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Evaluation of genotoxic and immunotoxic activities of potential glucose biosensor components: ferrocenes

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Three ferrocenes used in glucose biosensor construction were tested in the aspect of genotoxic and immunotoxic activities. All three ferrocenes were not mutagenic in the standard bacterial Ames test. Equally in the Sister Chromatid Exchanges test in human lymphocyte cultures, the genotoxic action of tested ferrocenes could be excluded. However, all three significantly decreased the rate of lymphocyte proliferation and especially diminished the numbers of B-lymphocytes and NK-cells after 72 hours of *in vitro* culture. Marked differences between the ferrocenes in their immunotoxic activities were noticed, and we were able to select those which would be relatively safe and those which should be avoided in further investigation of the glucose biosensor construction. Our results indicate the necessity to estimate immunotoxic effects as well as genotoxic effects, especially in biosensor components potentially used *in vivo*.

Keywords: glucose biosensors, ferrocenes, genotoxicity, immunotoxicity

Introduction

In vivo monitoring is a developing area of glucose biosensing (Fraser 1997). This type of biosensor gives a continuous, real time read-out, which is most desirable for monitoring blood glucose levels of diabetics. A needle-type, subcutaneous sensor containing glucose oxidase has been developed by many groups. A range of 'mediated' electrodes have been introduced, in which non-physiological, redox-active molecules serve as electron transfer mediators between the redox enzyme and the electrode. These 'mediated' electrodes have been used both *in vitro* (Kulys & Cenas 1983, Albery *et al.* 1985, Cooper *et al.* 1991, Zakeeruddin *et al.* 1992, Fraser *et al.* 1993) and *in vivo* (Boutelle *et al.* 1986, Matthews *et al.* 1988, Sakakida *et al.* 1993, Linke *et al.* 1994) in glucose sensor systems. In the case of *in vivo* biosensors, several technical problems arise, such as a difficulty in retaining usually low-molecular weight mediators at the electrode (Schuhmann *et al.* 1990). An especially important condition for *in vivo* biosensor components is a need for being non-toxic, non-genotoxic and non-immunotoxic to human cells because of long-lasting patients' exposure. In our previous studies, some complexes of the group VIII metals with tris-(4,4′-substituted-2,2′-bipyridine) exhibited a mutagenic activity in the standard bacterial assay – the Ames test, whereas the others were not mutagenic (Gąsiorowski *et al.* 1995). Even though these bipyridine complexes have not been used in *in vivo* sensors, it indicates that any mediator intended for such a use should be necessarily tested for its genotoxic activities.

Another important aspect of a possible biological effect of *in vivo* glucose sensor components is their possible immunotoxic activity. The immunotoxic

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influence may have both immunosupressive and immunostimulative consequences; the latter can appear in the case of a selective toxicity to several lymphocytes' subpopulations. It is obvious that in long-lasting exposures which take place in case of glucose biosensors implanted subcutaneously, both immunostimulation and immunosuppression could be harmful to patients' health. Therefore, it is necessary to check potential *in vivo* glucose biosensor components with respect to their influence on the immunological system.

The aim of the present study was the evaluation of three ferrocenes (all potential *in vivo* biosensor components) with regard to their toxic, genotoxic and immunotoxic activities. Genotoxicity was estimated both in the standard bacterial Ames test and in the Sister Chromatid Exchanges (SCEs) test with human lymphocyte cultures. Toxicity of the ferrocenes to bacterial cells and to human lymphocytes *in vitro* was assessed in the same battery of short-term genotoxicity-tests. Immunotoxicity was evaluated by the analysis of human lymphocyte proliferation as well as by enumeration of lymphocyte subpopulations after a short – term culture with the tested compounds.

Materials and methods

Chemicals

Ferrocenes were obtained from the Institute of Physical Chemistry in Lausanne Switzerland. The following ferrocenes were tested: Fer.1 (classic ferrocene), Fer.2 (ferrocene dicarboxylic acid), and Fer.3 (dimethylaminomethylferrocene methiodide).

