Immortalization of Human Lymphocytes by Fusion with Cytoplasts of Transformed Mouse L Cells

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Abstract. Fusion of mouse L929 cytoplasts with human peripheral blood lymphocytes induced lymphocyte proliferation that gave rise to lymphoid cell lines of B and T cell origin with unlimited growth potential. The immortalized cell lines were routinely grown in standard medium supplemented with fetal calf serum. Furthermore these cell lines could be propagated in chemically defined serum-free media. Each establishment of lymphoid cell lines was preceded by a proliferation phase 2 wk after cytoplast/cell fusion, which appears to be a necessary step in the immortalization process. The immortalized cells have a nearly normal human karyotype, do not form colonies in soft agar medium, and are not tumorigenic in nude mice. Cloned B cell lines produced human immunoglobulins of heavy and light chain types. No cross-reaction with DNA of herpes simplex virus, human cytomegalovirus, human T cell leukemia/lymphoma virus I and II, or polyoma virus was detected in the genome of immortalized cell lines by Southern blot hybridization. Furthermore B and T cell lines were established that appear to be free of Epstein-Barr virus genome.

THE mechanisms by which normal somatic cells acquire unlimited proliferation potential are not fully understood. The conversion of mammalian cells with limited life span to permanent proliferation is considered to be an important step in the progression from normal to malignant phenotype. It has been suggested (24, 25) that certain oncogenes ("immortalizing genes") may separately or in combination influence cellular functions that drive the cell to divide autonomously or in response to self-secreted growth-promoting factors (autocrine growth control).

Normal human lymphocytes from peripheral blood are quiescent cells with limited life span in vitro that enter S-phase only if stimulated differently, resulting in clonal proliferation and maturation. Resting B cells can be activated to a proliferating stage by mitogens, by B cell growth factors, or via a pathway that requires T cell recognition of B cells and accessory cells. Upon recognition of their specific antigen, activated helper T cells release a variety of lymphokines and hemopoietic growth factors (30). Long term cultures of normal lymphocyte subpopulations have been established that are dependent for proliferation on appropriate growth factors, e.g., B cell growth factor (28) or anti-IgM (27) in the case of human B cells, and T cell growth factor (14) in the case of T cells. In the absence of appropriate antigens or growth factors, these lymphocyte populations undergo senescence.

The only known human B cell mitogen independent of accessory cells for its action is Epstein-Barr virus (EBV)[1] which, after in vitro infection, immortalizes and transforms a subpopulation of human B lymphocytes. Only C3 receptor carrying B cells (33) can be infected by EBV. Like EBV, human T cell leukemia/lymphoma virus (HTLV) can transform normal lymphocytes in vitro to a stage of unlimited proliferation potential. Immortal T cell lines have been obtained by infection with both HTLV-I and HTLV-II (34, 44). Although B lymphocytes (5) and some nonlymphoid cells (6) can also be infected with HTLV, OKT4+ T cells appear to be the most permissive cells for viral replication and transformation. However, certain T cell lines remain T cell growth factor dependent upon HTLV infection.

In this paper, we describe a method for establishing human lymphoid cell lines of both B and T cell origin. Human lymphocytes were fused with isolated cytoplasts from mouse L929 cells. This procedure yielded lymphocyte populations 6–8 wk after fusion that can be routinely grown in standard medium or alternatively in chemically defined serum-free media without addition of hemopoietic growth factors. We isolated human B and T cell lines that appear to grow indefinitely without expressing the malignant phenotype.

Materials and Methods

Cells and Cell Lines

Mouse L929 cells were routinely grown in Dulbecco's modified Eagle's medium (DME) supplemented with 4 mM L-glutamine and 10% fetal calf

1. *Abbreviations used in this paper*: CMV, cytomegalovirus; DME, Dulbecco's modified Eagle's medium; EA, early antigen; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; HSA, human serum albumin; HSV, herpes simplex virus; HTLV, human T cell leukemia/lymphoma virus; PEG, polyethylene glycol; PWM, pokeweed mitogen; VCA, viral capsid antigen.

serum. Mouse embryo fibroblasts were prepared from 12-d-old BALB/c mouse embryos. Briefly the embryos were dissected, incubated with collagenase (0.1 U/ml, Boehringer Mannheim GmbH, Mannheim, FRG) and dispase (0.8 U/ml, Boehringer Mannheim GmbH) in PBS for 1 h at 37°C, and washed twice in PBS. The cells were cultured in DME, supplemented with 4 mM L-glutamine, 1 mM pyruvate, 15 mM Hepes buffer, pH 7.2, and 20% fetal calf serum.

Lymphoid cell lines Raji (ATCC CCL 68), B95-8 (ATCC CRL 1612), and Molt-4 (ATCC CRL 1582) were grown in RPMI 1640 medium, 4 mM L-glutamine, and 10% fetal calf serum.

Lymphocyte Isolation

Peripheral blood was collected from healthy adults, heparinized, and diluted 1:2 in 3 mM citric acid, 100 mM dextrose, 70 mM NaCl, 30 mM sodium citrate, pH 6.1. The lymphocytes were separated according to Bøyum (3) by centrifugation in a Metrizoat (Nyegaard Co., Oslo) gradient at 400 *g* for 35 min. After washing three times in PBS the lymphocytes were cultured in RPMI 1640 medium, 4 mM L-glutamine, 1 mM pyruvate, 1 mM oxalacetic acid, 0.1 U/ml insulin, 10 μg/ml transferrin, 15% fetal calf serum at 37°C in a humid atmosphere with 5% CO_2. For serum-free culture, lymphocytes were grown in Iscove's modified DME supplemented with SFM-2 (Boehringer Mannheim GmbH) or alternatively in HB 104 (New England Nuclear, Braunschweig, FRG). Both media were supplemented with human serum albumin (HSA; 0.5 μg/ml), pyruvate (1 mM), oxalacetic acid (1 mM), insulin (0.1 U/ml), transferrin (10 μg/ml), and Hepes (30 mM).

B and T lymphocytes were separated by affinity chromatography using monoclonal anti-human-pan B cell or anti-human-pan T cell antibodies, respectively, covalently coupled to Sepharose. Briefly, mouse monoclonal anti-human-pan B cell or pan T cell antibodies (Miles, Bayer AG, FRG) were dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5, and incubated with CNBr-activated Sepharose 6MB (Pharmacia Inc., Piscataway, NJ) (1 mg antibody/ml swollen gel) overnight at 4°C using an end-over-end mixer. To remove unbound antibodies the gel was washed three times for 30 min in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5, and three times for 30 min in 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5. To block the residual reactive groups the gel was incubated with 0.2 M glucosamine, 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5, for 2 h at room temperature. Finally the gel was washed twice with PBS containing HSA (0.2%). For preparation of B cells, 10^7 peripheral blood lymphocytes, washed in PBS containing 0.2% HSA, were applied onto a column of anti-human-pan B cell antibodies coupled to Sepharose (5 ml). Before elution the column was incubated for 15 min at room temperature. The nonadherent cell fraction was eluted with 50 ml PBS, 0.2% HSA (flow rate 2 ml/min). To recover the adsorbed cells, the column was incubated with 2 ml PBS, 0.2% HSA, 10 mg/ml IgG at 37°C without flow for 20 min, and mixed end-over-end. The B cell fraction was eluted with PBS, 0.2% HSA, 10 mg/ml IgG. The contamination of non-B cells was <2% as estimated by indirect immunofluorescence using anti-human-pan B cell antibodies. The cell viability was >90% as determined by trypan blue exclusion test. Using anti-human-pan T cell antibodies coupled to Sepharose the T cell fraction could be separated as described for B cells.

