Evidence against a key role for transforming growth factor-β1 in cytomegalovirus-induced bone marrow aplasia

Michaela Dobonici, Jürgen Podlech, Hans-Peter Steffens, Sabine Maiberger and Matthias J. Reddehase

Institute for Virology, Johannes Gutenberg-University, Hochhaus am Augustusplatz, 55101 Mainz, Germany

During immunodeficiency after sublethal haematopoietic ablative treatment, cytomegalovirus (CMV) infection interferes with haematopoietic reconstitution and can cause lethal bone marrow (BM) aplasia. The in vivo model of murine CMV infection has identified the BM stroma as the principal target site of CMV in the haematopoietic cord. The infected cell type is the reticular stromal cell which forms the stromal network and produces essential haemopoietins, such as stem-cell factor (SCF). The expression of SCF was found to be reduced in the infected stroma, but the stromal network was not disrupted and the number of infected stromal cells was too low to explain the functional deficiency. These facts call for a negatively regulating cytokine that is induced by the infection and that potentiates the direct effect of infection by down-regulating haemopoietins in un-infected bystander cells. Recent work has suggested that transforming growth factor (TGF)-β1 might be the cytokine involved in CMV-induced BM aplasia. We show here that murine CMV indirectly induces the accumulation of mature TGF-β1 in uninfected renal tubular epithelial cells and TGF-β1 transcription in BM stromal cells, whereas infected renal glomerular and interstitial cells, hepatocytes and BM stromal cells do not coexpress mature TGF-β1. Antiviral CD8 T-cell therapy prevented BM aplasia and also prevented the down-regulation of stromal SCF and interleukin-6 gene expression. Interestingly, however, the CD8 T cells did not preclude the up-regulation of mature TGF-β1, but proved to be inducers of TGF-β1 gene expression in BM stroma. These findings suggest that TGF-β1 is not the mediator of BM aplasia.

Introduction

During the immunodeficiency caused by haematopoietic ablative treatment, primary or recurrent infection with cytomegalovirus (CMV) can inhibit haematopoietic reconstitution in bone marrow (BM) transplant recipients with consequent graft failure and maintenance of the compromise (reviewed in Childs & Emanuel, 1993). BM aplasia is a pivot in CMV pathogenesis (Mutter et al., 1988), because a failure in the reconstitution of protective CD8 T cells provides the grounds for florid, cytolytic virus replication in a variety of vital organs, which is associated with tissue destruction and eventually results in death from multiple organ failure (Reddehase et al., 1985). The mechanism of CMV pathogenesis in BM is therefore of importance for the understanding of CMV pathogenesis in general.

The haematopoietic system can be subdivided into two compartments. The haematopoietic compartment consists of pluripotent stem cells capable of self-renewal, of committed lymphoid and myeloid lineage stem cells, of more differentiated progenitor cells, and of mature cells egressing into the blood. The second compartment is made up from a variety of cell types collectively referred to as stromal cells. Reticular stromal cells (RSC) form the stromal network that is essential for haematopoiesis to occur. This network provides the matrix for the homing of the pluripotent stem cells as well as haemopoietins operating as growth and differentiation factors for haematopoietic stem cells and progenitor cells (reviewed in Chabannon & Torok-Storb, 1992).

Studies with human and murine CMV have addressed the mechanism of myelosuppression by CMV in long-term BM cultures of myeloid lineage cells (reviewed in Childs & Emanuel, 1993). In essence, the studies concur in the conclusion that CMV can infect stromal as well as myeloid haematopoietic cell types. While the infection of monocyte and granulocyte progenitors does not inhibit growth and differentiation, but
generates a reservoir for latent CMV (Maciejewski et al., 1992; Kondo et al., 1994; Minton et al., 1994), infection of stromal cells was found to be productive and cytolytic, resulting in a decline in haemopoietin production with consequent cessation of in vitro haematopoiesis (Apperley et al., 1989; Simmons et al., 1990; Busch et al., 1991; Lagneaux et al., 1996). Thus, the in vitro studies had predicted a destructive infection of the BM stroma as the most likely pathomechanism of CMV in the BM.

In previous work, we have used a murine model of CMV-induced BM aplasia to test this prediction in vivo (Mayer et al., 1997). In accordance with the in vitro studies, the RSC in the haematopoietic cord were found to be in situ target cells for murine CMV, and the expression of genes encoding essential haemopoietins, such as stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), was significantly reduced in the BM stroma of infected mice. Accordingly, the reconstitution of haematopoietic stem and progenitor cells expressing SCF-receptor was deficient. However, the issue of CMV pathogenesis in the BM is not yet fully solved. An open question arises from the fact that the stromal network was not disrupted and that the number of infected RSC present in the haematopoietic cord during BM aplasia was too low to explain the functional deficiency by a direct cytopathic effect of CMV. Hence, we must propose a negative regulator that is induced by the infection and that down-regulates the expression of haemopoietins in uninfected bystander cells also.

