Productive Infection of Cultured Human Lymphoid Cells by Adenovirus

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We investigated infection of cultures from established human B- and T-cell lines by adenoviruses. Infection by adenovirus type 2 or 5 was productive by the criteria of viral DNA replication, RNA synthesis, immunofluorescent staining of viral proteins, and assembly of biologically active virions. Whereas the kinetics of infection were reproducible and characteristic for each cell line, there appeared to be no correlation between the kinetics of infection and the origin from which the cell lines were established. In a myeloma and a T-cell line, the kinetics of infection approached those in HeLa cells. The presence of the Epstein-Barr virus genome in B lymphoid cells was not a prerequisite for adenoviral infection. Furthermore, expression of the E1A gene was repressed in myeloma cells in comparison with HeLa cells.

Human adenoviruses were originally isolated from adenoids and tonsils of patients with acute respiratory diseases (6, 12, 26, 31). Although 75% of adenoid tissue is composed of lymphoid cells, isolation of the viruses from fresh lymphocytes purified from adenoids and tonsils was unsuccessful. Even after lymphocyte culturing, only very low yields of infectious virus, detected in approximately 1 in 10⁷ cells infected, were observed (29, 32). Replication of exogenous adenovirus type 2 (Ad2) in cultured lymphocytes purified from adenoids was also poor; only 1 to 3% of cells were found to produce virus (15). In their work superinfecting an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma B-cell line with adenovirus type 5 (Ad5), Faucon et al. (7) showed by electron microscopy that approximately 3 to 6% of EBV-positive Jijoye cells contained viral particles, twice that of an EBV-negative cell line. This low level of infection of lymphocytes by Ad5 was also maintained in human cord blood lymphoblastoid cell lines transformed by EBV after several months of cultivation (8).

The questions of whether and how lymphocytes are involved in the route of adenovirus infection remain unanswered. The viruses have been conventionally propagated in cell lines of nonlymphoid origin. Despite extensive studies of molecular interaction between adenoviruses and cells of nonlymphoid origin, little is known of the molecular biology of adenoviruses in a lymphoid cell background. Recent work by Chambon and colleagues pointed to the possibility of very interesting dialogues between adenoviral gene products and genes specifically expressed in B lymphoid cells. They showed (2, 3) that, in mouse plasmacytoma cell line MPC-11, a nonpermissive host for adenoviral DNA replication, expression of immunoglobulin genes γ_{2b} and κ was repressed after infection by Ad5.

To understand the molecular interaction between adenoviruses and human lymphoid cells better, we undertook the present study. We showed that, with one exception, established human B- and T-cell lines supported viral replication, RNA synthesis, and assembly of infectious particles productively. In a B-cell myeloma line, 2132, and a T-cell line, Jurkat, the kinetics of infection by Ad5 approached those in

HeLa cells. There appeared to be no correlation between the kinetics of infection on the one hand and the stage of differentiation or the presence of EBV genome in the cell lines on the other. Expression of viral early region genes and use of adenovirus as a vector in human lymphoid cells are discussed.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained as spinner cultures in Joklik modified minimum essential medium supplemented with 5% heat-inactivated fetal calf serum and L-glutamine (1.47 mg/ml) (23). A549 cells were maintained as monolayer cultures in Dulbecco minimum essential medium supplemented with 5% heat-inactivated fetal calf serum and L-glutamine. B and T lymphoid cell cultures were maintained in a humidified CO₂ incubator in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and L-glutamine. Adenoviruses were propagated and used for infection as previously described (4).

Southern blot analysis. After infection with Ad2 or Ad5 at 10 to 30 PFU per cell, 10⁵ cells from each cell culture were withdrawn immediately after viral adsorption (time zero) and at various times postinfection (p.i.). Total DNAs were isolated, digested with restriction endonucleases, separated by agarose gel electrophoresis, and blotted onto nitrocellulose membranes as previously described (23). The blots were analyzed by hybridization to nick-translated (25) ³²P-labeled total Ad2 or Ad5 DNA in the presence of 50% formamide (4). For detecting the presence of the EBV genome in B lymphoid cell lines, a cloned *Bam*HI W fragment of the EBV genome (kindly provided by W. Sugden) was used for Southern blot analyses.

