

Susceptibility of Human Bone Marrow Cells and Hematopoietic Cell Lines to Coxsackievirus B3 Infection

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Viremia is commonly observed in association with enterovirus infections, and during this phase viruses can be transmitted to secondary target organs in the body. It is not known, however, whether blood cells play a role in the pathogenesis of enterovirus infection supporting virus replication. Our earlier work (T. Vuorinen, R. Vainionpää, H. Kettinen, and T. Hyypiä, *Blood* 84:823–829, 1994) demonstrated that coxsackievirus B3 is able to replicate in representatives of B- and T-cell lines but not in a monocytic cell line or peripheral blood mononuclear cells, indicating that virus replication may depend on the differentiation and maturation stages of the cells. Therefore, we have broadened our studies and analyzed the susceptibility of granulocyte-macrophage CFU and hematopoietic cell lines with various differentiation and maturation stages to coxsackievirus B3 infection. Virus replication was detected in B- and T-cell lines with no direct correlation to the maturation stage. Granulocyte-macrophage CFU were also able to support virus multiplication.

Coxsackie B viruses (CBVs) are members of the *Enterovirus* genus in the picornavirus family. CBVs are important human pathogens and cause diseases varying from mild respiratory illness to severe meningoencephalitis and myocarditis (8). Primary replication of enteroviruses is thought to take place in the tissues of the nasopharynx and alimentary tract, from which the virus spreads to secondary target organs (e.g., the heart and central nervous system). In general, enteroviruses cause acute infection, but prolonged and persistent infections have also been reported in vivo and in vitro (2, 3, 15, 25, 26). An extensive viremia has been detected in mice infected with CBV3 (28), and enteroviruses have also been isolated from blood specimens collected from patients during acute infection (4, 24). The finding that the yield of enteroviruses from the leukocyte fraction may exceed that from plasma (24) suggests that enteroviruses may be capable of replicating in blood cells.

In general, the knowledge about the effects of enteroviruses on the human immune system is rather incomplete. Poliovirus infection in immune cells has been examined in a relatively detailed manner. The virus replicates in human peripheral blood leukocyte cultures (6, 7), and the production of infectious virus is enhanced by phytohemagglutinin stimulation (31). Poliovirus is also known to infect hematopoietic cell lines (14, 21) and to persist in both lymphoid and monocytic cell lines (5, 25).

CBVs are also able to replicate and persist in lymphoid cell lines (16), but the ability of CBV3 to infect human peripheral blood leukocytes differs from that of poliovirus. Although CBV3 has been reported to replicate in peripheral blood mononuclear cell cultures (11), we were unable to demonstrate CBV3 production in in vitro-infected peripheral blood mononuclear cells from several individuals (29). On the other hand, extensive CBV3 replication occurred in representatives of B (Raji)- and T (Molt-4)-cell lines and viral antigens were also detected in infected bone marrow aspirate cultures, suggesting that the maturation and differentiation stages of the immune cells may affect the permissiveness for CBV3 (29). To obtain a more comprehensive picture of the role of maturation and

differentiation stages on the susceptibility of blood cells to CBV3 infection we examined virus replication in granulocyte-macrophage CFU (CFU-GM) and in monocytic, granulocytic, B-, and T-cell lines with different maturation stages.

The cell lineages and maturation stages of the cell lines are summarized in Fig. 1. Hematopoietic cell lines representing different maturation stages (18, 19, 30) of granulocytic (KG-1 and HL-60), monocytic (THP-1 and U-937), T-cell (CCRF-CEM, Molt-4, and CCRF-HSB-2) and B-cell (Raji, CCRF-SB, and RPMI 8226) lineages were obtained from the American Type Culture Collection. CCRF-HSB-2 and KG-1 cell lines were maintained in Dulbecco's modified Eagle medium (IDMEM), and the other cell lines were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 50 µg of gentamicin per ml. Cells (10^6 cells per ml; 0.5×10^6 CFU-GM cells per ml) were inoculated with the Nancy strain of CBV3 at a multiplicity of infection of 1 in serum-free Hanks' balanced salt solution. After 1 h of incubation, the cells were washed three times in order to remove the unadsorbed virus.

