

Effect of a Streptococcal Preparation, OK-432, on Hematopoietic Spleen Colony Formation in Irradiated Mice¹

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ABSTRACT

In vivo effect of an immunostimulant, OK-432, on hematopoietic spleen colonies (CFU-S) was investigated in irradiated JCL/ICR mice. Administration of OK-432 i.p. at various times before and/or after irradiation resulted in a significant increase in endogenous CFU-S. This increase was further characterized microscopically by an increase in the number of megakaryocytic colonies. Transplantable exogenous CFU-S also increased when normal bone marrow cells were transplanted into irradiated recipient mice previously given OK-432 i.p. Treatment with OK-432 gave rise to an earlier recovery of granulocyte and, particularly, platelet counts in the peripheral blood after irradiation. All these findings indicate that an increase in CFU-S is associated with activated hematopoietic microenvironment by OK-432.

INTRODUCTION

An immunostimulant, OK-432 (14), is effective for immunotherapy of various malignant tumors, and the *in vivo* and *in vitro* tumor-inhibitory effect was shown to be derived from an activated host anti-tumor defense mechanism, e.g., activation of macrophages and lymphocytes (8, 16, 21).

There are numerous reports concerning the accelerated hematopoietic recovery or increased CFU-S³ in mice after irradiation or drug-induced myelosuppression by bacterial endotoxins (4, 5, 19) and such immunostimulants as BCG (9) or *Corynebacterium parvum* (10, 13). We also have observed an increase of murine transplantable exogenous CFU-S and CFU-C both in the bone marrow and spleen by treatment with OK-432 (6).

In the present study, we report the *in vivo* effects of OK-432 on endogenous CFU-S, irradiated recipient mice in the exogenous CFU-S assay, and hematopoietic recovery after irradiation.

MATERIALS AND METHODS

Animals. Female JCL/ICR mice, 10 to 12 weeks old, were used throughout.

OK-432. OK-432 (Picibanil, Lot A9D 016), a lyophilized preparation of attenuated strain Su of *Streptococcus haemolyticus* (Group A, type 3), was from Chugai Pharmaceutical Co. Ltd., Tokyo, Japan. The same batch of OK-432 was reconstituted in 0.9% NaCl solution, stored at -20°, and thawed

shortly before use in each experiment. The cell content of OK-432 was expressed as a Klinische Einheit (KE), i.e., 1 KE equals 0.1 mg of the dried cells.

Endogenous CFU-S. The endogenous spleen colony method of Marsh *et al.* (11) was used to assess the effect of OK-432 on endogenous CFU-S. Eight to ten mice were given 0.2 or 1 KE/0.5 ml of OK-432 i.p. at various times (Table 1) before and/or after 610 R total body irradiation. A compact ⁶⁰Co irradiation facility of The Institute for Chemical Research of Kyoto University was used to irradiate mice. The dose rate of irradiation in July 1980 was approximately 10.2 R/sec. Macroscopic colonies of the spleen fixed with Bouin's solution were counted on the eighth day after irradiation.

Transplantable Exogenous Spleen Colony (Exogenous CFU-S). Exogenous CFU-S was assayed by the transplantable exogenous spleen colony method of Till and McCulloch (20). Eight to ten recipient mice were given 810 R for each exogenous CFU-S assay. In previous work, we reported increased exogenous CFU-S in the bone marrow and spleen 3 days after i.p. administration of various doses of OK-432 (6). In the present study, the dose of OK-432 was fixed at 1 KE/0.5 ml, and the effect of OK-432 on the irradiated recipient mice for exogenous CFU-S assay was examined; i.e., normal bone marrow cells were transplanted into the irradiated recipient mice treated with 1 KE/0.5 ml of OK-432 at various times (Table 2) before 810 R total-body irradiation. In each case, the spleens were removed and fixed with Bouin's solution 8 days after irradiation, and the surface colonies were counted. Colony-forming cells in the spleen colonies were histologically examined using hematoxylin and eosin. The histological typing of colonies was carried out by testing 3 or 4 sections from each spleen, 100 μm apart from each other.

Effect of OK-432 on the Irradiated Recipient Mice in Assessing the "f factor." The "f factor," defined as the fraction (f) of injected bone marrow CFU-S recovered from the spleen, was studied in an attempt to clarify the effect of OK-432 on the irradiated recipient mice in the exogenous CFU-S assay. Measurement of the "f factor" of CFU-S recovered from the spleen was done according to the secondary transplantation technique (17). Normal bone marrow cells (1 × 10⁷) were transplanted into the irradiated recipient mice given 1 KE/0.5 ml of OK-432 i.p. on Days -6, -5, and -4 before irradiation. Two hr later, CFU-S in the spleen recovered from the OK-432-treated recipient mice was examined by transplanting the spleen cells (1 × 10⁶) into the irradiated mice given no treatment.

