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Hematological Effects of the New Immunosuppressive Drug 15-Deoxyspergualin

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Abstract. Since systematic hematological studies on blood and bone marrow changes after treatment with 15-Deoxyspergualin (DOS) are lacking, a quantitative assessment was performed fourteen or twenty eight days after intraperitoneal application of DOS to rats. Further observations done 7 and 14 days after discontinuation of DOS administration allowed analysis of bone marrow regeneration. DOS induced lymphocytopenia, granulocytopenia and anemia with a decrease of bone marrow cellularity due to suppression of cell maturation. The effect was dose-dependent and bone marrow as well as blood changes were observed in animals treated with doses from 0.5 to 10.0 mg/kg DOS. Within 14 days after termination of the treatment, rapid recovery with normalization of all hematological parameters was observed. In the light of our data, these hematological side effects may not be a major disadvantage, if DOS is used in doses below 2.5 mg/kg, and for a course of therapy which is limited to 7 to 14 days.

Key words: 15-Deoxyspergualin; hematology; immunosuppression; bone marrow; regeneration; experimental therapy.

Introduction

Deoxyspergualin (DOS), a substance composed of a guanidinic and a spergmidine moiety, was originally described as an antitumor agent[6,13]. Besides its documented activity against murine leukemias, it was shown to possess immunosuppressive properties[3]. DOS is presently being investigated as a new immunosuppressive drug in several transplantation models. It was demonstrated that DOS effectively prolongs the survival of transplanted kidneys[8,15], livers[4], hearts[12] and other organs[7,11] in doses ranging between 1.8 and 2.5 mg/kg given from day 0 to day 9 or 14 post-transplantation. DOS was also effectively used as a rescue drug for acute rejection of the kidney, in a dose of 2.4 mg/kg which could be reduced to 0.6 mg/kg within 7 days[5].

It has also been demonstrated that DOS given intraperitoneally in a dose of 2.5 mg/kg for 14 days induces specific immunological tolerance to the rat kidney allograft, as shown by indefinitely accepted donor type and normally rejected third party skin grafts[14]. Frozen tissue sections of surviving kidneys (treatment beginning on day +1, +2, or +3) show a remarkable downregulation of MHC class I (MRC-OX18) antigens, but not class II (MRC-OX6) antigens on various kidney cells. When treatment begins as late as day +5, DOS fails to inhibit upregulation of class I antigens as shown in rejected kidneys. DOS appears to reduce the expression of graft MHC class I antigens, i.e., of major target antigens, when given within the first days after transplantation. It also appears to interfere with

antigen presenting cells during the induction phase of allograft responses. Il-1 production by splenic and peritoneal exudate macrophages, treated *in vitro* with DOS, is reduced significantly at lower DOS dosages (0.005–0.5 mg DOS/ml culture medium)[14].

However, some observations showed that DOS may also exhibit toxic effects. In experiments on dogs treated with DOS, a dose of 4.0 mg/kg for 10 days caused severe gastrointestinal disturbances and anorexia[2,4,11]. Leukopenia, anemia and reduction of mean spleen weight were observed in mice receiving 5.0 mg/kg of DOS for 23 days or longer. Histologic examinations of spleen showed an atrophy of white pulp and a decrease of cells in red pulp[7]. It was also found that DOS suppressed proliferation ad differentiation of bone marrow cells when given in a dose of 3.0 mg/kg for 4 days[10].

No systematic hematological studies on peripheral blood and bone marrow changes during treatment with various doses of DOS have been published so far. Since future clinical application of this drug requires knowledge of its side effects, we performed a quantitative assessement of blood and bone marrow changes after a 14 or 28 day course of DOS applied intraperitoneally. Subsequent observation lasting 7 and 14 days after the end of DOS treatment allowed follow-up of bone marrow regeneration.

Materials and Methods

Immunosuppressive agent. 15-Deoxyspergualin produced by Nippon Kayaku Co. (Tokyo) was obtained from Behringwerke AG (Marburg). It was dissolved in sterile phosphate buffered saline (pH 7.2)

Animals. Thirty nine male LEW (RT1l) rats, 3 months old and weighing 250–300 g, bred in our Department, were used for the study.

