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Transfection of human lymphoblastoid cells with herpes simplex viral DNA

(Epstein-Barr virus/B lymphocytes/mitogens/infectious centers)

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ABSTRACT **The "calcium/dimethyl sulfoxide shock" method of transfection was adapted for use in human lymphoid cell cultures. One microgram of herpes simplex virus type 1 DNA regularly initiated virus replication in four lymphoblastoid cell lines. Per 10^5 cells exposed to 1 μg of DNA, 0.5–5 cells formed an infectious center. The minimal infective dose of DNA was approximately 500 ng.**

A reliable method for transfection of human lymphocytes by DNA could have many applications in the study of lymphotropic viruses and also might be useful in experimental immunology and genetics. To initiate studies of this problem we chose a system that would maximize the chances of demonstrating expression of genetic information after introduction of naked DNA into lymphoid cells. The DNA of herpes simplex virus (HSV) was selected because one assay for expression—namely, production of complete progeny—is simple and rapid and because several methods are already available for detection of infectious herpes DNA on monolayer tissue culture cells (1–3). The ability to assay HSV-1 DNA independently in permissive cells provided a check on the quality of the DNA and on the adequacy of the transfection technique. HSV DNA was chosen also because it is a good model for future studies with Epstein-Barr virus (EBV) DNA, a molecule of the same size.

MATERIALS AND METHODS

Virus Strain, Propagation, and Preparation of Viral DNA. The Lovering strain of HSV-1, originally from a fetal encephalitis case, was passed at low multiplicity three times in the AH-1 and once in the BSC-1 line of African green monkey kidney cells (4). The virus was plaque-purified in BSC-1 cells under 2% methylcellulose. Two batches of viral DNA were used: batch 4, prepared from virions propagated in BSC-1 cells; batch 6, derived from a subclone obtained in transfection of BSC-1 cells, was prepared from virions propagated in BHK-21 cells.

Viral DNA was obtained from virions by the procedure described by Geelen *et al.* (5) for preparation of infectious cytomegalovirus DNA. The starting material for each batch of DNA was 6–20 roller bottles of BSC-1 cells. The medium was removed from confluent cultures, which were inoculated with about 2 plaque-forming units (PFU) per cell. After 1 hr the inoculum was removed and 50 ml of Eagle's medium with 2% calf serum was added to each bottle. After 40 hr, when there was extensive cytopathic effect, the culture medium was decanted and centrifuged at 4000 rpm at 4°C for 10 min in the Sorvall GSA rotor. The supernatant fluids were separated from the pellets and centrifuged for 3 hr at 18,000 rpm at 4°C in the Beckman 19 rotor. The pellets were resuspended in 1/200th vol of 0.15 M NaCl/0.05 M Tris, pH 7.4 (TS buffer) containing 0.25 M sucrose and placed on top of a continuous 10–50% (wt/vol) sucrose gradient in TS buffer. The gradient was centrifuged in the SW 27 rotor for 1.5 hr at 23,000 rpm at 4°C. The virus band was harvested and dialyzed overnight against TS buffer.

The purified virus was concentrated by pelleting for 1.5 hr at 35,000 rpm in the SW 41 rotor. The pellet was resuspended in 0.05 M Tris/0.1 M NaCl/5 mM EDTA, pH 7.4 (TNE buffer) and viral DNA was released by addition of 1% sodium dodecyl sulfate and 2 mg of proteinase K per ml. The DNA extraction mixture was held at 37°C for 4 hr and then was gently pipetted on top of a 10–30% (wt/vol) sucrose gradient in TNE. The gradient was centrifuged for 3 hr at 35,000 rpm at 18°C in the SW 41 rotor and was fractionated by pumping 40% (wt/vol) sucrose in TNE into the bottom of the tube. The absorbance across the gradient was monitored with a Uvicord III (LKB Instruments) and recorded on a W and W recorder. High molecular weight DNA was collected and dialyzed against 0.01 M Tris/0.1 M NaCl/1 mM EDTA. The concentration of DNA was determined by absorbance at 260 nm. The yield of high molecular weight DNA was 6–34 μg per batch. The DNA used for transfection experiments was stored in aliquots at −20°C.

