Establishment and Characterization of an Epstein-Barr Virus Transformed Cell Line With Strong Phagocytic Activity


An immunoglobulin M (IgM)-positive cell line, Ms 28, apparently spontaneously transformed by Epstein-Barr virus (EBV) was established from peripheral blood cells of a patient with immature myeloblastic leukemia. It has been characterized according to phenotype, cytochemistry, and membrane antigen pattern. The cell line expresses lymphoid markers like CD 19, CD 22, and CD 30 and synthesizes and secretes IgM. Monocyte markers CD 11c, CD 14, and CD 15 are absent. Neither interleukin-1 (IL-1), nor tumor necrosis factor (TNF-α) are produced. But Ms 28 cells show strong phagocytic activity and engulf latex particles and sheep RBCs (SRBCs) that need not to be opsonized. The phagocytic activity can be inhibited by chloroquine. Both phagocytosis and EBV nuclearentigen (EBNA) expression can be observed in one and the same cell. Ms 28 cells might be useful to study immunologic activities like antigen processing and presentation.

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ELL LINES DERIVED from leukemic cells provide a tool to study antigenic properties, oncogen expression, and differentiation potential of leukemia cells. Usually leukemic cells, despite their seemingly unlimited growth potential in vivo, undergo only limited proliferation in liquid tissue culture. Nevertheless, a few cell lines have been established in the past 15 years.11 Because the cell lines known up to now do not cover the whole spectrum of antigenic properties and differentiation potentials of leukemic cells, we attempt to establish new lines. In the following, we describe a spontaneously transformed Epstein-Barr virus (EBV) immunoglobulin (Ig)-positive cell line with unusual properties, derived from a patient with acute myelogenous leukemia (AML). Because of their strong phagocytic activity, these cells can be of particular interest for immunologic investigations like antigen processing and presentation.

MATERIALS AND METHODS

Case description. In June 1987, a 60-year-old female patient was admitted to the hospital with anemia, thrombocytopenia, and a marked leukocytosis with 99% blasts in the peripheral blood. The disease was diagnosed as an acute undifferentiated leukemia (AUL according to French-American-British [FAB] criteria). In order to reduce the high leukocyte blood count, the patient was treated by leukapheresis to prevent an occlusion of the microcirculation. She then received a chemotherapy treatment with daunorubicin, 6-thioguanine, and cytarabine. The patient died seven days after onset of chemotherapy.

Tissue culture conditions. Separated blast cells were further purified by centrifugation through a Ficoll gradient. They were then cultivated in Dulbecco's modified Eagle medium (DMEM, 074-01600 Gibco-BRL, Eggenstein, FRG), with NaHCO3, (44 mM), glucose (18 mM), l-glutamine (2 mM), 2-mercaptoethanol (5 x 10-5 M), penicillin-streptomycin, nonessential amino acids, and 20% heat inactivated fetal calf serum (FCS; HyClone, Greiner, Nüttlingen, FRG) in a humidified atmosphere of 12% CO2 at 37°C. Medium was changed two to three times per week. Cultured cells were purified over a Ficoll gradient once a week to remove cell debris and washed three times before resumption in a volume of 10 mL medium in 50 mL tissue culture flasks (Greiner, Nüttlingen, FRG). For the first 4 months of cultivation, cell numbers remained unchanged. In some flasks, we observed occasionally a differentiation of round nonadherent cells into adherent macrophage-like cells, which did not further proliferate. From other flasks it was possible to establish nonadherent cells with continuous proliferation and rapid increase of total cell numbers. From that time on, the characterization of this cell line, Ms 28, began. So far, cloned cells have not been included in the studies.

