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Rescue of Myeloid Lineage-Committed Preprogenitor Cells from Cytomegalovirus-Infected Bone Marrow Stroma

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The effect of murine cytomegalovirus on myelopoiesis was studied in long-term bone marrow culture to find an in vitro correlate for the lethal virus interference with bone marrow reconstitution (W. Mutter, M. J. Reddehase, F. W. Busch, H.-J. Bühring, and U. H. Koszinowski, J. Exp. Med. 167:1645–1658, 1988). The in vitro generation of granulocyte-monocyte progenitors (CFU-GM) discontinued after infection of the stromal cell layer, whereas the proliferation and differentiation of CFU-GM to granulocyte-monocyte colonies remained unaffected. A protocol was established to probe the functional integrity of earlier hematopoietic cells. Pre-CFU-GM (the progenitors of the CFU-GM) could be recovered from an infected bone marrow donor culture by transfer onto an inductive recipient stromal cell layer. Thus, at least in vitro, infection of bone marrow stroma appears to be the only cause of the defect in myelopoiesis.

Clinical cytomegalovirus (CMV) infection endangers immunocompromised bone marrow transplant patients not only by causing interstitial pneumonia and other severe organ manifestations (8) but also by contributing actively by myelosuppression to the maintenance of an immunodepressed state and to graft failure (3, 11, 15). Perturbation of stromal cell functions critical for myelopoiesis as well as infection of cells of the myeloid lineage have been considered for an explanation. A recent study by Simmons and colleagues (18) on the effect of human CMV on in vitro hematopoiesis in long-term bone marrow culture (LTBMC) has shown that clinical CMV isolates can be grouped into two main classes. Two-thirds of the isolates resembled the laboratory strain AD169 in its tropism for fibroblastlike stromal cells (2, 18), whereas one-third affected myelopoiesis directly by infection of myeloid progenitors (18). Whether comparable mechanisms are operating in vivo is difficult to ascertain for human CMV disease.

We have been working on CMV pathogenesis in a mouse model (reviewed in reference 5) and previously documented a lethal in vivo interference of murine CMV infection with hematopoietic reconstitution (9). In that study, an inhibitory effect on myelopoiesis became manifested not only as a failure in the production of the direct progenitors of granulocytes and monocytes, the granulocyte-monocyte CFU (CFU-GM), but also as a failure in the regeneration of day 14 spleen colony-forming stem cells, which are considered to precede the CFU-GM in the myeloid lineage (reviewed in references 6 and 14). Therefore, infection of stem cells, infection of stromal cells that are required for supporting stem cell self-renewal and differentiation (1, 20), or both may account for the defective myelopoiesis. The specific aim of the present study was to distinguish between these possibilities in an in vitro model. To know whether a defect has a single cause or multiple causes is not only of theoretical interest but also of relevance for trials of a therapeutic intervention.

Cessation of CFU-GM production after CMV infection of LTBMC. LTBMC were established basically according to the Dexter protocol (4, 19) by seeding 3×10^6 bone marrow cells (BMC) in a volume of 3 ml in polypropylene tissue culture flasks. After 2 weeks, when an adherent cell layer composed of monocytic and endothelial-like cells as well as adipocytes and fibroblasts (1, 4, 12) had developed and clusters of proliferating hematopoietic cells in the form of cobblestone areas became discernible, cultures were infected with the ATCC VR-194 Smith strain of murine CMV. From then on, virus replication and CFU-GM content were monitored at 4-day intervals (Fig. 1). By day 4, after an initial drop in inoculum virus titer, intrinsic virus replication started and peaked around day 12, at which time the adherent stromal cell layer was visibly destroyed. As a coincident event, CFU-GM production ceased after day 4. By contrast, in the absence of CMV infection, CFU-GM content increased until by 1 month after the cultures were initiated, a plateau level was reached.