Preparation of L929 Cytoplasts

Exponentially growing L929 cells were trypsinized and washed twice in serum-free DME. For induction of cytoplast formation, L929 cells (10^6 cells/ml) were incubated in serum-free DME containing 50 μg/ml cytochalasin B at 37°C (1). Within 90 s blebs appeared on the cell surface. Before the blebs could aggregate at one pole of the cell surface, they were separated from the cells by vigorous vortexing for 40 s. After the cells were sedimented at 100 *g* for 10 min, the supernatant was centrifuged at 1,200 *g* for 15 min. The cytoplast pellet was resuspended in Ficoll (12.5%, Pharmacia Inc.) in serum-free DME and separated from nucleated cells according to Wigler and Weinstein (42) using a gradient with 2 ml 25% Ficoll, 2 ml 17% Ficoll, 0.5 ml 16% Ficoll, 0.5 ml 15% Ficoll, 2 ml 12.5% Ficoll in serum-free DME (from bottom to top). The suspension of cytoplasts was layered on top of the gradient and centrifuged at 100,000 *g* for 60 min at 30°C. The L929 cytoplasts were collected in the visible band at the 15–17% Ficoll region and washed twice in DME at 1,200 *g* for 15 min. Less than one nucleated cell per 1,000 cytoplasts was seen by fluorescence microscopy after staining with Hoechst 33 258. To prevent proliferation of contaminating L929 cells, the cytoplast fraction was treated with mitomycin C (400 μg/ml) for 15 min at 37°C.

Immortalization Procedure

To induce lymphocyte proliferation freshly prepared L929 cytoplasts (10^7/0.5 ml serum-free DME) were added to 10^7 human lymphocytes in 0.5 ml serum-free RPMI 1640. The cell/cytoplast suspension was incubated at 37°C for 15 min and sedimented for 20 s in an Eppendorf centrifuge. The sediment was resuspended in 1 ml 50% polyethyleneglycol (PEG, 4,000 mol wt; SERVA Feinbiochemica GmbH & Co., Heidelberg, FRG), 150 mM Hepes, pH 7.5, prewarmed to 37°C. After 90 s 5 ml warm serum-free RPMI 1640 medium was slowly added over a period of 5 min. The cell/cytoplast suspension was washed twice in RPMI 1640, and 2.5×10^6 cells were seeded in 0.5 ml RPMI 1640, 4 mM L-glutamine, 1 mM pyruvate, 1 mM oxalacetic acid, 0.1 U/ml insulin, 10 μg/ml transferrin, 15% fetal calf serum per well of a 24-well microtiter plate. For the next 2 wk 50 μl of the medium in each well was changed every 2 d. During the following weeks 250 μl of the culture medium was replaced by fresh medium twice a week. The culture was first split 5 wk after fusion.

To estimate the extent of cell-to-cytoplast fusion, an aliquot of L929 cytoplasts (10^5) was stained with Rhodamine 3B (10 μg/ml) (21) in serum-free RPMI 1640 for 30 min at room temperature. The lymphocyte nuclei were stained with Hoechst 33 258 (30 μg/ml serum-free RPMI 1640) for 10 min at 37°C. After cell/cytoplast fusion only cybrids carry a fluorescent nucleus surrounded by a Rhodamine 3B stained cytoplasm. The ratio of cybrids to nonfused cells was estimated by fluorescence microscopy.

Cell Cloning

Cell cloning was performed with a Cytofluorograf 50H interfaced with a model 2151 computer system (Ortho Diagnostic Systems Inc., Johnson & Johnson, Raritan, NJ) and equipped with an argon ion laser emitting 200 mW at 488 nm. Forward light scatter and 90° scatter were used to discriminate viable from nonviable cells. The flow rate was ~300 cells per second. The settings for sorting were deflection of one droplet per selected cell with rejection of coincidence over a span of three droplets. Cloning was done directly into 96-well culture plates by seeding a single cell per well containing 0.1 ml of lymphocyte culture medium.

The cloning efficiency in semi-solid agar medium was determined as described by MacPherson and Montagnier (26) using a 0.5% seaplaque agarose (Gibco, Grand Island, NY) base layer and a 0.32% seaplaque agarose layer in lymphocyte growth medium. After 3 wk colonies were counted under a Leitz stereo microscope.

Tumorigenicity

3-d-old NIH Swiss nu/nu mice were subcutaneously inoculated with 10^4–10^7 cells (in 100–500 μl PBS) from exponentially growing cell cultures. Any tumors obtained were explanted and the cells were grown in vitro to verify the tumorigenic cell type. An autopsy was performed after 5 mo when no tumor could be detected from outside.

Immunoglobulin Assay

Culture supernatants were analyzed for human immunoglobulins (Ig) by enzyme-linked immunosorbent assay (ELISA). Immunosorbent purified sheep antibodies to human IgM, IgA, or IgG (Boehringer Mannheim GmbH) and mouse monoclonal antibodies to human kappa- or lambda-chains (Seward Laboratory, Bedford, UK) were incubated in 96-well plates for 2 h at room temperature. The plates were coated with 1% BSA for 15 min, washed three times with PBS, and incubated with culture supernatants for 1 h. Then the plates were washed three times, incubated with peroxidase-conjugated sheep anti-human Ig (Boehringer Mannheim GmbH), for 2 h, washed four times, and 2,2' azino-di-(3'-ethylbenzthiazolinsulfonate [6]) (Boehringer Mannheim GmbH) as substrate was added. Color changes were quantified at 30 min using an automatic ELISA reader (SLT-Labinstruments, Salzburg). IgM production was measured with serial dilutions of culture supernatant using purified human myeloma IgM (Behringwerke, Marburg, FRG) as standard.

Immunofluorescence

Human B and T lymphocytes were detected by indirect immunofluorescence using a mouse monoclonal anti-human-pan B cell or pan T cell antibody, respectively (Miles). Washed lymphocytes (10^6 cells) were incubated with 400 μl anti-human-pan B cell or pan T cell antibody (1:100 diluted in serum-

free RPMI 1640) for 30 min at 4°C with occasional shaking. The cells were washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG_1 (affinity-purified, 1:100 diluted in serum-free RPMI 1640; Miles) for 30 min at 4°C. Finally the cells were washed twice in PBS and examined by fluorescence microscopy. Cells of the B cell line Raji and of the T cell line Molt-4 served as controls.

Epstein-Barr nuclear antigen (EBNA) was assayed by anti-complement immunofluorescence as described by Reedman and Klein (36). The EBV-associated early antigen (EA) and the viral capsid antigen (VCA) were tested as described (17, 18). B95-8 cells ($EBNA^+$, EA^+, VCA^+), Raji cells ($EBNA^+$, EA^+, VCA^-), and Molt-4 cells ($EBNA^-$, EA^-, VCA^-) served as controls.

Chromosome Analysis and Measurement of Total DNA Content

Metaphase chromosomes of cells in exponential growth phase were stained uniformly or differentially (Giemsa banding) according to standard techniques (43). A minimum of 50 metaphases were recorded for each individual cell line. Mouse chromosomes were assessed by differential staining of centromeres as described by Hilweg and Gropp (19).

