Systemic lupus erythematosus in mice, spontaneous and induced, is associated with autoimmunity to the C-terminal domain of p53 that recognizes damaged DNA

Johannes Herkel[1], Neta Erez-Alon[2], Avishai Mimran[1], Roland Wolkowicz[2], Alon Harmelin[3], Pedro Ruiz[4], Varda Rotter[2] and Irun R. Cohen[1]

[1] Department of Immunology, Weizmann Institute of Science, Rehovot, Israel
[2] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel
[3] Department of Preclinical Studies, Weizmann Institute of Science, Rehovot, Israel
[4] Department of Neurology, Stanford Medical Center, Stanford, USA

The tumor suppressor molecule p53 features a regulatory domain at the C terminus that recognizes damaged DNA. Since damaged DNA might be involved in activating anti-DNA autoantibodies, we tested whether autoimmunity to the C terminus of p53 might mark murine systemic lupus erythematosus (SLE). We now report that MRL/MpJ-Fas^{lpr} mice, which spontaneously develop SLE, produce antibodies both to the C terminus of p53 and to a monoclonal antibody (PAb-421) that binds the p53 C terminus. Anti-idiotypic antibodies to PAb-421 (sampled as monoclonal antibodies) could also bind DNA. Thus, the PAb-421 antibody mimics DNA, and the anti-idiotypic antibody to PAb-421 mimics the p53 DNA-binding site. This mimicry was functional; immunization of BALB/c mice to PAb-421 induced anti-DNA antibodies and antibodies to the C terminus of p53, and most of the mice developed an SLE-like disease. Immunization of C57BL/6 mice to PAb-421 induced antibodies to p53, but not to its C-terminal domain. The C57BL/6 mice also did not develop anti-DNA antibodies or the SLE-like disease. Thus, network autoimmunity to the domain of p53 that recognizes damaged DNA can be a pathogenic feature in SLE in genetically susceptible strains of mice.

Key words: p53 / Systemic lupus erythematosus / Anti-DNA antibody / DNA damage / Anti-idiotypic antibody

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1 Introduction

SLE is an autoimmune disease of unknown etiology, but antibodies to nuclear antigens and to DNA are believed to be major pathogenic features [1]. However, there is at present no generally accepted explanation for the prevalence of anti-DNA antibodies in SLE or in other autoimmune disorders. Immunity to DNA appears to be actively driven [2], but native self DNA is unlikely to be the driving antigen because mammalian DNA usually does not induce an anti-DNA immune response [3].

The p53 protein is a tumor suppressor that inhibits the growth of aberrant cells; functional p53 is believed to sense DNA damage [4] and, subsequently, to induce DNA repair, growth arrest, or apoptosis of the aberrant cell [5]. The p53 protein has at least two DNA-binding sites: (1) the core of the activated p53 protein, which interacts specifically with a defined DNA sequence in the promoters of p53-responsive genes [6], and (2) the C terminus of the p53 protein, which can recognize features common to damaged DNA [4].

Exposure to sunlight could be a factor linking p53 and SLE: p53 accumulates after sunburn [7] and sunburn exacerbates SLE [1]. We reasoned, therefore, that anti-DNA antibodies and the development of SLE might be related to DNA damage and to proteins like p53 that recognize DNA damage. We previously reported that immunization to autoantibodies to the central domain of p53 could induce immunity to p53 leading to resistance to tumors [8, 9]. The question was whether autoimmunity involving the C terminus of p53 might be related to anti-DNA antibodies and to SLE.

