Alteration of Cellular and Humoral Immunity by Mutant p53 Protein and Processed Mutant Peptide in Head and Neck Cancer

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Abstract

Purpose: To determine if serologic recognition of p53 mutations at the protein level depends upon the ability of mutant p53 to express new peptide epitopes that bind to human leukocyte antigen (HLA) class II molecules, we used anti-p53 antibody production as a marker for HLA class II – restricted T-cell involvement in head and neck cancer.

Experimental Design: An anti-p53 antibody response was correlated with specific p53 mutations and the patients' HLA class II alleles and haplotypes. HLA binding studies and *in vitro* stimulation (IVS) of peripheral blood mononuclear cells were done using a mutant versus wild-type HLA-DQ7-binding p53 peptide.

Results: Certain HLA-DQ and HLA-DR alleles were frequently present in p53 seropositive patients who produced serum anti-p53 antibodies. Selected mutated p53 peptides fit published allele-specific HLA class II binding motifs for the HLA-DQ7 or HLA-DR1 molecules. Moreover, a mutant p53 peptide bound with a 10-fold greater affinity than the wild-type p53 peptide to HLA-DQ7 molecules. IVS of $CD4^+$ T cells from seven healthy HLA-$DQ7^+$ donors using this mutant p53 peptide ($p53_{220C}$) was associated with a partial T helper type 2 phenotype compared with IVS using the wild-type $p53_{210-223}$ peptide.

Conclusions: Our results support the hypothesis that mutated p53 neoantigens can bind to specific HLA class II molecules, leading to a break in tolerance. This may lead to skewing of the $CD4^+$ T lymphocyte response toward a tumor-permissive T helper type 2 profile in head and neck cancer patients, as manifested by seropositivity for p53.

The immune system seems to recognize at least six categories of tumor-associated antigens: differentiation antigens, reactivated embryonic gene products, oncogene or suppressor gene products, mutated gene products, viral gene products, and idiotypic epitopes (1, 2). Tumor suppressor gene products, with mutations that may lead to new epitope formation, comprise another source of tumor antigens that are not on normal tissues. A member of this category of tumor antigens, the oncoprotein p53, may represent an attractive target for immunotherapy strategies (3) because this polypeptide chain of 393 amino acids is mutated in over 50% of all human cancers.

Mutated p53 peptides have been shown to bind to specific HLA class I molecules and generate CTLs capable of lysing head and neck squamous cell carcinoma cells containing p53 mutations in an HLA class I – restricted, tumor peptide – specific manner (4 – 7). There is also significant evidence that HLA class II – restricted antitumor responses are able to effectively contribute to antitumor immunity (8 – 10). Tumor burden and p53 seropositivity have been associated with a tumor permissive T helper type 2 (Th2) skewing of the $CD4^+$ T-cell response in cancer patients (11) as manifested by low secretion of IFN-γ and high secretion of interleukin-4 (IL-4), IL-5, and IL-13 (12, 13), However, the precise role of mutated tumor antigenic peptides in mediating such an effect on antitumor antigen immunity has not been sufficiently clarified.

Antibody production directed against mutated p53 has been found in the sera of patients with a wide variety of tumors, including breast, pancreas, lung, colon, and head and neck (14 – 18). The generation of these anti-p53 antibodies has previously been attributed to the presence of increased circulating levels of mutated p53 oncoprotein (19), binding of heat shock proteins to the mutated proteins (20, 21), and mutations in certain exons (22). Tumor antigen seropositivity is also thought to be reflective of a Th2 skewing of the $CD4^+$ T-cell

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response in cancer patients. Thus, we used anti-p53 antibody production as evidence for HLA class II–restricted Th cell activation and investigated the relationship between T helper profiles in healthy donor peripheral blood mononuclear cells (PBMC) stimulated with a mutant $p53_{210-223}$ peptide (Y→C at position 220). In this way, a form of immune escape mediated by p53 mutation may lead to presentation of a tumor antigen capable of shaping a tumor permissive immune response via HLA class II binding. Deleterious effects on antitumor immune responses may result from mutant tumor peptide specific, HLA class II antigen–restricted stimulation in cancer patients.

Materials and Methods

Sera. Serum was collected from each of 43 head and neck cancer patients during the perioperative period (within 3 months preoperatively or postoperatively). Blood was allowed to clot and then was spun for 15 min at 2,500 rpm in a chilled centrifuge. Serum was then decanted into a cryostat tube that was stored in a -80°C freezer.