Recovery of Granulocytes and Platelets in the Peripheral Blood after Irradiation. Five mice/group were given 1 KE/0.5 ml of OK-432 or 0.5 ml of 0.9% NaCl solution i.p. at various times (Chart 1) before or after 400 R total-body irradiation. Alterations in granulocyte and platelet counts in the peripheral

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³ The abbreviations used are: CFU-S, colony-forming unit-spleen; BCG, *Bacillus Calmette-Guérin*; CFU-C, colony-forming unit in culture.

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Table 1
Effect of OK-432 on hematopoietic spleen colonies in irradiated mice

The number of endogenous hematopoietic spleen colonies was counted on the eighth day after 610 R whole-body irradiation. Each arrow indicates i.p. administration of OK-432.

OK-432 treatment (KE)	Day of OK-432 administration																No. of colonies	No. of mice						
	-22	-21	-14	-13	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	+1	+2			+3	+4	+5	+6	+7	+8
1. 1					↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	9.0 ± 4.2 ^{a, b}	8
2. 1							↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	8.4 ± 5.6 ^b	10
3. 1	↓	↓									↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	6.0 ± 3.5 ^b	9
4. 1			↓	↓							↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	10.8 ± 7.9 ^b	10
5. 1							↓						↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	9.3 ± 4.3 ^b	10
6. 1														↓ ^c	↓	↓	↓	↓	↓	↓	↓	↓	10.1 ± 6.1 ^b	7
7. Irradiated control																							0.5 ± 0.9	10
8. 1											↓												6.8 ± 1.7 ^b	7
9. 1												↓											6.0 ± 4.1 ^b	6
10. 1													↓										5.2 ± 3.9	6
11. 1														↓ ^c									2.3 ± 3.7	8
12. 0.2											↓												1.8 ± 1.3	7
13. 0.2												↓											1.6 ± 1.0	7
14. 0.2													↓										1.2 ± 1.5	8
15. 0.2														↓ ^c									3.2 ± 3.5	8
16. Irradiated control																							0.4 ± 0.5	8

^a Mean ± S.E.
^b $p < 0.01$.
^c Administration of OK-432 i.p. within 2 hr after irradiation.

Table 2
Effect of OK-432 on the recipient mice for exogenous CFU-S assay

Normal bone marrow cell suspension ($5 \times 10^4/0.5$ ml) was transplanted into the irradiated recipient mice given 1 KE/0.5 ml of OK-432 or 0.5 ml of 0.9% NaCl solution i.p. at various times before irradiation. The number of exogenous hematopoietic spleen colonies was counted on the eighth day after 810 R whole-body irradiation. Each arrow indicates i.p. administration of 1 KE of OK-432 or 0.5 ml of 0.9% NaCl solution.

Treatment	Day of OK-432 administration								No. of colonies	No. of mice	
	-7	-6	-5	-4	-3	-2	-1	0			
1. 1 KE OK-432			↓	↓	↓				+ ^a	21.3 ± 6.7 ^{b, c}	12
2. 1 KE OK-432						↓			+	13.0 ± 5.1 ^d	7
3. 1 KE OK-432			↓					↓	+	11.7 ± 4.6 ^d	6
4. 1 KE OK-432			↓	↓	↓				-	0.4 ± 0.7	8
5. 0.9% NaCl solution			↓	↓	↓				+	5.9 ± 1.3	10
6. Irradiated control									-	0 ± 0	8

^a +, transplantation of normal bone marrow cells; -, no transplantation of normal bone marrow cells.
^b Mean ± S.E.
^c $p < 0.001$.
^d $p < 0.05$.

blood obtained by cutting the tail vein were examined until Day 20 after irradiation. Leukocyte and platelet counts were analyzed using a hemocytometer, while leukocyte differentials were analyzed using a cytosmear preparation of May-Grünwald-Giemsa.

RESULTS

Effect of OK-432 on Hematopoietic Spleen Colonies in Irradiated Mice. The number of endogenous CFU-S in 610 R irradiated mice given 0.2 or 1 KE of OK-432 i.p. at various times before and/or after irradiation is shown in Table 1. Irradiated controls gave rise to few endogenous CFU-S. In contrast, endogenous CFU-S increased significantly ($p < 0.01$) by the treatment with 1 KE of OK-432 both in successive injections and rechallenge groups. A single injection of OK-432 (1 KE) 2 and 3 days prior to irradiation also resulted in a significant increase in endogenous CFU-S. However, no significant increase was seen with a single injection of a low dose (0.2 KE) of OK-432 before or after irradiation.

