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# Effect of *Corynebacterium parvum* Stimulation on Granulopoiesis

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**Abstract**—*The intravenous administration of killed* Corynebacterium parvum (C. parvum) *into C57Bl mice lead to a significant granulocytopenia within* 7–15 min *after the injection, which was followed by a striking granulocytosis at* 2 hr. *A second granulocytopenic phase appeared at* 12 hr, *while normal blood neutrophil counts were observed at* 24 hr. *The bone marrow granulocyte reserves (MGR) decreased dramatically within* 2 hr. *A significant decline in the number of band cells and metamyelocytes was noted at* 12–72 hr *and of myelocytes (MC) at* 24 hr. *The number of promyelocytes (PMC) and myeloblasts (Bl) presented a significant rise at* 24–72 hr *after the injection of* C. parvum. *The labelling indexes were found to be increased at* 12–24 hr *in both MC and Bl-PMC compartments. The number of granulocyte–macrophage progenitor cells (GM–CFC) increased significantly at* 24–28 hr. *The proportion of DNA-synthesizing GM–CFC was also found to be elevated* 12–24 hr *after the injection of the bacterium. The sequence of events which were observed in these experiments indicates that the effect of* C. parvum *on granulopoiesis starts with a rapid release of MGR into the bloodstream and is followed by an acceleration of proliferation concerning simultaneously both differentiated and committed stem cell compartments of granulocytic series.*

## INTRODUCTION

One of the major problems which appear in the immunotherapy of cancer with killed *Corynebacterium parvum* (*C. parvum*) is the determination of the optimal timing of its administration in relation to the application of chemotherapy. This difficulty is probably due, at least in part, to some biological effects of the bacterium on normal bone marrow hemopoietic cells. Studies from several laboratories have already shown that the i.v. or i.p. injection of *C. parvum* into mice leads to significant changes in both pluripotent hemopoietic stem cell (CFUs) [1] and committed granulocyte–macrophage progenitor cell (GM–CFC) [2-7] compartments. The proportion of DNA-synthesizing CFUs and GM–CFC has also been found to be significantly increased after *C. parvum* stimulation [1, 8]. However, the effect of the bacterium on the various morphological compartments of bone marrow hemopoietic cells is not well known.

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The purpose of the present paper is to describe the alterations in granulopoiesis which were observed in the mouse after a single i.v. injection of *C. parvum*. The peripheral blood neutrophil counts, the bone marrow cellularity and the size and turnover of neutrophil precursor cells are studied in relation to the changes of GM–CFC after this single injection of the bacterium.

## MATERIALS AND METHODS

Adult C57Bl male syngeneic mice, 8–10-week-old, were used in all experiments. The animals were injected i.v. with 548 μg of killed *C. parvum* (Lot 0407, Mérieux Lab.) or with 10 μg of *E. coli* endotoxin (lipopolysaccharide W of 055:B5 strain, Difco Lab.) and studies were done at varying times thereafter.

Peripheral blood was obtained from the inner angle of the eye. The blood was collected directly in a leukocyte pipette and diluted 1:20 in Turk's solution. The total leukocyte counts were measured in a Malassez hemocytometer. Differential leukocyte counts were done by scoring at least 150 cells in each of the May–Grünwald–Giemsa stained blood smears.

Bone marrow cell suspensions were prepared by perfusing one femur with 1 ml of single-strength modified Eagle's medium [9]. The nucleated cell concentrations were measured using the same method as in the blood leukocytes. Marrow smears were prepared from the other femur and differential counts were done by scoring at least 500 cells in each of the May–Grünwald–Giemsa stained slides. The morphologic criteria for the classification of mouse bone marrow cells have been described in detail elsewhere [10].

Autoradiography was used to study the turnover of bone marrow cells in normal and in *C. parvum*-treated mice. The animals were injected i.p. with 1 $\mu$Ci/g body wt of tritiated thymidine ($^3$H-TdR) and were sacrificed 1 hr later. Smears taken from the femoral marrow were fixed in methanol, dipped in Ilford emulsion and exposed for 3 weeks at 4°C. Subsequently, they were developed in Kodak D-19b solution and stained with Giemsa. Labelling indexes on the various morphological compartments were determined by scoring at least 100 labelled cells per slide. Labelled cells were defined as cells presenting 4 or more grains on the nucleus.

