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Effect of Liposomal Muramyl Tripeptide Phosphatidyl-ethanolamine and Indomethacin on Hematopoietic Recovery in Irradiated Mice

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Summary

The effects of liposomal muramyl tripeptide phosphatidylethanolamine (MTP-PE/MLV, radioprotective immunomodulator; 10 mg/kg) and indomethacin (INDO, inhibitor of prostaglandin production; 2 mg/kg) on post-irradiation recovery of hematopoietic functions in mice were investigated. Two agents with distinct radioprotective mechanisms were administered alone or in combination 24 h and 3 h before exposure to 7 Gy ^{60}Co radiation. In the post-irradiation period (3-14 days) combined pre-treatment of mice accelerated recovery of bone marrow cellularity, weight of spleen and myelopoietic and erythropoietic activity in both hematopoietic organs, compared to treatment with MTP-PE/MLV or indomethacin alone. In the peripheral blood, improved radioprotective effects of combined drug administration were found in the recovery of reticulocytes and platelet count. No further significant differences in the recovery of leukocyte count were observed in the examined groups until post-irradiation day 14. Within the first 3-6 post-irradiation days, the bone marrow and peripheral blood smears of mice pre-treated with indomethacin alone or its combination with MTP-PE/MLV more frequently featured blast cells and large cells with abundant cytoplasm which could be considered the hematopoietic stem cells.

Key words

Radioprotection • Hematopoiesis • Muramyl peptide • Indomethacin

Introduction

Exposure to ionizing total body radiation suppresses hematopoiesis, resulting in decreased production of blood cells. New developments in radiobiology and radioprotection are becoming available for determining not only the mechanisms of radiation injury but also for controlling or preventing the acute effects of irradiation. There are several approaches to preventing death from bone marrow suppression following radiation. Reduction of the side effects, such as neutropenia, lymphopenia and thrombocytopenia, involve the most important factors for increasing the therapeutic effect. Attention has therefore been paid to various immunomodulatory and immunoenhancing agents which through the monocyte/macrophage system induce production of hematopoietic growth factors and cytokines, which can act radioprotectively (Neta 1988). It

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was found that muramyl peptides also stimulate their production (Sanceau *et al.* 1990, Suzuki *et al.* 1994). Their physiological effects are similar to the effects of endotoxins or other complex microbial polysaccharides, including the induction of colony-stimulating activity, modulation of myelopoiesis (Galelly and Chedid 1983) and stimulation of marrow colony-forming cells (Wuest and Wachsmuth 1982). As well as bacterial endotoxin, muramyl peptides and other agents with immunostimulating and inflammatory effects are radioprotective when administered before irradiation (Ainsworth 1988, Macková and Fedoročko 1993, 2000 Fedoročko 1994).

Similar protection against hematopoietic syndrome death can be achieved by the use of non-steroid anti-inflammatory drugs (NSAIDs) such as flurbiprofen or indomethacin, both of which are potent prostaglandin synthesis inhibitors. The administration of indomethacin to stimulate hematopoiesis in normal mice (Fontagne *et al.* 1980) as well as in mice irradiated with sublethal doses of irradiation (Kozubík *et al.* 1990, Nishiguchi *et al.* 1990, Pospíšil *et al.* 1992) were found. Because various radioprotective agents differ in the mechanisms of their action, more hematopoietic regeneration effects can be achieved by a combination of several protective agents in irradiated or chemotherapy-treated mice (Nishiguchi *et al.* 1990, Pospíšil *et al.* 1992). Experimental evidence reported in our previous paper (Fedoročko and Macková 1996) indicated that the beneficial action of the combined treatment could be a consequence of increased cell proliferation in the hemopoietic tissue and mobilization with redistribution of stem cells from bone marrow into the circulation. Combined treatment increased the number of bone marrow GM-CFC and numbers of pluripotent stem cells significantly when compared to mice treated with MTP-PE/MLV or indomethacin alone. Combined administration of both agents to mice prior lethal irradiation exerted an additional radioprotective effect and protected 100 % of mice. In the present study, we have further examined the effects of this combination on hemopoietic organs, namely the bone marrow and spleen, and on the recovery of peripheral blood cells after sublethal irradiation.

Methods

Mice

Female C57Bl/6 mice, 8-10 weeks old (weighing 20 g), were obtained from Velaz, s.r.o. (Praha, Czech Republic). Animals were quarantined for a period of 2 weeks and were housed in rodent cages with five to seven animals per cage at about 23 °C, and they were *ad libitum* given Velaz/Altromin 1320 St lab chow and tap water acidified to pH 2.4. Research was conducted according to the principles set out in the "Guide for the Care and Use of Laboratory Animals", prepared by the State Veterinary Office of the Slovak Republic, Bratislava.

