Immunization Against the Polyoma Virus-Induced Tumor-Specific Transplantation Antigen by Early Region Mutants of the Virus

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To investigate the relation between the polyoma tumor-specific transplantation antigen and the virus-coded proteins, mice were immunized by inoculation of a variety of viable polyoma virus mutants and then challenged with polyoma virus-induced tumors. Two classes of early region mutants were used. One class produces a normal small T-antigen and truncated middle and large T-antigens. The second class (*hr-t* mutants) forms a normal large T-antigen together with N-terminal fragments of small and middle T-antigens. All mutants, transforming as well as nontransforming, induced protection against polyoma virus tumors. However, there were quantitative differences between the mutants. The finding that an *hr-t* mutant could induce tumor rejection suggests that full-length middle and small T-antigens are not necessary for the induction of this response. Since intact middle T-antigen is the only virus-coded protein known to associate with the plasma membrane, the possibility must be considered that the polyoma virus tumor-specific transplantation antigen consists of cellular components.

Polyoma virus is ubiquitous in wild mice (30). It is highly oncogenic when inoculated into newborn mice that are not protected by maternal antibody. Nevertheless, under natural conditions it fails to induce tumors (24). The immune response of mice and other animals to polyoma virus and to polyoma virus-induced tumors has been studied in some detail (1-3, 14, 24, 30, 32, 35, 36). In immunologically competent animals, oncogenesis is prevented by a T-cell-dependent mechanism (1-3, 35, 36). Neonatal thymectomy (3, 29, 36) or anti-lymphocyte serum treatment (2, 35) increases the incidence of tumors in neonatally polyoma virus-infected mice and rats and permits tumor induction in mice at an age when they are otherwise resistant. The target of this highly efficient immune response, operationally designated as the tumor-specific transplantation antigen (TSTA), has not been defined chemically, nor has it been related to any of the known polyoma virus gene products.

Since TSTA is expressed *in vivo* by all polyoma virus-transformed cells and the viral early gene products are sufficient for the induction and maintenance of cellular transformation, TSTA is likely to be part of, or induced by, the polyoma virus early proteins. Three protein products of the early region are known (18). Large T-antigen, present mainly in the nucleus,

is necessary for lytic infection. Middle T-antigen, located in the plasma membrane, is required for transformation (17, 34). Small T-antigen, present mainly in the cytoplasm, has an unknown function. However, the presence of the DNA sequence coding for small T-antigen and middle T-antigen increases the spectrum of cells that can be infected by the virus (10). The apparent sizes of the three polypeptides are 100K, 55K, and 22K, respectively.

Two classes of mutants with deletions in the early region of the polyoma virus genome have been identified. The *hr-t* mutants have deletions in the area that codes exclusively for small and middle T-antigen (Fig. 1). NG-18, a prototype of these mutants, has a restricted host range for lytic growth and is transformation defective (4, 10). The second class of mutants have deletions affecting the over-lapping DNA sequences encoding middle and large T-antigen (Fig. 1). Their phenotype is variable (12, 13, 25, 26). Some of the variable mutants have a reduced growth rate in tissue culture, whereas others grow as well as the wild-type virus. Also their transforming ability varies. Some of these mutants have transformation properties indistinguishable from that of the wild type; others induce an either stronger or weaker transformation phenotype, whereas some do not transform at all.

FIG. 1. Map positions of polyoma virus early region mutants. The expression of the three early proteins, large, middle, and small T-antigens, requires splicing of RNA (9, 33). The positions of coding and intervening base sequences are shown in the figure. The cross-labeling indicates that the C-terminal parts of the sequences encoding large and middle T-antigen are translated in different reading frames (17). The sequences deleted or duplicated, respectively, by the mutants NG-18, *dl8*, *dl23*, *dl1013*, *dl1015*, and *ev1002* are indicated (4, 10, 12, 13, 25, 26).

In the present study, the two types of deletion mutants as well as the wild-type virus were used for immunization of mice. Subsequently, we compared the antiviral and anti-TSTA response of the immunized animals by using TSTA-positive, wild-type virus-induced tumors as the rejection targets.