Bone marrow mononuclear cells were obtained with the COBE 2991 model I Blood Cell Processor from Ficoll-Paque

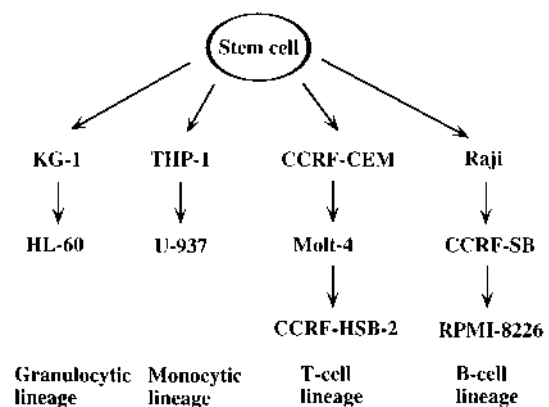


FIG. 1. Maturation and differentiation stages of hematopoietic cell lines (18, 19), modified from the study by Okada et al. (21).

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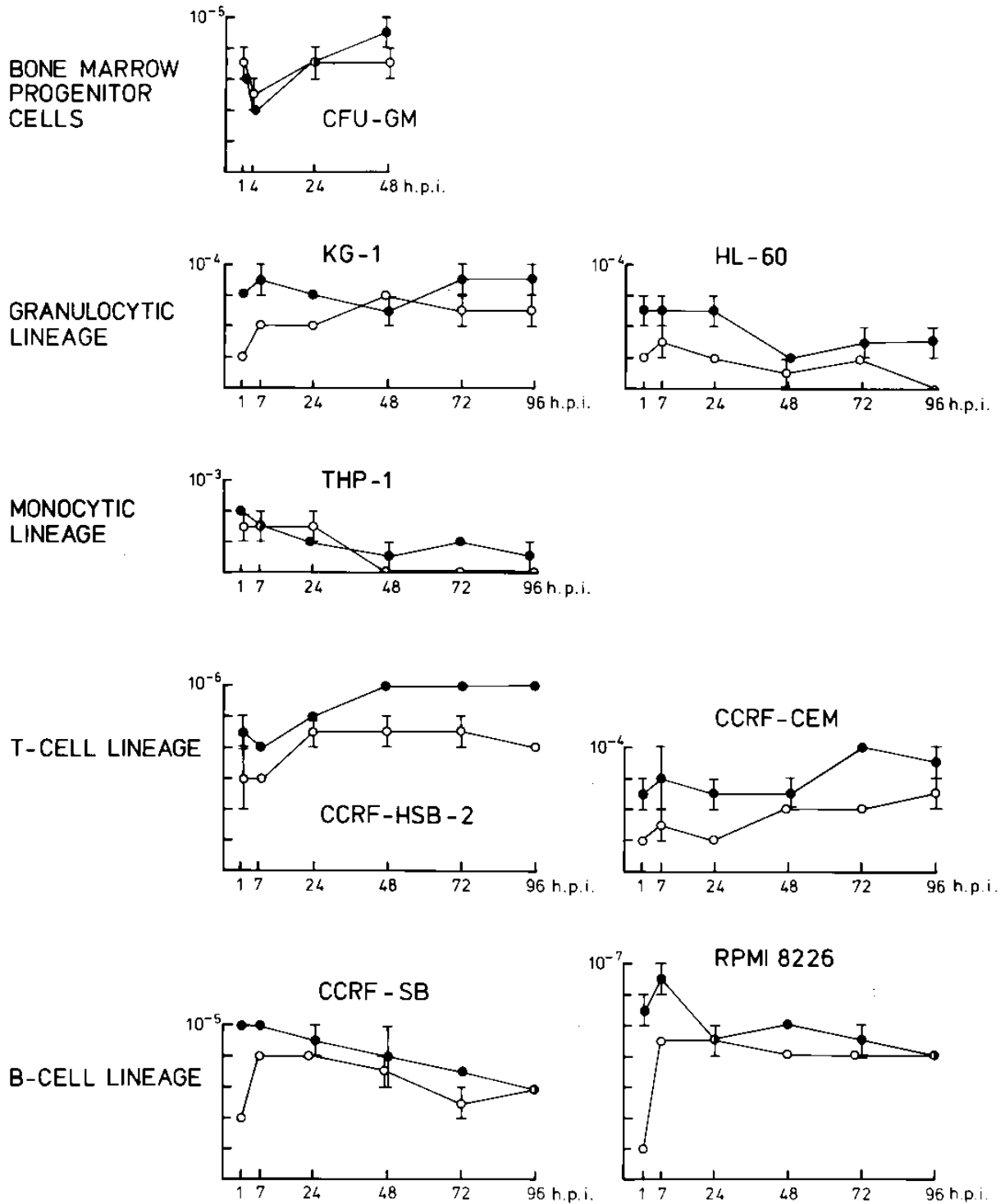


