Mycobacterium tuberculosis. 3 weeks later the skin reactions to 10 µg of ovalbumin were read.

Results and discussion. From the data presented in the table it is clear that the dipeptide (L-Ala-D-Glu-NH_2), and also the tetrapeptide (L-Ala-D-isoGlu-L-Lys-D-Ala) have no effect on delayed hypersensitivity in our tests. These results are in line with those published by Ellouz et al.[1] and Kotani et al.[3] who also reported no immunoadjuvant activity for these compounds. They suggested that N-acetyl muramic acid should be present to observe the adjuvant effect. Our results with the nonapeptide (L-Ala-D-isoGlu-L-Lys-D-Ala-$(Gly)_5$-OME), however, do not support the idea of a critical role of N-acetyl muramic acid. It is noteworthy that Nauciel et al.[9] originally reached the conclusion that the tetrapeptide subunit is already effective. However, they used the natural product in their experiments and azobenzenearsenate-N-acetyl tyrosine was administered instead of ovalbumin. Our present results would suggest that some longer peptides might be as effective as glycopeptides with a short peptide chain. This point certainly deserves more experimental work. From the table it is also evident that NAM-L-Ala-NH_2 showed no adjuvant effect and this is in agreement with results published by others[1-3]. Previous work also suggested that the amidation of the γ-carboxyl group instead of the α-carboxyl group of the glutamic acid residue abolished the adjuvant effect of muramyl dipeptide[3] in inducing delayed-type hypersensitivity. Also the derivatives where both α and γ-carboxyls of glutamic acid are esterified have lost most of their activity when administered in a water-in-oil emulsion[5]. The amidation of the γ-carboxyl group, in addition to the amidation of the α-carboxyl group of glutamic acid residue in our experimental design (NAM-L-Ala-D-isoGlu-NH_2), did not markedly affect the adjuvant potency. This is in agreement with results published by others[10]. On the other hand, the amidation of the γ-carboxyl of the D-iso-glutamine residue by butyl or stearyl groups, which gave muramyl dipeptide derivates with an increased hydrophobicity, clearly decreased the intensity of response of muramyl dipeptide. Kotani et al.[11] working with 6-0-stearoyl derivatives of N-acetyl muramyl dipeptide have recently noted that this compound is also adjuvant in water-in-oil emulsion, however, again less than the original compound. This, however, seems not to be the case when biodegradable liposomes are substituted for mineral oils. We have recently reported that Freund's incomplete adjuvant may be replaced by phosphatidyl choline-cholesterol liposomes. Muramyl dipeptide in aqueous suspension did not show any effect[12]. Stearyl amide of N-acetyl muramyl alanyl-D-isoglutamine injected in liposomes seems to be more effective in the induction of delayed hypersensitivity than muramyl dipeptide. The explanation for this finding might be that the introduction of a stearyl group increases the binding of muramyl dipeptide within the liposome. No experimental evidences to support this hypothesis, however, are at the present time available. Several papers published recently by the Chedid group[5,13] reported that in case of humoral immunity a potentiation can be achieved already with the compound in aqueous solution.

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Colony stimulating activity in acute and chronic endotoxinemia in man

W. Hinterberger, L. Fridrich, P. Ferenci and W. Paukovits

1st Department of Medicine, Department of Gastroenterology and Hepatology, and Institute of Cancer Research, University of Vienna, Lazarettgasse 14, A-1090 Wien (Austria), 12 December 1978

Summary. Blood granulocyte-macrophage colony stimulating activity (GM CSF) was measured in 6 normal individuals challenged with low-dose endotoxin and in 63 unselected patients with nonhaematological disorders. 5/63 patients were febrile and 5 other patients showed detectable endotoxin levels, as measured by the Limulus assay. CSA levels showed a rapid increase in normal individuals following endotoxin administration, but were in the normal range in patients with chronic endotoxinemia or in those with febrile disorders. Thus, unlike acute endotoxinemia, chronic endotoxinemia is not associated with elevated activity that promotes growth of myeloid commited stem cells. In addition, fever per se did not coincide with elevated blood CSA levels.

