DOPAMINE ACCELERATES RECOVERY FROM CYCLOPHOSPHAMIDE–INDUCED LEUKOPENIA

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УСКОРЕНИЕ ВОССТАНОВЛЕНИЯ КРОВЕТВОРЕНИЯ ПРИ ЛЕЙКОПЕНИИ, ИНДУЦИРОВАННОЙ ЦИКЛОФОСФАМИДОМ, С ПОМОЩЬЮ ДОПАМИНА

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Effect of dopamine (DA) on cyclophosphamide-induced hematotoxicity was evaluated in this study. DA was injected at a dose of 50 mg/kg/day for five consecutive days into Swiss mice that received 200 mg/kg cyclophosphamide (CY) 12 h before. The severity of CY-induced neutropenia was much less in DA-treated group. DA treatment resulted in total WBC and neutrophil recovery at day 7 and day 4, respectively, which were 3 days earlier than the animals that received CY-alone. Similarly, the decline in nucleated cells in the bone marrow (BM) and spleen were relatively less profound in DA treated-group. DA-treatment was successful in reducing the suppressive effect of CY on myeloid and erythroid cells in the BM. In addition, cytochemical staining with Sudan black B revealed 3-fold increase in the number of granulocytes in spleen of DA-treated mice in comparison with CY-only group. The results suggest that DA treatment after CY appreciably reduced the severity of CY-induced blood and BM cell counts. Moreover, DA treatment was effective in accelerating the recovery of blood and BM cells resulting in attainment of normal hematological profile much earlier. Thus, the results indicate appreciable protective effect of DA against cancer chemotherapy-related myelosuppression and leukopenia.
Key Words: **cyclophosphamide, myelosuppression, recovery, dopamine, mice.**

Изучали влияние допамина (ДА) на гематотоксичность, обусловленную циклофосфамидом (ЦФ). ДА вводили мышам линии Swiss через 12 ч после ЦФ (200 мг/кг) в дозе 50 мг/кг в сутки на протяжении 5 сут. Было показано, что при введении ДА нейтропения носит менее выраженный характер. В группе мышей, получавших ДА, суммарное количество лейкоцитов и нейтрофильных гранулоцитов возвращается к норме на 7-е и 4-е сутки соответственно, что на 3 сут опережает восстановление этих показателей в группе мышей, не получавших ДА. В группе мышей, получавших ДА, менее выражено уменьшение количества ядросодержащих клеток в костном мозге (КМ) и селезенке. Применение ДА снижает выраженность миелосупрессии, вызванной ЦФ. Кроме того, при цитохимическом исследовании с применением судана черного В было установлено, что количество гранулоцитов в селезенке мышей, получавших ДА, увеличивается в 3 раза по сравнению с таковым в селезенке мышей, подвергшихся иммуносупрессии ЦФ и не получавших ДА. Результаты исследования свидетельствуют о том, что ДА способствует восстановлению кроветворения, угнетенного под действием ЦФ, ускоряя возвращение гематологических параметров к норме. Таким образом, ДА может найти применение как препарат, восстанавливающий кроветворение, при цитотоксической химиотерапии пациентов с онкологическими заболеваниями.
***Ключевые слова*: циклофосфамид, миелосупрессия, восстановление, допамин, мыши.**

Dopamine (DA) is a catecholamine neurotransmitter of the central nervous system (CNS). Besides neurotransmission, DA has other biological activities. Exogenous DA has important use in clinical medicine for elevating blood pressure, raising cardiac output and in the treatment of shock in acute renal and pulmonary failure [1, 2]. In recent times, investigations in laboratory animals revealed the involvement of DA in the regulation of tumor growth. An inverse relationship between the concentration of DA in CNS and tumor growth has been demonstrated in experimental animals [3]. Administration of exogenous DA, on the other hand, resulted in inhibition of tumor growth and concomitant stimulation of host blood cell production [4]. Hematopoiesis–stimulatory activity of DA was later confirmed in normal mice [5]. DA was shown to be effective in stimulating multilineage hematopoiesis at the level of pluripotent hematopoietic stem cells, CFU–S [5].