MATERIALS AND METHODS

Non-polyoma virus tumors. SBfnHa is a mammary carcinoma that arose in a female CBA mouse foster nursed on a C3H female (22). S40C is a spontaneous mammary carcinoma that arose in an ACA female mouse (23). RBL-5 is a Rauscher virus-induced lymphoma of C57B1/6 origin (28). YWA and YBA are T-cell lymphomas induced by Moloney leukemia virus in an ASW and a CBA mouse, respectively (6, 21). P815 is a methylcholanthrene-induced mastocytoma of DBA origin (19).

Polyoma virus-induced tumors. SEBA is an undifferentiated sarcoma of CBA origin, SEBB is a CBA-derived fibrosarcoma, whereas SECA is a hair follicle tumor of ACA origin. All three tumors developed approximately 3 months after the intraperitoneal inoculation of newborn mice with wild-type polyoma virus. After explanation in vitro, the tumors were passaged in vivo by subcutaneous inoculation of mice of the original strain. The in vitro-cultured lines are designated with the postfix -TC. They were used for tumor rejection tests only after several months of in vitro culture. Confluent tumor cultures were tested repeatedly for virus production by plaque assay (26). SEBA-TC and SECA-TC, but not SEBB-TC, produced infectious virus. The T-antigen expression of the three tumors was checked by immunoprecipitation (17). The data in Fig. 2 show that SEBA expressed all three T-antigens. SEBB and SECA contained full-length small and middle T-antigens, together with truncated forms of large T-antigen. SEBB expressed a major large T-antigen species of approximately 70K and, in addition, a minor amount of full-length large T-antigen, whereas SECA produced a 39K polypeptide in addition to full-length large T-antigen.

Virus. Virus stocks were harvested from cultures of primary mouse kidney cells infected at low input

multiplicities. The virus titer was determined by both plaque assays and hemagglutinating ability. The ratio of infectivity and particle number was approximately 10^6 PFU per hemagglutination unit for all stocks (26). The wild-type polyoma virus was a plaque-purified derivative of the A-2 Pasadena large-plaque strain (8, 11) and the parent of the mutants *dl8*, *dl23*, *dl1013*, and *dl1015* (12, 13, 25, 26). Mutant NG-18 has been described previously (4, 10, 15). The mutant *ev1002* is an evolutionary variant with a duplication of approximately 170 base pairs, containing the origin of DNA replication (25a). The positions and sizes of the mutations are illustrated schematically in Fig. 1.

FIG. 2. T-antigen spectrum of the polyoma virus tumors SEBA-TC, SEBB-TC, and SECA-TC. Cultures of tumor cells were labeled with [35 S]methionine. Protein extracts were immunoprecipitated with rat anti-tumor (T) or control (C) serum and then separated by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide gels as described previously (26). Large (L-T), middle (M-T), and small (S-T) T-antigens induced in productively infected mouse cells are shown to the left. Molecular weight markers (200K, 92.5K, 69K, 49K, 46K, and 30K) are shown in lane MW.

TABLE 1. Specificity of polyoma virus-induced tumor immunity^a

Tumor line	Cell doses ^b inoculated	Mouse strain	Tumor takes ^c	
			Control mice	Immunized mice
SBfnHA	5 × 10 ³ , 5 × 10 ⁴	CBA	10/10	10/10
YBA	1 × 10 ³ , 1 × 10 ⁴	CBA	10/10	10/10
RBL-5	1 × 10 ³ , 1 × 10 ⁴	C57B1/6 × ASW	8/8	6/7
P815	1 × 10 ³ , 1 × 10 ⁴	A/Sn × DBA	8/8	8/8
S40C	5 × 10 ³ , 5 × 10 ⁴	ACA × CBA	9/9	10/10
SEBB	5 × 10 ⁴ , 1 × 10 ⁵	CBA	27/27	8/28
SECA	1 × 10 ³	ACA	4/5	1/8

^a Mice immunized with polyoma virus were challenged with two different cell doses of non-polyoma virus or polyoma virus tumors. All mice received 400 rads of whole body irradiation before the viable challenge.

^b No significant differences in tumor takes were observed for the two cell doses, and therefore the data were pooled.

