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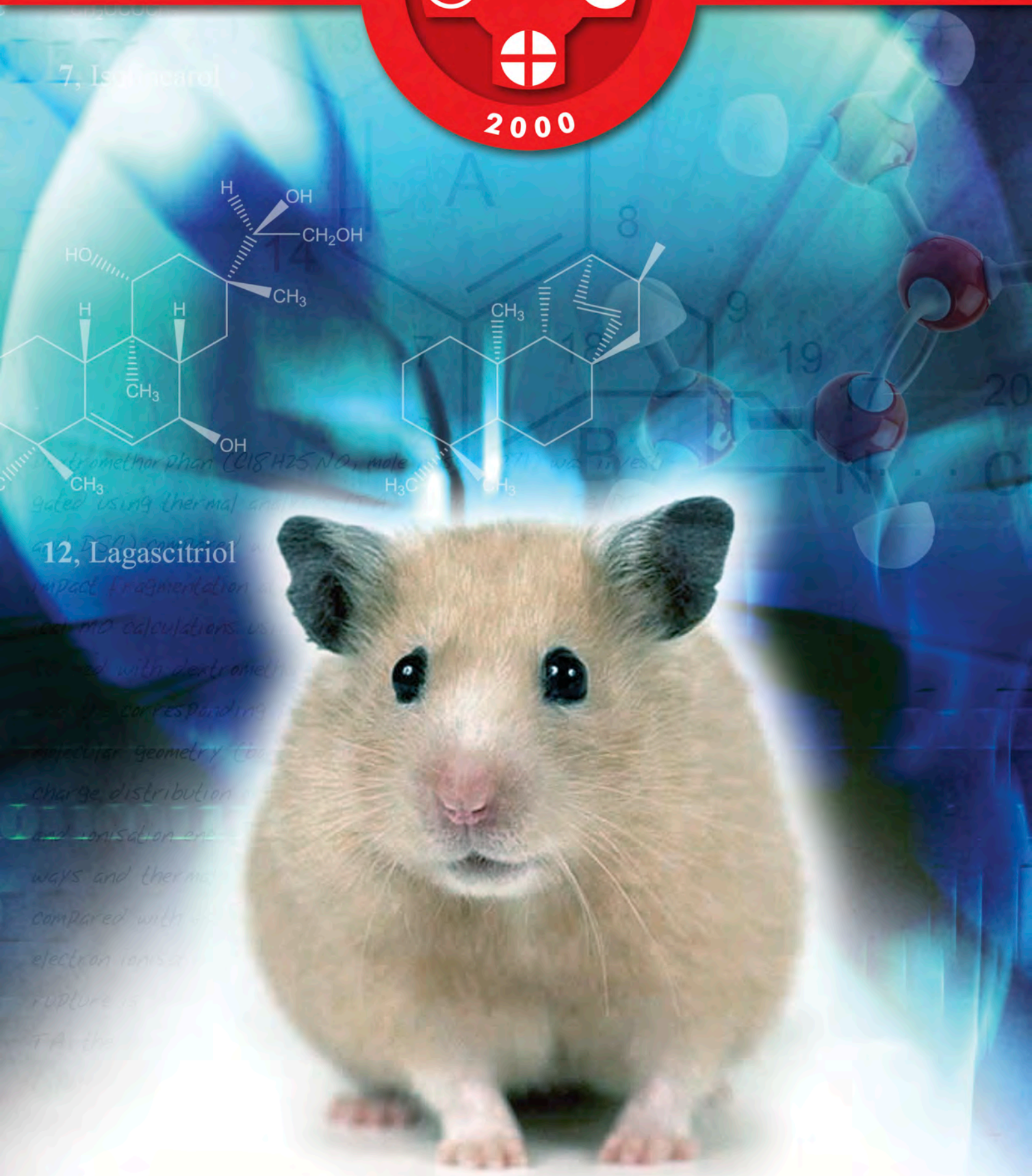

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DECREASED NK CELL CYTOTOXICITY AND INCREASED T REGULATORY CELLS FACILITATE PROGRESSION OF METASTATIC MURINE MELANOMA

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SMANJENA CITOTOKSIČNOST NK ĆELIJA I POVEĆANJE REGULATORNIH T LIMFOCITA UBRZAVA METASTAZIRANJE MALIGNOG MELANOMA MIŠA

Gordana Radosavljević[1], Ivan Jovanović[1], Katerina Martinova[1], Danijela Živić[2], Nada Pejnović[1], Nebojša Arsenijević[1], Miodrag L. Lukić[1]
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ABSTRACT

Malignant melanoma is the most aggressive form of skin cancer. Metastatic dissemination in distant organs is one of the hallmarks of melanoma progression. Immunosuppression and tumour escape from immune surveillance are thought to be the major factors responsible for the establishment and progression of melanoma; however, the exact mechanisms leading to decreased anti-tumour immunity are not completely understood. We aimed to analyse the anti-tumour immune response during hematogenous metastasis using a B16-F1 metastatic melanoma model in C57BL/6 mice. At 21 days after tumour cell inoculation, rapid metastatic melanoma growth was observed, reflected through the increased incidence, number and size of metastatic colonies in the lungs (B16-F1). Phenotypic analyses of splenocytes revealed an increased percentage of $CD3^+$T cells, a markedly reduced percentage of $CD19^+$ B cells and an increased percentage and absolute number of $CD4^+Foxp3^+$T regulatory cells. The cytotoxic activities of total splenocytes and isolated NK cells were significantly decreased in tumour-bearing mice. Thus, the metastatic progression of melanoma in this model is associated with diminished NK cytotoxicity, which may be due to an increased expansion of suppressive $CD4^+Foxp3^+$ T regulatory cells in the spleen.

Keywords: *B16-F1, malignant melanoma, metastasis, NK cells, T regulatory cells*

SAŽETAK

Maligni melanom je najagresivnija forma tumora kože. Diseminacija metastatskih ćelija u udaljene organe je glavna karakteristika progresije melanoma. Smatra se da su imunosupresija i izbegavanje imunskog nadzora glavni faktori odgovorni za uspostavljane metastaza, ali precizni mehanizmi odgovorni za oslabljen antitumorski imunski odgovor nisu u potpunosti razjašnjeni. U ovoj studiji, korišćenjem eksperimentalnog modela metastatskog melanoma (B16-F1) u C57BL/6 miševima analizirali smo antitumorski imunski odgovor u toku hematogenih metastatskih procesa. Dvadeset prvog dana nakon ubrizgavanja tumorskih ćelija detektovan je ubrzan rast metastaza malignog melanoma što se ogleda u povećanoj incidenci, broju i veličini metastatskih kolonija u plućima. Fenotipska analiza splenocita ukazuje na povećan procenat $CD3^+$T limfocita, značajno smanjene $CD19^+$ B limfocita i povećan procenat i apsolutan broj regulatornih $CD4^+Foxp3^+$T limfocita. Citotoksička aktivnost ukupnih splenocita i NK ćelija u slezini je statistički značajno smanjena u miševima kojima su ubrizgane ćelije malignog melanoma. Dobijeni rezultati u ovom eksperimentalnom modelu ukazuju da metastatskoj progresiji melanoma značajno doprinosi smanjena ubilačka sposobnost NK ćelija koja je najverovatnije posledica zabeležene ekspanzije imunosupresivnih regulatornih $CD4^+Foxp3^+$ T limfocita u slezini.

Ključne reči: *B16-F1, maligni melanom, metastaze, NK ćelije, regulatorni T limfociti*

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INTRODUCTION

Malignant melanoma is the most aggressive form of skin cancer. This disease arises from the malignant transformation of melanocytes, a complex process that involves the activation of multiple oncogenes and the inactivation of tumour suppressor genes (1,2). Metastatic tumour cells are characterised by their motility, ability to invade the surrounding tissues and enter the bloodstream, ability to survive the transit through the body and their ability to colonise distant organs (3). The invasion of malignant melanocytes, with subsequent metastatic dissemination and tumour growth in distant organs or tissues, is the hallmark of melanoma progression (4).

The metastatic spread of tumour cells involves interactions between tumour and immune cells (5), during which immune cells could either eliminate tumour cells and attenuate the metastasis or facilitate the metastatic dissemination (6). The role of anti-tumour immunity in metastatic melanoma growth is not completely understood. $CD8^+$T cell–mediated cellular immunity against melanoma-associated antigens has been shown to play an important role in the anti-tumour immune response in experimental melanoma models (7). However, in patients with melanoma, these melanoma-specific $CD8^+$ T cells are not efficient in controlling tumour progression. Thus, it appears that the role of $CD8^+$ cytotoxic T cells is variable and could be related to the immunosuppressed state associated with advanced tumours (8,9).

The immunosuppressive tumour microenvironment may be a major obstacle for the development of effective tumour-specific immune responses. Recent studies of malignant melanoma have demonstrated that the number of regulatory $CD4^+CD25^+Foxp3^+$T cells in the peripheral blood and within tumours is elevated, suggesting that these cells play a role in the induction of antigen-specific, local immune tolerance at tumour sites (10,11).

Natural killer (NK) cells, a key component of the innate immunity pathway, are cytolytic cells that recognise and kill malignant cells without prior sensitisation (12,13). NK cells are able to eliminate malignant cells from the circulation and thus serve as the earliest effectors against the dissemination of hematogenous metastasis [reviewed in (13)]. Natural Killer Group 2 Member D (NKG2D) is a powerful activating NK cell receptor that recognises various ligands on malignant transformed cells, such as MICA/B in humans and H60 and RAE-1 in mice (14). Natural regulatory T cells were shown to directly inhibit NKG2D-mediated NK cell cytotoxicity and suppress NK cell-mediated tumour rejection (15).

B cells are the effector cells of humoral immunity, and their role in antitumour immunity is not yet clear. Some studies suggest that these cells play a dual role in tumour-specific cellular immunity. For example, B cells can positively regulate cellular immune responses by serving as antigen-presenting cells and/or by providing costimulatory signals that can induce tumour-specific cytotoxic T cell activation (16-18). On the other hand, regulatory B cells (B10 cells) can negatively regulate inflammation and immune responses through the production of IL-10 (19-21). It has also been reported that B cells enhance premalignancy by potentiating chronic inflammation (22,23). The antibodies produced by activated B cells home to premalignant lesions and modulate chronic inflammation by cross-linking the FcR on resident leukocytes. This activity results in rapid degranulation and the release of proinflammatory mediators that further enhance the cascade of activation and recruitment of innate immune cells (23).

In the present study, we aimed to analyse anti-tumour innate and adaptive immune responses during hematogenous metastasis using the B16-F1 metastatic melanoma model in C57BL/6 mice.

MATERIALS AND METHODS

Mice

Eight to ten-week-old female and male C57BL/6 mice (purchased from the Military Medical Academy, Belgrade, Serbia) were used as model hosts for experimental metastatic melanoma. Mice were housed under standard laboratory conditions. The experiments were approved by the Ethics board of the University of Kragujevac Faculty of Medicine.

Murine melanoma cell line B16-F1

The murine skin melanoma cell line B16-F1, which is syngeneic to the C57BL/6 background, was purchased from the American Type Culture Collection (CRL-6323; ATCC, USA). The cells were routinely cultured as previously described (24,25).

Estimation of in vivo *metastasis in B16-F1 mouse melanoma model*

For inoculation, B16-F1 melanoma cells were harvested at ~90% confluency using 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS; PAA Laboratories GmbH). Cells were washed once in complete medium and twice in DMEM before inoculation. The viability of tumour cells was determined using the trypan blue assay, and only cell suspensions with ≥95% viable cells were used.

An experimental metastasis assay was performed by the intravenous injection of 5×10^4 B16-F1 cells, in a volume 0.2 ml, into the lateral tail vein of syngeneic C57BL/6 mice, as described previously (26). The mice were sacrificed on day 21 following melanoma cell injection, and lung, liver and brain tissues were removed for histological examination (24).

Splenic cell preparation

At 12 days after tumour cell injection, mice were sacrificed, and single-cell suspensions from spleens were obtained by mechanical dispersion through a cell strainer (BD Pharmingen, USA) in complete growth medium. Pellets were resuspended in red blood cell lysis solution, washed three times and resuspended in complete growth medium.

Phenotyping of splenocytes

The following anti-mouse mAbs were used: CD3, CD4, CD8, CD3e, CD19, F4/80 and NK1.1 (BD Pharmingen/

eBioscience, USA). Appropriate isotype control antibodies were used to assess the level of specific labelling. Dead cells were excluded by gating out propidium iodide-positive cells. For intracellular Foxp3 staining, cells were fixed and permeabilised with permeabilisation buffer (BD Pharmingen, USA). Permeabilised cells were stained with anti-mouse Foxp3 mAbs (BD Pharmingen). Stained cells were analysed using a FACSAria Flow cytometer (BD, USA). The gate used for FACS analysis was the mononuclear cell region in the FSC/SSC plot. The data were analysed using CELLQUEST software (BD, USA).

Adherent cell separation

Single-cell suspensions of the spleens were incubated for 2 h in complete media on plastic Petri dishes that had previously been covered with FBS. The non-adherent cells were removed by vigorously washing with DMEM, and the adherent cells were collected by gentle scraping with rubber policemen.