Standard mutagens, i.e. 4-nitroquinoline-N-oxide (NQNO), methylmethanosulfonate (MMS), and 2-aminofluorene (2AF) were purchased from Sigma (St.Louis, MO, USA). NADP and glucose-6-phosphate were also obtained from Sigma. Oxoid nutrient (Oxoid, Basingstoke, UK), Difco nutrient broth and Difco agar (Difco, Detroit, USA) were applied for bacterial growth. Phytohemagglutinin (PHA-P) was obtained from Serva (Heidelberg, Germany). Blood cell separation solution-Histopaque 1077, lymphocyte culture medium RPMI 1640, foetal calf serum (FCS), L-glutamine, 5-bromo-2-deoxyuridine (BrdUrd), demecolcine as well as the stains Azur II, Eosine B, Giemsa and trypan blue solutions were obtained from Sigma (St.Louis, MO, USA). Monoclonal antibodies as well as DAKO-LSAB Kit were purchased from DAKO A/S (Glostrup, Denmark). Other chemicals used for buffers and media preparation were obtained from POCH (Gliwice, Poland).

Mutagenicity

The mutagenic activities of three ferrocene complexes were estimated by means of the Ames test (Ames *et al.* 1975, Maron & Ames 1983) with *Salmonella typhimurium* TA97, TA98, TA100 and TA102 (kindly supplied by Dr. B.N. Ames) with and without a microsomal activation system (S9 fraction).

Tested compounds were dissolved in 75% ethanol and used in the doses of 100, 500, 1000 and 2000 μM per plate. The S9 fraction was prepared following Ames (Ames *et al.* 1975) i.e. from the liver of Aroclor 1254 treated male Wistar rats. Experiments including a tested compound, a negative control (solvent), and a positive control (the standard mutagens) were repeated three times.

Cytogenetic tests

The impact of ferrocenes on lymphocyte viability was evaluated by means of the standard trypan blue exclusion technique after four hours of culture with the tested compounds in concentrations ranging from 6.25 μM to 100 μM.

The influence of ferrocenes on human lymphocytes *in vitro* cultures was assessed by estimation of the cell proliferation indices and by counting the frequency of spontaneous (not induced) SCEs. The tests were carried out in accordance with the routine cytogenetic method (Perry & Wolffs 1974). Briefly, lymphocytes were isolated from the heparinized blood obtained by venipuncture of healthy volunteers. The cells were separated by centrifugation on the Histopaque – 1077 gradient solution (English & Anderson 1997). Lymphocytes obtained with this method were suspended to the density of 0.5×10^6 cells/ml in the culture medium (RPMI 1640, 10% FCS, 2mM L-glutamine), stimulated with PHA-P [10 μg/ml] and cultured for 72h at 37°C in CO_2 _ incubator. Ferrocenes were dissolved with DMSO/ethanol [7:3 (v/v)], and added to the culture medium at concentrations ranging from 6.25 – 100 μM in a volume of 25 μl. The tested ferrocenes were present in the culture medium for the whole culture time (72h). Control lymphocyte cultures contained the same volume of DMSO/ethanol (25 μl) instead of the ferrocene solution. The cultures were exposed to the thymidine analog, BrdUrd, added to the final concentration of 30 μM for the last 48h of culture. Proliferating lymphocytes were arrested at metaphase by demecolcine (0.1 μg/ml for 4h) and cultures were harvested with the standard cytogenetic method, and then stained following the procedures given in literature (Perry & Wolffs 1974, Antoshina & Poriadkova 1978).

The impact of ferrocenes on the proliferation of human PHA – stimulated lymphocytes was estimated by calculation of proliferative potentials in cultures containing BrdUrd. The replication indices (RI) were estimated by examination of the slides prior to their use to the SCEs analysis. In each culture, we examined 100 metaphase cells randomly found under a microscope in the aspect of the differential chromatid staining. The replication indices were calculated following the formula given in literature (Perry & Wolffs 1974). Simultaneously, we established the mitotic indices (MI) by counting the fraction of metaphase cells per 1000 of cells randomly found on the slides examined under a microscope.