Total DNA content was measured according to Crissman et al. (8). Briefly, cloned B and T cell lines in logarithmic growth phase were fixed with cold 95% ethanol, treated with RNase A (1 mg/ml), and stained with propidium iodide (50 μg/ml) for 30 min at 4°C. DNA analysis was done by using a Cytofluorograf 50H. During flow cytometry, cells were excited at 488 nm. Red fluorescence (600 nm) from propidium iodide was recorded as a measure of total DNA content. Human blood lymphocytes fixed on day 4 after lectin stimulation served as standard.

Electron Microscopy

Cells and cytoplasts were fixed in 2.5% glutaraldehyde in PBS for 30 min at room temperature, washed overnight in 0.1 M sodium cacodylate at 4°C, postfixed 1 h in 1% OsO_4, 0.1 M sodium cacodylate, dehydrated in a graded series of acetone, and embedded in Epon 812. Sections were post-stained with 0.5% lead citrate before examination with a Zeiss EM 10 transmission electron microscope.

Reverse Transcriptase Assay

Cell-free supernatant (15 μl) was incubated with 15-μl double concentrated reaction buffer (100 mM Tris, pH 8.3, 40 mM dithiothreitol, 1.2 mM $MnCl_2$, 120 mM NaCl, 0.1% Nonidet P-40, 30 μg/ml Poly(rA)·$p(dT)_{12-18}$ [Pharmacia Inc.], 0.2 μM dATP, 0.2 μM dGTP, 0.2 μM dCTP, 1 μM TTP) in the presence of 1 μCi α-^{32}P-TTP (3,000 Ci/mmol; Amersham Corp., Braunschweig, FRG) for 1 h at 37°C. The DNA was harvested on glass fiber filters (GF/C, Whatman Inc., Clifton, NJ), precipitated with cold TCA (5%) and washed with cold ethanol. Supernatants from Cll-1A cells (10) served as positive, and those from SV34-7F cells (2), as negative controls.

Nucleotide Incorporation

Lymphocytes (5×10^4) were cultured in RPMI 1640 medium (200 μl per well) in 96-well microtiter plates and labeled with 1 μCi [methyl-^{3}H]thymidine (78 Ci/mmol; New England Nuclear) for 6 h at 37°C. After washing the cells were harvested on glass fiber filters (Whatman GF/C). The [methyl-^{3}H]thymidine incorporation in TCA-insoluble material was determined by scintillation spectrometry. Each data point was obtained in duplicate.

For cell autoradiography 5×10^6 cells/ml were incubated with 5 μCi [methyl-^{3}H]thymidine/ml (78 Ci/mmol; New England Nuclear) for 30 min at 37°C. After washing three times in PBS the cells were stained for immunofluorescence. The cells were covered with autoradiographic film (Kodak AR 10) and exposed at 4°C for 25 d.

DNA Preparations

High molecular weight DNA was isolated from cells by lysis in 200 mM Tris, 100 mM EDTA, 0.2% SDS, pH 7.2, containing 100 μg/ml proteinase K (E. Merck, Darmstadt, FRG). After incubation at 37°C for 3 h nucleic acids were extracted with phenol and chloroform. The RNA was degraded by incubation with RNase A (100 μg/ml; Boehringer Mannheim GmbH) for 2 h at 37°C and the DNA was reextracted. Plasmid DNA was prepared, digested with restriction enzymes, and subjected to electrophoresis through a 0.8% seakem agarose gel (FMC Corp., Marine Coll. Div., Rockland, ME) according to standard methods (29). To isolate DNA fragments from a 0.8% low melting agarose gel, the band was excised, an equal volume of Tris/EDTA buffer was added, and the agarose was melted at 68°C for 5 min. DNA was extracted three times in phenol, twice in chloroform, and in ether.

DNA fragments were blotted by Southern transfer (39) onto a Genescreen plus membrane (New England Nuclear) or nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) alternatively. The membrane was prehybridized in 50% formamide, 0.2% polyvinylpyrrolidone (40,000 mol wt), 0.2% BSA, 0.2% Ficoll (400,000 mol wt), 0.05 M Tris, pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextransulfate (500,000 mol wt), 250 μg/ml salmon sperm DNA at 42°C for 24 h. Isolated DNA restriction fragments were labeled with ^{32}P by nick translation (29) to specific activities of 10^7–10^8 cpm/μg DNA. The hybridization was carried out with 100 ng labeled DNA at 42°C for 48 h in the prehybridization buffer (2×10^6 cpm/ml). The membrane was twice washed in 2× standard saline citrate (SSC), 0.1% SDS for 15 min at room temperature, and twice in 0.1× SSC, 0.1% SDS for 30 min at 52°C. The autoradiograph was exposed for 24 h at −80°C using intensifying screens (Titan 2HS, Siemens-Allis Inc., Cherry Hill, NJ).

Results

Human lymphocytes from the peripheral blood of healthy adults were mitogenically stimulated by incubation with pokeweed mitogen (PWM) 1 d after isolation. After 3–6 d [^{3}H]thymidine incorporation reached the first maximum, which is called proliferation phase I (Fig. 1). Nonstimulated lymphocytes remain quiescent in vitro as shown by [^{3}H]thymidine incorporation using parallel cultures supplemented with 20% autologous serum as control. At day 5 PWM-stimulated and nonstimulated, quiescent lymphocytes (10^7 cells each) were fused with cytoplasts from L929 cells (10^7 cytoplasts each) by incubation with PEG. Treatment with PEG without addition of L929 cytoplasts served as control. In addition the same number of quiescent and PWM-stimulated lymphocytes were co-cultivated with 10^7 L929 cytoplasts without PEG-mediated cytoplast/cell fusion. In all cases the induction of lymphocyte proliferation was monitored by [^{3}H]thymidine incorporation assay (Fig. 1) and phase-contrast microscopy (Fig. 2). 3 d after cytoplast/cell fusion, clusters of lymphoid cells were observed. During the next 5 d these clusters rapidly increased in size and cell number to ~300–500 cells per colony. This we call proliferation phase II. Proliferating colonies were initially mostly adherent; at later times large colonies detached and gave rise to small colonies that grew in suspension. Up to 2 d after addition of L929 cytoplasts, cell proliferation ceased and [^{3}H]thymidine incorporation decreased possibly due to cytotoxic effects of the cytoplast fraction. In the maximum of proliferation phase II, nonstimulated lymphocytes incorporated sixfold more [^{3}H]thymidine upon PEG-mediated fusion with L929 cytoplasts, twofold more upon co-cultivation with L929 cytoplasts compared with control cultures which had not been treated with L929 cytoplasts. PWM-stimulated lymphocytes increased [^{3}H]thymidine incorporation 100-fold upon PEG-mediated cytoplast/cell fusion and 50-fold upon co-cultivation with L929 cytoplasts. At this time no lymphocyte proliferation could be detected any more in parallel cultures not incubated with L929 cytoplasts.

