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Full Length Research Paper

Treatment for production of interferon-alpha (ifn-α) and interferon-gamma (ifn-γ) from the same purified suspension of leukocytes

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The basic aim of this work was to examine possibilities for introducing a new biotechnological treatment for preparation, storage and further purifying the suspension of leukocytes ("buffy coat" (BC)), intended for production of human interferon-alpha (hIFN-α) and human interferon-gamma (hIFN-γ). Three hundred and eighty four units of BC were examined. They were divided into three series of 128 BC units each - one control series (KSBC) and two experimental series to which 10% dextran solution (ESBC-I) or 6% hydroxyethyl starch solution (ESBC-II) were added. The lowest titer (297929 IU) of hIFN-α ensued from KSBC, a higher titer from ESBC-I (317184 IU) and the highest titer from ESBC-II (364406 IU). We also found that the lowest hIFN-γ titer was produced from KSBC (48142 IU), a somewhat larger one from ESBC-I (67571 IU), and the highest titer was obtained from ESBC-II (73428. IU). This study has demonstrated the positive influence of modified biotechnological treatments on the production of hIFN-α and hIFN-γ from BC.

Key words: Biotechnological treatment, buffy coat, interferon-alpha, interferon-gamma.

INTRODUCTION

Interferons (IFNs) are produced in the host after virus invasion and show antivirus activity on transfer to fresh tissues by inhibiting virus replication (Abbas et al., 1997; Balint, 2004; Boehring Ingelheim Bioproducts, 1996; Cassatella, 1996; Petz et al., 1996; Roit et al., 1998; Vucelic et al., 2000). Under normal conditions in the human body, IFN concentrations are below the detectable level, because cells neither synthesize nor contain them as constitutive elements (Balkwill, 1989; Boehring Ingelheim Bioproducts, 1996; Casstella 1996; Dipoala et al., 1994; Fields and Knipe, 1990; Mollison et al., 1993; Ress, 1992; Roit et al., 1998). Since the middle of the last century when they were discovered, IFNs have been divided into three classes: IFN-α, IFN-β and IFN-γ. IFN-α and IFN-β are produced by most mammalian cells after virus stimulation of other agents (Fensterl and Sen, 2009; Fields and Knipe, 1990; Gerrard et al., 1987). Initially the term "IFN type I" was used for the reactions of IFN-α and IFN-β, because they are very similar or identical. Different terms were used for IFN-γ ("IFN type II" or "immune IFN") depending on the cells of origin and demonstrated activities (Cassatella, 1996; Fields and Knipe, 1990; Stewart et al., 1980, 1997; The American Cancer Society, 1979; Vucelic et al., 2000; Warren, 1981; Yip et al., 1981; WHO, 1988).

In some countries, biotechnological processes for production of hIFNs for clinical use from human leukocytes isolated from whole blood (not more than 6 h

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old), were eliminated and the buffy coat was discarded (Akerlund et al., 1996; Dipoala et al., 1994; Pestka, 2007; Vucelic et al., 2000). Whole blood (WB) units were taken from non-selected, healthy donors (aged from 18 to 65 years) in plastic bags for exfusion and blood storage (Brozovic and Brozovic, 1986; Eriksson and Högman, 1990, 1997; Hirosue et al., 1988; Milenkovic et al., 1995; Mollison et al., 1993; Napier, 1987, 1995; Pietersz et al., 1987, 1988; Pisciotto et al., 1989; Price, 1991; Radovic, 1989; Stewart, 1980; Vengelen-Tyler, 1996). The suspension of leukocytes ("dry" BC) was rejected as a "harmful" product, because it could provoke negative effects in hemotherapy (therapy with blood products) (Balint, 2004; Mollison et al., 1993; Napier, 1987, 1995; Petz et al., 1996; Radovic, 1989; Robert et al., 1989). For production of hIFNs, the suspension of leukocytes was purified from erythrocyte contamination to obtain a fresh stock of human leukocytes to be exposed to virus infection for stimulation of human IFN-α synthesis. Among the different viruses used as inducers, "Sendai" virus was employed most often in practice. Induction of IFN-α synthesis was performed at 37°C and lasted 18 h (Akerlund et al., 1996; Anguē et al., 1991; Cantell and Hirvonen, 1978, 1981; Dipoala et al., 1994; Hirosue et al., 1988; Stewart and Gottlieb, 1997; Tóth et al., 1985; Zhang et al, 2010). It was also possible to produce a purified leukocyte suspension using plant substances (purified phytohemaglutinin-PHA). PHA sensitized human lymphocytes produced human IFN-γ which was later purified by a combination of chromatography and immune precipitation by monoclonal antibodies (Braude, 1983; Fields and Knipe, 1990; Vucelic et al., 2000; Warren, 1981; Yip et al., 1981; 1982).