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The first two authors contributed equally to this work

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2 Results

2.1 MRL/MpJ-Fas^{lpr} mice develop anti-p53 and anti-PAb-421 antibodies

To learn whether the spontaneous development of murine lupus is associated with anti-p53 autoimmunity, we examined sera of MRL/MpJ-Fas^{lpr} mice both for antibodies to the C terminus of p53 and for antibodies that mimic this domain (Fig. 1). At 9 weeks of age, the mice showed significant amounts of antibodies to whole p53 and to p53 peptide p363–382 derived from the sequence of the C-terminal DNA-binding domain ($p < 0.0001$). These antibodies increased significantly at 19 weeks ($p < 0.0002$). The mice did not manifest antibodies to a peptide derived from the N-terminal sequence of p53, p16–35. The presence of p53-mimicking antibodies was detected by testing the sera for antibodies binding to the PAb-421 mAb, which is specific for the p363–382 peptide of the C terminus of p53. These antibodies, as well shall next see, could bind DNA.

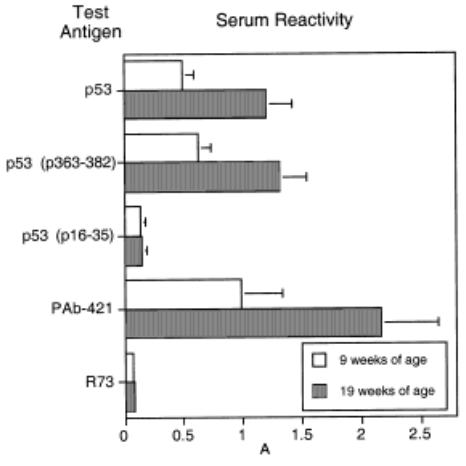

Fig. 1. Spontaneous development of anti-PAb-421 and anti-p53 antibodies in MRL/MpJ-Fas^{lpr} mice. At the age of 9 weeks and 19 weeks, sera of MRL/MpJ-Fas^{lpr} mice were tested for antibodies that bind to p53, to the N-terminal p53 peptide p16-35, to the C-terminal p53 peptide p363–382, which is recognized by mAb PAb-421, to the PAb-421 antibody molecule itself and to mAb R73 that does not bind p53.

2.2 Anti-PAb-421 antibodies bind DNA

We raised a monoclonal anti-PAb-421 hybridoma antibody from the spleen cells of 17-week old MRL/MpJ-Fas^{lpr} mice. Table 1 shows that this mAb, MRL-1, could bind both to PAb-421 and to double-stranded calf thymus DNA. Thus, we can conclude that the anti-PAb-421 antibody, like the C terminus of p53, recognizes DNA; in other words, PAb-421 mimics DNA, at least as seen by the anti-PAb-421 mAb. The question was whether the immune mimicry between antibody PAb-421 and DNA was a peculiarity of lupus-prone MRL/MpJ-Fas^{lpr} mice. We therefore used PAb-421 to immunize standard BALB/c and C57BL/6 mice, strains that do not spontaneously develop SLE.

2.3 Induction of anti-p53 immunity in standard mice

We immunized BALB/c and C57BL/6 mice with the PAb-421 mAb, and tested their sera for the appearance of antibodies to DNA and to p53. Additional mAb immunogens were PAb-246, which binds the central domain of p53 that recognizes the specific p53 responsive element, and PAb-248, which binds the N-terminal domain of p53 that does not recognize DNA. Both the BALB/c and C57BL/6 mice were found to produce significant amounts of anti-p53 antibodies in response to PAb-421 or to PAb-246 (Fig. 2 A; $p < 0.0001$).

Table 1. Anti-idiotypic mAb to PAb-421 are also anti-DNA antibodies

	(Fab')$_2$ fragments of		DNA		
mAb[a)]	PAb-421	R73	Double-stranded	Single-stranded	γ-irradiated (10000 rad)
MRL-1	0.625	0.030	0.850	0.896	1.212
Idi-1	1.514	0.024	0.671	0.805	1.451
Idi-2	0.676	0.015	0.490	0.760	1.497
R73	0.023	0.021	0.012	0.098	0.097

a) The Table shows the absorbance values produced in the ELISA assay by 5 μg/ml of the indicated mAb reacting with either DNA or with the (Fab')$_2$ fragments of the PAb-421 or R73 antibodies. The mAb MRL-1 was isolated from MRL/MpJ-Fas^{lpr} mice; the Idi-1 and Idi-2 antibodies were raised from BALB/c mice, R73 served as control antibody.