On the basis of this background information, we felt that it would be worthwhile to investigate whether DA could be effective in stimulating blood cell production in myelosuppressed individuals. In cancer research, investigation with this objective has obvious clinical relevance since a majority of anticancer drugs suppress hematopoiesis, especially the production of granulocytes [6], and chemotherapy–induced leukopenia could be life–threatening because of the possibility of opportunistic infection [7]. Accordingly, in this study in mice we have evaluated the effect of DA treatment on recovery from hematotoxic effects of cyclophosphamide (CY), a cancer chemotherapeutic agent with profound adverse effects

Received: October 12, 1999.
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Abbreviations used: AchE — acetylcholinesterase; BM — bone marrow; CY — cyclophosphamide; DA — dopamine.

on blood and the bone marrow [8]. Our results show that DA administration after CY significantly accelerates the recovery from CY–induced leukopenia.

MATERIALS AND METHODS

Mice. Normal Swiss mice of both sexes, 6–8 weeks of age and weighing 20–22 g were used. The animals were kept in alternate light and darkness cycles of 12 h each and they had free access to food (standard mouse pellet) and water.

Treatment. Cyclophosphamide (Endoxan–Asta, German Remedies Ltd, Mumbai, India) was dissolved in sterile water as directed by the manufacturer and was injected in a volume of 0,25 ml intravenously (i.v.) via a tail vein at a dose of 150 mg per kg body weight on day 0. Dopamine (DA, Dopamine hydrochloride, TTK Pharma Ltd. Chennai, India) was diluted in sterile 0.9% saline immediately before use and injected i.p. at a dose of 50 mg/kg body wt/day in a volume of 0.5 ml for five consecutive days beginning 12 h after CY injection (day 0–4). The animals which received only vehicles served as controls.

Hematology. Routine hematological parameters like hemoglobin, RBC, WBC and platelet count were done by standard procedure [9]. WBC differential count was done from Leishman–stained blood smears. Bone marrow (BM) cells were collected from femur bones into Dulbecco's modified Eagles medium (DMEM, Gibco–BRL, USA) following established procedures. Total nucleated cell count was done using hemocytometer after lysing non–nucleated erythrocytes by 3% glacial acetic acid. Single cell preparation of spleen were made in DMEM by gently teasing tile organ in steel wire mesh. Total count was done similarly as in case of BM [10]. Smears of BM and spleen cells were made on clean glass slides and stained with lineage–specific cytochemical stains for differential counts.

BM and spleen cytochemistry. Sudan black B staining was done for neutrophils and their progenitors [11] following the method of Smith and Bruton [12] using Sudan black B (Sigma Chem, USA) as substrate. For erythroid cells, methanol–fixed cell smears were stained with 1% solution of benzidine (Sigma Chem, USA) for 1 minute followed by 1 minute incubation in a mixture of 70% ethanol and 30% H_2O_2 (3 : 1, v/v) at room temperature [13]. Thereafter the cells were washed in distilled water, counter–stained with hematoxylin, dehydrated in alcohol, mounted in DPX and observed under oil immersion. Hemoglobin–containing erythroid cells stain golden brown [13]. The percentage of Sudan black B and benzidine–positive cells was calculated after counting at least 1000 cells per mouse. Megakaryocytes (MKs) and their progenitors were identified by the presence of marker enzyme acetylcholinesterase (AChE). Air–dried slides were stained for AChE for 3 h in the dark using acetylthiocholine iodide (Sigma Chem, USA) as the substrate following the procedure of Karnovsky and Roots [14]. AChE–positive cells stain golden brown.

Statistical analysis. The results were statistically analyzed for Students *t*–test and $P < 0.05$ was considered as significant.

RESULTS

Total and differential leukocyte count. CY injection in normal mice resulted in moderate leukopenia. WBC count declined from $11.8 \cdot 10^3/\mu l$ to $5.5 \cdot 10^3/\mu l$ and $4.0 \cdot 10^3/\mu l$ by day 2 and day 4 after treatment respectively (Table 1). Recovery from leukopenia started from day 6 and was completed by day 9. DA injection after CY elicited appreciable protective effect against CY–induced leukopenia. For instance, the circulating WBC count in CY plus DA treated group on day 2 and 4 after CY injection were 34.5% and 47.5% higher than that of CY–alone group, and the differences were statistically significant ($P < 0.05$). DA treatment also caused early and accelerated recovery from leukopenia and immature neutrophils like metamyelocytes and band cells were found in increased frequencies. WBC recovery started from day 5 as against day 6 in CY–only group, and it was complete by day 7 compared to day 9 in CY–only group. In fact, a rebound leukocytosis (2.5–fold over the normal WBC count) was observed in DA–injected animals a week after CY administration (Table 1).