The beneficial effects of h-IFNs were best shown for solid tumors and tumor metastases (such as kidney cancer, pancreatic cancer and cancer of the thoracic cavity) (Bakwill, 1989; Bukowski, 2009; Fields and Knipe, 1990; Hoffmann et al, 2008; The American Cancer Society 1979). The hematologic malignancies most sensitive to hIFN preparations were: hairy cell leukemia; myeloid chronic leukemia; cutaneous T-cell lymphomas; "non-Hodgkin 's lymphoma of low-grade malignancy and Kaposi's sarcoma; condilomata acumnata and other benign tumors associated with human papilloma virus infection (Burchert and Neubauer, 2005; Choi et al., 2000; Cook et al., 1994, Dai and Krantz, 1999; Dumer et al., 2006; Felli et al., 2005; Gisslinger et al., 2001; Kim et al., 2010; Kondo et al., 2010). hIFN products showed beneficial results in the treatment of systemic (lupus erythematosus) (Niewold et al., 2010) and fungal diseases (mycosis fungoides) (Peycheva et al., 2007).

hIFN was also very effective as an antiviral agent. Amounts of IFN-α as small as three picograms could protect approximately one million cells from ten million viral particles. Thus, IFN is effective at the fentomoleculular (10^{-15} mol) level (Fields and Knipe, 1990; Sedger et al., 1999). Although viral sensitivity varied, it has been shown that more than 50 IFN's molecules per cell establish an antiviral effect (Martins et al., 1999). The IFN-α preparation was particularly effective in virological myocarditis caused by coxsackie virus (Khakoo, 2005; Miric et al., 1994) infection with human immunodeficiency viruses (Tavel, 2010) and the treatment of hepatitis type C (Dubois et al., 2009).

The most significant advantage of hIFNs over recombinant IFNs was greater tolerance (that is human leukocyte IFN-α is the most natural, biologically least harmful and least immunogenic preparation of IFN). Even after long treatment, patients responded by synthesis of antibodies against hIFN-α very rarely (Pestka 2007; Stewart and Gottlieb, 1997; Vucelic et al., 2000). However, patients given rIFN for a long time, showed many adverse effects to the therapy (resistance to rIFN, production of autoantibodies and neurological difficulties) (Aus dem Siepen, 2007; Saadoun et al., 2010; Stübgen, 2009). At first there was an insufficient quantity of purified leukocyte suspensions as a starting source for hIFN production. In 1979 Taniguchi et al. succeeded in producing large quantities of recombinant IFN (rIFN) by genetic engineering (Fields and Knipe, 1990; Miller et al., 1982; Pestka, 2007; Senz et al., 2002; Vucelic et al., 2000; Wei et al., 2006). Pharmaceutical companies then invested much money in production and advertisement for rIFN to appear on the medicine market, which in many countries, including our own, halted the classical biotehnological production procedures.

Thus, the aim of this study was important from both fundamental and practical points. Fundamentally, the time course data were directed to the viability of leukocytes by introducing two new modified biotechnological procedures for preparation, storage and purification of BC, which were used as the unique stock for hIFN-α and hIFN-Ɣ induction. The ability of the same purified suspension of leukocytes to produce interferons under the influence of viruses in the proposed conditions was compared. Practically, to produce sufficient IFNs as biotherapeutic products from BC as "refuse" (an eliminated byproduct from whole blood in all transfusion centers involved in collecting and processing blood from voluntary donors).