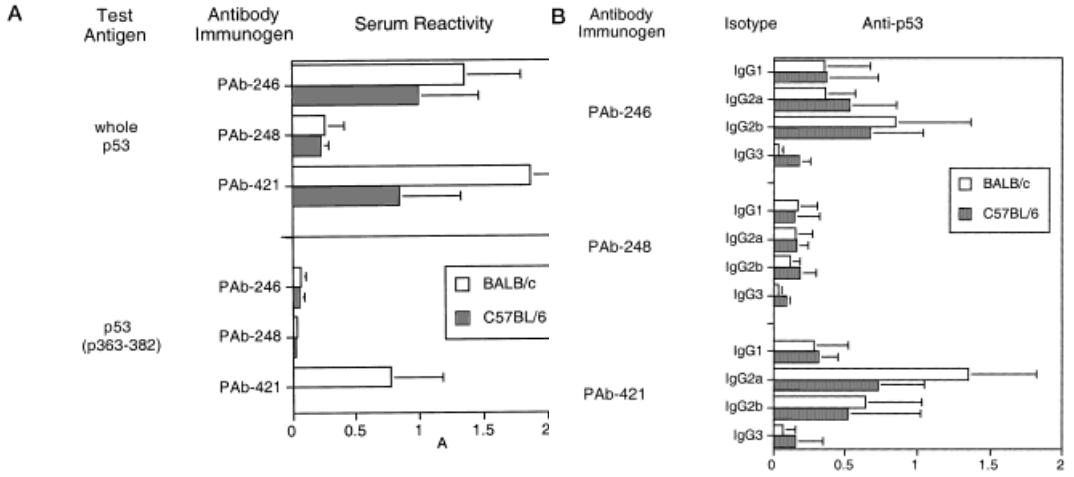

Fig. 2. Induction of anti-p53 antibodies in standard mice. (A) Sera of BALB/c and C57BL/6 mice immunized to the indicated anti-p53 mAb were examined for their serum IgG antibodies to whole p53, and to the p53 C-terminal peptide p363–382. (B) Anti-p53 antibodies were detected with secondary antibodies specific for the IgG1, IgG2a, IgG2b or IgG3 isotypes. The anti-p53 induced by PAb-246 or PAb-421 of both strains were a mixture of different isotypes.

We analyzed the specificity of the anti-p53 antibodies using the p53 C-terminal peptide p363–382; this peptide includes the p53 epitope recognized by the PAb-421 antibody used for immunization. Fig. 2 A shows that PAb-421, but not PAb-246, induced antibodies to this C-terminal p53 peptide in BALB/c but not in C57BL/6 mice ($p < 0.0001$). A control p53 peptide from the N-terminal domain, p16–35, was not recognized by antibodies in any of the immunized mice (not shown). Thus, the specificity of the anti-p53 antibodies induced by immunization to PAb-421 differed in the two mouse strains: the BALB/c mice made antibodies to the p363–382 peptide recognized by the PAb-421 immunogen, while the C57BL/6 mice made antibodies to p53 of an unknown fine specificity. Immunization to PAb-248 was not effective in inducing anti-p53 antibodies in either of the strains.

Analysis of the IgG isotypes of the anti-p53 antibodies showed a mixture of IgG1, IgG2a and IgG2b isotypes (Fig. 2 B). Since the mAb immunogens were either of the IgG1 (PAb-246) or IgG2a isotypes (PAb-421), it appears likely that the anti-p53 antibodies we detected were indeed produced actively by the immunized mice, and were not merely remnants of the injected antibody immunogens.

2.4 Anti-DNA antibodies

As we observed above, mAb PAb-421 mimicked DNA, at least for MRL/MpJ-Fas^{lpr} mice (Table 1). Hence, the immunization of BALB/c or C57BL/6 mice to PAb-421 might have induced immunity to DNA. To investigate this possibility, we examined the sera of the PAb-421-immunized mice for antibodies to DNA using three different assay systems.

