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Bone Marrow Fibroblasts from Children with Aplastic Anemia Exhibit Reduced Interleukin-6 Production in Response to Cytokines and Viral Challenge

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ABSTRACT

Defects of the bone marrow microenvironment have been implicated in the pathogenesis of aplastic anemia (AA). We examined granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-6 production in bone marrow fibroblasts from five children with AA in response to cytokines and viral challenge with cytomegalovirus (CMV). Cytokine-stimulated IL-6 production in patients' fibroblasts was significantly reduced, whereas GM-CSF production was found to be comparable to normal controls. Also, CMV-mediated stimulation of IL-6 production was significantly lower in bone marrow fibroblasts from patients with AA. During the late phase of CMV-infection, IL-6 production was suppressed in CMV-infected fibroblasts from patients with AA with 90% inhibition of IL-6 protein production and marked reduction in IL-6 mRNA accumulation. Defective IL-6 production in bone marrow fibroblasts might contribute to hematopoietic suppression in some patients with AA. (***Pediatr Res* 38: 716–721, 1995**)

Abbreviations

AA, aplastic anemia
NC, normal controls
GM-CSF, granulocyte/macrophage-stimulating factor
TNF-α, tumor necrosis factor-α
CMV, cytomegalovirus
CPE, cytopathic effect
MNC, mononuclear cells

AA is characterized by hypocellularity of the bone marrow and pancytopenia. The pathogenesis is thought to be multifactorial (1, 2). In acquired AA, immunologic suppression of hematopoiesis seems to be one pathogenetic mechanism (3) which may be mediated either by a disorder of cellular immunity or by the secretion of hematopoiesis-inhibiting soluble factors. An intrinsic defect of the hematopoietic stem cell has been suggested by studies with long-term bone marrow cultures (4, 5). Also, successful hematopoietic reconstitution after bone marrow transplantation (6) seems to imply that a stem cell defect plays a central role in the pathogenesis of AA. However, the comparatively high incidence of graft failure in patients with AA (6) seems to indicate that in addition a defect of the hematopoietic microenvironment may be of relevance. Abnormalities of the hematopoietic microenvironment in patients with AA have previously been described and might involve disturbance of stromal cell to stem cell contact (7) as well as altered secretion of growth factors (8–13).

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Among the growth factors produced by stromal cells are GM-CSF and IL-6 (14–16). GM-CSF stimulates proliferation and differentiation of myeloid progenitors (17, 18). IL-6 induces megakaryopoiesis (19), and in synergy with other cytokines enhances multilineage colony formation (20). As a pluripotent cytokine, IL-6 also functions as a mediator of the acute phase response (21) and host defense mechanisms. Release of GM-CSF and IL-6 from stromal cells is enhanced by IL-1 (22, 23) and TNF-α (24). Several viruses have also been shown to induce IL-6 production in infected host cells (25). *In vitro* infection of bone marrow stromal cells with CMV leads to perturbation of stromal cell function (26, 27) and has been postulated as a mechanism of marrow suppression in immunocompromised patients. In this study, we investigated the production of GM-CSF and IL-6 in bone marrow fibroblasts from three children with acquired AA and two children with Fanconi anemia in response to stimulation with IL-1, TNF-α, and viral challenge with CMV.

METHODS

Patients. Five children aged 4–15 y with AA were studied, three with acquired AA and two with Fanconi anemia. All

patients with acquired AA had suffered from longstanding disease (24–60 mo). Two of them were classified as patients with severe AA, and they had received red cell and platelet transfusions. At the time of bone marrow sampling one patient was treated with androgens and one with cortisone. The patients with Fanconi anemia suffered from nonsevere AA of short duration (1–12 mo).