Colonies proliferating in phase II required their own con-

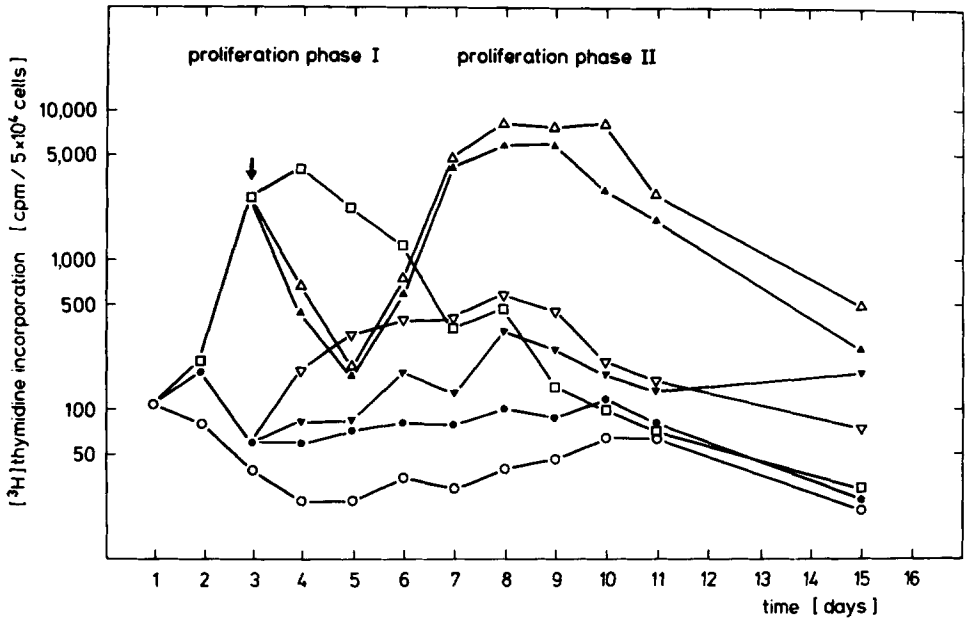

Figure 1. Induction of lymphocyte proliferation by L929 cytoplast fusion monitored by [³H]thymidine incorporation. Lymphocytes were prepared at day 0 and cultivated in lymphocyte growth medium supplemented with 20% autologous serum (○) or 20% fetal calf serum (●). At day 1 lymphocytes were stimulated with PWM (□). At day 3 (*arrow*) quiescent and PWM-stimulated lymphocytes were fused (▽ and △, respectively) or co-cultivated (▼ and ▲, respectively) with L929 cytoplasts.

ditioned medium. At the end of this proliferation phase, however, i.e., 17–24 d after cytoplast/cell fusion, the colonies grew in fresh culture medium provided that they were seeded at high cell densities (10^5 cells/ml). After three to five passages the cells could be diluted to 10^3 cells /ml and could be grown in serum-free media without addition of hemopoietic growth factors. Probably these cells had begun to produce their own growth factors which might have provided an autologous stimulus for self-renewal.

Proliferation phase II and subsequent infinite cell growth was only observed in lymphocyte populations treated with L929 cytoplasts. The resulting cell lines have been routinely grown in vitro for 1 yr (180 cell doublings) without any detection of growth arrest or senescence. Therefore we presume that these cell lines are immortalized. The maximum of [³H]thymidine incorporation during proliferation phase II and the frequency of immortalization was dependent on the extent of cybrid formation between L929 cytoplasts and lymphocytes (Table I). The rare induction of lymphocyte proliferation upon co-cultivation with L929 cytoplasts was possibly due to spontaneous cytoplast/cell fusions as monitored by fluorescence microscopy using Rhodamine 3B stained cytoplasts and Hoechst 33 258 stained lymphocytes as described in Materials and Methods.

In a second experiment we asked whether or not the L929 cytoplasts were mitogenic towards B or T lymphocytes and whether L929 cytoplasts could immortalize B or T lymphocytes. To analyze whether lymphocyte colonies in proliferation phase II were of B or T cell origin, parallel cultures were labeled with [³H]thymidine for autoradiography. The same cells were stained with monoclonal anti-human-pan B cell or pan T cell antibodies, respectively, for investigation of indirect immunofluorescence. 60% of all radioactively labeled colonies were of T cell origin, and 40% were of B cell origin (Table II). Colonies were either of B cell or T cell origin, suggesting that they were derived from single cells. 5–8 wk after cytoplast/cell fusion, rapidly proliferating lymphocyte cultures (cell doubling times 25–40 h) were 30–50% of B cell and 30–70% of T cell origin. B and T cell populations were cloned via dilution to single cells.

To measure the mitogenic activity of the cytoplast fraction towards purified B cell and T cell populations, the PWM-stimulated lymphocyte population was preparatively separated at day 6 by cell affinity chromatography on anti-human-pan B cell or pan T cell antibodies, respectively, coupled to Sepharose. Each cell fraction was fused or co-cultivated with L929 cytoplasts (10^7 cytoplasts per 10^7 lymphocytes). 7 d after addition of cytoplasts [³H]thymidine incorporation increased 400-fold in PWM-stimulated B cells upon PEG-mediated cytoplast/cell fusion and 300-fold upon cytoplast/cell co-cultivation compared with mock-treated PWM-stimulated B cell cultures. In contrast PWM-stimulated T cells increased [³H]thymidine incorporation only sevenfold in the maximum 5 d after cytoplast/cell fusion or co-cultivation (Fig. 3). The results show that the L929 cytoplast fraction is up to 20-fold more mitogenic towards B cells than towards T cells but gives rise to both B cell and T cell lines that grow independently from exogeneous purified growth factors in serum-free media.

Now we asked whether or not immortalization could also be induced by fusion with cytoplasts from primary mouse fibroblasts of limited life span. Therefore cytoplasts from BALB/c mouse embryo fibroblasts in logarithmic growth phase (passage 3) were obtained by incubation with cytochalasin B, purified, and fused with quiescent or PWM-stimulated human lymphocytes at day 5 after lymphocyte isolation. During the following weeks no induction of lymphocyte proliferation could be detected by microscopic observation or [³H]thymidine incorporation assay, suggesting that the immortalization activity is restricted to cytoplasts from cell lines with unlimited proliferation potential.

Transmission electron micrographs of L929 cells used for enucleation showed that these cells possessed the usual fine structure features that characterize mammalian cells in culture. Studies on L929 cytoplasts used for immortalization revealed a normal cytoplasm with one to three mitochondria per cytoplast, parts of the Golgi apparatus and the endoplasmic reticulum, and numerous ribosomes. Cells of the immortalized lines of B and T cell origin showed fine structures indistinguishable from normal lymphocytes in vitro (Fig. 4).