Lymphoid Cells. Three lymphoblastoid lines, designated X25, were derived after transformation of lymphocytes from one umbilical cord with a passage of the B95-8 strain of EBV (unpublished data); the fourth line (X50-7) originated from another umbilical cord. These lines are not spontaneous producers of extracellular EBV but transforming virus can be recovered from the lines by the technique of x-irradiation and cocultivation. All the cells have EBV nuclear antigen, and receptors for erythrocytes coated with antibody and complement are found on 90% of the cells in each of the lines. Some of the cells in the X25-9 line adhere to plastic surfaces, but adherent cells are not found in the X25-4, X25-6, or X50-7 lines. Raji and BJAB are Burkitt lymphoma lines; Raji has the EBV genome and BJAB does not (6). Mixed mononuclear leukocytes from umbilical cord blood were prepared for culture after isolation on a Ficoll/Hypaque gradient (7). The cell lines were maintained in medium RPMI 1640 plus 10% fetal calf serum and antibiotics.

Virus Assays. HSV plaque titrations were performed in BSC-1 cells under 0.9% agarose. Three days after infection, neutral red was added at a final concentration of 1:10,000 to

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Abbreviations: HSV, herpes simplex virus; PFU, plaque-forming units; EBV, Epstein-Barr virus; EE-10, Eagle's medium with Earle's salts and 10% fetal calf serum; Me_2SO, dimethyl sulfoxide.

Table 1. Sensitivity of various lymphoid cell preparations to infection with HSV type 1

Type of cell	Treatment or cell line	Infected centers, no./10^4 cells* Exp. 1	Exp. 2
Primary mixed human umbilical cord mononuclear leukocytes	None	12	18
	Aging†	170	161
	Mitogen‡	373	304
	Mitogen + aging	445	ND
EBV-transformed human umbilical cord leukocytes (lymphoblastoid cell lines)	X25-4	400	1250
	X25-6	495	726
	X25-9	430	ND
Burkitt lymphoma lines	Raji-EBV genome-positive	3	13
	BJAB-EBV genome-negative	8	23

* At 24 hr after infection with ≈2 PFU per cell, infected cells were incubated at 37°C in antibody for 1 hr, washed, and resuspended in medium. The cells were counted and serial dilutions were plated on VERO cells. A methylcellulose overlay was then added. After 3 days the overlay was removed and the cell sheet was fixed in methanol and stained with Giemsa. The wash fluid after antibody treatment was also plated and the number of plaques that developed was subtracted from the number of infected centers. ND, not done.

† Cells had been in culture for 5 days before they were exposed to virus.

‡ Cells were treated with staphylococcal phage lysate (150 μg/ml) and 2 days later were infected with HSV-1.

a second agarose overlay. Plaques were counted between days 4 and 7 after infection. HSV-infected centers in lymphocytes were measured on VERO or BSC-1 indicator cells under 1% methylcellulose. Plaques were fixed with methanol and stained with Giemsa after 3 or 4 days (8).

Transfection. A precipitate was formed by adding viral DNA, 5 μg of calf thymus DNA, and 125 mM $CaCl_2$ to a final volume of 0.5 ml in Hepes-buffered saline at pH 7.05. This reaction mixture was mixed gently and allowed to stand at room temperature for 0.5 hr. The infectivity of the DNA was assayed on freshly trypsinized BSC-1 cells in 60-mm petri dishes.

