represented by multiplex technology combining flow cytometry with ELISA technique [29]. Recently, by multiplex technology some serum biomarkers associated with apoptosis were linked to cancer [30]. The examples are numerous, urine biomarkers associated to prostate cancer [31], several concomitant serum markers for early detection of ovarian cancer [32], pancreatic cancer [33] and so on.

Western blot/immunoblot

It is a molecular biology method providing data about the size and amount of a protein isolated from cells or tissues [34]. Briefly, the first step is a SDS-PAGE electrophoresis that segregates the proteins on their size and charge basis. Subsequently, the separated proteins are transferred on a membrane and detection of the interest protein(s) is completed with specific antibodies labelled with enzymes (peroxidase). The method is extremely sensitive, for example using this method serum kallikrein 11 was suggested as a biomarker for prostate and ovarian cancer [35]. By blotting technique the Hsp 27 protein was demonstrated to be associated to hepatocellular carcinoma [36].

Protein microarray

An emerging technique that allows highly parallel quantitation of specific proteins in a rapid, low-cost and low sample volume format. It can be characterized as a type of "micro-FIA" that can investigate hundreds of proteins and, using standards, it can quantify them. With the help of this technology the screening or early diagnosis of head and neck squamous cell carcinoma was improved [37].

Multi-protein identification technology - MudPIT

The multi-protein identification technology - MudPIT - is a combination of proteome analysis and biomarkers detection [38]. MudPIT starts with a multidimensional chromatography followed by tandem mass spectrometry and the obtained data are analyzed with SEQUEST [22], a complex database

Table 1. Serum specific biomarkers for various types of cancer, validated or future candidates

Cancer type	Validated markers	Candidate biomarkers	References
Prostate	PSA	Acid prostatic phosphatase, CK18-Asp396, 94 aminoacids secretory protein, hepatocitary growth factor activator, insulin-like growth factor binding protein 1 and 3, E-cadherin, alpha-methylacyl-CoA racemase, kallikrein 11	[35, 42, 44, 46, 47, 48]
Breast	CA15.3, Her-2/neu, CA27-29	CEA, gene 5 for human kallikreine, colagene I carboxi-terminal telopeptide, MUC1, kallikrein-5 gene, isoforms of haptoglobin-1 precursor, haemoglobin-α/β, kallikrein 6 and 10, prostasine, osteopontine.	[41,49,50,51, 52,53,54,55]
Ovary	CA125	Isoforms of the haptoglobinei-1 precursor, various haemoglobins types, 6 and 10 human kallikreine, prostasine, osteopontine, trypsine inhibitor tumor associated, serum beta gonadotrophine, interleukin 8, collagen I carboxi-terminal telopeptide, transferine, kallikrein 11, MUC1, kallikrein-5 gene	[25, 35,49]
Cervix		haptoglobins, apolipoproteins, metalloproteinases, transthyretine, squamous carcinoma cell antigen, cytokeratine 19, HER1-3, uPA, VEGF	[14,57,58]
Lung		CEA, MCSF, peptide release of pro-gastrine	[59]
Pancreas		alpha-2 macroglobulin, ceruloplasmin and 3C component of the complement system	[28]
Liver		Alfa-fetoprotein, C3a complement component, Hsp 27 protein	[13, 36]
Thyroid	Tyroglobulin	MMP-2 and TIMP-2	[61,62]
Melanoma	LDH	S100, Melanocyte Inhibitory Antigen	[63]
Colorectum	CEA	gonadotropin-beta and dimer D	[64]
Pancreas	CA19-9	gonadotropin-beta	[65]
Kidney	cathepsin D	gonadotropin-beta	[66,67]

recognizing proteins and peptides on the basis of their fragments identified by mass spectrometry [39]. The International Organization of Human Proteome has discovered with the support of MudPIT about 500 serum proteins which could be developed into the next 10 years in biomarkers associated to different pathology including cancer [40].

SERUM BIOMARKERS ASSOCIATED TO CANCER

A validated biomarker must enclose the capacity to provide information regarding: patient survival, clinical management, therapy monitoring and patient's evaluation for clinical trials. Beside these, a biomarker must be specific, sensitive, must have a low cost, rapid detection method and be stable in terms of intra and inter-laboratory assessments. To be accepted as a new biomarker, it must provide additional clinical information compared with "classical" ones [41]. The biomarker's domain was inspired from the clinical definition of a tumoral marker, namely "a molecule, a process or a compound which is quantitatively and/or qualitatively modified in pre- or cancerous conditions, changes that can be detected by a method" [42]. The tumor itself and/or the bordering normal tissue develops an array of changes at both nucleic acids and proteins level, affecting thus the major cellular processes such as apoptosis, proliferation, angiogenesis etc. All this alterations can be measured by quantitative and/or quantitative tests. These investigations comprise a very large area of methods starting with immunoanalysis and ending with state-of-the-art technologies like mass spectrometry and microarray [43].