The number of GM–CFC per femur was evaluated using the double-layer soft agar culture technique of Bradley and Metcalf [11] slightly modified. The proliferative status of GM–CFC was estimated using high doses of hydroxyurea (HUR) according to the method proposed by Rickard *et al.* [12]. Both methods have been exposed in detail elsewhere [10].

Stitistical analysis was performed according to variance analysis ($F$ value) and Student's $t$-test.

## RESULTS

Figure 1 shows the changes in peripheral blood neutrophil counts. A significant decrease in the number of circulating neutrophils was observed 7–15 min after the injection of the bacterium. This granulocytopenia was followed by a striking granulocytosis 2 hr later. A second granulocytopenic phase appeared in the animals 12 hr after the injection, while normal values in blood neutrophil counts were noted at 24 hr and thereafter.

The changes in the number of nucleated cells per femur are presented in Fig. 2. A significant decline in bone marrow cellularity was observed as early as 2 hr after *C. parvum* reaching a nadir at 24 hr. It is evident that this decrease was due to the diminution of granulocyte elements and, to a lesser degree,

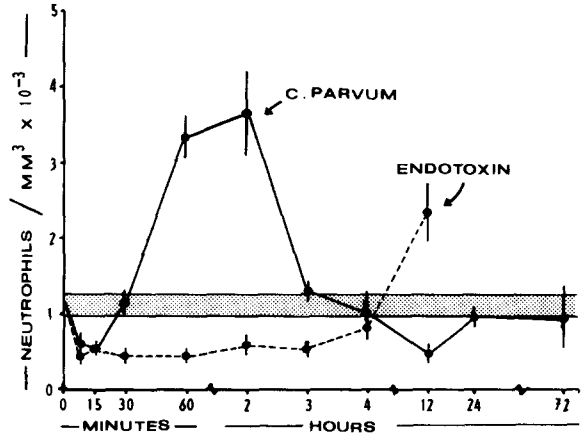


*Fig. 1. Changes in peripheral blood neutrophil counts after* C. parvum *or endotoxin treatment. The values, derived from 3–7 experiments with 3–5 mice in each experiment, are expressed as means* ± S.E. *Hatched area indicates control values* (2 S.E.).

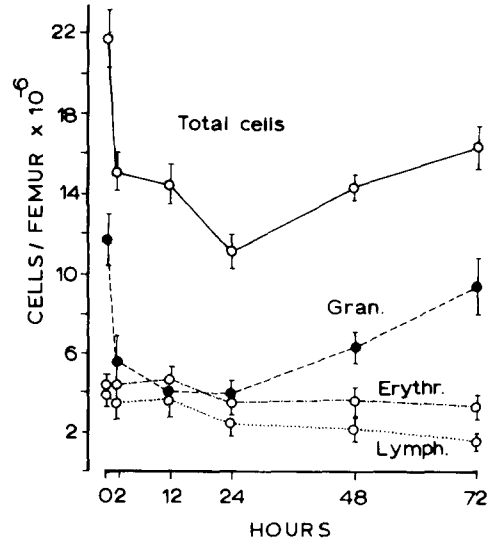


*Fig. 2. Changes in the numbers of bone marrow granulocytic cells, erythroblasts and lymphocytes in relation to the total number of nucleated cells per femur after* C. parvum *stimulation. Values expressed as means* ± S.E.

to the progressive fall of lymphocytes. The erythroblast counts presented only a slight and statistically insignificant decline.

The changes in the various morphological compartments of bone marrow granulocytic series are presented in Fig. 3. A statistically significant increase in the number of myeloblasts and promyelocytes was noted at 24, 48 and 72 hr and also in the number of promyelocytes at 12 hr. The number of myelocytes remained in the control range 2–12 hr after *C. parvum*, decreased at 24 hr and reached normal values at 48 hr. Dramatic