To transfect lymphocytes with DNA, the cells growing in suspension were removed from RPMI growth medium and washed twice in Eagle's medium with Earle's salts and 10% fetal calf serum (EE-10). The cells were resuspended to a concentration of 10^5–10^6 cells per ml and 5 ml was placed in a 60-mm tissue culture petri dish (Falcon). The preformed precipitate was added in droplets to various regions of the dish (9). The cells and the precipitate were incubated for 3.5–4 hr at 37°C. The suspended cells were removed from the petri dish, and washed once in EE-10; 25% dimethyl sulfoxide (Me_2SO) in Hepes-buffered saline was added to the resuspended cells. After 4 min at room temperature, 3 vol of Hepes-buffered saline was added to dilute the Me_2SO and immediately thereafter the cells were washed once with Hepes-buffered saline and once with EE-10. They were resuspended in EE-10. X25-9 cells that remained attached to the dish were also washed, treated with Me_2SO, and washed again. The cells that had been in suspension were then returned to the original dish with its adherent population.

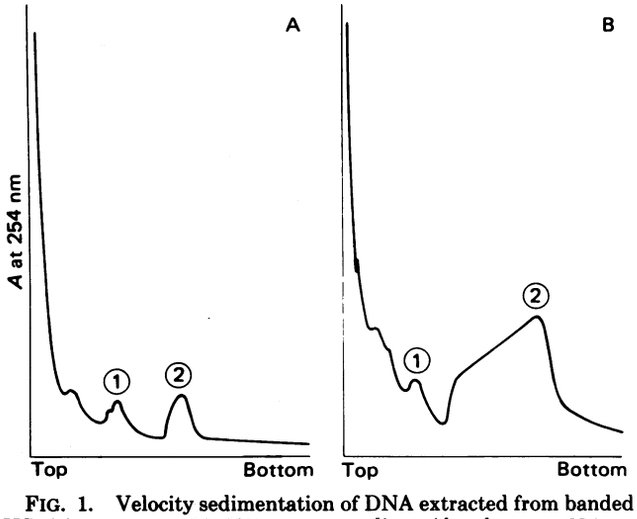

FIG. 1. Velocity sedimentation of DNA extracted from banded HS virions, across a 10–30% sucrose gradient. Absorbance at 254 nm was measured with a continuous flow spectrophotometer. (*A*) DNA prepared from virus harvested from eight roller bottles (batch 3); there was about 6 μg of DNA in peak 2 and about 3 μg in peak 1. (*B*) DNA prepared from virus harvested from 20 roller bottles (batch 4); approximately 13 μg of DNA was present in peak 2 and 7 μg in peak 1.

RESULTS

Susceptibility of Lymphoid Cells to Infection with HSV. Different types of lymphoid cells varied considerably in their sensitivity to infection with HSV. All were less sensitive than monolayer tissue culture cells. Some representative data are shown in Table 1. The number of infected centers was measured 24 hr after addition of 1–2 PFU per cell. Primary lymphocytes were relatively refractory to infection. However, if the primary cells were "aged" *in vitro* for a week they became 10- to 20-fold more susceptible. An increase in susceptibility to infection of similar or slightly greater magnitude was observed in cord leukocytes treated with staphylococcal phage lysate (Delmont Laboratories, Swarthmore, PA). This lysate is a mitogen of T and B lymphocytes and null cells (10). Lymphoblastoid lines resulting from *in vitro* transformation of umbilical cord cells by EBV appeared to be more susceptible to HSV than were primary cells from the same origin. Lines derived from Burkitt lymphoma were relatively refractory to production of complete virus (11). Thus, lymphoblastoid lines appeared most suitable for initial attempts at transfection with DNA.