Northern blot analysis. Total RNAs were isolated by the guanidinium thiocyanate method (5) at various times p.i. For prolonged periods of infection, infected cells were carefully pelleted at 30 h p.i. and gently suspended in fresh medium to maintain the optimum pH in the medium and prevent cell loss by lysis. Polyadenylated RNAs were selected by poly(U)-Sepharose chromatography and analyzed by Northern blot analysis as previously described (4). Hybridization to nick-translated probes in the presence of 50% formamide

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FIG. 1. Southern blot analysis of adenoviral DNA synthesis in cultured human B and T lymphoid cells. Cultured B and T cells were infected with Ad2 at 30 PFU per cell as were HeLa cells. At the days p.i. indicated (numbers at the tops of lanes), 10^5 cells (except for lane 1.5 of 2132, in which only 2×10^4 cells were used) were withdrawn, and total DNAs were isolated as previously described (23). The DNAs were digested with restriction enzyme *EcoRI*, separated by agarose gel electrophoresis, and analyzed by the Southern method with ^{32}P -labeled nick-translated Ad2 DNA as a probe. M, kb indicates *HindIII*-digested lambda DNA as size markers. The designation 0 represents 2 h from the beginning of viral adsorption, and $\times$ represents the time required for exposure of the autoradiograph, that of HeLa cells being $1\times$. The numbers at the right indicate molecular size in kilobases.

was performed essentially as described by Thomas (30). The probes included a cDNA clone specific for human class I major histocompatibility antigens HLA-B, pDP001 (28), a cDNA specific for the adenovirus E1A region, c131 (24), and a cloned *HindIII* D fragment of Ad2, pHindIII-D.

Indirect immunofluorescence microscopy. Infected or control cells were pelleted by centrifugation, washed three times with ice-cold phosphate-buffered saline (PBS; pH 7, supplemented with 1 mM MgCl_2), and suspended in PBS. In the subsequent steps, PBS was not supplemented with MgCl_2 . Samples of cell suspensions, 2×10^5 cells per 100 μl , were then spun onto acetone-cleaned glass slides at 500 rpm ($23 \times g$) for 2 min in a Cytospin centrifuge (Shandon-Southern). The attached cells were fixed in acetone at -20°C for 30 min, air dried, and quickly rinsed in distilled water. The fixed cells were then incubated with 100 μl of diluted (1:1,000 dilution in PBS, pH 7) rabbit antiserum to purified adenovirus virion (kindly provided by C. S. H. Young) for 45 min at 37°C in a humidified chamber. After incubation, the cells were washed three times, for 20 min each time, in PBS (pH 7) and then rinsed in distilled water, all at room temperature. Indirect immunofluorescent staining was carried out by blot drying the slides and incubating them with 100 μl of diluted (1:40 dilution in PBS, pH 8) fluorescein-conjugated goat antibody to rabbit immunoglobulin G (IgG; kindly provided by C. S. H. Young) for 45 min at room temperature in a humidified chamber. The stained cells were then washed three times in PBS, pH 8, for 20 min each time, rinsed in distilled water, and sealed in 90% glycerol under cover slips. We found that elevation of the pH to 8 greatly enhanced resolution and brightness of fluorescence. Photography under phase-contrast and UV microscopy was carried out with a Zeiss UV microscope (kindly provided by E. Wang).

Biological assay for production of infectious virus. Cells infected with Ad2 at 10 PFU per cell were collected at various times p.i. A portion was used for analysis of viral DNA replication, and lysates were prepared from the remainder. The lysates were then used to infect a second sample of uninfected cells from which line the lysates were prepared. DNAs isolated from the second round of infection were similarly analyzed by Southern blot analysis. Plaque

assays of the lysates were performed on A549 monolayer cells.

