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Original Contribution

COMPARATIVE STUDY OF THE EFFECT OF SPIN-LABELLED 1-ETHYL-1-NITROSOUREA AND CCNU ON THE SPECIFIC ANTIBODY PRODUCTION

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ABSTRACT

The aim of the present study was to investigate the influence of 1-ethyl-3-[4-(2,2,6,6-tetramethyl-piperidine-1-oxyl)]-1-nitrosourea (SLENU), spin - labelled structural analogues of the clinically used antitumour drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) on the specific antibody response. White mice were immunised with keyhole limpet haemocyanin (KLH). 24 h before antigen challenge the groups of mice were injected with different doses of SLENU, CCNU and combination of them. The control group mice were injected with KLH alone. The specific KLH IgM, IgG and polyvalent antibody production were determined on day 7 and 14 by ELISA. Results showed that KLH-specific IgM antibody production on day 14, KLH-specific IgG antibody production on day 7 and polyvalent antibody production on day 7 and 14 were significantly higher after treatment with 100 mg/kg SLENU, compared to 200 mg/kg SLENU, CCNU and CCNU+SLENU. The treatment with 200 mg/kg SLENU, CCNU and CCNU+SLENU significantly decreased IgM, IgG and polyvalent antibody productions compared to the control group. We conclude that SLENU exhibits lower immunosuppressive activity when compared to its structural analogues CCNU applied in therapeutic dose.

Key words: immunosuppression, IgM, IgG, chloroethylnitrosourea, SLENU

INTRODUCTION

Chloroethylnitrosoureas, such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, *lomustine*), and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU, *carmustine*) are chemotherapeutic agents and have been extensively used clinically for the treatment of a variety of human neoplasm. Chloroethylnitrosoureas have similar pharmacological and clinical properties. Their marked lipid solubility facilitates distribution into the brain; moreover, they can cross blood brain barrier and this is a reason for its application particularly against brain tumours (1, 2). These drugs, also, are active against chronic leukaemia, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and other cancers (3). In the human body chloroethylnitrosoureas decompose spontaneously and generate two types of cytotoxic intermediates – an alkylating and a carbamoylating moieties. The main target of the alkylating chloroethyl moiety is a nuclear DNA. As a result from interactions single-strand breaks and inter-strand cross-linkages between two chains of DNA are formed (4). The carbamoylating moiety, isocyanate, reacts with the -SH, -OH and -NH_2 groups of proteins and causes enzyme inactivation (5). The primary action of chloroethylnitrosoureas is the disruption of the cell cycle and cessation of cell division. Chloroethylnitrosoureas can kill cells in all phases on the cell cycle - their significant effects, however, is manifested mainly in $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transition of the cell cycle (3).

1-ethyl-3-[4-(2,2,6,6-tetramethyl - piperidine-1-oxyl)] -1-nitrosourea (SLENU) is a spin - labelled structural analogue of CCNU. SLENU possesses high carbamoylating and low alkylating activities in comparison with CCNU (6). Our recent study showed *in vitro* synergistic effect of the the spin-labelled nitrosourea SLENU on cytotoxicity of

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Bleomycin and Farmorubicin in human lymphoid leukaemia tumour cells (6).

Like other chemotherapeutic agents chloroethylnitrosoureas does not kill cancer cells preferentially over normal cells. Because a greater proportion of tumour cells are actively moving through the cell cycle, the sensitivity of tumour cells is highest than the sensitivity of normal non-cycling cells. The effect of chloroethylnitrosoureas on the rapidly dividing normal body cells can cause considerable toxicity. The universally accepted chloroethylnitrosoureas (CCNU, BCNU) produce noticeable toxic side effects on bone marrow cells and cells of the immune system and express strong immunosuppressive activity (7). There are presently very limited studies on the immunosuppressive activity of SLENU on the specific immune response. On account of this, the present study aims at investigating the influence of SLENU on the specific antibody production and to compare this effect with the immunosuppressive activity of CCNU.

MATERIALS AND METHODS

Mice: Eight ten-week old male and female white laboratory mice (bred in the animal facilities of the Medical Faculty, Trakia University), with individual body weight 23-25 g were used for this study. The animals were healthy and received food and water *ad libitum*.

Reagents: SLENU was synthesised in a research laboratory of the Department of Chemistry and Biochemistry, as described by Gadjeva and Koldamova (6). CCNU was kindly provided by Bristol-Myers Squibb Co. (Wallingford, CT). All other reagents (with exception of otherwise indicated) were purchased from Sigma.

