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Modulation of Myelopoiesis In Vivo by Chemically Pure Preparations of Cell Wall Components from Gram-Negative Bacteria: Effects at Different Stages

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Modulation of myelopoiesis by chemically pure preparations of different cell wall components from gram-negative bacteria was investigated in vivo. The effects of lipid A, outer membrane lipoprotein, and murein were evaluated at several distinct stages: induction of colony-stimulating activity (CSA) in the serum, increase in the number of committed splenic precursor cells (CFU-C) forming granulocyte-macrophage colonies in vitro, and triggering into the cell cycle of noncommitted hemopoietic stem cells (CFU-S) from bone marrow. The results reveal different patterns of activity of the bacterial cell wall components (BCWC) tested. (i) In C57Bl/6 mice and C3H/Bom mice, all three preparations were potent inducers of CSA. In C3H/HeJ mice, CSA was only induced by lipoprotein and murein and not by lipid A. After injection of lipid A or lipoprotein, but not murein, the number of CFU-C in spleens of C57Bl/6 mice was increased up to 100-fold. In C3H/Bom and C3H/HeJ mice, not only murein but also lipoprotein were much less potent in this respect. (iii) In C57Bl/6 mice, both lipid A and lipoprotein, but not murein, were capable of inducing the proliferation of CFU-S, as demonstrated by a hot thymidine cytocide technique. Thus, induction of CSA and changes in the pool size of splenic CFU-C after administration of BCWC may be unrelated events. On the other hand, the increase of CFU-C might reflect the mitogenicity of BCWC for CFU-S.

Myelopoiesis is known to be profoundly influenced by many bacteria and bacterial constituents (15). The effects of these agents are complex and may be produced at different levels of cell differentiation. Thus, in vivo bacterial lipopolysaccharide (LPS) was reported to affect myelopoiesis in several ways. First, it raises the serum and tissue levels of colony-stimulating activity (CSA) (14), i.e., the specific growth factor for committed progenitor cells of granulocytes and macrophages (CFU-C). Second, it increases the size of the CFU-C pool in bone marrow and even more so in the spleen (12). Moeover, LPS was shown to alter the cell cycle status of pluripotent hemopoietic stem cells (CFU-S) (8). However, the interrelationship of these phenomena has not yet been clarified.

Modulation of myelopoiesis by LPS may play a major role in the response to infection with gram-negative bacteria. Indeed, Quesenberry et al. suggested that under physiological conditions also, myelopoiesis is regulated symbiotically by the host's own microbiological flora via endotoxemia (18). It is, nevertheless, very unlikely that such regulatory effects are solely mediated by LPS. In fact, LPS-resistant C3H/HeJ mice, which exhibit no major defects in their antibacterial defenses or in their granulocyte and macrophage complement, fail to respond with elevated levels of serum CSA or an increased number of splenic CFU-C after administration of LPS (2).

In this context, it appeared important to compare the influence of chemically pure preparations of structurally unrelated bacterial cell wall components (BCWC). Three constituents from gram-negative bacteria were chosen for the present study: lipid A, which was previously reported to represent the active part of LPS with respect to myelopoiesis also (1), outer membrane lipoprotein, and murein.

In this communication we describe the effects of these constituents on the production of CSA, the size of the splenic CFU-C pool, and the proliferation of CFU-S. It was found that all three BCWC are powerful modulators of myelopoiesis, although they differ from each other with respect to their relative potency in the different systems. Moreover, no quantitative correlation could be found between CSA induc-

tion and the increase in splenic CFU-C. However, the size of the CFU-C pool appeared to be correlated with the cell cycle status of CFU-S.

MATERIALS AND METHODS

Preparation of BCWC. The heptoseless mutant strain *Salmonella minnesota* R595 was obtained through the courtesy of Otto Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, W. Germany. Endotoxic glycolipid from the R-form strain *S. minnesota* R595 (lipid A) was isolated by the phenol-chloroform-light petroleum ether procedure and checked for purity according to Galanos et al. (9). The protein content of the preparation was below 0.5%. Murein-lipoprotein complex and murein-free lipoprotein from *Escherichia coli* B were prepared as described by Braun and co-workers (6, 7). The lipoprotein was essentially free of lipid A, as shown by the absence of glucosamine in the lipoprotein hydrolysate. Purified murein was prepared as follows: 1 g of murein-lipoprotein complex from *E. coli* B was suspended in 200 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride-0.001 M $CaCl_2$ (pH 8) and digested with 20 mg of Pronase at 40°C for 24 h. Eight grams of sodium dodecyl sulfate was added, and the suspension was stirred at 90°C for 30 min. After cooling, the murein was sedimented by centrifugation at 25,000 × *g* for 2 h at 20°C. The sediment was washed twice with water and resuspended in 200 ml of hot (90°C) 4% sodium dodecyl sulfate solution. The suspension was stirred while cooling and centrifuged again as above. The sediment was washed four times with water and successively extracted with organic solvents in the following sequence: methanol, methanol-chloroform (2:1 and 1:2), chloroform, light petroleum ether, and acetone. The residue was suspended in water and freeze-dried. The absence of lipid A, lipoprotein, and phospholipids was shown by gas-liquid chromatographic analysis of fatty acids in a hydrolysate of the murein. Briefly, 20 mg of murein was hydrolyzed; 0.1 mg of pentadecanoic acid was added as internal standard. Based upon a comparison with the internal standard, the level of fatty acids in the murein preparation was below 0.05%. In an additional step, this preparation was subjected to alkaline hydrolysis. An alkaline hydroxylamine solution was prepared according to Snyder and Stephens (21): 3 volumes of 2.5% hydroxylamine hydrochloride and 4 volumes of 2.5% sodium hydroxide, both in 95% ethanol, were mixed, and the sodium chloride was removed by centrifugation. The hydroxylaminolysis was carried out as follows: 500 mg of purified murein was suspended in 100 ml of alkaline hydroxylamine solution, and the suspension was stirred under nitrogen for 1 h at room temperature. The murein was sedimented by centrifugation and washed three times with 95% ethanol, once with 0.02 N acetic acid in 95% ethanol, and once with 95% ethanol. The residue was suspended in water and lyophilized.