FIG. 2. Production of infectious CBV3 in human CFU-GM and hematopoietic cell lines infected at a multiplicity of infection of 1. The means and standard deviations (error bars) of dilutions (\log_{10}) are shown. Open circles are values obtained from the supernatants, while closed circles represent the cells.

(Pharmacia)-separated heparinized bone marrow collected from autologous transplantation patients (disease free at the time of collection). The in vitro colonies of hematopoietic progenitors were cultured by the methyl cellulose method (23) with the modification described by Guilbert and Iscove (9). Briefly, 2×10^5 mononuclear bone marrow cells per ml were

mixed with culture medium containing 1% methyl cellulose, 20% fetal bovine serum (HyClone), 1% delipidated and deionized bovine serum albumin (Sigma Cell Culture), 10^{-4} M mercaptoethanol, and 0.5 mg of iron-saturated human transferrin (Behringwerke) in IDMEM per ml and added to plastic petri dishes. The growth of granulocyte-macrophage precursors was

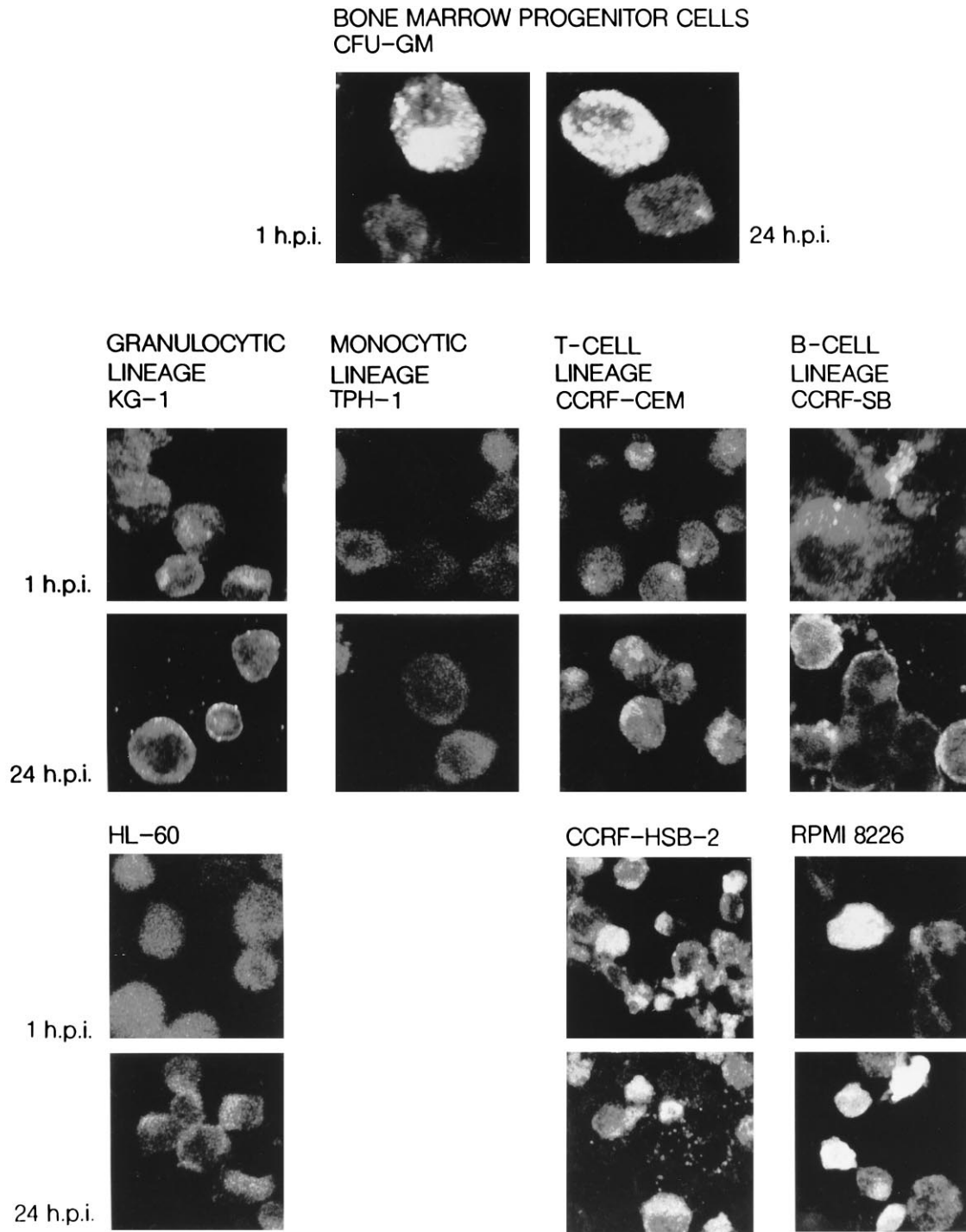


FIG. 3. Immunofluorescence staining of CBV3 proteins in human CFU-GM and hematopoietic cell lines with different maturation and differentiation stages examined by laser-scanning microscopy. Results after 1 and 24 h p.i. are shown.

stimulated with 10% conditioned medium from the human bladder carcinoma cell line 5637 (30). The plates were incubated at 37°C in humidified atmosphere with 5% CO₂. CFU-GM were counted on the 14th day, picked under a light microscope, washed, and suspended in the culture medium (without methyl cellulose).

The amount of infectious virus in the cells and supernatants

was determined by a 50% tissue culture infective dose assay on confluent monolayers of LLC-Mk₂ cells. Cells and supernatants for infectivity titration were collected 1, 7, 24, 48, 78, and 96 h postinfection (p.i.), except for CFU-GM, which were harvested 1, 4, 24, and 48 h p.i. CBV3 was able to replicate in the CFU-GM (Fig. 2; Table 1), and a small amount of virus was released into the culture medium. A slight increase was also

TABLE 1. Summary of the susceptibility of CFU-GM and hematopoietic cell lines to CBV3 infection by different methods

Cell	Virus production	Virus protein synthesis	Virus minus-strand RNA synthesis
CFU-GM	+	-	+
KG-1	±	-	-
HL-60	-	-	-
THP-1	-	-	-
U-937	- ^a	- ^a	-
Raji	+	+	+