Sequence analysis. Tumors from each of the 43 patients were immediately frozen in liquid nitrogen and stored in a -80°C freezer. To remove normal tissue and inflammatory cells, the frozen specimens were microdissected, and the DNA was isolated as previously described (23). Exons 5 through 9 of the p53 gene, contained in a 1.8-kb fragment, were amplified using the PCR, then cloned, and sequenced. All reactions were repeated to confirm the results.

Immunoprecipitation. Both mutant (248, 143) and wild-type (SN3) p53 plasmids (the kind gift of B. Vogelstein) were cloned into Bluescript plasmid vectors downstream from the T7 promoter site. After growth in *Escherichia coli*, the DNA was extracted using the boiling lysis method then linearized with restriction enzymes for *in vitro* transcription using T7 RNA polymerase (Riboprobe kit, Promega Biotech). The presence of RNA was confirmed with a MOPS minigel before *in vitro* translation in a rabbit reticulocyte lysate system (Promega). The protein generated was labeled with m7G(5′)ppp(5′)G (Pharmacia). Patient serum (5 μL) was mixed with the translation product (5-10 μL) in radioimmunoprecipitation assay buffer–bovine serum albumin buffer, vortexed, and incubated for 2 h at room temperature. Staph Protein A-Sepharose (Pharmacia; 1.5 mg/25 μL radioimmunoprecipitation assay buffer–bovine serum albumin) was then added, and this slurry was incubated on a shaker for 30 min at room temperature. After centrifugation, the supernatant was discarded, and the Sepharose beads were extensively washed in sequential buffer solutions (radioimmunoprecipitation assay buffer 1 mol/L urea, radioimmunoprecipitation assay buffer 1 mol/L NaCl, and radioimmunoprecipitation assay buffers). Sample buffer was added, and the Sepharose beads were then loaded onto a SDS-PAGE gel and run at 150 V. The gels were fixed, dried, and treated with Amplify (Amersham). The autoradiographs were exposed to film overnight in -80°C freezer, after which the film was developed. A murine monoclonal antibody (mAb) to I-CAM was used as a negative control, and a murine mAb NCL-p53-1801 (Novacastra Labortories Ltd., Newcastle upon Tyne, United Kingdom), which reacts with both mutant and wild-type p53, served as a positive control. All assays were repeated to confirm the presence of antibodies.

Western blotting. Various evolutionarily conserved domains of the p53 protein were used to create p53 fragments that were expressed as glutathione *S*-transferase fusion proteins in *E. coli*. Five of these constructs, which encompassed different combinations of these domains, and an unrelated protein, Oct 1 pou, were used in this study. The plasmid pET11GTK, containing the glutathione *S*-transferase gene and DNA encoding portions of p53, was transformed in *E. coli* BL21 (DE3) cells as previously described (24). Cultures were inoculated from frozen stock and grown at 25°C in M9ZB culture medium supplemented with 100 μg/mL ampicillin in 0.4 mmol/L isopropyl-β-D-thiogalactopyranoside for induction of the protein. The cultures were centrifuged and resuspended in ice-cold lysis buffer as previously described. The fusion proteins were extracted by treating the cultures with lysozyme (100 μ/mL), multiple freeze-thaw cycles, and then sonication. The extracts were centrifuged and then aliquoted for storage in -80°C freezer. These proteins were bound to glutathione-Sepharose beads (Pharmacia), and 100 μL of this slurry were then incubated with 10 μL of patient sera. After incubation for 1 h at 4°C, the beads were washed thrice in HNN buffer and then loaded onto an SDS-PAGE gel. The gel was transferred to a polyvinylidene difluoride membrane using the Milliblot-SDE Transfer system. Secondary antibody consisting of goat anti-human peroxidase was incubated with the membrane (1:5,000 in 5% Blotto) for 1 h. The unrelated protein, Oct 1 pou, was used as a negative control and sera with anti-p53 served as a positive control. Enhanced chemiluminescence solution (Amersham) was used to develop the chemiluminescence on the membrane. Film was placed over the membrane and exposed for 1 min and then developed.

Haplotype determination. DNA (1-10 μL) isolated from the tumors was PCR-amplified for the following class II loci: DRB1, DRB2, DQB1, DQB2. The DNA was then probed with horseradish peroxidase–labeled oligonucleotide as previously described (25).