Reagents

Liposomal muramyl tripeptide phosphatidylethanolamine (MTP-PE/MLV, CGP l9835A) is a synthetic muramyl tripeptide coupled to dipalmitoylphosphatidylethanolamine and it is N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[(1,2-dipalmitoyl-sn-glycero-3-(hydroxyphosphoryloxy)] ethylamide monosodium salt encapsulated in a multilamellar vesicle (MLV) - liposome. Liposomes containing MTP-PE and placebo liposomes (empty liposomes) were a generous gift of Ciba-Geigy Ltd. (Basel, Switzerland). Liposomes were prepared from dry lyophilisate composed of 250 mg of phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 (with or without 1 mg of MTP-PE) and shaken with 2.5 ml of suspension medium (Dulbecco buffer, pH = 7.2, without Ca^{2+} and Mg^{2+}- salts). After standing for 15 seconds, the reconstituted liposomes were suspended by vortexing for one minute. The drug was administered i.p. 24 h before irradiation at a dosage of 10 mg/kg MTP-PE encapsulated in liposomes. Control animals were administered the placebo in an adequate amount of saline.

Indomethacin (Sigma Chemical Co., St. Louis, USA) was prepared by dissolving 10 mg in 1 ml of 95 % ethylalcohol. This solution was then diluted to the working concentration with Dulbecco's phosphate-buffered saline (Ciba-Geigy, Basel, Switzerland) and injected i.m. in amounts of 2 mg/kg, in a volume of 0.2 ml, 24 and 3 h before irradiation. Both of the drugs were given either alone or in a combination.

Irradiation

Mice were placed in plexiglass containers and whole-body (unilaterally) exposed to 7 Gy of gamma rays (0.5 Gy/min). A Chisostat ^{60}Co source (Chirana, Czech Republic was used for all irradiations.

Collection and analysis of hematopoietic cells

Mice were anesthetized by Thiopental inj. (VUAB, Roztoky u Prahy, Czech Republic) immediately

before blood was obtained by cardiac puncture. The number of leukocytes was counted using an automatic Coulter Counter Model ZF and cell differentials were performed by counting 100 white blood cells on May-Grűnwald-Giemsa (MGG) stained smears. Reticulocytes were evaluated after staining blood smears with Brilliant cresyl blue and platelets were counted using a Bűrker chamber. After the removal and thorough rinsing of femurs with Hanks solution, the cellularity of bone marrow (BM) was determined by direct counting with Tűrk's solution. Bone marrow smears for morphological examination were stained with MGG. The spleen was immediately dissected out, weighed, immersed in Helly's fixative and then processed by routine histological methods. Histological sections were stained with hematoxylin-eosin (HE) and studied at the light microscope level.

Statistics

The values given in the figures represent the means ± standard error of the means (S.E.M.). The statistical significance of the differences was evaluated using Peritz's F-test. A value of $p<0.05$ was considered as the basis for a statistically significant difference.

Results

As indicated in Figure 1, gamma irradiation decreased the cellularity of bone marrow (BM) in all the examined groups (the mean value obtained in the non-irradiated control group was 21.0±1.74 x 10^6). The morphological picture of BM on day 3 after irradiation was characterized by the marked suppression of hematopoiesis. The main cell components were represented by neutrophil granulocytes, among which there were more frequently seen also blast cells and large cells with a round to oval nucleus. In the latter cells, one or more nucleoli were usually discernible, their cytoplasm was abundant and spreading with indefinite limits in many instances (Fig. 2A). The occurrence of these cells was more frequently seen in mice pre-treated with indomethacin alone and in combination with MTP-PE/MLV also at post-irradiation day 6. Besides these cells, there were also other cells of the immune system (plasmocytes, monocytes, histiocytes, macrophages) to be seen in BM smears in mice pre-treated with immunomodulators. A single administration of MTP-PE/MLV and its combined administration with indomethacin (MTP-PE/MLV+INDO) clearly accelerated

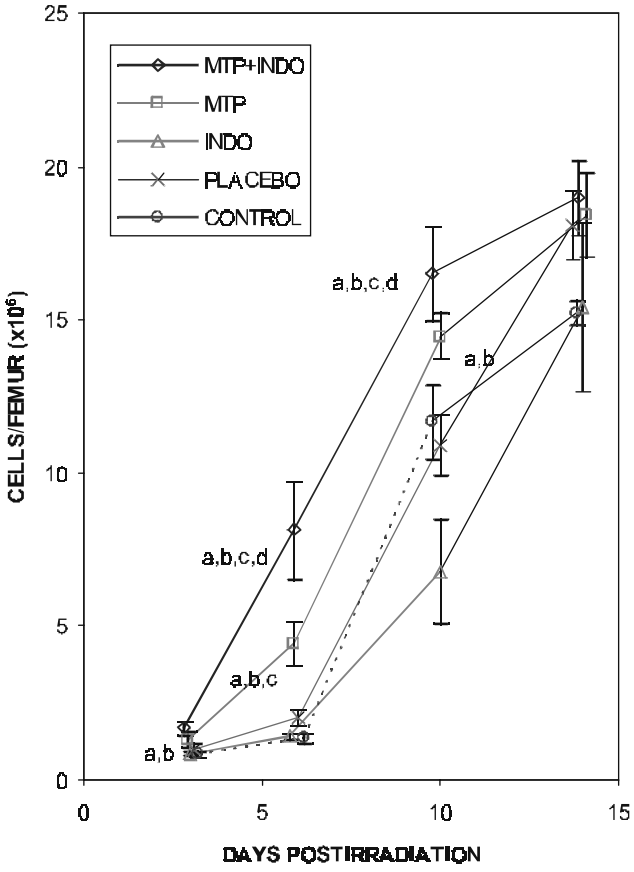

Fig. 1. *Numbers of nucleated cells in the femur on different days after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with controls (a), indomethacin group (b), placebo group (c), MTP-PE/MLV group (d). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 21.0±1.7 x 10^6.*