As we reported earlier [9], the serum antibodies of BALB/c mice that had been immunized to PAb-246 bound specifically to the oligonucleotide DNA sequence of the p53-responsive element in a band-shift assay. These antibodies were also specific for the inducing PAb-246 antibody. In the present study, we found that the BALB/c mice immunized to PAb-421 did not make these anti-oligonucleotide antibodies, although those immunized to PAb-246 did make anti-DNA antibodies specific for the p53-responsive oligonucleotide (not shown).

Secondly, we examined the test sera for binding to DNA by the *Crithidia luciliae* assay, which detects antibodies to native, histone-free DNA exposed at the base of the *Crithidia* flagellum (Fig. 3). This assay is used clinically for its diagnostic specificity for SLE in humans [10]. Both BALB/c and C57BL/6 mice immunized to PAb-246 (Fig. 3 A) or PAb-248 (B) were negative in this test, as were C57BL/6 mice immunized to PAb-421 (C). In contrast, BALB/c mice immunized to PAb-421 produced anti-DNA antibodies detectable by the *Crithidia* assay (D). Note that *C. luciliae* is a protozooic organism and does not have a p53 gene.

Thirdly, we examined the test sera for binding to single-stranded calf thymus DNA. Consistent with the *Crithidia* and the band-shift assays, significant ($p < 0.0007$) anti-

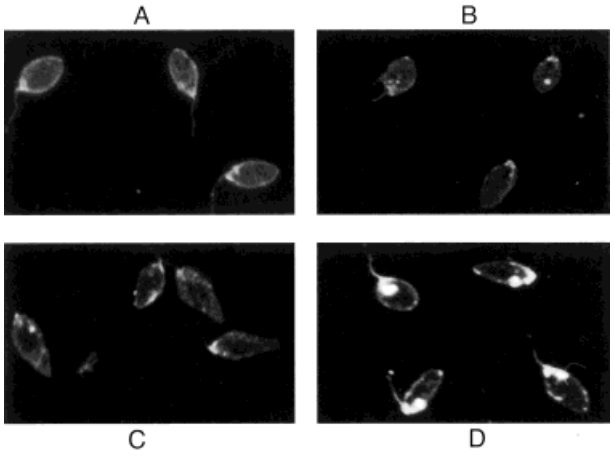

Fig. 3. Induction of antibodies to double-stranded DNA. Sera of BALB/c and C57BL/6 mice immunized to PAb-246, PAb-248 or PAb-421 were tested for antibodies to native DNA by the *C. luciliae* assay. BALB/c and C57BL/6 mice immunized to PAb-246 (A), or PAb-248 (B), and C57BL/6 mice immunized to PAb-421 (C) did not develop antibodies to native DNA. Only BALB/c mice immunized to PAb-421 developed anti-DNA antibodies detectable by the *Crithidia* assay (D).

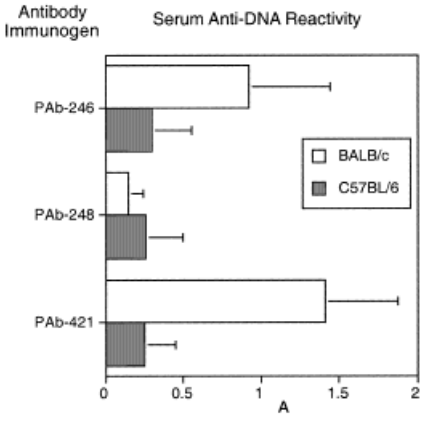

Fig. 4. Antibodies to single-stranded calf thymus DNA. Sera of BALB/c and C57BL/6 mice immunized to PAb-246, PAb-248 or PAb-421 were tested for antibodies to single-stranded calf thymus DNA. BALB/c mice immunized to PAb-246 or PAb-421 developed significant ($p < 0.0007$) titers of antibodies to single-stranded DNA when compared to control sera of naive mice. In contrast, BALB/c mice immunized to PAb-248, and C57BL/6 mice immunized to any of the antibodies did not develop significant anti-DNA reactivity.