RESULTS

Viral DNA replication in cultured lymphoid cells. Replication of adenoviral DNA in human lymphoid cells was assayed by Southern blot analysis with nick-translated total Ad2 DNA as a probe. Three T-cell lines, Jurkat (11), GM 2219 (Molt-4 [22]) and BM3671 (CCRF-CEM [9]), were infected with Ad2 as were control HeLa cells. Viral DNA replication was most efficient in Jurkat cells, in which the amount of Ad2 DNA synthesized at day 2 p.i. was comparable to that at day 1 p.i. in HeLa cells (Fig. 1). Replication of viral DNA in 2219 and 3671 cells followed slower kinetics. Repeated experiments showed, however, that the kinetics of DNA replication were reproducible and characteristic for each T-cell line.

Replication of Ad2 DNA was similarly analyzed in human B-cell lines with markers characteristic of various stages of B-cell differentiation (Fig. 1 and 2). Four of the cell lines studied here have been reported to contain EBV: Josh 7, a precursor B-cell line with Fc and C_3 receptors but no demonstrable immunoglobulin synthesis (10); SeD, a cell line producing predominantly membrane-bound and secreted IgM (20); 32a1, a cell line synthesizing both membrane-bound and secreted IgA (19); and RPMI 8866p, which produces predominantly secreted IgG (18). Three EBV-negative cell lines were used: GM 2132 (RPMI 8226), a λ light-chain-producing myeloma cell line (17); Manca, a cell line derived from a patient with gastric lymphoma (S. M. Fu, unpublished data); and BJA-B, a Burkitt's lymphoma cell line (21). Since EBV was used for establishment of most B lymphoid cell lines, we first confirmed the absence of the EBV genome in cell lines reported to be EBV negative by Southern blot analysis by using as a probe a cloned *BamHI* W fragment of the EBV genome (kindly provided by W. Sugden). Absence of the EBV genome in 2132, Manca, and BJA-B cells was confirmed as shown in Fig. 3 (lanes 2, M, and B, left panel), for which equal amounts of total cellular DNA of each line were analyzed (ethidium bromide-stained

FIG. 2. Southern analysis of adenoviral DNA synthesis during prolonged Ad5 infection. Manca, BJA-B, and 8866p cells were infected with Ad5 at 20 PFU per cell. Total DNAs were isolated after 1 h of adsorption (0) or the indicated number of days (1 to 6) p.i., digested with restriction enzyme *Hind*III, and analyzed by the Southern method with 32 P-labeled nick-translated Ad5 DNA as a probe. A $\times$ symbol represents the time required for exposure of the autoradiograph, that of Manca cells being 1 $\times$.

gel in the right panel), but only the DNA probe itself (lane E) and an EBV-positive reference cell line, Daudi (lane D) (14), were detectable by the EBV probe.

Adenovirus replicated in all of the B lymphoid cell lines studied, with the exception of cell line 8866p. Viral replication in the myeloma cell line 2132 was most efficient. The amount of adenoviral DNA present at day one p.i. was comparable to that in HeLa cells (Fig. 1). Viral replication in other B-cell cultures was considerably slower than in 2132 cells (Fig. 1). Analysis of prolonged infection suggested a 10-fold increase of viral DNA in Manca cells at 6 days p.i. and that, after a delay of 1 day, viral replication in BJA-B cells was well supported for at least 5 days (Fig. 2). Viral DNA could be detected in 8866p cells immediately after adsorption (Fig. 2, lane 0, panel 8866p) but increased only negligibly at day 4. We found in repeated experiments that, with the same multiplicity of infection, replication of aden-

oviral DNA in B lymphoid cells, as in T cells, was reproducible and characteristic for each cell line. Furthermore, there appeared to be no apparent correlation between the kinetics of viral replication and the developmental stage from which the B lymphoid cell lines were isolated. Contrasting with earlier reports by Faucon et al. (7), our data showed that adenoviral DNA replication was also not dependent on the presence of the EBV genome in B lymphoid cells.