NK cell and CD8$^+$ T cell separation

NK cells were isolated from splenocyte suspensions by magnetic cell sorting. Single-cell suspensions of splenocytes were labelled using microbeads conjugated to monoclonal anti-mouse CD49b (DX5) antibodies (Miltenyi Biotec, USA) and positively selected as previously described (24). CD8$^+$ T cells were negatively selected from single-cell suspensions of splenocytes using a Dynal mouse T cell negative isolation kit (Invitrogen) as previously described (27).

Cytotoxicity assay

The cytotoxic activities of splenocytes, adherent cells, CD8$^+$ T cells and NK cells were measured using a 4-h MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Isolated splenocytes, adherent cells, CD8$^+$ T cells and NK cells were used as effector cells (E), and B16F1 melanoma cells were used as target cells (T). MTT cytotoxicity assays were performed as previously described (28).

The percentage of cytotoxicy was calculated as: cytotoxicy (%) = [1-(experimental group (OD)/control group (OD))] × 100. The data were expressed as the mean±SD of triplicate wells. Cytotoxic capacity was also presented in lytic units, LU20/10^7 cells, which were calculated from the means of triplicates percentages of killing obtained at four different T:E ratios. The estimated numbers represent the mean values.

Statistical analysis

The data were analysed using SPSS version 13. Statistical significance was evaluated using the Student's t-test. The normal data distribution was evaluated by the Kolmogorov–Smirnov test. The results were considered significantly different when $p<0.05$.

RESULTS

The detection of metastatic melanoma growth in the lung

Metastatic melanoma was established in syngeneic C57BL/6 mice by the intravenous injection of 5×10^4 B16-F1 cells into the lateral tail vein. On day 21 following tumour cell injection, all mice were sacrificed, and the lungs, liver and brain were investigated for the presence of metastatic colonies. Metastatic colonies were evident in the lung tissue; eleven out of twelve mice (92%) developed numerous lung metastases, as shown in Figure 1A. Metastatic colonies were not observed in other parenchymal organs at this timepoint (data not shown).

The injection of B16-F1 malignant melanocytes causes an increase in the percentage of CD8$^+$ cells and a decrease of the percentage of CD19$^+$ B cells in the spleen

The proportions of splenocyte cell populations may be altered in a tumour-bearing host (29). Therefore, we characterised and quantified the immune cells in the spleen 12 days after melanoma cell injection in comparison with splenocytes from healthy mice.

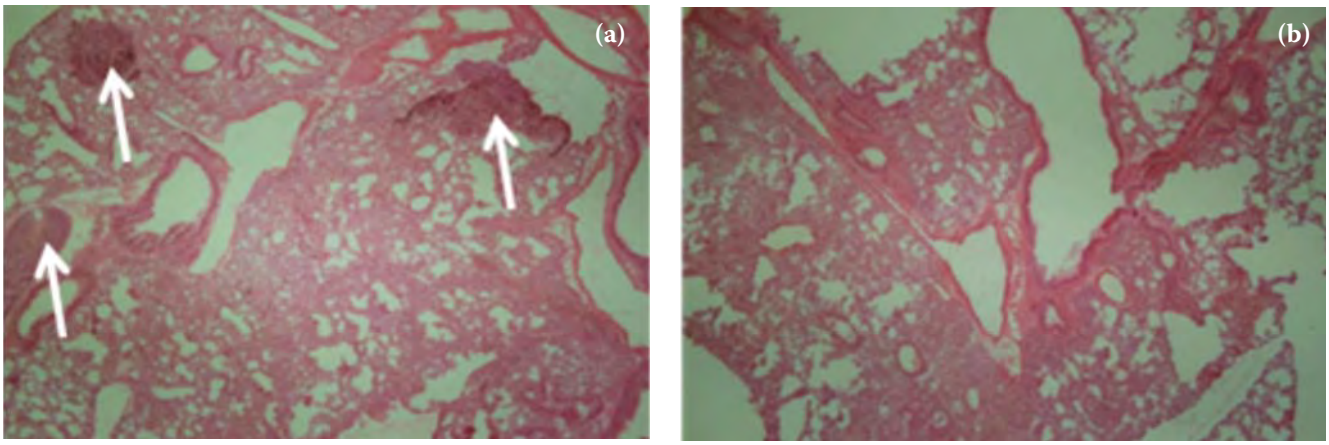

Figure 1. Metastatic melanoma growth in the lungs of C57BL/6 mice, 21 days after inoculation with B16-F1 cells
Fresh frozen lung tissues were stained with hematoxylin and eosin (H&E) and examined by light microscope for the number and size of metastatic colonies. H&E-stained sections (4 mm) from at least three different levels were analysed. A. Histological section across the lung of a mouse bearing B16-F1 melanoma cells (original magnification 10×) showing metastatic colonies (arrows). B. Histology of a lung section from a mouse without lung metastasis.

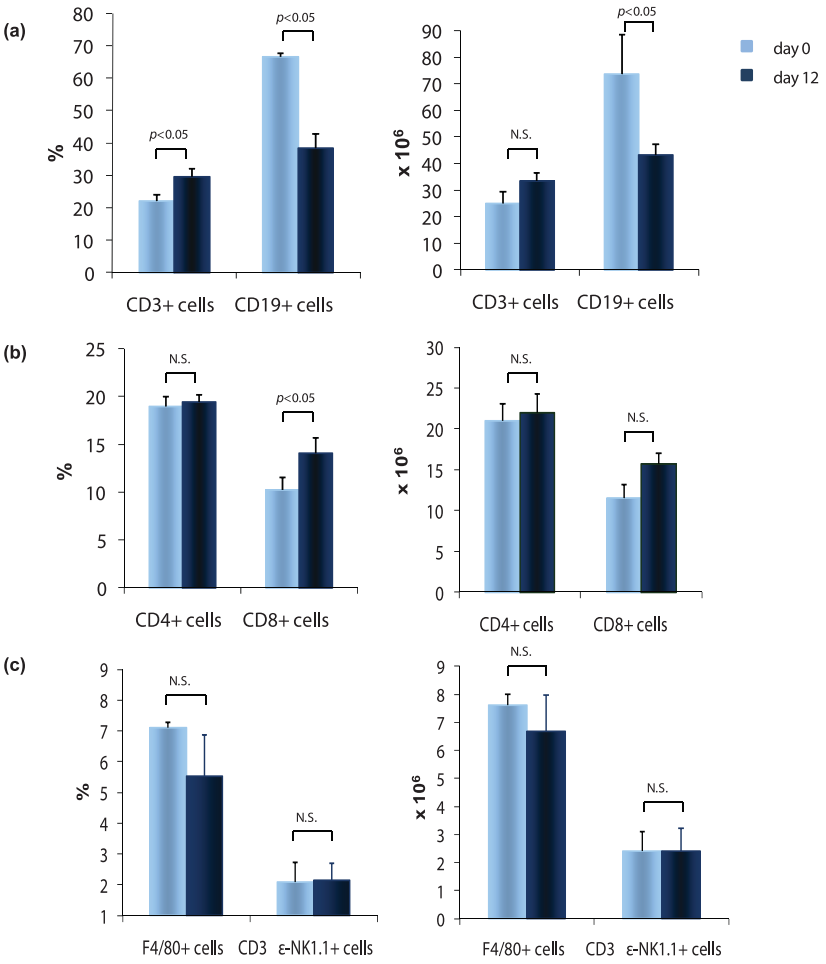

Figure 2. Flow cytometric analysis of splenocytes from naive and melanoma-injected C57BL/6 mice

A. and B. Injection of B16-F1 malignant melanocytes causes a statistically significant increase in the percentage of $CD3^+$T cells ($p<0.05$; M ± SD: 21.92± 1.05 vs. 29.59±1.17, left panel), which is most likely due to the increase in the percentage of $CD8^+$ cells ($p<0.05$; 10.23±0.81 vs. 14.04±0.92, left panel). The percentage of $CD19^+$B cells was drastically reduced ($p<0.05$; 66.70±0.46 vs. 38.33±2.19, left panel). There was no significant increase in the absolute number of $CD3^+$T cells (N.S; 25.02±4.15 vs. 33.37±3.14, right panel) or $CD8^+$ cells (N.S; 11.47±1.64 vs. 15.69±1.32, right panel), while the absolute number of $CD19^+$B cells was decreased after tumour cell inoculation ($p<0.05$; 73.52±15.00 vs. 43.07±4.32, right panel). In addition, there was no significant increase in the percentage (N.S; 18.96±1.03 vs. 19.46±0.75, left panel) or absolute number of $CD4^+$ cells (N.S; 21.01±2.09 vs. 22.01±2.31, right panel). **C.** There was no significant change in the percentage (N.S; 1.68±0.33 vs. 2.08±0.33, left panel) or absolute number of $CD3\varepsilon^-NK1.1^+$ cells in the spleen (N.S; 1.63±0.22 vs. 2.41±0.35, right panel) at 12 days after tumour inoculation. Similarly, there was no significant change in the percentage (N.S; 7.11±0.09 vs. 5.54±1.67, left panel) or absolute number of $F4/80^+$ cells in the spleen (N.S; 7.63±0.38 vs. 6.68±1.30, right panel) after tumour cell inoculation. The data are presented as the mean±SD of at least four mice per group. Statistical significance was tested by the Student's t-test. N.S. (not statistically significant).

The total number of mononuclear cells in the spleen was not significantly affected after the injection of B16-F1 malignant melanocytes (data not shown). As shown in the left panel of Figure 2A, there was a statistically significant increase in the percentage of $CD3^+$T cells ($p<0.05$) due to the increase in the percentage of $CD8^+$ cells ($p<0.05$; Figure 2B, left panel). Furthermore, the percentage of $CD19^+$B cells was drastically reduced ($p<0.05$; Figure 2A, left panel). The absolute number of subsets (Figure 2, right panels) correlates with these findings, while the absolute number of $CD19^+$B cells was again significantly reduced ($p<0.05$; Figure 2A, right panel). There were no significant changes in the percentages or absolute number of $CD4^+$ cells, NK ($CD3\varepsilon^-NK1.1^+$) cells, or macrophages ($F4/80^+$) in the spleen on day 12 after tumour inoculation (Figure 2B and 2C, left panel).

The number of $CD4^+Foxp3^+$ T regulatory cells in the spleen is elevated after melanoma cell inoculation

We next investigated whether the number of regulatory $CD4^+Foxp3^+$ T cells in the spleen is altered after melanoma cell inoculation. Flow cytometric analysis showed that the injection of melanoma cells resulted in a significant increase in the percentage and absolute number of $CD4^+Foxp3^+$ T cells ($p<0.05$, Figure 3). It appears that regulatory T cells may facilitate tumour metastasis by promoting an immunosuppressive environment.

A diminished NK cell-mediated anti-melanoma response in the spleen

We examined *in vitro* cytotoxic activity of splenocytes against tumour cells at the target–effector (T:E) ratios of 1:100, 1:50, 1:20 and 1:10. These cells were isolated before and on days 12 after i.v. injections of B16-F1 melanoma cells and were tested for cytotoxic activity against the melanoma cells using MTT assay. As shown in Figure 4A and 4B, cytotoxicity of total splenic cells was diminished after tumour cell injection.

To identify the type of effector cells that is responsible for the diminished cytotoxic capacity of splenocytes, we isolated adherent cells, $CD8^+$T cells and $CD49b^+$ NK cells and tested their anti-tumour cytotoxicity. We did not find any differences in the cytotoxicity of adherent cells between naive and melanoma-bearing mice (Figure 4C and 4F). We next tested the cytotoxic activity of $CD8^+$ T cells against tumour cells. As shown in Figure 4D and 4F, significant tumour-specific $CD8^+$T cell-mediated cytotoxicity was observed in control mice ($p<0.05$). Remarkably, we found that the cytotoxic activity of NK cells in the spleen was diminished after tumour cell injection (Figure 4E and 4F). The obtained results indicate that impaired NK cell cytotoxicity may be associated with diminished anti-melanoma immune response during hematogenous metastasis.

DISCUSSION

In the present study, we observed rapid metastatic melanoma dissemination in the lung tissue (B16-F1), reflected through the increased incidence, number and size of metastatic colonies. Our data show that hematogenous metastasis is accompanied by an increase in the percentage of $CD3^+$T cells, which is most likely due to an increased percentage of $CD8^+$ cells and a drastically reduced percentage of $CD19^+$ B cells in the spleen. In our tumour model, diminished cytotoxicity of total splenic cells and NK cells is associated with an increase in the percentage and absolute number of $CD4^+Foxp3^+$T regulatory cells. These results suggest that the spread of metastatic melanoma was mainly associated with decreased NK cell cytotoxicity, with a possible role for the suppressive activity of an increased proportion of Treg cells.