Stromal cell cultures. Bone marrow specimens from patients and healthy individuals donating for bone marrow transplantation were obtained from the posterior iliac crest. After informed consent, a small aliquot was used for the following investigations. Bone marrow MNC were isolated by Ficoll-Hypaque density gradient centrifugation. Erythrocytes contaminating the MNC fraction were lysed in ammonium chloride on ice for 5 min. Subsequently MNC were washed twice with PBS and suspended at a concentration of 2×10^9 cells/L in α-modified Eagle's medium (Sigma Chemical Co., Deisenhofen, FRG) supplemented with 10% (vol/vol) heat-inactivated FCS (GIBCO, Eggenstein, FRG) and 1.7×10^{-6} M methylprednisolone. MNC (4×10^5) were plated in 12-well culture plates (Costar, Cambridge, MA). Cell cultures were maintained at 37°C, 5% CO_2, and 98% humidity. Nonadherent cells were removed by weekly complete renewal of culture medium. After growth of the adherent cell fraction to near confluence, cells were treated with 0.5 g/L trypsin/0.53 mM EDTA·4Na and transferred to new culture plates. After four to five passages, stromal cell cultures consisted predominately of fibroblasts with 10–15% endothelial cells and less than 2% myeloid cells as assessed by flow cytometry. For stimulation of cytokine production, bone marrow fibroblasts of the fourth to fifth passage were incubated with 30 000 IU of IL-1-α/L (Genzyme, Cambridge, MA) or 50 000 IU of TNF-α/L (Sigma Chemical Co., Deisenhofen, Germany).

Infection of bone marrow fibroblasts with CMV. Bone marrow fibroblasts were infected with 1:1 mixture of the CMV laboratory strains AD 169 and Davies II. Fibroblasts were incubated with virus-containing supernatant at a tissue culture infection dosis 50 ($TCID_{50}$) of 10^{-5} for 1 h and washed. Fibroblasts were then stimulated with cytokines as described above with each experimental condition being set up in duplicates. Fibroblasts were either stimulated immediately after infection to assess IL-6 production during the early phase of CMV infection or at 10 d after initial infection for assessment of IL-6 production during prolonged CMV infection. At 3 d postinfection, no CPE could be observed in either bone marrow fibroblasts from patients with AA nor from NC. Ten days after CMV infection, the frequency of CPE per well was 34 ± 4% in bone marrow fibroblasts from patients with AA and 30 ± 7% in NC. Trypan blue stains of CMV-infected fibroblast layers from patients with AA and NC demonstrated that loss in cell viability generally corresponded to the frequency of CPE.

Cytokines. Three days after stimulation of bone marrow fibroblasts, GM-CSF and IL-6 concentrations in the supernatant were assessed by ELISA using the GM-CSF "Insight GM" test kit (Medical Resources Limited, Brookvale, Australia) and the "Quantikine" IL-6 kit (Research & Diagnostic Systems, Minneapolis, MN).

RNA purification. Four hours after stimulation of bone marrow fibroblasts with cytokines, total cellular RNA was isolated by phenol extraction in the presence of vanadyl ribonucleoside complex and precipitated with absolute ethanol. The recovery was 1–2 $\mu g/10^6$ cells as assessed by spectral photometry. Samples (10 μg) of isolated RNA were separated by agarose-formaldehyde gel electrophoresis. Concentration and integrity were verified by staining of ribosomal bands with ethidium bromide. Next, alkaline transfer (0.05 M NaOH) to Hybond N nylon membranes (Amersham Buchler, Braunschweig, FRG) was performed. Before hybridization the position of ethidium bromide-stained 28S and 18S rRNA was marked on the filter for subsequent calibration of cytokine mRNA signals. IL-6 mRNA measures 1.3 kb.