hematopoietic recovery as measured by the increase in BM cellularity. However, the recovery of BM cellularity in mice protected with the combined drugs was greatly accelerated on days 6 and 10 (Fig. 1). This reparative process of hematopoiesis in the bone marrow was first accompanied by vigorous proliferation of myeloid cells, which was expressed by frequent occurrence in MTP-PE/MLV+INDO pre-treated mice (Fig. 2B). On the 14th day after irradiation, the bone marrow cellularity of MTP-PE/MLV+INDO, MTP-PE/MLV alone and placebo pre-treated mice was almost normal (Fig. 1), and in co-treated mice lineage distribution with predominance of myeloid and erythroid elements was also found (Fig. 2C). In mice pre-treated with MTP-PE/MLV alone, the myelopoietic

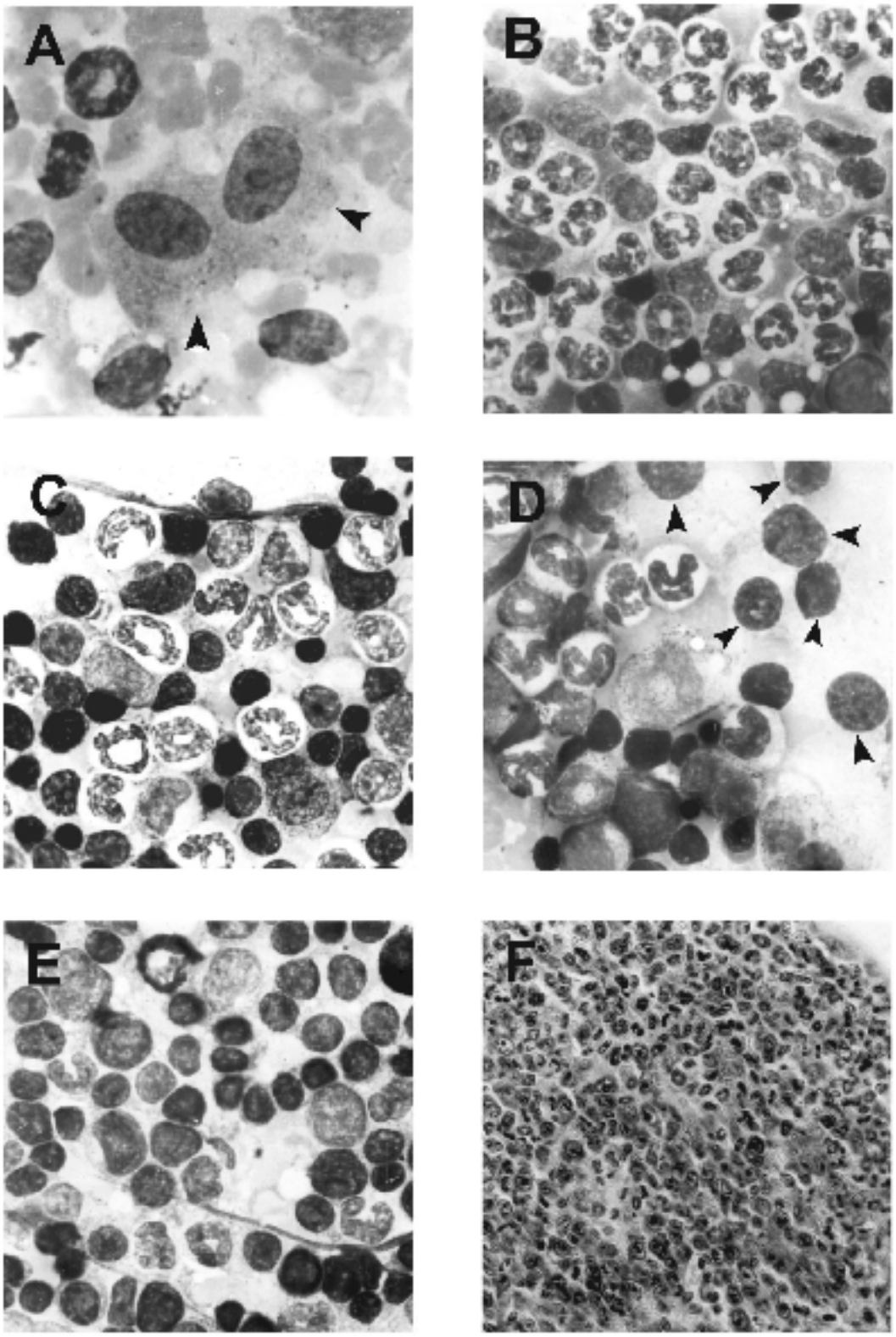

Fig. 2. *Morphologic pictures of hematopoietic recovery in bone marrow and spleen of irradiated mice pre-treated with MT-PE/ MLV, indomethacin and placebo. BM of mice pretreated with MTP-PE/ MLV+ INDO at post-irradiation days 3 (A), 6 (B) and 14 (C). BM at post-irradiation day 14 in mice pretreated with MTP-PE/ MLV alone (D) and placebo (E); MGG, x1000. Red pulp of spleen in mice pre-treated with MTP-PE/MLV+INDO at post-irradiation day 10 – proliferation of myeloid cells in the red pulp (F); HE, x1000.*