Rats were divided into the following experimental groups:

Group I: Intraperitoneal injections of DOS in doses of 0.5, 2.0, 2.5, 5.0 and 10.0 mg/kg for 14 consecutive days (n = 5 per experiment). Group II: Intraperitoneal injections of DOS in doses of 2.5, 5.0 and 10.0 mg/kg for 14 consecutive days with a subsequent interval of 7 days before hematological examination (n = 3 per experiment). Group III: Intraperitoneal injections of DOS in doses of 2.5, 5.0 and 10.0 mg/kg for 14 consecutive days with a subsequent interval of 14 days before hematological examination (n = 3 per experiment). Group IV: Intraperitoneal injections of DOS for 28 consecutive days in a dose 2.5 mg/kg (n = 3 per experiment). Group V: Intraperitoneal injections of DOS for 28 consecutive days in a dose of 2.5 mg/kg with a subsequent interval of 7 days before hematological examination (n = 3 per experiment). Group VI: Intraperitoneal injections of DOS for 28 consecutive days in a dose of 2.5 mg/kg with a subsequent interval of 14 days before hematological examination (n = 3 per experiment). Control groups: Rats were injected only with PBS (n = 5 per experiment).

Hematological examinations. Blood and bone marrow were used for hematological investigation. Hemoglobin, erythrocyte count, hematocrit, cell count, platelet count, blood and bone marrow smears were analysed. For estimation of bone marrow changes, 500 cells were counted. Cells of granulocyte maturation line identified as myeloblasts, promyelocytes and myelocytes were summed up and interpreted as bone marrow proliferative pool of granulocytes (BM-PPG). Other cells of the granulocyte line: metamyelocytes, bands, and morphologically mature polymorphonuclear granulocytes were summerically presented as bone marrow non-proliferative pool of granulocytes (BM-NPPG). All analyses were performed according to standard laboratory procedures.

Statistical analyses were done according to the Student's t – test for "small samples".

Results

Leukocytes

Absolute counts of leukocytes in peripheral blood were significantly lower after 14 days of DOS treatment than in the control group: 9806 ± 584 ($p < 0.02$) following 0.5 mg/kg and 3916 ± 2155 ($p < 0.01$) following 10.0 mg/kg DOS versus 15075 ± 1694 in the control group. Seven days later, a regeneration phase was observed in that leukocyte counts increased to 35733 ± 24135 following 2.5 mg/kg; 52516 ± 33494 following 5.0 mg/kg and 31833 ± 16064 following 10.0 mg/kg (group II). Two weeks after discontinuation of DOS treatment the leukocyte values were normalized (group III). Similar findings were observed when a dose of 2.5 mg/kg DOS was given for 28 days (group IV): the leukocyte count decreased from 11166 ± 280 to 6733 ± 1270 ($p < 0.01$), and was normalized two weeks after termination of treatment (group VI).

Lymphocytes

The value of lymphocyte count in the control group was 10856±1099. The mean absolute count of lymphocytes in rats treated with 0.5 mg/kg of DOS was 7452±137 ($p < 0.05$). In these rats the lymphocyte count exceeded the baseline level seven days after the last DOS injection and two weeks after cessation of treatment the count was normalized. In rats treated with higher DOS doses also a statistically significant reduction of lymphocyte counts was observed (group I). Again, seven days after the last DOS injection the lymphocyte counts exceeded that of controls (group II). The highest values were observed in animals treated with 2.5 mg/kg DOS. Fourteen days after termination of DOS treatment the count

