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Myelopoiesis in vitro is suppressed by hepatitis A virus*

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Summary. Perturbations of hematopoietic regulation ranging from transient granulocytopenia to rare cases of bone marrow failure are associated with infections due to hepatitis A virus (HAV). In an attempt to elucidate the pathogenetic mechanisms we had previously established that HAV has a direct suppressive effect on human bone marrow progenitors (CFU-GM, -GEMM, BFU-E). These studies were extended to long-term bone marrow cultures (LTBMC): Inoculation of bone marrow mononuclear cells with HAV did not interfere with the establishment of an adherent stromal layer, nor did the inoculation of already established layers cause any morphologically recognizable changes to the stroma. In contrast, a significant and progressive decline of the CFU-GM content in the culture supernatants was demonstrated. HAV antigen was detected by APAAP stain in a subpopulation of stromal cells, and sequential estimations of virus titers in the supernatants provided evidence for viral replication in primary bone marrow cultures. Interferon-gamma and tumor necrosis factor-alpha levels of infected cultures did not differ from those of uninfected controls. These findings argue for a direct suppression of (pre-) CFU-GM by HAV in a model system (LTBMC) lacking an immune defense which would limit viral replication.

Key words: Hepatitis A virus – In vitro myelopoiesis – Long-term bone marrow cultures

Introduction

A frequent observation in the early course of hepatitis A virus (HAV) infections is the decline of all blood-cell lineages except monocytes. These changes are transient, however, and usually resolve approximately 2 weeks after the onset of clinical hepatitis [11]. In contrast, a minority of hepatitis A cases [4] develop severe bone marrow failure, with a mortality exceeding 90% [5]. Early bone marrow transplantation seems to be the treatment of choice for these patients [13, 21]. In order to develop an in vitro model of HAV-associated myelosuppression, we studied the influence of hepatitis A virus on bone marrow progenitor cells such as CFU-GM, BFU-E, and CFU-GEMM. These experiments showed a dose- and duration of inoculation-dependent suppression of hematopoietic progenitors. Since depletion of accessory bone marrow cells did not alter the pattern of suppression, we postulated that HAV can directly inhibit proliferation and differentiation of progenitor cells [3]. In order to investigate the influence of HAV on more immature bone marrow cells in the context of a supporting stromal cell population, we here extend our studies to long-term bone marrow cultures (LTBMC), which probably maintain the proliferation of progenitors closely related to stem cells [20].

Materials and methods

Bone marrow cultures. Mononuclear bone marrow cells (BMNC) from four healthy consenting volunteers (two anti-HAV IgG positive, two negative) were obtained by density gradient centrifugation ($d < 1.077$ g/cm^3) over Percoll. For long-term culture, cells (2×10^6/ml) were suspended in Iscove's modified Dulbecco's medium (IMDM, Gibco), 12.5% fetal calf serum, 12.5% horse serum (Boehringer, Mannheim, FRG), 1% Pen-Strep (Gibco), and 10^{-6} *M* hydrocortisone sodium hydrogensuccinate (Upjohn), according to a modification of the method described by Gartner and Kaplan [10]. Triplicate cultures were set up in slide flasks (Nunc) in a 3-ml volume per flask and incubated in a fully humidified atmosphere of 5% CO_2 in air. Cultures were refed weekly by removal of half of the culture supernatant and replacement of an equal volume of fresh medium. The number of colony-forming units granulocyte-macrophage (CFU-GM) in the nonadherent cell fraction removed was determined weekly. Cells (1×10^5/ml or, if less, the content of the total 1.5-ml volume removed) were suspended in IMDM containing 0.9% methylcellulose, 20% FCS, and 10% human placenta-conditioned medium [15] as a source of colony-stimulating activity. Triplicate cultures were incubated in a fully humidified atmosphere/5% CO_2, and CFU-GM (colonies > 50 cells) were enumerated on day 14.

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Inoculation of bone marrow cultures with HAV. One single batch of mycoplasma-free hepatitis A virus (strain GBM, grown in human fetal fibroblasts [9] was employed for our studies. The virus/target cell ratio (multiplicity of infection, MOI) chosen was 0.5. This MOI had been proven to result in a 50% inhibition of CFU-GM when BMNC were inoculated as described [3]. BMNC were inoculated either before the initiation of long-term cultures or after the establishment of a confluent stromal layer, which was usually on day 14. Control cultures were set up after mock infection with virus-free fibroblast supernatant (HFS).

Determination of HAV titers in culture supernatants. Infectious HAV in LTBMC supernatants was estimated as described earlier [19]. Confluent embryonal fibroblasts were inoculated with log dilutions of LTBMC supernatants and, after 3 weeks of culture, were disrupted by repeated freeze/thaw. HAV in the lysate was estimated by RIA [8] and the tissue-culture infectious $dose_{50}$ ($TCID_{50}$) was calculated according to Kärber [12].