Immunisation and drug treatment: 10 groups of white mice (6 in a group) were immunised i.p. with 5 μg of keyhole limpet haemocyanin (KLH), dissolved in 0.5 ml 0.15 M phosphate-buffered saline (PBS), pH 7.2, as an antigen. The animals were treated i.p. with the antitumour drugs, dissolved in 20% ethyl alcohol *ex tempore*, 24 h before antigen challenge as follows: 2 groups with 100 mg/kg and 2 groups with 200 mg/kg SLENU; CCNU was applied in a single therapeutic dose of 33.3 mg/kg on 2 groups of mice also; the mice from 2 remaining groups were treated with CCNU (33.3 mg/kg) simultaneously with SLENU (100 mg/kg). Two control groups mice were injected with KLH alone.

Anti-KLH antibody detection: The specific KLH IgM, IgG and polyvalent (IgM, G, A) antibody production was determined on day 7 and day 14 after immunisation, in sera, by ELISA. The wells of Maxisorp flat-bottom immunomodules (Nunc, Roskilde, Denmark) were coated with 0.1 ml of 10 μg/ml solution of KLH in 0.05 M sodium carbonate buffer, pH 9.6 and incubated overnight at 4ºC. Then, the immunomodules were washed and blocked for 20 min with washing buffer (0.05 M Tris- HCl buffer, pH 7.2 containing 0.05% Tween). Next, the samples (0.1 ml per well) diluted 1:100 in sample buffer (0.15 M PBS, pH 7.2 containing 1% BSA and 0.2% Tween) were added and immunomodules were incubated for 1 h at room temperature. After three washes, the detecting antibodies, conjugated with peroxidase (0.1 ml per well) were added. For the detection of mouse KLH-specific IgM, goat anti-mouse IgM, μ-chain specific was used (diluted 1:10000 in washing buffer). Goat anti-mouse IgG, γ-chain specific antibodies were used for the detection of KLH-specific mouse IgG (diluted 1:15000). The total KLH-specific antibody production was revealed after addition of goat anti-mouse polyvalent antisera (1:15000), which contain antibodies against three mouse immunoglobulin isotypes IgM, IgG and IgA. After incubation for 1 h at room temperature the immunomodules were washed and 0.1 ml of substrate solution (containing 5 μl substrate - 35% H_2O_2, 2 mg chromogene - *o*-phenylenediamine, dissolved in 10 ml 0.1 M citric acid buffer, pH 5.0), was added to each well. The subsequent colour reaction was stopped with 10% H_2SO_4 and the optical density was measured at 492 nm on an ELISA plate reader (Rosys Anthos 2010, Austria). All samples were duplicated. The obtained results in OD 492 are presented as the percent (%) increase over the mean OD492 value of non-immune sera from 6 mice.

Statistical analysis: The data were expressed as a mean and standard deviation of the mean. Student's t-test was used to determine the statistical differences between groups. When the p-value was less than 0.05, the differences were considered to be significant.

RESULTS

Differences in serum levels of KLH-specific IgM production during immune response of mice: Changes in KLH-specific IgM production in mouse sera after immunisation

and treatment with chemotherapeutic agents are presented in **Figure 1**. Results showed significantly diminished IgM production in the group treated with CCNU+SLENU compared to the control group on day 7. On day 14, significantly decreased KLH-specific IgM production was detected in the group injected with 200 mg/kg SLENU. There were significant differences in comparison with both: the control group and the group treated with 100 mg/kg SLENU (**Figure 1**). Similarly, mice treated with CCNU and CCNU+SLENU produced significantly lower quantity of anti-KLH IgM than the control group and the group received 100 mg/kg SLENU.