The B16 cell line is derived from a spontaneous tumour isolated from a C57BL/6 mouse. It is a highly aggressive tumour, and more importantly, it is similar to human melanoma in its propensity for metastasis and low MHC expression (30). In the current study, on day 21 following i.v. injection of B16-F1 (murine melanoma variant cell line), we demonstrated rapid metastatic melanoma growth in the lung tissue, reflected through an increased incidence, number and size of metastatic colonies. Eleven out of twelve C57BL/6 mice (92%) developed numerous lung metastases (Figure 1A).

The spleen, a secondary lymphoid organ, may be involved in the anti-tumour immune response, and a relationship between splenectomy and lung metastasis has been reported (31,32). We noticed that at 12 days after inoculation, the percentage and absolute number of $CD3^+$T cells were significantly increased in the spleen. This change is most likely due to the increased frequency of $CD8^+$ cells, as the frequency and number of $CD4^+$ cells was not altered. Interestingly, we noticed a marked reduction in the percentage and number of $CD19^+$ B cells in the spleens of tumour-injected mice (Figure 2A), which could be required for optimal cellular immune responses against B16 tumours *in vivo* (33). DiLillo et al (33) reported that B cell depletion reduces the generation of effector/memory and cytokine-secreting $CD4^+$ and $CD8^+$ T cells as well as the activation and proliferation of tumour-specific $CD8^+$ T cells. These data suggest that impaired T cell activation and effector-memory cell generation in the absence of B cells is likely to promote tumour growth and metastasis. It has also been reported that the number of NK cells and B cells in the bone marrow and spleen of tumour-bearing mice are reduced. This correlates with a decrease in the number of common lymphoid progenitors, suggesting that the tumour growth can lead to reduced lymphopoiesis (29). However, in our tumour model, we found a reduced number of B cells and an increased number of T cells, and we did not find any differences in the number NK cells or macrophages before and after injection of melanoma cells (Figure 2C).

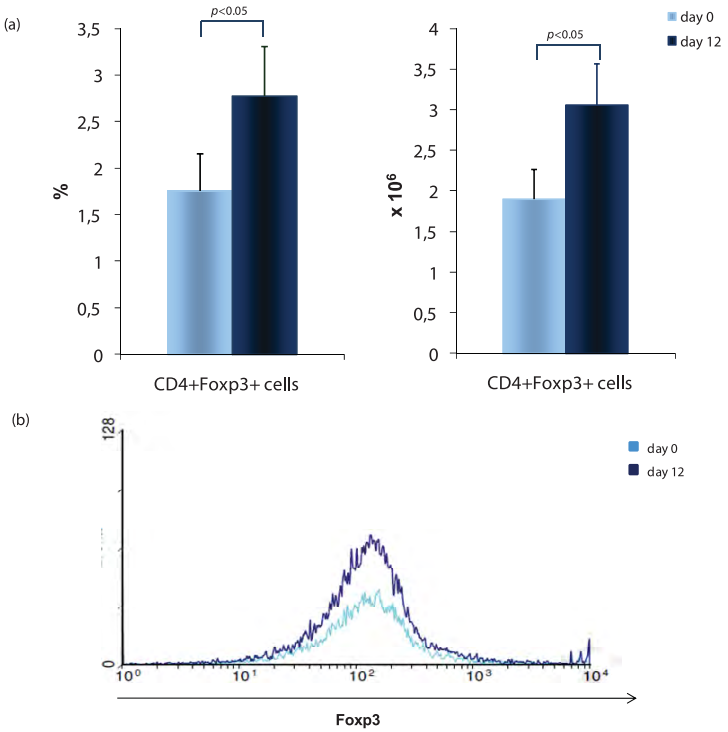

Figure 3. The injection of B16-F1 malignant melanocytes causes an increase in the percentage and number of $CD4^+$ $Foxp3^+$ T cells in the spleen of C57BL/6 mice

A. Melanoma cell-inoculated mice have a higher percentage ($p<0.05$; 1.75±0.4 vs. 2.77±0.54, left panel) and absolute number of $CD4^+Foxp3^+$ T regulatory cells than do naive mice ($p<0.05$; 1.9±0.36 vs. 3.06±0.5, right panel). B. A diagram illustrating the percentage of regulatory $CD4^+Foxp3^+$ T cells on the 0th (light blue) and 12th day after i.v. injection of B16-F1 cells (dark blue). The data are presented as the mean±SD of at least four mice per group. Statistical significance was tested by the Student's t-test.

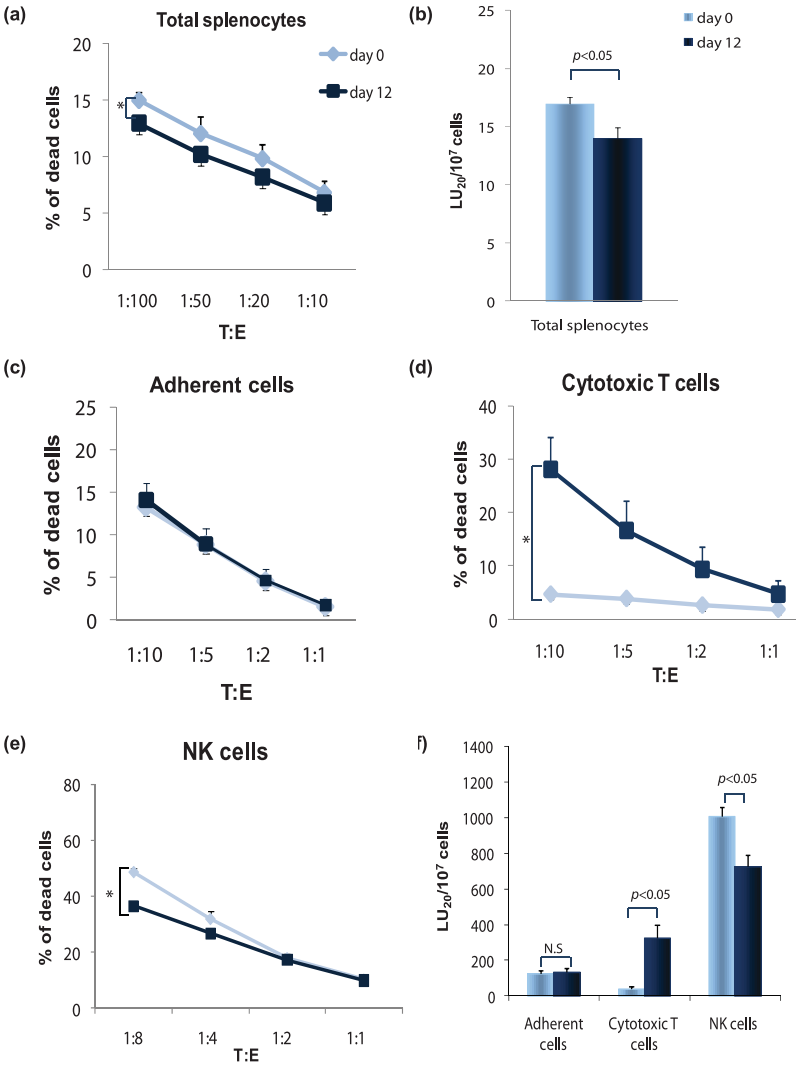

Figure 4. The cytotoxic activity of total splenocytes and different effector cells in the spleen.

The cytotoxic activity of effector cell populations was tested in a 4-h MTT assay against B16-F1 cell targets, at four different T:E ratios, on day 12. A. and B. The cytotoxicity of total splenic cells were diminished in melanoma cell-injected mice compared to naive mice. C. and F. There was no difference in the cytotoxicity of splenic adherent cells in naive and melanoma cell-inoculated mice. D and F. There was a significant increase in CD8⁺T cell-mediated cytotoxicity in the spleens of tumour cell-inoculated mice compared with naive mice. E. and F. The cytotoxic activity of NK cells in the spleen was diminished in melanoma cell-inoculated mice. The data are presented as the mean percentages of specific cytotoxicity and LU20/10^7 cells, which was calculated from the mean percentages of killing in four different T:E ratios and the percentages of effector cells found in the spleen. The data are presented as the mean±SD from at least four mice per group. Statistical significance was tested by the Student's t-test. N.S. (not statistically significant).

It has been suggested that the interactions between malignant cells and immune cells in the tumour microenvironment create an immunosuppressive network that protects the tumour from immune attack, permitting tumour progression (34-37). Recent studies suggest that T regulatory (Treg) cells are important cellular components of an immunosuppressive network that stimulates tumour growth and metastasis (38). We showed that the injection of melanoma cells resulted in a significant increase in the percentage and absolute number of CD4⁺Foxp3⁺ T regulatory cells (Figure 3). It appears that Tregs may facilitate tumour metastasis by promoting the formation of the immunosuppressive environment. The depletion of Treg cells was shown to facilitate tumour rejection in animal studies, implying that these cells suppress immune response against tumour cells (39,40).

Next, we also noticed that the cytotoxic activity of total splenic cells was diminished by day 12 after melanoma cell injection (Figure 4A and 4B). To define the effector cells responsible for the diminished cytotoxic capacity of splenocytes, we isolated adherent cells, CD8⁺T cells and NK cells and tested their cytotoxicity against tumour cells. We did not find any difference in the cytotoxicity of adherent cells of naive and melanoma cell-inoculated mice (Figure 4C and 4F), but CD8⁺ T cells from tumour-inoculated mice were more cytotoxic than those from naive mice (Figure 4D and 4F). Tumour immunity depends on factors other than T-cells, and numerous studies in hematopoietic and solid tumours have revealed that NK cell activation and cytotoxicity are related to patient outcome (41-43). Remarkably, we found that the cytotoxicity of NK cells in the spleen was diminished after melanoma injection (Figure 4E and 4F). Our results indicate that impaired NK cell cytotoxicity may be associated with diminished anti-tumour immune response during hematogenous metastasis. The diminished cytotoxicity of total splenic cells and NK cells may be due to an increase in the frequency and absolute number of splenic CD4⁺Foxp3⁺T regulatory cells. An inverse correlation between NK cell activity and Treg cell expansion is also found in cancer patients (44). There is evidence that Treg cells might hamper NK cell activation [reviewed in ref (45)]. For example, the suppressive effect of Tregs on the cytotoxicity of NK cells is in large part a result of the down-regulation of NKG2D mediated by TGF-ß (44), and Tregs seem to rather selectively inhibit NKG2D-mediated NK cell cytotoxicity (15,44).

Taken together, our results suggest that melanoma suppress innate anti-tumour immunity and facilitate metastasis, in part due to the increased expansion of $CD4^+Foxp3^+$T regulatory cells in the spleen.

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STUNTING, UNDERWEIGHT AND OVERWEIGHT: A MAJOR HEALTH PROBLEM AMONG CHILDREN UNDER 3 YEARS OF AGE IN URBAN AREAS OF WEST BENGAL, INDIA

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ABSTRACT

Background: *Malnutrition is still highly pervasive in developing countries, and pre-school age children may be a particular high-risk population. However, nutritional status of this group is poorly documented, particularly in urban areas.*

Aims: *To assess the stunting, underweight, thinness and overweight in urban Bengalee pre-school age children.*

Methods: *A total of 1060 children aged 1-3 years who attended the immunisation clinic of Midnapore District Red Cross Hospital of West Bengal, India during three years were enrolled in the study. The prevalence of underweight and stunting in pre-school age children were assessed using the SD classification based on the 2007 World Health Organization (WHO) child growth standards. The BMI classification was also used to assess thinness, overweight and obesity.*

Results: *Mean anthropometric variables were significantly higher among the boys than girls ($p \leq 0.05$). The results showed that the prevalence of undernutrition, particularly stunting (50.9%), was much higher than underweight (28.6%). The prevalence of underweight was more pronounced among boys. Conversely, girls tended to be more stunted than boys. The study revealed that approximately 14.4% of pre-school age children were overweight and that boys (16.6%) exhibited more overweight compared to girls (11.8%). The study also indicated the co-occurrence of stunting and overweight among the participants.*

Conclusions: *The present study emphasised that malnutrition is a growing public health issue, regardless of as stunting and overweight were highly prevalent among the Bengalee pre-school age children in urban areas of West Bengal.*

Key Words: *Stunting, Underweight, Overweight, Pre-school, Urban*

INTRODUCTION

In developing countries, the increasing prevalence of obesity along with the perseverance of undernutrition is referred to as the 'Double Burden of Malnutrition' (DBM).[1] In spite of the economic escalation of these countries, malnutrition is still highly prevalent, especially undernutrition.[2]

India represents a typical scenario in South-Asia, fitting the adage of the 'Asian Enigma'[3] where progress in childhood malnutrition seems to have sunken into an apparent undernutrition trap.[4] As per the latest estimates provided by the National Family Health Survey-3 (NFHS-3), the high overall levels of child undernutrition in India and its prevalence vary widely across the states and also across rural and urban areas.[5] Conversely, recent evidence suggests that overweight persist during the pre-school age children in India[6-8] and elsewhere.[9-12] In general, children living under better socio-economic conditions have consistently exceeded their counterparts living under worse conditions in growth and maturation, as an individual's genetic endowment can better manifest itself under better environmental circumstances.[13]

Children are a critical resource whose growth and well-being will determine to a large extent a country's social and economic future.[8] Pre-school age children are one of the most nutritionally vulnerable segments of the population. Nutrition during the first five years not only has an impact on growth and morbidity during childhood, but also acts as a determinant of nutritional status in adolescent and adult life.[14] Considerable evidence suggests that malnutrition affects human performance, health and survival, including physical growth, morbidity, mortality, cognitive development, reproduction, physical work capacity and risks for several adult-onset chronic diseases.[15]

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Assessment of growth is the single most important measurement for defining the nutritional and health status of children and provides an indirect measurement of the quality of life of the entire population.[16] The World Health Organization (WHO) has been monitoring child growth and malnutrition since 1986 and collecting data in the Global Database on Child Growth and Malnutrition, which aims to facilitate international comparison, the identification of populations in need, the evaluation of national public health interventions, and monitoring trends in child growth.[17] Therefore, an appraisal of a country's progress in healthcare can be made with the aid of growth studies.[8] In this respect, pre-school age children are the main target group for strategies and actions to fight malnutrition.