Determination of lymphokines. LTBMC supernatants of all four cultures inoculated by week 2 were screened weekly for interferon-γ (IFN-γ) by RIA (sensitivity 1 U/ml) as well as tumor necrosis factor-α (TNF-α) by IRMA (sensitivity 10 pg/ml, Medgenix, Belgium).

Immunocytological staining procedure. Stromal layers of inoculated LTBMC were assayed for the presence of HAV antigen by standard alkaline phosphatase/anti-alkaline phosphatase (APAAP) staining techniques [16], employing monoclonal antibody (MAB) 7E7 [14] as a HAV-specific reagent. MABs W6/32.HL (recognizing HLA class-I molecules [2]) and W6/32.HK (nonbinding variant [25]) served as positive and negative controls, respectively.

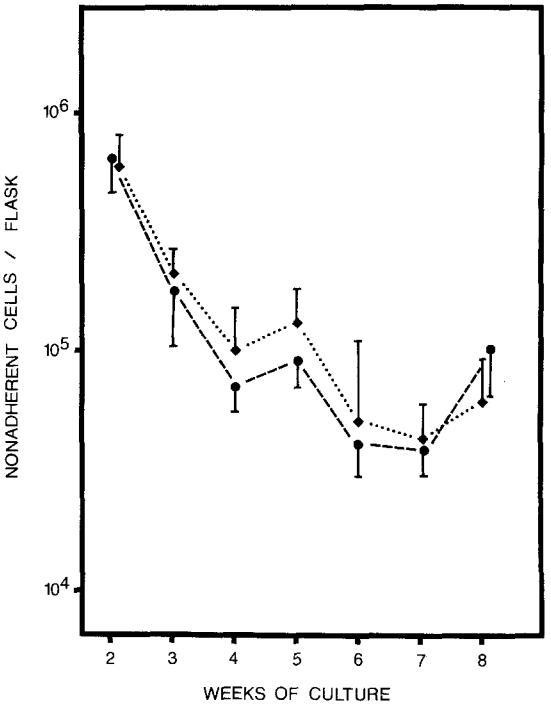

Fig. 1. Kinetics of nonadherent cells in long-term bone marrow cultures infected with HAV. Data represent the mean ± SEM of experiments performed with bone marrow samples from four different donors. ♦···♦, LTBMCs HAV infected at week 2; ●– – –●, LTBMCs mock infected at week 2

Results

In order to detect influences of HAV on the development and cellular composition of stromal layers, LTBMC were examined weekly by phase-contrast microscopy. Infected and control layers both reached confluence after week 2 of culture. The number of areas of active hematopoiesis (cobblestone areas) declined after week 4, followed by a progressive shift towards a predominantly monocyte/macrophage population, again with no noticeable difference between HAV-infected and control cultures. Even after 8 weeks of culture, a cytopathic effect in HAV-containing LTBMC was not observed. The number of cells present in the nonadherent fraction removed with each weekly refeeding did not differ between control and HAV infected cultures (Fig. 1). While by week 6 in the uninfected controls this nonadherent fraction consisted of 31% ± 10% cells of different maturational stages of granulocytes, 21% ± 12% cells with lymphoid or blast morphology, and 48% ± 9% monocytes, the cell production of the infected cultures had shifted to a predominantly monocytic population with only occasional (< 5%) granulocytes and lymphocytes. Independent of the bone marrow donor's anti-HAV status, the fraction of CFU-GM in the cultures inoculated with HAV after the establishment of a confluent stroma layer had already decreased to 24% ± 15%, one week after inoculation, and it declined further to 7% ± 4% 4 weeks later (Fig. 2) as compared to mock infected controls ($p < 0.01$ by the Friedman test).

That primary bone marrow stromal cultures are susceptible to infection by HAV was documented by the demonstration of HAV antigen in a subpopulation of cells with predominantly fibroblastoid morphology comprising 15%–25% of the cells in the adherent cell layer, independently of the time point of inoculation (week 0 or 2; Fig. 3). When cultures were infected at week 0, sequential determinations of virus titers in the culture supernatants provided evidence for productive infection, with an increase of the $TCID_{50}$ by a factor of 10^4 from week 1 to week 8 (Fig. 4). Infection of already established confluent stromal layers (week 2) also resulted in the production of infectious HAV (10^7 $TCID_{50}$/ml by week 8). Since $TCID_{50}$s were not corrected for the weekly replacement of half of the culture supernatant, these values certainly represent an underestimation of the actual rate of virus replication.