Immunocytochemical staining methods

An analysis of surface markers on the lymphocyte population after the culture in the presence of ferrocenes was performed following the procedure given in literature (Knutilla & Teerenhovi 1989). Shortly, lymphocytes cultured in the presence of the ferrocenes were harvested after 72h by suspending the cultures in a hypotonic solution containing a mixture of distilled water and the complete culture medium [4:1 (v/v)] with 0.4% glycerol. The hypotonic treatment time was 10 min. Afterwards the cells were centrifuged and smears on glass slides were prepared. Then the slides were air dried for 24 h and processed by the immunochemical staining procedure. They were immersed in fixing medium – a mixture of methanol, acetone, and formalin [19:19:2 (v/v)] – for 1 minute and subsequently stained following the immunoperoxidase technique with DAKO – LSAB Kit (Labeled Streptoavidin – Biotin) as described in the literature (Hsu *et al.* 1981, Warnke & Levy 1980). Mouse monoclonal antibodies were used to detect the following surface antigens: CD4 on helper lymphocytes (T4), CD8 on suppressor lymphocytes (T8), CD56 on lymphocytes NK and CD22 on B lymphocytes. The antibody–bound cells were visualized by the PAP-staining procedure using diaminobenzidine (DAB) as a chromogen. Smears were analyzed by counting the numbers of stained and non-stained cells among 2000 cells randomly found under a light microscope. Finally, the numbers of positively-stained cells in ferrocene cultures were compared to the numbers in the control culture [E/E_0], and histograms were drawn for each tested ferrocene in the selected doses: 6.25, 25 and 100 μM. The histograms of B lymphocytes, CD4, CD8 and NK – lymphocytes were given separately.

Statistical analysis

Regression equations and correlation coefficients were calculated using routine statistical methods (e.g. Campbell & Machin 1993).

Results

The tested ferrocenes were not toxic to bacterial cells in the range of applied doses: from 100 to 2000 μM per plate. The results obtained in the *Salmonella typhimurium* mutagenicity assay – the Ames test – showed that none of the tested compounds was active (mutagenic) in experiments with four tester strains both in the presence and in the absence of the microsomal – activation S9 fraction. The numbers of his+ revertants induced by them were at the level of spontaneous revertants typical for each *Salmonella typhimurium* tester strain (background level). The average of these background levels were: 149, 45, 169 and 437 revertants per plate for TA97, TA98, TA100 and TA102 strains, respectively.

The ferrocenes were not cytotoxic to human lymphocytes *in vitro* in the range of concentrations from 6.25 to 50 μM as was estimated with the standard trypan blue – exclusion test. At the highest concentration [100 μM] the toxic effects became visible – the percentages of dead cells were from 10% (Fer.1) to 20% (Fer.2) after a 4 h culture of lymphocytes with the ferrocenes. At the concentrations higher then 100 μM, the ferrocenes were cytotoxic to the lymphocyte cultures. Therefore, we established the 100 μM concentration as the highest tested ferrocene concentration. The influences of ferrocenes on a lymphocyte proliferation are shown in Figure 1: mitotic indices are given in Figure 1A, and replication indices are presented in Figure 1B.

The results are compared to relative results from control cultures (control culture =1.0) and expressed in Figure 1 as E/E_0 proportions.