activity decreased and the mononuclear cells of lymphoid type were more frequent (Fig. 2D). These cells were the main cell components in bone marrow in animals irradiated or pre-treated with indomethacin or placebo. On day 14, their frequency significantly exceeded that of the erythroid and myeloid cells, especially in mice pre-treated with placebo (Fig. 2E).

As indicated in Figure 3, gamma irradiation decreased spleen weight in all the examined groups (the mean value obtained in the non-irradiated control group was 75.1±3.1 mg). Placebo and indomethacin treatment as well as irradiation alone elevated the post-irradiation spleen weight, starting from day 10 after irradiation. The pre-irradiation administration of MTP-PE/MLV alone as well as its combination with indomethacin (MTP-PE/MLV+INDO) accelerated the increase of spleen weight within post-irradiation days 6-10. Morphological examination (the spleen is an important hematopoietic organ in mice throughout their life) on day 3 after the irradiation showed approximately the same damage in all irradiated groups, with the exception of a considerably roughened spleen capsule and the proliferation of reticular cells in mice pre-treated with MTP-PE/MLV+INDO. That is taken as a signal of mobilization of the reparative processes. Moreover, discrete colonies of myeloid cells were seen in the subcapsular zone of the

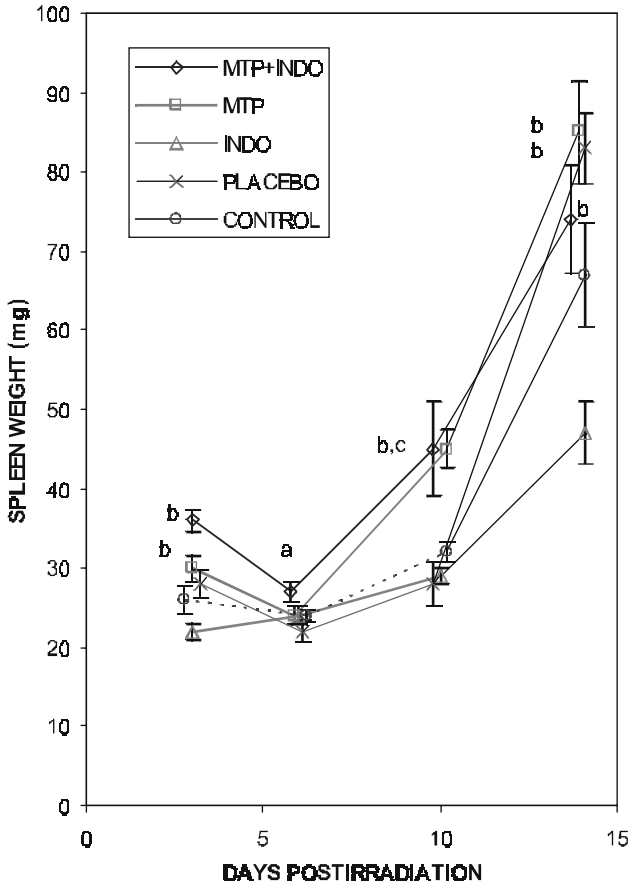

Fig. 3. *Spleen weight on different days after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with the control group (a), indomethacin group (b), placebo group (c). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 75.1±3.5 mg.*