Mice. Specific-pathogen-free mice, strains C57Bl/6, C3H/Bom, and C3H/HeJ, were purchased from Bomholtgård, Ry, Denmark, and maintained in a barrier-sustained isolation unit. All mice were males and weighed 18 to 22 g when used.

Preparation of MLCM. Standard mouse lung-conditioned medium (MLCM) was prepared according to the procedure described by Sheridan and Metcalf (19) and used as source of reference CSA. Briefly, C57Bl/6 mice were injected intraperitoneally with 25 μg of LPS and killed after 1 h. After excision of the lungs under standard aseptic conditions, clotted blood was removed, the lungs were washed in minimal essential medium (MEM) modified as specified below, and each pair of lungs was incubated after dissection into small pieces in 5 ml of modified MEM for 72 h at 37°C and 10% CO_2 in air in a humidified incubator. Sterile conditioned medium was pooled and spun for 5 min at 200 × *g*, and the supernatant was heated for 30 min at 56°C and centrifuged for 15 min at 4°C and 11,000 × *g*, the final supernatant being passed through a sterile 0.45-μm filter (Sartorius, Göttingen, Germany) and stored in aliquots at −20°C. The same procedure was used for preparation of MLCM from BCWC-treated C57Bl/6 mice.

Induction of serum CSA and splenic CFU-C. Mice of different strains were injected intraperitoneally with graded doses of the BCWC suspended in sterile, pyrogen-free, distilled water, containing 0.9% sodium chloride. The injection volume was 0.01 ml/g. For determination of serum CSA, mice were bled from the orbital plexus under ether anesthesia at different intervals. To remove probably nonspecific serum-lipoprotein inhibitors of CSA (17), 1 volume of serum was mixed with 4 volumes of chloroform and shaken for 2 min. The mixture was allowed to stand at room temperature for 60 min, and the thin surface layer of fat was discarded. The second layer was pipetted off and centrifuged at 5,000 × *g* for 20 min at 4°C. The extracted serum, forming a clear supernatant, was left overnight at 4°C and then tested for CSA (10). For evaluation of splenic CFU-C, mice were killed 6 days after injection of BCWC by cervical dislocation. The spleens were dissociated by teasing with sterile forceps in modified MEM. After dispersion of the cells with a Pasteur pipette, the remaining clumps were allowed to settle, and the cells in the supernatant were used without further manipulation.

Cell culture procedures. Bone marrow cells (BMC) were flushed from the femurs of mice with modified MEM (1 ml/femur). For determination of CSA in MLCM, 10^5 nucleated BMC were incubated in 35-mm plastic petri dishes in 1 ml of modified MEM, containing 15% horse serum, 0.8% methylcellulose, and 5% MLCM (11). For quantification of splenic CFU-C, usually 5×10^5 spleen cells were incubated under the same conditions, but with 5% standard MLCM. For quantification of serum CSA, sera were incubated in an 0.6% agar underlayer containing modified MEM (0.1 ml of extracted serum per ml of agar) to minimize the effect of inhibitory serum components not yet removed by the chloroform extraction (5). After gelling at room temperature, the agar was overlaid with 10^5 BMC in 1 ml of modified MEM containing 1% methylcellulose and 20% horse serum. The chloroform extraction in combination with this double-layer technique circumvents some difficulties commonly encountered when sera are tested for CSA in conventional colony assays: variability, lack of dose depend-

ency, and inhibition of colony formation at higher serum concentrations. Thus, as found in pilot experiments, colony numbers were almost linearly related to the logarithm of the serum concentration up to 5%. All cultures were incubated at 37°C with 10% CO_2 in air in a humidified incubator. Colonies containing more than 50 cells were counted 7 days later. For morphological analysis, individual colonies were harvested with a finely drawn Pasteur pipette, and smears were prepared on glass slides. After air drying, the preparations were stained with May-Grünwald-Giemsa.

Hot thymidine suicide technique for CFU-S. The fraction of CFU-S in deoxyribonucleic acid synthesis was determined by a modification of the technique of Becker et al. (4). Nucleated BMC, 10^7, were incubated at 37°C for 20 min in 2 ml of RPMI 1640 (50 U each of penicillin and streptomycin per ml, 20 mM HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid]) containing either 200 μCi of hot thymidine per ml alone or a mixture of 200 μCi of hot thymidine and 100 μg of cold thymidine per ml. The cells were then diluted with 28 ml of RPMI 1640 containing 100 μg of cold thymidine per ml and washed three times in the cold. Nucleated BMC, 10^5, in an injection volume of 0.5 ml were then injected intravenously into syngeneic recipient mice, which had received 850 rads, 3 h previously, from a Müller RT 250 X-ray machine (15 mA; 0.2 mm of Cu added filtration, 0.8-mm half-value layer; 60-cm focal distance; dose rate, 100 rads/min). Their drinking water contained chloramine T (87.4 mg/liter). After 9 days the mice were killed, their spleens were removed and incubated for 10 min in Bouin fixative, and the number of spleen colonies was counted by direct observation without magnification (22). Each experiment was repeated twice.

