Initiation of Autoimmunity to the p53 Tumor Suppressor Protein by Complexes of p53 and SV40 Large T Antigen

By Xingwen Dong, Kimberly J. Hamilton, Minoru Satoh, Jingsong Wang, and Westley H. Reeves

From the Departments of Medicine and Microbiology/Immunology, and UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7280

Summary

Antinuclear antibodies (ANAs) reactive with a limited spectrum of nuclear antigens are characteristic of systemic lupus erythematosus (SLE) and other collagen vascular diseases, and are also associated with certain viral infections. The factors that initiate ANA production and determine ANA specificity are not well understood. In this study, high titer ANAs specific for the p53 tumor suppressor protein were induced in mice immunized with purified complexes of murine p53 and the Simian virus 40 large T antigen (SVT), but not in mice immunized with either protein separately. The autoantibodies to p53 in these mice were primarily of the IgG1 isotype, were not cross-reactive with SVT, and were produced at titers up to 1:25,000, without the appearance of other autoantibodies. The high levels of autoantibodies to p53 in mice immunized with p53/SVT complexes were transient, but low levels of the autoantibodies persisted. The latter may have been maintained by self antigen, since the anti-p53, but not the SVT, response in these mice could be boosted by immunizing with murine p53. Thus, once autoimmunity to p53 was established by immunizing with p53/SVT complexes, it could be maintained without a requirement for SVT. These data may be explained in at least two ways. First, altered antigen processing resulting from the formation of p53/SVT complexes might activate autoreactive T helper cells specific for cryptic epitopes of murine p53, driving anti-p53 autoantibody production. Alternatively, SVT-responsive T cells may provide intermolecular–intrastructural help to B cells specific for murine p53. In a second stage, these activated B cells might themselves process self p53, generating p53-responsive autoreactive T cells. The induction of autoantibodies during the course of an immune response directed against this naturally occurring complex of self and nonself antigens may be relevant to the generation of specific autoantibodies in viral infections, and may also have implications for understanding the pathogenesis of ANAs in SLE. In particular, our results imply that autoimmunity can be initiated by a "hit and run" mechanism in which the binding of a viral antigen to a self protein triggers an immune response that subsequently can be perpetuated by self antigen.

Certain antinuclear antibodies (ANAs)[1] are specific markers for autoimmune disease subsets such as SLE, scleroderma, or polymyositis (1). Many of the antigenic targets of ANAs are multiprotein complexes or complexes of proteins with nucleic acids, and it has been suggested that the particulate nature of these antigens might be important for their antigenicity (2). In some respects, the immune responses to particulate autoantigens in SLE may be analogous to immune responses to viral particles. Viral particles are taken up and processed by APCs as a unit, and T cells specific for a single polypeptide component of a viral particle may provide help to B cell clones specific for several components of the same particle (3–9). This has been referred to as "intermolecular–intrastructural help" (10). In the special case of a complex consisting of both foreign and self antigens, T cells specific for nonself components might drive the production of autoantibodies to the self components (11).

Another way in which a complex of self and nonself antigens might induce autoimmunity is by altering the processing of a self antigen, with activation of T cells responsive to minor "cryptic" epitopes to which tolerance is incomplete

[1] *Abbreviations used in this paper:* AcMNPV, *Autographa californica* nuclear polyhedrosis virus; ANA, antinuclear antibody; MMB, mixed micelle buffer; Sf-9, *Spodoptera frugiperda* ovary cell line; SVT, SV40 large T antigen.

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(12–14). In view of recent evidence that antigen processing can be influenced greatly by protein–protein interactions, such as dimerization (15, 16), it is conceivable that a nonself antigen could alter the processing of a bound autoantigen by APCs, thereby stimulating T cells specific for cryptic self epitopes. In the present studies, we have examined the hypothesis that complexes of self and nonself antigens might trigger autoimmunity. We report that autoimmunity to the p53 tumor suppressor protein can be induced in BALB/c mice immunized with complexes of p53 and the SV40 large T antigen (SVT). Although p53/SVT complexes were essential for inducing autoimmunity, p53 alone was sufficient to maintain it, suggesting that the binding of SVT to p53 alters antigen processing and activates T cells responsive to cryptic epitopes of p53 which drive autoantibody production.

Materials and Methods

Cell Lines and Viruses. The Sf-9 (*Spodoptera frugiperda* ovary) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and maintained in Grace's insect tissue culture medium supplemented with 3.3 g/l TC yeastolate, 3.3 g/liter lactalbumin hydrolyzate (from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility), 10% fetal bovine serum, and penicillin/streptomycin (TNM-FH medium). Wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) was obtained from Dr. Elmer M. Price (University of North Carolina at Chapel Hill). The recombinant baculoviruses vEV55SVT (directing the expression of full-length SVT) and vEV55p53 (directing the expression of full-length wild-type murine p53) were provided by Dr. L. K. Miller (University of Georgia, Athens, GA) (17).

Monoclonal Antibodies. Hybridoma cells producing mAbs specific for SVT (PAb 101, IgG2a of BALB/c origin) (18) and p53 (PAb 122, IgG2b of BALB/c origin) (19) were obtained from the ATCC. The mAbs were partially purified from culture supernatant by ammonium sulfate precipitation. For some experiments, mAbs were purified from hybridoma culture supernatants onto protein A–Sepharose beads (Pharmacia, Piscataway, NJ). Isotype control mAbs 162 (IgG2a anti-Ku) and N3H10 (IgG2b anti-Ku) have been described previously (20).

Cell Labeling. Sf-9 cells were infected with baculoviruses at a multiplicity of infection of 20 as described (21), incubated for 24 h at 27°C, and metabolically labeled for an additional 18 h at 27°C in methionine-deficient TNM-FH medium containing 5% dialyzed fetal bovine serum, 3% regular TNM-FH medium (containing methionine) and 25 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Translabel; ICN, Costa Mesa, CA). The cells were detached by pipetting gently, collected by centrifugation, washed once with PBS, and sonicated at 5×10^5 cells/ml in ice-cold lysis buffer containing 0.15 M NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.3% NP-40, 0.5 mM PMSF, and aprotinin (0.3 trypsin inhibitor units/ml). The cell lysate was cleared by centrifuging twice for 10 min each at 11,000 *g* before immunoprecipitation.