Northern blot. An IL-6-specific antisense mRNA probe was generated in a two-step procedure. Initially a polymerase chain reaction with human genomic DNA was performed using a T7 promotor-coupled 3′ (GGA ATA CGA CTC ACT ATA GGA AGA TGG ACT GCA GGA ACT CCTT) and 5′ (AAC TCC TCC TCC ACA AGC) oligonucleotide. The 3′ oligonucleotide and the 5′ oligonucleotide used in the polymerase chain reaction stem from the exon 1/2 and exon 5 of the IL-6 gene (30), respectively. Subsequently, the IL-6 specific antisense mRNA probe is generated in the presence of [^{32}P]UTP (DuPont NEN, Dreieich, FRG) in a T7-polymerase assay (Boehringer Mannheim, FRG). Filters were prehybridized for 3 h at 58°C in 50% (vol/vol) formamide, 2.5 mM phosphate buffer (pH 6.5), 5 × SSC (0.75 M NaCl, 75 mM sodium citrate), 5 × Denhard's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1 g/L BSA), 1 g/L SDS, 60 mg/L salmon sperm DNA, 200 mg/L yeast RNA, and 10% (vol/vol) dextran sulfate. In addition, the hybridization buffer contained the labeled antisense mRNA. The filters were incubated for 20 h at 58°C. They were then washed in a multistep fashion with 2 g/L SDS/2 × SSC (0.3 M NaCl, 30 mM sodium citrate) at 58°C for 30 min, then 1 g/L SDS/0.2 × SSC (30 mM NaCl, 3 mM sodium citrate) at 60°C and at 68°C for 60 min each. Subsequently the filters were incubated in 2 × SSC containing 5 mg/L RNase A for 3 min at 37°C for further reduction of nonspecific binding. After a final wash with 2 × SSC for 3 min at 37°C, filters were exposed to 3M x-ray film with intensification screen at −70°C.

Statistical analysis. Patients' data were compared with data from five healthy age-matched bone marrow donors serving as NC. For descriptive statistics mean values and standard errors were calculated. The Mann Whitney U test was performed to detect differences between groups; the paired t test was used to evaluate differences within the NC. p values of 0.05 or less were considered significant.

RESULTS

Bone marrow stromal cell cultures, consisting predominately of fibroblasts, were established from three children with acquired AA, two children with Fanconi anemia, and five NC. Baseline GM-CSF production in bone marrow fibroblasts from patients with AA (44 ± 5 ng/L; mean ± SE) did not differ significantly from GM-CSF production in NC (91 ± 40 ng/L), neither did stimulated GM-CSF production in patients with AA

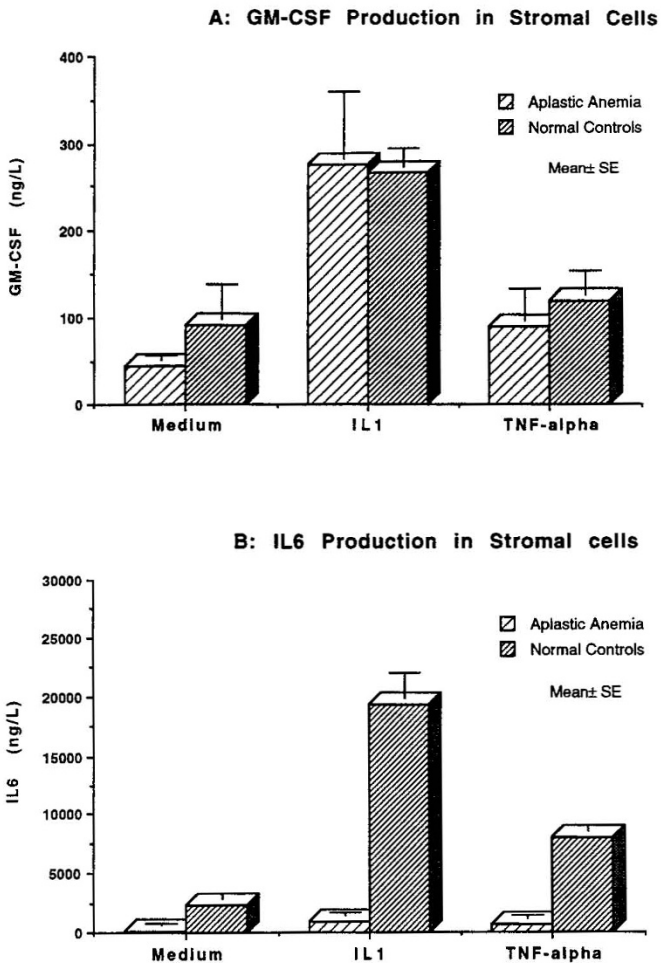

Figure 1. GM-CSF (*A*) and IL-6 (*B*) protein production in bone marrow fibroblasts from patients with AA and NC after culture for 3 d in the absence (*medium*) and presence of stimulation with 30 000 IU/L IL-1 or 50 000 IU/L TNF-α.

