Use of SV40 to immunize against hepatitis B surface antigen: implications for the use of SV40 for gene transduction and its use as an immunizing agent

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We have described a novel gene transfer system, in which replication-incompetent, T antigen-deleted simian virus-40 (SV40) is used as the transduction vehicle. We report here successful immunization using such an SV40-derived viral vector. Hepatitis B surface antigen (HBsAg) cDNA was cloned downstream of two tandem SV40 early promoters to yield a T antigen-deficient SV40 derivative, SV(HBS). Cultured TC7 cells were exposed to SV(HBS), and expression of HBsAg was detected 24 h later by Northern blot and RT-PCR analysis. Immunochemistry and Western blot analysis were also performed 24 h after infection to detect expression of HBsAg. Once it was ascertained that we could express HBsAg in this way, we used SV(HBS) to elicit anti-HBs. SV(HBS) was injected intraperitoneally or

subcutaneously into mice every 4 weeks. These mice were bled every 2 weeks and their sera assayed for antibody activity against HBsAg and SV40. Production of anti-HBs was measured by ELISA and confirmed by Western blot analysis, both of which demonstrated significant levels of anti-HBs after the second injection. We also tested production of anti-SV40 antibodies by the ability of sera to neutralize SV(HBS) infectivity. We found no evidence of neutralization of SV(HBS) infectivity even after eight inoculations. Thus, replication-incompetent SV40 is itself not a strong antigen. Our data suggest that SV40-based transduction systems may be a useful vehicle for immunization and for other gene transfer applications when a need for multiple inoculations is anticipated.

Keywords: gene transfer; papovavirus; transgene expression

Introduction

We have reported a novel gene transfer system, in which SV40 is used as a transduction vehicle.^{1–3} SV40 infects resting as well as cycling cells, and is not known to be inactivated by human complement. Its DNA may integrate into the genome of target cells, or it may express its genes episomally, usually as a minichromosome.⁴ We have found that SV40 can transduce firefly luciferase (luc) activity stably for >12 months into murine bone marrow cells *in vivo*.³ We have also shown that SV40-derived vectors can transduce resting human peripheral blood mononuclear cells (PBMC) using two different reporter genes, HBsAg and luc.⁵

Hepatitis B virus (HBV) infection is endemic to many parts of the world and is one of the most important chronic virus infections, with over 300 million HBV carriers worldwide.⁶ Infection is transmitted most commonly from chronically infected mothers to their newborns, who consequently often develop chronic hepatitis B. Many adults exposed to HBV suffer acute infections, a minority of which do not resolve but rather progress to chronic hepatitis B.⁷ Long-term sequelae of chronic hepatitis B include cirrhosis and hepatocellular

carcinoma, the latter being one of the most common cancers in the world.^{7,8}

Since there is no effective treatment available for chronic hepatitis B, prevention is indispensable. Recombinant hepatitis B vaccines were the first recombinant vaccines approved for human use.⁹ They were produced by subcloning the HBsAg gene into expression vectors that would direct the synthesis of large quantities of HBsAg in the host cells. The use of both yeast and mammalian host cells has been evaluated clinically. Approximately 20 to 25% of mammalian cell-derived HBsAg is glycosylated, while yeast-derived HBsAg is not.¹⁰ Both preparations elicit protective antibodies comparably, and the yeast-derived vaccine has been preferred.⁹ However, up to 10% of adults immunized against HBV still fail to respond to the currently used recombinant vaccines.¹¹

HBsAg is also one of the most suitable markers to test the efficiency of an expression vector in mammalian cells. *In vivo*, HBsAg synthesis allows straightforward immunohistochemical detection of HBsAg gene expression in animals. HBsAg also elicits antibodies, permitting indirect demonstration of effective transduction by measuring antibody activity.

We examined the ability of the SV40-vector system to express a protein that would induce an immune response and as such, act as a vaccine. We report successful use of this gene delivery system to immunize mice against HBsAg. Significant levels of anti-HBs serum antibodies were found after the second inoculation. Importantly, we also found that the large T antigen (Tag)-deleted SV40-

vector itself is not detectably immunogenic, even after eight inoculations.