Immunoprecipitation. PAb 101 or PAb 122 culture supernatant 100–300 μl was added to 200 μl (10^5 cell equivalents) of radiolabeled Sf-9 cell extract for 1.5 h at 4°C. For mouse sera, 10 μl of rabbit anti–mouse IgG antibodies (1 mg/ml, provided by Dr. Robert Eisenberg, University of North Carolina, Chapel Hill, NC) plus 1–5 μl of mouse serum was added to the same volume of cell extract. After centrifuging 15 min at 11,000 *g*, protein A–Sepharose beads (30 μl of a 50% slurry in water) were added for an additional 2 h at 22°C. Unless otherwise indicated, the beads were washed twice with mixed micelle buffer ([MMB] 150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.25 M sucrose, 0.5% SDS, 2.5% Triton X-100) at 4°C, and then with NET buffer (0.15 M NaCl, 2 mM EDTA, 50 mM Tris, pH 7.5). In some cases, the immunoprecipitates were washed twice with 50 mM Tris, pH 7.5, 2 mM EDTA, 0.3% NP-40 containing NaCl at 0.15, 0.5, or 1.5 M NaCl, or once with the same buffer containing 1.5 M NaCl, then with MMB, and then with NET buffer. Immunoprecipitated proteins were analyzed on 10% SDS-polyacrylamide gels which were fluorographed, dried, and exposed to x-ray film (22).

In other experiments, human K562 (erythroleukemia) and murine SP2/0 (nonsecreting myeloma) cells were labeled with [^{35}S]methionine and cysteine exactly as described (20). Extracts of the cells were immunoprecipitated using preimmune and immune sera from the mice immunized with p53/SVT complexes, p53, or SVT. The immunoprecipitates were washed with MMB and NET buffers as described above, and proteins remaining associated with the protein A–Sepharose beads were analyzed by SDS-PAGE and autoradiography.

Immunization of Mice. BALB/c mice (6–8-wk old female, four per group) were obtained from The Jackson Laboratory (Bar Harbor, ME) or Charles River (Wilmington, MA). Protein A–Sepharose beads (40 μl of a 50% slurry in PBS) were incubated for 1.5 h with 100 μl of hybridoma supernatant from PAb 101 or PAb 122, and then washed with PBS. The mAb-coated beads were then incubated for 1.5 h at 4°C with Sf-9 cell lysate (200 μl = 2×10^6 cell equivalents) containing the recombinant p53 (cells infected with vEV55p53), SVT (cells infected with vEV55SVT), or p53/SVT complexes (cells coinfected with both viruses). The beads were washed as before, resuspended in 100 μl of PBS, and emulsified with CFA for the initial immunization or IFA for subsequent injections. Mice were injected with beads every 2 wk. Serum samples were obtained at days 0 (preimmune), 14, 28, and 42. The amount of antigen attached to the affinity beads was estimated by Western blotting using antibodies specific for p53 or SVT as described below, and by Coomassie blue staining and comparison with standard amounts of BSA.

In some experiments, mice immunized three times with p53/SVT complexes were immunized once, 18 wk after the last p53/SVT injection, with murine p53 affinity purified on PAb 122 as described above. Serum was obtained 9 d later and tested for anti-p53 and anti-SVT antibodies by ELISA (see below).

Immunoblotting. Sf-9 cells infected with wild-type AcMNPV, vEV55p53, or vEV55SVT were solubilized directly in SDS sample buffer, and the lysates were fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose (20). Immunoblot analysis of the recombinant proteins was performed using sera collected from the mice at 2-wk intervals (1:500 dilution for 1.5 h), or PAb 101 or PAb 122 culture supernatants at a 1:5 dilution. Second antibodies were alkaline phosphatase–conjugated goat anti–mouse IgG (γ chain specific) antibodies (IgG plus L chain specific; Tago, Inc., Burlingame, CA) (1:1,000 dilution for 1.5 h). In some experiments, the second antibodies were alkaline phosphatase–conjugated goat anti–mouse γ 1, 2a, or 2b H chain–specific antibodies (Fisher Biotech, Pittsburgh, PA). Blots were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, Richmond, CA).

Affinity Purification of Antibodies. Antibodies specific for murine p53 or SVT were affinity purified from nitrocellulose strips cut from Western blots using the procedure of Smith and Fisher (23). Briefly, the affinity-purified antibodies were eluted from the nitrocellulose strips with 50 mM glycine HCl buffer (pH 2.5) plus 0.5 M NaCl, 0.5% (vol/vol) Tween 20, and 100 μg/ml BSA, and

neutralized immediately with 0.5 M Na_2HPO_4 buffer. The affinity-purified antibodies were diluted 1:15 and used to probe fresh Western blots of cell extracts derived from vEV55SVT or vEV55p53 infected Sf-9 cells.

ELISA for anti-p53 and anti-SVT. ELISAs for detecting antibodies to p53 or SVT in murine sera were a modification of the antigen capture assays described previously for Ku (24). Briefly, microtiter plates (Immunoplate MaxiSorp; Nunc, Inc., Naperville, IL) were coated overnight at 4°C with partially purified PAb 122 (anti-p53 ELISA) or PAb 101 (anti-SVT ELISA) at 10 μg/ml, washed once, and blocked with PBS containing 10% bovine calif serum. Cell extract from Sf-9 cells infected with vEV55p53 or VEV55SVT was added to the wells for 1.5 h (4×10^5 cell equivalents/well). The wells were washed and then incubated with murine sera at a 1:250 dilution for 1.5 h. After washing again, second antibody (alkaline phosphatase–conjugated goat anti–mouse γ 1 H chain–specific antibodies, 1:1,250 dilution) was added for 1.5 h. The wells were washed and developed with *p*-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO), and OD at 405 nm was determined as described (22). The serum binding to control wells (coated with antibody but no antigen) was determined in parallel, and subtracted from the optical density of the corresponding antigen-coated wells.

Results

Expression of Antigens. Full-length recombinant SVT and murine p53 were expressed in Sf-9 cells using recombinant baculoviruses vEV55SVT and vEV55p53, respectively (17). As shown in Fig. 1 *A*, Sf-9 cells infected with vEV55SVT expressed a ^{35}S-labeled protein of ~97 kD that was immunoprecipitated by PAb 101 (anti-SVT) but not PAb 122 (anti-p53) and was absent in extracts of Sf-9 cells infected with vEV55p53 or wild-type AcMNPV. In contrast, Sf-9 cells infected with vEV55p53 expressed a ^{35}S-labeled protein of ~53 kD that was immunoprecipitated by PAb 122 but not PAb 101, and was absent in extracts of cells infected with vEV55SVT or wild-type AcMNPV.