Characteristics of Infectious HSV DNA. Representative profiles of two sucrose gradients on which HSV DNA was purified are shown in Fig. 1. The DNA preparations used for lymphocyte transfection studies were full-length genomes harvested from peak 2. The mean contour length of DNA molecules in peak 2 was 15.9 times the length of the PM_2 marker. This corresponds to a size of 101.36×10^6 daltons. DNA in peak 1 was 4.15 times the size of the PM_2 marker and thus the molecules are approximately one-quarter full genome size. Most of the full-length HSV DNA molecules found in peak 2

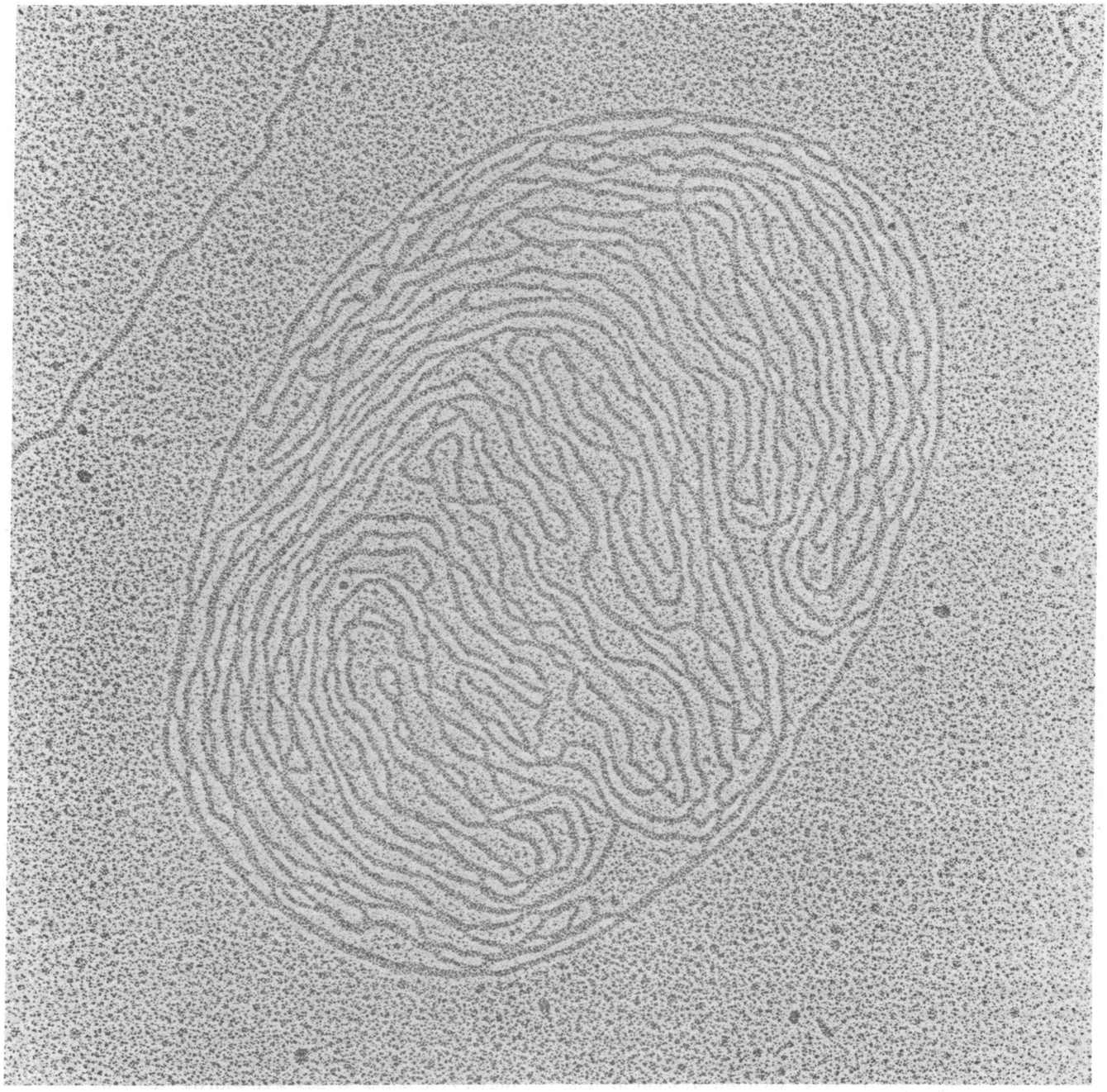

FIG. 2. Electron micrograph of a DNA molecule found in high molecular weight DNA prepared from purified HS type 1 virions. Based on comparative measurements with PM_2 DNA, the molecular weight was 108×10^6. Spreading of the DNA, photography, and measurements were performed by Mrs. Wil Maris, Laboratorium voor de Gezondheidsleer, University of Amsterdam. (×150,000.)