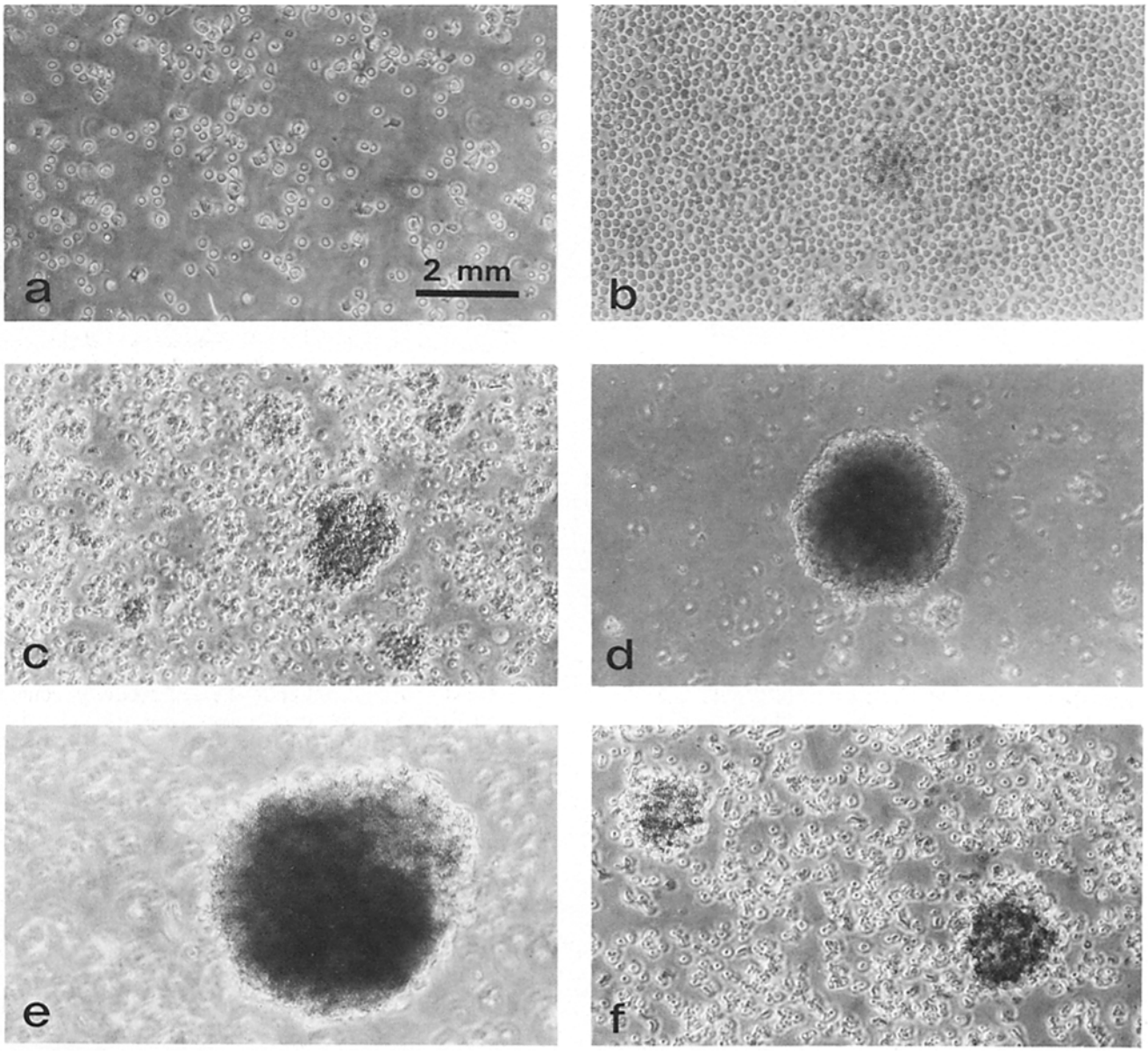

Figure 2. Immortalization of lymphocytes by L929 cytoplast fusion as observed by phase-contrast microscopy. Primary lymphocytes (*a*) were stimulated with PWM and gave rise to proliferation phase I (*b*, day 4). Upon fusion with L929 cytoplasts at day 6 rapidly growing lymphocyte colonies were observed (proliferation phase II) (*c* at day 8; *d* at day 12; *e* at day 14). 8 wk after fusion immortalized lymphoid cell lines were established (*f*).

Table I. Dependence of [³H]Thymidine Incorporation during Proliferation Phase II and the Frequency of Immortalization Events on the Extent of Cybrid Formation between L929 Cytoplasts and Lymphocytes

No. of lymphocytes	No. of L929 cytoplasts added	Cybrids	[³H]Thymidine incorporation at maximum of proliferative phase II	Immortalized lymphoid cell clones
		%	*cpm/5 × 10⁴ cells*	
10^6	10^7	35.2	230,500	14
10^6	10^6	32.7	210,000	12
10^6	10^5	2.43	151,000	5
10^6	10^4	0.17	10,200	1
10^6	10^3	0.07	5,700	1
10^6	10^2	⩽0.01	500	0
10^6	0	⩽0.01	200	0

Table II. Distribution of B and T Cells in Primary Lymphocyte Populations, during Proliferation Phase II, and in Immortalized Cell Lines 10 wk after Fusion with L929 Cytoplasts

	Primary lymphocytes		Proliferation phase II		Immortalized cell lines	
Cell type	B	T	B	T	B	T
	%		%		%	
Exp. 1	46	51	42	55	52	47
Exp. 2	35	64	35	62	33	65
Exp. 3	38	61	28	69	25	71
Exp. 4	31	66	40	57	68	30

The cell diameter varied between 80 and 140 μm. The nucleus with fine granular chromatin contained two to three prominent nuclei and the cytoplasm carried numerous ribosomes as well as a large endoplasmatic reticulum, indicating active protein synthesis of the cells. The immortalized cell lines exhibited morphological diversity ranging from adherent cells to free floating round cells.

To derive monoclonal B and T cell lines, lymphocytes were fused with L929 cytoplasts and cloned by limiting dilution, or preferentially by single cell sorting. In a representative experiment lymphoid cells had a cloning efficiency of 23% (224 clones/960 cells seeded) using single cell sorting at day 41 after cytoplast fusion. As tested by ELISA 41.5% of the clones produced detectable amounts of Ig:IgM-lambda (27.2%), IgM-kappa (12.9%), IgG-kappa (0.9%), and lambda-chains only (0.4%). Out of 10 of the Ig-negative clones four expressed pan T cell marker, one expressed pan B cell marker, and five clones could not be classified by immunofluorescence using antibodies to human B and T cell marker.

To estimate the stability of Ig secretion one IgM-kappa and two IgM-lambda producing clones were reexamined after a 4-mo period of continuous growth. The clones tested produced IgM at similar quantities as at the first screening.

Five monoclonal cell lines were subjected to karyotype analysis. All lines had a modal number of human chromosomes of 46 (range 36–50). No mouse chromosomes were detectable by mouse centromere staining.