Results

SV(HBS)

The principles used in the construction of Tag-deleted, replication-incompetent SV40 derivative viruses bearing transgenes such as hepatitis B surface antigen gene, SV(HBS), have been described.² Methods used to construct and propagate SV(HBS), as well as the specifics of the HBsAg gene included, are described in Materials and methods. A map of the SV(HBS) genome is shown in Figure 1.

Detection of HBsAg transcript in TC7 cells infected with SV(HBS)

TC7 cells were infected with SV(HBS) and control cells were mock-infected. Total RNA was extracted from infected cells, electrophoresed, transferred to a nylon membrane, and hybridized with a cDNA probe for the HBsAg gene. Expression of HBsAg was detected in SV(HBS)-infected cells but not in mock-infected cells (Figure 2). Two transcripts were observed: a 1 kb HBsAg transcript, plus a larger mRNA of 4.6 kb. The latter most likely represents read-through transcription, which we often observe in this system. As control, the same blot was stripped and hybridized with a cDNA probe for GAPDH, which showed expression from both treatment groups (Figure 2).

Expression of HBsAg was confirmed by RT-PCR analysis. Total RNA was extracted from SV(HBS)-infected and SV(luc)-infected TC7 cells and used as a template for RT-PCR. RT-PCR negative control was also performed without RNA template. The expected product of 438 bp was identified only from the cell sample infected with

Figure 1 Map of SV(HBS). This recombinant viral genome is excised from pSV5(HBS) as a NotI fragment, and then recircularized (see Materials and methods). It contains two head-to-tail SV40 EP followed by HBsAg gene and the SV40 polyadenylation signal.

Figure 2 Expression of HBsAg gene in TC7 cells. TC7 cells were transduced with SV40 derivative virus containing HBsAg gene (SV(HBS)) at MOI of 10. Mock-infected cells were treated similarly in control preparation without the virus. RNA was harvested from these cultures, 24 h later, then electrophoresed, blotted and hybridized with HBsAg probe (see Materials and methods). The second HBsAg transcript represents a read-through mRNA, as we have seen in many cases in this system. Its size is indicated. The same filter was stripped and reprobed with a control cDNA probe (GAPDH).

SV(HBS). It was not observed when RNA was pretreated with RNase before RT-PCR. RNA from TC7 cells infected with an unrelated SV40-derivative virus, SV(RGP) (see Materials and methods), was negative for HBsAg transcript (Figure 3).

Detection of HBsAg in TC7 cells in vitro

To determine whether detectable levels of HBsAg were produced in cultured cells on infection with SV(HBS), TC7 cells were infected at a MOI of 10. Control cells were mock-infected. SV(HBS)-infected and mock-infected cells were harvested 24 h later. Cell lysates were electrophoresed, blotted, and treated with anti-HBs rabbit polyclonal antiserum, followed by HRP-conjugated goat anti-rabbit antibody. HBsAg was detected by chemiluminescence in SV(HBS)-infected cells but not in mock-infected cells (Figure 4). HBsAg was also detected by immunostaining as described elsewhere.⁵

The sizes of the HBsAg protein bands produced by infecting with SV(HBS) were slightly different from the sizes of the bands detected within recombinant HBsAg. These differences may reflect different post-translational modifications (eg glycosylation). The presence of multiple bands may reflect the tendency of different preparations of HBsAg that were prepared differently to undergo variable oligomerization.

Detection of anti-HBs in mouse serum

To determine whether the SV40-derived virus could produce HBsAg *in vivo* and therefore elicit detectable anti-HBs in mice, BALB/cJ mice were treated in three groups of five mice each: intraperitoneal (i.p.) inoculation of 10^8

Figure 3 RT-PCR amplification of HBsAg transcript in TC7 cells. TC7 cells were transduced with SV(HBS) at a MOI of 10. Control cells were mock-infected or were infected with a different SV40-derived virus (SV(RGP)), containing the gene for the major glycoprotein of rabies virus, but lacking HBsAg. RNA was harvested from these cultures 24 h after infection. An aliquot of RNA from SV(HBS)-infected cells was treated with RNase before RT-PCR. RT-PCR was performed using primers within the HBsAg gene (see Materials and methods). RT-PCR negative control was performed without the RNA template. Molecular size markers (pGEM markers; Promega) are shown on the left.

