Abrogation of Simian Virus 40 DNA-Mediated Transformation of Primary C57BL/6 Mouse Embryo Fibroblasts by Exposure to a Simian Virus 40-Specific Cytotoxic T-Lymphocyte Clone

HENRY E. KARJALAINEN, MARY J. TEVETHIA, AND SATVIR S. TEVETHIA*

Department of Microbiology and Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Received 10 April 1985/Accepted 16 July 1985

Primary mouse embryo fibroblasts of C57BL/6 origin (B6/MEF) were transformed in vitro by transfection with simian virus 40 (SV40) DNA. The transformation frequency was markedly reduced if the SV40 DNA-transfected cultures were briefly exposed to K11 cells, an SV40-specific clone of cytotoxic T lymphocytes. This abrogation of SV40 transformation in vitro by cytotoxic T-lymphocyte clone K11 was specific, since transformation of B6/MEF cells by adenovirus type 5 DNA was not affected. The approach described here should serve as an ideal model of dissecting immunological events during in vivo tumorigenesis.

Simian virus 40 (SV40) codes for a multifunctional protein, the large T antigen, that mediates the transformation of a wide variety of mammalian cells (18, 30). While the majority of the T antigen synthesized in SV40-infected and -transformed cells is transported to the nucleus (22), a small amount is present in the cell membranes (10, 21). The physiological role of surface T antigen in transformation or the virus lytic cycle is not known. Two lines of evidence, however, suggest that surface T antigen in the virus-infected host acts as a target for immune surveillance against viral carcinogenesis. First, there is considerable evidence that T antigen purified to homogeneity immunizes mice against the transplantation of syngeneic SV40 tumor cells and can prime mice for the generation of cytotoxic T lymphocytes (CTL) (4, 5, 25). It has been shown that CTL play a definitive role in resistance to SV40 tumor growth (6). Second, the surface T antigen, in all probability, provides a target for CTL that are specific for the tumor-specific transplantation antigen (6, 14, 15, 25, 28).

It has been postulated that in vivo a large majority of virus-infected cells that are committed to undergo either abortive or stable transformation are eliminated by the immune response of the host, thus explaining the apparent nononcogenicity of SV40 in an immunocompetent host whose cells it transforms efficiently (24). Although the participation of SV40 tumor-specific transplantation antigen in immunological reactions involving CTL has been documented in vitro, the exact immunological events occurring in vivo during viral transformation have not been reproduced in vitro. We now describe a system for defining the role of virus-specific CTL in the abrogation of primary transforming events triggered by transfection of primary cells with SV40 DNA. The results show that a brief exposure of C57BL/6 mouse embryo fibroblasts (B6/MEF) transfected with SV40 DNA to an SV40-specific CTL clone results in nearly complete abrogation of SV40 transformation, possibly as a result of interaction between the CTL clone and surface T antigen.

MATERIALS AND METHODS

Virus and viral DNA. SV40 virus strain VA(45-54) was used to produce viral DNA for transfection experiments. Viral DNA was extracted from infected TC7 cells by the method of Hirt (9) followed by cesium chloride-ethidium bromide density gradient centrifugation. Plasmid DNA containing the adenovirus type 5 transforming regions E1A and E1B was also used to transfect B6/MEF cultures and was provided by David Spector. Carrier DNA was prepared from B6/MEF cells by a procedure described previously (7).

Cell lines. B6/WT-19 is derived from B6/MEF transformed by wild-type SV40 (26). KHTGSV and K5RSV are SV40-transformed mouse kidney cell lines provided by Barbara Knowles (11). B6/HCMV is a C57BL/6 cell line transformed by human cytomegalovirus DNA in this laboratory (M. J. Tevethia, unpublished data). B6/MEF cells used for transfection and transformation assays were prepared from 11- to 14-day-old C57BL/6 mouse embryos and used between passages 3 and 4 (26). All continuous cell lines were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum (FBS), 0.03% glutamine, 0.075% NaHCO₃, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. B6/MEF cells were grown in the same medium containing 10% FBS.