In order to exclude the possibility that HAV infection of LTBMC had induced the secretion of lymphokines with known inhibitory effects on in vitro hematopoiesis, levels of IFN-γ and TNF-α in the culture supernatants were determined weekly. Titers ranging from 2.1 ± 2.5 U/ml to 0.1 ± 0.2 U/ml IFN-γ and from 127 ± 104 pg/ml TNF-α by week 2, declining to under the threshold of detection during the culture period were measured. No differences between infected and control cultures were demonstrated, however.

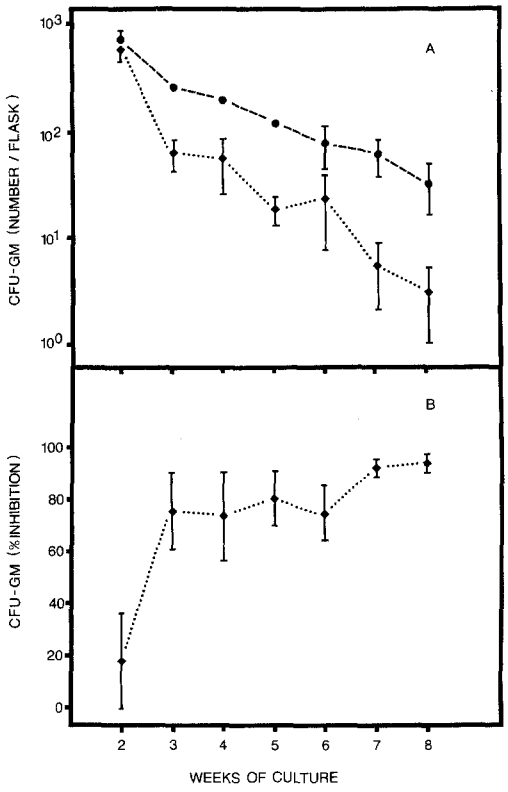

Fig. 2 A, B. Kinetics of CFU-GM in the nonadherent cell fraction of long-term marrow cultures infected with HAV. Data represent the mean ± SEM of experiments performed with bone marrow samples from four different donors. **A** Absolute numbers of granulocyte/macrophage progenitors (CFU-GM) in the nonadherent cell fraction. **B** Reduction (%) of CFU-GM by HAV compared with mock-infected LTBMCs. ♦···♦, LTBMCs HAV infected at week 2; ●- - - -●, LTBMCs mock infected at week 2

Discussion

The myelotropic properties of HAV have some important clinical cimplications: A transient suppressive influence on hematopoiesis can be regularly observed in the preicteric phase of hepatitis A. In addition, rare fatal courses of aplastic anemia associated with infectious hepatitis have been reported by several investigators with serological evidence of HAV as responsible agent [18, 21]. In an attempt to elucidate the pathogenesis of HAV-associated bone marrow suppression, we were previously able to demonstrate a virus-specific inhibition of stromal cell-depleted, CD 34^+ bone marrow progenitors (CFU-GM, BFU-E, CFU-GEMM) [3]. We now extended these studies to human Dexter-type LTBMC, a model which might support the proliferation of progenitors more closely related to pluripotent stem cells [20]. To the best of our knowledge, this is the first publication describing the effects of hepatitis viruses in that model system. In contrast to the severe damage usually observed after infection of stromal cells with cytomegalovirus (CMV) [1, 17], inoculation of BMNC with HAV-strain GBM did not influence the formation of a confluent stromal layer. Furthermore, inoculation of already established layers did not cause any cytopathic effects nor alterations of their cellular composition, at least as far as could be judged by phase-contrast microscopy. This finding is in accordance with the fact that, with the exception of some cytopathic variants, HAV generally induces an inapparent and persistent rather than a cytolytic infection in vitro [19]. While the numbers of nonadherent cells did not differ significantly between control and infected cultures, there was a shift towards a predominantly monocytic cell population in the infected cultures. This finding is well in accordance with previously published data suggesting a relative resist-