DNA reactivity could be detected in the BALB/c mice immunized with PAb-246 or with PAb-421 (Fig. 4). The BALB/c mice immunized to PAb-248 and the C57BL/6 mice immunized to either anti-p53 antibody, in contrast, made no significant anti-DNA antibody.

2.5 Anti-idiotypic mAb are anti-DNA antibodies

To confirm that the anti-DNA antibodies induced in the BALB/c mice by immunization to PAb-421 were anti-idiotypic antibodies, we isolated two mAb, Idi-1 and Idi-2, that specifically bound to the PAb-421 mAb immunogen and tested their ability to recognize DNA. Both Idi-1 and Idi-2 antibodies showed idiotypic binding to PAb-421, but not to other mAb, like R73 (Table 1). The anti-PAb-421 antibodies could also recognize DNA, single-stranded more than double-stranded DNA. Interestingly, the binding both of Idi-1 and of Idi-2 to single-stranded DNA was increased after gamma-irradiation of the DNA. Note that the C-terminal domain of p53 itself shows preferential binding to single-stranded DNA [11] and to gamma-irradiated DNA [12]. Thus, it seems that anti-idiotypic immunization to PAb-421 can generate antibodies that mimic the ability of the C-terminal domain of p53 to recognize DNA damage.

2.6 Induction of an SLE-like disease by immunization to PAb-421

To test whether the induction of anti-DNA by immunization to PAb-421 might lead to an SLE-like disease, we repeatedly immunized BALB/c and C57BL/6 mice with PAb-421, and observed the mice. PAb-246, which binds the central DNA-binding domain of p53, was used as a control immunogen; immunization to PAb-246 did not induce any signs of disease in either strain. However, each of 40 BALB/c mice immunized with PAb-421 developed proteinuria ranging from 1–3 g/l. The mice remained proteinuric throughout a period of 3 months after the last boost, after which they were killed. The protein content of the urine of ten unimmunized mice and of 20 BALB/c mice immunized with PAb-246 was below 0.3 g/l.

Histological examination of the kidneys of ten proteinuric BALB/c mice showed protein deposition in the tubuli and pathological changes in the glomeruli, marked by thickening of the glomerular basement membrane, “wire loop” formation, endothelial proliferation, neutrophilic exudation, deposition of hyaline material in the glomerular capillaries, and nuclear degeneration of cells. No such changes were seen in the kidneys of ten naive BALB/c or ten immunized C57BL/6 mice that were free of proteinuria. Examples of pathological glomeruli are shown in Fig. 5 A and B. Glomerular and vascular antibody depo-