Viral RNA synthesis. The presence of adenoviral RNA in lymphoid cells in the course of infection was assayed by Northern blot analysis with the following probes: c131 (24), a cDNA clone for the E1A mRNAs; p*Hind*III-D, a genomic clone specific for the L-2 family of the Ad2 late mRNAs; and, as a control, a probe specific for human class I major histocompatibility antigen (28).

Myeloma cells 2132 and HeLa cells were infected with Ad2 at 10 PFU per cell, and total polyadenylated RNA from 10^7 cells was isolated at various times p.i. for assay. At this multiplicity of infection, viral DNA replication was detectable after 30 h p.i. in 2132 cells. The three major L-2 mRNA species, identical to those accumulated at 18 h p.i. in HeLa cells, were seen in 2132 cells at 36 h p.i. By 48 h p.i., the L-2 mRNA levels were equal to or slightly higher than those in HeLa cells at 18 h p.i. (Fig. 4). These results demonstrated that late viral mRNAs were synthesized appropriately after viral DNA replication. Similar results were obtained for other late adenoviral mRNAs and in other lymphoid cell lines (data not shown).

Late in infection, the steady-state levels of E1A mRNAs in 2132 cells at 48 h p.i., however, were reduced to 1/20th of those in HeLa cells at 18 h p.i., when late mRNAs were at comparable levels (Fig. 4). A longer exposure of the autoradiograph presented in Fig. 4 showed that the 13S (1.2-kilobase) E1A mRNA was detectable early in infection in 2132 cells, at 12 h p.i. The differential levels of E1A mRNAs in lymphoid and nonlymphoid cell backgrounds suggested that viral early genes are differentially regulated in lymphoid cells. Experiments are in progress to investigate the molecular basis for these observations and the expression of other viral early genes in lymphoid cells.

As a control, the levels of steady-state mRNAs for the host human class I histocompatibility antigen HLA-B were assayed. In the course of an 18-h adenoviral infection, HLA

FIG. 3. Southern blot analysis of the EBV genome in B cell lines. *Bam*HI-digested DNAs (10 μ g) from the four B-cell lines (BJA-B [B], Manca [M], and 2132 [2] are EBV $^{-}$; Daudi [D] is reported to be EBV $^{+}$ [14]) were analyzed by the Southern method along with 100 pg of *Bam*HI W fragment (E; courtesy of W. Sugden) as a control. A nick-translated 32 P-labeled EBV *Bam*HI W DNA fragment was used as a probe for hybridization. The ethidium bromide-stained gel is shown on the right. λ represents *Hind*III-digested λ DNA as size markers. The numbers at the right indicate molecular size in kilobases.

FIG. 4. Synthesis of adenoviral mRNAs in human B lymphoid cells. Total poly(A)⁺ RNAs were isolated (5) from 10⁷ 2132 myeloma or HeLa cells at the indicated number of hours p.i. with Ad2 (10 PFU per cell). After formaldehyde denaturing agarose gel electrophoresis, RNAs on the same blot were analyzed by the Northern technique with the following probes: ³²P-labeled nick-translated inserts of clones pDP001 for HLA (28), c131 for E1A (24), and pHindIII-D for Ad2 L-2 mRNAs. The numbers at the right indicate molecular size in kilobases.

mRNA levels were maintained unchanged in HeLa cells. The slight increase in a 48-h infection period in 2132 cells may be attributed to the slower kinetics of infection, during which time cell mass also increased (Fig. 4).

Synthesis of viral proteins. Indirect immunofluorescent staining of adenoviral proteins with antisera directed toward purified Ad5 virion demonstrated that, in addition to synthesis of viral DNAs and RNAs, adenoviral proteins were translated in lymphoid cells. An example is shown in Fig. 5. Adsorption and internalization of infecting viral particles could be observed immediately after adsorption (O) and at early times p.i. (E) in myeloma 2132 cells as in HeLa cells.