Reagents. All reagents and solvents were of analytical grade. Pronase and trypsin (2× crystallized) were purchased from Serva (Heidelberg, W. Germany). Dulbecco-modified Eagle MEM (Grand Island Biological Co. [GIBCO] H21, Grand Island, N.Y.) was supplemented with 1% of a cocktail (2.5 mg of L-alanine, 5 mg of L-asparagine-water, 3 mg of L-aspartic acid, 7 mg of L-cysteine, 7.5 mg of L-glutamic acid, 4 mg of L-proline, 2.5 μg of vitamin B_{12}, and 3 μg of biotin per ml) and 1% of an antibiotic stock solution (5,000 U of penicillin and 5,000 U of streptomycin per ml). RPMI 1640 was purchased from GIBCO. Horse serum and HEPES buffer were obtained from Microbiological Associates (Bethesda, Md.). LPS *Salmonella typhimurium* W was a commercial preparation from Difco Laboratories (Detroit, Mich.). [6-^{3}H]thymidine (specific activity, 21.5 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, Buckinghamshire, England).

RESULTS

Conditioning of medium by lungs of BCWC-treated animals. In the first experiment, media conditioned by lungs from saline- or BCWC-treated C57Bl/6 mice were compared with respect to CSA content. MLCM from BCWC-treated animals induced about twice as many colonies as MLCM from saline-treated animals when tested in standard BMC cultures (10^5 C57Bl/6 BMC per dish) at a final concentration of 5% (Fig. 1).

Induction of serum CSA. In C57Bl/6 mice, intraperitoneal administration of graded doses of the three BCWC was found to induce serum CSA in a dose-dependent fashion in each case (Fig. 2). On comparison of the effects of the highest tolerated doses (lipid A and lipoprotein, 30 mg/kg; murein, 100 mg/kg), lipoprotein and murein appeared to be about equally potent, whereas lipid A was clearly less active. After administration of all three preparations, peak values of CSA were reached after 3 to 9 h. Sera of saline-treated mice showed no activity, even if BCWC were admixed in different concentrations (10 pg/ml to 10 μg/ml) before the colony assay was performed (data not shown). In a further experiment serum CSA induced by maximal doses of BCWC was determined in normal C3H/Bom mice and in LPS-resistant C3H/HeJ mice (Fig. 3). As expected, it was found that lipid A was perfectly capable of inducing CSA in C3H/Bom mice but was completely inactive in C3H/HeJ mice, thus confirming the data of Apte and Pluznik (3). Lipoprotein and murein were effective inducers in both strains. Nevertheless, in C3H/HeJ mice the peak CSA values were somewhat smaller and returned more quickly to background values than in C3H/Bom mice.

Induction of splenic CFU-C. The LPS-induced increase in splenic CFU-C in mice has been reported to be most pronounced on day 6 after treatment (2). In our own experiments, therefore, we chose the same interval for the evaluation of the three preparations. The results are summarized in Fig. 4 and 5. It can be seen that in C57Bl/6 mice both lipid A and lipoprotein caused a dose-dependent increase in the pool size of CFU-C in the spleen. With doses of 10 mg/kg (less than those required for maximal CSA induction), an approximately 100-fold increase was observed. In contrast, murein was much less active and only at the highest tolerated dose (100 mg/kg) did it have any discernible effect (Fig. 4). In strains C3H/Bom and C3H/HeJ neither murein nor lipoprotein was a good inducer of splenic CFU-C. Lipid A, on the other hand, was just as active in C3H/Bom mice as in C57Bl/6 mice. However, as expected, it failed to elicit a CFU-C response in LPS-resistant C3H/HeJ recipients (Fig. 5).

CSA requirements and cellular composition of BCWC-induced splenic colony-forming cells. The in vitro proliferation of CFU-C from the spleens of normal mice requires the presence of exogenous CSA. However, spleen cells from lipid A- and lipoprotein-treated donors cultured in the absence of exogenous CSA

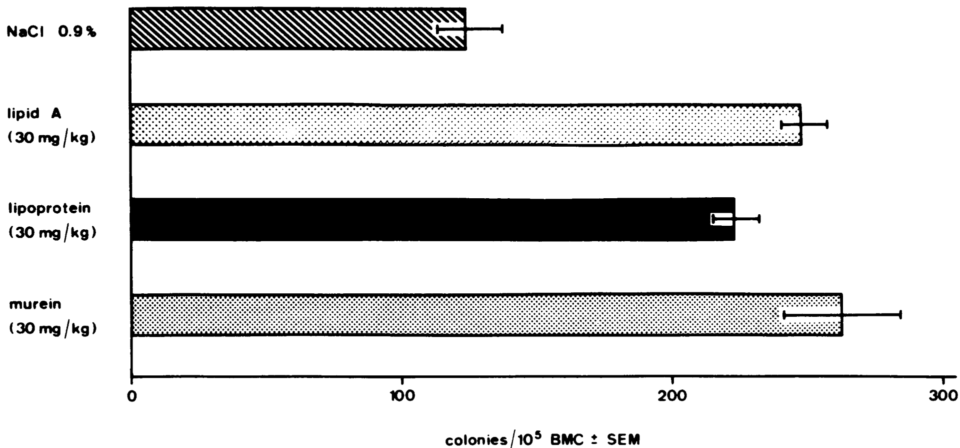

FIG. 1. *CSA in medium conditioned by lung fragments from BCWC-treated C57Bl/6 mice. MLCM was prepared as described in the text and tested for CSA in duplicate colony assays at a final concentration of 5%. Each bar represents the mean value of three to five MLCM preparations ± standard error of the mean (SEM).*