were completely unfolded. However, several intact molecules retained a pattern of folding (Fig. 2). Although this pattern may be an artifact of incomplete spreading, it provides a suggestion of the manner in which the DNA might be packaged in the virion.

The same eight DNA fragments were seen after digestion of DNA from peaks 1 and 2 with *Eco*RI restriction endonuclease and analysis by electrophoresis on 0.7% agarose gels 20 cm in length. Thus, peak 1 probably represents random breakage products of full-length DNA. The DNA found in peak 2 was infectious; the quarter-length molecules were not.

The specific infectivity of the two batches of DNA were 1550 PFU/μg for batch 4 and 300 PFU/μg for batch 6 when 100 ng of DNA was added per dish of BSC-1 cells. The calculation of specific infectivity was influenced by the amount of DNA added to the BSC-1 cells. At a DNA concentration of 1 μg per dish, the specific infectivity of all batches tested was lower (60–130 PFU/μg). No plaques were observed when 1 μg of HSV DNA was added to BSC-1 cells without $CaCl_2$ to facilitate transfection. The infectivity of the two batches of DNA for permissive cells was eliminated after 0.5-hr incubation with pancreatic DNase I at 10 μg/ml in 10 mM Tris/5 mM $MgCl_2$, pH 7.4. The addition of $MgCl_2$ alone or heating at 56°C for 1 hr did not affect the infectivity of the DNA. These results indicated that there was no residual virus in the preparation.

Replication of HSV after Transfection of Lymphoid Cells. When a mixture of viral and carrier DNA, $CaCl_2$, and Hepes-buffered saline was added to lymphoid cells suspended in their

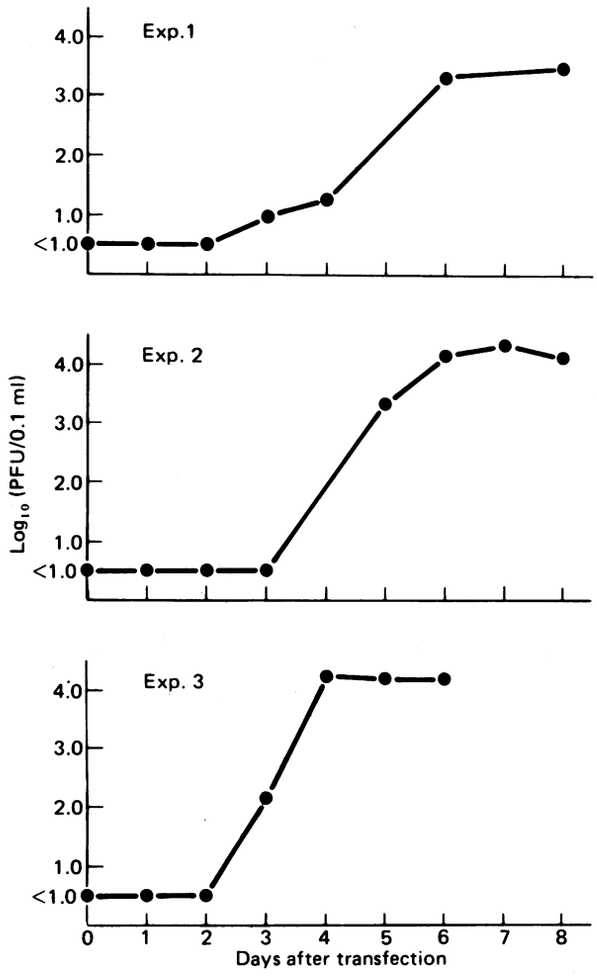

FIG. 3. Replication of HSV after transfection of line X25-9 with 1 μg of HSV DNA. Three separate experiments are shown. In Exp. 1 and Exp. 2, batch 4 DNA was used; in Exp. 3, batch 6 was used. Samples (100 μl) were collected daily from the culture and stored at −70°C. They were thawed, and plaque titrations of the mixture of cells and fluids were performed on BSC-1 cells.