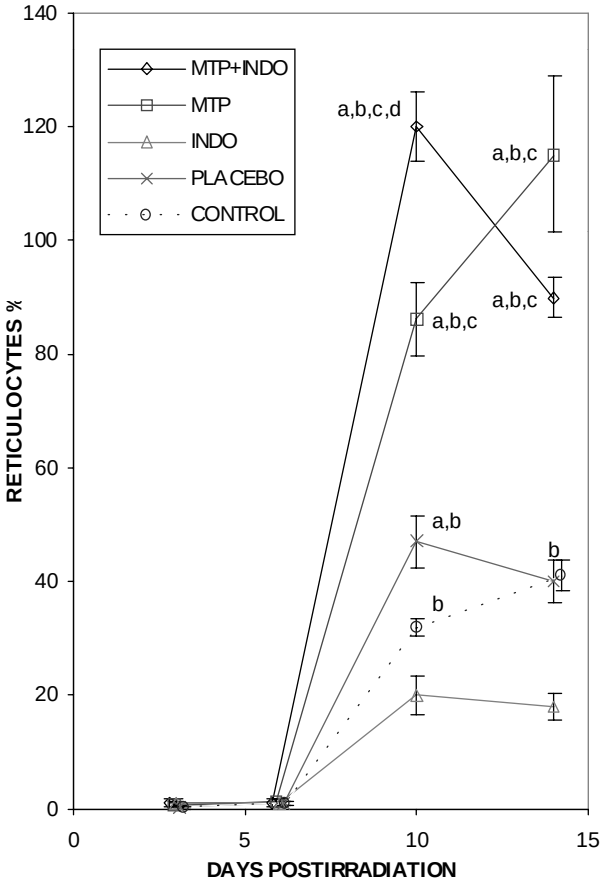

Fig. 4. *Numbers of reticulocytes in peripheral blood on different days after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with the control group (a), indomethacin group (b), placebo group (c), MTP-PE/MLV group (d). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 78.0±4.2.*

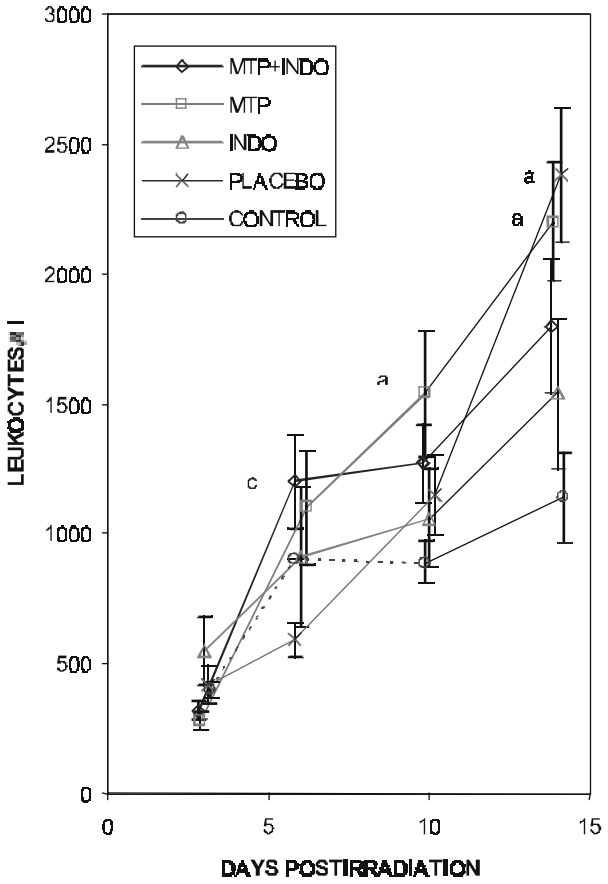

Fig. 5. *Numbers of leukocytes in peripheral blood on different days after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with the control group (a), placebo group (c). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 4.850±348.2.*

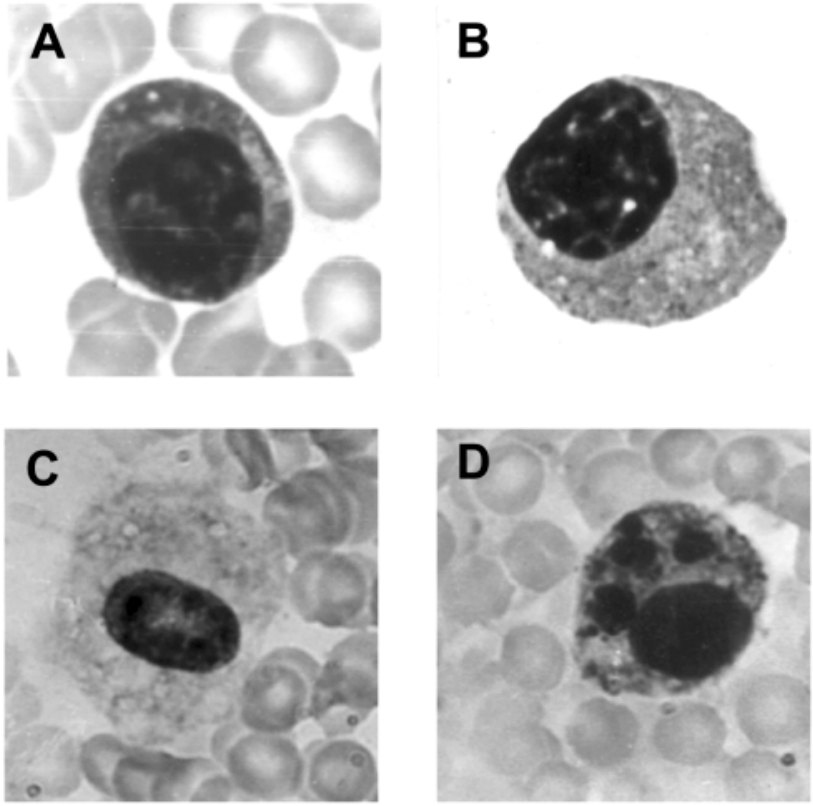

Fig. 6. *Peripheral blood cells in MTP-PE/MLV + INDO pre-treated mice at post-irradiation days 3 and 6. Blast cell (A), plasma cell (B), cell of dentritic type (C), histiocyte-macrophage (D). MGG, x1000.*

red pulp. As it was further evident, a more dramatic response was observed in these co-treated mice compared to mice treated with MTP-PE/MLV alone in the following postirradiation days as well. This was manifested by profound proliferation not only of erythroid but also of myeloid cells. Histological analysis of the spleen on day 10 showed large erythroid endocolonies and an extensive proliferation of myeloid cells in co-treated mice (Fig. 2F). In mice pre-treated with MTP-PE/MLV alone, smaller erythroid endocolonies and myeloid cells were found only sporadically spread throughout the red pulp of the spleen.