gave rise to considerable numbers of what appeared to be "CSA-independent," "spontaneous" colonies. This phenomenon was further analyzed with lipoprotein as inducing agent. The results are shown in Table 1 and Fig. 6. Clearly, the extent of spontaneous colony formation was dependent on the dose of lipoprotein previously administered to the donor animals (Table 1) and the number of spleen cells plated (Fig. 6). To determine the origin and composition of such spontaneous colonies, cells from individual colonies were harvested and stained smears were analyzed morphologically (Table 2). In all samples examined only myeloid cells could be detected, regardless of whether CSA had been added to the cultures. However, in the absence of exogenous CSA only pure macrophage and mixed macrophage-granulocyte, but no pure granulocytic, colonies were formed, whereas in the presence of exogenous CSA pure granulocytic colonies appeared. It is noteworthy that under the conditions chosen in this particular experiment, i.e., high expression of spontaneous colony-forming potential of spleen cells after pretreatment of cell donors with 10 mg of lipoprotein per kg and culture at high cell density (10^6 spleen cells per dish), the total number of colonies was barely increased by the addition of exogenous CSA, whereas the absolute and relative numbers of mixed colonies were actually decreased.

Cell cycle status of CFU-S after administration of BCWC. The administration of CFU-S has been reported to increase the proportion of CFU-S in the cell cycle. This effect is maximal 24 h after injection (8). The influence of the three BCWC preparations upon CFU-S from C57Bl/6 mice was therefore analyzed after the same interval. BMC from pretreated donors were exposed to a high concentration of [^{3}H]-thymidine before transfer into irradiated recipients. As judged by the number of spleen colonies on day 9, the number of CFU-S from saline- or murein-treated animals was not significantly reduced by a short incubation with hot thymidine as compared with that found in control cells incubated with both hot thymidine and an excess of cold thymidine. In contrast, more than 50% of CFU-S from lipid A- or lipoprotein-treated mice were killed by this procedure (Table 3). The results indicate that lipid A and lipoprotein, but not murein, are capable of shifting CFU-S into deoxyribonucleic acid synthesis.

DISCUSSION

In the present study modulation of myelopoiesis by three structurally unrelated BCWC was examined. In addition to lipid A, the activity of which is well documented in the literature (2), outer membrane lipoprotein and murein were also found to produce major effects on precursor cells along the pathway of granulocyte and macrophage differentiation. Since all three components were derived from similar starting material, i.e., gram-negative bacteria, it is important to exclude the possibility that some of the observed effects might have been due to cross-contamination of the individual preparations. This is highly unlikely for several reasons. The chemical purity of the three BCWC used was ascertained by careful chemical analysis. In the case of murein, any possible contamination with intact lipid A or lipoprotein was further minimized by alkaline hydrolysis, a procedure that

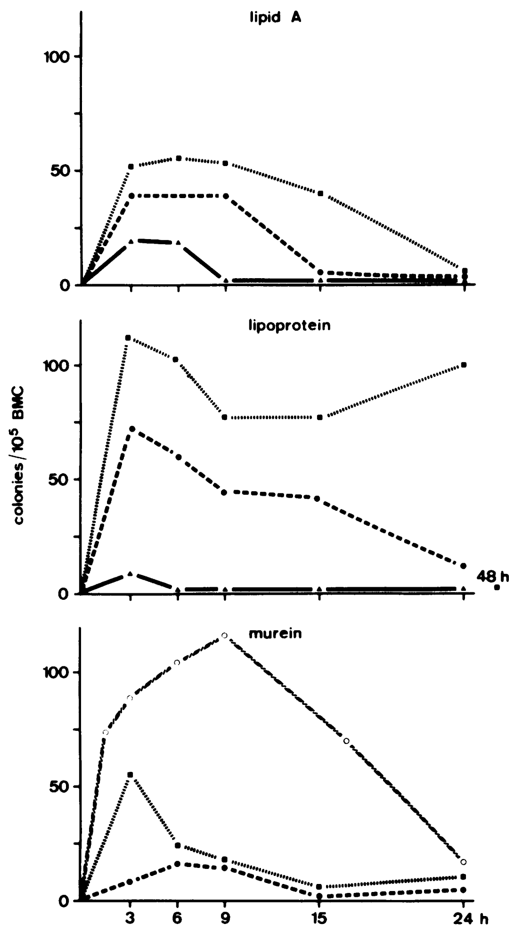

FIG. 2. *Induction of serum CSA by BCWC in C57Bl/6 mice. (Upper) Effect of lipid A; (Middle) effect of lipoprotein; (Lower) effect of murein. Dose of BCWC: (○) 100 mg/kg; (■) 30 mg/kg; (●) 3 mg/kg; (▲) 0.3 mg/kg. (Abscissae) Time of serum sampling (hours after intraperitoneal injection of BCWC). (Ordinate) Colonies per 10^5 C57Bl/6 BMC cultured in the presence of sera from pretreated mice. Each point represents the mean value of duplicate colony assays from pooled sera of eight mice. Each serum was tested by serial dilution. Since colony numbers increased up to the highest concentration tested (0.1 ml/dish), only these values are shown.*