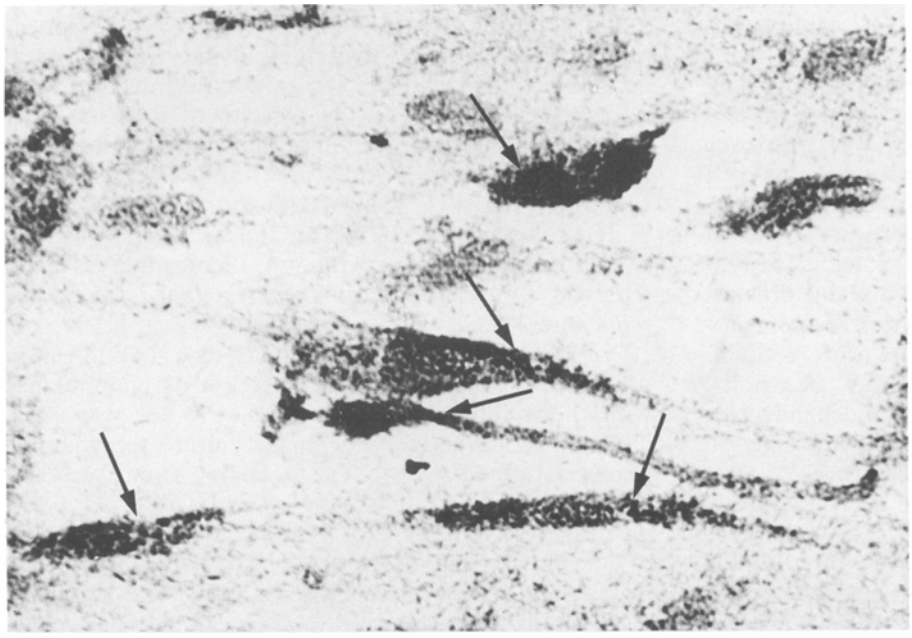

Fig. 3. APAAP stain of 6-week-old (*arrows*) HAV-infected stroma. HAV antigen is demonstrated in the cytoplasm of cells with predominantly fibroblastoid morphology. ×400

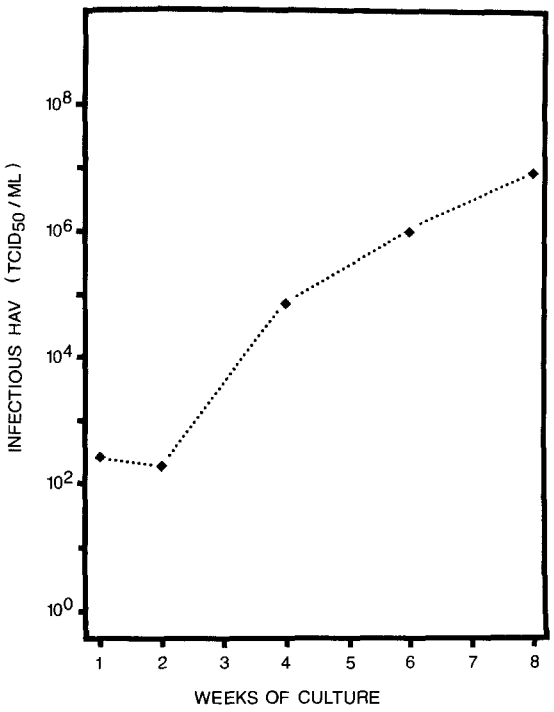

Fig. 4. Titers of infectious HAV in supernatants of long-term bone marrow cultures inoculated at the initiation of the culture (week 0)

ance of monocyte progenitors to HAV infection [3]. The hematopoietic activity as defined by the CFU-GM numbers declined significantly faster in infected LTBMC, independent of the bone marrow donor's anti-HAV serology. At the same time, viral replication took place in a subpopulation of stromal cells, and titers of infectious virus rose by a factor of up to 10^4. This yielded virus/non-adherent target cell ratios which would result – as demonstrated earlier [3] – in a direct inhibition of in vitro hematopoiesis at the maturational level of committed progenitor cells. Although the frequency of cobblestone areas seemed not to differ between infected and control cultures, suppressive effects of HAV on growth and differentiation of more immature hematopoietic cells cannot be excluded. Infection of stromal cells with HAV did not induce the secretion of the inhibitory lymphokines IFN-γ [6] or TNF-α [7], making humorally mediated hematopoietic suppression by these factors unlikely. Additional mechanisms such as reduced secretion of stimulatory molecules by stromal cells – as recently demonstrated in a CMV model [17] – cannot be ruled out and are the focus of current investigations. Our experiments were performed employing the GBM strain of HAV. Although this strain was isolated primarily in fibroblasts, viral attenuation may have influenced our results. Our findings thus await comparison with fresh clinical isolates of HAV. It is interesting, however, to note that bone marrow failure after viral disease is most commonly associated with viruses, particularly hepatitis A, B, and non-A, non-B (C) viruses [4, 21, 22], that are regarded as primarily hepatotropic. In vitro, these viruses rather similarly inhibit the proliferation and differentiation of hematopoietic progenitor cells [3, 23, 24]. Since the liver is the primary organ of fetal as well as extramedullary hematopoiesis in myeloproliferative diseases, this organ is, at least in that functional respect, related to bone marrow. These similarities in ontogeny may explain the myelotropism of hepatitis viruses.

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