Coxsackievirus B3 Infection in Human Leukocytes and Lymphoid Cell Lines

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Although coxsackie B viruses (CBVs) are known to cause viremia during acute infection, the role of the blood cells as a target for virus replication is poorly understood. We have analyzed the susceptibility of human peripheral blood mononuclear cells (PBMCs), granulocytes, bone marrow (BM) cells, and lymphoid cell lines to coxsackievirus B3 infection. Lymphoid cell lines with B- and T-cell characteristics (Raji and Molt-4, respectively) supported virus replication to high titers and virus protein synthesis was detected by metabolic labeling and immunoprecipitation. CBV3 synthesis in the U-937 cell line with mononuclear phagocytic characteristics was very limited. The virus was able to infect a small proportion of leukocytes and BM cells, and intracellular virus antigens were detected by immunofluorescent staining. However, only a diminutive amount of infectious virus was produced in isolated PBMCs and granulocytes, and no virus protein synthesis was detected by metabolic labeling and immunoprecipitation in these cells.

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COXSACKIE B viruses (CBVs), members of the enterovirus group in the Picornavirus family, are important human pathogens and cause a great variety of diseases varying from the common cold to severe and occasionally fatal infections, eg, myocarditis. After the initiation of enterovirus infection, the multiplication of the virus in the nasopharynx and in the alimentary tract is known to be often followed by a short viremic phase when the virus can be isolated from the blood. Despite the importance of this phenomenon in the pathogenesis of infection (the blood stream can transfer the virus to secondary target organs, eg, the heart) very little is known about CBV infection in human blood cells.

However, it is known that different blood cell types harbor enteroviruses in a different manner. Prather et al showed that in 28 patients with coxsackie or other enterovirus infections, 90% was isolated from blood in 11 cases and serum was positive in 7, mononuclear leukocytes were positive in 9, and granulocytes were positive in 3 cases. Recently, it was reported that coxsackievirus B3 is able to infect freshly harvested human monocytes and to affect the immunologic functions of the cells. On the other hand, Matteucci et al observed systemic lymphoid organ atrophy in coxsackievirus B3-infected mice, but were unable to show virus replication in thymus, spleen, and lymph nodes.

We report here that, although CBV3 antigens are found in human peripheral blood mononuclear cells (PBMCs), granulocytes, and bone marrow (BM) cells after in vitro infection, the production of newly synthesized viral polypeptides or infectious virus was not detected. However, the lymphoid cell lines with B- and T-cell characteristics are highly permissive to CBV3 infection.

MATERIALS AND METHODS

Preparation of virus stock. CBV3 (Nancy strain) was originally obtained from American Type Culture Collection (ATCC; Rockville, MD). The virus was propagated in roller cultures of LLC-MK2 cells (ATCC), which are Mycoplasma screen negative at regular intervals. When cytopathic effect (CPE) of 75% to 100% was observed, the cells and the culture medium were collected, frozen, and thawed three times to release the virus, and stored at −70°C. Before use, the cell debris was removed by low-speed centrifugation.

Cells and cell lines. Human PBMCs and granulocytes were isolated from healthy adults in Ficoll-Isopaque (Ficoll-Paque; Pharmacia Fine Chemicals, Uppsala, Sweden)/Histopaque-1119 (Sigma Diagnostics, St Louis, MO) double gradients. According to the FAC Scan flow cytometer analysis the mononuclear cell population of five persons consisted of 84% to 94% of lymphocytes and 1.5% to 7.5% of monocytes. The granulocyte fraction contained 47% to 66% granulocytes and 11% to 24% lymphocytes. Mononuclear cells were cultured either unstimulated or stimulated with phytohemagglutinin (PHA; 10 μg/mL, Difco Laboratories, Detroit, MI) 4 days before infection. Human BM cells were aspirated from patients at the Department of Hematology, Turku University Central Hospital. BM cells with morphologically normal appearance were used for virus studies. Cells from at least 4 donors were tested in each experiment. Raji, Molt-4, and U-937 cell lines were originally obtained from ATCC.

Infection of cells. PBMCs, granulocytes, and BM cells as well as Raji, Molt-4 and U-937 cell lines were infected with the Nancy strain of CBV3 at a low (1 or 5) and a high (100) multiplicity of infection (m.o.i.) in 0.2 mL of serum-free Hanks’ balanced salt solution. After the adsorption time of 1 hour, the cells were extensively washed to remove the unadsorbed virus inoculum. Infected and control cells (10⁶ cells/mL) were incubated at 37°C in RPMI 1640 medium, supplemented with 10% fetal calf serum (GIBCO BHC Europe, Glasgow, UK) and 50 μg/mL gentamicin. Lymphoblastoid cell lines, PBMCs, and their supernatants were harvested for infectivity titration and spot hybridization test after different time intervals postinfection (p.i.) during 96 hours, granulocytes were harvested during a 24-hour incubation period, and both were stored at −70°C until analyzed. The cells for the in situ hybridization and immunofluorescence (IF) tests were collected after the adsorption time and 1, 2, 3, 4, and 5 days postinfection. Uninfected cells were used as control material.