IU per mouse, subcutaneous (s.c.) inoculation of 10^8 IU per mouse, and vehicle (DMEM/2% FBS) only. Mice were bled every 2 weeks to collect sera. Inoculation was done after bleeding, every fourth week. Each serum sample was tested for antibody to HBsAg by ELISA. Levels of serum anti-HBs were increased significantly following the second injection, and remained elevated in both i.p.- and s.c.-injected mice (Figure 5).

The antibody, so produced was also characterized by Western blot analysis using commercially supplied HBsAg (Scantibodies Laboratories, Santee, CA, USA) (Figure 6).

In these blots, the murine antisera, as well as the rabbit antiserum, all recognized the 24 kDa recombinant protein. Higher molecular size derivatives or oligomers present in this recombinant protein preparation were also recognized by the control rabbit antiserum but not by sera from the mice receiving SV(HBS).

Neutralizing antibody vs SV40 is not detectable

We tested the same sera to see whether SV40-derived virus itself was an effective antigen *in vivo*. SV(HBS) was incubated with the same murine sera that tested positively for anti-HBs, and then used to infect TC7 cells. The treated cells were tested for SV40 infection by *in situ* PCR.

Figure 4 Expression of HBsAg in TC7 cells transduced with SV40 derivative virus containing HBsAg gene. SV(HBS) were used to transduce TC7 cells *in vitro* at a MOI of 10. Mock-infected cells were treated similarly in control preparation without the virus. Protein lysate was harvested from these cultures 24 h after infection (see Materials and methods). Sixty micrograms of cell lysate was electrophoresed by SDS-PAGE, blotted, then treated with anti-HBs rabbit polyclonal antibody followed by HRP conjugated goat anti-rabbit antibody. Signal was detected by chemiluminescence assay.

This assay, performed on microscope slides of virus-treated cells, allows direct visualization and both reproducible and rapid enumeration of SV(HBS)-infected cells. Sera from i.p.- and s.c.-inoculated mice did not neutralize SV40 infectivity differently from prebleed sera, even after eight inoculations (Figure 7). Control recipient sera, similarly, failed to neutralize SV40 infectivity. Simultaneously, rabbit antiserum to SV40 (diluted 1:400, see Materials and methods) neutralized >94% of virus infectivity, as our positive control. Pre- and post-inoculation sera from mice that received SVluc failed to neutralize SV(HBS) (not shown).

Discussion

Nonreplicating recombinant viruses have been generated for the purpose of vaccination, principally using poxviruses for veterinary applications. Most notably, vaccinia virus based-recombinant vaccines have been used against, for example, vesicular stomatitis, influenza, and rabies viruses.¹²⁻¹⁶ Additional poxviral vectors have also been used for immunization, including fowlpox,¹⁷⁻¹⁹ canarypox,²⁰ capripox²¹ and pigeonpox.²² In humans, canarypox-based vaccines expressing antigens from rabies virus²³ and HIV^{24,25} have been shown to be safe and immunogenic and other canarypox virus-based vaccines are being assessed.

