IMMUNOMEDULATORY EFFECTS OF SINGLE AND COMBINATORIAL ANTI-CANCER CHEMOTHERAPY IN A TUMOR MOUSE MODEL

ABSTRACT:
Anticancer chemotherapy is a successful treatment of tumor to kill or to low the multiplying rate of tumor cells. Although the immunomodulatory effect of single chemotherapy has been investigated, the effects of combinatorial chemotherapy are ill defined. Therefore the aim of this study was to evaluate effects of CTX and CIS alone or in combination as conventional chemotherapeutic drugs on the total and differential numbers of leukocytes. Groups of adult female Swiss albino (CD1) mice were treated with intraperitoneal (i.p.) injection of 0.5 x 10⁶ cells/mouse of Ehrlich’s ascites carcinoma (EAC) cells. After one day of injection, mice were i.p. treated either with PBS, (10 µg/mouse) CIS, (4 mg/mouse) CTX or in combination with different sequential treatment protocol, mice were bled on day11 and complete blood count (CBC) was assessed. Treatment with low dose of (10 µg/mouse) CIS or with high dose of (4 mg/mouse) CTX followed by (10 µg/mouse) CIS induced slight decreases in the total numbers of white blood cells (WBCs) and absolute numbers of neutrophils and lymphocytes. Treatment with high dose (4 mg/mouse) of CTX or with low dose of (10 µg/mouse) CIS followed by CTX (4mg/mouse) induced marked decreases in the total numbers of WBCs, relative and absolute numbers of neutrophils and lymphocytes. The data suggested that certain sequential treatment protocol of high dose of chemotherapy can induce lower lymphopenia, which have important implications in cancer treatment.

KEY WORDS:
Chemotherapy, Cyclophosphamide, Cisplatin, Ehrlich carcinoma, White blood cells, lymphocytes, neutrophils

INTRODUCTION:
Cancer represents the second cause of death nowadays all over the world after heart attack. Cancer can be treated with chemotherapy, radiotherapy, immunotherapy and other different investigational trials. Chemotherapy has a wide variety of applications in clinical studies for many cancer diseases from non-Hodgkin lymphoma, Lung, testicular cancers and other includes human malignancies (Bass and Mastrangelo, 1998; Zhou and Bartek, 2004). ACT is one of the successful methods for cancer treatment that depending on treated with immune cells, but it needed special conditions such as radiotherapy and chemotherapy (Salem et al., 2009a&b, 2010a-c & 2012).

Most studies used single anticancer drug, however patient in most cases are treated with combinatorial drug. Our resent studies have focused on identify the immunomodulatory effects of CTX (Salem et al., 2012).

Cyclophosphamide (CTX) is a nitrogen mustard alkylating agent, it is a widely used as a chemotherapeutic agent to treat various types of malignancies alone and in combination with other anticancer drugs as well as lymphoproliferative and autoimmune disorders (Bass and Mastrangelo, 1998; Brode and Cook, 2008).

CTX is reported to modulate the immune system in hosts (Bass and Mastrangelo, 1998;
Lake and Robinson, 2005) Examples for this include (i) elimination of T regulatory cells (Hoover et al., 1990; Andrade-Mena, 1994; Ikezawa et al., 2005; Lutsiak et al., 2005; Salem et al., 2010a&b) (ii) enhancement of dendritic cell based anti-tumor immunity by increased tumor antigens released from tumor cells dying of CTX induced apoptosis (Tong et al., 2001), (iii) increased type-I interferon production and evolution of CD44™ memory T cell response (Schiavoni et al., 2000; Matar et al., 2002; Salem et al., 2012), (iv) induction of homeostatic T cell proliferation by CTX-mediated lymphopenia that enhances some cancer vaccines (North RJ, 1982; Awwad and North, 1988), and (v) down regulation of T-cell derived IL-10 and TGF-β, productions (Matar et al., 2001). Cyclophosphamide (CTX) and Cisplatin (CIS) are widely used as anti cancer drugs alone and in combination in different tumor settings.