completely destroys the activity of both lipid A and lipoprotein (13, 20) but did not affect important biological activities of the murein preparation used (F. G. Staber et al., submitted for publication). (During the course of this study only alkaline-hydrolyzed murein was used.) The effectiveness of the pure lipoprotein and murein moieties is further underlined by our recent finding that low-molecular-weight degradation products of lipoprotein and synthetic muramylpeptides are potent inducers of CSA (Staber et al., submitted for publication). Cross-contamination of the BCWC used in this study can also be excluded on the basis of their distinct biological activity profiles, which clearly set them apart from each other. Thus, lipoprotein and murein, but not lipid A, were both capable of inducing CSA in LPS-resistant C3H/HeJ mice. Moreover, lipid A and lipoprotein, but not murein, greatly increased the numbers of splenic CFU-C in C57Bl/6 mice, whereas in strain C3H/Bom only lipid A proved to be a potent inducer in this respect. Finally, by administration of lipid A and lipoprotein, but not of murein, CFU-S were triggered to synthetize deoxyribonucleic acid. On the other hand, certain important properties were shared equally by the three BCWC used. All of them, for instance, proved to be potent inducers of CSA, both in serum and in lung tissue of susceptible mice.

The concept of physiological regulation of CSA release and myelopoiesis by LPS (18) and lipid A can therefore be extended to two other major bacterial constituents, namely, lipoprotein and murein. Hence, failure to recognize LPS, as is the case in some genetically deficient strains of mice, would in no way preclude normal homeostasis of the myeloid system. In addition, a rather different point emerges from these findings: bacterial antigens, such as flagellins (14), were reported to induce CSA; clearly, the possibility of contamination with BCWC other than LPS will have to be taken into account in such systems.

As is known from earlier reports (1, 12) and was confirmed and extended in the present study by the findings with lipid A and lipoprotein, LPS is capable of increasing the pool size of splenic CFU-C, which can be detected upon addition of exogenous CSA in a standard colony assay. Surprisingly, however, it was noted that the expression of the colony-forming potential of spleen cells from mice pretreated with lipid A or lipoprotein was largely independent of exogenous CSA. Since the proliferation in vitro of murine CFU-C exhibits a strong requirement for exogenous CSA, the possibility had to be considered that cells other than CFU-C were responsible for spontaneous colony formation. The prime suspects appeared to be B-cells, which under suitable conditions are known to form colonies in vitro (16). However, by morphological analysis only myeloid colonies could be detected. Nevertheless, these differed in their composition, depending on the presence or absence of CSA: spontaneous spleen cell colonies of lipoprotein-pretreated mice consisted either solely of macrophages or of both granulocytes and

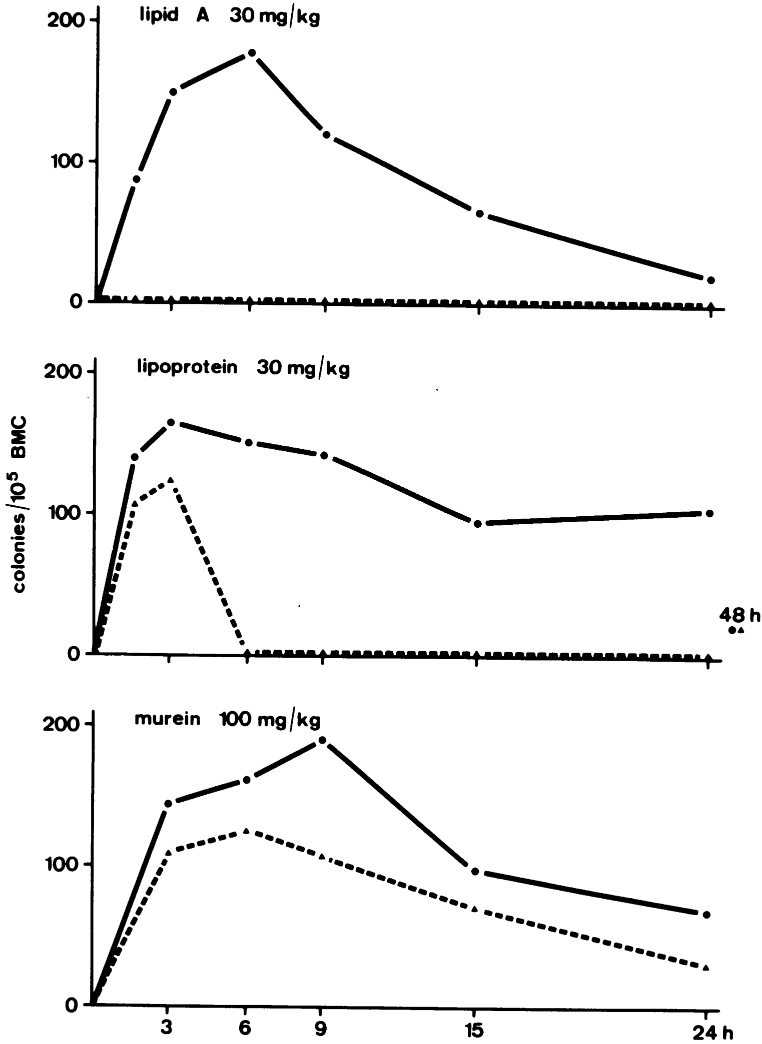

FIG. 3. *Induction of serum CSA by BCWC in C3H/Bom and C3H/HeJ mice. (Upper) Effect of lipid A (30 mg/kg); (Middle) effect of lipoprotein (30 mg/kg); (Lower) effect of murein (100 mg/kg). (Abscissae) Time of serum sampling (hours after intraperitoneal injection of BCWC). (Ordinates) Colonies per 10^5 C57Bl/6 BMC cultured in the presence of sera from pretreated C3H/Bom* (●) *or C3H/HeJ mice* (▲). *Sera were tested as described in the legend to Fig. 2.*