Infectivity titration. The release of CBV3 from infected cells to the culture medium and the amount of intracellular virus was determined on confluent monolayers of LLC-MK2 cell cultures in dilutions 10⁻¹ to 10⁻¹⁵ according to the appearance of a CPE. Before inoculation, the cell suspensions were frozen and thawed three times to release the virus and clarified by low-speed centrifugation. The cells were examined for the CPE for 7 days and the results were expressed as end-point titers.

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Submitted February 3, 1993; accepted March 28, 1994.

Supported by grants from the Sigrid Juselius Foundation and the Academy of Finland.

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**Fig 1.** Coxsackievirus B3 RNA in infected Raji (A), Molt-4 (B), and U-937 (C) cell lines 24 hours postinfection detected by in situ hybridization. D, E, and F represent corresponding dark fields. Original magnification x 25.

*In situ hybridization.* Ten to 20 µL of cell suspension was transferred onto polylysine-coated microscope slides and dried at room temperature (RT). The cells were fixed in 4% phosphate-buffered paraformaldehyde. Before hybridization, the slides were incubated in 0.2 mol/L HCl for 20 minutes; for 30 minutes in 2 × SSC at 70°C; proteinase K (1 mg/mL)-treated for 15 minutes at 37°C in 20 mmol/L TRIS-HCl, pH 7.4, 2 mmol/L CaCl₂; dehydrated in ethanol series; and air dried. The slides were washed in aqua between the treatments. The cDNA probe, covering most of the coxsackievirus B3 genome, was labeled in a nick translation reaction using ³²P-deoxytide triphosphate (³²P-dCTP) and ³²S-deoxyguanosine triphosphate precursors to a specific activity of 1.5 × 10⁸ cpm/µL. The hybridization solution contained 50% formamide, 10 mmol/L TRIS-HCl, pH 7.4, 600 mmol/L NaCl, 1 mmol/L EDTA, 10% dextran-sulfate, 0.2 mg/mL salmon sperm DNA, 0.1 mg/mL rabbit tRNA, 0.1 mg/mL total Vero cell RNA, 0.05% bovine serum albumin, and 20 mmol/L dithiotreitol (DTT), and the denatured probe. For each slide, 1.5 × 10⁶ cpm of the probe was used, and the hybridization was performed at 25°C for 48 hours. Then the slides were washed in 50% formamide, 600 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L TRIS-HCl, pH 7.4, 20 mmol/L DTT, first briefly four times, then twice for 5 minutes at RT and overnight at 30°C. Finally, the slides were washed for 60 minutes at 55°C, followed by two times for 5 minutes at RT in 2 × SSC, 20 mmol/L DTT and dehydrated in ethanol series containing 300 mmol/L ammonium acetate. The slides were dried at 42°C and coated for autoradiography with Nuclear Track emulsion, NTB3 (Eastman Kodak, Rochester, NY), diluted 1:1 with 0.6 mol/L ammonium acetate, and exposed at 4°C for 14 days. After developing the film, the cells were counterstained with hematoxylin and eosin. Plasmid vector pBR322 was used as a control probe.

**Fig 2.** Detection of coxsackievirus B3-specific RNA by spot hybridization in infected Raji, Molt-4, and U-937 cell lines, and in isolated granulocytes after different time intervals postinfection (h.p.i.). C shows uninfected control cells.
Fig 3. Synthesis of virus proteins detected by immunoprecipitation with rabbit anti-CVB3 serum. Uninfected Raji cell line (A), CBV3-infected Raji cell line (B), infected Molt-4 cell line (C), infected U 937 cell line (D), infected LLC-MK2 cells (E), and uninfected LLC-MK2 cells (F). Virus polypeptides VP0, VP1, VP2, and VP3 are indicated by arrows. Molecular-weight markers 46, 30, 21.5, and 14.3 kD are depicted by lines on the left.

5 minutes at RT in 2 × SSC containing 0.1% sodium dodecyl sulfate (SDS) and three times for 30 minutes at 42°C in 0.1 × SSC containing 0.1% SDS.