CY treatment caused neutropenia reaching a nadir at day 4 when the absolute neutrophil count was 78% below the normal level. The corresponding neutrophil count in CY plus DA treated animals was three times higher ($2.36 \cdot 10^3/\mu l$ against $0.84 \cdot 10^3/\mu l$ in CY–alone group) at this time point. Recovery from neutropenia was complete within a week in both the groups, but a remarkable rebound neutrophilia of a magnitude of 5.7–fold over control was observed in DA treated animals. In contrast to neutrophil count, total number of circulating lymphocytes did not recover completely from the suppresive effect of CY even after 10 days. But administration of DA following CY injection succeeded in complete lymphocyte recovery during this period (Table 1).

Table 1. Effect of DA on recovery from CY–induced leukopenia

	Control	Days after CY				
		1	2	4	7	10
Total WBC ($\cdot 10^3/\mu l$)	11.8 ± 0.9					
CY		6.5 ± 0.4	5.5 ± 0.5	4.0 ± 0.3	10.3 ± 2.1	19.3 ± 2.6
CY + DA		6.3 ± 0.2	7.4 ± 0.4*	6.8 ± 0.8*	29.1 ± 3.4*	29.5 ± 3.6*
Neutrophils ($\cdot 10^3/\mu l$)	3.8 ± 0.2					
CY		2.5 ± 0.2	1.8 ± 0.2	0.8 ± 0.1	5.0 ± 1.0	6.8 ± 1.7
CY + DA		3.6 ± 0.2*	2.8 ± 0.3*	3.8 ± 0.1*	21.6 ± 0.9*	20.5 ± 1.6*
Lymphocytes ($\cdot 10^3/\mu l$)	7.8 ± 0.2					
CY		3.8 ± 0.2	3.5 ± 0.2	3.0 ± 0.1	5.0 ± 1.0	12.0 ± 1.8
CY + DA		2.7 ± 0.2*	4.5 ± 0.3	2.9 ± 0.1	6.9 ± 0.9	8.8 ± 1.5

Results are mean ± S.E.
*$P < 0.05$ in comparison with CY–only group.
8–10 animals were used in each time point.

Erythrocyte and platelet count. An early and progressive decrease in circulating erythrocyte count was observed following CY injection, and RBC count did not return to normal level within the observational period (Table 2). In contrast, no significant decline in RBC count was recorded in DA treated mice during the first week after CY injection. A mild fall was observed afterwards, but the magnitude was much less than in the CY–only group. For example, the reduction in RBC count at day 10 was 23% in CY plus DA group while it was 43% in CY only group. Peripheral blood smears of both the groups showed normochromic red cells with anisopoi–

kilocytosis. Fragmented red cells were abundant in animals injected with CY alone, but fewer in number in mice that received DA later. The results therefore point to the possibility of CY–induced red cell damage and a marginal protective effect of DA in this regard. The number of circulating platelets was relatively unaffected by CY injection. But a significant elevation in platelet count was observed in CY plus DA injected animals (Table 2).

Table 2. Hematological changes in mice injected with CY followed by DA

	Control	1	2	4	7	10
RBC (· 10^6/μl)	7.9 ± 0.5					
CY		8.0 ± 0.6	6.4 ± 0.5	5.8 ± 0.4	5.9 + 0.2	4.5 ± 0.4
CY + DA		7.6 ± 0.5	7.9 ± 0.5	7.6 ± 0.3*	6.1 ± 0.2	6.1 ± 0.5*
Hemoglobin (g/dl)	15.3 ± 0.3					
CY		14.9 ± 0.3	14.8 ± 0.2	14.5 ± 0.3	14.8 ± 0.2	14.2 ± 0.6
CY + DA		14.8 ± 0.4	14.8 ± 0.5	14.8 ± 0.3	14.0 ± 0.7	13.9 ± 0.7
Platelets (· 10^5/μl)	5.7 ± 0.5					
CY		4.1 ± 0.2	7.5 ± 0.6	4.2 ± 0.5	5.6 ± 0.8	6.8 ± 0.5
CY + DA		7.6 ± 0.8*	6.6 ± 0.7	10.8 ± 1.2*	13.8 ± 1.2*	12.6 ± 0.7*

Results are mean ± S.E.
8–10 animals were used in each group.
*$P < 0.05$ in comparison with to CY–only group.