In all experimental groups, the treatment with sublethal doses of acute irradiation induced severe reticulocytopenia (Fig. 4) and leukopenia (Fig. 5). Reticulocytopenia persisted up to day 7 after irradiation. Afterwards treatment with MTP-PE/MLV and especially its combination with indomethacin clearly accelerated the recovery of reticulocytes in peripheral blood. On day 10 after irradiation, reticulocytes in MTP-PE/MLV+INDO pre-treated mice were significantly higher when compared with control mice and mice pre-treated with indomethacin, placebo, or MTP-PE/MLV alone (Fig. 4). The total WBC counts rapidly increased from day 3-6 in mice pre-treated with MTP-PE/MLV+INDO or MTP-PE/MLV alone, after which their number further increased until day 14 (Fig. 5). In peripheral blood smears, similarly to BM smears of co-treated mice and pre-treated with indomethacin alone, blast cells and large cells with abundant cytoplasm were seen within the first 6 post-irradiation days. Besides these cells plasmocytes and

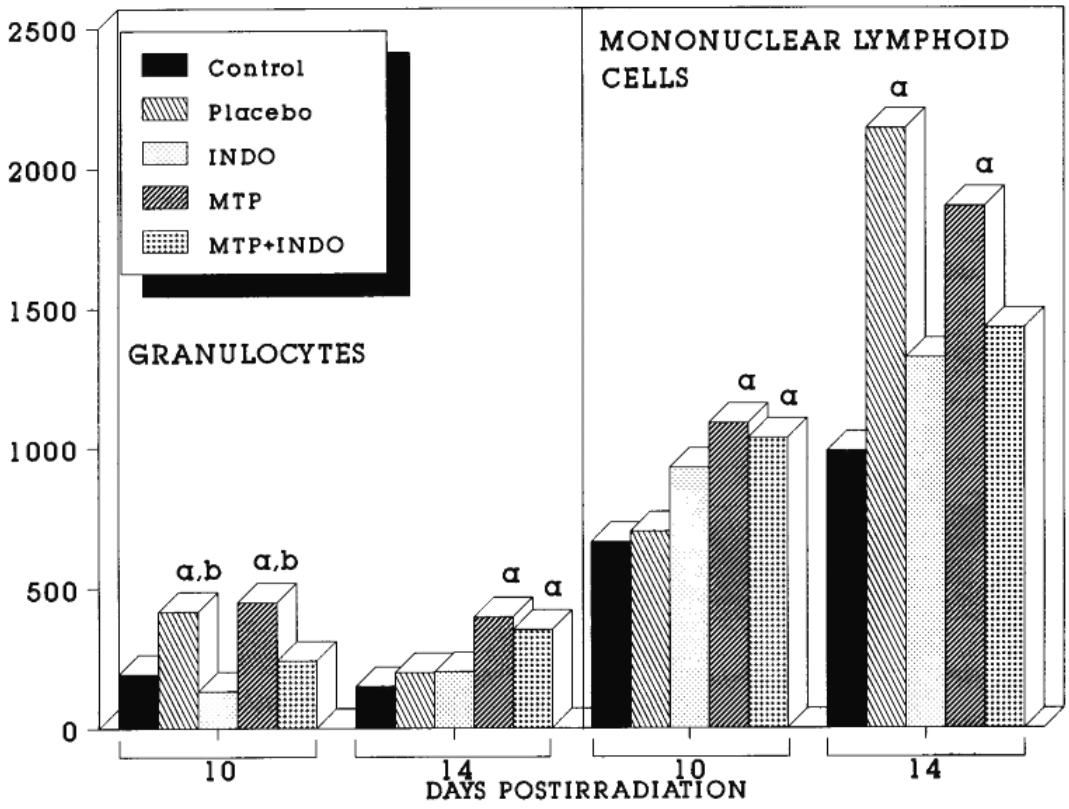

Fig. 7. *Numbers of granulocytes and lymphoid mononuclear cells in peripheral blood on days 10 and 14 after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with control group (a), indomethacin group (b). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 798.5±128.2 for granulocytes and 4050±425 for lymphoid mononuclear cells.*