Prostate cancer

Until now, only two biomarkers were validated for prostate cancer, namely prostatic acid phosphatase [44] and serum PSA [42], while many other proteins are under research and validation studies (see also Table 1). PSA is very useful for monitoring prostate cancer progression, but is not accepted in primary tumor diagnosis or in early detection [45]. For these reasons, the discovery of new biomarkers is extremely important, like citokeratin 18 cleaved by caspase (CK18-Asp396) that can be exploited as an apoptosis marker [46]. The secretory protein of 94 aminoacids and hepatocytic growing activator factor identified in sera can be associated to prostate cancer [47]. These markers along with the 80kDa fragment of e-cadherine and alpha-methylacyl-CoA racemase are on the point to acquire their clinical relevance for prostate cancer [48].

Breast cancer

The general tendency in biomarkers discovery is to find a panel of markers proper for depicting the early onset of cancer [2]. The classical serum carcinoembryonic antigen (CEA) is known to be elevated in 30-50% of symptomatic breast cancer patients. In conjunction with tumor tissue onset, the architecture of normal tissue is changed and MUC1, a normal glycoprotein, is also found elevated in patient's serum. The expression of kallikrein-5 gene could be also a potential diagnostic biomarker for patients suffering of breast or ovarian cancer [49]. In breast cancer prognostic, CA15.3 antigen [50], a protein involved in intercellular interaction and cell growth, possess important significances for becoming a biomarker. Since 2005, Her-2/neu (the membrane tyrosine-kinase involved in signalling pathways of cellular proliferation and differentiation) and MUC1, were both validated as biomarkers for breast cancer [41]. It seems that, the combined investigation of CA15.3 and serum CEA in breast cancer could increase the sensibility and specificity of the diagnostic [51]. On the waiting list for validation of breast cancer biomarkers are the following proteins: the isoforms of haptoglobin-1 precursor, haemoglobin-a/b [52], kallikrein 6 and 10 [53], prostasine [54], osteopontine [55].

Cervical cancer

In the last years, cervical cancer was the beneficiary of several European screening programs. The investigation of females by using the classical Papanicolau test has significantly reduced the incidence of this type of cancer in developed countries. The limitations of this test have conducted medical research to discovery of markers associated to cervical cancer [56]. Recently it was discovered an association between the disease progression and the expression of viral oncogenes E6 and E7 in infected basal and parabasal cells [56]. Also recently an elevation of CK19, MUC1, HER1-3, uPA and VEGF expression was determined at the cervical tumor tissue [57]. A simultaneously serum detection of the squamous carcinoma cells antigen (SCC-Ag) and cytokeratine-19 (CYFRA 21-1), could be useful for identification of early stages of cervical cancer patients [58].

Lung cancer

Serum CEA could follow-up the efficiency of gefitinib therapy of lung cancer with large cells. A recent study has revealed that serum stimulation factor of macrophage colony is an independent prediction marker for patients diagnosed with large cells lung cancer [59]. Nevertheless, there is not yet established a panel of validated biomarkers for this type of cancer.

Polanski and Anderson [60] have completed a list comprising 1261 proteins that are expressed in relation with cancer. Out of these proteins only nine biomarkers have been validated as cancer type specific by Food and Drug Administration. From this point of view, the research in biomarkers discovery has to complete the diagnostic panels for several cancers [61-67] and moreover to validate new biomarkers with predictive power in cancer.

CONCLUSIONS

The human serum is an extremely complex biological sample; therefore the proteomic technology could be applied in the identification and analysis stages of biomarker discovery. The translational research assisted by proteomic technology attempts to improve the diagnostic and prognostic of human cancer, specifically correlating the pathology with the biomarker. The number of validated biomarkers in human cancer is still small, and extended studies are trying to validate new biomarkers and/or to generate panels of classical biomarkers in order to identify as early and precisely the development of a neoplastic process. Many of the techniques are still not sufficiently sensitive and specific to identify certain proteins from a complex biological sample like serum or plasma. Thus, the clinical proteomics technology must focus on increasing sensitivity, reproducibility and specificity of novel biomarkers detection.

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