Transfection and transformation assay. DNA transfection was done by the calcium phosphate method of Graham and Van der Eb (8) with slight modifications as described previously (31). In all transformation assays duplicate flasks containing 2×10^5 B6/MEF cells were exposed to 1 µg of SV40 DNA and 10 µg of carrier DNA. Additional cultures received carrier DNA only as a control. After 24 h of exposure to the SV40 DNA precipitate, the medium was replaced with fresh Dulbecco modified Eagle medium supplemented with 10% FBS. Thereafter, the cultures were fed twice weekly with the same medium. The SV40-transformed colonies appeared usually at about 2 to 3 weeks after transfection, and the number of SV40-transformed colonies was counted directly under the microscope. The results were confirmed by counting the same flasks after crystal violet staining. The presence of SV40 T-antigen-positive cells was determined by the indirect immunofluorescence assay.

* Corresponding author.

TABLE 1. Abrogation of SV40 DNA-mediated transformation by CTL clone K11^a

Transfecting DNA	Treatment with lymphocytes	Day of lymphocyte treatment after transfection ^b		No. of colonies/flask	
		Expt 1	Expt 2	Expt 1	Expt 2
SV40	None			150 ^c	122
SV40	Normal B/6 T cells	12	10	153	116
SV40	K11	5	5	4	3
SV40	K11	12	10	0	1
SV40	K11	19	ND ^d	31	ND
SV40	K11	5, 12, 19	5, 10, 14	1	0
Carrier DNA	K11	12	10	0	0

^a For transformation of B6/MEF, 2×10^5 cells were seeded in 75-cm² flasks 1 day before transfection. Cells in each flask were transfected with 1 µg of SV40 DNA and 10 µg of carrier DNA or carrier DNA alone, as described previously (23).

^b On different days after transfection flasks were incubated with 4×10^6 (20:1) lymphocytes in RPMI 1640 medium overnight at 37°C, and thereafter the medium in each flask was changed twice a week.

^c In both experiments each number represents the average number of colonies in the two flasks.

^d ND, Not done.

Abrogation of transformation assay. At different time points after transfection with SV40 DNA, the transfected cells were treated with SV40-specific CTL clone K11. Accordingly, the medium was removed, and 4×10^6 K11 CTL clone or nylon wool-enriched normal B6 mouse spleen T cells in RPMI 1640 medium were added to the cultures. After 24 h of incubation at 37°C, the medium was removed and replaced with fresh Dulbecco modified Eagle medium with 10% FBS. Approximately 3 weeks after transfection, the transformed foci were counted, and the presence of T-antigen-positive foci was determined by the indirect immunofluorescence assay.

CTL clone. The origin of SV40-specific CTL clone K11 has been previously described in detail (3). The CTL were maintained in continuous culture in the presence of 5 U of purified human interleukin 2 (AMGen, Thousand Oaks, Calif.) per ml and irradiated (10,000 rads) B6/WT-19 stimulator cells in RPMI 1640 medium supplemented with 10% FBS, 0.03% glutamine, 0.22% NaHCO₃, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, 5×10^{-5} M β-mercaptoethanol, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 20 µg of gentamicin sulfate per ml. The CTL clone K11 is specific for SV40 tumor-specific transplantation antigen and lyses SV40-transformed B6 cells in an H-2-restricted manner. It recognizes SV40 tumor-specific transplantation antigen in association with H-2D^b. The K11 clone recognizes a specific site on SV40-transformed cells, and it does not lyse human papovavirus BK-transformed B6 cells (3).