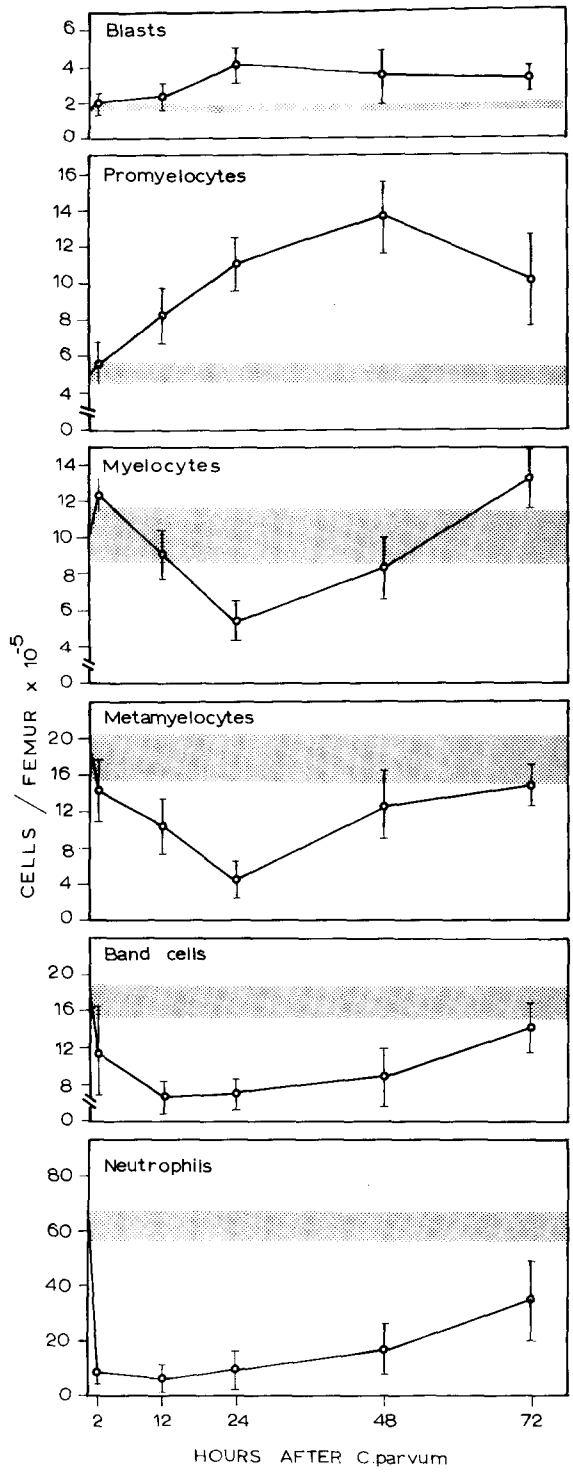


*Fig. 3. Alterations in the various morphological compartments of femoral granulocytic cells after* C. parvum *treatment. The data are derived from 3–7 experiments with 3–5 mine in each experiment, and are shown as means ± S.E. Hatched areas indicate corresponding control values* (2 S.E.).

changes were observed in the number of mature bone marrow neutrophils which were found to be 7.5 times lower than control values at 2 hr. This decrease persisted in varying levels until the 72nd hr. The number of band cells and metamyelocytes presented a significant decline 12–48 hr after the injection of *C. parvum*. An inverse statistically significant correlation was noted at 2 hr between circulating and bone marrow band cells and mature neutrophils (Fig. 4).

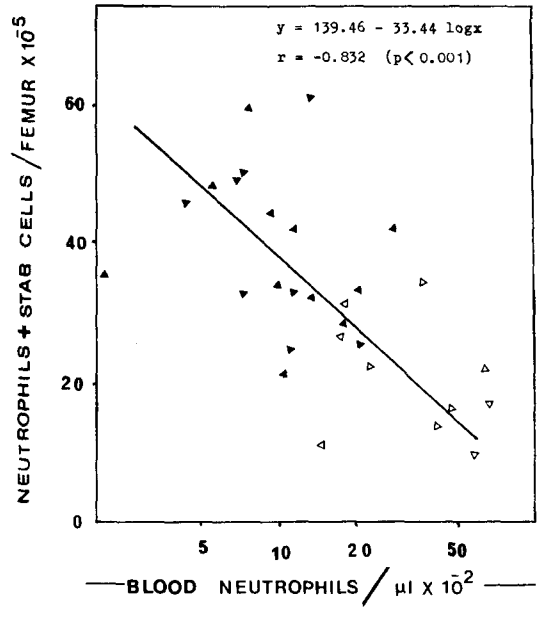


*Fig. 4. Relationship between the number of femoral band cells and mature neutrophils and the number of circulating granulocytes. Open triangles indicate animals treated with* C. parvum 2 hr *before blood and bone marrow cell collection. and closed triangles indicate normal untreated animals.*

The changes in the labelling indexes of differentiated granulocytic elements are given for the blast–promyelocyte (Bl–PMC)* and myelocyte (MC) compartments in Fig. 5. There was a significant increase in the labelling indexes of Bl–PMC at 12–24 hr ($P<0.05$ and $P<0.01$, respectively) and of MC compartment at 12, 24 and 48 hr ($P<0.01$, $P<0.001$ and $P<0.01$, respectively). The grain count distribution in the Bl–PMC and MC are presented in Fig. 6. An increased proportion of heavily labelled cells (more than 20 grains per nucleus) was observed in both Bl–PMC ($P<0.001$) and MC ($P<0.01$) compartments at 24–48 hr.