Significant published data are available[8, 18-20] on the prevalence of undernutrition among pre-school age children in different parts of India, but mostly for rural sectors. However in contrast, there is a paucity of studies in urban pre-school age children of West Bengal. In this context, this study has attempted to assess the prevalence of stunting, underweight and overweight among preschool children of under 3 years old.

MATERIALS AND METHODS

Settings

The present cross-sectional study was conducted among the urban pre-school age children of Bengalee ethnicity attending an immunisation clinic in Midnapore District Red Cross Hospital situated at Midnapore town, West Bengal, India. Midnapore is a district town, located at 22.25^0 N, 87.65^{0}E and is 23 meters above sea level. A total of 1060 children between the ages of 1-3 years participated in the study from January 2010 to December 2011. Data on age, sex, height and weight were recorded. A *pro forma* was designed to collect information on familial background. Household socio-economic and demographic variables such as father's occupation, family income and family size were included.

Anthropometric measurements

To assess the nutritional status of pre-school age children, anthropometric measurements, i.e., height and weight, were taken according to standard procedures describe by WHO.[22] Weight was measured to the nearest 0.1 kg on a weighing scale with children wearing no shoes and only light clothing. Individual height was measured to the nearest 0.1 cm with a wooden stadiometer placed on a flat surface. BMI was calculated from the formula weight/height^2 (kg/m^2).

Nutritional assessment

Nutritional assessment was carried out by using 2007 WHO Child Growth Standards[23] according to the standard deviation (SD) classification. Children who were more than 2 SD values below the reference median (<Median -2 SD) on the basis of weight-for-age, height-for-age and BMI-for-age indices were classified as 'underweight', 'stunted' and 'thinness', respectively, and children who were more than 3 SD values below the references median (Median -3 SD) were classified as 'severe underweight', 'severe stunted' and 'severe thinness', respectively. The overweight and obesity were defined as BMI-for-age above +1 and +2 SD scores, respectively.

Ethical clearance

The objective of the study was explained and verbal assent was obtained from the parents of each child.

Statistical analysis

Student's *t*-test was used to assess sex differences of mean anthropometric indices. Stunting, underweight and overweight/obesity were compared between sexes using a χ^2 test. In all analyses, p values less than 0.05 were considered statistically significant.

RESULTS

The majority of children came from relatively well-off families. The average family income of the children was more than eight thousand Rupees (Rs.8405.33, US$163.51) per month. Approximately half (49.5%) of the fathers were engaged in business and 24.6% worked in government sectors. The average number of persons per house was 3.3 and more than seventy percentage (72.6%) of families had three persons (Table 1).

Gender	No (%)	Family size	No (%)	Occupation	No (%)
Boys	578 (54.5)	Mean±SD	3.3±0.55	Govt service	261 (24.62)
Girls	482 (45.4)	1-3 persons	770(72.64)	Private service	195 (18.39)
		4-6 persons	285(26.8)	Business	525 (49.52)
		>7 persons	5(0.47)	Labour	54 (5.09)
				Others	25 (2.35)

Table 1: Socio-economic and demographic features of the families

Age (yrs)	Boys (n)	Girls (n)	Weight (kg)			Height(cm)		
			Boys Mean(SD)	Girls Mean(SD)	p-value	Boys Mean(SD)	Girls Mean(SD)	p-value
1.	146	122	8.28 (1.1)	7.78 (1.3)	0.008*	69.19 (4.1)	66.79 (4.5)	0.008*
2.	237	206	10.38 (1.7)	9.84 (1.5)	0.007*	78.48 (4.7)	76.9 (5.1)	0.009*
3.	195	154	12.46 (2.2)	11.85 (2.2)	0.013*	88.76 (5.1)	87.25 (5.5)	0.089*

Table 2: Mean anthropometric measurements of pre-school age children

Standard deviations are presented in parentheses.
*Significant sex differences ($p < 0.05$).

The anthropometric characteristics of the pre-school age children are presented in Table 2. The total sample included 1,060 children with a mean age of 1.46±0.49 years. Of these children, 578 (54.5%) were boys and 482 (45.4%) were girls. Overall, the mean values of weight (Boys 10.55±2.40 kg; Girls 9.96±2.34 kg) and height (Boys 79.60±8.86 cm; Girls 77.65±9.27 cm) were significantly higher in boys compared to girls ($p \leq 0.05$). However, there was no significant difference between sexes in mean height values of at 3 years of age. The children in this study had a mean height ranging from 69.19 cm to 88.76 cm in boys and 66.79 cm to 87.25 cm in girls. It is apparent from this table that the mean values of weight and height were progressively accelerating with increasing age.

The frequencies of underweight and stunting are presented in Table 3. The overall (age and sex combined) rates

Nutritional status	Boys				Girls			
	1 yrs	2 yrs	3 yrs	Total	1 yrs	2 yrs	3 yrs	Total
Weight-for-age[a]								
-3 SD	17(11.6)	18(7.5)	11(5.6)	46(7.9)	13(10.6)	36(17.4)	16(10.3)	65(13.4)
-2 SD	30(20.5)	51(21.5)	44(22.5)	125(21.6)	19(15.5)	3(1.4)	46(29.8)	68(14.1)
Total	47(32.1)	69(29.1)	55(28.2)	171(29.5)	32(26.2)	39(18.9)	62(40.2)	133(27.5)
Height-for-age[b]								
-3 SD	45(30.8)	77(32.8)	29(14.8)	151(26.12)	42(34.4)	65(31.5)	28(18.1)	135(28.0)
-2 SD	27(18.4)	59(24.8)	51(26.1)	137(23.7)	23(18.8)	48(23.3)	41(26.6)	112(23.2)
Total	72(49.3)	136(57.3)	80(41.0)	288(49.8)	65(53.2)	113(54.8)	69(50.6)	247(51.2)

Table 3: Age specific prevalence of underweight and stunting among the pre-school age children

Figures in parentheses are percentages of the total in each column.
[a] $\chi^2 = 9.901$, df=3, p=0.0194; [b] $\chi^2 = 2.704$, df=3, p=0.4394

Age	BMI classification					
	Thinness			Normal	Overweight[b]	Obesity[c]
	Moderate	Severe	Total[a]			
Boys						
1	51(34.9)	4(2.7)	55(37.6)	54(36.9)	27(18.4)	10(6.8)
2	67(28.2)	10 (4.2)	77(32.4)	85(35.8)	43(18.1)	32(13.5)
3	70(35.8)	25(12.8)	85(43.5)	69(35.3)	26(13.3)	15(7.6)
Total	188(32.5)	39(6.7)	217(37.5)	208(35.9)	96(16.6)	57(9.8)
Girls						
1	40(32.7)	29(23.7)	69(56.5)	31(25.4)	8(6.5)	14(11.4)
2	67(32.5)	7(3.3)	74(35.9)	78(37.8)	36(17.4)	18(8.7)
3	12(7.7)	1(0.64)	24(15.5)	106(68.8)	13(8.4)	10(6.4)
Total	119(24.6)	37(7.6)	156(32.3)	215(44.6)	57(11.8)	42(8.7)

Table 4: BMI classification of pre-school children

Figures in parentheses are percentages of the total in each column.
[a] $\chi^2 = 28.622$, df=3, p=0.000003; [b] $\chi^2 = 10.916$, df=3, p=0.0121; [c] $\chi^2 = 5.131$, df=3, p=0.1624

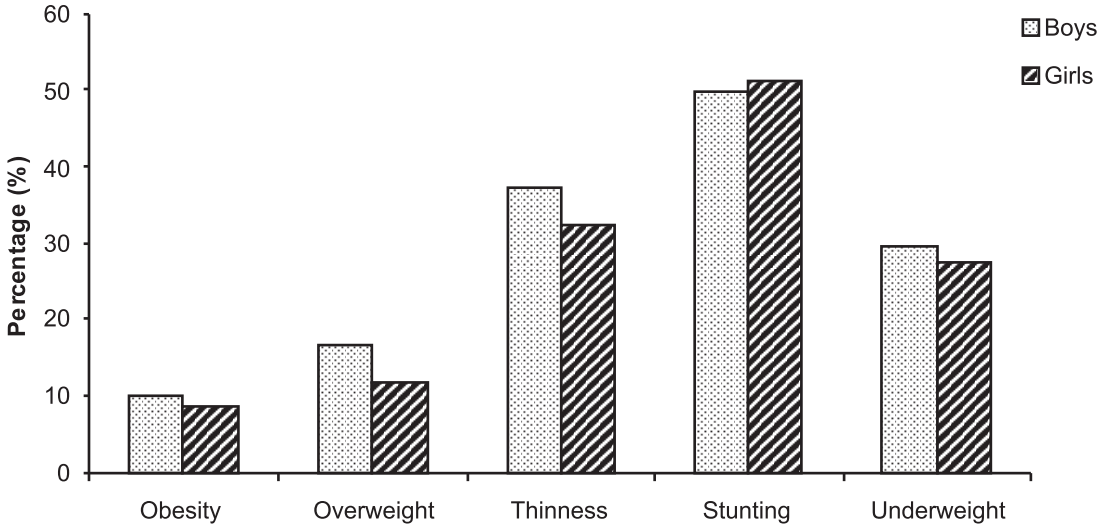

Fig. 1: Prevalence of overall malnutrition in urban Bengalee pre-school age children (N = 1060).

of underweight and stunting were 28.6% and 50.9%, respectively. The rate of underweight was higher among boys (underweight = 29.5% vs 27.5%), but stunting was higher among girls (stunting = 49.8% vs 51.2%). In both boys and girls, the highest percentages of stunting were exhibited at 2 years of age, followed by 1 and 3 years. Likewise, the extent of underweight decreased with increasing age in boys, but in girls the highest prevalence was observed in those 3 years of age. However, the extent of severe (< -3SD) underweight and stunting was comparatively higher among girls than boys. It is noted that the highest percentage of children were severely stunted.

Table 4 presents the BMI classification of participants according to gender and age. Overall, thinness affected approximately 35.1% of pre-school age children with most being moderate thinness (28.9%). The overall rates of overweight and obesity were 14.4% and 9.3%, respectively. Overweight was predominant at 2 years of age in both sexes. Normal BMI-for-age in boys and girls was 35.9% and 44.6%, respectively.

Fig. 1 depicts the nutritional status of urban Bengalee pre-school age children stratified by gender. A noteworthy point is that obesity was slightly higher in girls but overweight was significantly different ($p<0.05$) between boys (16.6%) and girls (11.8%). Boys tended to have more thinness compared to girls, and the difference was statistically significant ($p<0.05$). There was no significant difference between boys and girls in respect to stunting and underweight.

DISCUSSION

Malnutrition makes its principal impact on pre-school age children in developing countries. These nations face great difficulties in improving the standards of living of its population because of unequal distribution of its resources leading to widespread malnutrition.[21] There is a consensus that a wide variety of biological, behavioural and socioeconomic variables influence the health status of children in developing countries.[24]

The present study revealed that the major type of malnutrition was the seemingly high prevalence undernutrition (stunting and underweight) among the pre-school age children living in urban areas of Midnapore. In this study the overall prevalence of underweight was 28.6% among pre-school age children based on the 2007 WHO classification. Many studies in India[19] and West Bengal[20] have indicated a higher prevalence of underweight in pre-school age children. That a very high proportion (more than 50%) of children in this study was stunted is surprising.; stunting was more prevalent in two year old children of both sexes. Although it is well known that the prevalence of stunting is maximum in the first two to three years of life (due to high velocity of linear growth), certain reports have suggested that it varies by region.[20] There is sufficient evidence to demonstrate that the children in the general population of developing countries are markedly stunted.[25, 26] NFHS-3 indicates that approximately 46% of children under 5 years of age were moderately to severely underweight and that 38% were moderately to severely stunted.[5] In a study performed in different areas of Punjab, it was found that the prevalence of underweight and stunting was 55.6% and 40.9%, respectively.[2] Compared to our study, the prevalence of stunting were lower. In general, stunting and underweight are comparatively higher among Indian children.[26] Although determination of the main causal factors for stunting is beyond the scope of this study, Ying et al[25] mentioned that the high prevalence of stunting among pre-school age children is most likely related to environmental factors, mainly nutrition and slower socioeconomic development.