When Sf-9 cells were coinfected for 24 h with both vEV55SVT and vEV55p53 and labeled with [^{35}S]methionine, both p53 and SVT could be immunoprecipitated efficiently by either PAb 101 or PAb 122, consistent with the formation of a complex of the two proteins (Fig. 1 *B*). Both p53 and SVT remained associated with the beads after washing with 50 mM Tris, pH 7.5, 2 mM EDTA, 0.3% NP-40 containing 0.15 M (Fig. 1 *B*, lane *1*), 0.5 M (lane *2*), or 1.5 M (lane *3*) NaCl, or 1.5 M NaCl buffer followed by MMB (lane *4*), or MMB (lane *5*), indicating that the affinity beads could be washed under a variety of conditions without dissociating the p53/SVT complex. In other experiments, we found that this complex also formed readily in vitro (data not shown). Consistent with previous observations (17), there was no evidence that an insect homolog of p53 could be immunopre-

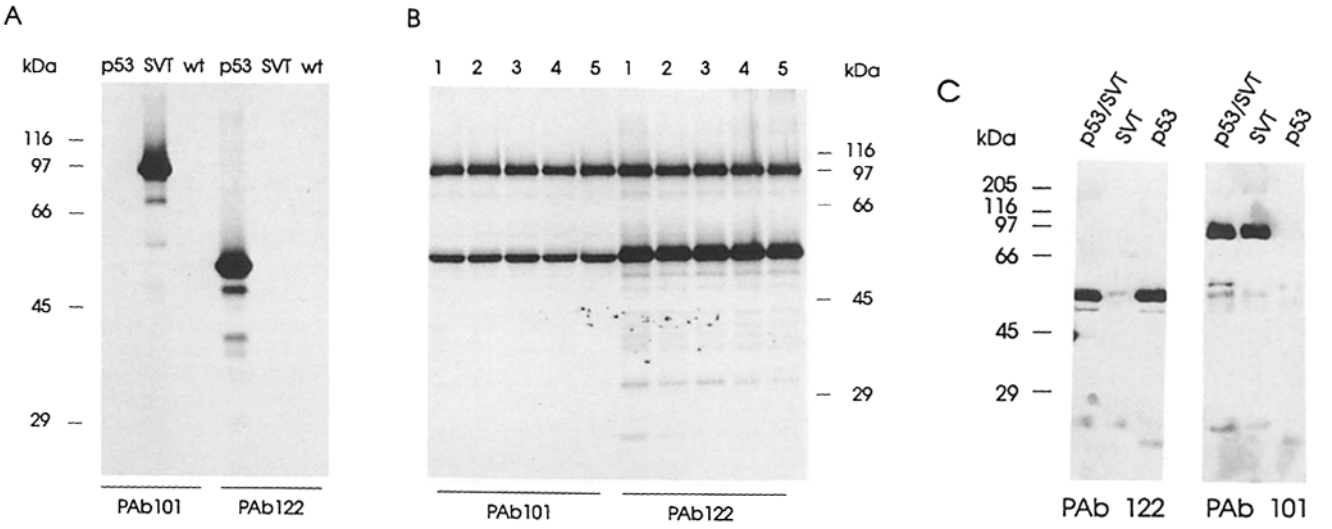

Figure 1. Immunoprecipitation analysis of protein expression in recombinant baculovirus infected insect cells and p53/SVT complex formation in insect cells. Sf-9 cells were infected with vEV55SVT (*SVT*), vEV55p53 (*p53*), wild-type AcMNPV (*wt*) (*A*), or coinfected with vEV55SVT and vEV55p53 (*B*). After 24 h, cells were labeled with [^{35}S]methionine and cysteine and extracts were immunoprecipitated with PAb 101 or PAb 122. (*A*) Single infection of Sf-9 cells. Immunoprecipitates were washed with 50 mM Tris, pH 7.5, 2 mM EDTA, 0.3% NP-40 containing 0.15 M NaCl, eluted by boiling in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography. PAb 101 immunoprecipitated a ~97-kD protein only from lysates of cells infected with vEV55SVT, whereas PAb 122 immunoprecipitated a ~53-kD protein present only in lysates of cells infected with vEV55p53. (*B*) Coinfected cells. Immunoprecipitates of coinfected Sf-9 cells using PAb 101 or PAb 122 were washed with 50 mM Tris, pH 7.5, 2 mM EDTA, 0.3% NP-40 containing: 0.15 M NaCl (lane *1*), 0.5 M NaCl (lane *2*), 1.5 M NaCl (lane *3*), 1.5 M NaCl followed by MMB (lane *4*), or MMB (lane *5*). Immunoprecipitated proteins were separated by SDS-PAGE, and the gel was fluorographed, dried, and exposed to XAR-5 film for 2 d. Positions of molecular weight standards (in kD) are indicated. (*C*) Estimation of the quantity of antigens. Antigens were affinity purified on protein A–Sepharose beads coated with either PAb 101 (*p53/SVT* complex and *SVT*) or PAb 122 (*p53*). Proteins were eluted from the beads by boiling in 25 μl SDS sample buffer, and 3 μl of each sample was analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were probed with PAb 122 (anti-p53) or PAb 101 (anti-SVT), followed by alkaline phosphatase–conjugated goat anti–mouse IgG (γ and L chain specific) antibodies. Note that small amounts of H and L chains were detectable on the blots, and that mouse γ H chain displays an electrophoretic mobility similar to that of the p53 protein.

cipitated by PAb 122, or coimmunoprecipitated by PAb 101 due to formation of a complex with SVT (Fig. 1 *A*).

The formation of a p53/SVT complex with a prolonged intracellular half-life compared with that of free p53 has been shown previously, and is thought to be a crucial step in transformation by SV40 and the related JC and BK viruses (25). Despite the short half-life of free p53 in mammalian cells, recombinant murine p53, SVT, and p53/SVT complex were all expressed at extremely high levels in Sf-9, making it possible to affinity purify large quantities of antigen from infected cells. The recombinant antigens comigrated on gels with the SVT antigen in Cos-1 cells, and the cellular p53 antigen, respectively (data not shown).