Figure 7 shows the alterations in the number and proliferative status of femoral GM–CFC. An increase in the absolute number of GM–CFC per femur was observed at 24–48 hr. The proportion of DNA-sythesizing GM–CFC was found to be increased as early as 12 hr after the injection of the bacterium ($P<0.05$).

*Because of some difficulties in the recognition of cells the Bl and PMC are grouped in one compartment, the Bl-PMC.

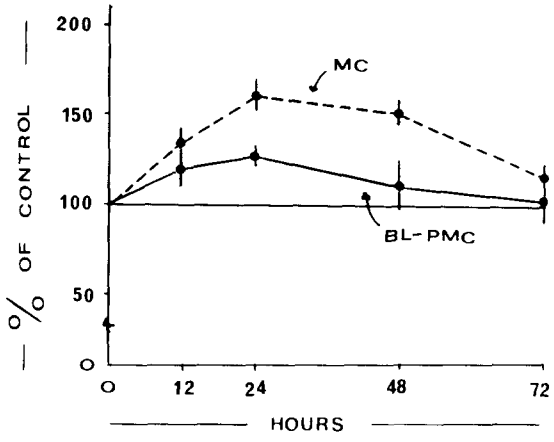


*Fig. 5. Labelling indexes in Bl–PMC and MC compartments after* C. parvum *administration. Values expressed as percentage of control* ± S.E. *Control labelling index for Bl–PMC was* 54.75 ± 2.25% *and for MC was* 38.25 ± 1.32%. *Each point represents data from four experiments with three mice in each experiment.*

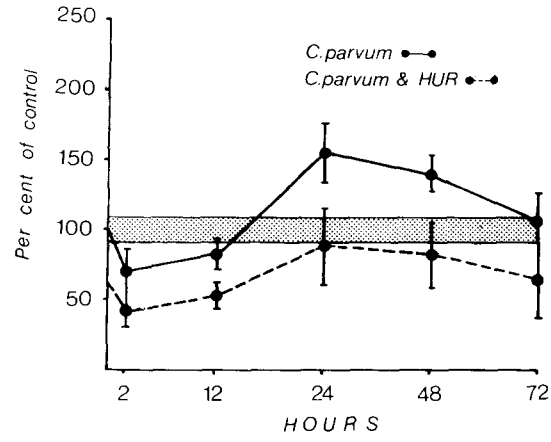


*Fig. 7. Femoral GM–CFC studied at varying times after* C. parvum *or* C. parvum *plus hydroxyurea treatment. The values are expressed as percentage of the control* ± 1 S.E. *Each point represents data from 3–7 experiments with three animals in each experiment. Hatched area indicates control values* (9580 ± 760 *GM–CFC per femur) considered as* 100% (2 S.E.).