MATERIALS AND METHODS

A total of 384 units of BC for hIFN-α and hIFN-γ production were examined in this study. They were divided into three series of 128 BC units - a control series (KSBC) and two experimental (ESBC) series (ESBC-I and ESBC-II). Each series consisted of 16 pools with 8 units in every pool. The BC units were prepared from whole blood (not older than 6 h after exfusion) taken from non-selected, healthy donors (aged from 18 to 65 years) into plastic bags for exfusion and blood storage (Brozovic and Brozovic, 1986; Eriksson and Högman, 1990, 1997; Hirosue et al., 1988; Milenkovic et al., 1995; Mollison et al., 1993; Napier, 1987, 1995; Pieters et al., 1987,

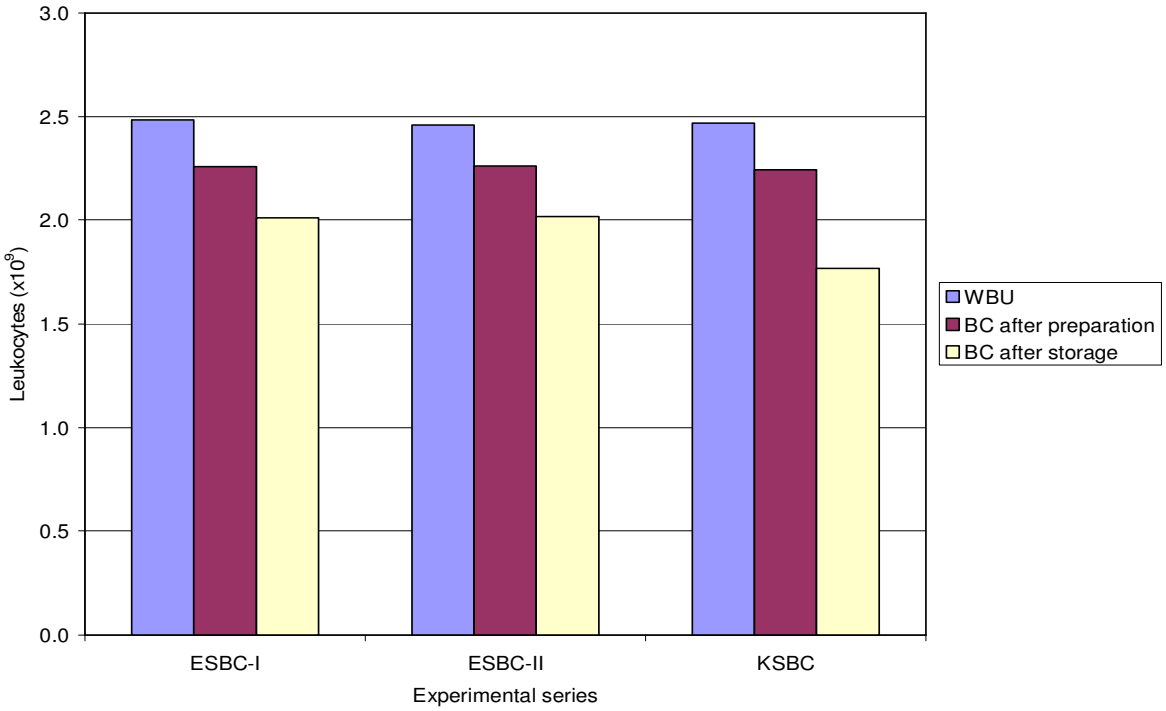

Figure 1. Mean values for number of leukocytes in WB units, BC after preparation and BC after storage for the control series (KSBC), and the dextran-treated (ESBC-I) and hydroxystarch-treated (ESBC-II) series.

1988; Piscioto et al., 1989; Price, 1991; Radovic, 1989; Stewart, 1980; Vengelen-Tyler, 1996). All units of whole blood were analyzed by enzymo-immune tests ("ELISA") (Busch and Courouce, 1997; Colin et al., 2001; Craske and Turner, 1990; Germer and Zein, 2001) and "PCR" testing (Ridgwell, 2004; Stramer, 2005; Yugi et al., 2005) for the presence of markers of transfusion-transmitted infections (hepatitis types B and C, human immunodeficiency-HIV viruses and syphilis). Only whole units of blood that were seronegative for markers of infection were used (Manitove, 1996; Vasiljevic, 2004; Vengelen-Tyler, 1996).