 Antibodies and reagents. The following commercially available reagents and monoclonal antibodies (MoAbs) against cell surface markers were used: HLA-ABC (IOT 2, no 0107) from Immuno- tech, Dianova, Hamburg, FRG; HLA-DR (no. 7360), HLA-DQ (Leu 10, no. 7450), Leu 4 (no. 7340), Leu 12 (no. 7540), Leu 14 (no. 7570), Leu M1 (no. 7420), Leu M3 (no. 7490), Leu M5 (no. 7630), H-Le1 (no. 7460), HPCA-1 (no. 7460), HPCA-1 (no. 7660) from Becton-Dickinson, Heidelberg, FRG; My 9 from Coulter Electronics, Krefeld, FRG; affinity pure F(abertura 2 fragments from goat antihuman IgM and IgG from Dianova, Hamburg, FRG; mouse MoAb antihuman IgM (CM 702) and alkaline phosphatase anti-alkaline phosphatase complex from Daco, Hamburg, FRG; goat antihuman Ig antisera (no. B9015) and streptavidin-biotinylated-β-galactosidase from Sigma, Deisenhofen, FRG; biotinylated sheep antiserum Ig from Amersham-Buchler, Braunschweig, FRG. Ki-1 MoAb recognizing activated T and B lymphocytes (CD 30) was given to us by Dr J.B. Gerdes, Forschungsinstitut Borstel, FRG. BA-1 was a gift from Dr B. Wörmann, Department of Medicine, University Münster, FRG. Recombinant γ-interferon (r-γIFN) was a gift from Boehringer, Ingelheim, FRG.

Cytochemistry and immunocytochemistry. Ms 28 cells were stained for myeloperoxidase, NaF-sensitive esterase, and periodic acid-Schiff (PAS) with standard methods. Intracellular and membrane-bound Ig was determined on cytocentrifuge preparations of Ms 28 cells with and without acetone fixation. Cells were incubated with affinity pure F(abutra 2) fragments of goat antihuman IgM and IgG and developed with the alkaline phosphatase-anti-alkaline phosphatase method. Controls were performed in absence of the F(abutra 2) fragments. Cells were counterstained with hemalaun.

Cellular enzyme-linked immunosorbent assay. For cellular enzyme-linked immunosorbent assay (cELISA), two preparations of target cells were used: Viable cells (trypanblue exclusion test) were incubated in 10 mL test tubes with the first MoAb for two hours.

Cells were then washed three times by centrifugation (400 g) in cold phosphate buffered saline (PBS). After that they were resuspended in PBS containing biotinylated sheep anti-mouse Ig and incubated for 90 minutes. After additional washing procedures, cells were incubated with streptavidin-biotinylated-β-galactosidase for one hour. After additional washings, 50 µL PBS containing 2 × 10^5 target cells were pipetted into flat-bottomed microtiter wells and incubated with 50 µL double concentrated substrate (1.8 mg ortho-nitrophenyl-β-d-galactopyranoside/mL, 4.0 mg MgCl₂/mL and 14 µL 2-mercaptoethanol/mL). Probes were set up in quadruplicates. After 30 minutes, the amount of ortho-nitrophenol produced was determined by measuring the extinction at 405 nm in a Dynareader (Dynatech, Denkendorf, FRG).

In other experiments, 2 × 10^5 target cells were fixed with 0.25% glutaraldehyde for five minutes. Cells were then washed and the wells were saturated with 5% FCS in PBS to prevent unspecific protein binding. ELISA procedure was then performed as described above. In all experiments, controls were performed with medium containing 10% FCS, an antimouse H-2\(^\text{D}^\text{d}\) MoAb (C34-1-2) as negative, and an anti-HLA-ABC as positive control.

Tests for cytokine production. Ms 28 cells were cultured at concentrations of 1, 2, 4, and 8 × 10^6 cells/mL under three different conditions: (1) in Dulbecco's modified Eagle's medium (DMEM) serum free; (2) in DMEM plus 15% FCS and stimulated with 1 µg/mL lipopolysaccharide (LPS) and 10 U/mL of r-γ-IFN; and (3) in DMEM plus 15% FCS with 10 µg/mL LPS and 10 U/mL of r-γ-IFN. Supernatants were harvested after 24 hours and tested for IL-1 and tumor necrosis factor (TNF-α) activities.