Induction of Autoantibodies to p53. The potential role of p53/SVT complexes in inducing autoantibodies to p53 was investigated by immunizing mice with complexes affinity purified on protein A–Sepharose beads. Extracts of Sf-9 cells infected with vEV55SVT or vEV55p53 alone, or coinfected with both viruses, were incubated with protein A–Sepharose beads coated with PAb 101 (vEV55SVT and vEV55SVT plus vEV55p53 extracts) or PAb 122 (vEV55p53 extract). Western blots indicated that comparable amounts of p53, SVT, and p53/SVT complex were injected into the different groups of BALB/c mice (Fig. 1 *C*), and Coomassie blue staining with comparison to a standard protein (BSA) showed that ~1 μg of p53, SVT, or both (in the case of p53/SVT complexes) could be purified on 40 μl of packed protein A–Sepharose beads coated with PAb 101 or PAb 122 (data not shown).

Mice were screened for the production of anti-SVT and anti-p53 antibodies by immunoprecipitating ^{35}S-labeled vEV55p53 or vEV55SVT infected Sf-9 cells using preimmune and immune sera collected at 2, 4, and 6 wk (Fig. 2). All four mice immunized with SVT/p53 complexes affinity purified using PAb 101 affinity beads produced antibodies that immunoprecipitated both p53 and SVT efficiently starting at 4 wk. Immunoprecipitations of p53 and SVT by serial sera from a representative mouse immunized with p53/SVT complexes are shown in the top and bottom panels, respectively, of Fig. 2. In contrast, four mice immunized with p53 did not develop antibodies to either p53 or SVT. All three mice immunized with SVT produced anti-SVT antibodies beginning 2 wk after immunization and, unexpectedly, two of the mice displayed a weak reaction with p53 at 6 wk by immunoprecipitation, whereas serum from the third mouse

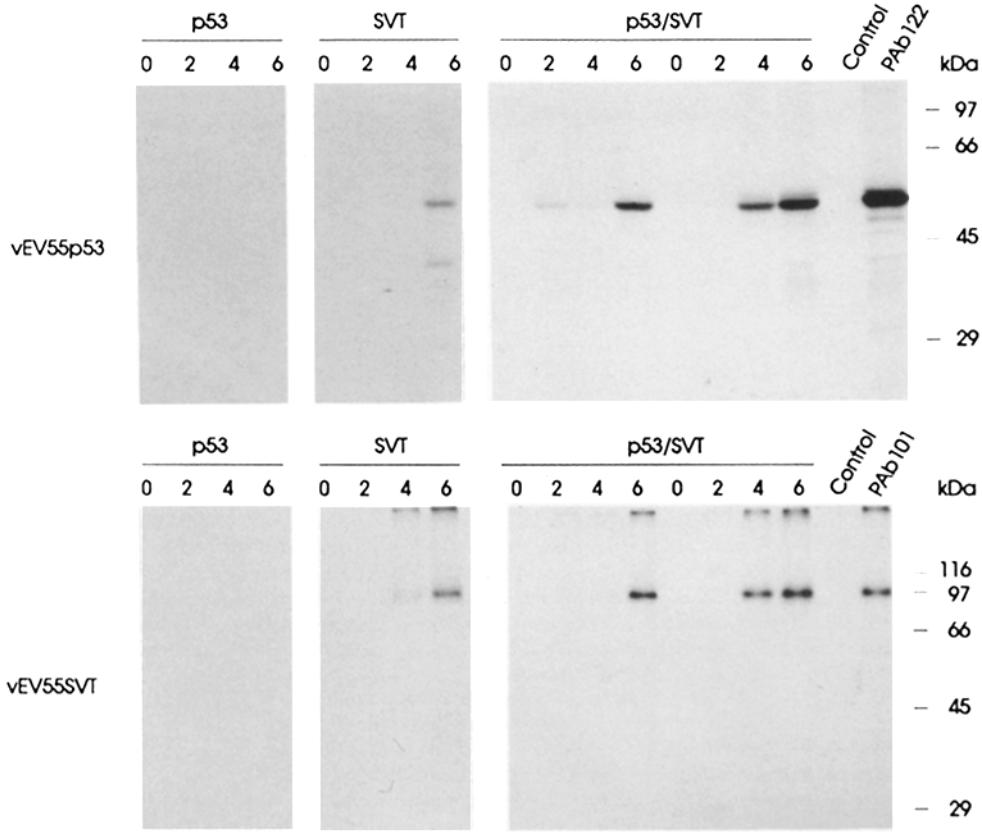

Figure 2. Immunoprecipitation analysis of sera from mice immunized with p53, SVT, or p53/SVT complex. BALB/c mice were immunized once with antigen-coated affinity beads in CFA, and twice more at 2-wk intervals with beads in IFA as follows: p53 from vEV55p53-infected cells purified on pAb 122 beads; SVT from vEV55SVT-infected cells purified on PAb 101 beads; and p53/SVT complex from coinfected cells purified on PAb 101 beads. Serum samples were collected at 0 wks (preimmune), and at 2, 4, and 6 wk after the first immunization. Sera were screened for antibodies to p53 and SVT by immunoprecipitating ^{35}S-labeled vEV55p53 (*top*) or vEV55SVT (*bottom*) infected Sf-9 cell lysates with 5 μl of serum collected at 0, 2, 4, and 6 wk. (*p53*) Sera from a mouse immunized with affinity purified p53; (*SVT*) sera from a mouse immunized with affinity purified SVT; (*p53/SVT*) sera from a mouse immunized with affinity-purified p53/SVT complexes. Control immunoprecipitates using protein A–Sepharose beads without serum are shown for each extract. Immunoprecipitation of recombinant p53 and SVT by PAb122 or PAb101, respectively, is shown for comparison. Immunoprecipitated proteins were analyzed by SDS-PAGE, and autoradiographed. Positions of molecular weight standards (in kD) are indicated.

was negative. The induction of autoantibodies to murine p53 was not dependent on the use of PAb 101 to affinity purify the antigen, since autoantibodies to p53 were also produced by mice immunized with p53/SVT complexes affinity purified on PAb 122 beads (data not shown).