BC units of KSBC were prepared in the Institute of Trans-fusiology in the Military Medical Academy. Every series of KSBC was stored at room temperature (18 to 22 °C) for not longer than 18 h from preparation of BC (Cantell and Hirvonen, 1978, 1981; Dipoala et al, 1994; Rubinstein et al., 1978, WHO, 1988). BC units of ESBC were subjected to horizontal shaking (80 rotations per minute) in an incubator kept at 22°C for not longer than 18 h after preparation. Each series of 128 units of BC was mixed manually in a sterile box, respecting the principles of asepsis and antisepsis:

The KSBC pool was stored for 1 hour in the cold compartment at 4 °C as previously described (Akerlund et al., 1996; Anguē et al., 1991; Cantell and Hirvonen, 1978, 1981). Rulo forming agents were added to ESBC, as follows:

a) 10% solution of dextran (molecular mass (MM) 250,000 Daltons (D)) (European Pharmacopoeia, 2004; The Merck Index, 2006) was added to the ESBC-I pool (Maryadele and O' Neil, 2006).
b) An equal volume of "Plasmasteril" (6% solution of hydroxyethyl starch (6% HES)), MM 450,000 D (European Pharmacopoeia, 2004; The Merck Index, 2006; Maryadele and O' Neil, 2006) was added. KSBC and ESBC leukocyte suspensions were further purified by a modified "Cantell's method" (usual procedure with 0.83% NH_4Cl solution) at 18 to 22°C (Cantell and Hirvonen, 1978, 1981).
c)After 2 h of mixing "Sendai" virus was added to purified KSBC and ESBC leukocyte suspensions. Induction lasted for 18 hours with constant mixing in a water bath at 37°C (Akerlund et al., 1996). Crude IFN was purified chemically by lowering the pH from 7.3 to 3.8 with continuous measurement on a pH-meter (Cantell and Hirvonen, 1978, 1981).

After complete induction of hIFN-α with "Sendai" virus, the same KSBC and ESBC leukocyte suspensions were used for induction of hIFN-γ. They were treated with plant substances (purified phytohemaglutinin-PHA) for 48 h at 37°C and 24 h at 40°C to induce synthesis of hIFN-γ. Crude hIFN-γ was purified by gel filtration on specific columns (Töth, 1985; Yip et al., 1981; 1982). The necessary laboratory tests (number of leukocytes in WB units, BC units, and leukocyte suspensions for induction) were done automatically by flow cytometry (Tehnicon H-1 System). The quality of hIFN-α and hIFN-γ prepared from the KSBC and ESBC leukocyte suspensions was examined according to the standard of the World Heath Organization (European Pharmacopoeia, 2004; Maryadele and O' Neil, 2006; WHO, 1988; Yip et al., 1981, 1982).

The results were processed statistically (Student's t-test with probability values of $p<0.05$ being taken as statistically significant) and are shown in the figures.

RESULTS

Mean values for the absolute number of leukocytes in WB prepared from BC for all examined series were very similar (about 2.4 x 10^9) as shown in Figure 1. Approximately 90% leukocytes were separated in each unit of BC. At the same time during storage the number of leukocytes decreased by about 9% in the BC units