Granulocyte-macrophage colony stimulating activity (CSA) is rapidly elevated in the blood of experimental animals and man upon administration of endotoxin[1,2]. Mice, exposed to repeated administration of endotoxin, became nonresponsive[3]. Abnormally low CSA levels in germ-free animals led to the assumption that endotoxin releasing bacteria are necessary for maintaining a certain CSA level[4]. Since most data were obtained in experimental animals and usually refer to acute endotoxinemia, we compared the effects of acute and chronic endotoxinemia on blood CSA levels in man.

Material and methods. The effect of acute endotoxinemia on blood CSA levels was tested in 6 normal individuals, who gave their informed consent to the administration of low-dose endotoxin (Pyrifer, step 1, Aristopharm). Blood from 63 unselected, nonhaematological patients was simultaneously assayed for CSA levels and the presence of endotoxin. Blood CSA levels were assayed in a monolayer

agar culture with 75,000 mouse bone marrow cells (C57bl mice) as target system. 1 ml cultures consisted of 0.1 ml patient serum and 0.9 ml agar-McCoy medium (0.5% agar), enriched with fetal calf serum (20% v/v).

Colonies were counted after a 1 week culture at 37 °C, 7.5% CO_2. Blood CSA levels were expressed as colonies/$75 \cdot 10^3$ bone marrow cells. This culture assay was calibrated with a potent conditioned medium (HLCM), prepared in our laboratory[5]. Mouse bone marrow cells cultured at 75,000 cells/ml in our experience never show spontaneous colony growth and were therefore a reliable indicator of exogenous CSF.

Blood endotoxin was assayed by the Limulus assay, described by Liehr[6]. Briefly, plasma was diluted 1:10 in pyrogen-free water and 0.1 ml was mixed at 1:1 with Limulus amoebocyte lysate (LAL-reagens, Pyrogent®, Byk-Mallinckrodt, Dietzenbach, Steinberg). Following a 24-h incubation in pyrogen-free test tubes the results were scored as follows: + + + complete gelation, + + incomplete gelation, + increase in viscosity and turbidity. No visible reaction was scored as negative. Each culture run included a negative (0.1 ml pyrogen-free water) and positive control (10 ng *Escherichia-coli* endotoxin/0.1 ml saline). The sensitivity of the Limulus assay is described to be 1 ng/ml[6].

Results. Figure 1 gives the calibration of the agar culture assay: 93 colonies/75,000 bone marrow cells were obtained with HLCM at 1:1. The same batch of fetal calf serum was used throughout the calibration experiment and the CSA assays.

Figure 2 shows the kinetics of blood CSA levels in 6 normal individuals following i.v. Pyrifer application: Maximal CSA levels ranged between 5.5 and 22 colonies and were detectable between 15 and 150 min.

63 patients suffered from cardiocirculatory disorders (18), renal diseases (8), endocrine disorders (21), pulmonary diseases (4), liver diseases (7) and disorders of the gastrointestinal tract (5). 5 patients showed detectable blood endotoxin levels (1 chronic hepatitis, 1 liver cirrhosis, 1 acute alcoholic hepatitis, 1 drug-induced cholestasis and 1 ulcerative colitis). 5 patients were febrile (2 pneumonia, 1 angina, 1 myocarditis and 1 thrombophlebitis). Blood CSA levels of these individuals are shown in figure 3: 53 afebrile, endotoxin negative patients activated 5.2 ± 3.8 colonies/75,000 bone marrow cells, febrile patients activated 4.5 ± 4.8 colonies and patients with detectable endotoxin levels disclosed 5.4 ± 1.1 colonies.

Discussion. Endotoxin, released from the cell wall of gram-negative bacteria, affects the granulocytic system at the stem cell and mature effector cell level. Our findings demonstrate a rapid CSA response in normal individuals challenged with low amounts of endotoxin. In contrast, patients in whom the underlying disease causes chronic endotoxinemia show normal blood CSA levels. The possibility that these patients generate concomitant inhibitory activity, which may mask an otherwise normal CSA response, cannot be safely excluded; the normal morphology of colonies activated with sera from endotoxinemic patients, however, makes nonspecific toxicity unlikely. Febrile patients without concomitant endotoxinemia show normal CSA levels too, indicating that fever per se does not induce blood CSA elevation.