^c (Number of mice dying with tumors)/(total number of mice).

HI tests. Portions (0.1 ml) of serially diluted antiserum were mixed with guinea pig erythrocytes (0.1 ml of a 1% guinea pig erythrocyte solution) and virus (0.1 ml of a 1:24 dilution of virus with an original titer of 1,000 hemagglutination units per ml) at 4°C. The reciprocal of the highest dilution that inhibited hemagglutination was designated as the hemagglutination inhibition (HI) titer.

Immunization procedure. Groups of mice were immunized weekly with a virus dose of 6 hemagglutination units. The HI titer was determined by using guinea pig erythrocytes. The first inoculation of 8- to 12-week-old mice was intraperitoneal, whereas the second through fifth inoculations were done subcutaneously and bilaterally. After the fifth week, the HI titers in the serum of immunized mice had reached a plateau (data not shown). Rejection tests were therefore performed on the sixth week.

Immune rejection tests. Before living tumor challenge, control and immunized mice received 400 rads of whole body irradiation. This proved necessary in previous studies (20), to distinguish between the non-specific boosting of natural resistance that follows the inoculation with many different tumors and a specific polyoma virus anti-TSTA response. The former, but not the latter, effect is abolished by whole body radiation. We have found that only the radioresistant response was specific for polyoma virus-induced tumors (T. Dalianis, manuscript in preparation). The viable tumor challenge was given 1 week after the last immunization, 1 day after the bleeding of the mice for HI tests. The cell doses were chosen near the mini-

mum cell number required for 100% tumor take in nonimmunized animals and determined individually for each tumor. This is important, since the resistance of the immunized animals is relative rather than absolute and can be overcome by increasing tumor cell doses (1, 31; unpublished results). Control and immunized mice were checked weekly for tumor growth, and the tumor diameters were measured. Tumor takes were evaluated finally 2 to 3 months after the viable challenge. By that time all tumors had grown to a minimum diameter of 15 mm, and the animals could be sacrificed. No tumors regressed after they had grown to 10 mm in diameter.

RESULTS

Specificity of tumor rejection after polyoma virus immunization. Mice immunized with wild-type polyoma virus were challenged with a panel of polyoma virus-induced and non-polyoma virus tumors. The two polyoma virus-induced tumors, SEBB and SECA, showed a significantly reduced incidence of takes in mice immunized with polyoma virus, as compared with untreated controls (Table 1). SEBB grew in 100% of the nonimmunized animals, but in only 29% of the polyoma virus-immunized mice. The corresponding figures for SECA were 80 and 12%, respectively. In contrast, the non-polyoma virus tumors SBfnHA, YBA, RBL-5, P815, and S40C showed no reduction of tumor takes after polyoma virus immunization. SEBB and SECA were therefore selected for analysis of the specific response against polyoma virus-induced tumors elicited by the inoculation of the various virus mutants (32).

Induction of immunity against polyoma virions and polyoma virus TSTA. Wild-type polyoma virus and the various mutants were compared for their ability to induce antiviral antibodies, measured by the HI technique. Pooled sera derived from groups of mice inoculated with wild-type or mutant virus were titrated. Figure 3A shows the median HI titer and the variations obtained in 4 to 7 separate experiments. The median HI titer of the sera of nonimmunized mice was 40. Wild-type polyoma virus, *d11013*, *d11015*, and *d18* induced high HI titers with a median value of 2,560 or more, whereas *d123*, *ev1002*, and NG-18 were less efficient. Sera from *d123*-infected mice had a median HI titer of 1,280. The corresponding value after the inoculation of *ev1002* and NG-18 was 320. When the NG-18 mutant was inoculated in doses 20 times larger, the HI titer rose up to 2,560 (data not shown). With the exception of the NG-18, further virus inoculations did not increase the HI titer.