Five B cell lines and nine T cell lines were subjected to

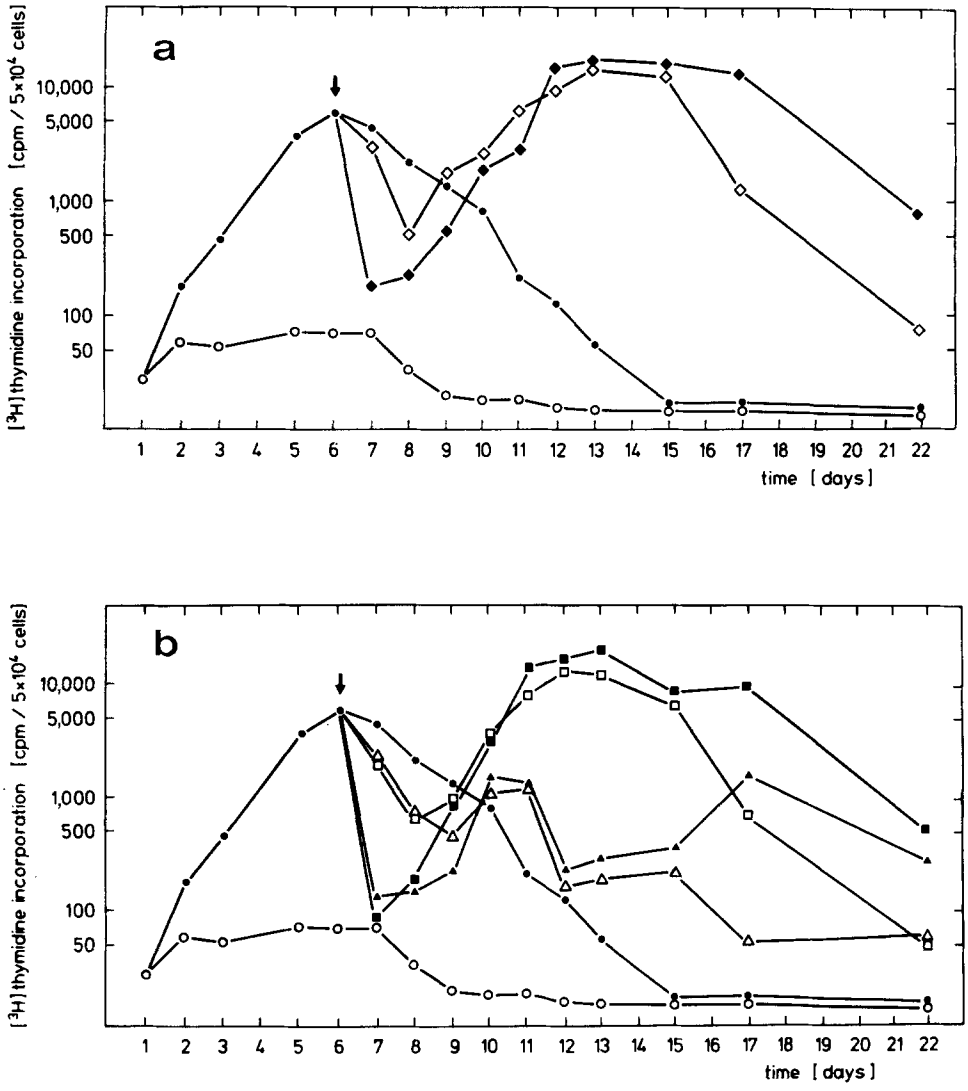

Figure 3. Induction of B and T lymphocyte proliferation by L929 cytoplast fusion monitored by [³H]-thymidine incorporation assay. Lymphocytes were prepared at day 0 (○), stimulated with PWM at day 1 (●), and (*a*) co-cultivated (◇) or fused (◆) with L929 cytoplasts at day 6 (*arrow*). A parallel culture of lymphocytes (*b*) was fractionated in B and T cells. The cell fractions were co-cultivated (B cells, □; T cells, △) or fused (B cells, ■; T cells, ▲) with L929 cytoplasts.

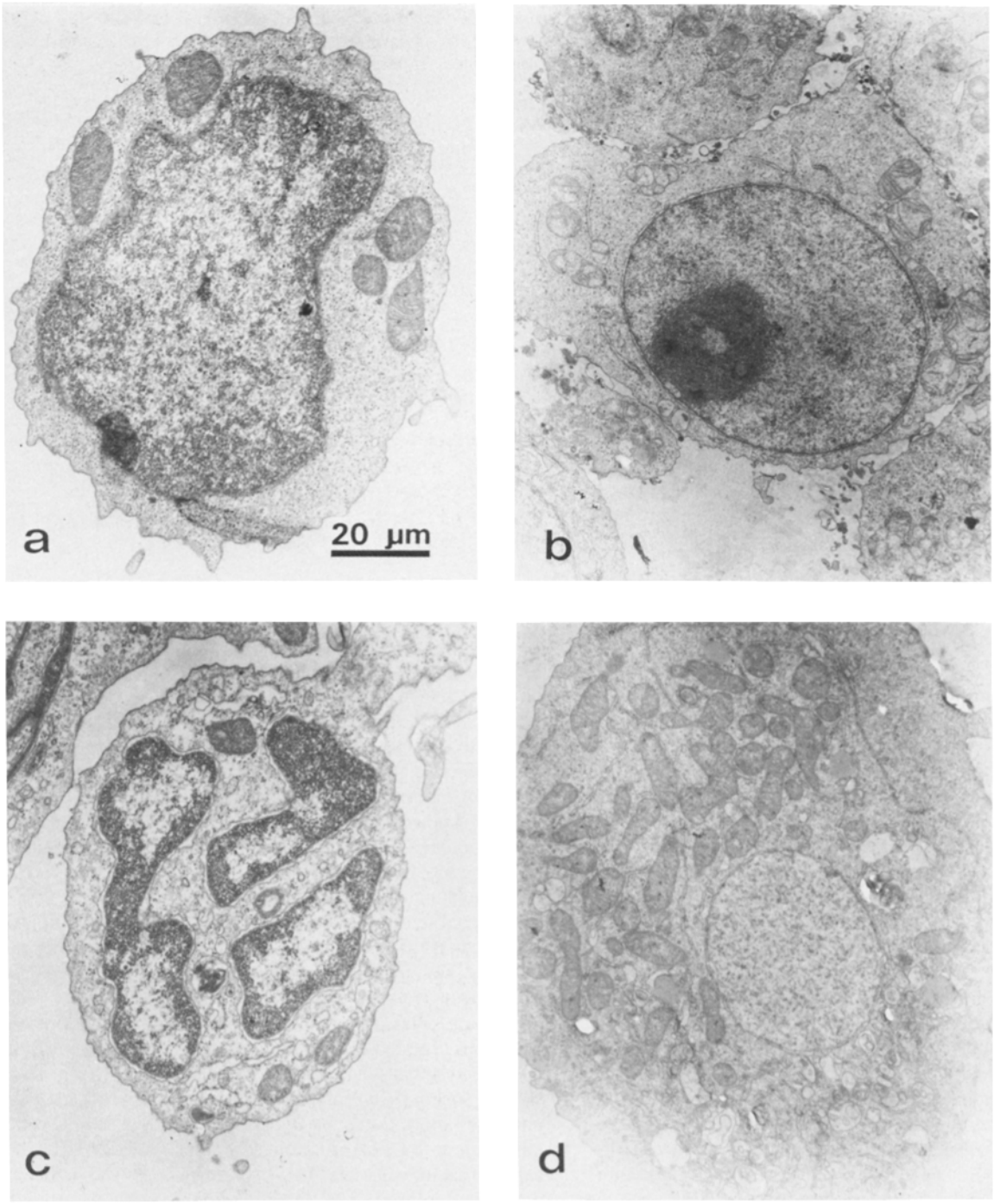

Figure 4. Transmission electron micrographs of a primary lymphocyte (day 3 in vitro) (*a*), lymphocytes incubated with L929 cytoplasts at day 6 (*b*), and mononuclear cells of immortalized cell lines (*c* and *d*) 12 wk after fusion with L929 cytoplasts.

cytometric DNA measurement. Their histograms revealed a DNA content similar to that of nontransformed human blood lymphocytes.

To characterize the transformed phenotype of the immortalized lymphoid cell lines the cloning efficiency in soft agar medium and the tumorigenicity in NIH Swiss nu/nu mice upon subcutaneous injection were tested. As shown in Table III, cells of the immortalized lines of B cell or T cell origin did not form colonies in soft agar medium (frequency $\leq 10^{-5}$) like quiescent lymphocytes. In contrast the malignantly transformed human Burkitt lymphoma line Raji and the T cell leukemia line Molt-4 exhibited cloning efficiencies of 10.8% and 9.8%, respectively. Upon subcutaneous injection in 3-d-old NIH Swiss nu/nu mice, cells of the immortalized lymphoid lines of B or T cell origin (10^4–10^7 cells per mouse) did not induce tumors at the injection site nor could tumors be detected at autopsy 5 mo after injection (Table III). When equal numbers of primary human lymphocytes were

Table III. Cloning Efficiency in Soft Agar Medium and Tumorigenicity in NIH Swiss nu/nu Mice of Immortalized Lymphoid Cell Lines of B and T Cell Origin

Cell lines	Cloning efficiency	Induction of tumors/injected mice
	%	
B-1	≤0.001	0/5
B-2	≤0.001	0/5
B-3	≤0.001	0/5
T-1	≤0.001	0/5
T-2	≤0.001	0/5
T-3	≤0.001	0/5
Primary lymphocytes	≤0.001	0/5
Controls		
Raji	10.8	5/5
Molt-4	9.8	5/5