Competitive binding assay to HLA-DQ7. To investigate the binding of synthetic mutant and wild-type p53 peptides to the purified HLA-DQ7 (DQ3.1; DQA1*0301, DQB1*0301) molecule, a competitive binding assay was done as previously described (26). p53 peptides were used only if the patient possessed the HLA-DQ7 genotype. The peptide AYK functioned as a positive control, as it binds well to the purified DQ7 molecule with a known IC_{50} of <0.1 μmol/L. The negative control, peptide 34P, bound with an IC_{50} of >10 μmol/L.

In vitro *stimulation of HLA-DQ7$^+$ healthy donor PBMC.* $CD4^+$ T-cell cultures were generated from HLA-$DQ7^+$ healthy donors identified by DNA-based typing at the University of Pittsburgh Immunogenetics facility, using PBMC as described (7, 27) with the following modifications. Monocytes were separated with a Percoll (Pharmacia) gradient, selected by plastic adherence for 2 h at 37°C, and cultured for 6 days in 10% human antibody serum (Mediatech) AIM-V with granulocyte macrophage colony–stimulating factor (100 ng/mL) and IL-4 (100 ng/mL). The resulting immature dendritic cells (DC) were matured for 24 h at 37°C in a medium supplemented with IFN-α (1000 units/mL), IFN-γ (1000 units/mL), IL-1β (25 ng/mL), and tumor necrosis factor-α (50 ng/mL). Wild-type $p53_{210-223}$ or mutant $p53_{220C}$ peptide was added at 1 μmol/L for the final 1 h of incubation. CD4+ PBMC obtained by negative selection using an antibody-coated MACS bead (Miltenyi Biotec) cocktail, including CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and glycophorin A. The isolated $CD4^+$ T cells (>97% purity) were resuspended in AIM-V medium supplemented with 2% human antibody serum and 7% fetal bovine serum.

Multiplexed ELISA (Luminex) assays. Twenty-four hours post restimulation of PBMC, supernatants were collected and analyzed using Luminex and the human cytokine Ten-plex panel kit (Invitrogen, Carlsbad, CA), which measured IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte macrophage colony-stimulating factor, tumor necrosis factor-α, and IFN-γ. LabMAP technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent bead–based technology, allowing multiplexed analysis of up to 100 different analytes in a single microtiter well (Biosource, Inc.). The differentially dyed polystyrene microspheres serve as a solid support for this "sandwich" immunoassay. Usual interassay variability within the replicates is 3.5% to 7%, and intraassay variability is 11% to 15%, as validated with appropriate ELISA with an 89% to 98% correlation (28, 29).

ELISPOT assays. IFN-γ or IL-5 ELISPOT assays were done in triplicate as described previously (7, 13). In all the experiments, autologous PBMC pulsed with an irrelevant peptide or with the p53 peptide (wild type or mutant) were used, respectively. Phorbol 12-myristate 13-acetate/ionomycin-treated CTL were used as a control for CTL secretion of IFN-γ. Plates were read using a Carl Zeiss VISION

Table 1. Correlation of patient's anti-p53 antibody production with p53 mutations and immunodominant epitopes

Patient no.	Mutation codon	p53 antibody	Western blot fragments
40	tyr→cys	+	2C, 23
53	tyr→cys	+	NC, N5, 2C, 35, 23
168	tyr→cys	+	N5, NC, 35
66	arg→gln	+	5, NC, 23
226	gly→glu	+	N5, NC
309	pro→ser	+	N5, NC
296	pro→leu	+	N5, 35
200	his→arg	-	—
192	arg→gly	-	—
69	ile→asn	-	—
245	cys→tyr	-	—
62	G insert	-	—
99	arg→leu	-	—
27	tyr→ser	-	—
71	glu→stop	-	—

NOTE: Patients' sera reacted with evolutionarily conserved domains of the p53 protein to determine possible immunodominant epitopes on the p53 molecule. Tumors from the patients were sequenced to determine the exact p53 mutation. Sera was tested for the presence of anti-p53 antibodies using a nondenaturing immunoprecipitation assay. Sera containing anti-p53 antibodies were incubated with various p53 fragments that were created using glutathione *S*-transferase fusion proteins in *E. coli*. This Western blot assay determined if the patient's sera bound to particular p53 fragments more frequently than others, thereby establishing if possible immunodominant portions7 of the p53 protein existed.