It became clear from these findings that CMV can productively infect bone marrow stroma and inhibit myelopoiesis in a system involving only hematopoietic and stromal BMC. Since in vitro hematopoiesis is known to be stroma dependent (1, 4, 19, 20), the observation that the production of CFU-GM in LTBMC did not continue on a destroyed stromal cell layer as shown here for murine CMV and previously for human CMV (2) could not come as a surprise. Yet, the question remained whether the stromal defect was the only reason for the failure in myelopoiesis.

CMV infection does not prevent the development of granulocyte-monocyte colonies from CFU-GM. Declining CFU-GM content in LTBMC (Fig. 1) could indicate not only a stop in production but also that previously generated CFU-GM were infected. CFU-GM cannot be quantitated directly but only by counting their clonal progeny, namely, granulocyte-monocyte colonies that develop in soft gel hematopoietic progenitor assay cultures supplemented with hemopoietins (7, 14). CMV could interrupt this development at any stage. This possibility was tested by incubating BMC in suspen-

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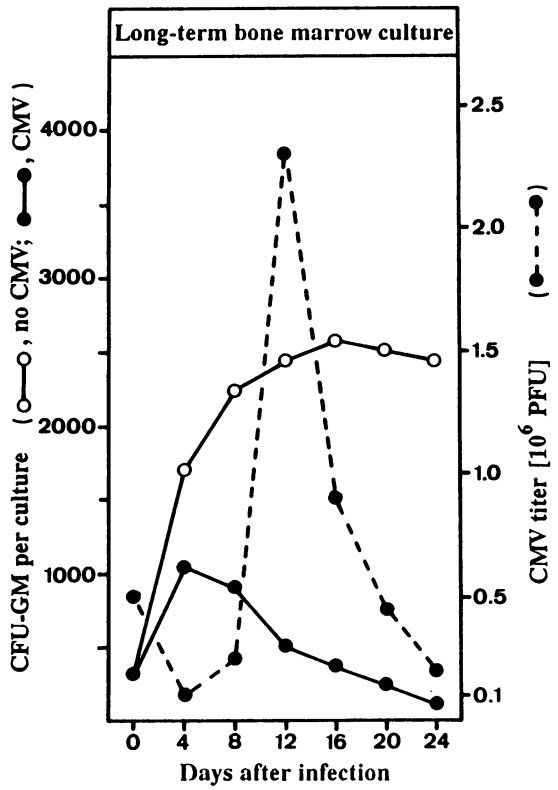

FIG. 1. Kinetics of virus production and CFU-GM content after infection of LTBMC. A total of 42 replicate Dexter LTBMC (4, 19) were established. Medium was renewed after week 1, and after 2 weeks, one-half of the cultures were infected. At 4-day intervals, nonadherent cells from three cultures of each of the two groups were harvested and CFU-GM were quantitated in a progenitor assay. Data represent granulocyte-macrophage colonies from 5×10^4 LTBMC cells counted in triplicate assay cultures and multiplied by the average yield of hematopoietic cells in the three LTBMC. For infected LTBMC, the virus titer was determined by a plaque assay (17) from the entire culture content after freezing and thawing. Data represent the median of three determinations.