(IL-1: 277 ± 77 ng/L; or TNF-α: 89 ± 37 ng/L) when compared with NC (IL-1: 266 ± 38 ng/L; TNF-α: 119 ± 27 ng/L) (Fig. 1*A*). In contrast, baseline IL-6 production in bone marrow fibroblasts from patients with AA (184 ± 49 ng/L) is significantly reduced in comparison to NC (2349 ± 459 ng/L, $p = 0.003$) (Fig. 1*B*). Also, after stimulation with IL-1 (935 ± 257 ng/L) or TNF-α (678 ± 203 ng/L) IL-6 production in bone marrow fibroblasts from patients with AA is significantly lower than in NC under the respective stimulatory conditions (IL-1: 19354 ± 2115 ng/L, $p = 0.002$; TNF-α: 7970 ± 475 ng/L, $p = 0.003$). IL-6 measurements from each patient with AA studied are specified in Table 1.

Several viruses have been shown to modulate host cell IL-6 production. We investigated baseline and cytokine-induced IL-6 production in CMV-infected bone marrow fibroblasts from patients with acquired AA and NC. Effects of CMV infection on cytokine production could not be studied in fibroblasts from patients with Fanconi anemia, as cellular destruction was pronounced shortly after CMV infection due to the pathognomonic cellular fragility.

Three days after infection with the CMV laboratory strains AD 169 and Davies II, IL-6 concentrations in stromal cell supernatants were assessed (Fig. 2). CMV-infected bone marrow fibroblasts from patients with AA exhibit some increase in baseline (975 ± 169 ng/L) and cytokine-induced IL-6 production (IL-1: 5332 ± 1934 ng/L; TNF-α: 3522 ± 1324 ng/L) when compared with uninfected bone marrow fibroblasts (baseline: 221 ± 79 ng/L; IL-1: 1318 ± 192 ng/L; TNF-α: 858 ± 304 ng/L). However, stimulation of IL-6 production in response to viral challenge is significantly lower in bone marrow fibroblasts from patients with AA than in CMV-infected bone marrow fibroblasts from NC (baseline: 2114 ± 156 ng/L, $p = 0.02$; IL-1: 29 958 ± 2635 ng/L, $p = 0.01$; TNF-α: 11 802 ± 1708 ng/L, $p = 0.03$).

Table 1. *IL-6 protein production (ng/L) in bone marrow fibroblasts from three children with acquired aplastic anemia (aAA) and two children with Fanconi anemia (FA) after culture for 3 d in the absence (medium) and presence of stimulation with 30 000 IU/L IL-1 or 50 000 IU/L TNF-α*

Patients	Medium	IL-1	TNF-α
aAA	375	1700	300
aAA	116	1103	1348
aAA	172	1150	925
FA	145	330	590
FA	110	390	275