Detection of SV40 T antigen. For detection of SV40 nuclear T-antigen-positive colonies, the culture flasks were first fixed with cold ethanol at -70°C for 45 min. Flasks were air dried and incubated with a 1/100 dilution of a mixture of monoclonal anti-T-antigen antibodies Pab 901 and Pab 902 (one plus one) at 37°C for 1 h. Pab 901 recognizes determinants in the carboxy-terminal region of T antigen, whereas Pab 902 recognizes determinants in the amino-terminal region of T antigen (S. Tevethia, unpublished data). After rinsing three to four times with Tris-buffered saline, fluorescein isothiocyanate-conjugated goat anti-mouse gammaglobulin (Cappel

Laboratories, West Chester, Pa.) was added. After a 45-min incubation at 37°C, the flasks were rinsed with Tris-buffered saline and received Tris-buffered glycerol. The T-antigen-positive colonies were counted with a fluorescence microscope.

Cytotoxicity assay. The target cells were labeled with ⁵¹Cr and reacted with the CTL clone at various CTL/target cell ratios for 4 h. The percent specific lysis was determined as described previously (3).

RESULTS

Specificity of CTL clone K11. The specificity of CTL clone K11 has been documented previously by a ⁵¹Cr release assay (3, 27). Before using K11 as effector cells in the abrogation of transformation assay, we compared the specificity of K11 in the in vitro ⁵¹Cr release assay with its growth-inhibiting activity. The results showed that the activity of K11 was H-2D^b restricted since K5RSV cells, which are SV40-transformed cells of a K^b and D^d haplotype, were not lysed whereas KHTGSV cells (K^d D^b) were susceptible to lysis by the CTL clone K11 (data not shown).

The growth-inhibitory activity of K11 followed a similar pattern. Only the SV40-transformed B6/WT-19 (K^b D^b) and KHTGSV (K^d D^b) cells were destroyed by K11. No growth-inhibitory effect was observed on SV40-transformed K5RSV (K^b D^d) and human cytomegalovirus-transformed B6/HCMV (K^b D^b) cells (data not shown). These results show that the activity of K11 is highly specific and that they are appropriate effector cells for the abrogation of transformation.

CTL abrogation of SV40 DNA-mediated cell transformation. Previous studies have shown that 1 µg of SV40 DNA when transfected into primary B6/MEF cells induced the formation of about 50 to 100 transformed foci (23) and that all cells in the transformed foci synthesized SV40 T antigen (data not shown).

The transfected B6/MEF cells were treated either with normal T cells or with CTL clone K11 at different times, only once or multiple times, as indicated in Table 1 and Fig. 1. One group of transfected cells remained untreated. After incubation the lymphocytes were removed by washing the cultures. The total number of SV40 transformants in untreated SV40 DNA-transfected cultures varied from 150 to 122 per flask in two separate experiments (Table 1). Addition of CTL clone K11 5 days after SV40 DNA transfection markedly reduced the number of transformed foci to four and three per flask in two separate experiments. Similar results were obtained when the transformed cultures were treated once with K11 on either 10 or 12 days after DNA transfection. In two separate experiments, the number of transformed foci was reduced to 0 and 1, respectively. Treatment of transfected cultures three times (days 5, 12, and 19 in experiment 1 and days 5, 10, and 14 in experiment 2) also reduced the number of transformed colonies to 1 and 0, respectively. The possibility was considered that T-antigen-positive cells that were unable to form transformed foci might remain after treatment of transfected cultures with CTL. The cultures were fixed with absolute ethanol and reacted with a mixture of monoclonal antibodies Pab 901 and Pab 902. No immunofluorescence-positive cells indicative of the presence of T antigen were seen in CTL-treated cultures devoid of visible transformed foci (data not shown).

Treatment of transfected cells with CTL clone K11 on day 19 after transfection, when most of the transformed foci had appeared and were readily visible without the aid of a microscope, resulted in the survival of 31 colonies. All of

these remaining transformed foci contained T-antigen-positive cells (data not shown), suggesting that the cells in large colonies may be protected or that it might take longer than 24 h of CTL treatment to destroy all of the cells in large colonies. Normal T cells had no significant effect on the efficiency of transformation by SV40 DNA.