Cisplatin (CIS) is a prominent member of the effective broad-spectrum antitumor drugs. CIS is the most effective chemotherapeutic agent chemotherapeutic drug (Veronique et al., 2012) that used alone or in combination with other chemotherapeutic agents to treated many solid tumors and metastatic cancers, including ovarian, testicular, bladder, head and neck, lung, cervical, and breast cancers (Slattery and Warchol, 2010). The biochemical mechanisms of CIS involve inhibition of DNA synthesis, suppression of RNA transcription and binding of this drug to DNA and non-DNA targets and subsequent induction of cell death through apoptosis, necrosis, or cell cycle arrest (Hanigan and Devarajan, 2003; Rudin et al., 2003; Ozdogan et al., 2008; Zamay et al., 2011).

Since in most cases patients are treated with CIS or CTX or combination, it is important to study the immunomodulatory effect of these drugs individually and in combination on immune cells. Therefore, the aim of this study was to investigate the single or combined effects of these drugs on the total numbers of white blood cells (WBC), relative and absolute neutrophils and lymphocytes.

Certain sequential treatment protocol of high dose of chemotherapy can induced lower lymphopenia, which have important implications in cancer treatment.

MATERIAL AND METHODS:

Mice:

Adult female Swiss albino mice (CD1 strain) weighting 20 ± 2 g were used in this study were purchased from National Research Center (NRC, Cairo, Egypt). Animals were housed (5 animals per cage) at the animal facility at Zoology Department, Faculty of Science (Tanta University, Egypt) in clean and dry plastic cages, in 12h/12h dark/light cycle under laboratory condition of temperature and humidity. The mice were fed with rodent pellets and tap water ad libitum. This study was performed in accordance to guidelines of the use of experimental animals in research at Zoology Department, Faculty of Science, Tanta University, Egypt.

Tumor Cells:

Ehrlich ascites carcinoma (EAC) cell line was obtained from the National Cancer Institute (Cairo University, Egypt). The tumor line was established in female mice, and then the cells were harvested by sterile syringe and diluted with phosphate buffer saline (PBS).

Chemicals:

Cisplatin (CIS) or CDDP and Cyclophosphamide (CTX) were purchased from Sigma (Aldrich-Sigma Company, CA, USA) and reconstituted in PBS in stock solution and kept at -80°C until use. They were diluted in PBS to the required concentration before injection.

Tumor challenge and anticancer treatment:

EAC were inoculated into mice through intraperitoneal (i.p.) injection of (0.5 x 10^6 cells). Eight days after inoculation, mice were sacrificed and EAC cells were collected and washed twice with PBS. EAC cells were harvested and diluted with PBS and the viability of total number were counted using trypan blue exclusion assay.

Mice (n = 5) were i.p. inoculated with 0.5 x 10^6 cells EAC cells (day 0) and then treated for 4 days with 10µg/mouse CIS alone, followed with 4 doses of injection of 4 mg/mouse CTX. On day 11, mice were bled to harvest blood for complete blood count (CBC), as shown in figure 1.

**Fig. 1. A diagram which shows the experimental design of tumor inoculation and treatment with chemotherapy**
Preparation and counting of peripheral blood mononuclear cells:

Mice were anesthetized by inhalation of isoflurane (1-chloro-2, 2, 2-trifluoroethyl difluoromethyl ether; Hospira, Inc. Lake Forest, IL, USA) and bled from the orbital sinus using heparinized microhematocrit tubes into 1.5-ml Eppendorf tubes. Samples were analyzed for the total number of leukocytes using an automated instrument for complete blood counts (CBC) ( Vet-Scan HM2™ Hematology System, Abaxis, Union City, CA) to determine WBCs, platelets, relative and absolute number of neutrophils and lymphocytes.

Statistical analysis:

Data obtained from each experiment were analyzed using Microsoft Excel (Seattle, WA). The differences between the experimental groups were assessed using the Student’s t-test. P > 0.05 was considered to indicate statistical significance by using GraphPad Prism version 4.0 software (Graph Pad).