In this study, a significant difference between the two sexes with regard to prevalence of undernutrition was not found. However, prevalence of underweight was more pronounced in boys and stunting was more pronounced

in girls. These findings support of previous observations. This concurs with the results of our previous study performed in rural areas of Kharagpur, West Bengal by Chatterjee & Paul.[19] In general, it was observed that boys suffer less undernutrition than girls (NFHS, 1998-99).[4]

In this study, BMI-for-age was utilised as an indicator of thinness and overweight. The WHO expert committee[22] has recommended BMI-for-age as the best indicator to assess undernutrition (thinness) or overweight. Thinness usually describes acute malnutrition. Table 4 shows the prevalence of thinness was 35% and was higher among boys (37.5%) than girls (32.3%). When the prevalence of thinness between boys and girls of each age was compared, the differences were statistically significant ($p<0.05$). A similar trend was reported by Bisai & Manna[27] who conducted a study in the urban area of West Bengal and showed that approximately 47% of the pre-school age children suffered thinness according to the Cole et. al. classification.

The present study demonstrated the alarmingly higher prevalence of overweight (14.4%) and obesity (9.3%) among the Bengalee pre-school age children. This rate were much higher than that found in previous studies conducted in different parts of India.[6-8] Increasing obesity is already a major concern for pre-school age children in developed countries[12] as well as urban India.[6,7] Affluence and urban lifestyle are associated with a higher prevalence of overweight in lower and middle income developing countries.[28]

This study provides evidence that stunting concurrent with overweight or obesity is an important public health problem among urban pre-school aged children in West Bengal, and it is in agreement with previous studies that show stunting is associated with overweight in children of developing nations that are undergoing nutritional transition.[9-11]

The interpretation of our findings indicates that both undernutrition and overnutrition coexist in our study areas among pre-school age children. Few studies have reported the presence of obesity and growth retardation within the same children.[9, 10] Wang et al[9] mentioned that it is a major public health problem that is most likely growing and should be given due attention. The "nutrition paradox"[29], the coexistence of nutritional deficit and excess, underlines the difficulty of designing interventions aimed at reducing undernutrition while addressing the increasing problem of overweight and obesity at the same time. Future studies should address causes of overnutrition and undernutrition among Bengalee pre-school age children. There is an urgent need to address nutrition problems among pre-school age children in developing countries, without neglecting urban areas.

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CYTOTOXIC EFFECTS OF SELECTED GOLD(III) COMPLEXES ON THE MURINE BCL-1 B LINEAGE LEUKAEMIA CELL LINE

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CITOTOKSIČNI EFEKTI IZABRANIH KOMPLEKSA TROVALENTNOG ZLATA NA ĆELIJSKU LINIJU MURINE BCL-1 B LEUKEMIJE

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ABSTRACT

In recent years, gold(III) complexes have attracted great interest because of their cytotoxicity to cancer cells.

We investigated the cytotoxic effects of three newly synthesised gold(III) complexes, $[Au(en)Cl_2]^+$ (dichloride (ethylendiamine) aurate(III)-ion), [Au(dach)Cl_2] (dichloride (1,2-diaminocyclohexane) aurate(III)-ion) and $[Au(bipy)Cl_2]^+$ (dichloride (2,2'-bipyridyl) aurate(III)-ion), on the murine BCL-1 B lineage leukaemia cell line.

The cytotoxicity of these gold(III) complexes was evaluated by cytotoxic assay (MTT test).

The results showed that all of the tested gold(III) complexes displayed a cytotoxic effect on BCL-1 cells. The concentration decrease was followed by a marked increase in BCL-1 cell viability. At a concentration of 125 μM, which we suppose could be used in vivo, the $[Au(bipy)Cl_2]^+$ complex showed the greatest cytotoxic effects among the tested gold(III) complexes and similar cytotoxicity asto the cisplatinum that we used as control. Among the tested gold(III) complexes, $[Au(en)Cl_2]^+$ was the least cytotoxic to BCL-1 cells.

In line with the obtained results, we suggest that the $[Au(bipy)Cl_2]^+$ complex should be tested in vivo in experimental models of B cell leukaemia.

Key words: gold(III) complexes, cytotoxicity, BCL-1 cells

SAŽETAK

Poslednjih nekoliko godina rade brojna istraživanja u cilju ispitivanja citotoksičnosti jedinjenja zlata radi njihove eventualne primene u onkologiji.

Mi smo ispitali citotoksičnost novosintetisanih jedinjenja zlata [Au(en)Cl2]+ (dichloride (ethylendiamine) aurate(III)-ion), [Au(dach)Cl2] (dichloride (1,2-diaminocyclohexane) aurate(III)-ion) i [Au(bipy)Cl2]+ (dichloride (2,2'-bipyridyl) aurate(III)-ion) na BCL-1 liniji V ćelijske mišje leukemije.

Citotoksičnost je analizirana primenom MTT testa.

Naši rezultati pokazuju da sva novosintetisana jedinjenja zlata pokazuju citotoksičan efekat na BCL-1 liniji koji je dozno zavistan (smanjenje koncentracije korelira sa porastom proliferacije BCL-1 ćelija). Pri koncentraciji 125 μM, za koju smatramo da treba testirati in vivo, najbolji citotoksični efekat je pokazao kompleks [Au(bipy)Cl2]+. Citotoksičnost ovog kompleksa je bila približna citotoksičnošću cisplatine koju smo koristili kao kontrolu. Među ispitivanim kompleksima najslabiju citotoksičnost na liniji V ćelijske mišje leukemije je pokazao [Au(en)Cl2]+ .

U skladu sa dobijenim rezultatima, smatramo da in vivo treba ispitati terapijski efekat [Au(bipy)Cl2]+ u eksperimentalnom modelu V ćelijske leukemije.

Ključne reči: *jedinjenja zlata, citotoksičnost, BCL-1 ćelije*

INTRODUCTION

The success of cisplatin, carboplatin and oxaliplatin, which now play a major role in established medical treatments of cancer, has aroused great interest in the study of the cytotoxic effects of metal complexes that are isostructural to these platinum complexes [1-3].

During the last twenty years, much research has focused on gold(III) complexes, which are square-planar d8, isoelectronic and isostructural to platinum(II) complexes. Many *in vitro* and *in vivo* studies have been conducted to investigate and precisely describe the mechanism underlying the anti-tumour effects of gold(III) complexes [3-7]. Although the results were encouraging and gold(III) compounds appeared to be very good candidates for anticancer drugs [4-7], because of their reductive potential, these complexes were not stable under physiological conditions [8]. Therefore, the selection of a suitable ligand to stabilise the complex became a foremost challenge in the de-

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sign of new gold(III) complexes with one or more multidentate ligands that enhance the stability of the complex.

We investigated and present here the cytotoxic effects of selected gold(III) complexes, $[Au(en)Cl_2]^+$ (dichlorido(ethylendiamine)aurate(III)-ion) $[Au(dach)Cl_2]$ (dichloride(1,2-diaminocyclohexane)aurate(III)-ion) and $[Au(bipy)Cl_2]^+$ (dichlorido(2,2'-bipyridyl)aurate(III)-ion), on the murine BCL-1 B lineage leukaemia cell line .

BCL-1 is a murine B lineage leukaemia cell line that was first described by Slovin and Straber [9]. BCL-1 leukaemia arose spontaneously in a 2-year-old BALB/c mouse and is easily transplanted in syngeneic recipients by injection of spleen or peripheral blood lymphocytes previously obtained from leukaemic animals [10]. BCL-1 leukaemia represents an experimental model for human chronic prolymphocytic leukaemia (PLL) [11]. The analysis of BCL-1 cell morphology showed that these cells closely resemble the prolymphocytes obtained from patients with prolymphocytic leukaemia [10-11]. Further, BALB/c mice injected with BCL-1 cells develop enlarged spleens diffusely infiltrated by BCL1-prolymphocytes [10-11]. In accordance with massive splenomegaly, the murine BLC-1 leukaemia is characterised by leukocytosis (white blood cell counts $>10^8$/ml), hepatomegaly and little or no lymphadenopathy [11].

This study could shed elucidate the *in vitro* anti-cancer properties of selected gold(III) complexes and indicate the value of investigating some of these newly synthesised gold(III) complexes in future studies.

MATERIALS AND METHODS

Chemicals and ligands

The ligands 2,2'-bipyridyl (bipy) and (1R,2R)-1,2-diaminocyclohexane (dach) were obtained from Acros Organics, while the ligand ethylenediamine (en) was obtained from Sigma-Aldrich (Munich, Germany). The starting potassium tetrachloridoaurate(III) complex, $K[AuCl_4]$, was purchased from ABCR GmbH & Co (Karlsruhe, Germany), while cisplatin (cisdiamminedichloroplatinum(II), cis-$[Pt(NH_3)_2Cl_2]$) was purchased from Sigma-Aldrich. All chemicals were of the highest purity commercially available and were used without further purification.

For the cytotoxicity determination, further chemicals were used, including foetal bovine serum (FBS), growth medium RPMI 1640, penicillin G, streptomycin, (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT), phosphate buffered saline (PBS), dimethylsulfoxide (DMSO), trypan blue stain (all from Sigma Chemicals, Germany) and Haemaccel (Theraselect Gmbh, Germany). The assays were performed in 96-well plates (Sarstedt, Germany).

Synthesis of the complexes

The complexes $[Au(en)Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$ were prepared according to the published procedure [12-14]. The $[Au(dach)Cl_2]Cl$ complex was synthesised starting from $KAuCl_4$. Salt (0.2 g, 0.5 mmol) was dissolved in a small amount of water and was added to the solution obtained by dissolving (1R,2R)-1,2-diaminocyclohexane (0.057 g, 0.5 mmol) in a mixture of MeOH/H_2O (1:1, v/v). The reaction was stirred for 5 h at room temperature. The yellow solution obtained was left to evaporate in darkness. After a few days, the yellow crystals that had formed were filtered, washed with cold water and dried. Found: H, 4.91; C, 13.66; N, 2.84; Calc. for $AuC_6H_{14}N_2Cl_3$: H, 5.34; C, 13.80; N, 2.71 %.

Cell culture

The BCL-1, murine B lineage leukaemia cell line, syngeneic in BALB/c mice, was purchased from the American Type Culture Collection (ATCC) Manassas, VA, USA. The BCL-1 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol containing 15% FBS, 100 IU/mL penicillin G and 100 µg/mL streptomycin (Sigma-Aldrich chemical, Munich, Germany). BCL-1 cells from the third passage were used throughout these experiments.

Cytotoxicity assay

The effects of $[Au(en)Cl_2]^+$ $[Au(dach)Cl_2]$ and $[Au(bipy)Cl_2]^+$ complexes on BCL-1 cell viability were determined using the MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric test [15].

BCL-1 cells were diluted with medium to 1 x 10^5 cells/mL, aliquots (1 x 10^4 cells/100 µL) were placed in individual wells in 96-well plates, and 100 µL of complexes diluted in medium in selected concentrations were added. Cells were treated with selected concentrations of complexes for three days. Control wells were prepared by adding culture medium. Wells containing culture medium without cells were used as blanks. The MTT solution was prepared as 5 mg/ml in PBS just before use and filtered through a 0.22-µm filter. After incubation, the cells were pelleted, and the drug-containing medium was discarded and replaced with serum-free medium containing 15% MTT solution. After an additional 4 h of incubation at 37 °C in a 5% CO_2 incubator, the medium with MTT was removed, and DMSO (150 µL) with glycine buffer (20 µL) was added to dissolve the blue formazan crystals. The plates were shaken for 10 min. The optical density of each well was determined at 595 nm.

The percentage of cytotoxicity was calculated using the following formula:

% of viable cells = (E–B)/(K–B) × 100

where B is for the background optical density of the medium alone, K is for the total viability/spontaneous death of untreated target cells, and E is for the experimental well.

STATISTICAL ANALYSES

Where appropriate, the data were presented as means +/- SD. Statistical analyses were performed by ANOVA followed by the Bonferroni test. The level of significance was set at $p < 0.05$.

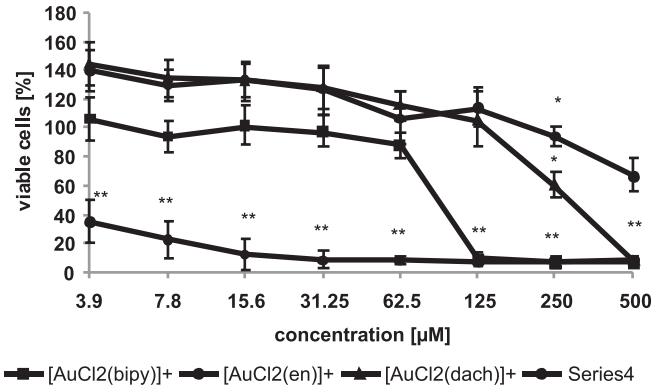

Figure 1. Toxicity of the $[Au(bipy)Cl_2]^+$, $[Au(dach)Cl_2]^+$ and $[Au(en)Cl_2]^+$ and cisplatin $[PtCl_2(NH_3)_2]$ complexes, using BCL-1 cells as the target cells.
BCL-1 cells were cultured with different doses of the tested complexes, ranging from 3.9 to 500 μM. Cell viability was determined based on the MTT assay. Each point represents the mean value of three experiments with three replicates per dose. The data are presented as mean +/- SD (*$p<0.05$: **$p<0.01$).