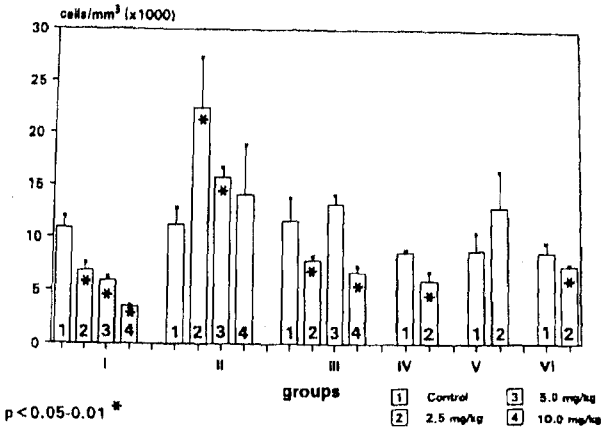

Fig. 1. Absolute values of lymphocytes in peripheral blood of rats treated with various doses of DOS: 0, 2.5, 5.0 or 10.0 mg/kg. After 14 days of DOS-treatment a significant reduction in lymphocyte counts was observed (group I). A similar result was observed after 28 days of DOS application (group IV). In the regeneration phase 7 days after the last DOS injection the lymphocyte count exceeded that of the controls (groups II and V). The highest counts were obtained in animals treated with 2.5 mg/kg. Two weeks after cessation of treatment, the count normalized (groups III and VI)

was normalized only in rats injected with 5.0 mg/kg DOS (group III). In other rats the values were significantly lower than in the control group. The results are presented in Figure 1. DOS given in a dose of 2.5 mg/kg for 28 days (group IV) induced also a statistically significant reduction of lymphocytes in peripheral blood (Fig. 1).

Granulocytes

In rats treated 14 days with a dose of 0.5 mg/kg of DOS the mean count of granulocytes in peripheral blood decreased from 3167±372 to 1612±386 ($p < 0$). Rats treated with higher doses of DOS had lower counts of granulocytes in peripheral blood (group I). The results presented in Figure 2 show

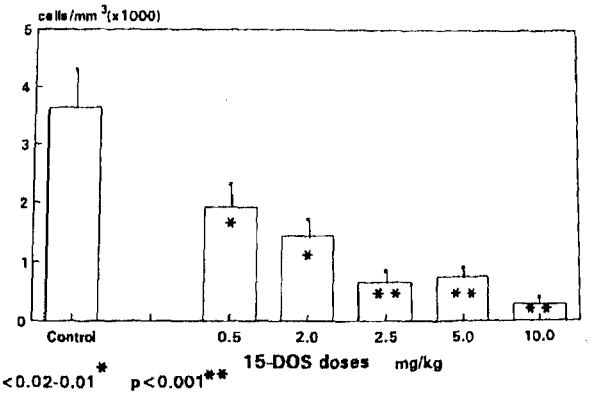

Fig. 2. Absolute values of granulocytes in peripheral blood of rats treated for 14 days with various doses of DOS: 0, 0.5, 2.0, 5.0 or 10.0 mg/kg. The results show that DOS treatment induced a significant and dose-dependent reduction of granulocyte counts

that DOS treatment induces significant reduction of granulocyte counts in peripheral blood in a dose-dependent manner. Seven days after the last DOS injection high numbers of granulocytes were observed (group II). Fourteen days after cessation of treatment the count of granulocytes was normalized (Fig. 3). In groups IV, V and VI similar changes in granulocyte counts were observed as in rats treated with the same dose for 14 days.

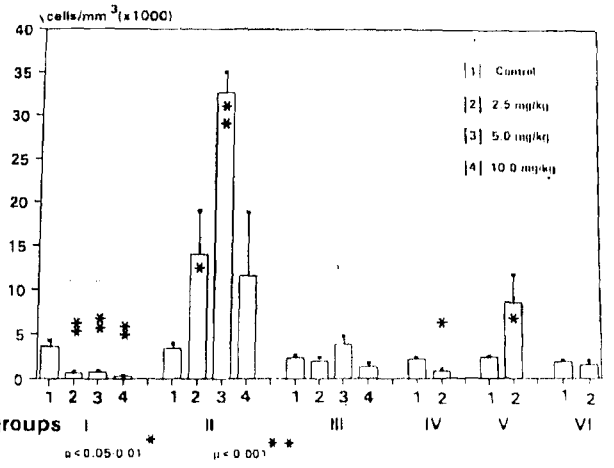

Fig. 3. Time-dependent changes in granulocyte count in peripheral blood of rats treated with various doses of DOS: 0, 2.5, 5.0 or 10.0 mg/kg. DOS-therapy, continued for 14 (group I) or 28 days (group IV), induced a significant reduction of granulocyte count at all doses used in this study. Seven days after termination of treatment very high proliferation of granulocytes were observed (groups II and V). The granulocyte count normalized after 14 days (group III and VI)