As may be seen in Fig. 1A, the tested ferrocenes markedly differ in their influence on lymphocyte mitotic indices. Fer.1 stimulates mitotic indices at the whole range of the tested concentrations, the relatively strongest effect is observed at the concentration of 50 μM (stimulation by about 15%), but also at the highest tested concentration [100 μM] a stimulation of the mitotic index was noticeable. Fer.3, on the contrary, inhibits mitotic indices in all concentrations used. The dose–effect relations could be described by a polynominal regression function in the case of both ferrocenes presented above. Fer.2 showed diverse, strongly concentration – dependent effects on lymphocyte proliferation: at the lower concentrations [6.25–50 μM] the stimulation of mitotic indices was noticed, whereas at the highest concentration [100 μM] the marked inhibition was found (by more than 25%). In the case of Fer.2 the dose–response relation was described by linear regression equation, given in Figure 1A.

As may be noticed in Fig. 1B, the replication indices (RI) are, in general, slightly below the level of the control culture replication index, although in the case of Fer.2 a weak stimulation (by about 5%) is found at lower concentrations of the tested compound [6.25–25 μM]. The inhibition of lymphocyte replication indices is rather weak – a decrease by about 10% as compared to the control at the highest of tested concentrations [100 μM]. The dose response relations of replication indices are described by a regression equation given in Fig. 1B.

The effect of ferrocenes on the frequency of spontaneous (not induced) SCEs in human lymphocytes is presented in Figure 2.

The data presented in Figure 2 are expressed in proportion to the control culture SCEs frequency

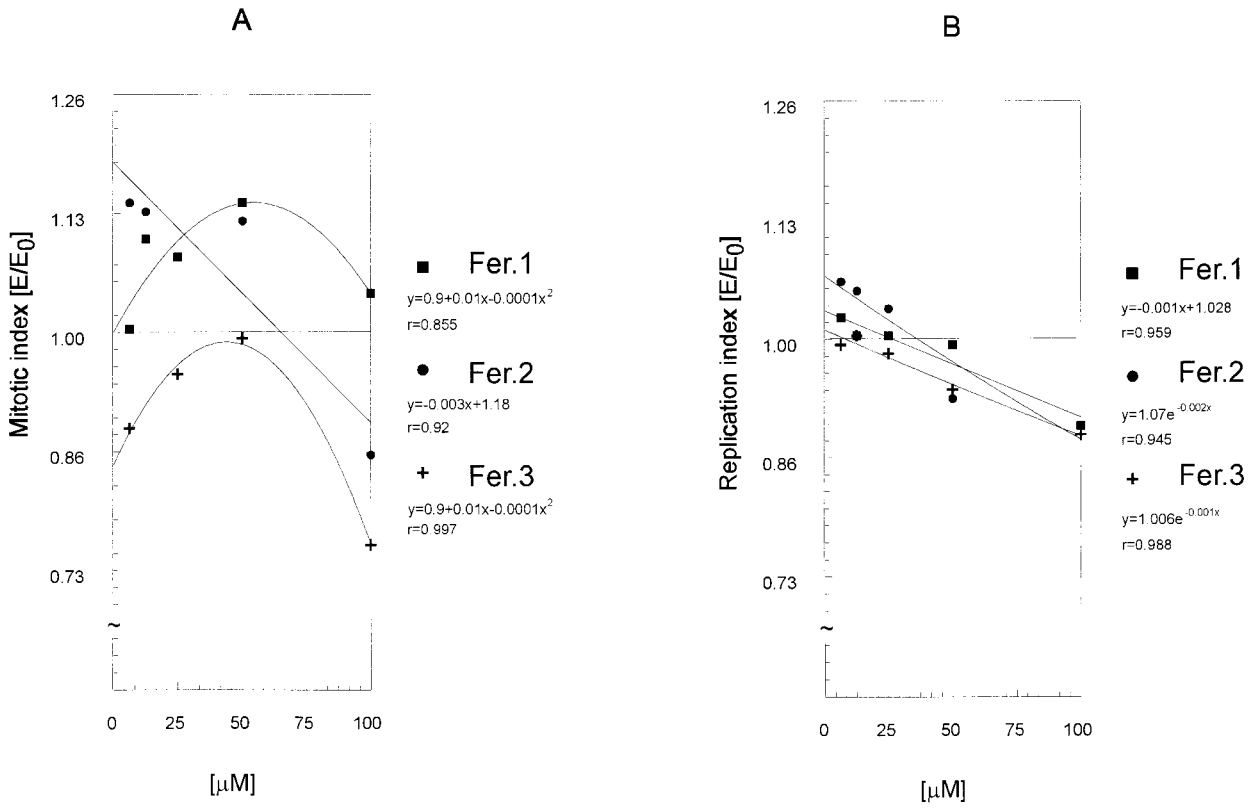
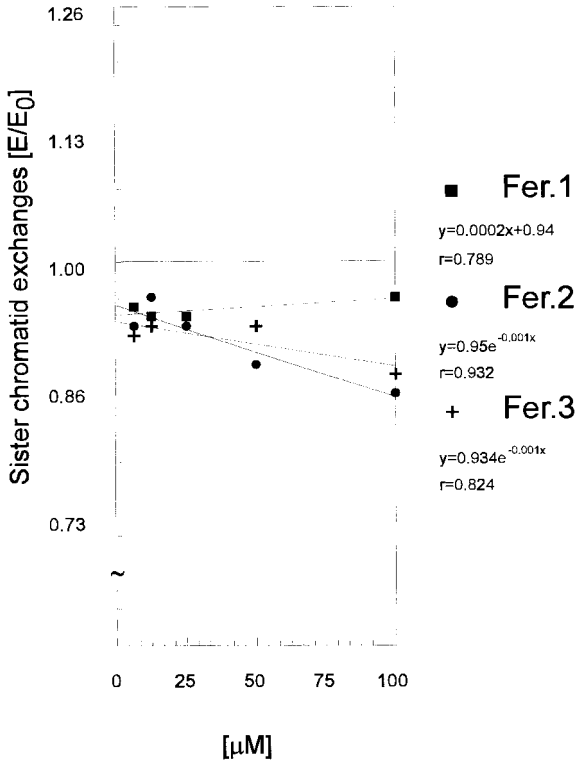