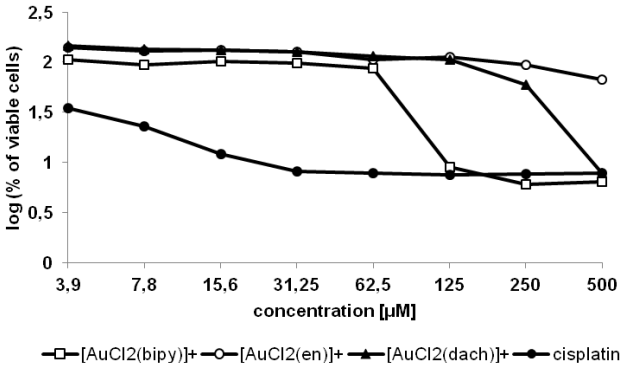

Figure 2. Semi-logarithmic plot of cytotoxic effects of $[Au(bipy)Cl_2]^+$, $[Au(dach)Cl_2]^+$ and $[Au(en)Cl_2]^+$ and cisplatin [PtCl2(NH3)2] complex on BCL-1 cells.
Each point represents a mean value of three experiments with three replicates per dose.

RESULTS

All three of the gold(III) complexes showed cytotoxic effects on BCL-1 cells (Figures 1 and 2).

At concentrations from 3.9 μM to 125 μM, all of the tested complexes showed similar and low cytotoxic effects. Furthermore, BCL-1 cells that were treated with $[Au(en)Cl_2]^+$ and $[Au(dach)Cl_2]^+$ complexes (at concentrations from 3.9 μM to 125 μM) managed to proliferate.

The cytotoxic effects of gold(III) complexes on BCL-1 cells differed at the concentration of 125 μM ($p<0.05$). The $[Au(bipy)Cl_2]^+$ complex showed high cytotoxicity, killing almost 100% of the cells, while the other two tested complexes had very low cytotoxic effects. Interestingly, BCL-1 cells proliferated after treatment with $[Au(en)Cl_2]^+$ and $[Au(dach)Cl_2]^+$ complexes (at a concentration of 125 μM).

The cytotoxicity of $[Au(en)Cl_2]^+$ and $[Au(dach)Cl_2]^+$ complexes to BCL-1 cells differed significantly at 250 μM ($p<0.05$). At this concentration, both complexes showed cytotoxic effects on BCL-1 cells. The percentage of viable BCL-1 cells was about 60% after treatment with $[Au(dach)Cl_2]^+$ and about 94% after treatment with $[Au(en)Cl_2]^+$, suggesting that among the tested gold(III) complexes, $[Au(en)Cl_2]^+$ was the least cytotoxic to BCL-1 cells. On the contrary, approximately 5% of BCL-1 cells were viable after treatment with $[Au(bipy)Cl_2]^+$, confirming the high cytotoxic potential of $[Au(bipy)Cl_2]^+$ on BCL-1 cells.

These results were confirmed at the concentration of 500 μM. $[Au(bipy)Cl_2]^+$ and $[Au(dach)Cl_2]^+$ complexes showed high cytotoxicity (almost 100% of BCL-1 cells were killed), while the percentage of viable BCL-1 cells after treatment with $[Au(en)Cl_2]^+$ was still high (approximately 67%).

It is interesting to note that the cisplatin complex showed high and dose-independent cytotoxicity on BCL-1 cells (Figures 2 and 3).

DISCUSSION

We, here, for the first time, report the cytotoxic effects of newly synthesised $[Au(bipy)Cl_2]^+$, $[Au(dach)Cl_2]^+$ and $[Au(en)Cl_2]^+$ gold(III) complexes on the BCL-1 murine B lineage leukaemia cell line. Our results showed that all of the tested gold(III) complexes displayed cytotoxic effects on BCL-1 cells (Figures 2 and 3). The concentration decrease was followed by a marked increase in BCL-1 cell viability (Figure 3).

At the concentration of 125 μM, which we suppose could be used *in vivo*, the $[Au(bipy)Cl_2]^+$ complex showed the greatest cytotoxic effects among the tested gold(III) complexes and similar cytotoxicity compared to the cisplatinum control. At 125 μM, only 24 hours after treatment with $[Au(bipy)Cl_2]^+$, almost all of the BCL-1 cells were dead (Figure 3).

Recently, described activation parameters for kinetic reactions important for the synthesis of the tested complexes [16] suggest that an associative substitution mechanism is responsible for the different cytotoxic effects of $[Au(bipy)Cl_2]^+$, $[Au(dach)Cl_2]^+$ and $[Au(en)Cl_2]^+$ gold(III) complexes. As previously described [14, 16-17], the first reaction step occurs via nucleophilic attack of the N7 donor atom of the purine base in 5'-GMP, resulting in the formation of a product by the departure of one chloride ion. The second step includes the substitution of another chloride ion from the starting complex, when 1:2 complexes are formed. Both the first and second steps of the substitution of the $[Au(bipy)Cl_2]^+$ complex are faster than those in the case of the $[Au(dach)Cl_2]^+$ and $[Au(en)Cl_2]^+$ complexes, suggesting higher efficacy of the $[Au(bipy)Cl_2]^+$ complex.

Although the cisplatin complex shows high cytotoxicity on BCL-1 cells, it was previously reported [4] that gold(III) complexes are better tolerated *in vivo* because of the different anticancer mechanisms utilised by gold complexes and cisplatin. The main anticancer mechanism of the cisplatin

complex is its interaction with DNA. The cisplatin complex forms an adduct that interferes with transcription and replication, which is followed by apoptosis of the cancer cell [18]. The interactions of cisplatin with DNA result in a Pt-GG intrastrand crosslink that is the critical lesion leading to cisplatin toxicity dominantly manifested by dysfunction of gastrointestinal and haematological systems [18-19]. Although the main intracellular target for gold(III) complexes and the precise mechanisms responsible for their anticancer effect are still unknown, some recently published data suggest that their mechanisms of action, such as modification of surface protein residues and inhibition of proteasome function [20], are substantially different from that of the cisplatin complexes.

In view of our results, we suggest that the $[Au(bipy)Cl_2]^+$ complex should be tested *in vivo* in experimental models of B cell leukaemia.

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THE EFFECTS OF AN ADAPTED BASKETBALL TRAINING PROGRAM ON THE PHYSICAL FITNESS OF ADOLESCENTS WITH MENTAL RETARDATION: A PILOT STUDY

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EFEKATI SPECIJALNO PRILAGOĐENOG PROGRAMA KOŠARKAŠKOG TRENINGA NA FIZIČKU PRIPREMLJENOST ADOLESCENATA SA MENTALNOM RETARDACIJOM: PILOT STUDIJA

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ABSTRACT

Introduction: *Previous studies have established a direct connection between levels of physical fitness and the time needed to perform daily tasks in adults with intellectual disabilities. These findings indicate that physical activity can improve the quality of life of individuals with intellectual disabilities. The aim of this pilot study was to evaluate the effects of an eight-week specially adapted basketball training program on the physical fitness of adolescents with mental retardation.*

Methods: *Twelve adolescents (6 males and 6 females, mean age 15.1±1.5 yrs) with mental retardation participated in the study. A specially adapted basketball training program was conducted four times per week over eight consecutive weeks. Each training session lasted approximately 30 minutes. Anthropometric measurements included height, weight, and percentper cent body fat. Exercise testing included monitoring of heart rate (HR at rest and HR at the end of the 6-MWT) and the six-minute walk test (6-MWT).*

Results: *The obtained results showed that the specially adapted training program improved the physical fitness of adolescents with mental retardation (6-MWT distance 473.7 m ±74.5 pre vs. 672.6 m ± 76.1 post, p<0.05; HR at the end of the 6-MWT 122.1 beats/min ± 16.5 pre vs. 116.8 beats/min ± 9.4 post, p<0.05); however, this type of training did not decrease body weight or percentper cent body fat in the adolescent participants.*

Conclusion: *Considering the small number of participants who were involved in the study, the obtained results provide only limited information on the sources and magnitude of the variation in response measures, but these results support the design of a full-scale experiment on this topic.*

Key words: *mental retardation, basketball, training, physical fitness.*

SAŽETAK

Uvod: *Prethodne studije su utvrdile direktnu povezanost između nivoa fizičke pripremljenosti i vreme potrebnog za izvršavanje svakodnevnih zadataka kod odraslih osoba sa mentalnom retardacijom. Ovi nalazi ukazuju da fizička aktivnost može da poboljša kvalitet života osoba sa mentalnom retardacijom. Cilj sprovedenog pilot istraživanja bio je procena efekata osmonedeljnog specijalno prilagođenog programa košarkaškog treninga na fizičku pripremljenost adolescenata sa mentalnom retardacijom.*

Metode: *Dvanaest adolescenata (6 muškog i 6 ženkog pola, prosečne starosti 15,1 ± 1,5 god) sa mentalnom retardacijom su učestvovali u studiji. Specijalno prilagođen program košarkog treninga sproveden je četiri puta nedeljno, tokom osam uzastopnih nedelja. Svaki trening je trajao oko 30 minuta. Antropometrijska merenja obuhvatila su određivanje telesne visine, telesne težine i procenta masnog tkiva, dok je testiranjem određivana frekvencija rada srca (FS u mirovanju i FS na kraju 6 MTH) i pređena razdaljina tokom šestominutnog testa hodanja (6 MTH).*

Rezultati: *Dobijeni rezultati su pokazali da je specijalno prilagođen program košarkog treninga doveo do poboljšanja fizičke pripremljenosti adolescenata sa mentalnom retardacijom (6 MTH razdaljina 473.7 m ±74.5 pre vs. 672.6 m ± 76.1 post, p<0.05; FS na kraju 6 MTH 122.1 otkucaja/min ± 16.5 pre vs. 116.8 otkucaja /min ± 9.4 post, p<0.05). Međutim, ova vrsta treninga nije dovela do smanjenja telesne težine ili procenta masnog tkiva ispitanika.*

Zaključak: *Obzirom na mali broj učesnika uključenih u studiju, dobijeni rezultati daju samo ograničene informacije o efektima sprovedenog programa, ali dozvoljavaju i usmeravaju planiranje celovitog eksperimentalnog postupka na ovu temu.*

Ključne reči: *mentalna retardacija, košarka, trening, fizička pripremljenost.*

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INTRODUCTION

Previous studies have indicated that individuals with intellectual disabilities score lower on standardised tests of physical fitness during all the phases of their life than do individuals with no out an intellectual disability (1,2). For this reason, individuals who are intellectually disabled are often unable to adequately perform everyday activities and are limited in their work-related duties (3).

One of the latest studies in this area established a direct connection between levels of physical fitness and the time needed to perform daily tasks in adults with intellectual disabilities (4). These findings indicate that physical activity can improve the quality of life of individuals with intellectual disabilities. Furthermore, some studies have shown that physical inactivity and obesity among individuals with intellectual disabilities cause serious problems for their general health. For this reason, it is recommended that experts begin to include this population in various programs and initiatives for the promotion of health, including greater participation in physical activities (5). Muscle endurance and aerobic capacity can be so greatly reduced in intellectually disabled individuals as to impede the daily functioning of these persons (6). It is well known that muscle strength and balance decrease in adulthood in individuals with intellectual disabilities; at this same time,simultaneously, other health risks, such as weight gain and obesity, also develop (7). These factors additionally have a negative effect on physical fitness and increase the risk of a fall if, e.g., the stability of the surface under the subject were to be disturbed (8). Although certain studies have shown that differences in the level of intellectual disability can influence the level of physical ability and that individuals with a higher IQ show greater progress in terms of motor skills over a longer period of time (9), today it is believed that a lower level of physical fitness among individuals with intellectual disabilities is the consequence of a sedentary lifestyle and the lack of opportunity for these individuals to participate in any form of planned physical activity.

Regular physical activity can not only improve muscle strength and aerobic endurance but also balance and self-perception among individuals with intellectual disabilities (10). The participation of children and adults with intellectual disabilities in recreational activities and sports often improves their social inclusion and the overall quality of their lives (11). Nevertheless, obstacles often hinder participation in physical activities among individuals with intellectual disability. These obstacles include certain functional limitations of individuals, the lack of suitable objects, terrain or specialised programs, as well as the high cost of organising such forms of physical activity (12).

During adolescence, daily physical activity is essential for proper growth and development, improving health and decreasing the risk of cardiovascular and metabolic disorders in adulthood. The existing guidelines recommend at least 60 minutes of moderate-to-intense physical activity for adolescents several days a week (13). It is thus necessary to establish the preconditions for the physical activity of adolescents with intellectual disabilities, allowing them opportunities equal to those given of their peers.