Several recombinant vaccinia viruses have been con-

Figure 5 Production of antibody against HBsAg in mice. SV(HBS) (10^8 IU) were used to inject mice every 4 weeks; five each of control, intraperitoneally and subcutaneously injected mice. These mice were bled every 2 weeks. Serum samples were diluted to 1:10 and antibody versus HBsAg were measured by ELISA assay (see Materials and methods). Individual serum samples were averaged for each treatment group and time point, $\pm$ s.e.m. IP; intraperitoneal injection, SQ; subcutaneous injection. *, $P < 0.05$, compared with control sera drawn at the same time point. Arrows indicate when SV(HBS) or control injection occurred after bleeding.

structured that direct the synthesis of HBsAg *in vivo*.^{26,27} Chimpanzees infected with vaccinia virus-HBV recombinant were protected against HBV challenge.²⁸ Recombinant adenovirus also produced HBsAg *in vitro*¹³ and elicited anti-HBs in rabbits²⁹ and hamsters.³⁰

The HBV genome consists of a small (approximately 3.2 kb), partially double-stranded, circular DNA molecule.³¹ HBsAg is made up of a group of viral structural proteins that constitute the principal components of subviral particles, filaments and the viral envelope.^{25,31} The HBsAg gene is one of the four major open reading frames (ORF). It contains three in-frame translation initiation codons that divide the gene into three coding regions known as preS1, preS2 and S. The three polypeptides produced are known as the major (S), middle (preS2 + S), and large (preS1 + preS2 + S) proteins. The major S protein is the most abundant form, and exists as a glycoprotein of 27 kDa (GP27) and as a nonglycosylated 24 kDa protein (P24). PreS1(P39/GP41) proteins and preS2 (GP33/GP36) proteins constitute approximately 2% and 15% of the HBsAg proteins produced from this ORF.¹⁰ Due to high cysteine content, these HBsAg proteins form mixed dimers or oligomers after dissociation with mercaptoethanol, which may yield complicated patterns and slower migration on SDS-PAGE.²⁶ Repeated freezing and thawing can also cause spontaneous aggregation. This tendency to mixed oligomerization may

Figure 6 Characterization of antibody against HBsAg elicited by SV(HBS). Commercial HBsAg was electrophoresed on SDS-PAGE gel and blotted. Membranes were hybridized to serum from mice inoculated with SV(HBS), and then to HRP-conjugated sheep anti-mouse Ig. Signal was detected by chemiluminescence assay. IP; intraperitoneal injection, SQ; subcutaneous injection.

Figure 7 Production of antibody against SV40 in mice. SV(HBS) was incubated with mice sera diluted to 1:100, before infection to TC7 cells. The proportions of TC7 cells infected was determined by *in situ* PCR (see Materials and methods). IP; intraperitoneal injection, SQ; subcutaneous injection. Within each group, the fraction of infected cells was not significantly different in comparing prebleed and post-inoculation samples. Rabbit anti-SV40 serum (kindly provided by Dr JS Butel) served as the positive control. This serum was tested at a 1:400 dilution, and the fraction of cells infected following treatment with this antiserum was significantly different ($P < 0.01$) from all other groups tested.

account for the diverse banding patterns noted in our Western blots, including slower migration seen in some of our studies.

Different methods of preparation may be reflected both by different banding patterns seen when the same sera were used to probe HBsAg produced by TC7 cells and HBsAg produced by recombinant methods in bacteria (eg Figure 5). The fact that HBsAg delivered to eukaryotic cells may differ somewhat in structure from HBsAg derived from bacteria may also account for the differences in banding patterns seen when recombinant HBsAg is probed by our mouse antisera, compared to commercial rabbit antisera.

SV40 is a small (5.2 kb) nonenveloped double-stranded DNA virus of the papilloma virus group. A number of features of SV40 are advantageous for use as a gene transfer vehicle: (1) It infects and express its genes in cells from variety of tissues and of different animals. (2) Its genome may persist in infected cells, either by integration or as a minichromosome.⁴ (3) Live, wild-type SV40 was an unrecognized contaminant in early lots of polio vaccines, and was inadvertently administered to many polio vaccine recipients. However, extensive epidemiologic studies on polio vaccine recipients have not shown any significant adverse effect of live wild-type SV40 in humans.^{32,33} (4) In addition, as shown in the present work, the administered SV40 virus particle itself elicits no detectable neutralizing antibody response by itself. Animals in which the virus replicates may produce antibodies against these proteins. In the absence of virus replication, we did not detect antibodies capable of neutralizing the virus' infectivity.