Effect of SV40-specific CTL on transformation of primary B6/MEF cells by human adenovirus type 5. To rule out the possibility that the SV40-specific CTL clone K11 abrogates SV40 DNA-mediated transformation of mouse cells nonspecifically, we investigated the effect of CTL clone K11 on transformation by adenovirus type 5. Accordingly, B6/MEF cells were exposed to the E1 region of the adenovirus type 5 genome cloned in plasmid pBR322 (provided by

TABLE 2. Effect of CTL clone K11 on adenovirus type 5 DNA-mediated transformation^a

Transfecting DNA	Day of lymphocyte treatment after transfection	Average no. of colonies/flask
SV40	None	154
SV40	5	0
SV40	8	1
Carrier DNA (B6/MEF) alone	None	0
Adenovirus type 5 (E1A-E1B)	None	40
Adenovirus type 5 (E1A-E1B)	6	30

^a Transformation was performed as described in Table 1.

David Spector) in place of SV40 DNA. The E1 region is sufficient to transform cells fully in vitro (20). Whereas CTL clone K11 abrogated SV40 DNA-mediated transformation when cultures were exposed on days 5 or 8 after transfection, the transformation by adenovirus type 5 DNA was not significantly affected (Table 2). These results show that the CTL clone K11 is specific for SV40 in its ability to search out and destroy the potentially emerging transformants.

DISCUSSION

SV40 T antigen mediates cell transformation and also provides a target for the immune effector mechanisms at the cell surface. Cell surface expression of T antigen is not limited to transformed cells only. By 24 h after infection of permissive cells, T antigen is detectable serologically in the nucleus as well as at the cell surface (19). In addition, virus-infected nonpermissive cells are lysed by SV40-specific CTL (14, 16), indicating that the expression of T antigen at the cell surface occurs during both productive and nonproductive virus infections as well as in fully transformed cells. The role, if any, that cell surface T antigen plays in either the transforming or the lytic cycle is not known.

The expression of T antigen at the cell surface provides a target for the immune effector mechanisms. That the cellular immune response affects the outcome of virus-induced carcinogenesis in vivo is well known (24). The results described in this study provide conclusive evidence that an interaction of a well-defined effector cell with a determinant on the surface of cells undergoing transformation will result in the abrogation of virus-induced transformation in vitro. This provides a glimpse of events that may actually be occurring in vivo. The CTL clone K11 used in our studies is specific for SV40 (3). The determinant that it recognizes maps in the amino-terminal half of the T antigen as cells transformed by SV40 DNA which contain the coding sequences between 0.65 and 0.37 map units and synthesize a 48,000-dalton amino-terminal T-antigen fragment are susceptible to lysis by K11 (27). Whether other CTL clones that recognize different determinants on the T antigen will also abrogate transformation of primary B6/MEF cells efficiently is not known.

Perhaps observations more relevant to our investigations have been made in studies which describe transformation of human B lymphocytes with Epstein-Barr virus. It has been reported that lymphocytes from individuals with a prior history of Epstein-Barr virus infection undergo transient viral transformation upon Epstein-Barr virus infection in vitro, followed by the regression of transformed cells (13). Regression is mediated by T lymphocytes (17, 29) with

FIG. 1. Cultures of B6/MEF cells were transfected with 1 µg of SV40 DNA (B, C, and D) or carrier DNA alone (A) as described in Materials and Methods. After DNA transfection, cultures were treated once (C) or three times (D) with CTL clone K11 as described in Table 1, experiment 2. The cultures were fed twice weekly and scored for transformed foci as described in Materials and Methods.

cytotoxic activity (12). Analogous observations have also been made in the lymphocytic choriomeningitis virus system which show that the lymphocytic choriomeningitis virus-specific CTL clones when reacted with lymphocytic choriomeningitis virus-infected syngeneic cells in vitro or transferred to lymphocytic choriomeningitis virus-infected mice in vivo inhibit virus replication (1, 2).

