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Effect of *Corynebacterium Parvum* on Bone Marrow Cell Cultures (38557)

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Bone marrow progenitors can proliferate *in vitro* culture forming distinct colonies containing granulocytic and monocytic cells (1–4). A colony stimulating factor (CSF) is essential for the *in vitro* survival of the proliferating hematopoietic precursors which have the potential of forming colonies (3–5). CSF has been extensively studied and its biological activity and biochemical properties have been established (5–7). The variations in the activity of the factor have been described as depending on the pathological or experimental conditions (4, 8, 9).

In the present study evidence is presented from experiments in mice that *Cornynebacterium parvum* exercises a stimulatory effect on the proliferating precursor cells and an inhibitory effect on the CSF.

Material and Methods. Adult C57Bl mice (10–12 wk old) were used in the experiments.

Experimental groups. The animals were divided into four experimental groups: Group I—Serum and bone marrow specimens were collected 4 hr after injection of *Corynebacterium parvum* (one injection 500 μg ip); Group II—Serum and bone marrow specimens were collected 24 hr after injection of *C. parvum* (one injection 500 μg ip); Group III—Serum and bone marrow specimens were collected 48 hr after the first injection of *C. parvum* (two consecutive injections 500 μg ip, the second injection applied after 24 hr); Group IV—Serum and bone marrow specimens were collected 72 hr after the first injections (three consecutive injections 500 μg ip applied in interval of 24 hr); Group V—Control.

Procedures. Mice anesthetized with nembutal (1.5 mg in 0.20 mg saline ip), were bled through the angle of the eye followed by intracardiac aspiration. The bone marrow was obtained by the perfusion of the femur and suspended in Eagle's medium. The blood was centrifuged and serum separated and dialyzed for use as a source of CSF.

The bone marrow cultures were performed using the agar culture technique as previously described (2, 3). The cultures were made in duplicate using 35 mm plastic petri dishes (Falcon plastics, Los Angeles). Each petri dish contained 1×10^5 nucleated cells/ml and 0.1 ml pooled serum/ml. After 7 days incubation at 37° in humidified 5% CO_2 in air, the colony was counted using a dissecting microscope with indirect illumination.

Each experiment consisted of four combinations: (a) Bone marrow and serum from nontreated control animals); (b) bone marrow from control animals and serum from animals treated with *C. parvum*; (c) bone marrow from animals treated with *C. parvum* and serum from control animals); (d) bone marrow from animals treated with *C. parvum* and serum from animals treated with *C. parvum.*

The serum was passed through a millipore filter and standardized with mouse bone marrow against a specimen of mouse CSF kindly supplied by Dr. D. Metcalf of the Walter and Eliza Hall Institute, Melbourne, Australia.

Small clusters of less than 50 cells were not counted as colonies. Colonies were counted on duplicate plates and the colony value expressed as a mean of the two counts.

Results. For the determination of the optimal experimental conditions, we performed preliminary studies using various concentrations of mouse serum as a source of CSF and various numbers of bone marrow cells. Our results indicated that the greatest number of colonies were obtained when 0.1 ml

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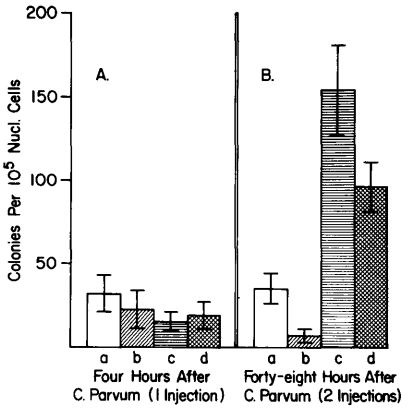

FIG. 1. Diagram of distribution of colonies during bone marrow cultures using different conditions: (a) Normal bone marrow cells and normal serum; (b) normal bone marrow cells and serum from animals previously injected with *C. parvum*; (c) bone marrow cells from animals previously injected with *C. parvum* and normal serum; (d) bone marrow cells and serum from animals previously injected with *C. parvum*. The results represent mean ± SD from four experiments.

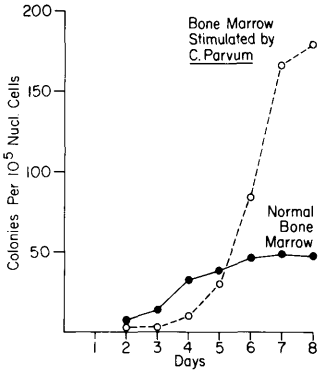

FIG. 2. Distribution of colonies counted 7 consecutive days. The results represent the mean of three experiments.

serum and 1×10^5 cells per ml of culture medium was used. These amounts were used as a standard procedure in all experiments.

The results from the first group showed that there is no significant effect of *C. parvum* on bone marrow cell cultures and CSF when the specimens are taken 4 hr after the injections of the vaccine. Colony-forming capacity in all experimental subgroups was similar and did not differ from the control subgroup (Fig. 1 A).

The results from the second group (Fig. 1 B) indicated that serum from mice injected twice with 500 μg *C. parvum* during 48 hr, inhibited the colony formation of normal mouse bone marrow. Normal mouse serum used as a source of CSF stimulates significantly colony formulating capacity of bone marrow previously injected twice with *C. parvum* (Fig. 1 A–c). Similar stimulation but to a lesser extent was also observed when the serum and the bone marrow were collected from mice previously injected with *C. parvum* (Fig. 1 A–d).