SV40 encodes five major proteins, including three structural genes and large and small T antigens.³⁴ The transforming property³⁵ and the principal target of immunity vs SV40-infected cells is the large T antigen.³⁶ T antigen is necessary for late virus gene expression and for virus DNA replication. However, we have found that T antigen-deficient SV40 can be grown easily to a relatively high titer ($>10^9$ infectious units per milliliter) in COS cells, which supply the missing T antigen *in trans*.^{1,2} (5) The efficiency of transduction and level of expression are known to be very high. Thus, using HBsAg, we examined the use of this SV40 system for the introduction of genes *in vitro* and *in vivo*.

The purpose of these studies was to test the ability of SV40 to deliver DNAs encoding important antigens, such as HBsAg. Our data show that SV(HBS) elicited high levels of antibody against HBsAg. Mice receiving a single inoculation did not induce detectable antibody within 4 weeks. While additional bleeds at later times may have allowed us to detect serologic immune responses, a second inoculation was performed 4 weeks after the first and it elicited detectable levels of antibody. Anti-HBs antibody activity in immunized mice remained above control and prebleed levels thereafter. Antibody activity increased with additional inoculations. We did not test cell-mediated immunity.

Our data show no evidence of neutralizing antibody against SV40. We used BALB/c mice, which have been shown to produce high levels of antibody when inoculated with wt SV40.^{37,38} Most antibody responsiveness against wt SV40, and the most studied antibody responses, are against Tag.^{39,40} Antibody activity against the structural proteins of SV40 is most readily measured

by virus neutralization assays, similar to those done here. In some cases, wt SV40 and related papovaviruses have been reported to elicit neutralizing antibodies, usually at low levels.⁴¹ However, these responses are generally small in comparison to antibody responses to Tag⁴² and the extent to which antibody responses vs virus structural proteins may require virus replication and/or Tag expression is not clear.

We found no evidence of such antibodies in our studies. SV40 is not phagocytosed like other large particulates. Rather, the virus interacts with a cell membrane receptor, thought to be class I MHC,^{43,44} to yield an endocytic vesicle that goes directly to the nucleus, where the virus uncoats.⁴⁵ Antigen processing in phagolysosomes is not clearly documented for SV40. This observation, together with the lack of Tag and the consequent lack of late gene expression in infected cells, may account for the absence of detectable antibody responses to capsid antigens.

In considering the potential for Tag-deleted SV40 as a gene transfer vehicle, the most important operational issues to be resolved regarding immunogenicity center on the antigenicity of infected cells and the antigenicity of the inoculated virus particle. In the one case, immunogenicity of infected cells will limit the longevity of transgene expression in immunocompetent hosts. In the other, immune responses against the virus particle may restrict the potential for multiple inoculations. Our studies to date suggest that Tag-deficient SV40 may be useful as a gene transfer vehicle on both accounts.

Materials and methods

Cell lines

Two monkey cell lines were used: COS-7 cells (ATCC, Bethesda, MD, USA) which contain an origin-defective SV40 genome, and TC7 cells which do not. Both cell lines were maintained by serial passage in Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA).

Mice

Retired female bleeders, BALB/cJ (Jackson Laboratories, Bar Harbor, ME, USA), were fed and housed in accordance with American Association for Accreditation of Laboratory Animal Care standards.

Generation of recombinant SV40

The HBsAg gene was cloned into pSV5, which yielded pSV5(HBS). The details of the construction of this plasmid is described elsewhere.³ Briefly, pSV5 consists of the SV40 genome, minus the T antigen gene, plus a polylinker immediately downstream of two tandem SV40 early promoters (EP). This viral genome was cloned, using an engineered *NotI* site in the polylinker, into pGEM13. A 739 bp DNA fragment containing the coding sequences of the HBsAg major protein gene (see Discussion) was amplified by polymerase chain reaction (PCR) using oligonucleotide primers that contained orienting restriction sites; HBVS1 (5'-TTCTCGAGGAT TGGGGACCC-3') and HBVS2 (5'-GGACCGCGGCTC TTTGTTTTGTTA-3'). This HBsAg DNA encoded a 24 kDa protein of 226 amino acids.