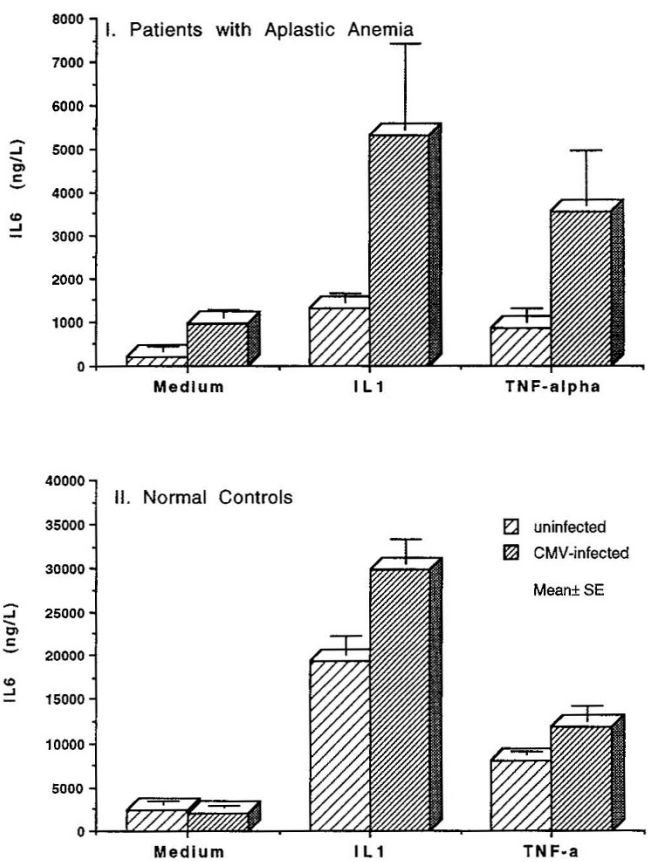

Figure 2. IL-6 protein production in bone marrow fibroblasts from patients with AA (*I*) and NC (*II*) 3 d after infection with a mixture of CMV laboratory strains ADA169 and Davies II and subsequent culture in the absence (*medium*) or presence of stimulation with 30 000 IU/L IL-1 or 50 000 IU/L TNF-α. Please note the change of scale in the *y* axis between patients with AA and NC.

Ten days after CMV-infection, stromal cell cultures were restimulated with cytokines. After additional 3 d of culture, IL-6 concentrations in stromal cell supernatants were assessed (Fig. 3). In patients with AA, cytokine-induced IL-6 production in CMV-infected bone marrow fibroblasts (IL-1: 93 ± 49 ng/L; TNF-α: 71 ± 14 ng/L) was markedly lower than in uninfected cell cultures (IL-1: 923 ± 441 ng/L; TNF-α: 2074 ± 514 ng/L). In contrast in NC, baseline (4114 ± 1054 ng/L) and TNF-α-induced (9116 ± 1026 ng/l) IL-6 production remained elevated in CMV-infected bone marrow fibroblasts when compared with their uninfected controls (baseline: 1588 ± 434 ng/L, $p = 0.02$; TNF-α: 6556 ± 803 ng/L, $p = 0.007$). In NC, CMV-mediated inhibition of IL-6 production was observed only upon stimulation with IL-1 (CMV-infected: 14 059 ± 2671 ng/L; uninfected: 21 773 ± 5340 ng/L, $p = 0.04$). Yet, inhibition was only 35% in comparison to 90% in patients with AA. As described in Methods, there was no difference in CMV-induced CPE between bone marrow fibroblasts from patients with acquired AA and NC. Inhibition of IL-6 production during prolonged CMV infection was further demonstrated by Northern blot analysis (Fig. 4). Ten days after CMV infection, bone marrow fibroblasts from patients with AA and NC were stimulated with IL-1. After 4 h, mRNA was extracted for analysis. The ethidium bromide-stained rRNA bands document RNA integrity. In patients with AA, the IL-6 mRNA specific signal is markedly reduced in CMV-infected bone marrow fibroblasts when compared with uninfected fibroblasts. In bone marrow fibroblasts from NC, CMV-mediated reduction of IL-6-specific mRNA accumulation is less pronounced than in patients with AA.