The fibroblast proliferation assay used for detection of interleukin-1 (IL-1) has been described previously. Briefly, human foreskin fibroblasts were grown in flat-bottom microtiter plates at a concentration of 5 × 10^3 cells/mL in DMEM containing 10% FCS, t-glutamine, and antibiotics. After 24 hours, the medium was replaced by test samples diluted in DMEM. After culture for 72 hours, cells were pulsed with tritiated thymidine (¹H-TdR) (0.2 µCi/well, 7.4 kBq/well; specific activity 2 Ci/mmol, Amersham-Buchler, Braunschweig, FRG) and incubated for another 24 hours. Cells were harvested by treatment with trypsin/EDTA solution. ¹H-TdR incorporation was measured in a liquid scintillation counter. The detection limit for IL-1 is 10 pg/mL (10 pg = 1 U).

Supernatants were also tested in the EL4-6.1 assay. This murine thymoma subline produces IL-2 in dependence of IL-1. IL-2 is determined by proliferation of murine CTL L6 cells. The detection limit for IL-1 is 1 pg/mL.

TNF activity was determined essentially as described. Briefly, serially diluted culture supernatants were placed in flat-bottom microtiter plates (Falcon 3072 Becton-Dickinson) with actinomycin D (1 µg/mL) L929 cells precultured for three days were harvested with trypsin, washed, and 4 × 10^5 cells/well were added to the samples. The test was carried out in volumes of 200 µL in RPMI 1640 containing 5% FCS. After 24 hours, the supernatants were removed, the cells were fixed in 3% formaldehyde, and after washing, staining with 0.2% crystal violet in 10% methanol. After additional washing, the stained cells were lysed with 100 µL of 0.5% sodium dodecyl sulfate (SDS) in distilled water and evaluated in a Dynareader at 540 nm. Cytotoxic activities in the samples were determined using a standard of human recombinant TNF-α (provided by BASF Knoll AG, Ludwigshafen, FRG), and comparing the dilutions giving 50% cytotoxicity by probit analysis.

Biosynthetic labeling of immunoglobulin and immunoprecipitation of immunoglobulin M. Five times 10^7 Ms 28 cells were incubated and washed two times with methionine-free minimum essential medium (MEM), GIBCO-BRL, Karlsruhe, FRG). They were then suspended in 1.75 mL of the above MEM containing 15 µL of concentrated glutamine, 75 µL of dialysed FCS and 500 µCi ³⁵S-methionine (specific radioactivity 37 T Bq/mmol ~ 10² Ci/mmol; Amersham-Buchler). Cells were incubated for four hours in a humidified atmosphere of 5% CO₂ at 37°C. They were then washed three times with PBS. Cells were lysed on ice for 30 minutes in lysis buffer (1% Nonidet P 40 in 0.1 mol/L Tris buffer pH 7.4, containing 0.15 mol/L NaCl and 2 mol/L protease inhibitor phenylmethyl-sulfonylfluoride. Cell debris were removed by centrifugation (ten minutes at 13,000 g). The supernatant was cleared with a mouse antiserum T-cell MoAb of irrelevant specificity. Then the affinity-purified F(ab')₂ fragments of goat antihuman IgM and IgG or mouse MoAb antihuman IgM were incubated with supernatant aliquots at 4°C for 16 hours. Afterward, cellulose-labeled second antibodies against goat or mouse Ig were added and incubated at room temperature for one hour. These immunoprecipitates were centrifuged and washed three times in washing buffer. After that the immunoprecipitates were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Demonstration of Ig secretion by Ms 28 cells into the labeling medium followed essentially the above immunoprecipitation procedure.