The PCR product was digested with *XhoI* and *SacII* and

subcloned directionally to the pSV5 polylinker. Recombinant SV40 derivative virus, SV(HBS), was produced by excising the recombinant virus genome from pSV5(HBS) with *NotI*, band-purifying it, then recircularizing the modified viral genome, and transfecting it into packaging cell line (COS-7 cells) (Figure 1). The anticipated transcript length for this transgene is 1 kb, including the untranslated 3' end of the HBsAg transcript which was joined to 0.2 kb of untranslated SV40 sequences before the polyadenylation signal. The entire SV(HBS) virus genome is 3.6 kb.

Two weeks later, crude virus was obtained by three rounds of freezing–thawing the cells, followed by 2.5 min sonication. This lysate was used to produce working stocks of virus by infecting more COS-7 cells.

Infection, 2 h at room temperature, was done in a small volume of culture medium (DMEM/2% FBS). After infection, more medium was added and cultures were incubated for 2 weeks at 37°C, 7% CO₂ until approximately 50% of cells were infected as judged by cytopathic effect. Virus was harvested by freezing–thawing and sonication, as described above.

SV(RGP) was used as a control virus in some of these studies. It lacks HBsAg. Instead, SV(RGP) contains the gene for the major glycoprotein antigen of rabies virus (a gift from Dr H Koprowski, Thomas Jefferson University, PA, USA), cloned downstream from the cytomegalovirus intermediate–early promoter. Production and expansion of SV(RGP) stocks was carried out similarly to that for SV(HBS). SVluc, an additional control virus used in some of our studies, contains the firefly luciferase transgene instead of HBsAg. Its production and characteristics have been published.^{2,3}

Measuring infectivity of SV(HBS) by in situ PCR

Virus was titrated using TC7 cells, by *in situ* PCR, an approach we devised specifically to measure the infectivity of replication-incompetent DNA viruses.⁴⁶ Appropriate dilutions of SV(HBS) in DMEM/2% FBS were used to infect TC7 cells in 2.5-cm wells. Each well was layered with 100 µl of diluted virus stock and 400 µl of DMEM/2% FBS, and was incubated for 2 h at room temperature, shaking. More medium was added and cultures were incubated for 24 h more. Cells were trypsinized and placed on 3-amino-propyl-triethoxy-silane treated Teflon slides (Erie Scientific, Erie, PA, USA). Slides were fixed with 4% paraformaldehyde, then treated with proteinase K (6 µg/ml). Each slide was covered with PCR cocktail (250 mM each of dNTP, 1 µM each of primer, 0.1 U/µl of *Taq* polymerase, 500 mM KCl, 100 mM Tris pH 8.3, 15 mM MgCl₂ and 0.01% gelatin). The primers were BP3-1 (5'-ACTGTGACTGGTGTGAGCGCTG-3') and BP3-2 (5'-TGGACCCCAATGTCTGGGGTC-3'). The slide was covered with cover slips, sealed with nail polish, and incubated as follows: 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min for 35 cycles (PT-100; MJ Research, Watertown, MA, USA). After removing the coverslip, the slide was hybridized (50% formamide, 2×SSC, 10×Denhardt's solution, 1 mg/ml salmon sperm DNA, 0.1% SDS, 32 mM 5' biotinylated probe) overnight at 37°C. The color of the slide was developed by incubation at 37°C with 330 mg/ml streptavidin-peroxidase and then with a reaction solution (50 mM sodium acetate pH 5.0, 0.15% H₂O₂ and 5% 3-amino-9-ethyl-carbazole (Sigma, St Louis, MO, USA).