Figure 1. Influence of ferrocenes on the mitotic indices (A) and replication indices (B) of human lymphocyte cultures. The results were compared to those of the control culture and presented as E/E_0 ratios. The dose–response relations were estimated by computing the regression equations.



$[E/E_0]$. It should be noticed in Figure 2 that all three of the tested ferrocenes lowered frequency of the spontaneous SCEs; all three regression lines are below the level of the control culture (1.0). The effect is dose-dependent in the case of Fer.2 and Fer.3, whereas that of Fer.1 appears to be very weak, almost negligible. The effect is relatively strongest in the case of Fer.2 (decrease by about 13% at the concentration of 100 μM) and slightly weaker in the case of Fer.3 (about 10% decrease).

Figure 3 comprises the results obtained with the immunochemical method, describing the influence of three ferrocenes on the lymphocyte subpopulation numbers after 72 h of culture *in vitro*. The estimated lymphocyte subpopulations were: CD4, CD8, B, and NK. The histograms presented in Figure 3 shows the results obtained with three concentrations

Figure 2. Influence of ferrocenes on the frequency of spontaneous (not induced) SCEs in human lymphocyte cultures. The results obtained in cultures with ferrocenes were compared to those of the control culture $[E/E_o]$. The regression equations were calculated with the obtained results.