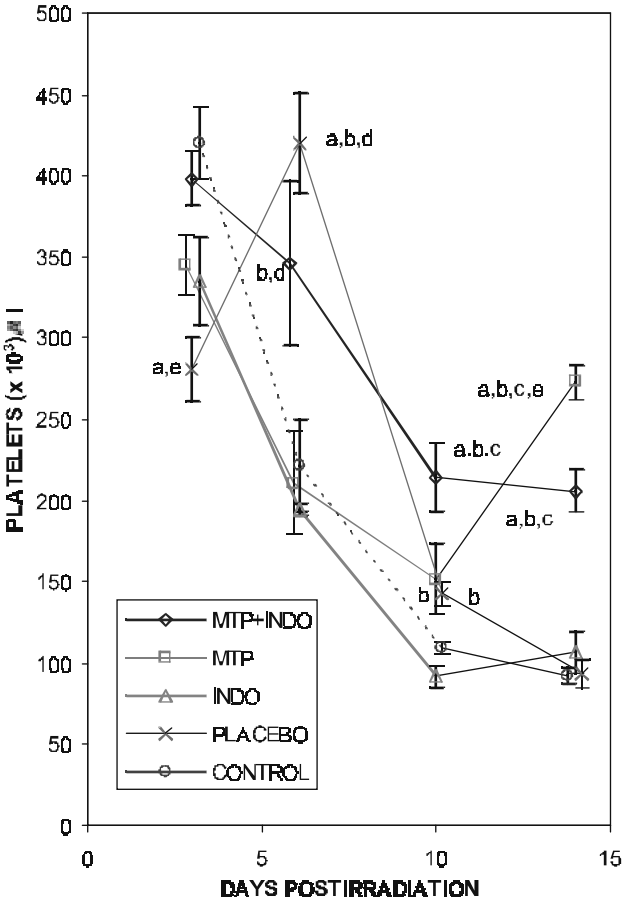

Fig. 8. *Numbers of platelets in peripheral blood on different days after 7 Gy irradiation in control mice (saline, placebo) and mice treated with MTP-PE/MVL and indomethacin alone or in combination. Ten mice per point were used. Symbols denote statistical significance when compared with the control group (a), indomethacin group (b), placebo group (c), MTP-PE/MLV group (d) and MTP-PEM/LV+INDO group (d). For the sake of simplicity significance at the 0.05 level was used for all the comparisons. The mean value obtained in the non-irradiated control group was 380±45.3 x10^3.*

sometimes cells of the dentritic type or histiocytes (macrophages) were seen (Fig. 6 A, B, C, D). Just as monocytes may be found in the tissues, so histiocytes may be found in the blood under pathological conditions. At the end of the experiment, differential leukocyte analysis showed that in both groups of mice protected with MTP-PE/MLV alone or with MTP-PE/MLV in combination with indomethacin significantly higher granulocyte numbers were found when compared with mice which were only irradiated (Fig. 7). Mononuclear lymphoid cells were significantly higher on day 14 in mice pre-treated with placebo or MTP-PE/MLV alone, compared with mice that were only irradiated (Fig. 7). The platelet count decreased gradually in control-irradiated mice, in mice pre-treated with indomethacin alone, or MTP-PE/MLV alone, and reached its nadir on day 10 and day 14, respectively (Fig. 8). The placebo induced a temporary increase of platelets on day 6, but thereafter their numbers clearly decreased (Fig. 8). Treatment with MTP-PE/MLV alone accelerated recovery of platelets from day 10 and co-treatment with MTP-PE/MLV+INDO prevented more profound platelet decrease (Fig. 8).

Discussion

Administration of MTP-PE/MLV alone induced bone marrow cell recovery in irradiated mice. In addition, the results suggest better radioprotective effects of the combined administration of MTP-PE/MLV and indomethacin, as the recovery was then accompanied by marked and continuous proliferation of BM myeloid cells. These results correlate in general with the increase of GM-CFC, the number of which was significantly higher on post-irradiation days 6-14 than in mice pre-treated with MTP-PE/MLV alone (Fedoročko and Macková 1996). The better radioprotective effect of the combined administration of MTP-PE/MLV+INDO on recovery of hematopoiesis was also seen in the spleen. This was manifested not only in endoCFU-S, whose number was significantly higher in co-treated mice than in mice pre-treated with MTP-PE/MLV alone (Fedoročko and Macková 1996), but also in the earlier mobilization of reparative processes and higher extramedullary hematopoiesis. Our data correspond with those reported by Nishiguchi and co-workers (1990), who used WR-2721 and indomethacin. Both agents were protective, but combined treatment was more effective, including the stimulation of endoCFU-S. The role of the spleen, at least in the mouse, is relegated to that of an auxiliary site for hematopoietic expansion. At the time of irradiation (24 h after administration of MTP-PE/MLP with INDO), bone marrow cellularity decreased and the radioresistant GM-CFC population as well as spleen endoCFU-S increased (Fedoročko and Macková 1996). It is possible that a combination of drugs causes a mobilization of stem cells and their redistribution over the bone marrow, peripheral blood, and spleen. We assume on the basis of these notions that the blast cells and large cells with abundant cytoplasm which were more frequently seen in co-treated