Table IV. Expression of EBNA, EA, and VCA in Immortalized Lymphoid Cell Lines of B and T Cell Origin Monitored Immunofluorescence

Cell lines	EBNA	EA	VCA
	%	%	%
B-1	5	≤0.1	≤0.1
B-2	≤0.1	≤0.1	≤0.1
B-3	3	≤0.1	≤0.1
B-4	19	≤0.1	≤0.1
B-4/1	96	≤0.1	≤0.1
B-4/2	≤0.1	≤0.1	≤0.1
T-1	≤0.1	≤0.1	≤0.1
T-2	≤0.1	≤0.1	≤0.1
T-3	≤0.1	≤0.1	≤0.1
T-4	≤0.1	≤0.1	≤0.1
Primary lymphocytes	≤0.1	≤0.1	≤0.1
Proliferation phase II			
B cells	≤0.1	≤0.1	≤0.1
T cells	≤0.1	≤0.1	≤0.1

B cell line B-4 has been subcloned by dilution to single cells (B-4/1, B-4/2).

injected as controls no tumors were obtained. Under the same conditions the human cell lines Raji and Molt-4 gave rise to vigorously proliferating tumors, killing the host within 6–10 d after injection.

Various members of herpes virus group can infect leukocytes, lymphocytes, and lymphoreticular cells, e.g., EBV, herpes simplex virus (HSV), and cytomegalovirus (CMV). Furthermore HTLV can infect and transform human lymphocytes. In addition the polyoma virus genome could be transferred to human lymphocytes via L929 cytoplasts. We have searched for the presence of these viral genomes in the continuously proliferating cell lines obtained in this study.

EBV transforms human lymphocytes into proliferating blast cells that can be easily established in vitro as infinitely proliferating cell lines. In nearly all cells infected by EBV, the EBNA was found to be expressed (36) and in productively infected cells the Epstein-Barr EA (18) and VCA (17) could be detected in the cytoplasm. The EBV DNA is present as multiple unintegrated molecules as well as linear molecules integrated into the chromosomal DNA (16). In the lymphoid cell lines immortalized by L929 cytoplast fusion, expression of EBNA was tested by anti-complement immunofluorescence. EA and VCA were assayed by indirect specific immunofluorescence. 0–19% of the immortalized noncloned cell populations of B cell origin expressed EBNA dependent on the lymphocyte donor (Table IV), thus demonstrating the presence of the EB viral genome in these cells. EA and VCA could not be detected, however. In the immortalized T cell populations no expression of EBNA, EA, or VCA was found. Furthermore no expression of EBNA, EA, or VCA could be detected in primary lymphocytes and isolated colonies of proliferation phase II upon fusion with L929 cytoplasts. EBNA-negative B cell clones and several T cell clones were tested for the presence of the EB viral genome by Southern blot hybridization. Total cellular DNA was isolated, digested with Bam HI, and separated in a 0.8% agarose gel. Upon Southern blotting the filter was hybridized with the cloned 3.1-kbp Bam HI fragment W of the EBV internal repeat sequence (15) isolated from pBR 322 sequences before nick translation. Plasmid DNA (6 pg, equivalent to about one EBV internal repeat copy/cell or to about 0.1 EBV genome equivalent/cell) served as positive control. No EBV DNA could be detected in total lymphocyte cultures or in purified B and T cell colonies during proliferation phase II or in immortalized EBNA-negative B cell clones or in T cell clones (Fig. 5). This suggested that the EBV genome is unlikely to be involved in the immortalization of B and T cell clones by fusion with L929 cytoplasts.

Since HSV replicates upon infection in both mitogen-stimulated B and T lymphocytes and in myeloid cell lines (22), we checked whether the HSV genome was present in the DNA of human lymphocytes. The HSV-2 Bgl II N-fragment is known to establish transformed rodent fibroblast lines upon DNA transfection (11). Therefore we probed by Southern blot hybridization total cellular DNA isolated from L929 cells, primary human lymphocytes, B and T cell colonies in proliferation phase II, and from noncloned lymphoid cell lines and from clones of B and T cell origin immortalized by L929 cytoplast fusion with the cloned HSV-2 Bgl II N-fragment isolated from pBR 322 sequences before nick-translation. Plasmid DNA (12 pg, equivalent to about one copy/cell) digested with Bgl II served as positive control. No positive hybridization could be detected in DNA from L929 cells or primary lymphocytes or from immortalized lymphoid cell lines. Since the Bgl II N-fragment of HSV-2 is homologous to the corresponding fragment of HSV-1 (11), we can also exclude that the HSV-1 genome is involved in the immortalization process of human lymphocytes by fusion with L929 cytoplasts.

Human CMV causes latent, nonproductive infections in lymphoid cells (41) harboring the CMV genome (20). Transfections of rodent cells with overlapping cloned fragments of the CMV genome (strain AD 169) identified a transforming 490-bp fragment near the right end of the genome (32). By Southern blot hybridization with the cloned probe of the transforming region (pCM 5018) (31) we could not detect any hybridization with the cellular DNA of the immortalized lymphoid cell lines of B cell or T cell origin or with DNA from L929 cells, lymphocytes in proliferation phase II upon fusion with L929 cytoplasts or primary lymphocytes. Plasmid DNA (4 pg, equivalent to about one copy/cell) served as positive control.

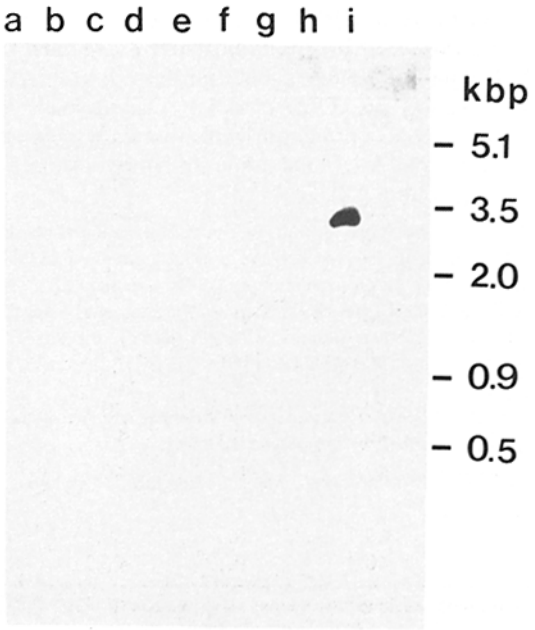

Figure 5. Southern blot hybridization of EBV internal repeat sequence with DNA of lymphoid cells. Cellular DNA (15 μg) was digested with Bam HI, separated in a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with the cloned 3.1-kbp Bam HI fragment W of EBV internal repeat sequence (15) isolated from pBR 322 DNA before nick-translation (10^8 cpm/μg DNA). Lane *a*, DNA of lymphocytes from peripheral blood; lane *b*, B lymphocyte clone during proliferation phase II; lane *c*, T lymphocyte clone during proliferation phase II; lanes *d* and *e*, immortalized, EBNA-negative B lymphoid cell clones; lanes *f* and *g*, immortalized, EBNA-negative T lymphoid cell clones; lane *h*, L929 cells; lane *i*, pEB-W DNA, digested with Bam HI (cloned EBV internal repeat sequence, Bam HI-fragment W) (6 pg; equivalent to about 1 EBV internal repeat copy/cell or to about 0.1 EBV genome equivalent/cell). Eco RI/Hind III fragments of λ DNA served as molecular weight standard.