ELISPOT reader. Background was considered as the number of spots secreted by CD4+ T cells alone. T-cell reactivity, as measured by the ELISPOT assay, was considered positive if the number of spots in test wells was significantly higher than that in background wells when using a two-tailed permutation test at $\alpha \leq 0.05$. Precursor frequency of unstimulated cells was 10 to 15 cells per well for IFN-γ and negligible (0-2 cells per well) for IL-5. To determine the HLA class II antigen restriction of the CTL reactivity, target cells were incubated with either HLA-A, HLA-B, HLA-C–specific mAb W6/32 (30) or HLA-DR, HLA-DP, HLA-DQ antigen–specific mAb (LGII-612.14; ref. 31) or without any mAb. The precursor frequency of unstimulated cells for IFN-γ was 10 to 15 cells per well, and IL-5 was negligible (0-2 cells per well). Each mAb was used at the final concentration of 10 μg/mL and was incubated for 30 min at 37°C before the addition of the effector CTL.

Results

Correlation of antibody production with p53 mutations. To determine whether the mutated p53 protein could express new epitopes that would be predicted to bind the patient's HLA class II molecules, the p53 gene was sequenced (exons 5-9) in a series of 43 tumors from patients with head and neck squamous cell carcinoma. This revealed that 15 patients had mutations, mostly missense mutations, although one patient had a frameshift mutation (patient 62; Table 1). In our patient population, the mutations were present in multiple locations, including exons 6, 7, and 8.

The ability of these patients to generate anti-p53 antibodies served as a marker for class II $CD4^+$ Th2 helper lymphocyte activation. Immunoprecipitation and Western blot assays allowed identification of the p53 mutations that were associated with an immune response. These techniques did not cause denaturation of the p53 protein before the addition of the patient's sera. Both wild-type and mutant forms of p53 were reacted with the serum in the immunoprecipitation assay. The reactive serum determined by immunoprecipitation had antibodies that reacted with both mutant and wild-type forms of p53 (Fig. 1). Seven patients (of 43; 16%) had circulating anti-p53 antibodies as detected by the Western blot technique (Table 2). Of the patients with p53 mutations, approximately half (7 of 15 or 47%) had detectable antibody to p53 (Table 1).

Determination of immunodominant p53 epitopes. The evolutionarily conserved domains of the p53 protein were used as fusion proteins bound to glutathione-Sepharose beads in a sensitive Western blot assay. This technique allowed identification of possible immunodominant epitopes on the p53 molecule because the sera reacted with fragments of the p53 molecule. Sera from seven patients bound to various p53 constructs. No immunodominant epitopes could be localized in this patient population because antibodies to p53-mutated oncoproteins reacted with all domains of the p53 molecule, not just the amino and/or carboxy terminus. As seen in Fig. 2 and Table 1, internal domains (2C, 23), as well as more terminal domains (N5, NC), bound p53 antibody.

Class II alleles and anti-p53 antibody production. The possible association between class II HLA major histocompatibility antigen expression and the immune response to p53 oncoproteins was assessed by genotyping 43 patients with head and neck squamous cell carcinoma (Table 3). The HLA-DQ, HLA-DR, and HLA-DP alleles and haplotypes were determined. The DPB1 alleles did not seem to have any association with antibody production. However, many of the patients who produced antibodies were found to have the DQB1*0301 (three of seven patients), DRB1*0701 (three of seven patients), DQB1*0501 (three of seven patients), and DRB1*0101 (two of seven patients) alleles. Patients with p53 mutations who did not produce antibodies frequently had the DQB1*0201 (four of eight patients) and the DRB1*0701 (four of eight patients) alleles.

Matching of p53 mutation sequences to HLA class II binding motifs. Several studies (32) have elucidated the detailed allele-specific peptide binding motifs for some of the class II