Bone marrow and splenic cellularity. CY administration resulted in a sharp decline in nucleated cell count of femoral marrow (Table 3). A nadir was recorded within 48 h when the cell count was as low as 3.8 · 10^6 nucleated cells per femur compared to 19.1 · 10^6 cells per femur in matched controls. Lowest BM cell count was observed on day 2 in CY plus DA group also, but the cellularity was 42% higher than the CY only group. Recovery from BM suppression was achieved within a week but yet another fall in BM cell count was observer there after.

Table 3. Effect of dopamine treatment on recovery from CY–induced myelosuppression

	Control	1	2	4	7	10
TNC/femur · 10^6/μl	19.1 ± 0.8					
CY		6.1 ± 0.4	3.8 ± 0.8	9.8 ± 1.2	19.5 ± 2.0	8.6 ± 1.3
CY + DA		5.7 ± 0.2	5.4 ± 1.2	16.9 ± 1.2*	19.4 ± 1.8	12.0 ± 1.1
TNC/spleen · 10^7/μl	11.2 ± 0.7					
CY		8.1 ± 1.3	4.0 ± 0.6	3.8 ± 0.9	12.5 ± 1.4	10.9 ± 1.3
CY + DA		16.9 ± 1.5*	6.2 ± 1.1	8.2 ± 1.6*	18.7 ± 0.9*	14.0 ± 1.6
Spleen weight (mg)	106 ± 8					
CY		42 ± 7	34 ± 6	68 ± 8	108 ± 11	142 ± 10
CY + DA		56 ± 8	72 ± 8*	99 ± 10*	170 ± 14*	204 ± 12*

Results are mean ± S.E.
DA treatment completed on day 3.
TNC — total nucleated cell count.
*$P < 0.05$ in comparison with CY alone group.
5–8 mice were used at each time point.

Like the BM, marked reduction was also observed in splenic cell count and organ weight following CY injection. Splenic hypocellularity was maximum at 48 h after treatment and recovery was nearly complete within a week. DA injection elicited a distinct rise in nucleated cell count and spleen weight in CY–induced myelosuppressed animals.

Cytochemical studies. In order to evaluate the number of hematopoietic cells of different lineages, a panel of lineage specific cytochemical stainings was employed. Sudan black B staining for granulocytes particularly the neutrophils and their progenitors showed a greater percentage of granulocytic cells in the BM of CY plus DA group compared to the CY–only group (Table 4). The number of cells of the neutrophil series returned to normal level within a week in this (CY plus DA) group, while it was still far bellow the normal level in animals that received only CY. Benzidine positive erythroid cells were drastically reduced following CY injection. DA showed a mild protective effect on these cells.

Table 4. Lineage–specific staining of bone marrow and spleen cells

Staining	Control	Days after CY injection	
		2	7
Sudan black, BM	43.7 ± 2.2		
CY		10.6 ± 1.6	40.6 ± 3.7
CY + DA		18.5 ± 1.8*	53.9 ± 2.6*
Sudan black, Spleen	4.1 ± 0.3		
CY		1.8 ± 0.5	2.6 ± 0.3
CY + DA		5.3 ± 0.5*	7.4 ± 1.2*
Benzidine, BM	24.5 ± 1.6		
CY		8.1 ± 1.2	8.2 ± 1.2
CY + DA		11.6 ± 1.3	10.5 ± 1.5
Benzidine, Spleen	3.6 ± 0.4		
CY		3.5 ± 0.2	2.0 ± 0.2
CY + DA		3.2 ± 0.3	3.1 ± 0.3*
AChE, BM	0.1 ± 0.02		
CY		0.1 ± 0.02	0.2 ± 0.01
CY + DA		0.1 ± 0.03	0.2 ± 0.01
AChE, Spleen	0.05 ± 0.01		
CY		0.03 ± 0.01	0.06 ± 0.02
CY + DA		0.10 ± 0.02*	0.20 ± 0.02*

Results are expressed as % of positive cells ± S.E.
*$P < 0.05$ compared to CY–only group.
8–10 animals were used in each group.