Recent work by Haagmans et al. (1997) has provided evidence for the induction of transforming growth factor (TGF)-β1 in the BM and in a variety of tissues by rat CMV infection. Likewise, Lagneaux et al. (1996) reported an in vitro induction of TGF-β1 in human BM stroma by human CMV. TGF-β1, a member of a cytokine superfamily including activins, inhibins and bone morphogenic proteins (reviewed in Roberts & Sporn, 1990; Massague, 1990; Gleizes et al., 1997), can inhibit in vitro haematopoiesis by repression of SCF gene transcription as well as by decreasing the stability of SCF-receptor transcripts (Dubois et al., 1994; Heinrich et al., 1995). Since these effects of TGF-β1 would perfectly explain our previous in vivo data (Mayer et al., 1997), it could be suggested that TGF-β1 might be the postulated negative regulator cytokine. The experiments shown here were aimed at testing this attractive hypothesis.

Methods

- **Induction of BM aplasia.** Animal experiments were approved by the Ethics Commission, permission no. 177-07/941-4, according to German federal law. Throughout, female mice of the inbred strain BALB/c (H-2b haplotype) were used at the age of 8 weeks. Haematoblastic treatment was performed by sublethal total-body irradiation with a single dose of 6 Gy delivered by a 125I source. Murine CMV, strain Smith ATCC VR-194, which represents the cloned and sequenced prototype (Smith, 1954; Ebeling et al., 1983; Rawlinson et al., 1996) was purified as previously described (Kurz et al., 1997), and mice were infected subcutaneously at the left hind footpad with 10^5 p.f.u., corresponding to 10^5 infectious particles (Kurz et al., 1997), at 8 h after irradiation.

  - **Histological analysis of TGF-β1 expression and infection of tissues.** Histopathology in BM, in kidney and in liver was studied at day 14 post-infection (p.i.), which is the time of maximum mortality rate in the infected group. Tissue was fixed in 4% formalin, buffered at pH 7.4. In the case of the femurs, the bone substance was decalcified with either 20% (w/v) EDTA for 18 h for subsequent haematoxylin–eosin (HE) staining or with 5% (w/v) trichloroacetic acid (TCA) for 18 h for the double-staining immunohistochimistry (IHC). Paraffin sections (2 µm) were dewaxed in xylene and processed for HE staining according to established procedures.

  DNA–DNA in situ hybridization (ISH) specific for viral genome was performed precisely as previously described with new fuchsin as the substrate yielding a brilliant red colour (Mayer et al., 1997). Negative controls included sections from uninfected tissue as well as DNase pretreatment of the sections or omission of the hybridization probe in the staining of infected tissue.

  In the two-colour IHC, a red staining was chosen to display the intranuclear viral immediate early 1 (IE1) protein pp89 (Kel and others, 1985) and a brown staining was chosen to display mature TGF-β1. After deparaffinization, sections were incubated for 15 min at 37 °C in 0.1% (w/v) trypsin followed by 15 min at 20 °C in 5% (w/v) TCA. Endogenous peroxidase was blocked with 0.5% (v/v) hydrogen peroxide in PBS–methanol (1:1, v/v). Unspecific binding sites were blocked with a 1:10 dilution of normal goat serum in Tris-buffered saline (TBS) for 30 min at 20 °C. The sections were then labelled for 60 min at 37 °C with a mixture of the two specific antibodies: (i) polyclonal rabbit IgG directed against the mature, active TGF-β1 (cat. no. G1221; Promega) and (ii) monoclonal antibody (MAb) CROMA 101 (IgG1 isotype) specific for the murine CMV IE1 protein pp89 (supplied by S. Jonjic, University of Rijeka, Croatia), diluted in TBS 1:50 and 1:500, respectively. The incubation with the mixture of secondary antibodies was performed for 30 min at 20 °C: (i) biotinylated goat antibody anti-rabbit IgG (cat. no. B-8895; Sigma) and (ii) goat antibody anti-mouse IgG (cat. no. M-5899; Sigma), diluted in TBS 1:500 and 1:20, respectively. Enzyme complexes were added for 30 min at 20 °C as a mixture: (i) avidin–biotin–peroxidase complex (Vestastain ABC kit standard PK 4000; Vector Laboratories) and (ii) alkaline phosphatase–anti-alkaline phosphatase (APAAP) complex (cat. no. A-7827; Sigma), diluted in TBS 1:100 and 1:50, respectively. Finally, brown staining of TGF-β1 was achieved with 3,3'-diaminobenzidine and hydrogen peroxide, whereas new fuchsin (cat. no. K596; Dako) was used as the substrate dye system for the red staining of IE1. The counterstaining was performed with haematoxylin, applied for 5 s in the case of kidneys and liver, and for 5 min in the case of femurs decalcified with TCA. Negative controls included the staining of uninfected tissue as well as substitution of CROMA 101 and anti-TGF-β1 rabbit IgG by mouse IgG1 (cat.no. X0931; Dako) and normal rabbit IgG (cat. no. L-5006; Sigma), respectively, in the staining of infected tissue.