Immunoprecipitation. Raji, Molt-4, and U-937 cell lines, LLC-MK2 cells, as well as stimulated and unstimulated PBMCs were infected with CBV3 at a high multiplicity of infection (MOI). After 3 hours p.i., the cells were cultured for 1 hour in methionine-free medium and then labeled with 50 μCi/ml 35S-methionine. After overnight incubation, lymphoid cell lines and PBMCs were washed with phosphate-buffered saline (PBS) and stored at −70°C until lysed in 0.05 mol/L TRIS-HCl, pH 7.4, 0.15 mol/L NaCl, and 0.001 mol/L EDTA, containing 1% Tween and 1% sodium desoxycholate. The lysates were sonicated for 1 minute and centrifugated for 10 minutes at 8,000 rpm to remove insoluble proteins. Protein A-Sepharose CL-4B beads and 20 μL of rabbit CBV3-antiserum were incubated at RT for 60 minutes. The labeled cell lysate was added and the mixture was incubated overnight at 4°C, washed four times with 0.1 mol/L TRIS-HCl containing 0.01% Tween and eluted with 0.1 mol/L TRIS-HCl, 2% SDS, 5% mercaptoethanol. The immunoprecipitates were analyzed by electrophoresis in 12% SDS-polyacrylamide gels. The results were shown by autoradiography on an x-ray film for 4 days and by using Phosphor Analyst (Bio-Rad Laboratories, Richmond, VA).

Immunofluorescence. The cells were fixed on slides in acetone at 4°C for 10 minutes and stored at −20°C until tested. The fixed cells were incubated for 45 minutes at 37°C with rabbit anti-CBV3 serum, diluted 1:50 in PBS and further stained with fluorescein isothiocyanate (FITC)-conjugated sheep antirabbit IgG (Dako, Glostrup, Denmark). For the double-staining, cells were first incubated with the FITC-conjugated anti-Leu-M3 antibodies (Beckton Dickinson, San Jose, CA), fixed in acetone at 4°C, incubated with rabbit anti-CBV3 serum and finally stained with tetramethylrhodamine isothiocyanate-labeled sheep-antirabbit IgG. The fluorescence was examined using a Leitz Dialux 22 microscope (E. Leitz, Wetzlar, Germany).

RESULTS

Lymphoblastoid cell lines. The in situ hybridization test was used to show the proportion of cells containing viral

Fig 4. Production of infectious coxsackievirus B3 in the Raji, Molt-4, and U-937 cell lines, in unstimulated and stimulated PBMCs and in granulocytes. Mean and SD of end-point titers from cells (○) and the culture medium (□) are shown. For PBMCs and granulocytes, results with m.o.i. of 1 and 100 are shown.
RNA after the adsorption time of the virus and 24 hours p.i. (Fig 1). Uninfected cell lines were used as controls. The Molt-4 and Raji cell lines were highly permissive to CBV3 infection, whereas the monocytic cell line U-937 did not show any positive signal. Viral RNA synthesis in the cell cultures was also followed by analyzing the total quantity of CBV3 RNA by spot hybridization (Fig 2). Also in this assay, the Raji and Molt-4 cell lines were observed to support virus RNA synthesis, whereas the monocytic cell line U-937 did not.

The synthesis of virus polypeptides VP0, VP1, VP2, and VP3 was detected by immunoprecipitation from Raji and Molt-4 cell lines after metabolic labeling, but no virus-specific proteins were detected in the U-937 cell line (Fig 3).

The human lymphoblastoid cell lines with both B- and T-cell characteristics also supported growth of infectious virus to high titers (Fig 4), and the replication cycle was similar to that observed in standard cell cultures used to propagate the virus. The production of the virus started to increase during the first 4 hours p.i. On the other hand, CBV3 production in the U-937 cell line was very limited or negative (Fig 4).

**PB leukocytes and BM cells.** By the in situ hybridization test (Fig 5), only individual positive cells (=1/1,000) could be detected in cultures of CBV3-infected PBMC and BM cells, and in the spot hybridization test, the signals were under the detection level (data not shown). However, immunofluorescent staining showed the presence of CBV3-antigen-positive cells already after the adsorption time. The proportional number of the positive cells was between 10% and 20%. The number of CBV3 positive leukocytes, both in unstimulated and PHA-stimulated cells remained at a similar level (Fig 6). The same proportion of the cells was positive when low or high m.o.i. was used. Although individual variation (5% to 25% of positive cells) was observed, when CBV3-infected mononuclear cell cultures of six donors representing different HLA-types were examined, comparable results were obtained. Double staining showed that some cells of the monocyte population contained virus antigens (Fig 6).

No release of infectious virus from PBMC infected with either a low or high m.o.i. was detected, and PHA stimulation had no effect on virus production (Fig 4). When 100 m.o.i. was used, the end-point titers were at a higher level, obviously because of the high concentration of the inoculum virus. No synthesis of virus polypeptides was detected in infected unstimulated or stimulated PBMCs after metabolic labeling and immunoprecipitation (not shown).