The megakaryocytes (MK) in the BM and spleen were relatively unaffected by CY treatment. But DA treatment was associated with threefold increase in the number of mature megakaryocytes and their immature forms (assessed as small AChE positive cells) in the spleen, suggesting stimulatory effect of DA on splenic megakaryocytopoiesis.

DISCUSSION

Chemotherapy–induced neutropenia often prevents dose escalation and optimal tumor cell killing [6]. Hematopoietic growth factors (HGFs) like granulocyte–, and granulocyte–macrophage colony stimulating factors (G–CSF and GM–CSF) are now being used in clinical practice to circumvent the hematological toxicity of cancer chemotherapy [15]. But the growing concerns about the use of HGFs are manifold. For instance, G–CSF and GM–CSF promote granulocytopoiesis at the expense of other cell lineages which may result in thrombocytopenia and hemorrhage [16, 17]. Even more important criticism against the use of HGFs is the fact that they promote the growth of tumor cells as well since several human cancers express receptor for HGFs [18, 19]. Further, it has recently been demonstrated that G–CSF promotes metastasis of human head–and–neck cancer [20]. Moreover, HGFs are expensive particularly for the developing countries [21]. Considering these, a search is going on for suitable alternative compound(s) for the protection against chemotherapy–induced myelosuppression. Our results showing accelerated recovery from CY–induced leukopenia in mice by DA underscores the usefulness of DA in this regard.

The mechanism of DA action in increasing the number of leukocytes and erythrocytes is not clearly under–

stood. We have previously demonstrated that DA increased the number of pluripotent hematopoietic stem cells (CFU-s) in mice [5]. Differentiation of CFUs towards granulocytopoiesis was observed to be stimulated [5] and erythropoiesis was found to be enhanced quantitatively in DA-treated mice [4]. It is possible therefore that DA administration after CY has increased the number of CFUs and stimulated their differentiation particularly towards granulocyte lineage. Since CFUs are generally non-proliferating and quiescent [21], they escape the cytotoxic effects of CY. It is also possible that DA mediates the premature release of granulocytes from the bone marrow to the circulation as we have reported earlier in tumor bearing mice [4]. The appearance of metamyelocytes and band cells in increased numbers in the circulation of DA treated animals of this study supports this argument. This may also explain, in part, the reduction in nucleated cell count in BM of DA treated animals as observed in this study. It is also apparent from the results that the spleen plays an important role in DA-mediated recovery from leukopenia. We included spleen in this study because of the fact that murine spleen actively participates in hematopoiesis in adult life and may accelerate it when the situation demands [22]. In any case, DA has successfully enhanced the number of granulocytes in the circulation resulting in complete leukocyte and neutrophil recovery within a relatively shortened period compared to CY-injected animals that did not receive DA afterwards.

The advantage of using DA in the recovery from myelosuppression is multiple. The rationale for selecting the present dose schedule of DA is that it causes significant inhibition of experimental tumors in mice [4]. Therefore, administration of DA in this concentration along with established anti-cancer drugs is expected to augment their efficacy concomitant with the protection against hematotoxicity. Hence, the hematoprotective effect of DA in present dose schedule has obvious clinical relevance. This is further supported by the fact that hematological toxicity in mice accurately predicts the effects in man [23]. The other advantage of using DA is the cost which is negligible compared to the HGFs. In addition, besides granulocyte and neutrophil recovery, DA was able to enhance appreciably the production of RBCs and platelets. It is concluded from the present results that DA stimulates multilineage hematopoiesis in CY-induced myelosuppressed animals resulting in accelerated recovery from drug-induced leukopenia.

ACKNOWLEDGEMENT

The authors are thankful to Indian Council of Medical Research, New Delhi for supporting this project through a grant-in-aid.

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