macrophages, but were never purely granulocytic. Colonies of this last type appeared only when exogenous CSA was added to the spleen cell cultures. Thus, under these conditions, the absence or presence of exogenous CSA affected mainly the frequency of the different colony types, whereas the total number of colonies was much less influenced. It would appear, therefore, that lipoprotein-induced CFU-C are influenced preferentially in their differentiation and much less in their proliferation, if exogenous CSA is added or omitted. The spontaneous expression of the colony-forming potential of splenic CFU-C from BCWC-treated mice may be most readily explained in terms of release of endogenous CSA from mature BCWC-stimulated spleen macrophages, which were carried over into culture. This explanation is consistent with the finding that spontaneous colony formation is dependent on the dose of lipoprotein administered to the test animals and on the number of spleen cells plated. To test whether release of endogenous CSA was indeed the causative factor for formation of "spontaneous" colonies, two experimen-

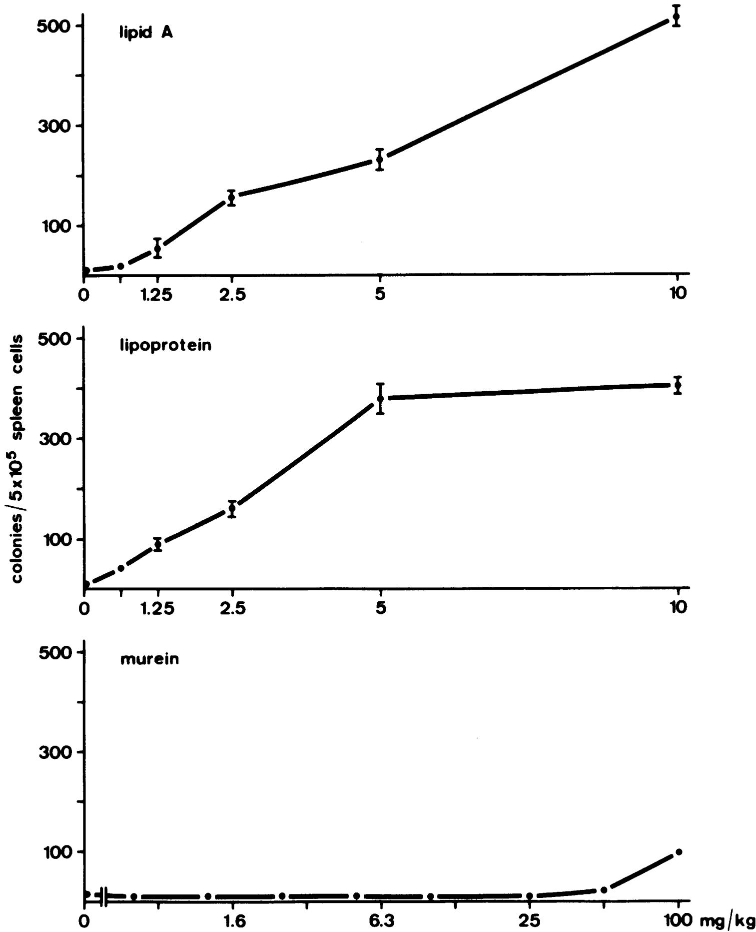

FIG. 4. *Induction of splenic CFU-C by BCWC in C57Bl/6 mice. (Upper) Effect of lipid A; (Middle) effect of lipoprotein; (Lower) effect of murein. (Abscissae) Dose of BCWC (intraperitoneally) administered 6 days before culture of spleen cells. (Ordinates) Colonies per 5×10^5 C57Bl/6 spleen cells cultured in the presence of 5% reference MLCM. Each point represents the mean value ± standard error from four spleens treated separately in duplicate cultures. Optimal assay conditions were controlled in each experiment by simultaneous culture of normal BMC. Note that the lower panel (murein) represents a semilog plot.*

tal approaches were chosen. First, spleen cells from animals pretreated with optimal doses of BCWC were cultured in medium for different periods and the supernatants were tested for the presence of endogenous CSA on normal BMC. Second, irradiated spleen cells from BCWC-treated animals were added directly to normal BMC, both in monolayer and double-layer cultures. However, CSA could not be detected in either case (unpublished data). Hence, the question as to the cause of spontaneous colony formation must remain unanswered for the time being.