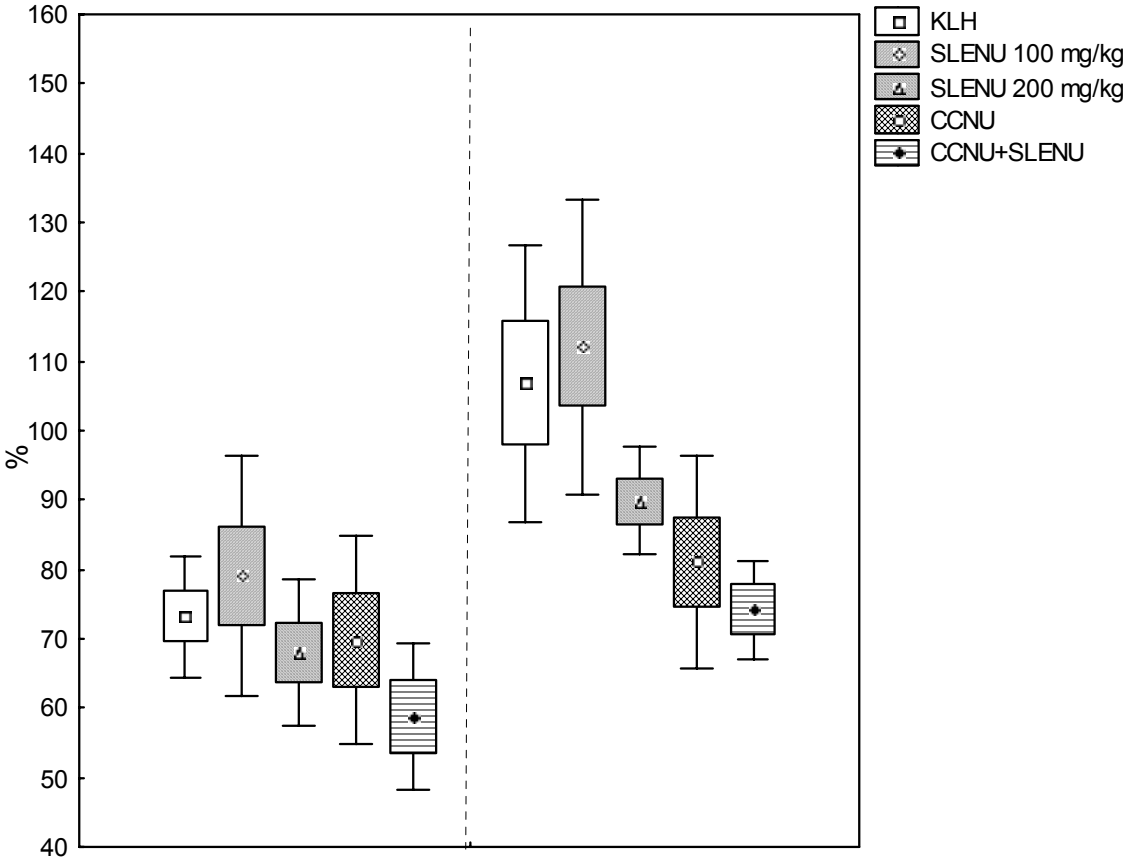

Figure 1. *Changes of KLH-specific IgM production in immunised mice. Mice were immunised with 5 µg KLH and treated with 100 mg/kg SLENU, 200 mg/kg SLENU, 33.3 mg/kg CCNU or 33.3 mg/kg CCNU simultaneously with 100 mg/kg SLENU, 24 h before antigen challenge. The control groups of mice were immunised with 5 µg KLH. The obtained results in OD 492 are presented as the percent (%) increase over the OD value of nonimmune sera.*

**p<0.05 vs. KLH; ◊ p<0.05 vs. 100 mg/kg SLENU.*

Differences in serum levels of KLH-specific IgG production during immune response of mice: Changes in KLH-specific IgG production in mouse sera after immunisation and treatment with chemotherapeutic agents are presented in **Figure. 2**. Decreased level of anti-KLH IgG in the group treated with 200 mg/kg SLENU was detected on day 7. The statistical analysis showed significant differences compared to both: the control and 100 mg/kg SLENU treated group. We found that the injection of CCNU and CCNU+SLENU also reduced specific IgG production. Significant differences compared to the control group and the group treated with 100 mg/kg SLENU were determined. Similar low level of KLH-specific IgG in groups injected with 200 mg/kg SLENU, CCNU alone and CCNU+SLENU were detected, without significant differences between groups. The results on day 14 followed up the results from day 7 (**Figure 2**). Significantly diminished IgG production in mice after treatment with 200 mg/kg SLENU, CCNU alone and CCNU+SLENU compared to the control group were determined. KLH-specific IgG production in group treated with 100 mg/kg SLENU was decreased compared to control group; no significant differences between groups.