Effect of OK-432 on the Recipient Mice for Exogenous CFU-S Assay. The number of exogenous CFU-S increased significantly as compared to the recipient control treated with 0.9% NaCl solution when normal bone marrow cells were

transplanted into the 810 R irradiated recipient mice treated previously with 1 KE of OK-432 (Table 2). Particularly, about a 4-fold increase of exogenous CFU-S should be noticed in the group treated with OK-432 for 3 successive days on Days -6, -5, and -4. A 2-fold increase was also observed in 2 groups, i.e., the group receiving a single injection on Day -2 and that of the 6-day interval rechallenge. Irradiated controls with or without OK-432 treatment gave rise to no CFU-S.

Effect of OK-432 on the Irradiated Recipient Mice in Assessing the "f factor." The "f factor" was studied by the secondary transplantation technique. As shown in Table 3, the "f factor" in normal recipient groups was 0.16 ± 0.04 . In contrast, that in the OK-432-treated recipient groups increased to 0.26 ± 0.08 , while there was no significant change in the number of cells recovered per spleen between the 2 groups.

Histology of Hematopoietic Spleen Colonies. Fifty to 150 spleen colonies/group were histologically examined (Table 4). Exogenous spleen colonies grown in the OK-432-pretreated recipient mice were mostly erythroid. However, megakaryocyte colonies were unexpectedly increased to about one-half of the endogenous spleen colonies formed in the OK-432-treated mice. This finding did not vary with the schedule of OK-432 administration.

Recovery of Granulocyte and Platelet Counts in the Peripheral Blood after Irradiation. Chart 1 indicates the change in granulocyte and platelet counts in the peripheral blood in each group after 400 R irradiation up to Day 20. An earlier recovery in the granulocyte counts after irradiation was observed in the OK-432-treated groups, as compared to the control. In the 6-day interval rechallenge group (Group C) and those injected after irradiation (Group D), the commencement of recovery was early; *i.e.*, increases were seen on Day 6 and, in Group C, achieved the highest level of over 7000/ μ l on Day 20. A single injection on Day -2 (Group B) showed earlier

recovery on Day 8, while granulocyte counts were not sufficiently restored on Days 16 and 20. However, such a recovery was not so marked. Particularly, granulocyte counts in the group given injections on Days -6, -5, and -4 (Group A) remained at the lower level until Day 12, despite a steep rise at the convalescent stage of Days 16 to 20. In contrast, an earlier recovery in the platelet counts was definite in the OK-432-treated groups (Chart 1). Platelet counts in the OK-432-treated groups were consistently higher throughout than those in the control. A transient rise in the platelet counts was seen on Day 2 in the group given injections after irradiation (Group D). Significant restoration began on Day 8 in Group C and on Day 12 in other OK-432-treated groups. In the OK-432-treated groups, platelet counts returned to the initial levels on Day 16, but remained at about half-level in the control. Particularly, Groups B and C showed an overshooting on Days 16 and 20. The 6-day interval rechallenge (Group C) was most favorable among the OK-432-treated groups for an early recovery in both the granulocyte and platelet counts.