Similarly, the mechanism underlying the increase of CFU-C after administration of BCWC has not yet been clarified. Earlier authors proposed that the LPS-induced enlargement of the pool size of CFU-C could be mediated by CSA (2, 3). If this were true, one would expect that any inducer of CSA should also increase the number of CFU-C. However, this is clearly not the case: whereas murein proved to be an effective stimulant of serum CSA formation, in all three mouse strains tested it almost completely failed to increase the number of splenic CFU-C. Likewise, lipoprotein was a potent inducer of CSA in both C57Bl and C3H/Bom mice, but its stimulatory effect on CFU-C was largely restricted to strain C57Bl/6. Alternatively, the increase of splenic CFU-C after administration

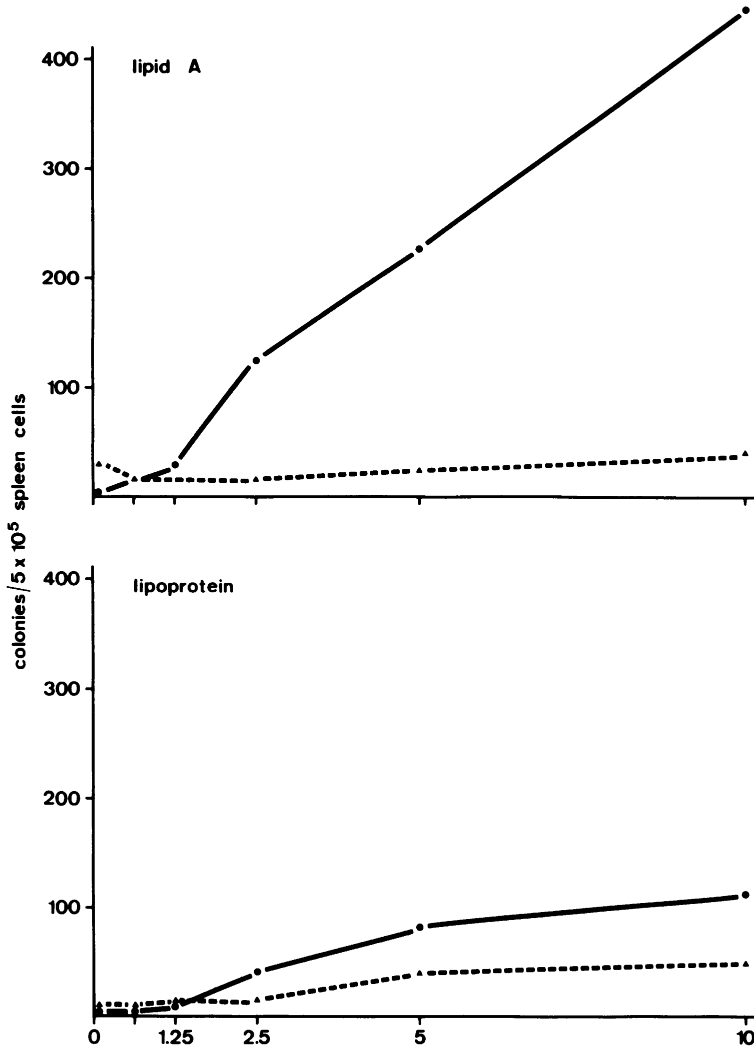

FIG. 5. Induction of splenic CFU-C by BCWC in C3H/Bom and C3H/HeJ mice. (Upper) Effect of lipid A; (Lower) effect of lipoprotein. (Abscissae) Dose of BCWC (milligrams per kilogram, intraperitoneally) administered to C3H/Bom (●) or C3H/HeJ (▲) mice 6 days before culture of spleen cells. (Ordinates) Colonies per 5 × 10^5 spleen cells cultured in the presence of 5% standard MLCM. Each point represents the mean value from four spleens tested separately in duplicate cultures. Optimal assay conditions were controlled as described in Fig. 4.

of BCWC may reflect proliferation and differentiation of pluripotent hemopoietic stem cells. Upon assessment of the cell cycle status of CFU-S after administration of the different BCWC, a good correlation was, in fact, found between the in vivo mitogenicity for CFU-S of BCWC and their effect on the pool size of CFU-C. Although these data are quite suggestive, other mechanisms, such as self-replication of CFU-C or migration of CFU-C from the bone marrow to the spleen, are by no means excluded. That the increase in CFU-C might in fact be a complex phenomenon is suggested by the different slopes of the dose-response curves of lipid A and lipoprotein in C3H/Bom mice. Therefore, it must be considered that different BCWC cause an increase of CFU-C by partially different mechanisms.

Together with recent evidence to be published elsewhere, the data presented in this communication concur to indicate that BCWC can influence myelopoiesis at several distinct levels (Fig.

TABLE 1. *Spontaneous colony formation by spleen cells from C57Bl/6 mice pretreated with lipoprotein*

Dosage of lipoprotein (mg/kg, i.p., day −6)[a]	No. of colonies/10^6 spleen cells[b] in the presence of: 5% Standard MLCM[c]	Control medium	% Spontaneous colonies
None	10 ± 0.9	1 ± 0.2	10
0.625	71 ± 13	23 ± 6	32
1.25	133 ± 14	64 ± 6	48
2.5	251 ± 14	165 ± 13	66
5.0	535 ± 45	385 ± 35	72
10.0	672 ± 58	483 ± 57	72

[a] i.p., Intraperitoneally.

[b] Each number represents the mean value of six replicate cultures ± standard error of the mean.

[c] CSA concentration sufficient to induce plateau colony numbers in cultures of normal BMC.

TABLE 2. *Morphology of colonies from lipoprotein-induced C57Bl/6 splenic colony-forming cells*[a]

5% Standard MLCM	No. of colonies/10^6 spleen cells (%): Total[b]	Granulocytic[c]	Mixed[c]	Macrophage[c]
Present	492	197 (40)	261 (53)	34 (7)
Absent	425	0	383 (90)	42 (10)

[a] 10 mg of lipoprotein per kg injected intraperitoneally 6 days before sacrifice.