The objective of this pilot study was to evaluate the effects of eight weeks of a specially adapted basketball training program on physical fitness in adolescents with mental retardation. It was hypothesised that adapted basketball training would provide significant training gains in adolescents with mental retardation.

METHODS

The participants

Twelve adolescents with mental retardation (mean age: 15.1±1.5 yrs) participated in the study. All of the participants (n=12, 6 males and 6 females) were classified as having mild mental retardation and lived at home; none were institutionalised. The study was approved by the Institutional Board of Special Schools for Elementary and Secondary Education "14 October" in Nis. Written informed consent was obtained from all the participants and from their parents or legal guardians (if indicated) after a detailed description of the procedures was provided. The procedures presented were in accordance with ethical standards set for human experimentation.

All of the participants underwent a physical examination for athletic eligibility, which was performed by a specialist in sports medicine. None of the participants showed any evidence of recent injury in their anamnesis or clinical report.

Training procedures

A specially adapted basketball training program was conducted four times per week over eight consecutive weeks. Each training session lasted approximately 30 minutes. The first 5 minutes were spent in a dynamic warm-up to set the tone for the training session, and the last 5 minutes were spent doing stretching exercises to help relax the body. Over the eight-week period, the subjects had 32 training sessions in total. The duration of the training sessions during the first four weeks ranged from 25 to 30 min, extending to 30 to 35 min after fourth week. The main training activities in the first four weeks of the program included ball handling, reception and passing. From the fifth week until the end of the program, shooting and playing basketball were added.

Measurements and exercise testing procedures

Anthropometric measurements included height, weight, and percentper cent body fat. Heights were measured using an anthropometer (GPM, Switzerland) in accordance with standardised procedure (14). The results were accurate within 0.1 cm. Weights were measured using electronic scales (Tefal, France) with an accuracy within 0.1 kg. Body compositions were measured by bioelectrical impedance analysis using the BF 300 (Omron, Japan) according to the manufacturer's instructions. PercentThe percentages of body fat were read off the display with an accuracy of 0.1%. The resting heart rates and heart rates during the six-minute walk test (6-MWT) were determined continuously using an automated telemetric monitoring system (Polar, Finland).

To evaluate the general and integrated responses of the organ systems involved in physical activity, the six-minute walk test was used (15). The test does not offer any concrete information regarding the function of each of the various organs and systems involved in physical activity, as is possible with standardised laboratory load tests using suitable equipment for cardio-pulmonary studies. Nevertheless, as most of the daily activities take place below maximal intensity, the 6-MWT offers sufficient insight into the functional state of the bodies of the participants for daily physical activities (15,16). This test was used in previous research involving individuals with special needs, including those suffering from mental retardation (17).

The six-minute walk test, conducted at the participant's own pace on a flat surface 30 m in length, in a gym, was carried out according to a standardised protocol while adhering to the existing recommendations (15,16). The participants were assigned the task of crossing as great a distance as possible in a period of 6 min, walking (but not running) at a tempo that suited them. The participants received instructions and were continuously motivated during the test. In addition, the participants were allowed to stop at any point during the test but were encouraged to resume in the shortest possible time. The distance covered in 6 min was measured to the closest meter. To simplify measurement, alternating fluorescent green and orange markers were placed at 1-m intervals of along the edge of the surface on which the test was being carried out.

Statistical Analyses

The Kolmogorov-Smirnov test of normality was performed on all variables. All of the data were normally distributed, and a paired t-test was used to compare physical fitness and specific motor skills before and after the eight weeks of the specially adapted basketball training program. The data were described as the means ± standard deviation (SD). Statistical significance was set at $p<0.05$ for all statistical analyses.

RESULTS

The results of this pilot study are presented as the means and standard deviation in Tables 1 and 2. The eight-week specially adapted basketball training program did not result in any statistically significant changes in body weight and body fat percentage (Table 1).

Variables (unit)	Before		After		p value
	Mean	SD	Mean	SD	
Body height (cm)	155.5	14.8	155.9	15.1	ns
Body weight (kg)	55.6	16.6	55.1	15.8	ns
Body fat mass (%)	21.1	6.8	20.4	6.3	ns

Table 1. The anthropometric variables of the participants (n=12) before and after eight weeks of a specially adapted basketball training program.

Significant changes in heart rate at the end of the 6-MWT were recorded for the participants after eight weeks of a specially adapted training program (Table 2).

Variables (unit)	Before		After		p value
	Mean	SD	Mean	SD	
Resting heart rate (beats/min)	84.4	9.5	82.3	8.4	ns
Heart rate at the end of the 6-MWT (beats/min)	122.1	16.5	116.8	9.4	p<0.05

Table 2. The participants' heart rates at rest and at the end of the 6-MWT (n=12) before and after eight weeks of a specially adapted basketball training program.

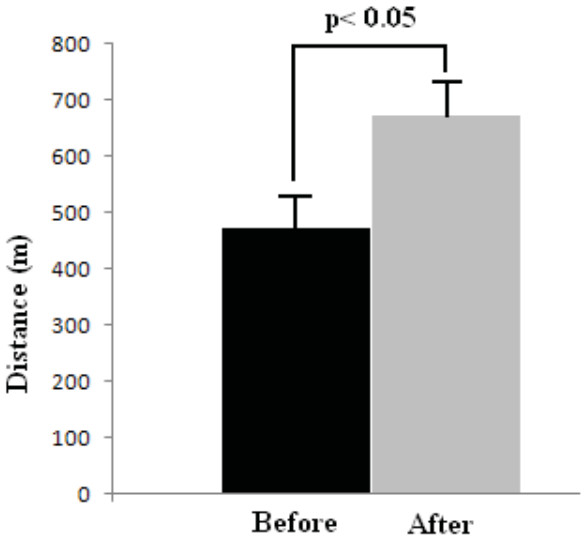

Figure 1. The significant differences in the distance covered during the six-minute walk test (6-MWT) before and after eight weeks of a specially adapted basketball training program.

DISCUSSION

Young individuals with intellectual disabilities (aged 20-30) usually have cardio-respiratory endurance 8-12% lower and maximum heat rates 15 beats/min lower than the expected values for non-disabled individuals of the same gender and age (18, 3). Approximately 20% of adults with intellectual disabilities were classified as obese, with a strong inverse relationship between intelligence and the percentage of body fat (19). A comparison of muscle strength at the elbow and knee joints of young individuals with intellectual disabilities with the values obtained from a non-disabled population of the same age and gender found that these values were 35-40% lower in disabled individuals (20); this finding is likely connected to the predominantly sedentary lifestyle of individuals with intellectual disabilities. One of the few studies examining flexibility indicated that there

are no significant differences between individuals with intellectual disabilities and a non-disabled population of the same gender and age (21). As a consequence, the lower level of physical preparation among individuals with intellectual disabilities is considered to be "related to the type of disability ", even though a sedentary lifestyle is the primary cause (22). For this reason, whatthe extent to which the level of physical fitness among individuals suffering from intellectual disabilities reflects their full potential is unclear.

In the case of physically healthy individuals, the function of the cardiovascular system represents a limiting factor for taking part in physical activities. The parameters of the function of the cardiovascular system, especially heart rate, are strongly correlated with oxygen uptake and energy consumption, especially below maximal work. The results of the pilot study indicate that eight weeks of a specially adapted basketball training program initiated a process of physiological adaptation, resulting in a statistically significant decrease in heart rate at the end of the 6-MWT (Table 2).

On the basis of the statistically significant differences in the covered distance during the 6-MWT (Figure 1), we conclude that the specially adapted basketball training program led to an improvement in function. Nevertheless, the level of physical activity and/or its duration were not sufficient to lead to statistically significant changes in body weight and percentage of body fat. Nevertheless, the results show a difference, and so we assume that an increase in program duration or work intensity could lead to a statistically significant decrease in body fat.

Sport can play an important role in the lives of individuals with intellectual disabilities, as it presents a good foundation for the development of physical abilities and improvement of the quality of life of individuals with mental retardation. Objective problems such as a lack of suitable equipment, fields, or specialised programs, as well as the high cost of organising such forms of physical activity, can be overcome through a variety of different adapted basketball training programs, under the guidance of a dedicated and qualified physical -education teacher with regular medical monitoring. The tests proposed in this pilot study could be useful for monitoring early improvements in adolescents with mental retardation.

Considering the small number of participants who were involved in the study, more research is needed to establish the effectiveness of adapted basketball training programs for adolescents with mental retardation before specific training recommendations can be made.

CONCLUSION

This pilot study demonstrated that eight weeks of a specially adapted basketball training program improved physical fitness in adolescents with mental retardation. Such physical activity and the nature of basketball, which necessitates interaction and decision-making in a variety of situations, can be a means of improving interaction and promoting connections among members of this population. Such programs also help to form a good foundation for the development of physical abilities and improve quality of life. The results of this pilot study provide only limited information on the sources and magnitude of the variation of the response measures, but these results support the design of a full-scale experiment on this topic.

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MEANINGFUL LIFE IS POSSIBLE WITH LOCKED - IN SYNDROME THE PERSONAL ACCOUNT OF A SURVIVOR

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NORMALAN ŽIVOT JE MOGUĆ SA "LOCKED-IN" SINDROMOM LIČNO SVEDOČANSTVO JEDNOG BOLESNIKA

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ABSTRACT

Locked-in syndrome (LIS) is a rare condition characterised by quadriplegia and anarthria and is usually caused by a bilateral ventral ischemic pontine lesion. Patients are normally fully conscious, but their only mode of communication is with vertical eye movements and/or blinking. Although the mortality rate is high, it has been shown that patients can survive for a significant period of time. Once an LIS patient becomes medically stable, given appropriate medical care, his or her life expectancy may be several decades. LIS patients may suffer appreciably if they are treated by hospital staff as nonresponsive. Medical professionals and lay people often assume that the quality of life of an LIS patient is so poor that it is not worth living. However, the reported overall quality of life of LIS patients is not significantly different from that of healthy subjects. In this case report, we describe a 60-year-old retired man living in a locked-in state due to a brainstem infarct. His personal account vividly reveals his inner thoughts, a great deal of suffering, and his ability to cope with his condition throughout seven years of illness. LIS patients' early referral to specialist rehabilitation services and strong social support from family greatly improves LIS patients'their quality of life. Even limited physical recovery can improve quality of life and enable LIS patients to become active members of society and return to living with family.

Key words: *locked-in syndrome, brain stem infarction, quality of life*

SAŽETAK:

"Locked-in" sindrom (LIS) ili sindrom "zarobljenosti u sopstvenom telu", je redak hronični poremećaj u kome bolesnici nisu u stanju da se pomeraju ili da govore, ali imaju potpuno očuvanu svest. LIS najčešće nastaje zbog opsežne lezije ventralnog dela ponsa, izazvane trombozom bazilarne arterije. Mortalitet je visok u prvim mesecima nakon doživljenog insulta ali bolesnici po stabilizaciji stanja mogu da prežive i nekoliko desetina godina, ako im je obezbeđena adekvatna medicinska nega. Osobe sa ovim sindromom mogu veoma da pate ukoliko medicinsko osoblje ne prepozna da se radi o nepokretnim bolesnicima koji su potpuno svesni. Zdravstveni radnici i laici često smatraju da je kvalitet života u LIS veoma loš, ali se on ne razlikuje značajno u odnosu na zdrave osobe. U ovom prikazu opisujemo bolesnika, starog 60 godina, koji živi u "locked-in" stanju poslednjih sedam godina nakon doživljenog infarkta moždanog stabla,. Njegovo lično svedočanstvo, otkriva na impresivan način, unutrašnja razmišljanja, veliko odricanje i patnju kroz koju prolaze ovi bolesnici, uz istovremenu rešenost da se bore sa svojom bolešću. Rano uključivanje bolesnika u rehabilitacioni tretman, kao i snažna podrška članova porodice veoma značajno utiču na kvalitet života osoba sa LIS jer čak i minimalno poboljšanje motornih funkcija značajno poboljšava kvalitet života i omogućava im da žive u sklopu svoje porodice i postanu korisni članovi društva.