Late in infection, the virion proteins which reacted with the antisera were dramatically increased in 2132 cells as in HeLa cells. With our experimental protocol, at late times nearly all HeLa cells were immunofluorescence positive, and approximately 60% of 2132 cells stained positive for virion proteins. Similar positive results, albeit at lower percentages, were obtained in other B and T lymphoid cell lines.

Production of biologically active viruses. A two-cycle infection assay was used to determine whether active virions were produced in lymphoid cells. Ad5 virion prepared from HeLa cells and purified by cesium chloride gradients (33) was used to infect 2132 myeloma cells and Jurkat T cells at

FIG. 5. Indirect immunofluorescence and phase-contrast microscopy of Ad2-infected cells. Cells of an established myeloma line, 2132, and HeLa cells were infected with Ad2 at 20 PFU per cell and subjected to immunofluorescence as described in Materials and Methods. Samples were incubated first with rabbit antisera directed toward purified adenovirus virion and then incubated with fluorescein-conjugated goat antibody to rabbit IgG. Designations: IFM, immunofluorescent microscopy; Control, uninfected cells; O, cells immediately after 30 min of adsorption at 37°C; E, cells at early times p.i. (5 h p.i. for HeLa and 7 h p.i. for 2132 cells); L, cells at late times p.i. (18 h p.i. for HeLa and 24 h p.i. for 2132 cells). All photographs were taken at $\times 630$ magnification.

20 PFU per cell. Total DNAs immediately after adsorption and at 43 (Jurkat) and 48 (2132) h p.i. were analyzed by Southern blot analysis with nick-translated total Ad5 DNA as a probe (Fig. 6, lanes 1°). Lysates were made from infected Jurkat and 2132 cells and used to infect an uninfected sample of the respective cell line from which the lysates were prepared (lanes 2°). Viral DNA synthesis in lymphoid cells during secondary infections was similarly assayed. Replication of adenoviral DNA during secondary infection demonstrated that biologically active virions were assembled in Jurkat and 2132 cells during primary infection and could be subsequently used to infect lymphoid cells.

A plaque assay was performed to quantify the yield of viruses from 2132 and Jurkat cells in the course of a 4-day infection with Ad2 at a multiplicity of infection of 10 PFU per cell. Taking into consideration the slight increase in cell number during the experimental period, 1.3-fold for 2132 cells and 2-fold for Jurkat cells, and the percentage of immunofluorescence-positive cells in the cultures, the virus produced per cell was 3,000 PFU for 2132 cells at 72 h p.i., 2,200 PFU for Jurkat cells at 96 h p.i., and 5,000 PFU for HeLa cells at 24 h p.i. (Fig. 7). Hence, the yield of viruses from lymphoid cells was comparable to that of nonlymphoid cells.

FIG. 6. Synthesis of infectious viral particles in Ad2-infected human B and T cells. Human 2132 myeloma cells and Jurkat T cells were infected with Ad5 at 20 PFU per cell. At 48 h p.i. of 2132 and 43 h p.i. of Jurkat cells, DNAs from 4×10^5 cells were isolated as previously described (23). Lysates were made from the remainder of the infected 2132 or Jurkat cells and used for infecting 2132 and Jurkat cells, respectively. The multiplicity of the secondary infection was estimated to be 20 PFU per cell. DNAs were isolated from the secondary (2°), as well as the primary (1°), infection. Each DNA sample was digested with restriction enzyme *KpnI* and analyzed by the Southern blot method with ^{32}P -labeled nick-translated Ad2 DNA as a probe. $\times$ indicates the autoradiograph exposure time, that of 2132 cells being $1 \times$.

FIG. 7. Plaque assay of viruses produced in 2132, Jurkat, and HeLa cells. Cultures at the log phase of growth were concentrated to 10^7 cells per ml, infected with Ad2 at 10 PFU per cell, and diluted to 2×10^5 cells per ml after a 30-min adsorption period. Lysates were prepared immediately after adsorption and on the days p.i. indicated. Plaque assay was performed on human A549 cells. Symbols: Δ , HeLa cells; $\circ$, 2132 cells; $\times$, Jurkat cells.