Analysis of Autoantibodies by Western Blot

The production of autoantibodies to p53 in mice immunized with SVT/p53 complexes was confirmed by Western blot (Fig. 3 *A*). Sera from the mice immunized with p53/SVT complexes, but not those from mice immunized with p53 or SVT alone, showed a strong reaction with recombinant murine p53 expressed in Sf-9 cells and its proteolytic degradation fragments. The sera appeared to recognize the degradation fragments more strongly than intact p53, in contrast to PAb 122, which recognized the intact murine p53 protein as well as its degradation products (Fig. 3 *A*, *right*). Antibodies to SVT were, in general, less reactive on immunoblots, but the expression of SVT in the baculovirus system was also somewhat less efficient than that of p53, suggesting that this may have been partially related to a lower amount of SVT than p53 loaded on the gel. Using subclass-specific second antibodies in the Western blot assay, the autoantibodies to p53 as well as the antibodies to SVT were found to be primarily of the IgG1 isotype (data not shown). IgG2b and IgG3 antibodies to p53 and IgG2b antibodies to SVT were also detected by Western blot in sera of mice immunized with p53/SVT complexes, but at considerably lower levels.

To exclude the possibility that the autoantibodies to murine p53 were a population of cross-reactive anti-SVT antibodies, the specificities of affinity-purified antibodies were examined. Antibodies were affinity purified from nitrocellulose strips as described (23). To permit affinity purification of anti-SVT antibodies from nitrocellulose strips, the amount of cell extract from Sf-9 cells infected with vEV55SVT was increased relative to the amount of cell extract from vEV55p53 infected cells (Fig. 3 *B*, *left*). Using approximately four times as much SVT extract, serum from a mouse immunized with p53/SVT complexes could be shown to bind on Western blots to SVT as well as p53 (Fig. 3 *B*, *serum*). Antibodies from that serum were affinity purified on recombinant murine p53 and were highly specific for p53 when used to probe a second immunoblot (*p53 affin*), whereas antibodies affinity purified on SVT were highly specific for SVT, and displayed no cross-reactivity with p53 (*SVT affin*). These studies suggested that autoantibodies to p53 were a different population than the antibodies to SVT in the sera of mice immunized with p53/SVT complexes.

Analysis of Autoantibody Production by ELISA

Antigen capture ELISAs for IgG1 antibodies to p53 (based on the IgG2b mAb PAb 122) and SVT (based on the IgG2a mAb PAb 101) were used to examine the titer and time course of autoantibody production in this model. The assays were similar in design to an anti-Ku ELISA in use in our laboratory (24), but utilized an IgG1 specific second antibody.

Antibody titers. As shown in Fig. 4 *A*, a representative

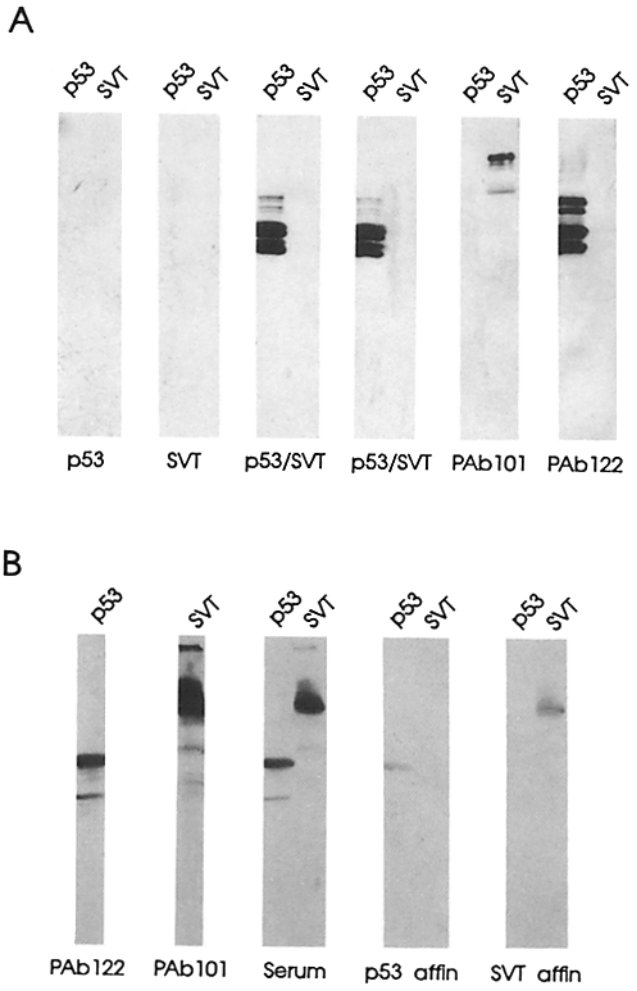

Figure 3. Western blot analysis of antibody production in mice immunized with p53, SVT, and p53/SVT. (*A*) Western blots using whole sera. Pellets of Sf-9 cells infected with vEV55p53 (*p53*, left lane of each panel) or vEV55SVT (*SVT*, right lane of each panel) were solubilized in SDS-sample buffer, and subjected to SDS-PAGE and Western blot analysis using 1:500 diluted mouse sera collected 6 wk after the first immunization followed by alkaline phosphatase–conjugated goat anti–mouse IgG antibodies. Sera from mice immunized with p53, SVT, or p53/SVT complexes (two mice) were analyzed. Immunoblots with PAb 101 (anti-SVT) and PAb 122 (anti-p53) were performed for comparison. Autoantibodies reactive by Western blotting with p53 were more readily detectable than those to SVT. This is in part due to the smaller amount of recombinant SVT than p53 loaded on the gel. However, in other experiments, the level of anti-SVT antibodies reactive on Western blot appeared to be lower than the level of anti-p53 antibodies (data not shown). (*B*) Specificity of affinity-purified antibodies. Immunoblots were performed using serum of a BALB/c mouse immunized with p53/SVT complexes (*serum*) that contained antibodies to SVT as well as autoantibodies to p53. Autoantibodies to p53 were eluted from the blot as described in Materials and Methods. The affinity-purified antibodies were neutralized and used to probe a similar Western blot (*p53 affin*). Likewise, antibodies to SVT were affinity purified from the serum, and used to probe another blot (*SVT affin*). Antibodies affinity purified from the p53 band were specific for p53, whereas those purified from SVT were specific for SVT. Immunoblots with mAbs PAb 122 (anti-p53) and PAb 101 (anti-SVT) are shown for comparison.

mouse serum collected 1 wk after the third immunization with p53/SVT complexes displayed strong reactivity with SVT in the anti-SVT antigen capture ELISA at a titer of ~1:25,000. Weaker reactivity (titer ~1:250) with PAb 101 alone, which was used to affinity purify the p53/SVT complexes for immunizing the mice, was also apparent. Binding to an irrelevant IgG2a (anti-Ku mAb 162) was comparable, suggesting that the weak binding to PAb 101 is attributable to rheumatoid factor (anti-Fc) activity. This argues against the possibility that the autoantibodies to p53 are internal image–type antibodies antiidiotypic to PAb 101. Moreover, p53/SVT complexes affinity purified with either PAb 122 or PAb 101 induced autoantibodies to p53, and PAb 101 was found to immunoprecipitate p53/SVT complexes efficiently (Fig. 1 *B*), further supporting the interpretation that the anti-p53 autoantibodies were not antiidiotypic (internal image) antibodies induced by PAb 101.