Phagocytosis. Sheep RBCs (SRBCs, Behringwerke, Marburg, FRG) were washed three times in Eagle's balanced salt solution. Then they were incubated in DMEM without FCS at 37°C for ten minutes to destroy fragile cells, washed again, and subsequently used in the assay. Two times 10⁶ Ms 28 cells were incubated with 1 × 10⁶ SRBCs in 2 mL medium for 20 hours in 24-well costar plates. Cells were then harvested at different times and nonphagocytosed SRBCs were lysed with 0.84% NH₄Cl. Then Ms 28 cells were washed three times and phagocytosis was examined under light and transmission electron microscopy. For comparative control experiments, Raji EBV-transformed lymphoblastoid cells were used. Phagocytosis was also determined quantitatively after incorporation of SRBC by Ms 28 cells. For that purpose, cells were lysed in hypotonic hemolysis buffer and the amount of internalized hemoglobin was measured spectrophotopically by absorption at 412 nm. Control experiments were performed measuring phagocytosis by adherent peritoneal exudate cells of BALB/c mice and by BALB/c X63-Ag8 myeloma cells. Phagocytosis inhibition experiments were performed in presence of 300 µm chloroquine (Sigma, Deisenhofen, FRG) in culture. In other experiments phagocytosis of Latex particles (7 µm diameter, Serva, Heidelberg, FRG) was followed up by electron microscopy.

**Fig 1.** Light microscopic appearance of Ms 28 cells in tissue culture. Single cells and clusters of different size can be observed (magnification ×120).
Epstein-Barr virus transformation. Transformation of Ms 28 cells by EBV was tested by indirect immunofluorescence using human antiserum against Epstein-Barr nuclear antigen (EBNA) by Dr. H. Müller, Landesuntersuchungsanstalt Münster, FRG. EBNA was visualized in the majority of the Ms 28 cells.

Electron microscopy. Ms 28 cells from suspension culture were fixed with Karnovsky’s fixative for one hour, postfixed with OsO₄ for 30 minutes, dehydrated in ethanol and embedded in Epon (Serva). The specimens were sectioned with a diamond knife on a Reichard microtome, counterstained with uranylacetate and lead citrate, and micrographed with a Philips electron microscope 301 at 60 kV.

Cytogenetic examination. Cytogenetic examination of Ms 28

Fig 2. Transmission electron microscopy of Ms 28 cells. (a) The cells are of different size, but all are capable to phagocytose SRBCs. Large cells with light cytoplasm and phagocytic vacuoles possess many pseudopodia (left). The smallest cells (middle) have little and dark cytoplasm and possess no or few pseudopodia. A dead cell is present at the top margin and an intermediate-sized cell at the lower right end below (magnification × 5,500). (b) A cell having phagocyted several SRBCs, the remnants of which are faintly discernable in the large vacuole. (c) A cell with a large number of phagocytic vacuoles showing the typical aspect of cells fed with SRBCs for 20 hours. The nucleus is extensively lobed. This cell shows signs of beginning necrosis as is typical after extensive phagocytosis.
cells with standard methods, performed by Prof Dr C. Fonatsch, Institut für Humangenetik, Universität Lübeck, FRG, revealed a normal female karyotype (data not shown).

RESULTS

Growth and differentiation characteristics. Ms 28 cells grow under the culture conditions described with an approximate doubling time of 72 hours and reach a saturation density of $4 \times 10^6$ cells/cm² in 10 mL of medium. This corresponds to $1 \times 10^6$ cells/mL. They grow in suspension and tend to aggregate in clusters; but single cells are also observed (Fig 1). Dead cells are often found in the center of the clusters. Microscopic examination of Pappenheim-stained cells shows blasts with dark basophilic cytoplasm and lobed nuclei. Occasionally one sees a discrete azurophilic granulation. With standard cytochemistry methods, cells are negative for myeloperoxidase and positive for NaF-sensitive esterase. Ten percent of the cells are PAS positive.