The activity of CTL clone K11 depends upon simultaneous recognition of T antigen and *H-2D^b* glycoprotein. In the *H-2^b* haplotype both *H-2K^b* and *H-2D^b* act as restriction elements in the recognition of SV40 T antigen by CTL (3, 11). Spontaneous mutations in *H-2D^b* glycoprotein which prevent association with SV40 T antigen do not occur frequently, since no colonies that survived exposure to K11 at early times after transfection expressed T antigen. The results also indicate that in *H-2^b* SV40-transformed cells there is no preferential association of *H-2K^b* glycoproteins with SV40 T antigen, since transformed colonies resistant to *D^b*-restricted K11 were not detected. We are now in the process of determining whether CTL clones that are *K^b* restricted or which recognize other determinants on the surface of SV40-transformed cells similarly inhibit transformation.

The approach described here should be useful in screening for viral mutants that are unable to insert T antigen into the cell membrane. Such mutants may elucidate the role of surface T antigen in the transformation process. These studies are in progress.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants CA 25000 to S.S.T. and CA 24698 to M.J.T. from the National Cancer Institute and in part by a grant to the Cancer Research Center.

We would like to thank Patricia Thompson for her excellent assistance in the preparation of this manuscript.

LITERATURE CITED

- Anderson, J., J. A. Byrne, R. Schreiber, S. Patterson, and M. B. A. Oldstone. 1985. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus and in vitro properties. *J. Virol.* 53:552-560.
- Byrne, J. A., and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. *J. Virol.* 51:682-686.
- Campbell, A. E., L. F. Foley, and S. S. Tevethia. 1983. Demonstration of multiple antigenic sites of the SV40 transplantation rejection antigen by using cytotoxic lymphocyte clones. *J. Immunol.* 130:490-492.
- Chang, C., R. Chang, P. T. Mora, and C.-P. Hu. 1982. Generation of cytotoxic lymphocytes by SV40-induced antigens. *J. Immunol.* 128:2160-2163.
- Chang, C., R. G. Martin, D. M. Livingston, S. W. Luborsky, C.-P. Hu, and P. T. Mora. 1979. Relationship between T-antigen and tumor-specific transplantation antigen in simian virus 40-transformed cells. *J. Virol.* 29:69-75.
- Gooding, L. R. 1982. Characterization of a progressive tumor from C3H fibroblasts transformed *in vitro* with SV40 virus. Immunoresistance *in vivo* correlates with phenotypic loss of *H-2K^b*. *J. Immunol.* 129:1306-1312.
- Graham, F. L., S. Bacchetti, R. McKinnon, C. Stanners, B. Cordell, and H. M. Goodman. 1980. Transformation of mammalian cells with DNA using the calcium technique, p. 3-26. *In* R. Baserga, C. Croce, and G. Rovera, (ed.), *Introduction of macromolecules into viable mammalian cells*. Alan R. Liss, Inc., New York.
- Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
- Klockman, H., and W. Deppert. 1983. Acylated simian virus 40-specific proteins in the plasma membrane of HeLa cells infected with adenovirus 2-simian virus 40 hybrid virus Ad2⁺ ND2. *Virology* 126:717-720.
- Knowles, B. B., M. Koncar, K. Pfizenmaier, D. Solter, D. P. Aden, and G. Trinchieri. 1979. Genetic control of the cytotoxic T cell response to SV40 tumor-associated specific antigen. *J. Immunol.* 122:1798-1806.
- Moss, D. J., A. B. Rickinson, and J. H. Pope. 1979. Long term T-cell mediated immunity to Epstein-Barr virus in man. III. Activation of cytotoxic T cells in virus infected leukocyte cultures. *Int. J. Cancer* 23:618-625.