  The viral 36–38 kDa early protein E1 (Buhr and others, 1990) was detected in liver tissue by labelling for 60 min at 37 °C with the E1-specific MAB CROMA 103 (IgG1 isotype; supplied by S. Jonjic, University of Rijeka, Croatia), diluted 1:500 in TBS, followed by the APAAP–new fuchsin method for red staining. Methods were as described above for the staining of IE1, except that trypsin treatment was followed by an incubation for 5 min in 10 mM trisodium citrate dihydrate, pH 6.0, in a microwave oven at 300 W. This procedure is required for unmasking the E1 protein in formalin-fixed tissues.

  - **Pre-emptive CD8 T-cell immunotherapy.** The establishment of a polyclonal, antiviral short-term T-cell line, the depletion of CD4 T cells
from that line, and the two-colour (CD4 versus CD8) cytofluorometric monitoring of CD8 T-cell purity after CD4 depletion have been described (Reddehase et al., 1987; Balthesen et al., 1994) and the cytolytic T lymphocyte (CTL) specificities represented by such a CD8 T-cell population have been the subject of many reports (reviewed in Koszinowski et al., 1990). For pre-emptive immunotherapy of murine CMV infection, CD8 T cells were injected intravenously into the tail vein 6 h after the irradiation of the mice and 2 h before intraperitoneal infection.

**Analysis of stromal gene expression by RT–PCR.** For restricting the analysis to the gene expression in radiation-resistant stromal cell types, transcription in radiation-sensitive haematopoietic cells was abolished by a 7 Gy γ-irradiation of the mice performed approximately 24 h before the isolation of poly(A)⁺ RNA from femoral BM cells. The efficacy of that strategy was documented in a previous report by the absence of SCF-receptor transcripts in the BM (Mayer et al., 1997). The purification of poly(A)⁺ RNA from the BM as well as protocols, primers and probes for the RT–PCRs specific for murine CMV *ie1* gene transcripts (CMV-IE1), transcripts specifying hypoxanthine phosphoribosyltransferase (HPRT), SCF transcripts and IL-6 transcripts were previously reported (Mayer et al., 1997). In the case of TGF-β1 transcripts, oligonucleotides 5'-n693–724 and 5'-n1217–1180 were used as forward and reverse primers, respectively, yielding a fragment of 525 bp. Oligonucleotide 5'-n881–911 served as the probe (Derynck et al., 1986; GenBank accession no. M13177). Amplificates were separated on a 1.5% (w/v) agarose gel and visualized by autoradiography after Southern blotting and hybridization with the respective γ-32P-end-labelled internal oligonucleotide probe. In the case of TGF-β1 transcription, a relative quantification was performed with a digital phosphoimaging system (Fujifilm bioimaging system BAS 2500). Radioactivity per band is expressed as phosphostimulated luminescence (PSL) units. Data analysis was performed by using Tina 2.10 software (Raytest, Straubenhardt, Germany).

**Quantification of colony-forming units spleen (c.f.u.-S) in reconstituting BM.** The early myeloid progenitor cell, the c.f.u.-S, was detected by its property, upon intravenous transplantation and within 14 days, to form a clonal colony in the spleen of recipient mice that were γ-irradiated with a dose of 7 Gy (Magli et al., 1982). With that dose of irradiation, endogenous colonies were not formed. BM cells were pooled from the femurs of three donor mice per time-point, incubated for 1 h at 37 °C in a 1:5 dilution of native anti-CMV antiserum in PBS to neutralize contaminating virus; washed and graded numbers were then transferred intravenously into the tail vein of four recipients per titration step. In the case of the infected group as well as for days 6 and 10 of the uninfected group, three log₂ dilutions started with the cellular yield of one femur, whereas for days 14 and 16 of the uninfected group the titration started with one-tenth of this yield. After 14 days, spleens were fixed in Bouin’s solution. Except in the groups with no or few colonies, colony counting was done for the dilutions that gave > 5 and < 20 colonies, and the number of c.f.u.-S per donor femur was then calculated from the mean spleen colony number of the four indicator recipients. Absence of infection of the indicator recipients was verified by the absence of IE1 antigen in the immunohistochemical analysis of the spleen. It is worth noting that even an extensive infection of the recipient’s spleen would not prevent the formation of colonies (not shown).