[b] Mean values of duplicate cultures.

[c] Calculated values obtained by analysis of smears from 30 randomly selected colonies of each type of culture.

7). (i) They induce the proliferation of CFU-S and (accordingly) increase the pool size of CFU-C. (ii) They stimulate the functional differentiation of immature bone marrow-derived macrophages (as shown by the ability of in vivo-generated macrophages to facilitate antibody synthesis to a T-dependent antigen) (Staber and Gisler, unpublished data). (iii) They directly activate mature macrophages to release CSA (Staber et al., submitted for publication), which in turn causes the proliferation and differentiation of CFU-C.

TABLE 3. *Effect of BCWC in vivo upon cell cycle status of CFU-S*

Pretreatment of donors (−24 h, i.p.)[a]	CFU-S/spleen (mean value ± SEM)[b]: Hot + excess cold thymidine	Hot thymidine	% Loss of CFU-S
Expt 1			
NaCl, 0.9%	15.9 ± 3.3	15.8 ± 3.13	0.6
Lipid A, 10 mg/kg	14.9 ± 0.9	6.0 ± 1.9	59.7
Lipoprotein, 10 mg/kg	8.2 ± 2.0	3.5 ± 0.9	57.3
Expt 2			
NaCl, 0.9%	19.3 ± 3.0	17.8 ± 3.0	7.8
Murein, 10 mg/kg	18.3 ± 4.0	19.3 ± 2.3	+5.5
Murein, 50 mg/kg	15.4 ± 1.5	11.8 ± 2.1	23.3

[a] Two C57Bl/6 mice per group. i.p., Intraperitoneally.

[b] Fifteen C57Bl/6 recipients per group. SEM, Standard error of the mean.

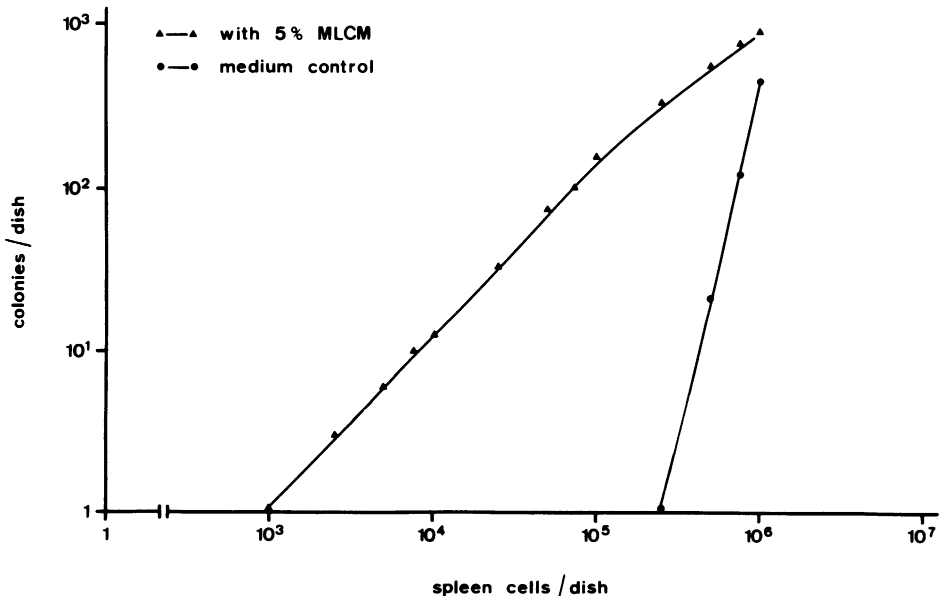

FIG. 6. *Spontaneous colony formation by spleen cells from lipoprotein-pretreated C57Bl/6 mice. (Abscissae) Number of spleen cells seeded per plate. (Ordinate) Number of colonies obtained in the presence (▲) or absence (●) of 5% standard MLCM. Each point represents the mean value of duplicate cultures. Donors were treated with 10 mg of lipoprotein per kg intraperitoneally 6 days before collection of spleen cells.*

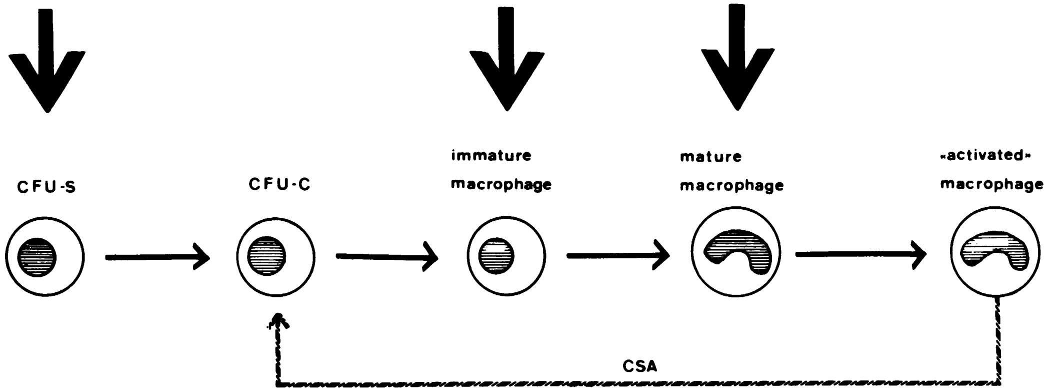

FIG. 7. *Effects of BCWC on myelopoiesis at different levels of cell differentiation. Vertical arrows indicate targets of BCWC.*

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