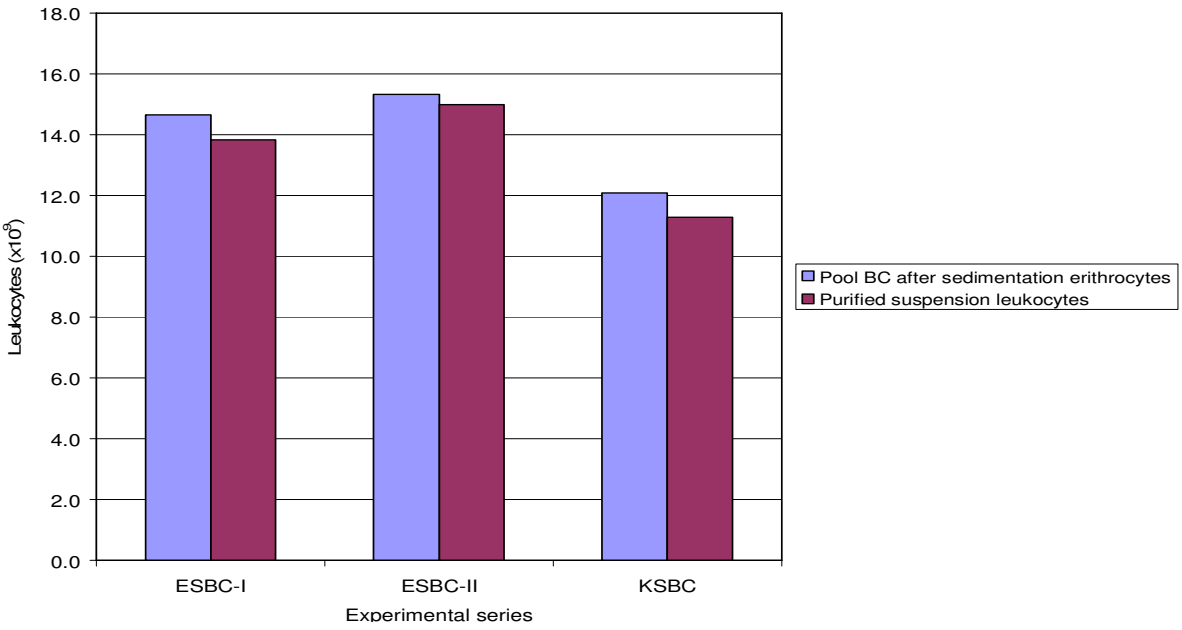

Figure 2. Mean values for leucocyte counts in BC pools after complete erythrocyte sedimentation and after further purification for the control series (KSBC), and the dextran-treated (ESBC-I) and hydroxystarch-treated (ESBC-II) series.

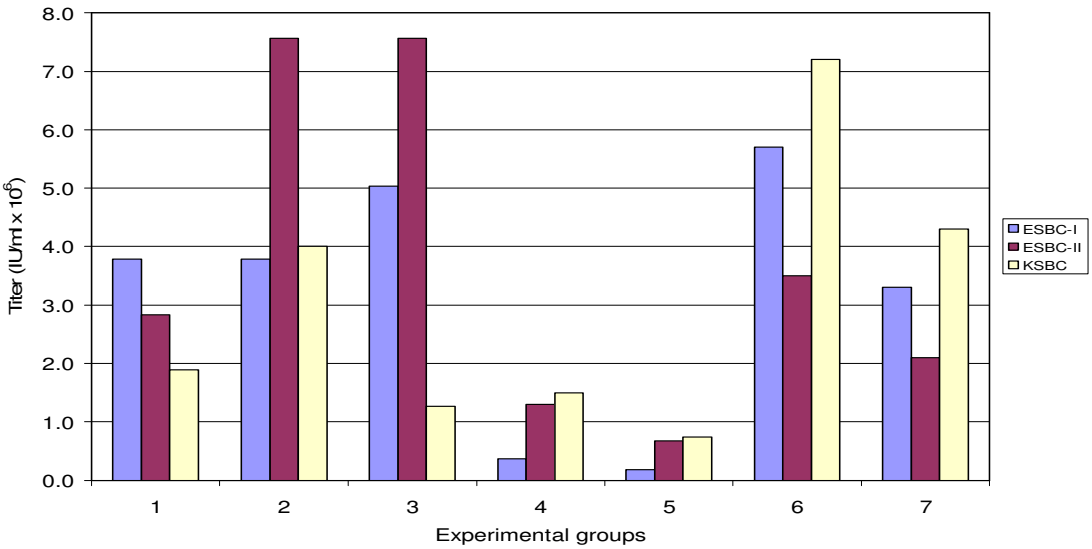

Figure 3. Titration values for IFN-α obtained for the experimental series ESBC-I and, ESBC-II and the control series KSBC.

(ESBC-I and ESBC-II) that were kept at constant temperature, and were significantly higher in BC kept at room temperature (KSBC) (Figure 1). There were highly significant differences in the mean number of leukocytes between WB and BC after preparation, as well as in BC after storage. Statistically significant differences were found between the mean number of leukocytes in ESBC-I and KSBC, and between ESBC-II and KSBC (Figure 1). Moreover, there were also marked differences between mean values for leukocytes in each examined series, as they were significantly higher in WB than in BC (after preparation and after storage), as well as in BC after preparation compared to BC after storage (Figure 2). Using the "Reed-Muench" formula and the "Carber" method, LD_{50} titers of hIFN-α were calculated and the results are shown in Figure 3.