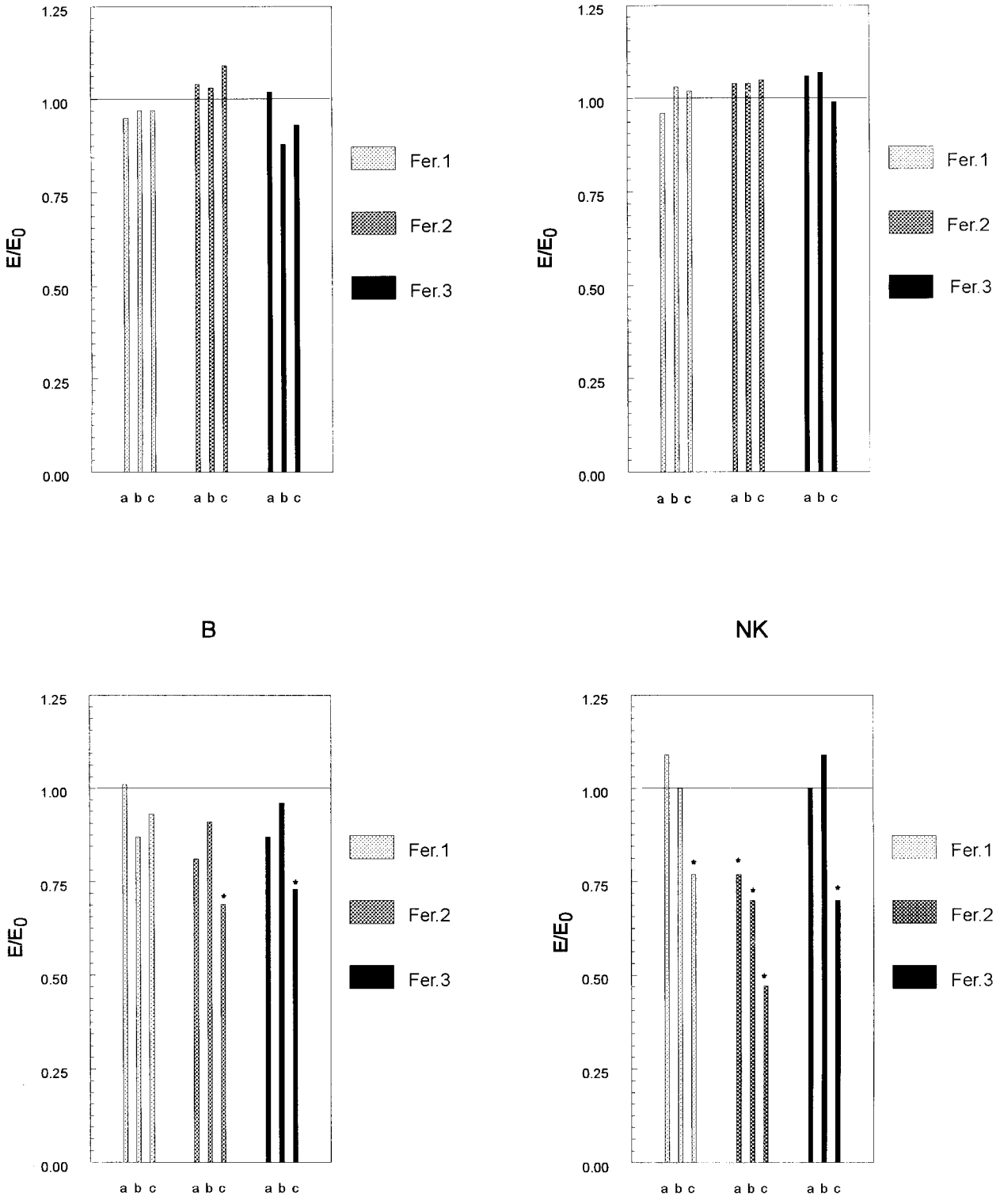


Figure 3. Influence of tested ferrocenes on the number of main lymphocyte sobpopulations after 72 hours of culture. The percentual representation of analysed lymphocyte subpopulations was compared to those in the relative control cultures and histograms were drawn on the basis of the E/E_0 ratios.
Ferrocene concentration at culture medium: a – 6.25μM; b – 25 μM; c – 100 μM
*Statistically significant, $0.01 < p < 0.05$

of ferrocenes: 6.25 μM (a), 25 μM (b) and 100 μM (c), and the results were expressed in proportion to relative control cultures [E/E_0].