The femurs of rats treated 14 days with DOS showed bone marrow hypocellularity, especially in rats treated with 5.0 or 10.0 mg/kg of DOS. The quantitative bone marrow analyses demonstrated suppression of granulocyte maturation manifested by the increasing percentage of cells from BM-PPG. This even was strictly dose-related. Seven days after the last DOS injection cellularity of the femurs bone marrow significantly improved with concomitant increase of granulopoetic cell maturation. It was manifested by the increase of BM-NPPG 73.2% by 2.5 mg/kg, 71.4% by 5.0 mg/kg and 69.1% by 10.0 mg/kg versus 61.3% in the control group. Fourteen days after termination of treatment, the bone marrow granulopoietic system was almost normal (Fig. 4). The more prolonged treatment with DOS

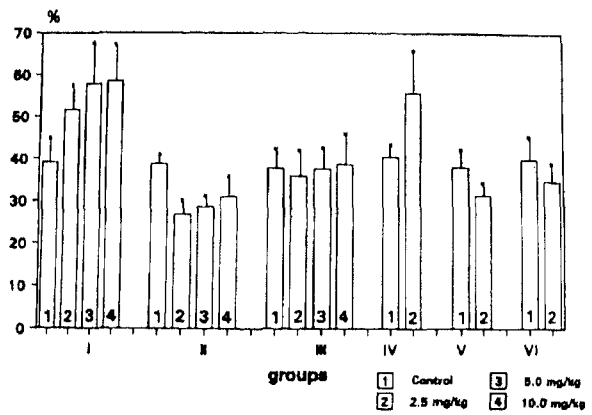

Fig. 4. Percentage values of granulocyte precursors in the bone marrow proliferative pool of granulocytes in rats treated with various doses of DOS: 0, 2.5, 5.0 or 10.0 mg/kg. DOS was applied for either 14 (group I) or 28 (group IV) consecutive days. After that time an increased percentage of granulocyte precursors was observed in bone marrow (groups II and V). Seven days after termination of DOS treatment the percentage of granulocyte precursors decreased below the normal value. These values normalized after 14 days (group III and VI)

in a dose of 2.5 mg/kg (groups IV, V and VI) induced similar changes, but the normalization of granulopoiesis lasted significantly longer.

Red blood cells (RBC)

The mean erythrocyte count in the control group was 8.776.000 ± 489.000. The absolute values of erythrocytes in rats treated with DOS for 14 days were within the normal range (group I). A decrease of erythrocyte counts was observed in animals treated for 28 days with a dose of 2.5 mg/kg (group IV). These decreased values significantly improved but did not return to normal level after 14 days (group VI) (Fig. 5).

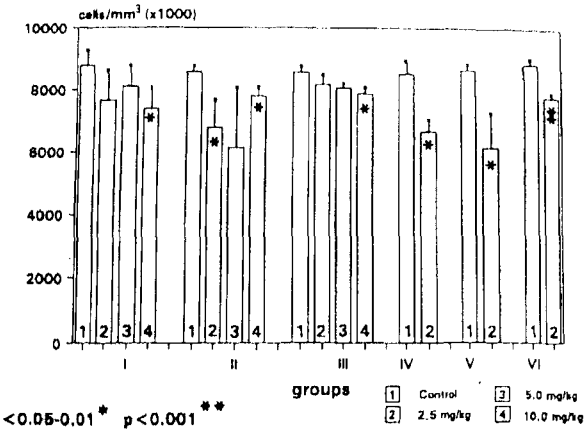

Fig. 5. Absolute values of erythrocytes in peripheral blood of rats treated with various doses of DOS 0, 2.5, 5.0 or 10.0 mg/kg. After 14 days of DOS therapy the reduction of erythrocyte count was observed only in the group of rats that had been treated with 10.0 mg/kg. Animals treated for 28 days showed also reduced erythrocyte count, althoguh the dose was only 2.5 mg/kg (group IV). Seven and fourteen days after termination of treatment the count did not yet normalize in those groups (groups V and VI), but significantly improved (group VI vs. IV)