usual growth medium, RPMI 1640, a dense precipitate formed and was toxic to the lymphocytes. We thought that this precipitate might be due to the 5-fold higher concentration of phosphate in RPMI medium than in Eagle's medium with Earle's salts. Therefore, the latter medium was substituted. Successful transfection then occurred in all successive trials with the X25-9 cells.

In the three experiments shown in Fig. 3, samples of cells and fluids were harvested daily and the numbers of PFU in frozen and thawed samples were titrated on BSC-1 cells. Virus was first detected at 48 hr after transfection in one experiment and at 72 hr in the other two. Maximal titers of about 10^5 PFU/ml were reached after 4–6 days.

The number of infected centers responsible for the viral growth curves was measured (Table 2). There was one infected center per 5×10^4 cells at 24 hr after transfection of X25-9 cells. Because there was no virus in the supernatant fluid at the time of appearance of these early infected centers, they represent the minimal number of cells that were transfected by DNA. In three other experiments (not shown) the number of cells initially transfected and competent to produce mature progeny was 0.5, 3, and 5 per 10^5 cells exposed to DNA. Infectious virus was found in the supernatant fluids of the X25-9 cultures 24 hr after the first infected centers were detected. Therefore, the number of infected cells measured later in the experiment may result either from cells initially transfected by DNA in which virus production was delayed or from cells infected by virus released during the first round of replication.

To compare the sensitivity of the X25-9 line to infection by virus and to transfection by DNA, both virus infection and transfection were done simultaneously under the conditions of calcium precipitation and Me_2SO shock. Serial 1:10 dilutions of virus or DNA were added to individual cultures. Aliquots were harvested daily and tested for their content of infectious virus. The minimal infective dose of virus was 250 PFU; 25 PFU did not initiate infection. The minimal infective dose of DNA was 500 ng of batch 6, an amount equivalent to 150 PFU measured on BSC-1 cells. The virus growth curve after infection of the lymphoid cells with 250 PFU was similar to that seen after transfection with 1 μg of DNA (Fig. 3); however, a higher titer of virus (4×10^6 PFU/ml) was reached 4 days after infection.

Other Lymphoblastoid Lines. The X25-9 line has certain properties not seen in most other lymphoblastoid lines derived after transformation of umbilical cord cells by EBV. The cells are larger than normal and they are hyperdiploid: there are 70–90 chromosomes per cell due to triploidy or tetraploidy of many chromosomes. Furthermore, some of the cells adhere to plastic or glass surfaces. Therefore, we examined two other lymphoblastoid cell lines from the same infant, X25-4 and X25-6, which have a normal chromosome number and which grow totally in suspension. Both the X25-4 and the X25-6 line were susceptible to transfection with 1 μg of HSV DNA (Table 2). The time course of replication and amounts of virus produced were similar to those seen with the X25-9 line. Successful transfection also occurred with the X50-7 line from another infant.

Table 2. Transfection of four different lymphoblastoid lines with HSV type 1 DNA

Days after transfection	Line X25-9		Line X25-4	Line X25-6	Line X50-7
	No. IC per 10^4 cells*	PFU/0.1 ml supernatant	PFU/0.1 ml supernatant	PFU/0.1 ml supernatant	PFU/0.1 ml supernatant
0	0	0	0	0	0
1	0.2	0	0	0	0
2	3.8	10	0	8	ND
3	20.0	30,000	43	50	ND
4	60.7	20,000	TNTC	TNTC	28,000
5	69.0	50,000	ND	ND	100,000
6	206	25,000	ND	ND	900,000

Transfection was with 1 μg of HSV-1 DNA, batch 6. TNTC, too numerous to count. ND, not done.
* Infected centers (IC) were assayed as described in Table 1, except BSC-1 cells were used as indicator monolayers.