CCRF-SB	±	±	+
RPMI 8226	+	+	+
CCRF-CEM	±	+	+
Molt-4	+ ^a	+	+
CCRF-HSB-2	+	+	+

^a Data from reference 23.

observed in virus titers determined from the CCRF-HSB-2 cells and culture medium. Because interpretation of the susceptibility of the other cells to the CBV3 infection on the basis of infectivity titration was difficult because of the high background level originating from the virus inoculum, immunological and molecular methods were used for further analysis of the presence and synthesis of viral components in the cells.

Immunofluorescent staining was performed in order to determine the presence of CBV3 antigens in infected CFU-GM and hematopoietic cell lines after the adsorption and at 24 h p.i. For confocal microscopy, the CBV3-infected CFU-GM were fixed in 4% formalin at room temperature for 15 min, incubated with the CBV3 antiserum (diluted 1:30), prepared by immunizing rabbits with highly purified virus (29), and then stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (diluted 1:150; Cappel). The fluorescence was examined with a laser scanning confocal microscope (CCM; EMBL) at the Department of Medical Physics and Chemistry, University of Turku.

Intensive fluorescence in CFU-GM was seen already after the adsorption, whereas no viral antigens were detected in the monocytic cell line THP-1 (Fig. 3). CBV3 antigens were found in the KG-1 and HL-60 cells of granulocytic lineage, with an increase in the intensity during the first 24 h p.i. possibly due to reorganization of the antigen-positive clusters. The B- and T-cell lines were also positive after the adsorption, and no increase in the intensity of the fluorescence could be detected above this background.

Synthesis of viral capsid polypeptides was analyzed by immunoprecipitation after metabolic labeling between 4 and 22 and 73 and 91 h p.i. The CFU-GM, the hematopoietic cell lines (10^6 cells), and the LLC-Mk₂ cells were infected at a multiplicity of infection of 5. At 3 and 72 h p.i., the cells were preincubated in methionine-free medium for 1 h and labeled for 18 h with [³⁵S]methionine (50 μCi/ml; Amersham). Immunoprecipitation was done with rabbit polyclonal antiserum against purified CBV3 and protein A-Sepharose beads as described previously (29). The precipitated proteins were analyzed in 12.5% polyacrylamide gels, and the dried gels were exposed to X-ray films.