DOS treatment induced a significant reduction of cells of the erythroblastic system of bone marrow. In rats treated with 2.5 mg/kg percentage value of erythroblasts was observed 1.6 ± 1.1 versus 22.0 ± 7.8 in controls. However, this was recovered after the termination of treatment. Seven days after the last DOS injection, a significant increase of erythroblasts in bone marrow and the appearance of single nucleated red blood cells in peripheral blood were observed. But the mean values were lower than in controls. In groups IV, V and VI a similar reduction of erythroblast percentage values was observed. However, normalization of erythroblast values in bone marrow was found two weeks after the end of DOS treatment.

Thrombocytes

The value of thrombocyte count in the control group was 697.000 ± 19.200. After 14 days of DOS treatment platelet counts were lower than those of the control group: 434.000 ± 130.000, 370.000 ± 70.000, 208.000 ± 19.000 and 170.000 ± 98.000 in rats receiving doses of 0.5, 2.5, 5.0, 10.0 mg/kg DOS respectively. Seven days after the last DOS injection the platelet counts were significantly higher and reached the normal limit (565.000 ± 186.000 in animals treated with 10.0 mg/kg). In rats treated with a dose of 2.5 mg/kg for 28 days normal value of platelet count was observed after the end of injections and during the recovery phase.

Hemoglobin (Hb)

The mean Hb level in the control group was 15.8 ± 0.5 g/dl. In rats treated with small doses of DOS, 0.5 and 2.5 mg/kg for 14 days, the mean Hb level was significantly decreased: 13.8 ± 0.3 and 13.8 ± 0.1 ($p < 0.01$) respectively. In rats injected with 5.0 and 10.0 mg/kg these values were nearly within normal range: 14.7 ± 1.4 and 14.7 ± 0.7 respectively. However, a decrease of Hb level was observed 7 days after termination of treatment 11.2 ± 3.2 and 12.7 ± 0.6 ($p < 0.01$) respectively. Normalization of Hb level was detected 14 days after the last DOS injection. Similar reduction of Hb level was observed in rats treated for 28 days with 2.5 mg/kg DOS.

Hematocrit (Ht) and body weight

The mean Ht value in the control group was 0.448 ± 0.006. Ht values were significantly reduced on day 14 in rats treated with 0.5 and 2.5 mg/kg: 0.423 ± 0.11 and 0.418 ± 0.008 ($p < 0.001$). In rats treated with 5.0 and 10.0 mg/kg Ht and Hb values were within normal limits, however, a reduction of body weight connected with diarrhoea was observed: 166 ± 5 g ($p < 0.01$) in animals treated with 10.0 mg/kg versus 255 ± 25 g in controls. Seven days after termination of treatment, Ht values were significantly lower in all groups: 0.364 ± 0.028 ($p < 0.02$), 0.337 ± 0.078 ($p < 0.05$), 0.375 ± 0.017 ($p < 0.01$) in rats receiving doses of 2.5, 5.0 and 10.0 mg/kg DOS respectively (group II). Two weeks after termination of DOS, normalization of Ht and body weight were observed (group III). In rats treated for 28 days with 2.5 mg/kg DOS decrease of Ht was more prominent at the end of treatment up to seven days later (0.336 ± 0.017 and 0.367 ± 0.074). The value normalized two weeks after termination of DOS injections. During this mode of treatment no body weight changes were observed (groups IV, V and VI).

Discussion

Experimental studies in rats and dogs showed that although DOS is an effective drug for the prolongation of graft survival, it has a negative influence on cell counts in peripheral blood. These observations were confirmed lately in clinical studies where renal graft patients had received DOS as a rescue drug[1]. The reduction of white blood cells other than lymphocytes was the most notable adverse reaction. Moreover, reduction of red blood cells and platelets was observed.