Ključne reči: *locked-in sindrom, infarkt moždanog stabla, kvalitet života*

LIST OF ABBREVIATIONS:

ALIS - Association du Locked-in Syndrome
CT - Computed tomography
ICU - intensive care unit
LIS - locked-in syndrome

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INTRODUCTION

Locked-in syndrome (LIS) is a rare condition characterised by quadriplegia and anarthria and is usually caused by bilateral lesions of the ventral part of the brainstem. The patient is fully conscious and aware but unable to communicate intelligibly, except by using vertical eye movements or blinking. The most common aetiology of LIS is an atherosclerotic basilar artery disease leading to ischemic lesions that disrupt corticospinal and corticobulbar pathways passing through the basis pontis. The term "LIS" was coined in 1966 by Plum and Posner, emphasising the state in which the victim is fully alert but virtually "locked" inside an immobile body (1). Formal criteria for the diagnosis of LIS in adult patients with severe brain injury were proposed by the American Congress of Rehabilitation Medicine. The authors defined LIS as a condition in which all of the following are present: 1) eye opening is well sustained (bilateral ptosis should be ruled out); 2) basic cognitive abilities on clinical examination are preserved; 3) evidence of hypophonia or aphonia is present; 4) quadriparesis or quadriplegia is present; and 5) the primary mode of communication is through eye movements or blinking (2). Based on clinical findings, Bauer subdivided LIS patients into three groups: a) classical LIS (only conjugated vertical eye movements or blinking are retained), b) total LIS (complete loss of any voluntary movement including eye movement), and c) incomplete LIS (signs of classical LIS plus remnants of other voluntary muscle action). In addition, Bauer separated transitory from chronic LIS forms (3). In addition to vascular pathology (occlusion of the basilar artery or a pontine haemorrhage), there are sporadic reports in the medical literature of other causes of LIS. The most important nonvascular conditions that may resemble LIS are brain stem injury (traumatic LIS), primary or secondary malignant infiltration of the basis pontis (neoplastic LIS), and central pontine myelinolysis (metabolic LIS) (4, 5). In addition, there are case reports of inflammatory, infective, toxic, and various other causes of LIS (6, 7). A transitory locked-in state has occasionally been observed in subjects with an acute inflammatory polyradiculoneuropathy, with a postinfectious neuropathy, or under general anaesthesia when receiving an insufficient amount of a muscle relaxant drug (8-10). Finally, a state that is identical to LIS can be observed in the final stage of motor neuron disease (11). In this case report, we describe a patient with ischemic LIS and his personal account of the disease.

CASE OUTLINE

It was an ordinary cloudy autumn day in Belgrade, the economic and political capital of Serbia. A 53-year-old male accountant was going to his job, deeply immersed in thoughts about the day ahead. Upon arriving at the parking lot in front of his office, he suddenly felt debilitating pain in the neck and posterior part of the head.

Later, he vividly described his condition at that moment: "It was the worst pain I ever had. I thought that something was tearing me apart from the inside. It was like watching TV and the program suddenly went out completely. Suddenly, all I could see was black and white dots, as if there was no TV signal, only the lingering static in my head. I was fully conscious but fell down on the ground shaking all over from the sheer frustration and that terrible pain". Soon afterwards, people passing nearby called emergency services. Within 20 minutes of the onset of symptoms, he was transferred by an ambulance to the emergency department of a university hospital. One hour later, his vision cleared, and he found himself lying on a cold flat surface, completely weakened, with no ability to move his limbs or utter a word. From the ambient sounds and the medical jargon used by the people around him, he deduced that he was in a busy emergency department. His past medical history, obtained from relatives, was unremarkable except for untreated mild arterial hypertension. He also had a healed fracture of the jaw, stabilised with a metal implant, from a traffic accident that took place ten years prior. He lived with his wife and children. He did not smoke but drank alcohol occasionally. Computed tomography (CT) of the head performed without the administration of contrast medium was normal. Blood pressure was 220/120 mmHg, pulse was 140 beats per minute, and axillary temperature was 38.5°C. His respiration was shallow but regular, with 10 breaths per minute. The general physical examination was otherwise normal. On neurologic examination, the neck was supple and the pupils of equal size and reactive. He was lethargic, opening his eyes on request, but there were no other spontaneous or voluntary ocular movements. No facial movements were detectable, and he had total paralysis of the tongue and oropharyngeal musculature. Voluntary limb movements were not observed, but painful stimulation of either the arm or leg caused a decerebrate posture. Both plantar responses were extensor. A limited sensory examination revealed no abnormalities. After the initial evaluation, the trachea was intubated for airway protection, and a nasogastric tube was inserted. Thrombolytic therapy was not considered as a possible treatment because it was not a standard procedure at the time. Aspirin was administered through the nasogastric tube, low-molecular-weight heparin was given subcutaneously, and the patient was transferred to the neurology department for further diagnostic workup. Basic blood chemistry tests were normal, except for mild hyperlipidemia with total cholesterol of 6, 02; low-density lipoprotein cholesterol of 3,62; high-density lipoprotein cholesterol of 1,27; and triglycerides of 2,05 millimoles per litre. The white cell count was 14,300 per mm^3; the hematocrit 44,6%, and the platelet count 230,000 per cubic millimetre. Routine tests of blood coagulation were normal. Lumbar puncture revealed clear cerebrospinal fluid, with 40 erythrocytes per mm^3 and 1 leukocyte per mm^3. The protein level was 0,22 grams per litre, and the glucose level was 4,0 millimoles per litre. An electrocardiogram was normal. A chest radiograph re-

vealed clear lungs and a normal cardiomediastinal silhouette. Additional cerebral and neurovascular imaging (CT, transcranial Doppler) performed 3 and 7 days later showed bilateral ischemic lesions in the basis pontis and stenosis in the middle part of the basilar artery.

He recalled from his first days of hospitalizisation, "The first few weeks spent in the hospital were the most horrifying period in my life. In the intensive care unit, the busy medical staff was proceeding with their daily routine treating me as any other unresponsive patient with a severe brain injury. All I could detect from their indifferent faces and manners was the revelation of approaching doom. I wanted to scream out my helplessness and frustrations, but all was in vain. The inability to sleep and the agonising pain, my constant and faithful companions, made death seem a good alternative to me. The tiny thread that kept my sanity in check was the precious moment during visiting hour when I could hear comfort from my loved ones."

After three weeks, he was transferred, due to a shortage of beds, to another hospital for further treatment. At discharge, he was lying motionless on a stretcher, still intubated, looking straight ahead as if at an invisible dot in front of him. To a casual observer, only the voluntary use of vertical eye movements and blinking revealed that a fully conscious human being was present. He had fulfilled all clinical criteria for the diagnosis of classic LIS.

Thinking about the time spent at the second intensive care unit (ICU) brings him somewhat more congenial memories: "I was lying in a hospital bed, surrounded by monitors and machines, with their incessant beeping and buzzing as a constant reminder that I was once again in the ICU. However, with each passing day, I learned bit by bit to cope with my situation. I tried to find comfort in small things, like a smile or a few calming words from the attending nurse. The particular moment when, for the first time, one doctor looked into my eyes and spoke to me as a person engraved itself into my memory. Her soothing and gentle words were balm to my soul. After a several weeks I was extubated. What a relief, breathing ambient air again!" Two months after the brain stem stroke, with a diagnosis of LIS due to bilateral infarct of the ventral part of brain stem, he was moved to a rehabilitation clinic, where he spent an additional six weeks in intensive physical therapy.

He concluded his narration, " The bed sores caused me much discomfort and affected my ability to cooperate. The constant pain that I felt in my knees and heels from being in the same position for hours was almost unbearable. If people could only imagine how a small, seemingly trivial part of the regular nursing routine, such as the manoeuvre that keeps near joints from rubbing each other while moving the patient from their back onto their side, may be of such enormous importance for the completely paralysed person."

Three months after the stroke, he was discharged to go home with no visible functional improvement. He stated firmly that his family's support and caring was a turning point in his ability to cope with the disease. "The discharge to in-home care was the decisive moment in my illness. I was mentally and physically very weak, but with the unconditional love and support of my loved ones, every passing day provides a new ray of hope and gives me the reason to fight back even more. It was not an easy journey, but within months, my bed sores healed and the pain, my constant companion for months, was almost gone. Gradually, I managed to feebly move my head to the left side, and maintain a sitting position in a wheelchair with back support. I still couldn't speak, but after sixth months I managed to communicate by blinking and using tiny movements of the head and right hand. With the help of my daughter, I devised a code, using for each letter of the alphabet a previously determined combination of various signs. After a little bit of practice, she could easily, from two or three of my incompletely spelled words, make a meaningful sentence by guessing the missing parts. At last, I was able to accurately convey my inner thoughts to someone else." With this, he concluded his remarkable story.

In the first year of his illness, he suffered from occasional periods of insomnia that could last for up to four nights in a row. The resulting emotional lability and drooling made it difficult for him to create and retain social contacts, and his inability to voluntarily control breathing frustrated him. Fortunately, he was free of any serious medical complications, with the exception of recurrent crural deep vein thrombosis, which was successfully treated with low-molecular-weight heparins. Now, almost seven years after the stroke, he has regained some movement of the tongue and the neck but is still unable to produce meaningful speech or sit unsupported. He has gradually regained a degree of independence, allowing him to use an electric wheelchair for mobility. He uses a computer to access the Internet, keep up with social contacts through e-mail, and play games (Figures 1 and 2). Nonetheless, due to prolonged immobility and lack of no verbal communication, he has been able to remain in contact with only a few of his former friends and colleagues. In the warm months, he en-

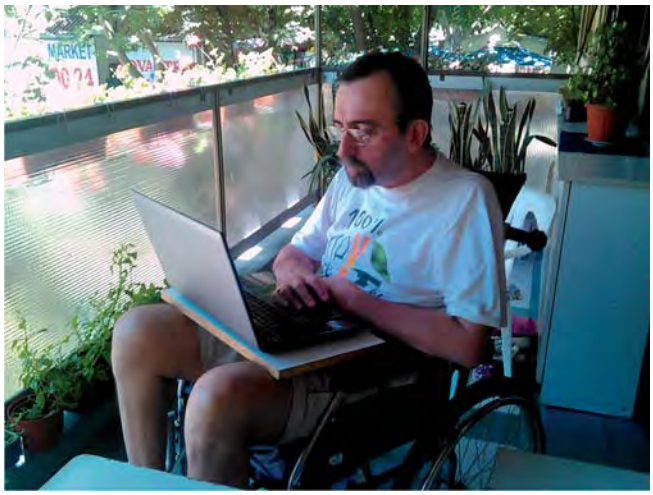

Figure 1. The patient works at a computer, which has become an indispensable part of his daily life.
Consent was obtained for publication of the story and figures.

Figure 2. The patient shops at the supermarket. The patient now finds time to enjoy and appreciate day-to-day activities.
Consent was obtained for publication of the story and figures.

joys spending time outdoors with his family and relatives. He concludes that his quality of life has improved primarily due to his willingness to cope with his disease, but it would all be in vain if it were not for the unconditional love and understanding of his family.

DISCUSSION

The outcome for patients with LIS is directly related to the disease aetiology and the patient's age at disease onset. A vascular aetiology is found in 86% of 320 subjects listed in the world's largest database of subjects with LIS ("Association du Locked-in Syndrome", ALIS), and brain stem infarct is reported as the most frequent cause (12). The overall prognosis and the degree of functional recovery are much worse for patients with vascular LIS than non-vascular LIS. Systemic and pulmonary infections are the leading causes of death in patients who survive the acute phase. Functional recovery from vascular LIS is very limited. However, there are case reports of substantial motor improvements after intensive and early rehabilitative treatment applied in the first months following the disease onset. Recently, it has been shown that the prognosis in chronic LIS (more than one year in a locked-in state and medically stabilised) is much more favourable than previously thought (13). The 20-year survival rate ranges from 31 to 40% (14). Insomnia and emotional lability (87% of patients) are well-known complications in patients with chronic LIS (15). LIS patients with lesions in the ventral pons and superior medulla are prone to problems with voluntary control of breathing because the respiratory brainstem centres are often damaged as well (16). Despite a widespread belief to the contrary, profound motor deficits and a limited capability for social interaction do not preclude these patients from having a rich and meaningful life. Almost half of the patients listed in the ALIS database return home. For those who are unfamiliar with chronic LIS patients, the reported scope of social participation and recreational activities is surprising. Some surveys show that more than 70% enjoy going out, and 80% of patients diagnosed with LIS meet with friends several times per month. Nearly half of them describe their mood as good, and up to 30% report active sexual relations. Furthermore, nearly 75% are able to participate in recreational activities, such as hobbies, sports, and games. It has been noted, as in our case report, that the factor that most helps LIS patients to cope with their disease is social support from family and close friends. On the other hand, caring for patients in a locked-in state places an enormous burden on the caregivers. Interestingly, in a group of patients with LIS caused by brainstem infarct (17 patients, mean LIS duration 6 ys.), the subjective quality of life was not related to physical limitations, nor could it be predicted by the degree of motor impairment. The public's negative view of LIS patients' quality of life may be explained by the idea that healthy people may have difficulty imagining the emotions and experiences of severely impaired patients (see the excellent discussion of psychosocial adjustment to LIS, from Lulé et colleagues , 2009). The authors conclude that the overall quality of life in a locked-in state is not significantly different from that of healthy subjects (17).

CONCLUSION

Patients in a locked-in state may return to live at home and begin a new, very different, but satisfactory life, if they have adequate medical treatment and physical rehabilitation. Significant and continuous social support from the family is of the outmost importance for LIS patients. We hope that this case report emphasises the fact that the majority of LIS patients have the potential to achieve a meaningful quality of life and become productive members of society.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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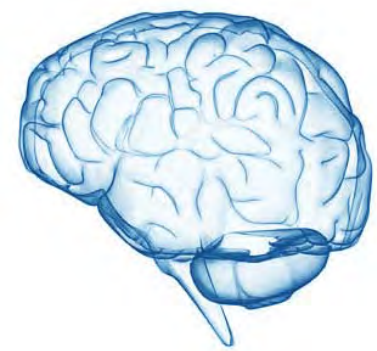

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