No virus-specific protein synthesis was detected in human CFU-GM or in the granulocytic (KG-1 and HL-60) and monocytic (THP-1) cell lines, whereas virus-specific proteins were

immunoprecipitated with the CBV3 antiserum from the CCRF-HSB cells at 22 h p.i. (Fig. 4). In the other T-cell line (CCRF-CEM) no viral polypeptides were detected until 91 h p.i. Synthesis of viral proteins was also seen in the RPMI 8226 and CCRF-SB cells; the signals in the CCRF-SB cells were weak at 22 h p.i., and only a faint, evidently nonspecific band comigrating with VP3 was detected at 91 h p.i.

The proportional amount of CBV3 RNA in the infected cells was determined by spot hybridization as described earlier (28) with the following modifications: the CBV3 cDNA probe (13) was labeled with [³²P]dCTP by using a nick translation kit (Bethesda Research Laboratories) to a specific activity of 2×10^7 cpm/μg. The prehybridization solution contained 50% formamide, 1 M NaCl, 5× Denhardt's solution, 9% dextran sulfate, 100 μg of denatured herring sperm DNA per ml, 1% sodium dodecyl sulfate (SDS), and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.3]). After hybridization at 42°C overnight, the membrane was washed three times for 5 min each at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS and three times for 30 min each at 42°C in 0.1× SSC containing 0.1% SDS and exposed to an X-ray film. Hybridization signals were also analyzed with a PhosphorImager (GS-250 Molecular Imager; Bio-Rad).

No viral RNA was detected in the granulocytic and monocytic cell lines (Fig. 5). Infected CCRF-CEM cells appeared to be positive only at 96 h p.i., while CCRF-HSB-2 cells supported detectable viral RNA synthesis during the first 24 h. A clear increase in the amount of CBV3 RNA was seen in the RPMI 8226 cells, while the CBV3 RNA synthesis in the CCRF-SB cells was less extensive.

When different methods were used for the detection of virus replication in the cells and cell lines (Table 1), there were difficulties in the interpretation of the results obtained by infectivity titration and immunofluorescence due to the high

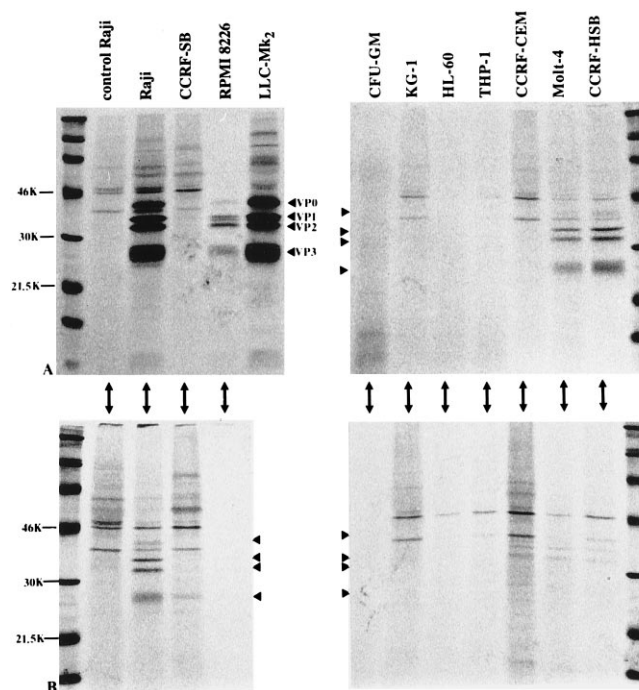


FIG. 4. Synthesis of viral proteins detected by immunoprecipitation with rabbit antiserum against purified CBV3 at 22 (A) and 91 (B) h p.i. Virus polypeptides VP0, VP1, VP2, and VP3 are indicated by arrowheads.