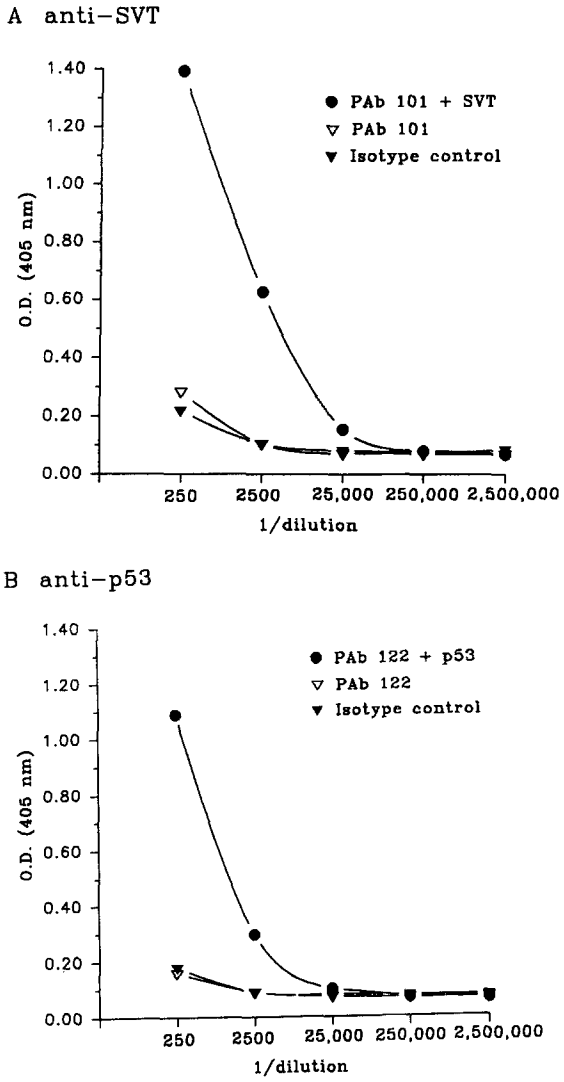

Figure 4. Antibody titers by ELISA. (*A*) Titer of IgG1 anti-SVT antibodies. Serum from a mouse immunized three times with p53/SVT complexes was tested for antibodies to SVT by antigen capture ELISA based on PAb 101 (IgG2a). Second antibody was 1:1,250 alkaline phosphatase–conjugated goat anti–mouse IgG1. (●) Binding to wells coated with PAb 101 plus recombinant SVT (values were corrected for binding to PAb 101 alone); (▽) binding to PAb 101 alone; (▼) binding to anti-Ku mAb 162 (IgG2a isotype control). (*B*) Titer of anti-p53 antibodies. Serum from the same mouse shown in *A* was tested for antibodies to p53 by antigen capture ELISA based on PAb 122 (IgG2b). Second antibody was 1:1,250 alkaline phosphatase–conjugated goat anti–mouse IgG1. (●) Binding to wells coated with PAb 122 plus recombinant p53 (values were corrected for binding to PAb 122 alone); (▽) binding to PAb 122 alone; (▼) binding to anti-Ku mAb N3H10 (IgG2b isotype control).

Examination of the binding of serum to p53 in the anti-p53 antigen capture ELISA also revealed a high titer humoral immune response (titer ~1:25,000) (Fig. 4 *B*). Weaker binding to PAb 122 alone was again detected, and was comparable with the binding to an irrelevant IgG2b isotype control (anti-Ku mAb N3H10). Consistent with the ELISA results, the titers of antibodies to p53 and SVT as determined by Western blot ranged from 1:25,000 to 1:100,000 (data not shown).

Time Course of Antibody Production. A striking increase in IgG1 anti-p53 autoantibodies in the sera of the mice immunized with p53/SVT complex was observed from day 14 to 42 (Fig. 5 *A*). In contrast, IgG1 autoantibodies to p53 were undetectable by ELISA in the sera of mice immunized with p53. Sera of the mice immunized with SVT alone were either negative (Fig. 5 *A*) or weakly positive (data not shown) for autoantibodies to p53.

To examine whether the production of autoantibodies to p53 could be maintained by endogenous murine p53 once initiated by immunization with p53/SVT complexes, serum samples were obtained from the mice for 18 wk after the last immunization with p53/SVT complexes. The levels of anti-p53 and anti-SVT antibodies in these sera were determined by ELISA. As shown in Fig. 5 *B*, the level of autoantibodies to p53 fell significantly and in parallel with the level of antibodies to SVT. However, the drop in anti-p53 appeared more marked than that of anti-SVT. Approximately 60 d after the last p53/SVT injection, the levels of both antibodies reached a steady state. However, autoantibodies to p53 never reached preimmune levels.

Immunization with affinity-purified p53 alone at day 156 (127 d after the last p53/SVT injection) resulted in a dramatic increase in anti-p53 antibodies within 9 d (Fig. 5 *B*, **). The level of anti-p53 on day 165 was comparable with that seen on day 42 after three injections of p53/SVT complexes. In contrast, antibodies to SVT displayed a modest decline from day 156 to 165, indicating that the anti-p53 response was specific for p53, and not a consequence of polyclonal activation. Moreover, the levels of anti-Ig antibodies increased only slightly (Fig. 5 *B*).

Autoantibody Specificity

The specificities of the autoantibodies to p53 induced in mice immunized with p53/SVT complexes were examined further by immunoprecipitating lysates of ^{35}S-labeled K562 and SP2/0 cells. The murine anti-p53 sera did not immunoprecipitate other proteins from extracts of either cell line, suggesting that the autoimmune response was specific for p53 (data not shown).