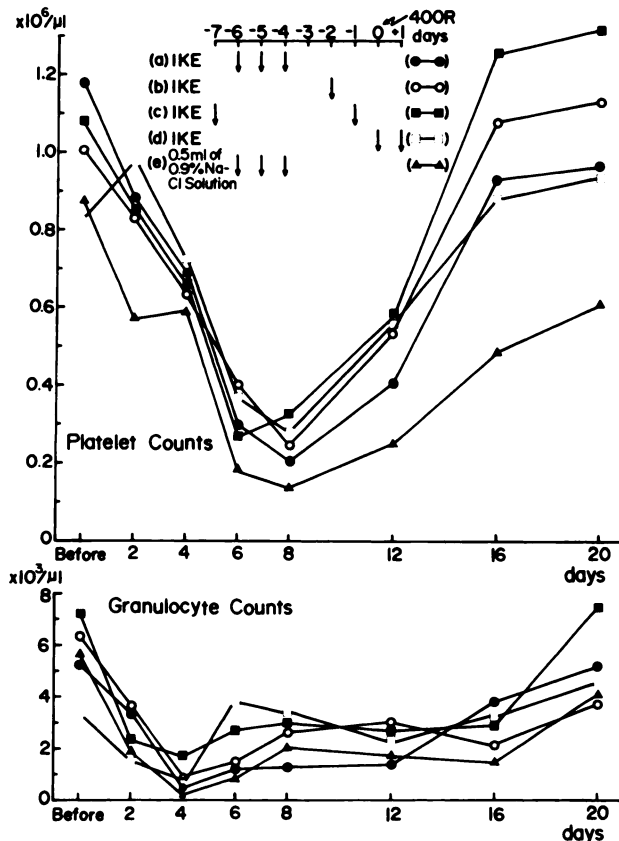


Chart 1. Recovery of granulocyte and platelet counts in the peripheral blood after irradiation. Five mice/group were given 1 KE/0.5 ml of OK-432 or 0.5 ml of 0.9% NaCl solution i.p. at various times before or after 400 R total-body irradiation. ●, injections of OK-432 on Days -6, -5, and -4 before irradiation (Group A); ○, on Day -2 (Group B); ■, on Days -7 and -1 (Group C); □, within 2 hr and on Day +1 after irradiation (Group D); ▲, injections of 0.9% NaCl solution on Days -6, -5, and -4 before irradiation (Group E).

DISCUSSION

The increase of endogenous CFU-S as reported here is nonspecific; *i.e.*, similar increases were observed in the irradiated mice treated with such immunostimulants as BCG (9) or *C. parvum* (10, 13), androgen (22) or testosterone, bacterial endotoxins (4, 5, 19), foreign plasma, vaccines, and erythropoietin (3, 12). However, the mechanism underlying an increase of endogenous CFU-S, although still ambiguous, is divergent among various influencing factors. There have been reports of the effect of 2 immunostimulants in clinical use, *i.e.*, *C. parvum* and BCG, on CFU-S. *C. parvum* evoked an increase of both exogenous and endogenous CFU-S in the spleen and peripheral blood (10), and a marked increase in the percentage of exogenous CFU-S in the S phase was noted after i.v. administration of BCG, despite no increase in the total number per femur (15). No appreciable increase in exogenous CFU-S was observed by the other agents. Previously, we found a slight increase in the total number of exogenous CFU-S in the femoral marrow and a marked one in the spleen following treatment with OK-432. This increase was particularly marked by i.p. administration of over 1 KE (6). Thus, the effect of OK-432 on CFU-S is more equivalent to that of *C. parvum*. Boggs *et al.* (1) suggested that endogenous CFU-S reflects a stem cell population of exogenous CFU-S in the cell cycle. The kinetics of CFU-S in response to various stimuli is complex, and the mechanism involved in the increase of endogenous

Table 3

Effect of OK-432 on the irradiated recipient mice in assessing the "f factor"

Normal bone marrow cells (1×10^7) were transplanted into the irradiated recipient mice given 1 KE/0.5 ml of OK-432 i.p. on Days -6, -5, and -4. Two hr later, CFU-S in the spleen ($CFU-S/5 \times 10^6$ transplanted bone marrow cells, 12.0 ± 1.63 ; total CFU-S transplanted/recipient mouse, 2400 ± 326.6) recovered from the OK-432-treated and normal control recipient mice was examined by transplanting the spleen cells (1×10^6) into the irradiated mice given no treatment.

	No. of CFU-S recovered from the spleens of normal and OK-432-treated recipient mice 2 hr after transplantation			
	No. of cells re-covered/spleen (mean of 8 mice)	CFU-S/ 1×10^6 recovered spleen cells	Total CFU-S recovered	f factor
Normal	8.94×10^7	4.25 ± 1.09^a	379.84 ± 97.39	0.16 ± 0.04
Treatment with OK-432	1.17×10^8	5.38 ± 1.58	626.88 ± 183.83	0.26 ± 0.08

^a Mean \pm S.E.

Table 4
Histology of hematopoietic spleen colonies

Endogenous CFU-S indicates hematopoietic spleen colonies formed in 610 R-irradiated mice treated with 1 KE OK-432, and exogenous CFU-S indicates colonies formed when normal bone marrow cells were transplanted into 810 R-irradiated recipient mice treated previously with OK-432. Various periods of time of i.p. administrations of OK-432 are shown in Tables 1 and 2.