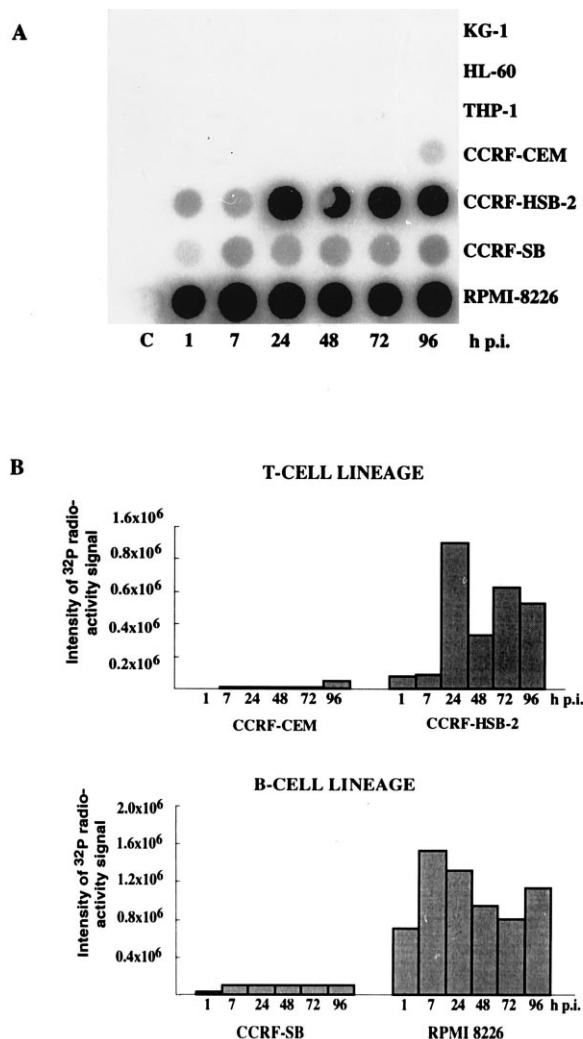


FIG. 5. (A) Detection of CBV3 RNA by spot hybridization in hematopoietic cell lines after different incubation periods p.i. C, uninfected control cells. (B) Intensity of ^{32}P signals in the spot hybridization assay in the hematopoietic cell lines determined by PhosphorImaging analysis. The intensities of the ^{32}P signals of the uninfected control cells have been deducted from the intensity of infected cells.

background originating from the inoculum virus. Both immunoprecipitation and nucleic acid hybridization suffered, to some extent, from the lack of sensitivity. Therefore, the results were confirmed by a sensitive reverse transcription (RT)-PCR for the detection of genomic (positive) and complementary (negative) RNA strands by protocols described earlier (1, 12). The oligonucleotide primers for the RT-PCR were from the conserved parts of the 5'-untranslated and the VP2 capsid-protein coding regions of the enterovirus genome (1, 12). Extraction of RNA from the cells was performed by using the Ultraspec (Biotecx) RNA isolation system with slight modifications. In brief, 200 μl of chloroform was added to the Ultraspec mixture containing the cells; the mixture was incubated on ice for 5 min and centrifuged $12,000 \times g$ at 4°C for 15 min. RNA was precipitated by an equal volume of isopropanol and 0.04 μg of glycogen at -20°C overnight and centrifuged $12,000 \times g$ at 4°C for 20 min, and the precipitates were washed twice with 75% ethanol. Finally, the pellets were dried at room temperature, dissolved in RNase-free water, incubated at 55°C for 15 min, and stored at -70°C until used in the RT-PCR.

In principle, negative strands should represent de novo synthesis, thus being the most informative indication for virus replication. Uninfected control cells representing each cell line were also analyzed by RT-PCR, but no specific signals were detected (data not shown). Some background, obviously caused by the presence of negative strands in the virus inoculum, was occasionally observed (Fig. 6). Therefore, the samples collected late during the infection were compared with those collected immediately after inoculation. This approach clearly confirmed that both B- and T-cell lines are susceptible to CBV3 infection while monocytic and granulocytic cell lines are not (Fig. 6; Table 1). The cells of monocytic and granulocytic lineage did not support CBV3 replication as already shown by infectivity titration, immunoprecipitation, and analysis of viral RNA synthesis by spot hybridization. Viral antigens detected by immunofluorescence in the KG-1 and HL-60 cells could originate from the virus inoculum ingested into the cells by phagocytosis. It appears that negative-strand RT-PCR offers the best alternative for the detection of low levels of enterovirus replication.