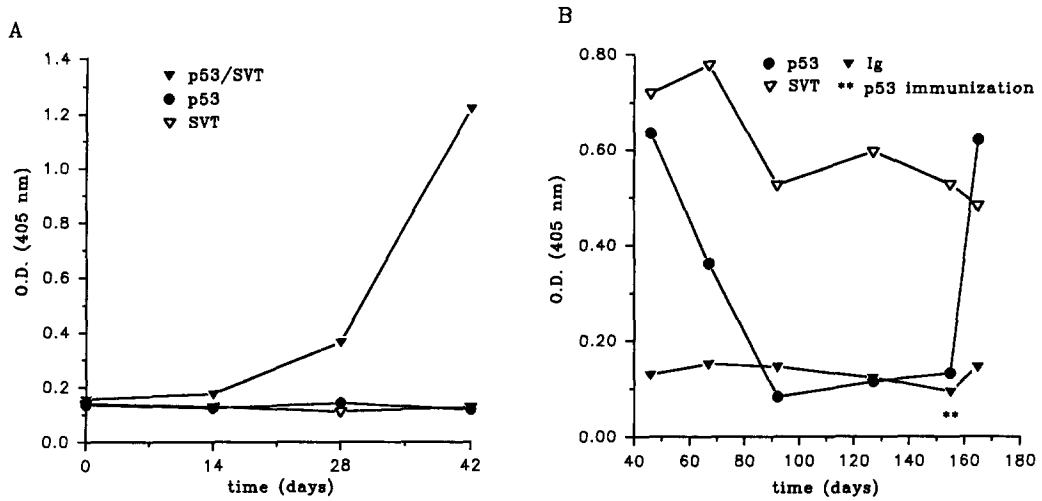

Figure 5. Analysis of anti-p53 autoantibodies in mouse sera by ELISA. (*A*) Onset of autoantibody production. Microtiter plates were coated with PAb 122 (anti-p53), followed by Sf-9 cell lysate containing recombinant murine p53 or lysis buffer alone, as described in Materials and Methods. Sera were tested at 1:250, followed by alkaline phosphatase–conjugated goat anti-mouse IgG1 subclass-specific antibodies at 1:1,250 and *p*-nitrophenyl phosphate substrate. Binding to the wells coated with antibody alone was subtracted from the binding to wells coated with antigen plus antibody. OD (405 nm) is plotted as a function of time (*days*) after the first immunization. (▼) Sera from a mouse immunized with p53/SVT complexes; (●) sera from a mouse immunized with p53; (▽) sera from a mouse immunized with SVT. (*B*) Immunization of p53/SVT primed mouse with murine p53. Levels of IgG1 autoantibodies to murine p53 (●) and antibodies to SVT (▽) in serial serum samples of a mouse previously injected three times (day 0, 14, and 28) with p53/SVT complexes were determined by antigen capture ELISAs as described in Materials and Methods. On day 156 the mouse was immunized with affinity-purified murine p53 (**) and serum obtained 9 d later was tested for anti-p53 and anti-SVT antibodies. Binding to Ig (PAb 101 and PAb 122, respectively) coated wells was subtracted in each assay. The binding to PAb 101 coated wells in the absence of antigen is also shown (▼).

Discussion

Autoantibodies are detected at increased frequency during certain viral infections (26–30) and in some virally induced neoplastic diseases (31). These autoantibodies may sometimes be a consequence of nonspecific polyclonal B cell activation, whereas in other cases, more specific mechanisms appear to be involved. For example, rodents carrying SV40-induced tumors develop autoantibodies specific for the p53 tumor suppressor protein, and it has been proposed that the binding of p53 to SVT in these tumors may render p53 immunogenic, as if it were a hapten with SVT as the antigenic carrier protein (32, 33). However, autoantibodies to p53 have also been reported in human breast cancer and murine sarcomas that are not induced by SV 40 (34, 35). Since somatic mutations in p53 represent one step in the pathogenesis of many neoplasms (36), the possibility that a T cell response directed at somatically mutated p53 might induce autoantibodies to p53 in mice carrying SV40 induced tumors could not be excluded. Indeed, there is recent evidence that the development of autoantibodies to p53 in human neoplastic disease is dependent on missense mutations (37, 38). The present studies suggest that the binding of SVT to wild-type (i.e. unmutated) p53 may induce autoimmunity by activating T cells specific for cryptic epitopes of p53.

Neoplastic Disease and p53 Mutations Are Not Required to Induce Autoantibodies. The development of autoimmunity to p53 does not require SV40-transformed tumor cells or somatic mutation of p53, since a strong, specific autoantibody response to p53 was induced by immunizing mice with biochemically purified p53/SVT complexes. Repeated immunization with murine p53 alone did not induce autoantibodies to p53, consistent with immune tolerance to this self antigen. Moreover, since the present studies employed recombinant wild-type murine p53/SVT complexes instead of tumor cells, which might undergo somatic mutation, it is likely that neoantigenic determinants resulting from mutation of p53 did not trigger autoimmunity in this model. Thus, our data suggest that the association of certain self proteins with foreign antigens may induce autoantibodies specific for the cellular components.

Autoimmunity Is Unlikely to Be Due to Coimmunization with Self and Foreign p53. It has been shown recently that autoantibodies to cytochrome *c* can be induced by coimmunizing mice with self and foreign cytochrome *c*, suggesting that autoreactive B cells activated during an immune response to foreign cytochrome *c* can present self antigen to autoreactive T cells (39, 40). Several lines of evidence argue that the autoantibodies to p53 were not generated by a similar mechanism. An insect homolog of p53 has not been reported, and even if insect p53 exists, there was no evidence that it bound to SVT (Fig. 1), in agreement with previous observations (17). In addition, a hypothetical insect p53 homolog was not immunoprecipitated by PAb 122, even though p53/SVT complexes affinity purified using PAb 122, like those purified using PAb 101, induced autoantibodies to p53. Moreover, mice immunized with p53 expressed in Sf-9 cells did not develop autoantibodies, suggesting that the complex of murine p53 with SVT was required for inducing autoantibodies. The previous observations that mice carrying SV40-induced tumor cells develop autoantibodies to p53 (32, 33) provide additional indirect evidence that the autoantibodies to p53 were not a con-

sequence of immunizing with a mixture of foreign and self p53.