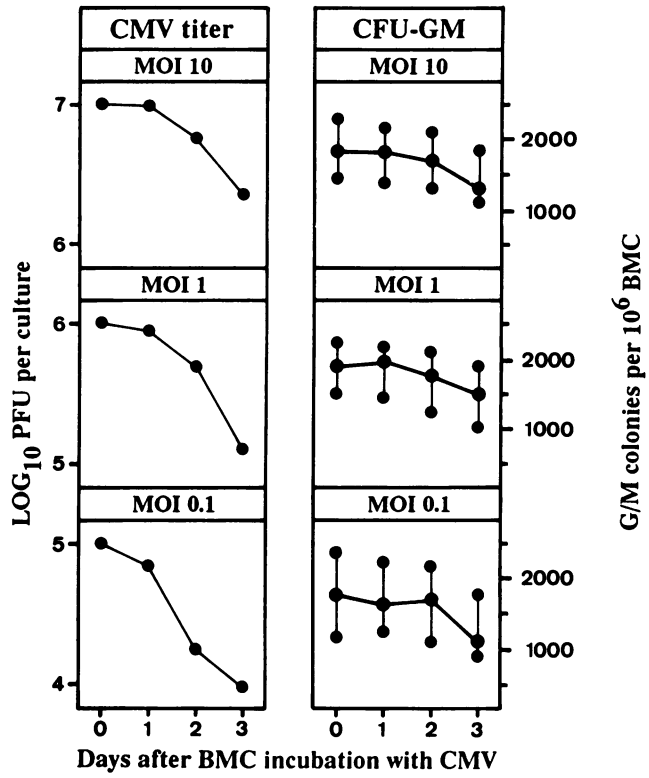

FIG. 2. Exposure of BMC to CMV. Samples of 10^6 femoral BMC were exposed to CMV at the indicated multiplicities of infection (MOI) for up to 3 days. CFU-GM numbers were determined in triplicate. Median value and range are indicated. G/M, Granulocyte-macrophage.

sion for up to 3 days with CMV at multiplicities of infection ranging from 0.1 to 10 before CFU-GM were quantitated in progenitor assay cultures supplemented with WEHI 3B-conditioned medium as a source of colony-stimulating factors. At no dose of CMV and after no period of preincubation of BMC could an inhibition of GM colony formation be observed (Fig. 2).

Salivary gland preparations of murine CMV are myelosuppressive in the absence of viral infectivity. The message of Fig. 2 was inconsistent with previous data of Petursson et al. (13), who did conceptually the same experiment but found a significant reduction in granulocyte-macrophage colony formation, which led them to conclude that murine CMV can cause myelosuppression by direct infection of progenitor cells. These investigators had used the Osborn derivative of the Smith strain of murine CMV propagated in salivary glands and added to BMC as diluted tissue homogenate, while we worked with the ATCC virus propagated in mouse embryo fibroblasts and purified. In the light of the recent experience that clinical isolates of human CMV differ in their cell tropism (18), the reported difference in virulence between salivary gland isolates of murine CMV and the tissue-culture virus (10) had to be considered as a possible reason for the discrepant results.

We therefore repeated the experiments with a salivary gland preparation of the ATCC CMV and also confirmed with this virus a dose-dependent inhibition of granulocyte-macrophage colony formation that was significantly stronger than the negative effect seen with control preparations from salivary glands of age-matched noninfected donors (Table 1). Yet, a preparation in which viral infectivity was abolished by inactivation with UV light remained as suppressive as the infectious preparation. UV light treatment of the tissue culture virus did not induce a suppressive property (data not shown).

It is beyond the scope of this report to identify the suppressive principle that is clearly not infectious CMV and that is present in infected but not, or to a lesser extent, in noninfected salivary gland tissue. One might think of cytokines that are released only by infected cells or by leukocytes infiltrating the infected tissue. The fact that salivary gland preparations of murine CMV are myelosuppressive in the absence of viral infectivity should be considered whenever such preparations are used or have been used for studying the pathogenesis of murine CMV.

Myeloid lineage-committed preprogenitor cells can be recovered from infected LTBMC. The question of whether inhibition of myelopoiesis is caused by infection of hematopoietic stem cells has not yet been adequately addressed for either murine or human CMV. The experimental regimen of our approach to the problem is outlined in Fig. 3.