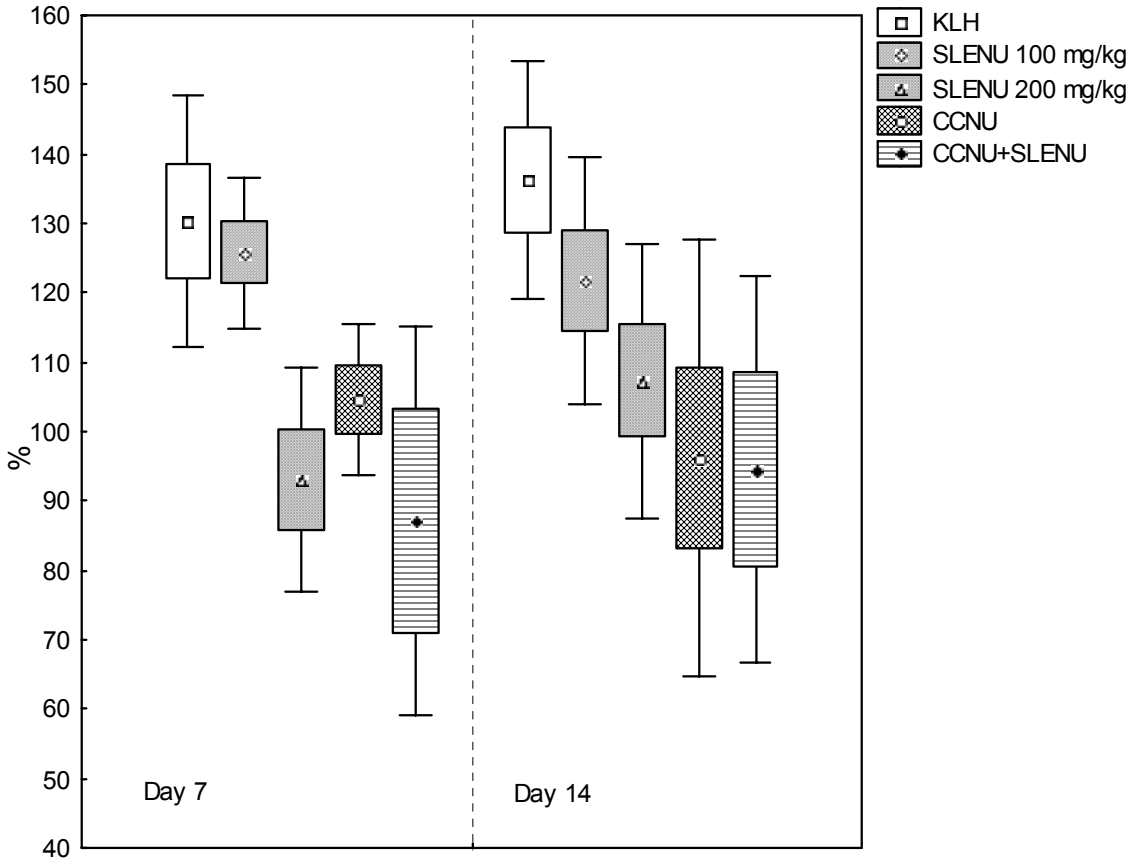

Figure 2. *Changes of KLH-specific IgG production in immunised mice. Mice were immunised with 5 µg KLH and treated with 100 mg/kg SLENU, 200 mg/kg SLENU, 33.3 mg/kg CCNU or 33.3 mg/kg CCNU simultaneously with 100 mg/kg SLENU, 24 h before antigen challenge. The control groups of mice were immunised with 5 µg KLH. The obtained results in OD 492 are presented as the percent (%) increase over the OD value of nonimmune sera.*

**p<0.05 vs. KLH; ◊ p<0.05 vs. 100 mg/kg SLENU.*

Differences in serum levels of KLH-specific total (IgM, IgG, and IgA) immunoglobulin production during immune response of mice: Changes in total KLH-specific antibody production in mouse sera after immunisation and treatment with chemotherapeutic agents are presented in **Figure 3**. Results demonstrated that the application of 200 mg/kg SLENU leads to a clearly manifested suppression of the specific antibody response on days 7 and 14. Significant differences in comparison with the control group as well as with the group treated with 100 mg/kg SLENU were detected. The injection with CCNU alone or CCNU+SLENU decreased in a similar degree the specific total antibody production on both studied days. Significant differences compared to both, that is, the control group and group treated with 100 mg/kg SLENU, were detected. The application of 200 mg/kg SLENU, CCNU alone and CCNU+SLENU led to a similar low level of KLH-specific polyvalent antibody production; there were no significant differences between groups.