RESULTS:

Effect of CIS and CTX on the Total numbers of WBC:

As shown in figure 2, EAC-bearing mice showed increases in the numbers of WBC by 1.5-fold as compared with normal mice. The group that treated with 10 µg/mouse CIS from days 7-10 showed decreases in the numbers of WBC by 1.3-fold as compared with EAC mice. Treatment with 4 mg/mouse CTX from days 7-10 showed decreases in WBC by 7.9-fold as compared with EAC mice.

![Figure 2. Effect of single and combined chemotherapy of CIS and CTX on WBC. Swiss albino mice that treated with PBS, CTX 4mg/mouse or CIS 10 µg/mouse alone or in combination. On D 11, mice were bled and blood was collected for CBC analysis to determine the total number of WBC (n = 5). *: p < 0.001: versus normal control group; #: p < 0.001: versus EAC control group. The number of each column represent the percentage of EAC to naïve and the percentage of the other groups to EAC. Treatement with 4 mg/mouse CTX from days 1-4 followed by 10 µg/mouse CIS from days 7-10 showed decreases in the number of WBC by 1.6-fold as compared with EAC mice. Treatment with 10 µg/mouse CIS from days 1-4 followed by treatment with 4 mg/mouse CTX days 7-10 resulted in decreases in the number of WBC by 4.8-fold as compared with EAC mice.](http://www.egyseb.org)

Effect of CIS and CTX on the Total numbers of neutrophils:

Figure 3 showed the numbers of neutrophils in EAC-bearing mice with or without chemotherapy. EAC bearing mice showed decreases in the relative numbers of neutrophils by 1.3-fold and increases in their absolute numbers by 1.5-fold as compared with normal mice. Treatment with 10 µg/mouse CIS from days 7-10 induced increases in the relative and absolute numbers of neutrophils by 1.1 and 1.3-fold, respectively as compared with normal mice. However, treatment with 4mg/mouse CTX from days 7-10 induced decreases in the relative and absolute numbers neutrophils by 3.6 and 75-fold, respectively, as compared with untreated EAC mice.

![Figure 3. Effect of chemotherapy on the number of neutrophils, A: Absolute neutrophile and B: Relative neutrophile. Swiss albino mice (n = 5) inculcated (i.p) with 0.5 x 106 Ehrlich ascites carcinoma (EAC) on d0 and started treatment with PBS, CTX 4mg/mouse or CIS 10 µg/mouse alone or in combination on days 1-10. on D11 mice were bled for blood CBC. *: p < 0.001: versus normal control group; #: p < 0.001: versus EAC control group. Treatment with 4 mg/mouse CTX from days 1-4 then 10 µg/mouse CIS from days 7-10 induced increases in relative numbers of neutrophils by 1.1-fold but it induced decreases in absolute numbers of neutrophils by 3-fold as compared with EAC mice. Treatment with 10pg/mouse CIS from days 1-4 then 4 mg/mouse CTX induced decreases in the relative and absolute numbers of neutrophils by 2 and 15-fold, respectively, and absolute numbers of neutrophils by 1.7 and 15-fold, respectively, as compared with EAC mice.](http://www.egyseb.org)

Effect of CIS and CTX on the Total numbers of lymphocytes:

Figure 4 showed the effect of treatment with PBS, CIS or CTX on the numbers of lymphocytes in EAC-bearing mice. Untreated EAC-bearing mice showed increases in the
relative and absolute numbers of lymphocytes by 1.1 and 1.2-fold, respectively, as compared with normal mice. Treatment of EAC-bearing mice with 10 µg/mouse CIS from days 7-10 induced increases in the relative and absolute numbers of lymphocytes by 1.1 and 1.3-fold, respectively, as compared with EAC mice. In contrast, treatment of EAC-bearing mice with 4mg/mouse CTX from days 7-10 induced decreases in the relative and absolute numbers of lymphocytes by 1.8 and 2.1-fold, respectively, as compared with EAC mice.