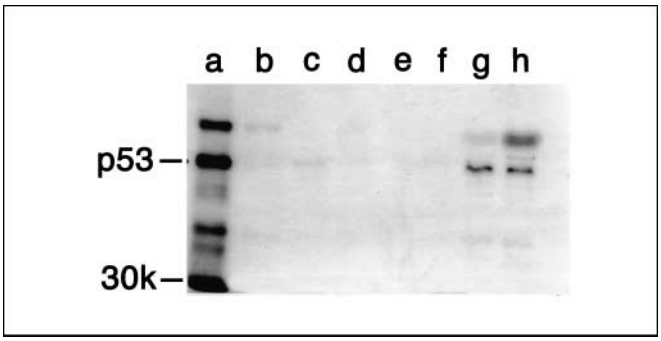

Fig. 1. Patient's anti-p53 antibodies in immunoprecipitation assays react with both mutant (248) and wild-type (SN3) forms of the p53 protein. As a positive control, a murine monoclonal antibody to p53 was reacted with the wild-type p53 (*lane a*). Monoclonal antibody to I-CAM was reacted with mutant p53 as a negative control (*lane b*). Patient's sera without anti-p53 did not bind the labeled p53 (*lanes c-f*); however, sera from patients with p53 missense mutation bound both mutant and wild-type p53 (*lanes g-h*), respectively.

Table 2. Correlation of patients' antibody production and p53 mutations with class II haplotypes

Patient no.	Anti-p53 antibody	p53 mutation codon/exon	DR-DQ1	DR-DQ2	DPB1	DPB2
27	No	Yes/220/6	1302-0609	0103-0501	0401	0201
40	Yes	Yes/220/6	1201-0301	1201-0301	0101	3601
46	No	No	1601-0502	0301-0201	0401	0402
49	No	No	1501-0602	0101-0501	0401	0401
53	Yes	Yes/220/6	1502-0601	0701-0303	1501	0301
55	No	No	0701-0201	1305-0301	—	—
56	No	No	1501-0602	1101-0301	3301	4101
58	No	No	1301-0603	0701-0303	-	-
59	No	No	0103-0301	0301-0201	0401	0201
62	No	Yes/279/8	1501-0503	1401-0503	0401	1001
63	No	No	0103-0501	0301-0201	0401	0401
66	Yes	Yes/248/7	0101-0501	0701-0303	3701	4601
69	No	Yes/251/7	1101-0301	1101-0301	3701	3701
71	No	No	0701-0201	0301-0201	0201	1101
75	No	No	1503-0602	0302-0402	0201	0101
77	No	No	1302-0609	0701-0303	0402	0101
85	No	No	0101-0501	0701-0303	0401	0401
93	No	No	0701-0201	0701-0201	-	-
98	No	No	0301-0201	0402-0301	0402	0301
99	No	Yes/306/8	1201-0301	0701-0201	—	—
100	No	No	0301-0201	0301-0201	0201	0402
102	No	No	0301-0201	1602-0502	—	—
107	No	No	0801-0301	0701-0201	0402	0301
168	Yes	Yes/220/6	1503-0602	1102-0301	0401	0201
180	No	No	0701-0201	0402-0301	—	—
191	No	No	0401-0301	0101-0501	5101	1101
192	No	Yes/249/7	0701-0201	0701-0201	1301	1401
200	No	Yes/193/6	1401-0503	1503-0502	0301	1301
217	No	No	0401-0302	0404-0303	0401	0201
226	Yes	Yes/272/8	0403/06-0302	0407-0301	0401	0301
230	No	No	1501-0602	0301-0201	0401	0301
233	No	No	0701-0201	1302-0501	1801	0101
242	No	No	0101-0501	1104-0301	0402	0402
244	No	No	0101-0501	0101-0501	0401	0402
245	No	Yes/275/8	0101-0501	0701-0201	0401	0401
253	No	No	1602-0502	1303-0301	0201	1701
269	No	No	1502-0601	1502-0601	2301	4801
270	No	No	0102-0501	1103-0301	0401	0402
273	No	No	0301-0201	1302-0501	1101	4001
278	No	No	1302-0609	0901-0303	0101	1701
289	No	No	0901-0303	0102-0501	0401	0201
296	Yes	Yes/278/8	0701-0201	0101-0501	0401	0301
309	Yes	Yes/278/8	1101-0602	0102-0501	0201	0101

NOTE: Tumor DNA was isolated PCR-amplified for the class II loci listed above and then probed with horseradish peroxidase–labeled oligonucleotides as described in Materials and Methods (—, not determined).

molecules. Therefore, a search was undertaken for the best possible fit of mutant p53 proteins with these published class II ligand motifs. In Falk's study, the allele DQB1*0301 ligand motif was derived from pool sequencing natural peptides of the DQA1*0501-DQB1*0301 molecule. In this particular motif, there are three important hydrophobic or aromatic anchor residues at positions 1, 5, and 7. In addition, peptide ligands usually possess charged and polar residues in clusters, as well as proline residues at positions 2, 11, 12, 13, and 14 (19). Because every patient with a known class II haplotype had their tumor DNA sequenced for p53 gene mutations, the mutant amino acid residue sequences (14-mers) could be matched with some of the allele-specific class II ligand motifs for best possible fit. All the mutant p53 peptides that fit this class II allele binding motif were also the mutant p53 molecules associated with antibody production (Fig. 3A). The converse was also true.