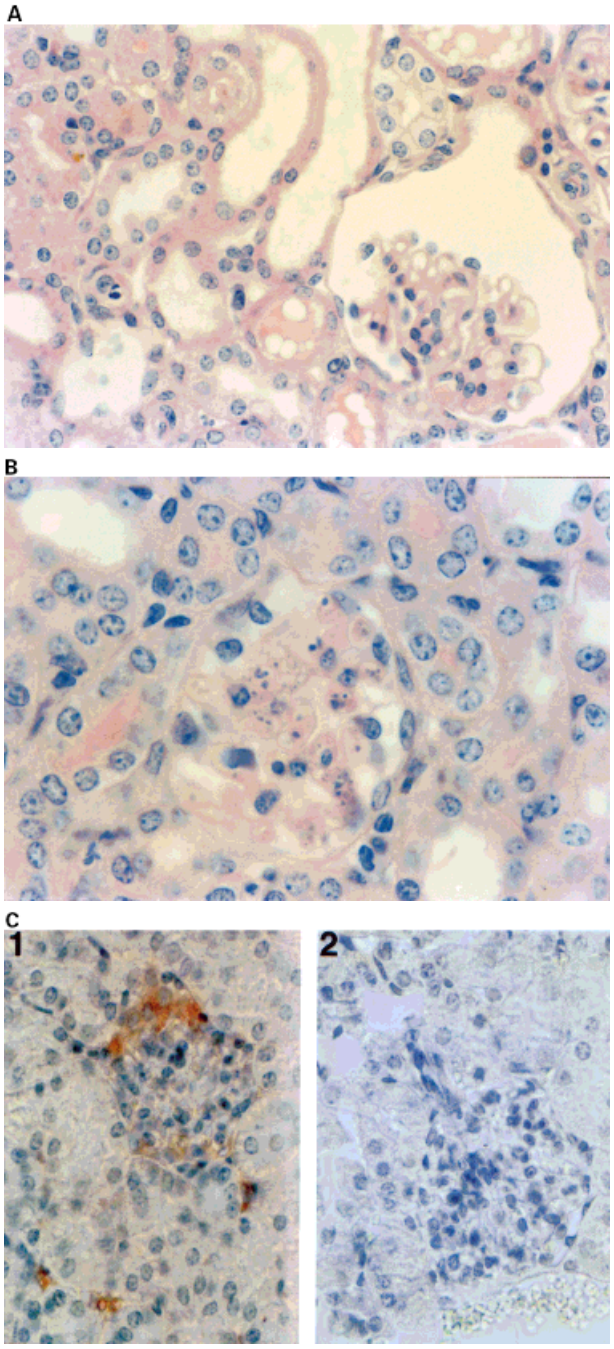

Fig. 5. Histology of BALB/c kidney lesions. BALB/c mice were immunized with PAb-421 and the kidneys of mice that subsequently developed proteinuria (1–3 g/l) were examined. (A) A shrunken glomerulus showing thickening of the glomerular basement membrane and tubular protein deposition (× 400). (B) A glomerulus showing degeneration of nuclei (× 400). (C) Glomerular Ig deposits (× 400): a kidney section from an immunized mouse (1) shows glomerular and vascular antibody deposition detected by DAB staining. No such deposits were detected in the kidneys of naive mice or of mice immunized with the other antibodies (2).

sition is evident (Fig. 5 C). Blood samples of ten proteinuric BALB/c mice were examined and seven of the mice were found to be leukopenic (< 4000 leukocytes/μl) and lymphopenic (< 1200 lymphocytes/μl). Ten non-proteinuric control mice, in contrast, showed a mean leukocyte count of 6820 (± 1480) and a lymphocyte count of 4600 (± 1400)/μl. Thirty BALB/c mice immunized with PAb-421 were tested for anti-histone antibodies and 10 (33 %) were found to develop significant titers of anti-histone antibodies, suggesting that either the immune response to PAb-421 could spread to DNA-binding molecules other than p53, or that the induced antibodies were cross-reactive to different DNA-binding proteins. In contrast to the BALB/c mice, none of 40 C57BL/6 mice immunized with PAb-421 developed signs of murine lupus. Thus, an immune response to an antibody specific for the DNA damage-recognizing domain of p53 can lead to murine lupus in genetically susceptible mice. A comparison of relevant features in three mouse strains, immunized BALB/c and C57BL/6 mice and unimmunized MRL/MpJ-Fas^{lpr} mice, is shown in Table 2.

Table 2. Comparison of the lupus manifestations induced by PAb-421 immunization in BALB/c and C57BL/6 mice, and appearing spontaneously in MRL/MpJ- Fas^{lpr} mice.