**Results**

**Microanatomical localization of CMV-infected cells and of cells expressing mature TGF-β1**

Previous work has indicated that rat CMV infection induces immunohistochemically detectable TGF-β1 in a variety of tissues, including the BM (Haagmans et al., 1997). Furthermore, the human CMV protein IE2 was shown to transactivate the TGF-β1 promoter *in vitro* in fibroblasts (Michelson et al., 1994; Yoo et al., 1996). Taken together, these data have suggested a production of TGF-β1 by the infected cells. We used double-staining immunohistology to test the predicted colocalization of viral gene expression and TGF-β1 in kidneys, liver and BM.

(i) **Glomerular infection and detection of TGF-β1 in medullary tubules of the kidney.** The kidney is a known site of CMV replication and CMV latency, transmitting CMV upon transplantation (Ho et al., 1975; Hamilton & Seaworth, 1985; Chou, 1986; Grundy et al., 1987). Furthermore, all nephron segments were found to express TGF-β1 mRNA and latent forms of TGF-β1, but not the mature form (Ando et al., 1995). Specifically, glomerular cells secrete a large latent TGF-β1, in which the active dimer is non-covalently associated with a prepropeptide dimer, known as latency-associated peptide (LAP), that is covalently linked to the latent TGF-β-binding protein (LTBP; Massague, 1990; Ando et al., 1995; Gleizes et al., 1997). In contrast, tubular epithelial cells secrete a small latent TGF-β1 that lacks the LTBP (Ando et al., 1995). In agreement with this knowledge, by using an antibody specific for active TGF-β1, we did not detect TGF-β1 in the kidney of uninfected mice, as is documented for the tubules in the kidney medulla (Fig. 1A1). Notably, this negative result was true for normal mouse kidney (not shown) as well as for kidney tissue analysed at day 14 after a 6 Gy γ-irradiation (Fig. 1A1), which indicates that the cell decay associated with the haematoblast treatment does not deliver signals that would induce mature TGF-β1 expression or mobilize the latent pool in renal tissue. In contrast, mature TGF-β1 was localized in the kidney medulla of mice at day 14 after 6 Gy γ-irradiation and infection (Fig. 1A2 for overview). The TGF-β1-positive cells are clearly identified as uninfected tubular epithelial cells, lining the tubules (Fig. 1A2, arrow; shown enlarged in panel A3). They display a basal–apical polarity in that TGF-β1 is located at the membrane and in a submembranal zone of the cytoplasm directed towards the lumen of the tubules (Fig. 1A3). That infection and TGF-β1 do not colocalize became evident from the observation that the few infected renal interstitial cells did not express mature TGF-β1 (Fig. 1A4; arrows). Likewise, glomerular cells in the kidney cortex are infected by CMV, but mature TGF-β1 was not detectable in the infected glomeruli (Fig. 1A5 for overview and A6 for detail) and was absent also from the proximal cortical tubules, the site of protein resorption (Fig. 1A5). In conclusion, CMV infection of the organism is associated with the presence of mature TGF-β1 in the kidney medulla. However, infected glomerular and interstitial cells do not express mature TGF-β1, and the TGF-β1-positive tubular epithelial cells in the medulla are not infected.