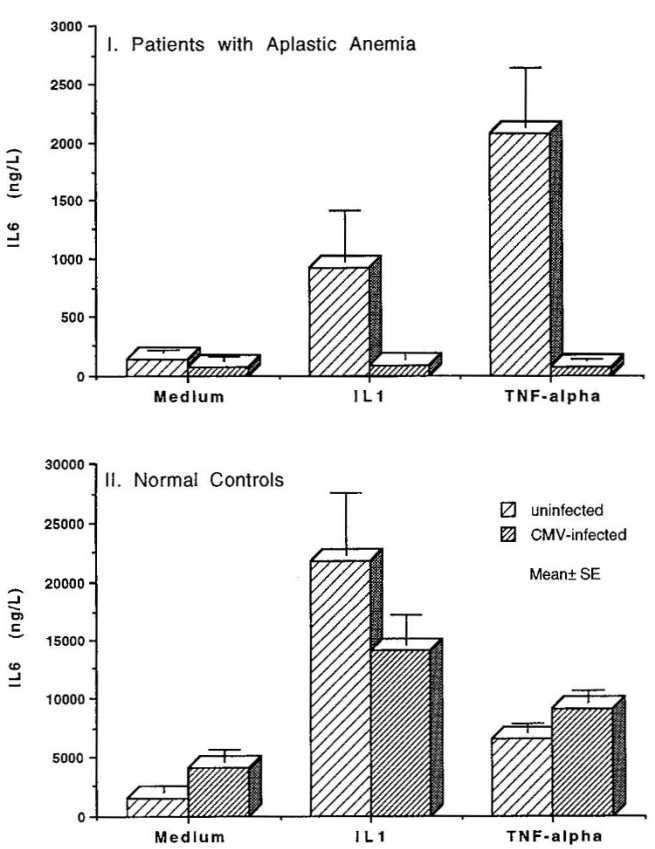

Figure 3. IL-6 protein production in bone marrow fibroblasts from patients with AA (*I*) and NC (*II*) 10 d after infection with CMV and subsequent culture for 3 d in the absence (*medium*) or presence of stimulation with 30 000 IU/L IL-1 or 50 000 IU/L TNF-α. Please note the change of scale in the *y* axis between patients with AA and NC.

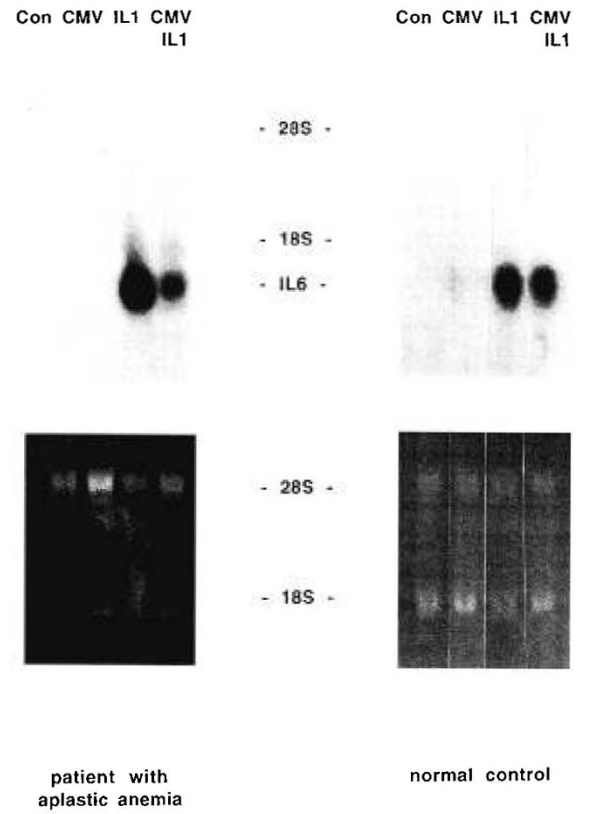

Figure 4. IL-6 mRNA production in bone marrow fibroblasts from patients with aplastic anemia and normal controls ten days after infection with CMV and subsequent culture for four hours in the absence or presence of stimulation with 30 000 IU/L IL-1. Uninfected fibroblasts cultured in medium alone (*Con*) or in the presence of IL-1 were also assessed for IL-6 mRNA production. Accumulation of IL-6 mRNA was assessed by Northern blot analysis. The IL-6 specific mRNA signal (*upper panel*) is indicated. Ethidium bromide stain of ribosomal 18S and 28S RNA bands (*lower panel*) indicate the total amount of RNA loaded.