As may be seen in Figure 3, the influence of ferrocenes on the numbers of the main T lymphocyte subpopulations i.e. CD4 – and CD8 – cells, differs only slightly (by ±5%) from those counted in the control cultures. The impact of ferrocenes on the number of lymphocytes B can be seen as the marked decrease in the case of Fer.2 and Fer.3 (by about 30% and 25% respectively, in the concentration of 100 μM), and significantly weaker diminution in the case of Fer.1 (by about 15%). In the case of NK lymphocytes Fer.2 exhibits a strong inhibitory effect on the NK cell numbers, even by more than 55% at the highest tested concentration [100 μM]. Fer.1 and Fer.3, on the contrary, enlarges the NK numbers (by about 10% at lower concentrations), and decreases their numbers at the highest concentration (by about 25%).

Discussion

Ferrocenes are undoubtedly much–studied redox mediators in glucose biosensing systems in the aspect of their electrochemical characteristics (e.g. Cass *et al.* 1984, Cooper *et al.* 1991). They have been used commercially in disposable electrodes *in vitro* connected with a hand–held meter for home–testing of glucose level by diabetics (e.g. the Exactech' sensor series from MediSense, Cambridge, MA). However, the results of their interference with biological systems are still controversial and even conflicting. There is evidence that ferrocenes can exhibit toxic effects (Leung *et al.* 1987), especially after an inhalation exposure of mice and rats (Nikula *et al.* 1993). The results obtained with the ferrocenes in a short–term genotoxicity–evaluation test are contradictory. For instance, even by means of the same protocol of the standard Ames test the ferrocenes were found mutagenic to *Salmonella typhimurium* tester strains in some labs, whereas did not exhibit mutagenic activity as reported by other groups (Haworth *et al.* 1983; Marcher *et al.* 1988). In Chinese hamster ovary cells *in vitro* (CHO line) it was noticed that the ferrocene did not generate the chromosomal aberrations, but enhanced the frequency of sister chromatid exchanges in those cells (Galloway *et al.* 1985). In our research presented in this paper, we found that three ferrocenes were not mutagenic in the Ames test with four *Salmonella typhimurium* tester strains, regardless of the presence of the activating microsomal S9 fraction. In cultured human lymphocytes *in vitro,* all three ferrocenes decreased the frequency of spontaneous (not induced) sister chromatid exchanges by 5–15%. It is difficult to explain precisely the contradiction of our results and those cited above (Galloway *et al.* 1985), in which the ferrocenes were found positive in the SCEs test in Chinese hamster ovary cell lines. A difference in the types of the tested cells might be the main reason for differences in the obtained results. There is an obvious dissimilarity of hamster's self–propagated cell line and freshly–prepared human lymphocytes stimulated to proliferate with lectin PHA.

The cause of these differences may also be linked with the differences in the cell–culture proliferation rates. Susceptibility of cells to the action of genotoxic agents depends, to a large extent, on the efficiency of detoxifying–repairing cellular systems, which exhibit a limited efficiency at higher cellular proliferation rates (Tomatis 1993, Cunningham & Matthews 1995, Ames & Gold 1996). Our tested ferrocenes slightly stimulated the replication indices of human lymphocyte cultures at lower concentrations, and markedly lowered the replication rates at higher concentrations. Also it is well known that established, self–propagated cell lines usually exhibit a markedly higher rate of cellular proliferation in vitro than human blood–derived lymphocytes stimulated with lectin PHA.

We consider, the different cellular source as well as the probable differences in the cells proliferation rates as an explanation of the dissimilarity of our results and those found in the literature.