Our previous work showed that CBV3 replicates in human hematopoietic cell lines with B (Raji)- and T (Molt-4)-cell characteristics (29). In the present study the permissiveness of the more mature T-cell (CCRF-HSB-2) and B-cell (RPMI 8226) lines for CBV3 was greater than that of the more immature cell lines. One explanation for these differences could be that the virus receptors are expressed on the cell surfaces in a variable manner depending on the maturation stage of the cells. Recently, the decay-accelerating factor (CD55) has been

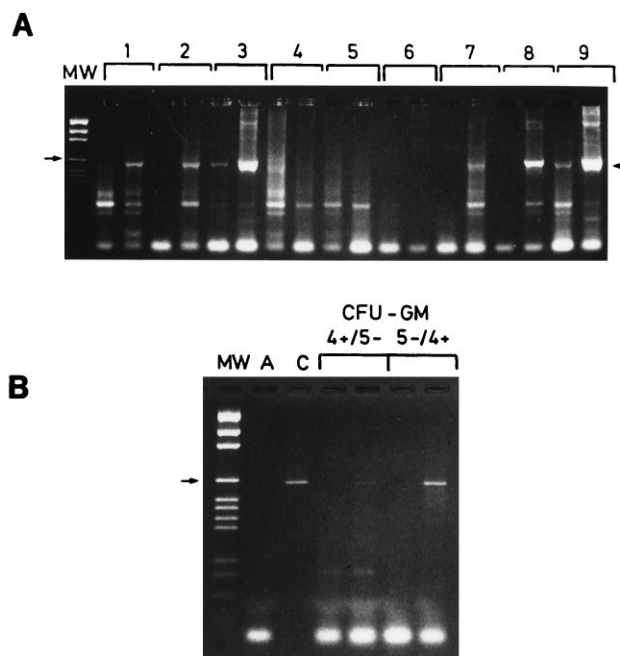


FIG. 6. (A) Detection of viral RNA synthesis by negative-strand RT-PCR in infected hematopoietic cell lines of granulocytic, monocytic, B-cell, and T-cell lineages after adsorption and at 2 days p.i. Lanes: MW, molecular weight marker; 1, Raji; 2, CCRF-SB; 3, RPMI (B-cell lines); 4, KG-1; 5, HL-60 (granulocytic cell lines); 6, THP-1 (monocytic cell line); 7, CCRF-CEM; 8, Molt-4; 9, CCRF-HSB (T-cell lines). (B) Negative- and positive-strand RT-PCR from infected CFU-GM. Lanes: MW, molecular weight marker; A, negative control (uninfected LLC-Mk₂ cells); C, positive control (LLC-Mk₂ cells infected with CBV3). The 4+/5- primer pair (1) was used for negative-strand RT-PCR, and 5-/4+ primer pair used for positive-strand RT-PCR. Arrows indicate the CBV3-specific amplification products.

reported to be a cellular receptor for CBV3 (27). It is expressed on blood cells, and expression is increased during the cell maturation (20, 22). However, when our earlier results of the CBV3 infection in Raji and Molt-4 cells are also considered (29) this cannot be directly explained by the maturation of the cells, because virus replication in these cell lines, representing earlier maturation stages, is more effective. Replication of measles virus in peripheral blood mononuclear cells is dependent on the cellular activation stage (10); thus, an alternative explanation could be the differences in the metabolic activity of the cell lines.

An increase in the levels of both complementary and genomic RNA could be detected in the CFU-GM, indicating virus replication (Fig. 6). CBV3 antigen-positive cells were also detected in CFU-GM, and the infectivity titration indicated some virus release. Because we were unable to detect synthesis of CBV3 proteins by immunoprecipitation, it is possible that CBV3 replicates only in a certain subpopulation of the CFU-GM and the low number of the infected cells does not allow sufficient labeling.

The cell tropism of CBV3 is different from that of poliovirus, which can infect hematopoietic cell lines with monocytic and granulocytic characteristics (21). The most likely explanation is the diverse expression of virus receptors, since the poliovirus receptor, a member of the immunoglobulin superfamily, is expressed on human peripheral mononuclear phagocytes (17), although intracellular factors may also play a role. Further studies characterizing the presence of enterovirus receptors in different human hematopoietic cell lines, and transfection experiments using viral RNA to determine the level of the restriction in the replication cycle would provide important information about the pathogenesis of enterovirus infections.

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