Autoantibodies to p53 Are Not Cross-reactive with SVT. Although not completely excluded, the autoantibodies to p53 are also unlikely to have been a cross-reactive population of antibodies directed primarily at SVT. Affinity-purified autoantibodies to p53 from the sera of mice immunized with p53/SVT complexes were highly specific for p53, whereas affinity-purified antibodies to SVT displayed no cross-reactivity with p53 (Fig. 3 *B*). Thus, at least the antibodies reactive with p53 and SVT on immunoblots were distinct populations. Moreover, immunization of p53/SVT primed mice with p53 alone boosted the anti-p53, but not the anti-SVT, response (Fig. 5 *B*), providing additional evidence that the antibodies reactive with p53 and SVT were distinct.

Despite the fact that the affinity-purified antibodies to p53 and SVT represented distinct populations, some mice immunized with SVT alone produced low levels of autoantibodies to p53. Most likely, this was related to the formation of p53/SVT complexes in vivo after immunizing the mice. The p53 antigen bound readily to SVT in vitro, suggesting that cellular p53 released by necrotic cells could bind to the affinity beads carrying SVT. Moreover, injection of 250 ng or less of the p53/SVT complexes induced autoantibodies to p53, suggesting that even small amounts of the complex can trigger autoimmunity (Dong, X., unpublished data).

p53/SVT Complexes May Induce Autoreactive T Cells Specific for p53. Although immunization with p53/SVT complexes was necessary to break tolerance to p53, a subsequent injection of p53 alone elicited a brisk secondary autoantibody response (Fig. 5 *B*). This might be explained in several ways. The possibility that autoantibodies to p53 were induced in response to the mAb used for affinity purifying p53/SVT complexes is unlikely, in view of the low level of antibodies specific for PAb 101 and the strong secondary response to immunization with affinity-purified p53 on PAb 122 beads (Fig. 5 *B*). However, a potential role for autoantigen-mimicking idiotypes (41) in the pathogenesis of autoantibodies to p53 in this model cannot be excluded completely.

Another possible explanation for our data is the initiation of autoantibody production by a mechanism involving intermolecular–intrastructural help (11), and its subsequent maintenance by activated p53-specific B cells. Although the production of autoantibodies in response to a complex of self and nonself might be expected to depend on continued availability of that complex (3), a role for intermolecular–intrastructural help in initiating autoimmunity is not excluded by our data if a two-step mechanism is invoked. In the first stage, T cells specific for SVT may activate B cells specific for murine p53 as envisioned by the intermolecular–intrastructural help model, leading to the expression of costimulatory molecules on their surface. In a second stage, these activated autoreactive B cells might present murine p53 peptides to autoreactive T cells in a manner analogous to that proposed in the cytochrome *c* model (39, 40), thus accounting for the immune response to murine p53 alone after priming with p53/SVT complexes. However, the simplest explanation for our data is a model involving altered antigen processing of self p53.

The expressed repertoire of T cells responsive to an exogenous antigen is focused onto a limited number of major T cell–inducing determinants rather than being broadly directed against all portions of the antigen (42, 43). This is controlled, in part, by MHC-linked genes through a determinant selection mechanism in which immunodominant peptides bind to MHC molecules that can present the peptide (42, 44). Another factor influencing T cell repertoire expression is the existence of hindering structures on naturally processed fragments that differentially affect presentation by different MHC molecules (42, 45). These may include structural constraints imposed by the tertiary structure of a protein (46) as well as intermolecular contacts between subunits of an oligomeric antigen (15, 16).

It has been suggested that T cells responsive to minor antigenic determinants can evade tolerance because of the relatively low amounts of peptides suitable for MHC binding after in vivo processing of the intact antigen (12). Autoreactive T cells can be activated under special circumstances, such as immunization with artificially processed peptides (13). Altered antigen processing induced by changing the quaternary structure of a complex may represent a second mechanism for activating autoreactive T cells. Thus, the binding of SVT to p53 might alter processing of p53, resulting in the presentation of "cryptic" T cell epitopes of self to which tolerance is incomplete or nonexistent (12–14, 47).

A similar mechanism may underlie autoimmunity to p53 in patients with breast or lung cancer, in whom autoantibodies are correlated with missense mutations in exons 5 and 6 (which, perhaps not coincidentally, are located within the SVT binding domain of p53) and with binding of the mutant protein to hsp70 (37, 38, 48). We speculate that altered antigen processing, as a consequence of either the missense mutations themselves or binding of hsp70 to the mutant protein, may trigger autoimmunity to p53. However, a role of T cells specific for the mutant p53 protein cannot be ruled out at present.

Antigen processing might also be altered by autoantibodies to p53 in a manner analogous to that proposed above for SVT. In this scenario, autoantibodies induced by a mechanism involving intermolecular–intrastructural help might themselves facilitate the presentation of cryptic T cell epitopes to p53-responsive autoreactive T cells. There is evidence for altered processing of antigens bound to different Igs (49), and indirect evidence that the binding of autoantibodies to antigens can induce new autoantibodies (50–52). Thus, the intermolecular–intrastructural help and altered antigen-processing models may not be mutually exclusive, and both mechanisms could play a role in perpetuating autoimmunity.

Relevance to Virally Induced Autoimmunity. The induction of ANAs by immunization with p53/SVT complexes may have relevance for the induction of specific autoantibodies in viral infections (4) or collagen vascular disease. For example, the papovaviruses JC and BK are closely related to SV40, produce large T antigens analogous to SVT, and are common human pathogens (53). It would not be surprising to find autoantibodies to p53 in sera of patients infected acutely with these viruses, or possibly during reactivation of latent infec-

tion, for instance, in progressive multifocal leukoencephalopathy (54). ANAs specific for histones and DNA, both of which may be physically associated with the p53 protein in chromatin, have been described previously in papovavirus infection, although specificity for p53 was not investigated (55). It is also of interest that progressive multifocal leukoencephalopathy may complicate SLE, presenting a clinical picture similar to that seen in central nervous system lupus (56). It remains to be established, however, whether either disorder is associated with autoantibodies to p53. In view of the wide variety of viral antigens known to form complexes with specific cellular proteins, and previous data suggesting that reovirus-induced autoantibody production is specific for virally infected cell types or tissues (28), it is tempting to speculate that other complexes of self and nonself might trigger autoantibody production in certain patients with acute or chronic viral infections or systemic autoimmune diseases.

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Address correspondence to Dr. W. H. Reeves, Division of Rheumatology and Immunology, University of North Carolina at Chapel Hill, 932 FLOB, CB# 7280, Chapel Hill, NC 27599-7280.

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