mice, not only in bone marrow but also in peripheral blood, may be stem cells (totipotent and pluripotent stem cells). This consideration seems to be supported by the experiment of Mišúrová *et al.* (1989), who investigated nucleic acids in peripheral blood cells of irradiated rats after treatment with indomethacin. In protected animals, the stimulative indomethacin effect on hematopoietic cells manifests itself as temporary increase in incorporation of ^{3}H-thymidine into blood nucleated cells. Previous studies had shown that at least a part of DNA synthesizing cells in the blood of irradiated rats represents circulating hemopoietic stem cells (Mišúrová and Kalina 1982). Because the immune system is evidently activated by muramyl peptides and probably also by indomethacin, it is possible that dentritic cells, that are initiators of the immune response (Melief 1989, Steinman 1991) and producers of cytokines (Lung *et al.* 2000), can also be among the observed cells. They originate from hematopoietic progenitors in bone marrow and differentiate principally in response to GM-CSF (Zorina *et al.* 1994). The content of GM-CSF was significantly increased in the serum from mice treated with MTP-PE/MLV alone or with a combination of the drugs (Fedoročko and Macková 1996).

Thrombocytopenia in the peripheral blood is still a serious problem after radiation treatment or anticancer chemotherapy. Here we have shown the protective efficiency of MTP-PE/MLV alone and a better radioprotective effect in co-treated mice (MTP-PE/MLV+INDO) on experimental thrombocytopenia induced by irradiation. The therapeutic efficiency of muramyl peptide derivatives on the thrombocytopoietic system supports the reports of other authors (Yano *et al.* 1995, Namba *et al.* 1996). Although the mechanism of platelet production has not been fully understood so far, IL-6 is believed to play an important role in this mechanism (Ishibashi *et al.* 1989). Its production was stimulated by MDP (Senceau *et al.* 1990). A clearly better radioprotective effect of the combined administration of MTP-PE/MLV and indomethacin in the peripheral blood was also manifested in the acceleration of recovery of reticulocytes, which is a result of erythropoiesis recovery not only in bone marrow but also in the spleen. During the period until 14 days after irradiation, no more significant differences were observed in the examined groups in the recovery of leukocytes, in contrast to the reticulocyte recovery. The significant increase of mononuclear lymphoid cells in the peripheral blood of mice pre-treated with MTP-PE/MLV alone and placebo by day 14 after irradiation will be connected with accumulation of these cells in the bone marrow after post-irradiation days 10-14. From the experiments in animal models we know that major histopathological changes, which occurred in the spleen and bone marrow 1-3 months after MTP-PE/MLV administration, were predominantly of an inflammatory nature with slight leukocytosis (Schumann *et al.* 1989). It is possible that combination with indomethacin could reduce these side effects of MTP-PE/MLV.

The precise mechanisms of muramyl peptide radioprotection is still unknown. On the other hand, it is known from many experiments that muramyl peptides have a powerful effect on the immune system by way of macrophage and monocyte activation, and by stimulating other cells of the immune system which play a crucial role in cytokine production (Macková and Fedoročko 1993, 1997, Namba *et al.* 1996, Pabst *et al.* 1999). The biochemical signal transduction mechanisms responsible for macrophage activation by muramyl peptides are only partly known. First of all, there is a plasma membrane receptor for these immunomodulating agents (Silverman *et al.* 1986, Golovina *et al.* 1994). Photoaffinity labeling also shows an intracellular binding protein for MDP (Tenu *et al.* 1989). Membrane and intracellular receptors might both have good access to liposome encapsulated MDP, which could be a reason why MDP (or MTP-PE) in liposomes is so effective (Daemen *et al.* 1989). Bearing in mind the sequence of changes induced by the encapsulated muramyl tripeptide, it can be assumed that its effects are first mediated through induction of hematopoiesis-regulating cytokines, the production of which is stimulated by muramyl peptides. Other factors that play a role in hematopoiesis are prostaglandins (PGs) of the E series, which are involved in a negative regulatory pathway of hematopoiesis by blocking the proliferation of hematopoietic cells (Pellus 1989). An inhibitor of PGE_2 synthesis is indomethacin, repeated administration of which has been found to enhance murine hematopoiesis (Pospíšil *et al.* 1986, Nishiguchi *et al.* 1990) and to protect hematopoietic tissue indirectly through stimulation of hematopoietic cells in the spleen (Serushago *et al.* 1987, Nishiguchi *et al.* 1990). The level of GM-CFC in the spleen is significantly higher than in the bone marrow following indomethacin administration (Gallicchio *et al.* 1989). The inhibition of PG production by indomethacin could be a way of enhancing positive hematopoietic control. Regarding the sequence of changes induced by encapsulated muramyl tripeptide with indomethacin in the post-irradiation period, it can be assumed that the radioprotective action of the combined

treatment may be the consequence of increased cell proliferation in the hematopoietic tissue induced by the immunomodulator and suppression of the negatively acting prostaglandin production.

In general, our results suggest that different hematostimulatory mechanisms through which MTP-PE/MLV and indomethacin appear to mediate their hematopoietic-enhancing effects might further accelerate hematopoietic regeneration if used in combination in irradiated mice. The positive effect of the combination therapy on post-irradiation recovery of hematopoiesis was evident not only in the bone marrow but also in the spleen, which could be connected with stem cell mobilization and redistribution in the bone marrow, peripheral blood and spleen.

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