DISCUSSION

Patients with cancer, especially those undergoing therapy for malignancies, are extremely susceptible to infections (5). Several factors determine the inefficiency of the patients' immune system. They include immunosuppressive factors derived from cancer cells, myelosuppression induced by chemo- and radiotherapy and cytotoxic effect of the chemotherapeutic agents on the antigen specific rapidly dividing hosts T and B lymphocytes (2, 7, 8). Previously published data have showed that SLENU does not affect the *in vitro* viability of unstimulated peripheral lymphocytes from healthy persons, but possess cytotoxicity against lymphocytes from patients with acute and chronic myeloleucosis, chronic lympholeucosis and non-Hodgkin's lymphomas (6). This result may be explained by the fact that lymphocytes in the blood of healthy individuals are resting into G_0 phase of the cell cycle. In this study we investigated the dose dependent effect of the spin – labelled 1-ethyl-1-nitrosourea (SLENU) on the humoral immune response, *in vivo*, after antigen challenge, when a

specific immune response are mounting, including cell division of stimulated lymphocytes. We also compared immunosuppressive activity of SLENU with the effect of its analogue CCNU, applied in a therapeutic dose.

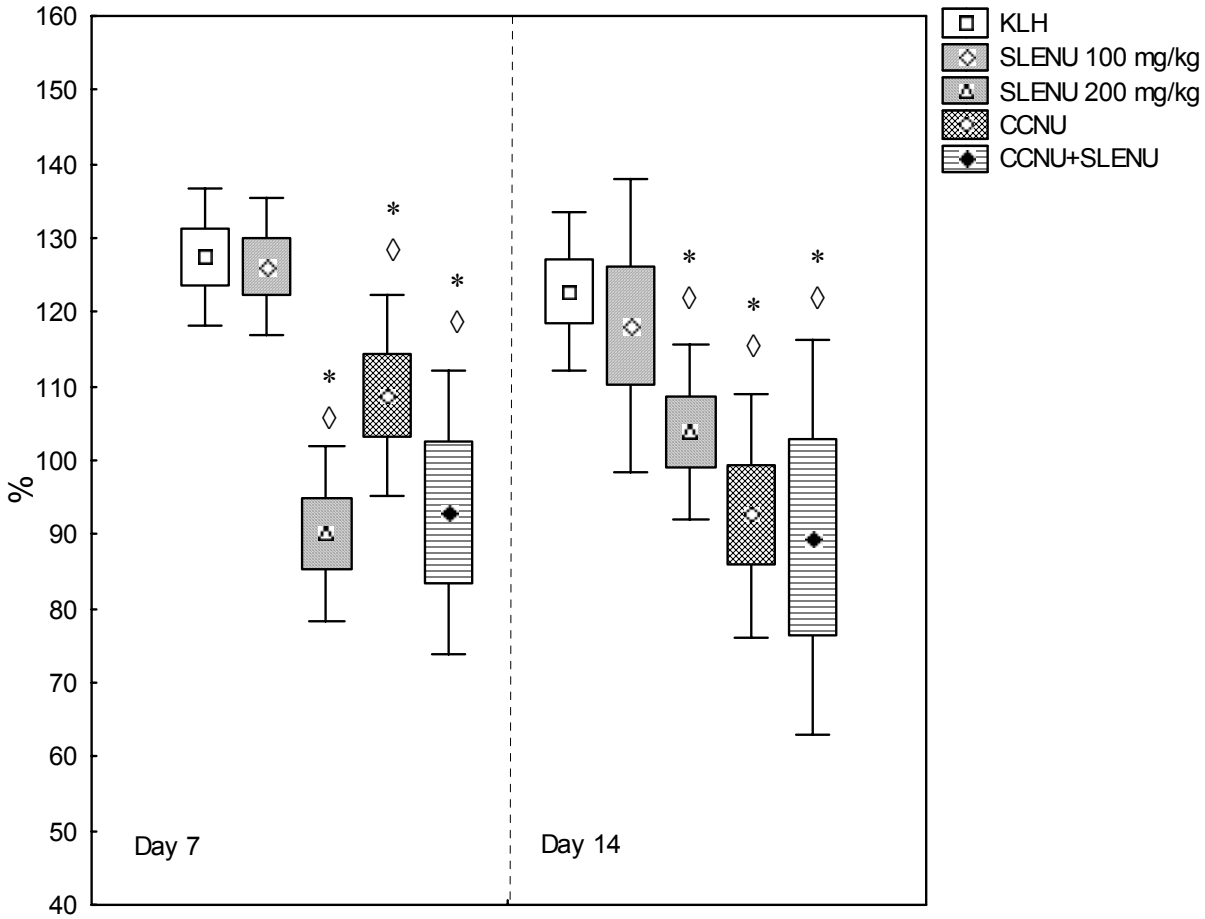

Figure 3. *Changes of KLH-specific total (IgM, IgG and IgA) immunoglobulin production in immunised mice. Mice were immunised with 5 µg KLH and treated with 100 mg/kg SLENU, 200 mg/kg SLENU, 33.3 mg/kg CCNU or 33.3 mg/kg CCNU simultaneously with 100 mg/kg SLENU, 24 h before antigen challenge. The control groups of mice were immunised with 5 µg KLH. The obtained results in OD 492 are presented as the percent (%) increase over the OD value of nonimmune sera.*