Three of the patients with known p53 mutations possessed the same DRB1*0101-DQB1*0501 haplotype. The peptide sequences containing the mutations were matched via the best possible fit to the allele-specific HLA-DR1 (DRB1*0101) binding motif published by Falk et al. (32). Anchor residues were located at positions 1, 4, and 9 on this binding motif. In Fig. 3B, the mutated p53 peptides (from patients 66 and 296) that matched two of three residues on the DRB1*0101 binding motif were associated with antibody production. The peptide (patient 245) that was predicted to bind only one of three anchor residues was not associated with anti-p53 production.

Competitive binding of p53 mutant peptides to HLADQ7 (DQA1*0301, DQB1*0301, DQ 3.1). Two patients (patients 40 and 168) with the same DQA1*0301-DQB1*0301 haplotype had tumors with a p53 missense mutation (tyr→cys) in codon 220 (exon 6). Their mutated p53 peptide (14-mer) was

sequenced and used in a competitive binding assay using the DQ7 molecule DQ3.1. It would be predicted that binding of the synthetic mutated p53 peptides with the DQ7 molecule would be seen if class II presentation was involved in determining antibody production. Not only was this the case but also the mutated peptide sequence (NTFRHSVVVPCEPP) bound to the DQ7 molecule with higher affinity than the wild-type p53 peptide (NTFRHSVVVPYEPP; Fig. 3A). The substitution of a cysteine for a tyrosine in the mutated p53 peptide resulted in a 10-fold greater binding to the DQ7 molecule as shown by an IC_{50} of 0.58 μmol/L. The wild-type peptide had an IC_{50} of 4.5 μmol/L. In addition, patient 69 possessed the DQB1*0301 allele (DRB1*1101-DQB1*0301) but did not produce anti-p53 antibody. The mutated p53 peptide (MNRRPNLTIITLED) was also sequenced then used in the same competitive binding assay. As predicted by this model, the p53 mutated peptide (at codon 251) and the wild-type peptide (MNRRPILTIITLED) did not function as a ligand for this class II molecule. IC_{50} values were both >10 μmol/L. Patient 226 (DRB1*0407-DQB1*0301) produced anti-p53 antibodies and possessed a p53 mutation at codon 272. When the mutated peptide sequence (14-mer) was used in the competitive binding assay to the DQ7 molecule, no binding was found by either the mutated (NSFEERVCACPGRD) or wild-type p53 peptide (NSFEVRVCACPGRD). In this case, the substitution of a glutamic acid residue for a glycine residue did not alter binding to the DQ7 molecule.

HLA-DQ7–binding mutant $p53_{220C}$ peptide stimulation induces a partial Th2 cytokine phenotype in healthy donor $CD4^+$ PBMC. The cytokine phenotype induced by stimulation of $CD4^+$ T cells with either the wild-type ($p53_{210\text{-}223}$) or mutant ($p53_{220C}$) peptide was compared in ELISPOT assays. Healthy donor PBMCs were used because tolerance to p53 is similarly induced in the thymus of healthy donors and cancer patients, and the HLA-$DQ7^+$ head and neck squamous cell carcinoma patients were deceased and PBMC were unavailable for these studies. Thus, $CD4^+$ T cells from seven healthy, HLA-$DQ7^+$ donors were magnetic-bead isolated, and *in vitro* stimulation (IVS) was done for 1 week using the wild-type $p53_{210\text{-}223}$ or mutant $p53_{220C}$ peptide. To ensure that results were not skewed by the induction conditions, autologous DC or PBMC stimulator cells were used. IFN-γ or IL-5 ELISPOT assays were then done, as shown in Fig. 4A and B, using autologous PBMC target stimulators pulsed with either p53 peptides or irrelevant surface peptides, respectively. As shown in Fig. 4A, significantly lower IFN-γ release was observed in $CD4^+$ T cells stimulated with the mutant $p53_{220C}$ peptide compared with the same $CD4^+$ PBMC stimulated after one round of IVS using DC pulsed with the wild-type $p53_{210\text{-}223}$ peptide ($P \leq 0.05$, one-tailed permutation test). To determine whether this effect was influenced by the induction conditions, IVS was also done using autologous HLA-$DQ7^+$ PBMC as stimulators, pulsed with either wild-type ($p53_{210\text{-}223}$) or mutant $p53_{220C}$ peptides. These experiments yielded a similar pattern of cytokine release as IVS using DC stimulators, but lower overall spots were observed using PBMC (not shown). IL-5 ELISPOT assays using $CD4^+$ T cells stimulated with the mutant ($p53_{220C}$) peptide yielded HLA Class II–restricted IL-5 spots ($P \leq 0.05$). The precursor frequency of unstimulated cells for IFN-γ was 10 to 15 cells per well, and IL-5 was negligible (0-2 cells per well). These results were observed in separate HLA-$DQ7^+$ PBMC IVS cultures repeated at least twice for each donor tested ($n = 7$).