By IF test, CBV3 positive granulocytes were detected already after 1 hour p.i. (Fig 6), and after 24 hours p.i.,
the IF and the in situ hybridization tests (Fig 5) showed a proportional number (1/1,000) of CBV3 positive granulocytes. The signals in the spot hybridization test (Fig 2) were weak, obviously detecting only RNA originating from the adsorbed particles. No release of infectious virus to the culture medium was observed (Fig 4).

**DISCUSSION**

Viremia is associated with infections caused by a great variety of viruses. In some cases, eg, parvovirus B19 and human immunodeficiency virus infections, it is known to be caused by replication of the virus in defined subgroups of blood cells. Although the target cells responsible for virus replication have not yet been definitely identified, viremia is also common in enterovirus infections.

Our earlier studies using a mouse model for coxsackievirus B3 infection showed that extensive viremia occurs during the first days after infection. However, our attempts to show viral infection of mouse leukocytes were unsuccessful, indicating that the replication may rather take place in local lymph nodes or in secondary target organs. Indeed, the course of the viremic phase correlates well with the destruction of the exocrine pancreas, which could be the source of the virus detected in the blood. However, in human infections, the destruction of the target organs is usually less extensive, and therefore, other explanations for viremia are needed.

CBV3 infection in human PBMCs was observed to be highly restricted. No distinct release of infectious virus was detected in unstimulated or PHA-stimulated PBMCs (Fig 4). This is different from other infectious viruses, eg, measles virus, where PHA-stimulation extensively increases the synthesis of infectious virus. The release of infectious CBV3 from the granulocytes was also minimal and the small amount of virus detected during the first 24 hours incubation time may originate from the inoculum. The relatively low proportion of virus-containing PBMCs can be explained by infection of a subpopulation expressing the virus receptor. It is also evident that the virus enters some cells by phagocytosis or related mechanisms, but does not replicate. Although
Henke et al. observed CBV3 production in infected human monocytes, we have been unable to detect active virus replication in these cells. The IF test also showed CBV3 positive polymorphonuclear cells (Fig 6). It is not known whether these cells have receptors for CBVs or if the viruses are taken into the cells by phagocytosis. For instance, members of another enterovirus group, echoviruses, have been found to change cell membrane structure of granulocytes and alter their adhesion properties.

It is possible that infected granulocytes or monocytes may transfer viruses through the circulation to different target organs.

Virus infections are known to cause BM failure and the role of hematopoietic cells as a target of virus infections has arisen interest. Parvovirus is able to cause aplastic crisis in patients with hemolytic anemia and the virus is able to infect precursors of mature erythrocytes in vitro. Dengue virus replicates in BM mononuclear cells in vitro, whereas cytomegalovirus infects stromal cells. We observed that human BM cells were infected in vitro by CBV3, but the number of infected cells was low, suggesting that BM does not represent any main target for the replication of the coxsackieviruses.

When replication characteristics are concerned, CBV3 differs from polioviruses, which infect Molt4 and U-937 cells, whereas no virus production is observed in Raji cells. An important determinant in susceptibility to viral infection is the cell surface receptor. Some of these molecules have already been identified and it is known that the poliovirus receptor is a member of the Ig superfamily that is expressed in many cell types. Although the CBV receptor molecule has been partially characterized, its exact nature is not known to date.

The variability in the susceptibility of human lymphoid cell lines to these viruses could, at least partly, be explained by the presence of different functionally active receptors on the cell surface. However, other factors may also play an important role because expression of the human poliovirus receptor gene in the developing T-lymphocytes in the thymus of transgenic mice is not sufficient for making these cells susceptible to infection.

We show here that a small proportion of in vitro infected human PBMCs absorb CBV3 antigens, and granulocytes are also able to harbor virus. According to the double-staining IF, the PBMCs finding can be partly explained by the presence of the viral material in a monocyte population. Production of virus polypeptides in PBMCs was not observed by immunoprecipitation even though a high concentration of inoculum virus was used and no clearly detectable synthesis of infectious virus in the cultured cells occurred. Therefore, the transient viremia cannot be explained by the replication of the virus in these cells. However, it is evident that absorption/phagocytosis of CVB3 to the blood cells takes place and it is possible that a small population supports active virus replication. The results are unchanged when very high virus concentrations are used. Further studies using new approaches will be needed to shed light on the possible role of lymph nodes and vascular epithelium as the origin of enteroviruses found in blood during the viremic phase of infection.

ACKNOWLEDGMENT

We thank Marjut Sarjovaara and Marita Maaronen for technical assistance, Taina Kivelä for secretarial help, Dr Reinhard Kandolf for providing us with the CBV3 cDNA probe, and Dr Tarja-Terttu Pellinieni for the examination of the BM aspirates.

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