**p<0.05 vs. KLH; ◊ p<0.05 vs. 100 mg/kg SLENU.*

First, we demonstrate that SLENU exhibits immunosuppressive activity on the specific antibody production in a dose dependent manner. This dependence is clearly underlined by the differences in total antibody production, which present the immunoglobulin production containing specific anti-KLH antibody from IgM, IgG, and IgA isotypes. As is well-known, the primary immune response to T-cell dependent antigens, like KLH, is initiated after antigen recognition by antigen specific B lymphocytes. In brief, after antigen capture B cells engulfed, processed, and presented peptide fragments derived from the antigen on the cell surface in association with class II MHC molecules. Thereupon, cell-cell cooperation is achieved between antigen specific B and Th2 cells. As a consequence of this direct interaction and in the presence of appropriate type 2 cytokines (IL -4, -6, -10), B lymphocytes are activated, proliferated and differentiated to effector plasma cells secreting IgM, IgG, or IgA antibody in the blood (9). During proliferation stage B cells are rapidly moving through the cell-cycle and are highly susceptible of alkylating and/or carbamoylating cytotoxic action of the nitrosoureas (3, 4, 5). Because the dividing B cells are definitely dependent on successful DNA replication, the irreversible damage of DNA arrests the cells in G1/S or G2/M checkpoints of the cell-cycle and initiates a process of programmed cell death, apoptosis, which results in cellular suicide (10). The significantly diminished specific IgM and polyvalent antibody production in mice after introduction of high dose SLENU is an indication that the target of its cytotoxic action is the proliferation stage of antigen-

specific B lymphocytes. Furthermore, our previously published comparative study on the immunosuppressive activity of CCNU and other glycine containing (ChCNUgly) analogues of CCNU, showed complete abrogation of IgM secreting plasma cells in mouse spleens after treatment with both CCNU and ChCNUgly (11).

We have emphasised that SLENU exhibits lower immunosuppressive activity when compared to its structural analogues CCNU, applied in therapeutic dose. In our experiments the immunosuppressive effect of the highest dose SLENU (six-fold more than therapeutic dose of CCNU) was comparable with the immunosuppression induced by CCNU. Moreover, our results indicate that the dose of 100 mg/kg SLENU does not exhibit immunosuppressive effect in these experimental condition as well as additive effect over the immunosuppressive activity of CCNU. We observed similar lower immunosuppressive activity on the specific IgM secreting plasma cells in relation to other spin-labelled (SLCNUgly) analogue of CCNU (11). These data suggest that the stable nitroxyl radical present in the chemical structure of SLENU is probably involved in the lowered immunosuppressive activity of this drug.

Further, we suppose that the immunosuppressive action of SLENU and CCNU may affect the isotype class switching of B cells and subsequently their differentiation to IgG secreting plasma cells, because the level of the specific IgG antibody production in drug treated groups are similar on day 14. The class switching is performed in the nucleus of the activated B cells and involves rearrangement of DNA regions of Ig heavy chain genes by precise action of cell enzyme system (9). We suppose that the damages of DNA caused by the alkylating moiety and the inactivation of the class switching enzyme system caused by the carbamoylating moiety may be a reason for the unsuccessful class switching to γ-heavy chain followed by reduced number of IgG secreting plasma cells and decreased serum level of anti-KLH IgG.

In conclusion, we have demonstrated that: i) the spin - labelled 1-ethyl-3-[4-(2,2,6,6-tetramethyl - piperidine-1-oxyl)] -1-nitrosourea (SLENU) expressed immunosuppressive activity on the specific antibody production in a dose dependent manner; ii) SLENU exhibits lower immunosuppressive activity when compared to its structural analogues 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) applied in therapeutic dose; iii) immunosuppressive activity of SLENU and CCNU may affect the class switching of B cells and their differentiation to IgG secreting plasma cells.

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