It is commonly accepted that the exchange of sister chromatids is a normal physiological process associated with DNA replication and represents the interchange of DNA replication products at apparently homologous loci (e.g. O'Neill *et al.* 1983, Tucker *et al.* 1986). The frequency of spontaneous (not induced) SCEs can be regearded as a mirror reflecting the level of spontaneous DNA damages, which has a potential role in gene rearrangement, recombination and amplification (Kanda 1982, Pinero *et al.* 1993). Additionally, several physiological processes, such as glucose and other oxidative sugar metabolism, oxygen free radicals and other metabolic byproducts generation, body heating etc., usually cause DNA damages which are quickly and efficiently repaired by the cellular repair system. As the DNA – damaging actions are consistently present in every cell, and DNA damages are almost unavoidable, they are called 'spontaneous' genotoxic activities. It is well established that the frequency of spontaneous SCEs increases with age in a statistically significant mode (Franceschi 1990, Franceschi *et al.*

1992). On the other hand, it is also well known that the frequency of SCEs in mammalian cells increases after an exposure to a variety of genotoxic agents both mutagenic and potentially carcinogenic (e.g. Carrano *et al.* 1978, Perry 1980, Pinero *et al.* 1993). The efficiency of repair processes belongs to the factors determining susceptibility of cells to genotoxic agents and designating the stability of cellular genome. The efficiency can be evaluated also with the SCEs test (Sasaki 1977, Franceschi *et al.* 1992).

The decrease of the spontaneous SCEs frequency by ferrocenes observed in our research appears to be an important result which can be interpreted in the aspect of improving the cells' genetic stability. Considering the arguments presented above, one can perceive lowering of the SCEs frequency by the ferrocenes in cultured human lymphocytes as a beneficial activity which should result in decreasing the cells susceptibility to variety of genotoxic agents probably by enhancing the genetic stability of cellular genome.

As the lymphocytes are the main components of the human immunological system, the impact of ferrocenes on lymphocyte proliferation could obviously influence human immunity. Therefore, it seems to be important to check in more detail their immunotoxic activity. We carried out the experiments intended to evaluate the effect of ferrocenes on the major lymphocytes subpopulations in a 72 hours – culture. It was shown that B and NK–lymphocytes but not T–lymphocytes were susceptible to the presence of the ferrocenes. In particular, the number of NK cells decreased significantly after the culture with the presence of Fer.2 – a statistically significant decrease was noticed at all tested doses of this ferrocene. In the case of Fer.1 and Fer.3, the lowering of lymphocyte frequencies was observed only at the highest ferrocene concentration [100 μM]. We could also observe Fer.2 and Fer.3 lowering the level of B cells in a culture. Therefore, it could be concluded that Fer.2 and especially Fer.3 exhibited immunotoxic activities in human lymphocyte cultures.

The explanation of the differences observed between tested ferrocenes in their genotoxic and immunotoxic activities should comprise also the differences in physico–chemical properties of the tested compounds, i.e: the differences in the global charge, quantitative differences in their solubility, and the ability to penetrate inside the cell. The only physico–chemical condition we observed was an equally good solubility of the tested ferrocenes in DMSO/ethanol [7:3 (v/v)]. The complex physico–chemical studies exceed the aim of the present paper, which was to evaluate and describe the biological effects of the ferrocenes on human lymphocytes and on the standard bacterial strains.

Summing up the results presented above, we suggest that Fer.2 (ferrocene dicarboxylic acid) should be necessarily avoided in construction of an *in vivo* glucose biosensor since it is immunotoxic especially to human NK–cells and B–lymphocytes *in vitro.* From this point of view, Fer.1 (classic ferrocene) can be recommended for such a sensor, if the use of such freely diffusable molecules *in vivo* can be justified overall. Fer.3 (dimethylaminomethylferrocene methiodide) should be used with care, as at the highest tested concentration it decreased significantly both NK–cells and B–lymphocytes.

Although not being genotoxic, the tested ferrocenes differ significantly in their immunotoxic activities. It seems to be especially important and dangerous in the case of an *in vivo* glucose biosensing system in which a patient is exposed continuously to such mediators. We recommend the testing of any compound intended for use in a glucose biosensor component with respect to its genotoxic and immunotoxic activities.

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