Groups of CBA mice immunized with wild-type or mutant viruses were challenged with syngeneic SEBB tumor cells. Figure 3B shows the incidence of progressively growing tumors,

FIG. 3. Immunity against polyoma viruses and TSTA after titration of antiviral antibodies (A) of sera from mice inoculated with polyoma virus mutants or wild-type virus. The animals were challenged with 5×10^4 or 1×10^5 SEBB tumor cells. The formation of palpable tumors was scored (B) and was found to be independent of cell dose. The data were therefore pooled. The figures within the bars indicate the number of tumor takes per total number of mice within each group.

in the same groups as in Fig. 3A. The take incidence was high (96%) in untreated controls. Mice immunized with wild-type virus had a significantly reduced take incidence (32%), as expected. All virus mutants induced tumor protection, although at various degrees. The mutants *d1015*, *d18*, and *ev1002* induced a better protection than did mutants *d1013* and *d123*. The immunogenicity of NG-18 against TSTA was poor, but significant ($P < 0.025$, Fischer's exact

test). For some of the mutants there was a relationship between the antiviral antibody induction and the anti-TSTA response. However, two mutants, *d1013* and *ev1002*, deviated from this pattern, since *d1013* elicited a weaker, and *ev1002* elicited a stronger, anti-TSTA response than predicted from the antiviral antibody titer.

The anti-TSTA response against other tumor target cells was also investigated. The in vitro-cultured SEBA-TC, SEBB-TC, and SECA-TC lines were used for this purpose. We also included mice that had been immunized with 20 times more NG-18 virus than in the first experiment. Table 2 is a summary of the take incidence of the three tumors in control and immunized mice. In the controls the take incidence ranged between 84 and 100%. The wild type and all mutant viruses tested induced various degrees of protection. As a rule, all viruses induced better protection against the TC tumor lines (Table 2) than against the SEBB tumor (Fig. 3B). All three tumors were sensitive to the rejection induced by the wild type, *d1015*, and *d18* (Table 2) as described in Fig. 3B. Mutants *d123* and *d1013* were less efficient in their anti-TSTA induction, with the exception of the immunizing effect induced by *d123* against SECA-TC and by *d1013* against SEBB-TC.

The NG-18-immunized mice cannot be compared directly with the groups immunized with the other virus stocks, since a higher dose of virus particles was used. However, the incidence of takes was clearly reduced ($P < 0.01$, Fischer's exact test) for SEBA-TC and SECA-TC within the relatively small experimental groups.

DISCUSSION

Here we report the result of experiments on polyoma virus mutants with alterations in the early region with regard to their ability to induce syngeneic transplantation immunity against polyoma virus tumors. The specificity of the immunity elicited by inoculation with wild-type polyoma virus was reaffirmed by comparing a

TABLE 2. Take incidence of the in vitro-cultured polyoma virus tumor lines SEBA-TC, SEBB-TC, and SECA-TC in virus-immunized and control mice^a

Cell line	Cell dose inoculated ^b	Mouse strain	No. of mice with tumors/total no. of mice (%) after immunization with:						
			Controls	Wild-type virus	<i>d1013</i>	<i>d1015</i>	<i>d18</i>	<i>d123</i>	NG-18
SEBA-TC	1×10^5	CBA	36/40 (90)	7/32 (22)	9/26 (35)	1/27 (4)	5/30 (7)	13/29 (45)	3/10 (30)
SEBB-TC	1×10^5	CBA	21/25 (84)	1/25 (4)	0/18 (0)	0/18 (0)	0/10 (0)	2/7 (29)	6/9 (67)
SECA-TC	5×10^2	ACA	14/14 (100)	3/14 (21)	8/19 (42)	4/15 (27)	1/10 (10)	3/11 (27)	5/14 (36)
	1×10^3								

^a All mice received 400 rad total body irradiation prior to the viable challenge.

^b No significant difference in tumor takes was observed for the two cell doses, and therefore the data were pooled.

panel of polyoma virus and non-polyoma virus tumors (Table 1). A clear polyoma virus specificity was seen, but only when the mice received 400 rads of whole body irradiation before the tumor challenge. In non-irradiated, polyoma virus-immunized animals the take incidence of some non-polyoma virus tumors was also reduced. This effect was entirely radiosensitive (manuscript in preparation). It has also been shown previously in other systems (20) that irradiation before viable challenge can serve to discriminate between specific immunization and nonspecific boosting of host resistance to the viable challenge.