The highest mean titer of IFN-α was found in ESBC-II samples (364406 IU/ml), a somewhat lower mean titer in

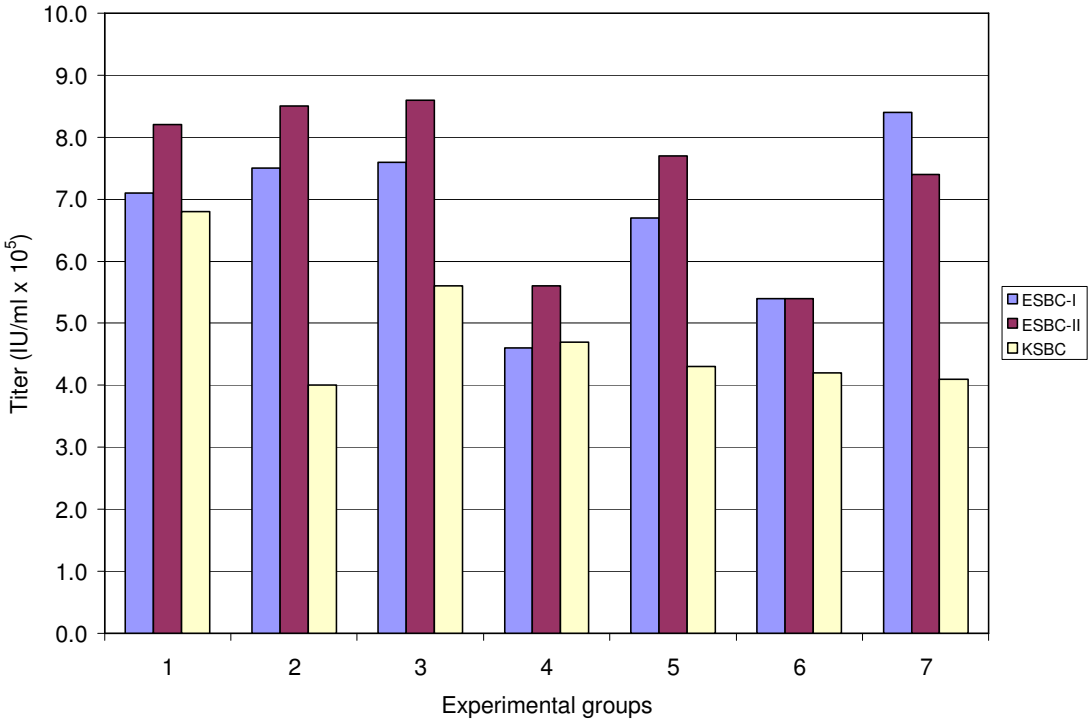

Figure 4. Titration values for IFN-γ obtained for the experimental series ESBC-I and ESBC-II and the control series KSBC.

ESBC-I samples (317181 IU/ml), while the lowest mean titer of IFN-α occurred in KSBC samples (297929 IU/ml). Thus, mean titers of IFN-α from KSBC samples were 18% lower than those in ESBC-II samples, and 5% lower than those in ESBC-I samples. Using the "Reed-Muench" formula and the "Carber" method LD_{50} titers of hIFN-γ were obtained and the results are given in Figure 4. The highest mean titer of IFN-γ was found in ESBC-II samples (73428 IU/ml), a somewhat lower mean value in ESBC-I samples (67571 IU/ml), while the lowest mean titer of IFN-γ was observed in KSBC samples (48142 IU/ml). Thus, mean titers of IFN-γ for KSBC samples were 34.44% lower than those in ESBC-II samples, and 28.75% lower than those in ESBC-I samples.

DISCUSSION

In our study the greatest mean number of leukocytes per unit BC was found after one hour sedimentation of red blood cells with 6% hydroxyethyl starch in the ESBC-II pool (1.915×10^9). Somewhat fewer leukocytes per unit BC were seen in the ESBC-I pool (1.832×10^9) where 10% dextran was used as the "rulo-forming agent". The smallest number of leukocytes per unit of BC occurred in KSBC pools (1.511×10^9) with no added "rulo-forming agent". Using modified biotechnological procedures, we found that production of hIFN-α was more effective, giving higher yields than for hIFN-γ. The lowest titer of hIFN-α was derived from KSBC, a higher one from ESBC-I and the highest from ESBC-II. The biotechnological production process was less effective for hIFN-γ and the titers achieved were about 4 to 5 times lower than those for hIFN-α obtained from KSBC and ESBC, respectively. The lowest mean titer of hIFN-γ was derived from KSBC, a slightly higher one from ESBC-I and the highest from ESBC-II.