TABLE 1. Inhibition of granulocyte-macrophage colony formation by salivary gland isolate of murine CMV[a]

CMV prep[b]	PFU (MOI[c])	Granulocyte-macrophage colonies from 10^6 BMC	
		Day 1	Day 3
No CMV	0 (0)	1,660	1,260
TC-CMV	1×10^7 (10)	1,780	1,340
SGH-CMV (1:10)	2×10^6 (2)	0	0
SGH-CMV (1:100)	2×10^5 (0.2)	780	640
SGH (1:10)	0 (0)	540	300
SGH (1:100)	0 (0)	1,160	660
SGH-CMV$_i$ (1:10)	0 (0)	0	0

[a] BMC (10^6) from normal BALB/c donors were incubated with CMV or control preparations. CFU-GM assay cultures were set up at day 1 or day 3 postinfection, and granulocyte-macrophage colonies were determined 8 days later. Data represent colonies from 5×10^4 BMC counted in triplicate cultures and multiplied by a factor of 20.

[b] TC-CMV, Sucrose gradient-purified tissue culture (mouse embryo fibroblast) CMV; SGH-CMV, 1:10-diluted salivary gland homogenate from infected 3-week-old BALB/c donors (the titer of the virus stock was 2×10^7 PFU/ml); SGH, 1:10-diluted salivary gland homogenate from noninfected donors; SGH-CMV$_i$, SGH-CMV inactivated by UV light.

[c] MOI, Multiplicity of infection.

LTBMC were established and either infected after 2 weeks or left uninfected as a control (Fig. 3, top line). As documented in the original report (4), LTBMC consist of an adherent stromal cell layer, of hematopoietic progenitor cells that per definition cannot self-renew, and of hematopoietic stem cells. Because we identified stem cells not by spleen colony formation but by their ability to give rise to CFU-GM, a progenitor cell type, we named them preprogenitors or pre-CFU-GM to describe more accurately what had been measured. Within 4 days of infection, the production rate of CFU-GM had already declined (recall Fig. 1), which could result from infection of the stroma, of the pre-CFU-GM, or both. If the functional integrity of pre-CFU-GM was not affected by either infection or a deprivation of stromal support during the 4 days of exposure to CMV, the pre-CFU-GM should be able to generate CFU-GM after transfer onto a functional stromal layer (Fig. 3, center line). The recipient inductive stroma was established in a regular LTBMC, from which the nonadherent hematopoietic cells were washed off after they had been inactivated by irradiation (20). Residual intrinsic hematopoiesis was not detectable in the recipient cultures (data not shown). To preclude infection of the recipient stroma by free CMV, nonadherent hematopoietic donor culture cells were incubated with CMV-neutralizing antiserum and sedimented through a serum cushion before they were transferred. CFU-GM production in the recipient cultures was assessed by quantitating CFU-GM (Fig. 3, bottom line) before (CFU-GM input) and after (CFU-GM output) an 8-day period of in vitro hematopoiesis.

The results compiled in Table 2 make it clear that the rate of CFU-GM production on the recipient stroma is independent of an infection in the donor cultures. Thus, apparently, pre-CFU-GM were neither destroyed by productive infection nor was their functional integrity damaged by a short-term deprivation of stromal support. The functional data do not exclude latent infection of pre-CFU-GM that may result in reactivation at later stages of differentiation. The cellular site of CMV latency has not yet been identified in any tissue or, specifically, in bone marrow. Consequently, the physical state of the viral genome and viral gene expression during latency are still undefined. Testing this for pre-CFU-GM is not trivial, because these cells have a very low frequency in bone marrow and because there is to date no protocol for purifying them preparatively. We can, however, draw an important conclusion from the findings presented here. If pre-CFU-GM were indeed latently infected, then latent infection did not disturb their capacity to give rise to colonies of differentiated progeny.