Period of OK-432 treatment (days)	% of hematopoietic spleen colonies						
	E ^a	G	Mgk	Immature cells	E + G	E + Mgk	G + Mgk
Endogenous CFU-S							
1. -9 to +7	40.7	3.7	37.1	7.4	0	7.4	3.7
2. -7 to -0 ^b	38.1	28.6	23.7	4.8	4.8	0	0
3. -22, -21, -3 to -0	38.7	0	48.4	12.9	0	0	0
4. -14, -13, -3 to -0	26.2	21.4	33.3	4.8	0	14.3	0
5. -7, -1	24.1	8	54	2.3	1.3	8	2.3
6. +0 to +3	23.1	9.6	55.8	7.7	0	1.9	1.9
7. -3	13.5	2.7	56.8	10.8	0	16.2	0
8. -2	40.9	4.5	36.4	18.2	0	0	0
9. -1	22.5	9.7	58.1	6.5	0	3.2	0
10. +0	23.8	4.8	66.6	4.8	0	0	0
Exogenous CFU-S							
1. -6, -5, -4	63.1	10	7.7	14.6	1.5	2.3	0.8
2. -2	65.9	2.4	9.8	19.5	0	2.4	0
3. -7, -1	71.4	2.9	8.6	17.1	0	0	0

^a E, erythroid; G, granuloid; Mgk, megakaryocyte.

^b -0, shortly before irradiation on Day 0; +0, within 2 hr after irradiation on Day 0.

CFU-S remains to be clarified in reference to the altered radiosensitivity of CFU-S (13) or some activation of hematopoietic stromal microenvironment with OK-432 for lodging CFU-S, as described later.

Histological examination of endogenous spleen colonies disclosed a notable increase in the number of megakaryocytic colonies. Boggs *et al.* (2) reported an early rise in microscopic granulocytic spleen colonies by injection of 25 μ g *Salmonella typhosa* endotoxin just after 600 R irradiation. A consistent increase in megakaryocytic spleen colonies may be specific to OK-432 in reference to an earlier recovery of platelet counts after 400 R irradiation; however, the biological function of OK-432 as exerted on megakaryocytopoiesis remains unknown. We have no explanation why endogenous but not exogenous megakaryocytic spleen colonies are increased.

The effects of OK-432 on irradiated recipient mice in the exogenous CFU-S assay were investigated in an attempt to elucidate the interaction between stem cells and hematopoietic microenvironment. When normal bone marrow cells were transplanted into irradiated recipient mice previously given 1 KE of OK-432 i.p., the exogenous CFU-S increased significantly, as compared to the findings in the irradiated recipient mice given no treatment. Furthermore, an increase in the "f factor" indicates that more CFU-S lodged in the spleen of OK-432-treated recipient mice than in normal ones given no treatment. Thus, there appears to be some activation of the hematopoietic stromal microenvironment, e.g., tissue macrophages, with OK-432 favorable for lodging CFU-S. Endogenous CFU-S was not detected when the bone marrow cells were not transplanted into the 810 R irradiated mice treated previously with OK-432. We have also found that bone marrow CFU-S was sustained longer *in vitro* on the peritoneal exudate cells, largely consisting of macrophages, activated with OK-432, than on the nonactivated ones (7). In contrast, there was no significant difference in sustaining CFU-C *in vitro* on the activated or nonactivated peritoneal exudate cells. These *in vitro* findings are compatible

with the increased *in vivo* effect of OK-432 on irradiated recipient mice for exogenous CFU-S assay. Thus, the increase of endogenous CFU-S by an immunostimulant, OK-432, can probably be ascribed to a substantial increase of CFU-S itself with an activated hematopoietic stromal microenvironment favorable for lodging CFU-S, regardless of whether the occurrence is cell to cell or cell to humoral factor interaction.

An apparent earlier recovery of platelet counts in the OK-432-treated mice after 400 R irradiation may reflect increased endogenous megakaryocytic spleen colonies. However, granulocyte counts in the group given injections on Days -6, -5, and -4 (Group A) were lower than in the control during the period of 12 days after irradiation, which did not reflect a 4-fold increase of exogenous CFU-S. A recovery of granulocyte counts may be modified by some complex mechanism in Group A during the acute stage after irradiation. An earlier recovery of granulocyte counts in the peripheral blood after myelosuppression has also been found in mice treated with other agents (9, 18, 22). Earlier recovery of granulocyte counts in other OK-432-treated groups, although not as marked, may be due to an earlier recovery of the less damaged stem cells in relation to an activated hematopoietic microenvironment, earlier recruitment of CFU-C to granulocytes by elevated colony-stimulating factor (6), and/or mobilization of granulocytes from the marginal pool to the circulation.

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A. Hiraoka et al.

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