DISCUSSION

The present experiments were stimulated by the need to define precisely the conditions for consistent expression of viral DNA introduced into lymphoid cells. We have shown that EBV-transformed human lymphoblastoid cells of umbilical cord origin can regularly be transfected with HSV DNA by using the calcium technique first described by Graham and van der Eb (9). According to one preliminary report, the Raji and Molt-4 lines have also been transfected with HSV-1 DNA by means of the DEAE-dextran method, and the Raji cells formed a carrier culture (12, ¶).

Several factors may have contributed to the success of these trials. One was the selection of a suitable cell/virus system. It was also important to use viral DNA of high specific infectivity; thus, the full-length genomes obtained by gentle disruption of purified virions may have been especially suitable. Modification of the lymphocyte culture medium to avoid excessive precipitation and damage to the cells appeared necessary for successful transfection. In one experiment, 1000 PFU of HSV was included in the transfection mixture of Hepes buffer, $CaCl_2$, and carrier DNA. Half of the inoculum was added to cells resuspended in RPMI and half to cells in Eagle's medium. Infected centers ($10/10^5$ cells) were detected only in the culture in Eagle's medium. Toxicity of the Me_2SO was minimized by diluting it immediately after the treatment. The lymphocytes were spread over a large area when they were exposed to DNA. This allowed maximal contact of the viral DNA with the target cells without the need for vigorous shaking which might fracture the DNA.

The extension of transfection methods to human lymphocytes will permit many new experimental approaches in the study of lymphotropic viruses. One area which can now be studied is the effect of superinfection of EBV genome carrier cells by EBV DNA and specific EBV DNA fragments. For example, it may now be possible to define the region of the HR-1 EBV genome that is responsible for early antigen expression (13). When lymphoblastoid cells transformed by conditional lethal EBV mutants are available, it should be possible to perform classical marker rescue experiments with EBV DNA fragments. Conversion of EBV genome-negative lines to expression of EBV nuclear antigens may be attempted with EBV DNA fragments, and thus the region coding for these nuclear antigens may be defined directly (14). Because EBV is a plasmid, it has been considered as a potential vehicle for cloning eukaryotic genes. Transfection of lymphoid cells is a step on the way to developing such technology.

The availability of lymphoblastoid lines proved to be competent in transfection may aid in the cultivation of viruses that have resisted growth *in vitro*.

Some new investigations will require that primary lymphocytes, which have not been first transformed by EBV, be shown to be susceptible to DNA. It seems likely that stimulation of cellular DNA synthesis or cellular activation will be necessary to demonstrate transfection of primary lymphoid cells with HSV DNA. These events seem to enhance susceptibility of umbilical cord leukocyte cultures to HSV infection (Table 1; detailed studies will be published elsewhere). Assuming that the techniques presented in this report will be applicable to primary lymphocytes, several further studies can be contemplated. Because lymphoid lines exist that do not contain EBV, it will be of interest to learn whether lymphoid cell immortalization can occur after delivery of specific DNA fragments from other viruses such as HSV. It may now be possible to delineate portions of the EBV genome needed for immortalization, provided that a virion-associated polymerase is not needed for the infectivity of EBV DNA. Another objective would be to learn whether the strict B cell tropism of EBV, which seems to be determined by an interaction between the virus and a specific receptor on B lymphocytes, can be overcome by using EBV DNA (15). It would then be possible to learn whether the highly efficient transforming capacities of the EBV genome can be expressed in other hematopoietic or tissue cell types.

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