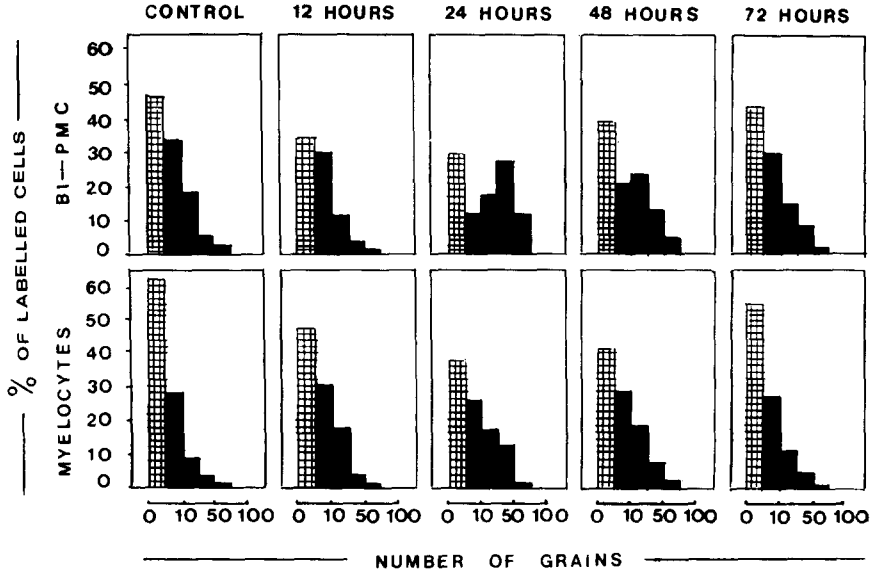


*Fig. 6. Grain count distribution in Bl–PMC and MC compartments at varying time points after* C. parvum *stimulation. Data resulted from four experiments at each time point.*

## DISCUSSION

The experimental data presented here indicate that the i.v. administration of killed *C. parvum* into mice leads to important changes in granulopoiesis. It seems that these changes start with a rapid release of marrow granulocyte reserves (MGR) into the bloodstream and are followed by an accelerated proliferation in both committed stem cells and differentiated cells of granulocytic series.

Although the mechanisms involved in the regulation of granulopoiesis are not well known yet, one could discuss the possible mode of action of *C. parvum*. The rapid release of MGR into the bloodstream could be attributed to humoral releasing factors liberated in the circulation during the early granulocytopenic phase induced by *C. parvum* 7–15 min after the injection. The existence of such humoral factors for neutrophils is actually well documented [13, 14]. Previous studies in our laboratory offered some evidence that a neutrophil releasing activity has to be present in the serum of *C. parvum*-treated mice shortly after the injection of the bacterium [10]. On the other hand, the mentioned above early

granulocytopenic phase is probably due, at least in part, to the accelerated ingestion of injected bacteria by circulating neutrophils and the subsequent migration or sequestration of these cells into the various organs and tissues. Fifteen min after the injection of *C. parvum* almost all circulating neutrophils were seen to have ingested one or more bacteria. This rapid ingestion might be explained by the fact that *C. parvum* can generate chemotactic factors for neutrophils by activating complement through the alternative pathway [15].

The profound decrease in bone marrow cellularity and the striking granulocytosis which was observed 1–2 hr after the injection of *C. parvum* could be attributed to the evacuation of MGR into the blood circulation. A 50% decline in femoral cellularity after *C. parvum*-treatment has also been reported recently by MacVittie [6].

The stimulatory effect of *C. parvum* on differentiated and committed stem cell compartments is indicated by a significant increase in the number of GM–CFC and proliferating cells of granulocytic series, and also by an increase in the labelling indexes for the Bl-PMC and MC compartments, and in the proportion of DNA-sythesizing cells for the GM–CFC compartment. A shortening of the generation time of proliferating granulocytic cells could be assumed from the pattern of grain count distribution of thymidine-labelled cells. It is interesting that in this stimulation of granulocytic precursor cells by *C. parvum* there is no apparent wave of proliferation beginning from GM–CFC and resulting to MC, but it seems that all compartments present a simultaneously appearing increase of proliferation which starts a few hours after the diminution. of MGR.

The mechanism by which *C. parvum* stimulates granulopoiesis has not been elucidated. Nevertheless, a direct effect of the bacterium on the granulocytic precursor cells seems to be unlikely, while the small amounts of circulating colony-stimulating activity (CSA) which were observed 12 hr after the injection [10] cannot certainly explain the profound changes in granulopoiesis induced by *C. parvum*. Increased serum CSA levels for up to 14 days after *C. parvum* have been reported recently by Foster *et al.* [5], but studies from other laboratories did not reveal any significant changes in serum CSA after *C. parvum* stimulation [2, 6, 16]. If CSA is really prerequisite for granulopoiesis *in vivo*, one could assume that in the case of *C. parvum*, an increased production of this stimulatory material into the bone cavity has to take place just after the migration of MGR into the bloodstream.

All these alterations in granulopoiesis induced by *C. parvum* might have some value when a combined chemo-immunotherapy schedule has to be applied. Indeed, Foster [8] presented experimental data indicating that *C. parvum* sensitises hemopoietic system to 5-fluorouracil. An increased sensitivity of bone marrow GM-CFC to methotrexate and cytosine arabinoside after *C. parvum* has also been observed recently in our laboratory [17].

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