The anti-TSTA response induced by the wild-type virus and the mutants was analyzed. Initially, equal quantities of wild-type and mutant viruses were used for immunization in an attempt to compare the anti-TSTA responses induced by the different viruses. Immunization with wild-type virus protected a substantial fraction of the animals against the polyoma virus tumor cells. The early region mutants *dl1015*, and *dl8* induced an almost equally good protection, whereas *dl23* and *dl1013* were less efficient, with the exception of the protection induced by *dl1013* against SEBB-TC. The NG-18 mutant induced an extremely poor anti-TSTA response at the same virus dose level, but when 20 times more NG-18 virus was used for immunization the anti-TSTA response improved.

The anti-TSTA activity induced by the different mutants must be mainly related to the number of infected cells, but also must be related to the concentration of TSTA induced per cell. Since neither of these two parameters can be measured *in vivo*, we instead assayed antiviral antibody production, which is an indirect measure of virus growth *in vivo* (27). Our purpose was to study whether the differences in anti-TSTA response observed for the various mutants were correlated to the antiviral antibody titer, i.e., indirectly correlated to virus growth *in vivo*.

The mutants differed with regard to their ability to induce antiviral antibodies (Fig. 3A). Wild-type virus, *dl1013*, *dl1015*, and *dl8* produced a good antibody response, whereas *dl23* induced an intermediate response, and NG-18 and *ev1002* induced a poor response.

For some of the mutants there was a correlation between the antiviral (HI) and the anti-TSTA response. However, two mutants did not show this behavior. The mutant *dl1013* induced a high HI titer, but only a moderate tumor protection, whereas *ev1002* showed the opposite discordance. Interestingly, the latter mutant shows an analogous behavior *in vitro* (25a). In both permissive and semipermissive cells, its gene expression and DNA replication is more

efficient than that of wild-type virus. However, its growth is unbalanced, resulting in a reduced progeny yield.

NG-18 induced a low HI titer and only minimal tumor protection. The 20-fold increase in dose mentioned above raised the level of both.

Our results also show that the ability to elicit an anti-TSTA response is not dependent on transforming ability. TSTA can be expressed by nontransformed or abortively transformed cells, since tumor protection was induced by the transforming mutants *dl8*, *dl1013*, *dl23*, and *ev1002* as well as by the nontransforming mutants *dl1015* and NG-18.

It is noteworthy that the two mutants *dl23* and *dl1013*, which have overlapping deletions (Fig. 1) but different phenotypes, both elicited a reduced TSTA response.

The uniqueness of polyoma virus TSTA for polyoma virus-induced tumors suggests (but does not prove) that TSTA is coded by the viral genome, or, alternatively, induced by some viral function. Since it serves as a rejection target, TSTA must be expressed on the cell surface. Middle T-antigen is the only known virus-coded polypeptide associated with the plasma membrane of infected and transformed cells, and therefore it has often been speculated that it may be responsible for part or all of polyoma virus TSTA. All mutants we have tested, except NG-18, induced middle T-antigen bound to the plasma membrane (4, 10, 18; S. M. Dilworth, personal communication). The fact that the *hr-t* mutant NG-18 could induce an anti-TSTA response suggests, however, that middle and small T-antigens are not necessary for an anti-TSTA response. The possibility cannot be excluded, however, that unstable N-terminal fragments of small and middle T-antigens are synthesized by the NG-18-infected cells and contribute to the expression or induction of TSTA. Such fragments are unlikely to bind to the plasma membrane, since they lack the hydrophobic C-terminal portion of the antigen. Alternatively, large T-antigen, or a fragment of the protein, may constitute TSTA or may suffice to induce a cellular protein that serves as TSTA. Polyoma virus large T-antigen, however (18), in contrast to simian virus 40 large T-antigen (5), has not been identified at the cell membrane. Furthermore, Israel et al. (16) demonstrated that the C-terminal part of the large T-antigen is not essential for the expression of TSTA.

The resolution of question of whether any of the three T-antigens can serve as TSTA must await the availability of abundant quantities of purified antigen for immunization experiments. The present study suggests, however, that the T-antigens may not necessarily serve directly as a rejection target, but that they still may be impor-

tant for the induction or presentation of a cellular TSTA.

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