- Moss, D. J., A. B. Rickinson, and J. H. Pope. 1978. Long term T-cell mediated immunity to Epstein-Barr virus in man. I. Complete regression of virus induced transformation in cultures of seropositive donor leukocytes. *Int. J. Cancer* 22:622-668.
- O'Connell, K. A., and L. R. Gooding. 1984. Cloned cytotoxic T lymphocytes recognize cells expressing discrete fragments of the SV40 tumor antigen. *J. Immunol.* 132:953-958.
- Pan, S., and B. B. Knowles. 1983. Monoclonal antibody to SV40 T antigen blocks lysis of cloned cytotoxic T cell line specific for SV40 TASA. *Virology* 125:1-71.
- Pretell, J., R. S. Greenfield, and S. S. Tevethia. 1979. Biology of simian virus (SV40) transplantation rejection antigen (TrAg). V. *In vitro* demonstration of SV40 TrAg in SV40 infected nonpermissive mouse cells by the lymphocyte mediated cytotoxicity assay. *Virology* 97:32-41.
- Rickinson, A. B., D. J. Moss, and J. H. Pope. 1979. Long term T-cell mediated immunity to Epstein-Barr virus in man. II. Components necessary for regression in virus infected leukocyte cultures. *Int. J. Cancer* 23:610-617.
- Rigby, P. W. J., and D. P. Lane. 1983. The structure and function of the simian virus 40 large T antigen. *Adv. Viral Oncol.* 3:31-57.
- Santos, M., and J. Butel. 1984. Antigenic structure of simian virus 40 large tumor antigen and association with cellular protein p53 on the surfaces of simian virus 40-infected and -transformed cells. *J. Virol.* 51:376-383.
- Shenk, T., and J. Williams. 1984. Genetic analysis of adenoviruses. *Curr. Top. Microbiol. Immunol.* 111:1-39.
- Soule, H. R., R. E. Lanford, and J. S. Butel. 1980. Antigenic and immunogenic characteristics of nuclear and membrane-associated SV40 tumor antigen. *J. Virol.* 33:887-901.
- Tegtmeyer, P., M. Schwartz, J. K. Collins, and R. Rundell. 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. *J. Virol.* 16:168-178.
- Tevethia, M. J. 1984. immortalization of primary mouse embryo fibroblasts with SV40 virions, viral DNA, and a subgenomic DNA fragment in a quantitative assay. *Virology* 137:414-421.
- Tevethia, S. S. 1980. Immunology of simian virus 40, p. 581-601. *In* G. Klein (ed.), *Viral oncology*. Raven Press, New York.
- Tevethia, S. S., D. C. Flyer, and R. Tjian. 1980. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). VI. Mechanism of induction of SV40 transplantation immunity in mice by purified SV40 T antigen (D2 protein). *Virology* 107:13-23.
- Tevethia, S. S., R. S. Greenfield, D. Flyer, and M. J. Tevethia. 1980b. Simian virus 40 (SV40) transplantation rejection antigen. Relationship to SV40 specific proteins. *Cold Spring Harbor Symp. Quant. Biol.* 44:235-242.
- Tevethia, S. S., A. J. Lewis, A. E. Campbell, M. J. Tevethia, and P. W. J. Rigby. 1984. Simian virus 40 specific cytotoxic lymphocyte clones localize two distinct TSTA sites on cells synthesizing a 48 kD SV40 T antigen. *Virology* 133:443-447.
- Tevethia, S. S., M. J. Tevethia, A. J. Lewis, V. B. Reddy, and S. M. Weissman. 1983. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). IX. Analysis of TrAg in mouse cells synthesizing truncated SV40 large T antigen. *Virology* 128:319-330.
- Thorley-Lawson, D. A., L. Chess, and J. L. Strominger. 1977.

- Suppression of *in vitro* Epstein-Barr virus infection. A new role for adult human lymphocytes. J. Exp. Med. **146**:495–508.
30. **Tooze, J.** 1980. Transformation by simian virus 40 and polyoma virus, p. 205–296. In J. Tooze (ed.), DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyl-transferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.