(ii) **Absence of mature TGF-β1 from the liver during all stages of the CMV replicative cycle.** Hepatocytes *in situ* are reportedly
Fig. 1. *In situ* localization of infected cells and of cells expressing TGF-β1. Except for panel A1, tissues were analysed at day 14 after 6 Gy γ-irradiation and intraplantar murine CMV infection. Throughout, viral products are stained in red and mature TGF-β1 is stained brown. A1–A6, Double-staining IHC for TGF-β1 and viral IE1 protein of kidney tissue, with haematoxylin counterstaining. A1, Kidney medulla at day 14 after a 6 Gy γ-irradiation with no infection; the arrows point to renal tubules. A2, Analogous section in an infected kidney; the arrow points to a renal tubule expressing TGF-β1. A3, Detail of A2, showing the apical localization of TGF-β1 in tubular epithelial cells of the kidney medulla. A4, Arrows point to infected cells in the medullary interstitium. A5, Infected glomeruli in the kidney cortex. A6, Detail of an infected glomerulus. B1–B6, Three serial, neighbouring sections of infected liver tissue. Note the central vein and adjacent bile duct (upper right quadrant) that can serve as a landmark. Haematoxylin counterstaining throughout. B1, Double-staining IHC for TGF-β1 and viral IE1 protein. B2, Detail, revealing the subnuclear localization of IE1. B3, IHC for viral E1 protein. B4, Detail, revealing the spotty subnuclear localization of E1. B5, DNA–DNA ISH specific for viral DNA represented by the gB gene. B6, Detail, revealing the typical intranuclear inclusion body characteristic of the late stage of the CMV replicative cycle. C1, Empty stromal network spanning between bone trabeculae in the epiphysial region of an infected femur, illustrating BM aplasia. HE staining. C2, Cluster of infected, reticular stromal cells in the diaphysial region of a femur. Double-staining IHC for TGF-β1 and viral IE1 protein, with haematoxylin counterstaining. Bar markers represent 25 μm throughout.
negative for mature TGF-β1 in IHC, but it can be mobilized in cultured hepatocytes in the absence of transcription or translation, thus indicating that hepatocytes are a source of latent TGF-β1 (Gressner & Wulbrand, 1997). It was therefore worth asking whether mature TGF-β1 is either induced de novo or mobilized from the latent pool in the liver of the infected mice. Double-staining IHC specific for viral intranuclear IE1 protein and for mature TGF-β1 revealed an extensive, cytolysic infection of the liver resulting in plaque-like necrotic areas. Notably, however, the infected hepatocytes did not express mature TGF-β1 nor did the uninfected neighboring hepatocytes and bile duct epithelial cells (Fig. 1B1). Since the IE1 protein is present from the IE phase throughout the CMV replicative cycle, IE1-staining detects infected cells of all stages. The stage can be recognized from the subnuclear localization of the IE1 protein. During the IE and early (E) phase, the IE1 protein is homogeneously distributed within the nucleus, whereas in the late phase an inclusion body is demarcated by more intense staining (Fig. 1B2). As a plaque spreads centrifugally, the infected tissue area necessarily contains cells in all stages of the viral replicative cycle, with cells in the late phase predominating in the centre of the focus and cells in the IE phase extending to the periphery. This is formally documented by serial neighbouring sections stained by IHC for the viral early protein E1 (Fig. 1B3 and B4) and by DNA–DNA ISH for viral DNA that indicates the late phase of the replicative cycle when nucleocapsid assembly and DNA packaging occur in intranuclear inclusion bodies (Fig. 1B5 and B6). Note that cells in the E phase and the late phase occupy the centre of the infected focus. The subnuclear localization of the 36–38 kDa E1 protein in hepatocyte nuclei in situ reveals the same spotty distribution as was shown previously by immunofluorescence staining of infected fibroblasts in cell culture (Bühler et al., 1990) (Fig. 1B4). The viral DNA is confined to an intranuclear inclusion body, surrounded by a lucent halo followed by a rim of haematoxylin-stained chromatin with polar bodies (Fig. 1B6). In conclusion, mature TGF-β1 is not induced or mobilized in hepatocytes at any kinetic stage of the viral replicative cycle.

(ii) Infected reticular BM stromal cells do not coexpress mature TGF-β1. Previous work has postulated a coexpression of CMV antigen and TGF-β1 in BM stromal cells (Haagmans et al., 1997), but the conclusion was not based on a double-staining analysis, nor on serial sections. Since we did not see a coexpression in kidney and liver, we reinvestigated this question by double-staining IHC of BM during lethal BM aplasia. As we have shown recently, the RSC that make up the stromal network are the target cells of CMV infection in the haematopoietic cord, but the number of infected RSC is low and the majority of the infected RSC do not reach the stage of virus production (Mayer et al., 1997). The infection was associated with BM aplasia, characterized by complete absence of haematopoietic islands and by the radiation-resistant RSC spanning an empty stromal network between the bone trabeculae, shown by HE staining of an epiphysial section of a femur (Fig. 1C1). A focus of infected, IE1 antigen-expressing RSC located in the diaphysial region of a femur did not show any coexpression of mature TGF-β1 (Fig. 1C2). Notably, some of the infected RSC show an IE1 protein-containing intra-