Conclusion. With respect to the in vitro effects on bone marrow stroma and on hematopoietic progenitor cells, the

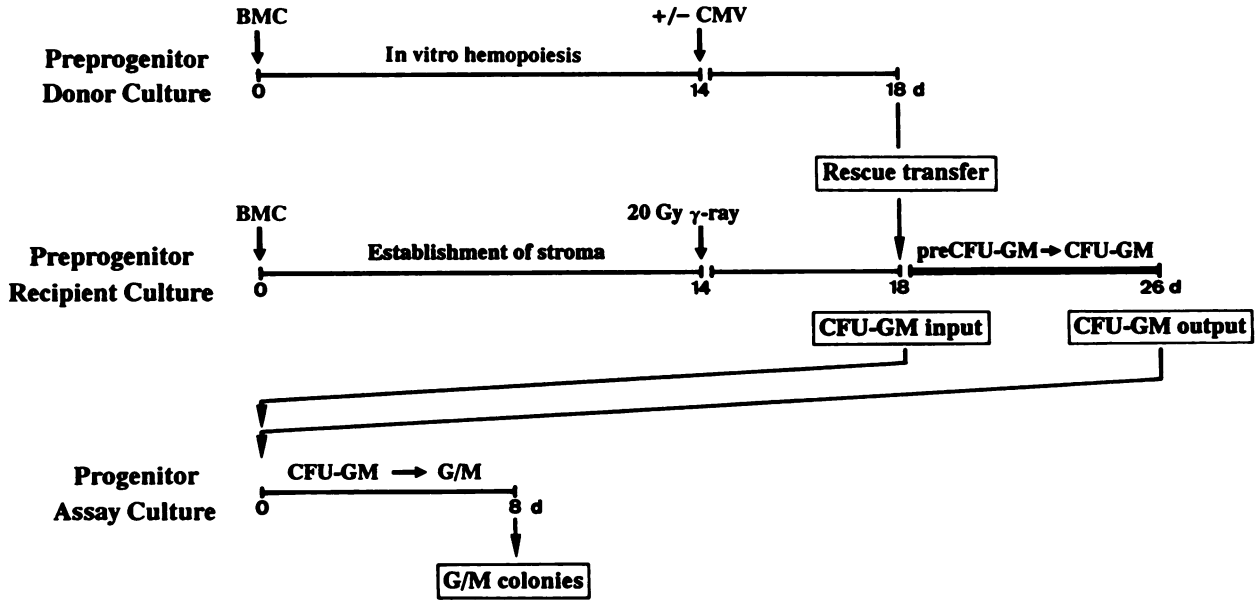

FIG. 3. Experimental regimen and time schedule for preprogenitor transfer onto inductive bone marrow stroma. d, Days; G/M, granulocyte-macrophage.

TABLE 2. Rescue of pre-CFU-GM from CMV-infected LTBMC[a]

Expt (no. of cultures)	Preprogenitor recipient culture		% Amplification
	Input CFU-GM	Output CFU-GM	
+CMV			
1 (1)	561	1,338	239
2 (1)	441	1,278	290
3 (6)	549	840	153
		963	175
		1,737	316
		693	126
		954	174
		1,080	197
Mean			209
Median			186
−CMV (3 (3))	882	2,115	240
		1,341	152
		1,803	204
Mean			199
Median			204

[a] For experimental regimen and time schedule, see Fig. 3. Data represent granulocyte-macrophage colonies from 5×10^4 nonadherent LTBMC cells counted in triplicate assay cultures and multiplied by the yield of nonadherent cells per donor (input CFU-GM) or recipient (output CFU-GM) culture.

data obtained with murine CMV are in line with the properties described for the human CMV laboratory strain AD169 and the majority of clinical isolates (2, 18). This fact encourages the use of the mouse model for studying CMV-mediated myelosuppression in the infected host, in which, in addition to intrinsic effects of CMV within bone marrow, tissue lesions in several organs (17) including hormonal glands (16) may also account for extrinsic negative influences on hematopoiesis. The new information is that murine CMV does not interfere with proliferation and differentiation of preprogenitors of the myelopoietic lineage. The in vitro hematopoiesis transfer protocol used here to probe preprogenitor function should also be applicable for further investigating the inhibition of human myelopoiesis by CMV.

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