Transmission electron microscopy shows three morphologically distinct cell types (Fig 2a): large cells with light cytoplasm and many pseudopods, intermediate-sized cells with darker cytoplasm and pseudopods, small cells with little dark cytoplasm and few pseudopods. The large cells have some dark phagocytotic vacuoles, probably from digesting dead cells. All three cell types are able to phagocytose SRBCs. Two examples at different stages of digestion of SRBCs are shown from Ms 28 cells cultured with SRBCs for 20 hours (Figs 2b and c).

Membrane antigen characteristics. cELISA binding studies using various antimembrane MoAbs are summarized in Table 1. They reveal a strong expression of an HLA-ABC monomorphic determinant on Ms 28 cells. In addition, HLA-DR and HLA-DQ antigens are expressed. All cells are Ig positive as determined by immunocytochemistry and cELISA. One hundred percent of the Ms 28 cells stain positive for intracellular Ig. In addition they express typical B lymphocyte markers like CD 19 and CD 22, as well as CD 30, a marker for activated lymphocytes. On the other hand, mono- and myelocytic markers like CD 11c, CD 14, CD 15, and CD 33 are missing.

Phagocytosis. Phagocytosis can be observed in over 80% of the Ms 28 cells after 20 hours of cocultivation with SRBCs. It is important to note that SRBCs did not have to be opsonized in these assays (Figs 2b and c). Nonopsonized latex particles (0.45 to 11 μm diameter) are internalized to a similar extend (Fig 3a). Quantitative studies on the kinetics of phagocytosis are given in Fig 4. The data show that Ms 28 cells phagocytose a mean of three SRBCs within two hours at 37°C. Marine adherent peritoneal exudate cells are twice as effective; but it has to be noted the adherent v nonadherent cells are compared in these assays. Phagocytosis is completely inhibited by chloroquine. For comparison, we have included an experiment in which SRBCs were presented to Raji EBV-transformed lymphoblastoid cells under the same culture conditions for four hours. Phagocytosis is only observed in Ms 28 but not in Raji cells (Figs 3b and c).

Metabolic properties. The ability to produce IL-1 and TNF-α is a characteristic property of cells of the monocyte/macrophage lineage. To categorize Ms 28 cells, it was tested whether they produce IL-1 or TNF-α (Table 2). Ms 28 cells produce IL-1 neither spontaneously nor when stimulated with LPS plus r-γ-IFN. However, when supernatants were tested for cytotoxic activity on murine L929 cells, there was cytotoxic activity that was not neutralized by an antiserum against human TNF-α. This finding clearly demonstrates that Ms 28 cells cannot be induced to produce TNF-α. Whether the cytotoxic factor produced is lymphotoxin (TNF-β) remains to be determined.

On the other hand, biosynthesis of IgM can be shown by labeling the cells with 35S-methionine. Subsequent immuno-

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<th>Table 1. Membrane Antigen Expression of Ms 28 Cells</th>
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<td>HPCA-1</td>
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<td>Anti-human IgM*</td>
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*Also tested and found to be positive in immunocytochemical preparations; 100% of Ms 28 cells stain positive for intracellular Ig.
Fig 3. Transmission electron microscopy of the phagocytosis process (bars = 10 μm). (a) Internalization of a nonopsonized Latex particle (7 μm diameter) by an Ms 28 cell. (b) Phagocytosis of SRBCs by an Ms 28 cell in a four-hour cocultivation experiment. (c) Lack of phagocytosis of SRBCs by Raji EBV-transformed lymphoblastoid cells in a four-hour cocultivation experiment. Raji cells do not phagocytose SRBCs even after 20 hours of cocultivation.