Fig. 2. Prevention of CMV infection of BM stroma by adoptive transfer of antiviral CD8 T cells. Mice were γ-irradiated with a dose of 6 Gy. Four experimental groups were defined by infection with murine CMV and adoptive transfer of 10⁸ antiviral CD8 T cells in the indicated combinations of treatment. At day 13 p.i., transcription in radiation-sensitive haematopoietic cells was abolished by a second irradiation with a dose of 7 Gy. After 24 h, poly(A)⁺ RNA was isolated from radiation-resistant, femoral BM stromal cells, and analysed for HPRT and CMV-IE1 transcripts by the respective RT–PCRs. Log₂ titrations started with the yield of poly(A)⁺ RNA representing the BM stroma of one femur. Specific amplification products of 163 bp and 280 bp, respectively, were visualized by autoradiography after Southern blot and hybridization with the respective 𝜈-32P-end-labelled, internal oligonucleotide probes.

Fig. 3. Haemopoietin gene expression in BM stroma. Femoral poly(A)⁺ RNA, derived from the experiment described in Fig. 2, was analysed by RT–PCR for SCF and IL-6 transcripts. Specific amplification products of 543 and 519 bp, respectively, were visualized by hybridization with the respective internal oligonucleotide probes.
nuclear inclusion body and haematoxylin-stained polar bodies, indicative of the late stage of the viral replicative cycle.

Effect of infection and of a pre-emptive CD8 T-cell therapy on gene expression in BM stromal cells

The finding that RSC do not express the mature form of TGF-β1 does not exclude an induction of TGF-β1-gene transcription and an overexpression of latent TGF-β1 by the infection. Since an adoptive transfer of antiviral CD8 T cells controls infection in essentially all infected tissues (for a review see Koszinowski et al., 1990), we tested whether this applies also to the infection of the BM stroma and what influence pre-emptive therapy would have on stromal gene expression.

(i) Antiviral CD8 T cells prevent viral gene expression in BM stroma. The analysis of gene expression was restricted to the expression in BM stroma by a strategy of repeated irradiation previously discussed and documented (Mayer et al., 1997). All four possible combinations of infection and CD8 T-cell therapy were included in the analysis. The amount of HPRT housekeeping gene transcripts was found to be the same for all groups (Fig. 2, left panel), showing that the subsequent comparison of gene expression was based on the same amount of poly(A)+ RNA. That the infection in the BM does not reduce the number of stromal cells has been previously verified in detail (Mayer et al., 1997). In accordance with the IHC analysis of IE1 protein expression (recall Fig. 1C2), ie1 gene transcripts were detected in the infected BM stroma. This expression was completely abrogated by antiviral CD8 T cells (Fig. 2, right panel).

(ii) Antiviral CD8 T cells prevent the down-regulation of stromal haemopoietin gene expression. If the infection is responsible for haemopoietin down-regulation, as previously postulated (Mayer et al., 1997), the antiviral CD8 T cells should prevent this. We therefore compared the expression of SCF (Fig. 3, left panel) and of IL-6 (Fig. 3, right panel) for the four experimental groups. The data reproduced the previous result of a down-regulation, as previously postulated (Fig. 2, upper panel). We recently described the results of an independent experiment in which a tenfold lower number of CD8 T cells had been transferred (Fig. 5). In essence, this experiment has reproduced all findings in a qualitative sense. We conclude that CD8 T cells can prevent the viral up-regulation of TGF-β1 transcription but are by themselves inducers.

CD8 T cells in the BM of the infected group may result from trapping of CD8 T cells in infected organs. That the level of TGF-β1 transcription in the BM stroma indeed depends upon the dose of inducing CD8 T cells is shown by an independent experiment in which a tenfold lower number of CD8 T cells had been transferred (Fig. 5). In essence, this experiment has reproduced all findings in a qualitative sense. We conclude that CD8 T cells can prevent the viral up-regulation of TGF-β1 transcription but are by themselves inducers.