Normally, liver macrophages clear portal blood of endotoxin released by gastrointestinal bacteria. The detection of

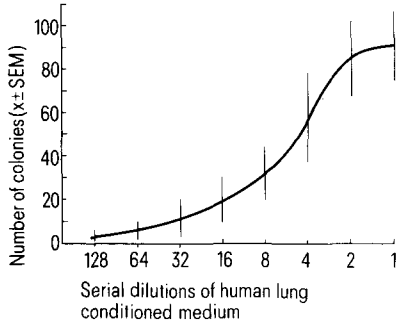

Fig. 1. Mouse bone marrow cultures (75,000 cells/ml) stimulated with HLCM in serial dilutions.

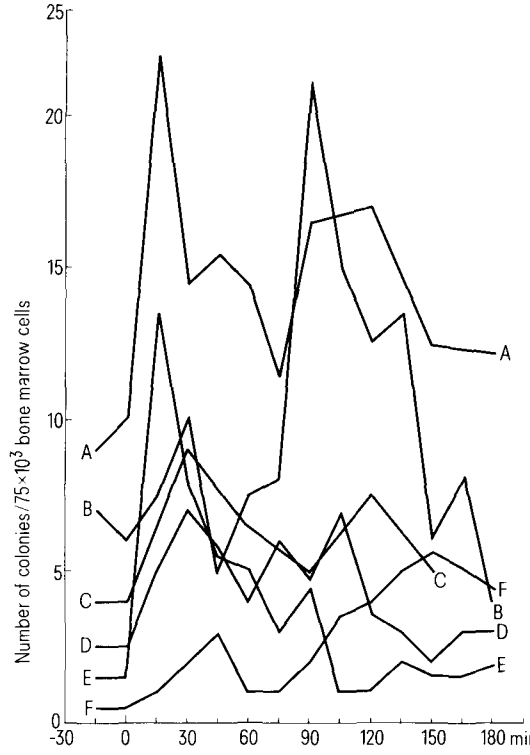

Fig. 2. Blood CSA levels in 6 normal individuals given endotoxin i.v. (Pyrifer, step 1).

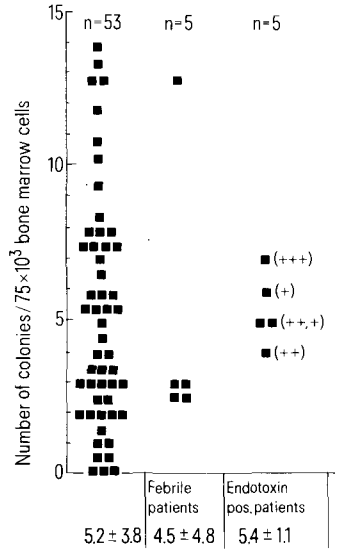

Fig. 3. CSA levels in febrile (5), endotoxinemic (5) and control patients (53).

endotoxin in patients with liver diseases strongly suggests an abnormality of clearance by liver macrophages. Hence, with respect to the chronic character of these disorders, endotoxinemia in these patients resembles the situation in experimental animals in which endotoxin tolerance takes place following repeated administration of endotoxin.

Thus, the complex relationship of stem cell activation and defence against bacterial infection in man appears similar to that in mice. Since however the term 'CSA' comprises various factors differing in biological and chemical qualities, the direct assessment of these may still exhibit some correlations with certain pathological conditions.

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Dose-related enhancement of erythropoiesis by sulfhydryl compounds and its reversal with a thiol inhibitor

Natalie Pourreau-Schneider[1]

Institut de Pathologie Cellulaire (INSERM 48), Hôpital de Bicêtre, F-94270 Kremlin-Bicêtre (France), 15 December 1978