Fig 4. Kinetics of phagocytosis of SRBCs by phagocytic cells of different origin in the absence (■—■) or presence (▲—▲) of chloroquine in culture. (a) Human cell line Ms 28. (b) Adherent mouse peritoneal exudate cells. (c) Murine plasmacytoma line X63-Ag8 as control for a nonphagocytic cell line. In each case, 2 x 10⁶ nucleated cells were cocultivated with 2 x 10⁶ SRBCs. Various times after culture, nonphagocytosed SRBCs were removed, the nucleated cells lysed, and the content of incorporated sheep hemoglobin spectrophotometrically determined by measuring absorption at 412 nm. The numbers of SRBCs internalized was determined using a calibration curve with lysed SRBCs (10⁷ SRBCs having an extinction of 0.75 at 412 nm).
Table 2. Ms 28 Cells Lack the Capacity to Produce IL-1 and TNF-

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<th>Cell Concentration (µg/mL)</th>
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<th>TNF-α Assays (U/mL)</th>
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<td>Medium control</td>
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*In the presence of anti-TNF-α; neutralizing capacity of rabbit anti-human recombinant TNF-α antiserum against TNF-α is 1,240 U.

The observed slight differences in the molecular weight of intracellular and secreted Ig are due to posttranslational modifications. In the controls, there is some crossreactivity of the μ chains with the anti-T-cell MoAb and unspecific coprecipitation of smaller polypeptides.

DISCUSSION

The cell line Ms 28 is derived from peripheral blood cells of a patient with AML. The FAB classification according to standard methods of these cells was M1 (immature myeloblastic). In the first 3 weeks of culturing, one occasionally observed in some flasks differentiation into adherent macrophage-like cells with no further proliferation, an observation that previously has been made in AML cultures of various FAB types. The nonadherent cell line Ms 28 was established from other flasks. The cytogenetic data showing a diploid female karyotype exclude the possibility that Ms 28 cells are a fusion product of two different cell types. The cells appear to be monocytic with respect to cell morphology in Pappenheim staining and enzymatic reactivity with NaF-sensitive esterase. On the other side, Ms 28 cells are EBNA positive, stain all positive for intracellular Ig, and synthesize IgM de novo as demonstrated by biosynthetic labeling. Strong phagocytosis of nonopsonized SRBCs and Latex particles by more than 80% of the cells can be observed. This process can be inhibited by chloroquine, indicating an active process of internalization.

According to cytochemical phenotype and phagocytic activity, one would expect expression of monocytic differentiation antigens, especially the CD 14 cluster, in Ms 28 cells. However, cELISA binding studies failed to demonstrate CD14, CD14, and CD15. Monoblastic cell lines as U 937 and THP 1 fail to express CD 14 antigens and thus compare to our results. Only the recently described cell clone, MonoMac 6, which can phagocytose opsonized SRBCs, expresses spontaneously this antigen cluster and, according to immunophenotyping, might be the most differentiated monocytic cell line so far known. From our data it would seem that a cell with complete phagocytic properties does not require expression of CD 14 antigens. Spontaneous EBV transformation of cells is not a rare event in cultures of human B lymphocytes and Ms 28 cell line might be derived from residual B lymphocytes of the AML patient. Not only the EBV transformation and IgM biosynthesis but also CD 19, CD 22, and CD 30 expression support the notion that Ms 28 cells belong to the B-cell lineage. The failure to demonstrate IL-1 and TNF-α production as well as the lack of expression of mono-/myelocytic cell surface markers are in accordance with the above idea. According to this view, Ms 28 cells would be B lymphocytes that have acquired phagocytic capacity stronger and more pronounced than one would expect from B-cell antigen presentation.
studies. Acquisition of new unexpected properties has recently been described for B cells expressing Thy-1 antigen and T lymphocytes in culture shifting from T4-"T8" to T4+"T8" phenotype.

The expression of CD 24 determinants opens another possibility. It could be that Ms 28 cells are derived from an early intermediate differentiation stage of B and myeloid cells. This would explain their strong phagocytic activity. It will be interesting to describe lineage origin and phagocytic properties in more detail and further to prove immunologic activities as antigen processing and presentation in adequate assays.

ACKNOWLEDGMENT

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