DISCUSSION

AA is a disease of diverse etiologies (1, 2). Defects of the bone marrow microenvironment have been suggested as one possible pathogenetic mechanism (8–13). Yet, its actual contribution to hematopoietic insufficiency in man is still controversial. Bone marrow stromal cells exert their regulatory effects on hematopoiesis in part by the production of cytokines. We investigated constitutive and induced production of GM-CSF and IL-6 by adherent bone marrow stromal cells from patients with AA. Cytokine production was assessed after several passages of bone marrow stromal cells yielding an adherent cell layer of predominately fibroblasts deficient of myeloid cells. In our experimental system, there was no difference in both baseline and induced GM-CSF production between bone marrow fibroblasts from patients with AA or NC. In contrast, constitutive IL-6 production in bone marrow

fibroblasts from patients with AA was less than 10% of IL-6 production in NC. IL-6 production in these patients was also severely impaired upon stimulation of stromal cell cultures with either IL-1 or TNF-α with less than 22% of IL-6 production seen in NC. There was no difference in the IL-6 production defect between patients with acquired AA and Fanconi anemia.

A specific IL-6 production defect has previously been demonstrated for patients with Fanconi anemia in skin fibroblastoid cell lines (28, 29), whereas for patients with acquired AA, IL-6 production in stromal cells grown under long-term bone marrow culture conditions has been shown to be largely comparable to NC (30, 31). However, in these studies, cytokine production in patients with acquired AA did show considerable variation. Variation in cytokine production among patients with acquired AA may in part be due to different patient characteristics. The three patients with acquired AA in our study had all suffered from longstanding disease, two of them from severe AA. Also, differences in the experimental systems used may account for the variation in stromal cell function observed in patients with AA. Thus, in cross-over studies using a long-term bone marrow culture system, bone marrow stromal cells from patients with acquired AA were shown to give sufficient support to normal progenitor growth (4, 5, 7), whereas supernatants produced soley by bone marrow fibroblasts were found to exhibit defective colony-stimulating activity (8–10). Our data provide evidence for a specific IL-6 production defect in bone marrow fibroblasts of some patients with AA in the presence of normal GM-CSF production. IL-6 is critical in inducing megakaryopoiesis (19) and potentiating IL-3-mediated stimulation of stem cell proliferation (20). Impaired production of IL-1 in patients with AA (32) in combination with an IL-6 production defect in bone marrow fibroblasts might lead to a deficient proliferative response of progenitor cells of particular relevance in high demand situations such as acute infections.

Increased IL-6 production in stromal as well as T cells represents an early antiviral defense mechanism (25). We examined the IL-6 response to viral challenge with CMV in bone marrow fibroblasts from patients with acquired AA and NC. During the early phase of infection, CMV stimulates constitutive as well as induced IL-6 protein production. However, bone marrow fibroblasts from patients with AA produce significantly less IL-6 protein in response to viral challenge than bone marrow fibroblasts from NC. During the late phase of CMV infection, cytokine-mediated IL-6 protein production was reduced by 90% in bone marrow fibroblasts from patients with AA, whereas in NC CMV-mediated inhibition of IL-6 production was 35% and could be demonstrated in IL-1-stimulated fibroblasts only. As there was no difference in CMV-induced CPE between bone marrow fibroblasts from patients with acquired AA and NC, reduced IL-6 production in the three patients studied seems to be constitutive. In addition, reduction of IL-6 production during the later stage of CMV-infection was demonstrated on the mRNA level. Again inhibition of IL-6 mRNA accumulation was more profound in IL-1-stimulated bone marrow fibroblasts from patients with AA than from NC.

We demonstrated that, in some patients with acquired AA, IL-6 production in bone marrow fibroblasts is not only reduced constitutively and in response to stimulation with cytokines, such as IL-1 or TNF-α, but also in response to viral challenge. This indicates that a functionally abnormal microenvironment does play a role in some cases of AA. Viral infections of bone marrow fibroblasts in association with inhibition of IL-6 production might potentiate hematopoietic suppression in these patients.

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