Detection of HBsAg transcript by Northern analysis

TC7 cells were infected with SV(HBS) at MOI of 10. Twenty four hours later, total RNA was prepared from cultured cells (RNAzolB; Molecular Research Center, Cincinnati, OH, USA), and electrophoresed (10 µg per lane) in formaldehyde gels, blotted to nylon membrane (Amersham, Arlington Heights, IL, USA). *XhoI* to *SacII* HBsAg fragment was labeled with α³²P-dCTP (DuPont NEN) for DNA probe. The membrane was baked for 2 h at 80°C, prehybridized and hybridized at 42°C (solutions include 50% formamide, 0.1% SDS), and washed stringently (final wash: 0.1×SSC, 0.1% SDS, 50°C). Binding of radiolabeled probes was visualized by autoradiography.⁴⁷

Detection of HBsAg transcript by RT-PCR

Total RNA from cultured cells was obtained as above. PCR was done in 50 µl containing 1 µg of total RNA, 1 µM each of primers HBVS3 (5'-CTAGACTCGTGG TGGACTTC-3') and HBVS4 (5'-CACTAGTAACT GAGCCAGG-3'), 0.1 U/µl each of AMV reverse transcriptase and *Tfl* DNA polymerase (Promega, Madison, WI, USA), 0.2 mM each of dNTP, 1 mM MgSO₄ and AMV/*Tfl* reaction buffer (Promega, Madison, WI, USA). It was incubated as follows: 45 min at 48°C, 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 1 min.

Detection of HBsAg protein by Western blot analysis

Cells were lysed with lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µg/ml pepstatin). Protein lysate was loaded on a 12% SDS-polyacrylamide gel, electrophoresed and blotted to a PVDF membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was blocked with 5% skim milk for 2 h, treated with rabbit polyclonal anti-HBs (1:1000 in 1% BSA/PBST; Accurate Chemical and Scientific, Westbury, NY, USA) overnight at 4°C, and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000 dilution with 1% BSA/PBST; Transduction Laboratories, Lexington, KY, USA) for 2 h at room temperature. Signal was detected with chemiluminescence reagent (DuPont NEN, Wilmington, DE, USA).

Detection of anti-HBs by enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated with 1 µg per well synthetic peptides (surface antigen peptide 28)⁴⁸ in PBS/10% FBS. Fifty microliters of diluted serum samples (1:10 in PBS/10% FBS) were transferred into corresponding wells for overnight incubation at 4°C. Each well was washed six times with PBS, treated with HRP-conjugated goat anti-mouse Ig (1:1000 dilution). The plates were incubated for 1 h at 37°C, washed six times in PBS and then substrate (1.5 mg/ml *o*-phenylenediamine dihydrochloride in 0.05 M phosphate-citrate/0.014% H₂O₂) added. Spectrophotometric readings at 450 nm were taken after 15 min of color development (Dynatech MR5000 ELISA plate reader, Alexandria, VA, USA).

Detection of anti-HBs by Western blot analysis

The above protocol for Western blotting was applied. Purified HBsAg protein (0.4 µg) (Scantibodies Laboratories) per lane was electrophoresed (SDS-12%

PAGE) and blotted to PVDF membrane (Schleicher and Schuell), treated with test mouse serum (1:50 in 1% BSA/PBST) and then to HRP-conjugated sheep anti-mouse antibody (1:5000 in 1% BSA/PBST; Amersham). Signal was detected with chemiluminescence reagent (DuPont NEN).

Testing for neutralizing activity versus recombinant SV40 by *in situ* PCR

One microliter of SV(HBS) (10^9 IU/ml) was incubated with 99 μ l of each mouse serum (1:100 dilution in DMEM/2% FBS) for 2 h at 37°C. This was added to TC7 cells in a 60 mm dish containing 400 μ l of DMEM/2% FBS for *in situ* PCR (described above) to deduce the proportion of infected TC7 cells.

Anti-SV40 serum

Rabbit anti-SV40 serum was the kind gift of Dr J Butel (Baylor College of Medicine). It is capable of neutralizing >50% of wt SV40 plaque forming activity at dilutions >1:400, the dilution used in our virus neutralization assay.

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