Lupus manifestations[a)]	MRL/MpJ-Fas^{lpr}	BALB/c	C57BL/6
Proteinuria (> 0.3 g/l)	+	+	–
Nephritis	+	+	–
Leukopenia (< 4000/μl)	+	+	–
Lymphopenia (< 1200/μl)	+	+	–
Anti-p53 antibodies	+	+	+
Anti-p53 (p363–382) antibodies	+	+	–
Anti-PAb-421 antibodies	+	+	+
Anti-DNA antibodies	+	+	–
Anti-histone antibodies	ND	+	–

a) The responses of the BALB/c and C57BL/6 mice are summarized from the results reported here. The manifestations of lupus in MRL/MpJ-Fas^{lpr} mice are summarized from our antibody results and from the literature [22].

3 Discussion

The results presented here show that the spontaneous development of murine SLE in MRL/MpJ-Fas^{lpr} mice is accompanied by the appearance in the blood of antibodies to p53, including antibodies to a peptide from the sequence of the C-terminal domain of the p53 molecule, p363–382. Moreover, the MRL/MpJ-Fas^{lpr} mice also produced anti-idiotypic antibodies to the PAb-421 antibody, which itself recognizes p363–382 (Fig. 1). PAb-421 mimics DNA immunologically: mAb MRL-1 to PAb-421 also recognizes DNA (Table 1). mAb isolated from immunized BALB/c mice also confirmed the immunological mimicry between PAb-421 and DNA, particularly damaged DNA (Table 1). A connection between these p53-associated antibodies and murine SLE was suggested by the induction of an SLE-like disease in BALB/c mice by immunization to PAb-421 (Table 2), accompanied by anti-DNA antibodies (Fig. 3), and by antibodies to peptide p363–382 of p53, also recognized by PAb-421 (Fig. 2 A).

The association noted here between p53-related autoimmunity, anti-DNA and murine SLE is intriguing. Genotoxic stress has been found to induce the accumulation of p53 in the stressed cells; high levels of p53 are observed in tumors [13] and in activated inflammatory cells [14]. The accumulation of p53 can trigger an autoimmune response; humans [15] or mice with tumors [16] and subjects suffering from chronic inflammation [17, 18] show elevated levels of anti-p53 antibodies. Moreover, it has been reported that SLE patients show elevated levels of antibodies to p53 [19]. In addition to p53, some of the other autoantibodies characteristic of SLE appear to recognize other molecules involved in apoptosis [20].

We have shown that an autoimmune network directed to the central domain of p53 can generate anti-p53 immunity and resistance to tumors [8, 9]; here we find the C-terminal domain involved in murine SLE. Thus p53 may be a member of the set of dominant self antigens, termed the immunological homunculus, to which autoimmunty can be either beneficial or detrimental, depending on the circumstances [21].

4 Materials and methods

4.1 Mice, antibodies and assessment of lupus

Female MRL/MpJ-Fas^{lpr} mice were obtained from the Jackson Laboratories, Bar Harbor, ME, at the age of 6 weeks. They were bled twice, at the ages of 9 and 19 weeks, marking the beginning and an advanced stage of their spontaneous development of murine lupus [22].

Female mice of the BALB/c or C57BL/6 strains were obtained from the animal breeding facilities at the Weizmann Institute of Science, Rehovot, Israel, and used at the age of 8–10 weeks. Groups of at least ten mice each were immunized with anti-p53 antibodies PAb-246, PAb-248 [23], or PAb-421 [24], which were purified from ascitic fluid by protein A affinity chromatography (Sigma, Rehovot, Israel). For immunization, 50 μg antibody emulsified in CFA were injected i.d. into the hind footpads. To induce anti-DNA and anti-p53 antibodies, the mice were boosted 3 weeks later s.c. in the flank with 20 μg of the antigen in IFA, and sera were taken for study 10 days later. Individual mice were not bled repeatedly to avoid artifacts. Thus, for detection of anti-histone antibodies, 30 BALB/c mice were boosted three times at 3-week intervals with PAb-421 and were bled 2 weeks after the last boost. For induction of an SLE-like disease, the mice were boosted four times at 3-week intervals with 50 μg antibody emulsified in CFA. Ten mice each were bled 2 weeks later to study their leukocyte counts (heparinized blood diluted 1:10 in 1 % acetic acid). Proteinuria was detected with Albustix strips (Bayer, Slough, GB). Degrees of proteinuria were as follows: + (0.3 g/l), ++ (1 g/l), +++ (3 g/l), ++++ ($\geq$ 20 g/l).