The current studies were undertaken to investigate the changes in peripheral blood cell counts as well as cell maturation in bone marrow in rats receiving differet doses of DOS intraperitoneally. Our studies confirmed earlier observations of granulocytopenia during DOS treatment. However, in contrary to observations made by AMEMIYA et al.[1] and by NISHIMURA and TOKUNAGA[10] who injected mice with a dose of 3.0 mg/kg DOS and described that peripheral lymphocytes underwent only minor changes during and after treatment, we have observed a significant reduction of absolute numbers of lymphocytes. This effect was seen even in rats treated with a dose as low as 0.5 mg/kg. This difference in lymphocyte reduction may depend on longer periods of treatment in our experimental design. We have injected animals for fourteen days, whereas AMEMIYA et al.[1] and NISHIMURA and TOKUNAGA[10] for five and four to nine days, respectively.

Similarly to AMEMIYA et al.[1], we observed a reduction of platelet and red blood cell counts. It appears probable that DOS causes thrombocytopenia by suppressing platelet production through a toxic effects on the megakarocytes. The cause of anemia, however, could be more complex. It is well known that some anemic crises may be related to drug toxicity, but gastroenterological side effects of the DOS manifested as diarrhea may induce protein malnutrition and deficiences of folic acid, ascorbinic acid and riboflavin which have also been implicated in the etiology of anemic crises. Taking into cosideration that changes in the erythroblastic system appeared very early in DOS treatment and that the changes were reversible shortly after discontinuation of this drug it is more likely that a toxic drug effect, rather than malnutrition, was the most important factor for induction of anemia.

Repeated intraperitoneal injections of DOS resulted in a depressed granulopoietic and erythropoietic function of bone marrow. This effect was dose-dependent. DOS did not decrease cell proliferation, but suppressed the maturation of granulocyte precursors of the bone marrow proliferative pool of granulocytes. The percentage of immature granulocyte precursor was increased. The consequence of depressed granulocyte maturation was reduced percentage of mature cells in bone marrow with its hypocellularity. Our results confirmed those of NISHIMURA and TOKUNAGA[10] that DOS blocked maturation of bone marrow cells, whereas functionally responsive stem

cells remained in bone marrow. The discontinuation of DOS treatment led quickly to recovery of bone marrow cellularity and normalization of the granulopoietic system.

The erythropoietic system appears to be particularly sensitive to DOS, as the percentage values of erythroblastic cells decreased significantly even in rats treated with small doses of DOS. The DOS-dependent reduction of erythrocyte numbers in peripheral blood in rats treated with high doses was masked by diarrhea, body weight loss and hemoconcentration. The most intensive anemia was seen seven days after termination of treatment. At this time the diarrhea disappeared and the body weight increased. As a consequence of body hydration, the Ht values decreased. After termination of treatment a sharp increase of erythroblast number in the bone marrow was observed and the animals recovered very rapidly from anemia. These observations allow us to suggest that DOS has only minor side effects at doses below 2.5 mg/kg used not longer than 7–14 days.

The results of our study suggest dose-dependent drug-induced granulocytopenia and anemia, which are probably due to DOS interference with cell proliferation. The drug action is not selective and is likely to involve pluripotent cells. However, the hematological recovery commences already a few days following cessation of drug treatment. The cell counts rise rapidly to levels exceeding the normal range.

These effects are minor in doses below 2.5 mg/kg applied not longer than for fourteen days. Termination of treatment leads to normalization of most changes within two weeks.

There is little published data on the mechanisms of these reversible toxic effects of DOS. It was shown that DOS requires metabolic activation to express its cytotoxic effects and it was suggested that its active products are aldehydes[13]. Studies performed in dogs, in which a toxic cystitis was observed (a typical effect of aldehyde toxicity), may support this suggestion. Spergualin which is a polyamine antibiotic, isolated from cultures of *Bacillus laterosporus*, may be transformed to toxic aldehydes by oxidase. KUNIMOTO et al.[9] demonstrated that an anti-proliferative influence of spergualin *in vitro* depended on the oxidase concentration in the medium.

Further studies are required to clarify the mechanism of DOS toxicity. They are especially needed in respect of future use in human organ transplantation. Our studies show that hematological side effects may developed after DOS therapy and that it is necessary to collect observations in patients treated with DOS, to estimate the level of toxicity and to control whether this effect is also reversible.

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