Pre-emptive CD8 T-cell therapy prevents the CMV-inducible BM aplasia

If the enhanced TGF-β1 transcription within the BM would lead to active TGF-β1 of pathogenic relevance, the CD8 T cells should be unable to prevent the aplasia despite their documented antiviral function. Clearly, this is not the case. CD8 T cells prevent mortality (Fig. 6, top panel), permit the reconstitution of the early myeloid lineage progenitor c.f.u.-S (Fig. 6,

---

**Fig. 4.** Induction of TGF-β1-transcription in BM stroma. Top panel, femoral poly(A)+ RNA, derived from the experiment described in Fig. 2, was analysed for TGF-β1 transcripts by RT–PCR. The specific amplification product of 525 bp was visualized by hybridization with an internal oligonucleotide probe. Bottom panel, log–log plot of poly(A)+ RNA dilution (abscissa) versus radioactivity per band, expressed as phosphostimulated luminescence (PSL) units (ordinate). The comparison of the linear portions of the dilutions indicates a ca. 16-fold increase in TGF-β1 transcription by the infection (arrow).
105 CD8 T cells, but otherwise analogous to the one shown in Fig. 4, respective internal oligonucleotide probes. HPRT transcription (left panel). Specific amplification products of 525 bp TGF-β1 transcription in BM stroma (right panel) is shown in comparison to HPRT transcription (left panel). Specific amplification products of 525 bp and 163 bp, respectively, were visualized by hybridization with the respective internal oligonucleotide probes.

**Pre-emptive CD8 T-cell therapy does not preclude the accumulation of mature TGF-β1 in tubular epithelial cells of the kidney medulla**

Previous work has indicated increased serum levels of TGF-β1 after CMV infection (Haagmans et al., 1997). It could hence be argued that BM aplasia may not result from locally produced, but rather from systemic TGF-β1. It was therefore of importance to know whether or not the protecting, antiviral CD8 T-cell therapy would prevent the accumulation of mature TGF-β1 in the renal tubular epithelial cells. This question was answered by TGF-β1-specific IHC of renal tissue from infected mice that had received CD8 T cells. Clearly, while the absence of CMV-antigen-positive cells in the kidney cortex (not shown), as well as in the liver (Fig. 7, top panel), documents the success of the antiviral therapy, tubular epithelial cells in the kidney medulla still contained mature TGF-β1 (Fig. 7, bottom panel).

**Discussion**

Bone marrow aplasia induced by CMV (Mutter et al., 1988; Reddehase et al., 1992) is not primarily caused by cytokines that negatively control the growth and differentiation of haematopoietic cells, but by a deficiency in stromal cell-derived haemopoietins that are essential as positive regulators of proliferation and differentiation of haematopoietic cells (Mayer et al., 1997). Specifically, a deficiency in stromal SCF, the kit-ligand encoded at the Steel locus (reviewed in Williams et al., 1992), has its counterpart in a deficiency in SCF-receptor-expressing haematopoietic stem cells and early progenitor cells (Mayer et al., 1997). In addition, the expression of other haemopoietins positively involved in haematopoiesis, such as IL-6 and G-CSF, is also down-regulated in BM stroma during lethal BM aplasia. While the haemopoietin down-regulation was clearly associated with CMV infection, the local infection of the stromal network was too low to explain the deficiency by direct infection (Mayer et al., 1997). A negative regulator cytokine affecting haemopoietin gene expression in uninfected stromal cells might explain haemopoietin deficiency and consequent BM aplasia. The TGF-β1 induced by CMV infection in vitro and in vivo (Michelson et al., 1994; Yoo et al., 1996; Lagneaux et al., 1996; Haagmans et al., 1997) was a promising candidate.

The in vivo data presented herein have shown that murine CMV infection induces TGF-β1-gene transcription in BM stroma and induces immunohistochemically detectable, mature TGF-β1 in tubular epithelial cells of the kidney medulla. Viral genes and mature TGF-β1 were never seen coexpressed in situ in the same cell. Specifically, the infected cells in BM stroma, in the liver and in the renal glomeruli and interstitium were negative for mature TGF-β1, and conversely, the TGF-β1-positive tubular epithelial cells in the kidney medulla were not infected. It should be noted that infected cells in spleen, adrenal glands and lungs were also negative for mature TGF-β1 (not shown). This lack of coexpression was not a problem of viral gene expression kinetics, since serial sections of a CMV plaque in the liver have documented the absence of mature TGF-β1 in infected hepatocytes during all stages of the CMV replicative cycle.

At first glance, these findings appear to be in conflict with results in the rat CMV model (Haagmans et al., 1997). In that work, IHC staining of TGF-β1 was seen in a variety of infected tissues, and it was postulated that the TGF-β1 is expressed by the infected cells. Notably, these authors also mentioned having seen positive TGF-β1 staining in uninfected kidneys. In contrast, with an antibody specific for active TGF-β1, we did not see staining of renal tissue or any other tissue in uninfected mice. Most tissues contain cells that secrete either of the two latent forms of TGF-β1. Specifically, renal tubular epithelial cells and glomerular cells constitutively secrete the small and large latent forms of TGF-β1, respectively (Ando et al., 1995). Cytoplasmic in situ staining of TGF-β1 in the uninfected kidney therefore indicates detection of latent TGF-β1 that might be overexpressed in infected tissues. Accordingly, fibroblasts infected in vitro with rat CMV did not secrete active TGF-β1, but the latent complex in the culture supernatant had to be heat-dissociated for measuring activity (Haagmans et al., 1997).