4.2 Recombinant p53 and p53 peptides

Escherichia coli BL21 (DE3) cells were transformed with the T7 expression vector containing mouse p53 cDNA [25]; purification of p53 was done as described [26]. The p53 peptides (p16–35) and p363–382) were prepared using an automated synthesizer (Abimed AMS 422; Langenfeld, Germany) according to the manufacturer. Peptide purity was tested by analytical reverse-phase HPLC and mass spectroscopic analysis. The sequence of p16–35 is: PLSQETFSGLWKLLPPEDIL; that of p363–p382 is: HSSYLKTKKGQSTSRHKKTM.

4.3 ELISA

ELISA were done in 96-well Maxisorp plates (Nunc, Roskilde, Denmark), which were coated with 10 μg/ml test antigen in PBS. After washing and blocking with 1 % BSA in PBS for 1 h at 37 °C, diluted test sera (1:100; 0.1 ml per well) were added for 1 h at 37 °C. There then followed 1 h of incubation with goat anti-mouse IgG Fc-specific, or IgG isotype-specific secondary antibodies conjugated to alkaline phosphatase (Jackson, Philadelphia, PA), diluted 1:5000. A substrate solution containing 0.6 mg/ml p-nitrophenyl-phosphate (Sigma, Rehovot, Israel) in diethanolamine-H_2O, pH 9.8 was added, and the plates were read at 405 nm. For the measurement of anti-DNA antibodies, a secondary antibody conjugated to horseradish peroxidase, and, as substrate, 1 mg/ml 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) in 0.2 M citric acid, 0.2 M Na_2HPO_4, and 3‰ H_2O_2 (all from Sigma) was used. The test antigens used were calf thymus DNA after pre-coating with methylated

BSA, histones (all from Sigma, $(Fab')_2$ fragments of the different mAb, which were prepared as described [27], recombinant p53, or the p53 peptides p16–35 or p363–382. The antibody titers are shown as the mean absorbance. The error bars in the figures represent the SD of the mean.

4.4 mAb

The mAb MRL-1, Idi-1 and Idi-2 were raised at the Department of Biological Services of the Weizmann Institute of Science as described [27]. In brief, NS0/1 myeloma cells [28] were fused with spleen cells derived either from 17-week old MRL/MpJ-Fas^{lpr} mice, or from proteinuric BALB/c mice that had been immunized to PAb-421. Clones were selected for the production of anti-idiotypic antibodies to PAb-421 using the ELISA assay. Idiotypic specificity was controlled by testing for reactivity to the PAb-246 and R73 [29] mAb.

4.5 Band shift assay

A band shift assay was performed as described [9] to detect antibodies specific for the radiolabeled p53-responsive element oligonucleotide TCGAGAGGCATGTCTAGGCATGTCTC [6].

4.6 *C. luciliae* assay

To detect anti-DNA antibodies associated with lupus, slides with *Crithidia* were purchased from Immco (Buffalo, NY) and incubated with the test sera according to the manufacturer's protocol.

4.7 Histology

Paraffin sections of tissue samples from ten proteinuric BALB/c mice immunized with PAb-421 and from ten control mice were either stained with hematoxylin and eosin, or immunostained with a peroxidase-labeled anti-mouse-IgG antibody (Jackson, Philadelphia, PA) and 3,3'-diaminobenzidine (DAB; Sigma, Rehovot, Israel) according to the manufacturer's instructions.

4.8 Statistics

The differences between experimental groups were tested for significance using the Mann-Whitney test.

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Correspondence: Irun R. Cohen, Department of Immunology, The Weizmann Institute of Science, 76100 Rehovot, Israel

Fax: +972-8-9344103

e-mail: Irun.Cohen@weizmann.ac.il