According to the results of other authors (Maryadele and O' Neil, 2006; Pestka, 2007; Pietersz et al., 1987, 1988) the yield per unit BC after treatment with "6% hydroxyethyl starch solution" was about 25% lower than after red blood cell destruction using 0.83% NH_4Cl. The titer of hIFN-α obtained from 1×10^7 leukocytes treated with 6% hydroxyethyl starch solution was about 3.5 times higher than that after treatment with 0.83% NH_4Cl. This shows that leukocyte suspensions obtained from ESBC (using "rulo-forming agents" for red cells), were more purified than those obtained from KSBC (where the released contaminating red blood cells were destroyed by 0.83% NH_4Cl). According to others (Akerlund et al., 1996; Cantell and Hirvonen, 1978, 1981; European Pharmacopoeia, 2004; Maryadele and O' Neil, 2006; Robert et al., 1989) the use of dextran of different molecular weight as a "rulo-forming agent" was less effective than 6% hydroxyethyl starch solution (The Merck Index, 2006) and the leukocyte yield was lower and more variable, which was confirmed by our findings. Although many biotechnological procedures for production of

hIFNs are used all over the world, including our country, the available quantity of leukocyte suspensions (BC) cannot satisfy the need for hIFNs in third world countries. Therefore, these needs are fulfilled by manufacture of hIFN by genetic engineering. Unfortunately, compared to the hIFNs derived by biotechnological procedures, rIFN preparations are much more expensive and therefore available to a very small number of countries (Ivanovic, 2010; Pestka, 2007).

Extensive clinical experience over more than 30 years has confirmed that hIFN-α is natural and the least immunogenous IFN product in the drug market (BNF, 2010; Boehring Ingelheim Bioproducts, 1996; Fields and Knipe, 1990; Lucy, 2010; Vucelic et al., 2000). Even after long treatment with biotechnological rIFNs, patients rarely reacted by synthesis of antibodies against it. Nevertheless, the use of rIFN-γ was shown to lead to intensified secretion of activated T-lymphocytes, thus contributing to the pathogenesis of some autoimmune disorders (Aus dem Siepen et al., 2007; Cook et al., 1994; Macijewski et al., 1995; Saadoun et al., 2010). In present day scientific circles, great attention is paid to biotechnological procedures for production of hIFN. Namely, in 2006 Pestka (rIFN creator) received a prestigious award (European Pharmacopoeia, 2004; Langlois et al., 1976; Pestka, 2007). During the past three decades insufficient quantities of hIFN products for clinical use were the main reason for fewer clinical investigations. Due to fear of disease transmission, many patients were deprived of hIFNs obtained from leukocytes, even when they were available. Modern tests such as "ELISA", the most recent nucleic acid testing and "PCR" have decreased the risk to a minimum, thus reducing transfusion-transmitted infections (Manitove, 1996; Saura et al., 1999, Vasiljevic, 2004).

All in all, the contribution of our results is relevant in three respects:

(1) In obtaining a cleaner suspension of human leukocytes in a higher yield, so increasing their viability, which resulted in the production of significantly greater amounts of hIFNs, than so far achieved by conventional biotechnological processes used here and in the other countries, (2) in the use of the suspension of human leukocytes, dismissed as a "harmful" product after removal from units of whole blood, to implement two new biotechnological production processes for hIFN-α and hIFN-γ, and (3) in lowering the cost of IFN products compared to their rIFN counterparts produced by rDNA technology.

This is especially important for the numerous „third world“ countries, where much cheaper preparations of hIFN-α and hIFN-γ purified from the same human leukocyte suspension can be made, which would make them independent of imports of expensive rhIFNS manufactured by foreign pharmaceutical companies. If produced in amounts sufficient for domestic use, such countries may even become exporters of these important medicines necessary to treat several severe diseases, including malignomas.

Conclusions

The results of this study may contribute to solving the fundamental problem of increasing the viability of leukocytes, which can then produce significantly more hIFNs. This was achieved by modifying the classic biotehnological process used nowadays in many countries. hIFN-α and hIFN-γ have significant advantages over rIFNs in:

(1) being better tolerated; (2) using the same suspension of human leukocytes as a unique normally rejected starting material from whole blood donors for biotehnological production and (3) lowering the cost of hIFN-α and hIFN-γ preparations.

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