HTLV, types I and II, transform normal human lymphocytes to infinite proliferation upon infection in vitro (29, 37). Although OKT4$^+$ T cells appear to be the most permissive cells, certain B lymphocytes (5) and nonlymphoid cells (6) can also be infected by HTLV. In the genome of the lymphoid cells immortalized by L929 cytoplast fusion, we could not detect the HTLV-I or HTLV-II genome by Southern blot hybridization using the cloned 8.3-kbp SstI-proviral fragment (pMT-2; HLTV-I) (37) and the 3.5-kbp Bam HI-proviral fragment (pMOIA; HTLV-II) (13), respectively, as probe. Plasmid DNA (24 pg and 14 pg, respectively, equivalent to about one copy/cell) served as positive control.

In addition we could not detect hybridization to the polyoma virus genome in DNA of the immortalized cell lines using the cloned complete polyoma virus genome (pPB21) (12) as probe.

These results suggest that EBV, HSV types 1 and 2, human CMV, HTLV I and II, and polyoma virus may not be involved in the process of immortalization by fusion with L929 cytoplasts.

Mouse cells are known to harbor endogeneous retroviruses and to produce retroviruses under certain conditions. To check if the lymphoid cell lines immortalized by fusion with L929 cytoplasts produce retroviruses, we tested the activity of reverse transcriptase in the culture supernatant of these cell lines. No reverse transcriptase activity could be detected in the supernatant of the immortalized human cell lines tested, showing that no retroviruses were produced by these cells.

Discussion

In this paper we describe the induction of human lymphocyte proliferation by fusion with cytoplasts from mouse L929 cells generating permanently growing lymphoid cell populations of both B and T cell origin. These cell lines can be routinely grown in vitro in serum-supplemented media and in chemically defined serum-free media without addition of hemopoietic growth factors, suggesting that these cells possibly secrete their own growth factors. The cell lines have been maintained in culture for 1 yr (≥180 cell doublings) without any detection of growth arrest or senescence. We presume that these cell lines are immortalized since quiescent or lectin-stimulated human control lymphocytes undergo senescence at day 3 or at day 10 in vitro, respectively, and human fibroblasts achieve ~50 cell doublings in culture. While the cell doubling time of 25–40 h is similar to other malignant lymphoid cell lines, these immortalized cells do not form colonies in soft agar medium (frequency $\leq 10^{-5}$) and do not induce tumors upon subcutaneous injection of up to 10^7 cells in nude mice.

Stable human B cell and T cell lines could be established by fusion with L929 cytoplasts and could be cloned by single cell cloning. These clones contained a nearly diploid set of human chromosomes. Murine chromosomes were regularly absent as revealed by centromere staining. Neither a systematical loss of duplication of single human chromosomes, nor systematical rearrangements could be recorded.

Cloned B cell lines produced human Ig with monoclonally restricted heavy chain classes and light chain types. Over a period of 4 mo B cell clones secreted continuously Ig in microgram quantities per 10^6 cells. These results suggest that the immortalization method described will be useful in the generation of monoclonal human antibodies with predefined specificity.

Earlier attempts to generate immortalized human immunoglobulin-producing cells involved the fusion of human lymphoid cells with mouse myeloma cells (4). The interspecies hybridomas tended to cease human immunoglobulin production due to the selective loss of human chromosomes (9) or to disturbances of gene expression (35). However, intraspecies hybridomas generated by fusion of antigen-primed human B lymphocytes with EBV-transformed B lymphoblastoid cell lines (40) secrete immunoglobulin molecules derived from both fusion partners (23), and the amount of immunoglobulin secreted can vary 100-fold among several clones (7). Our results show that human B lymphocytes immortalized by L929 cytoplast fusion give rise to cell lines that stably produce human homogeneous antibodies and do not express the malignant phenotype.

Cytoplasts derived from transformed mouse L929 cells transfer an immortalizing activity on quiescent or lectin-prestimulated human B and T lymphocytes in contrast to cytoplasts from primary mouse embryo fibroblasts with limited life span. This suggests that the immortalization ac-

tivity is restricted to cytoplasts from cell lines with unlimited proliferation potential.

Furthermore the L929 cytoplast fraction harbors a mitogenic activity inducing lymphocyte proliferation phase II 5–15 d after cytoplasts/cell fusion. The cytoplasts act on both quiescent and lectin-prestimulated lymphocytes and are 20-fold more mitogenic for PWM-stimulated B cells than for T cells. We only observed lymphocyte immortalization after the proliferation phase II had occurred, suggesting that this proliferation phase is a necessary step in the immortalization process.

Because of the following findings we think it very unlikely that the immortalized cell lines were derived from whole somatic cell hybrids: (*a*) All lymphoid cell lines tested have a near diploid human karyotype. (*b*) The immortalized cell lines are lymphoid in origin since they grow in suspension, are recognized by monoclonal anti-human B cell or T cell antibodies, and produce homogeneous human immunoglobulins. Mouse L929 cells do not react with the anti-human B cell and T cell antibodies used.

Conditioned medium of L929 cells failed to immortalize human lymphocytes. Cytoplast/cell contact or PEG-mediated fusion of cytoplasts with cells is required to induce continuous lymphocyte proliferation. During the proliferation phase II some of the competent cells fused with L929 cytoplasts gain an additional proliferation potential. At this time many cells of finite life span will continue to divide slowly. After more than 6 wk the fused cell population becomes homogeneously clonogenic and steadily proliferative. Our observations based on light microscopy and [^{3}H]thymidine incorporation suggest that the proliferation phase II does not directly lead to immortalization but is followed by a crisis of proliferation. At least three hypothetical mechanisms can be envisaged to explain the results: (*a*) A minimum number of proliferation initiations transferred by a trans-acting cytoplasmic factor of L929 cytoplasts is needed for establishment of a lymphoid lineage. During the critical period of proliferation, this factor(s) must achieve a level sufficient for immortalization, and suboptimal concentrations of such a factor may instead result in transient cell activation. (*b*) Alternatively initiation of proliferation may occur very frequently followed by reversions to normal senescent cells after some cell doublings. Normal diploid cells may synthesize an inhibitor that blocks the initiation of DNA synthesis at the time the cell has completed its in vitro life span (38). During proliferation the synthesis of the inhibitor may be repressed. L929 cytoplasts could harbor a factor that maintains the repression of inhibitor genes of DNA synthesis or that alters the expression of inhibitor gene(s) or functionally neutralizes the inhibitor gene products in indefinitely proliferating lymphocytes. (*c*) Alternatively genetic material may be transferred by cytoplast fusion from L929 cells into human lymphocytes. The genome of several mouse endogeneous DNA or RNA viruses may induce lymphocyte proliferation and give rise to continuously growing cell lines. Due to our results described above we think it unlikely that the genome of EBV, HSV types 1 and 2, human CMV, HTLV I and II, or polyoma virus is transmitted to or induced in the recipient cells by L929 cytoplasts. These viruses are probably not involved in the induction of lymphocyte immortalization. In addition, no activity of reverse transcriptase could be detected in the culture supernatant indicating that no retroviruses are produced by the immortalized cell lines.

Although the immortalizing agent transferred by L929 cytoplasts is unknown yet, L929 cytoplast fusions may be used to generate a panel of nontumorigenic human lymphoid cell lines of B and T cell origin for studies of lymphocyte heterogeneity and function.

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