The relationship between the time and appearance of colonies is presented in Fig. 2. Normal bone marrow cultures with normal serum as a source of CSF is compared to bone marrow obtained from mice previously treated with two 500 μg injections of *C. parvum* during a 48-hr period with 24-hr intervals between the two injections. As shown in Fig. 2, the colony-forming capacity of the normal bone marrow was slightly increased during the third and fourth day of the culture compared to the marrow stimulated by *C. parvum*. After the fifth day the colony count was identical and the number of colonies in the normal bone marrow cultures showed no significant change up to the last day of the culture. The bone marrow cultures from mice injected with *C. parvum* showed progressive increase in the colony-forming capacity and at the seventh day the colony count revealed a threefold increase.

Discussion. The enhancing effect of some antigens on *in vitro* colony formation by normal bone marrow cells mediated through CSF has been previously reported (9–11). Bacterial endotoxin, heterologous erythrocytes and bacterial flagellin preparations can stimulate the colony-forming capacity in cultures of normal mouse bone marrow (9). Usually, the effect of endotoxin and related antigen is upon the CSF (11) showing a rise in the colony stimulating activity 4–6 hr after a single intravenous injection.

The results of our experiments showed that a single ip injection of *C. parvum* had no effect either on CSF, or on bone marrow cells. This indicates that the mechanism of action of *C. parvum* on CSF is different than that of endotoxin and the other antigens capable of stimulating CSF (9–11). Moreover, the delayed effect of *C. parvum* on CSF

appears to be inhibitory rather than stimulatory (Fig. 1 B–b). The most important result of our studies is the enhancement of the colony forming capacity of the bone marrow by *C. parvum*. Since the marrow culture contains normal mouse serum as a source of CSF and bone marrow cells from mice pretreated with *C. parvum*, one may conclude that the effect of *C. parvum* is on the precursor cells capable of forming colonies in culture medium. The colonies consist of granulocytes and monocytes with a preponderance of the latter type. The representatives of all granulocyte precursors were present. The stimulation of colony formation by *C. parvum* is a time related function and occurred 5 days after the initiation of the cultures (Fig. 2). This indicates that colony-forming cells require a certain time for adaptation to the culture conditions. At this time it is difficult to speculate on the stimulation of the bone marrow by *C. parvum* which may occur *in vivo*. However, Dazord *et al.*, reported that *C. granulosum* which has similar properties as *C. parvum* (13) is capable of stimulating colony formation in the spleen of mice previously treated with the immunostimulant (12). The stimulatory effect reported by these authors began at the second day after the injection of *C. granulosum*. These results and the results of our study indicate that corynebacteria appear to be stimulants of bone marrow cells capable of proliferation. This further suggests that *C. parvum* may be used as a valuable adjuvant during the radiation or chemotherapy which are well known as bone marrow depressants. Since *C. parvum* has practically no side effects its therapeutic implication as a bone marrow protector could be of great value. Further studies are necessary for thorough evaluation of the stimulatory effect of *C. parvum* on bone marrow cells *in vitro* and *in vivo*, including the effect of BCG and endotoxin.

Summary. The effect of *C. parvum* on *in vitro* colony-forming cell was evaluated. A single ip injection of the vaccine had no effect during the first 4 hr either on colony stimulating factor or on colony-forming cells. The enhancement of the colony-forming capacity of the bone marrow by *C. parvum* occurred after two injections of the vaccine within 48 hr. The stimulation of colony formation by *C. parvum* was time related function and occurred 5 days after the initiation of the cultures.

The results of this study indicate that *C. parvum* appears to be a stimulant of bone marrow cells capable of proliferation and may be used as a valuable adjuvant against myelosuppressive agents.

1. Bradley, T. R., and Metcalf, D., Aust. J. Exp. Biol. Med. Sci. **44,** 287 (1966).
2. Metcalf, D., J. Cell. Physiol. **74,** 323 (1969).
3. Paran, M., and Sach, L., J. Cell Physiol. **72,** 247 (1968).
4. Quesenberry, P., Morley, A., Stohlman, R. J., Richard, K., Howard, D., and Smith, M., N. Engl. J. Med. **286,** 227 (1972).
5. Metcalf, D., J. Cell Physiol. **76,** 89 (1970).
6. Stanley, E. R., Robinson, W. A., and Ida, G. I., Aust. J. Exp. Biol. Med. Sci. **46,** 715 (1968).
7. Metcalf, D., J. Cell Physiol. **76,** 89 (1970).
8. Robinson, W., Metcalf, D., and Bradley, T. R., J. Cell Physiol. **69,** 83 (1967).
9. McNeill, T. A., Immunology **18,** 39 (1970).
10. McNeill, T. A., Immunology **18,** 61 (1970).
11. Metcalf, D., Immunology **21,** 474 (1971).
12. Prevot, A., "Manual for the Classification and Determination of the Anerobic Bacteria", 1st Amer. Ed., pp. 349 Lea and Febiger, Philadelphia (1966).
13. Dazord, L., Toujaa, L., Ramee, M. P., and Guelfi, J., Ann. Immunol. **124C,** 375 (1973).
14. Israel L., and Halpern, B., Nouv. Presse, Med. **1,** 19 (1972).

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