DISCUSSION

Our data demonstrated that infection of established B lymphoid cell lines by Ad2 or Ad5 was characteristic of each cell line regardless of its origin or the presence of the EBV genome. Results obtained from indirect immunofluorescent staining of infected cells and the cytopathic effect caused by infection with an Ad5 mutant (D. Lavery and S. Chen-Kiang, manuscript in preparation), as well as the yields of viruses, suggested to us that the differences in infectivity among the cell lines were most likely due to the number of cells infected as opposed to the yield of infection by each cell in the culture. Work is in progress to subclone the cell lines to verify this hypothesis. Replication of viral DNA in cell line 8866p was marginal. The basis for this exception is not understood at present. It appears, however, that the defect cannot be attributed to viral adsorption (Fig. 3).

With regard to natural infection of human lymphoid tissue by adenoviruses, previous investigators have found periph-

eral human lymphocytes to be largely refractory to infection by Ad2 or Ad5 (8, 29, 32). Our results with cultured lymphoid cell lines and peripheral blood lymphocytes (D. Lavery, S. M. Fu, and S. Chen-Kiang, unpublished data) suggest that, whereas the entire peripheral lymphocyte population may not be productively infectable, an infectable subpopulation exists. Whether there is any functional relationship between the activation state of lymphocyte and its susceptibility to infection remains to be determined. This infectable subpopulation of lymphocytes may serve to carry adenoviral infection to other sites, where acute infection takes place, a role previously suggested for both lymphocytes and fibroblasts (8, 29).

The presence of the EBV genome was postulated as enhancing the infectability of B lymphocytes by adenoviruses (7, 8). Our results with more detailed investigations of many cell lines, however, indicated that there was no requirement for the presence of EBV. In particular, 2132, an EBV-negative myeloma cell line, showed the most rapid kinetics of infection of all of the lymphoid cells thus far studied.

The levels of infection in other cell lines ranges from 6 to 60% of cells infected, much greater than those reported previously (7). This must be in part due to the culture conditions we maintained, in which infected cells were not diluted by successive feeding, which would create a bias in favor of uninfected, dividing cells. Rapid and uniform infection of 2132 presents the intriguing possibility that regulation of the cellular genes specifically expressed in B lymphoid cells by adenoviral genes can be addressed. Recent results obtained in our laboratory suggested that the Ad2 and Ad5 early viral transcription units themselves are expressed differentially in lymphoid cells as in HeLa cells (D. Lavery and S. Chen-Kiang, unpublished data).

We demonstrated here for the first time that established T-cell lines could be infected by Ad2 with kinetics characteristic of each cell line as in lymphoid lines of B-cell origin. This would permit studies of regulation of genes specifically expressed in host T cells by adenoviruses in cell lines such as Jurkat, which is highly infectable by the virus. Independently, H. Ginsberg and colleagues have also obtained results indicating that the Molt-4 (2219) cell line supports viral growth (personal communication).

A recent report by Weber and colleagues showed that, by Southern blot analysis, several established cell lines contained very low levels of endogenous adenovirus genome, approximately one copy per cell (13). However, Arrand et al. (1), using stringent washing procedures after Southern hybridization, found that the endogenous viral genome bands were homologous but not identical to those of adenoviruses. Using stringent hybridization conditions (see Materials and Methods) and a 28S ribosomal clone to compete for nonspecific binding sites, we detected no discrete virus-specific DNA or RNA species in any lymphoid cell lines before infection by exogenous adenoviruses.

The well-characterized molecular genetics of adenovirus, as well as its high copy number and episomal replication, have allowed it to be used by others as an effective vector system (16). Successful infections of established human B- and T-cell lines, which are difficult to transfect, prompted us to modify adenovirus as an expression vector for lymphoid cells. This proved successful. Under control of the early region 1B promoter, the mouse immunoglobulin heavy-chain gene was found to be expressed as cell type-specific mRNAs, independent of the immunoglobulin heavy-chain enhancer, and regulated posttranscriptionally (27).

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