Since murine CMV infection was found to be associated with the presence of mature TGF-β1 in uninfected tubular epithelial cells of the kidney medulla, one must either assume resorption at an unphysiological site or postulate an ‘inducer’ that activates the latent pool and/or up-regulates TGF-β1.
expression in these cells. One could speculate that the postulated inducer is produced in the infected glomerular cells of the kidney cortex and operates in the medullary tubules. However, this is not the case, since we have also seen examples of poor infection of the kidney with almost no infected cells in the glomeruli, but nonetheless a significant accumulation of mature TGF-β1 in the medulla (not shown). Likewise, the up-regulation of TGF-β1 transcription in the BM stroma is so significant that it cannot be explained by the low number of infected cells in the BM stroma. The postulated inducer is thus likely to operate systemically and to induce TGF-β1 transcription in uninfected cells. Furthermore, it is not necessarily a direct viral product. As we have shown here, upon adoptive transfer, CD8 T cells also induce TGF-β1 transcription in BM stroma. We are not aware of any previous report having shown this effect of CD8 T cells on stromal or parenchymal TGF-β1 producers, but that CD8 T cells are principally capable of inducing TGF-β1-gene expression has been documented for the interaction between CD8 T cells and NK cells (Gray et al., 1994). The induction of TGF-β1 by CD8 T cells in the absence of infection is an interesting side aspect of our study. This function of CD8 T cells is clearly unrelated to their antigen specificity, since it was observed also in uninfected recipients. Whether it depends on pre-activation of the CD8 T cells and

---

**Fig. 6.** CD8 T cells protect against lethal BM failure. Top panel, Kaplan–Meier survival plots showing the survival rates (ordinate) as a function of time after 6 Gy γ-irradiation and intraplantar infection with murine CMV (abscissa) for groups of 20 mice. Pre-emptive CD8 T-cell therapy (right panel) was performed with $10^6$ antiviral CD8 T cells. Centre panel, quantification of the c.f.u.-S, an early myeloid lineage-committed progenitor cell. Bottom panel, histological analysis of femoral BM at day 14 p.i. (arrows). Note the empty stromal network in the group with no therapy (left panel), and the beginning of endogenous reconstitution in the group with therapy, visible as haematopoietic islands in the stroma (right panel); HE staining. The bar marker represents 25 μm.

**Fig. 7.** Protective CD8 T cells do not preclude TGF-β1-expression in medullary renal tubular epithelium. At day 14 of the experiment shown in Fig. 6, liver (A1, no therapy; A2, therapy) and kidney medulla (B1, no therapy; B2, therapy) were analysed by IHC for the in situ expression of viral IE1 protein and mature TGF-β1, respectively; haematoxylin counterstaining. The bar marker represents 25 μm.
whether it can be performed also by CD4 T cells, either Th1 or Th2 cells, are obvious questions for future research.

In essence, regarding the expression of TGF-β1, CMV infection and CD8 T cells have shown parallel effects. In contrast, the effects on BM reconstitution after hematopoietic ablative treatment were opposite. CMV infection down-regulates stromal haemopoietic gene expression and blocks the reconstitution of early haemopoietic progenitors with consequent BM aplasia. CD8 T cells do not impair stromal haemopoietin gene expression in the absence of infection, and they prevent adverse effects of the infection. Apparently, the TGF-β1 expressed in the infected group after CD8 T-cell therapy was not of pathogenic relevance. This suggests that the TGF-β1 present in the renal medullary tubules does not contribute to systemically active TGF-β1, and that TGF-β1 transcription in the BM stroma does not generate active TGF-β1. The process of latent TGF-β1 activation is critical for the in vivo function of TGF-β1, and is an important element of control (for a review see Gleizes et al., 1997). Glycosidases, proteases such as plasmin, and interaction with thrombospondin-1 are thought to be central to the release of active TGF-β1 from the latent complex. As a consequence, induction of TGF-β1 gene expression during CMV infection does not inevitably imply a key role of TGF-β1 in viral pathogenesis.

We thank Stipan Jonjic and his group (University of Rijeka, Croatia) for the supply of MAbs CROMA 101 and CROMA 103. This work was supported by a grant to M.J.R. by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 311.

References


Received 4 August 1997; Accepted 5 December 1997