**DISCUSSION:**

CIS and CTX at high dose are the most effective chemotherapeutic agent chemotherapeutic drug (Veronique et al., 2012) when they injected individually. Our recent studies have shown interesting antitumor effects of certain sequential treatment with CIS and CTX (Salem et al., 2014). So our study focused on using CTX and CIS alone or in combination with different doses and sequential treatments to study their immunomodulatory effects on the relative and absolute numbers of leucocytes in blood as an indicator of the immune response at the cellular level.

We have reported recently that treatment with high dose of CTX resulted in lymphopenia that induced decreases in the number of WBC, lymphocytes, neutrophils, Treg cells, dendritic cells (DCs) and myloid derived suppressor cells (MDSC) in peripheral blood (PBL) (D3-15) and in spleen and bone marrow at days 3-6 during the lymphopenic phase (Chakraborty et al., 2009; Salem et al., 2012). However, all the previous types of cells returned back to control level in recovery phase at days 6-15. this lymphopenic phase can play an important role in ACT since it acts as a niche with more avilable survival cytokines that together indeed a homeostatic proliferation, especially if the host is primed with specific antigen treatment. As aresult of the immature MDSC that followed by expansion of DCs in the peripheral blood, spleen, bone marrow, and liver in recovery phase, we have used this phase for a vaccination with a tumor antigen and an adjuvant to induced potent anti tumor T cell responses (Salem and David, 2010).

As a result of lymphopenic effect of treatment with high dose of CIS, it induced increases in the number of blood neutrophils with decreases in the number of bone marrow cells (Miller et al., 2010). Our data further showed that treatment with a low dose of CIS induced slight increases in the numbers of lymphocytes and neutrophils with slight decreases in the number WBC. Taken together, these results confirm that CIS at low dose can have a low leukenic effect.

We have reported recently that treatment with high dose of CTX resulted in lymphopenia that induced decreases in the total numbers of peripheral blood (PBL) (D3-15) and in spleen and bone marrow at days 3-6 during the lymphopenic phase during the lymphopenic phase (Chakraborty et al., 2009; Salem et al., 2012). These studies could explain our result that demonstrated decreases in the number of blood lymphocytes, neutrophils, WBC when we treated with CTX alone. Taken together with these result, we suggested that CTX in high dose is a dose leukopenic dependant.

As compared with CTX alone which induced more than 90% lymphopenic effect, treatment with CTX (4 mg/mouse) followed by CIS (10 µg/mouse) induced slight decreases in the number of WBC and decreases in absolute numbers of neutrophils and lymphocytes, with increases in the relative numbers of neutrophils and lymphocytes. These data suggested that combined treatment induced less lymphopenic effect.

When we reversed the treatment and started treatment with CIS (10 µg/mouse)
followed by CTX (4mg/mouse), it resulted in highly decreases in the number of WBC, lymphocytes, neutrophils with increases in relative lymphocytes. In contrast treatment with low dose of CIS followed by high dose of CTX induced high lymphopenic effect analysed on recovery phase (D11).

Conclusion:

This study further showed that treatment with low dose of CIS or treatment with high dose of CTX followed by low dose of CIS showed low lymphopenic effect is opposite to treatment with single high dose of CTX or low dose of CIS followed by high dose of CTX showed high lymphopenic effect.

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دراسة التأثيرات المناعية للعلاج الكيميائي الفردى والمشترك على نموذج للورم في فئران العامل

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بعد العلاج الكيميائي لمعرض السرطان من المكملات الناجحة لقتل أو تقليل معدل الزيادة في الخلايا السرطانية. على الرغم من أنه قد تم بالفعل دراسات التأثيرات المناعية عند استخدام علاج فريد، فإن تأثير الدمج بين العلاجات المختلفة لم يتم معالجة تأثيرها. ومن هنا كان هدف هذه الدراسة تعزيز التأثيرات المناعية عند استخدام علاج فريد. أظهرت النتائج أن استخدام علاجات متعددة في الخلايا السرطانية في الفئران أن طماً لتباطئ نمو الخلايا السرطانية بالفيروسات اللازم لخصوص الدراسة.

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