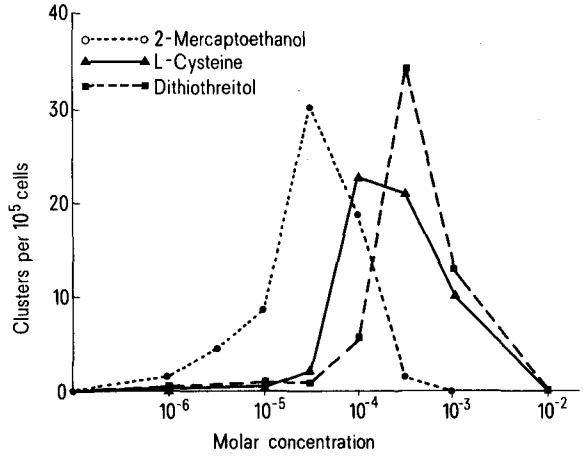
Summary. Erythropoietin-mediated erythrocyte development from bone marrow of hypertransfused rats was significantly greater when the culture medium contained an optimal dose of certain sulfhydryls. This stimulatory action was attributed to the presence of SH groups because erythroblast numbers fell to control levels when the culture contained the thiol inhibitor, p-hydroxymercuribenzoate.

Bone marrow of rats rendered polycythemic by hypertransfusion is practically devoid of morphologically identifiable erythroblasts[2,3]. We have previously reported that by using such polycythemic marrow we were able to follow the orderly progression of erythroid proliferation and maturation[4]. We described the development of erythroid cells which formed tight aggregates of quite homogeneous composition and which could be easily harvested for cytological examination because of the liquid system. These observations on the amplification of erythropoiesis have been the subject of a further report[5].

In the original paper, we mentioned that the culture medium contained 2-mercaptoethanol and L-cysteine. Although the use of thiol additives to promote erythropoiesis in culture is not new[6,7], insufficient emphasis has yet been placed on the necessity of SH protection for erythrocytic development. The present report provides dose-response curves for three thiols, 2-mercaptoethanol (2-ME), L-cysteine (L-CY) and dithiothreitol (DTT), in the polycythemic bone marrow system in vitro and shows that their stimulatory action on erythroid cell cluster formation is reversed by thiol inhibition.

Materials and methods. Femoral bone marrow was taken 5 days after hypertransfusion (packed red blood cells) from 8- to 12-week-old female Wistar rats (hematocrit > 70%). The marrow was mechanically dissociated into a single cell suspension and plated 10^5 nucleated cells/ml in a modified NCTC medium[8] containing 30% fetal bovine serum, and when desired 0.2 IU erythropoietin (Connaught Step III), and microliter doses of 2-ME, L-CY, DTT, or the thiol inhibitor, p-hydroxymercuribenzoate (p-HMB). After 48 h of incubation at 37 °C, the cell suspension was harvested onto glass slides by cytocentrifugation and stained by May-Grünwald-Giemsa or Benzidine. The erythroid groups were counted and classed according to size and to stage of maturation.

Results and discussion. At the time of plating, the bone marrow suspension contained less than 5% nucleated erythroid cells. After 48 h of culture without erythropoietin, regardless of the presence of thiol, erythroid development was nil. With erythropoietin and without thiol additive (EPO controls), a mean number of 28.7 erythroid cell clusters formed. In the experimental groups, containing erythropoietin and an optimal concentration of one of the 3 thiols, erythroblastic differentiation and proliferation resulted in a 10-fold increase in the number of clusters and in the total number of erythroblasts with 2-ME or DTT, and a 5-fold increase with L-CY (table). In paired observations with corresponding erythropoietin controls, the thiol cultures showed significant erythrocytic cluster formation ($p \leq 0.01$ for 2-ME and L-CY and $p \leq 0.05$ for DTT). t-test showed significant erythrocytic development in the 3 thiol groups over the corresponding EPO controls ($p \leq 0.01$). Dose-response curves for the 3 thiols (figure) showed that for erythroid development 2-ME was most effective at



Relationship between the dose of sulfhydryl compounds and the number of erythroid clusters formed from 10^5 bone marrow cells after 48 h in the presence of erythropoietin. Each point is the mean count of erythrocytic groups in thiol-containing medium divided by the corresponding erythropoietin control in 3 to 6 individual experiments. In paired observations with the corresponding erythropoietin control p-values were found to be: $p \leq 0.05$ for 2-mercaptoethanol at the concentration of 5×10^{-5} M, and for dithiothreitol at 5×10^{-4} M; $p \leq 0.01$ for L-cysteine at 5×10^{-4}.