Multiplex Luminex cytokine assays were then done to measure the global cytokine profiles secreted by $CD4^+$ T cells, as listed in Materials and Methods, stimulated with either the mutant or wild-type p53 peptide. Although IL-4 secretion was consistently higher in a subset of donor cells after mutant ($p53_{220C}$) peptide stimulation, this difference was not statistically significant due to the sample size. Secretion of IL-4 and IL-5 (Fig. 4B) support a partial Th2 bias under these conditions. Interestingly other Th1 cytokines (IFN-γ, tumor necrosis factor-α) were not detected in these cultures (data not shown), confirming

Table 3. Competitive binding assay of p53 mutant peptides to DQ7 (DQA1*0301, DQB1*0301, DQB 3.1)

Peptide	IC_{50} (umol/L)
AYK (positive control)	<0.1
34P (negative control)	>10
p53 mutant patient 168	0.58
p53 wild-type patient 168	4.5
p53 mutant patient 69	>10
p53 wild-type patient 69	>10
p53 mutant patient 226	>10
p53 wild-type patient 226	>10

NOTE: p53 peptides, both mutant and wild type, were sequenced (14-mers), and IC_{50} was determined.

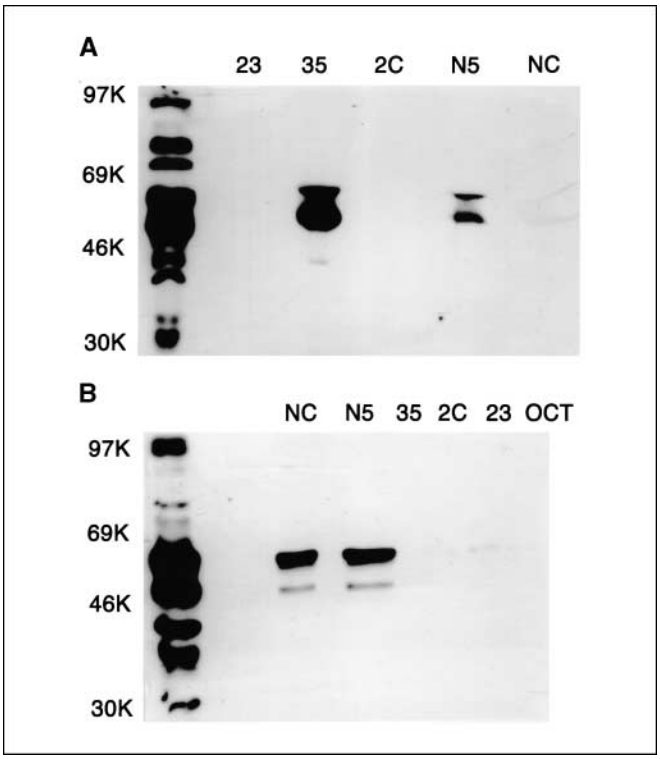

Fig. 2. Western blot assay using fragments of p53 as fusion proteins. No immunodominant regions were found. Examples of two patient's reactions. *A,* patient 296 reacted with the 35 and N5 fusion proteins. *B,* patient 309 had antibodies that recognized NC and N5 proteins. Oct 1 pou was an unrelated protein that served as a negative control.

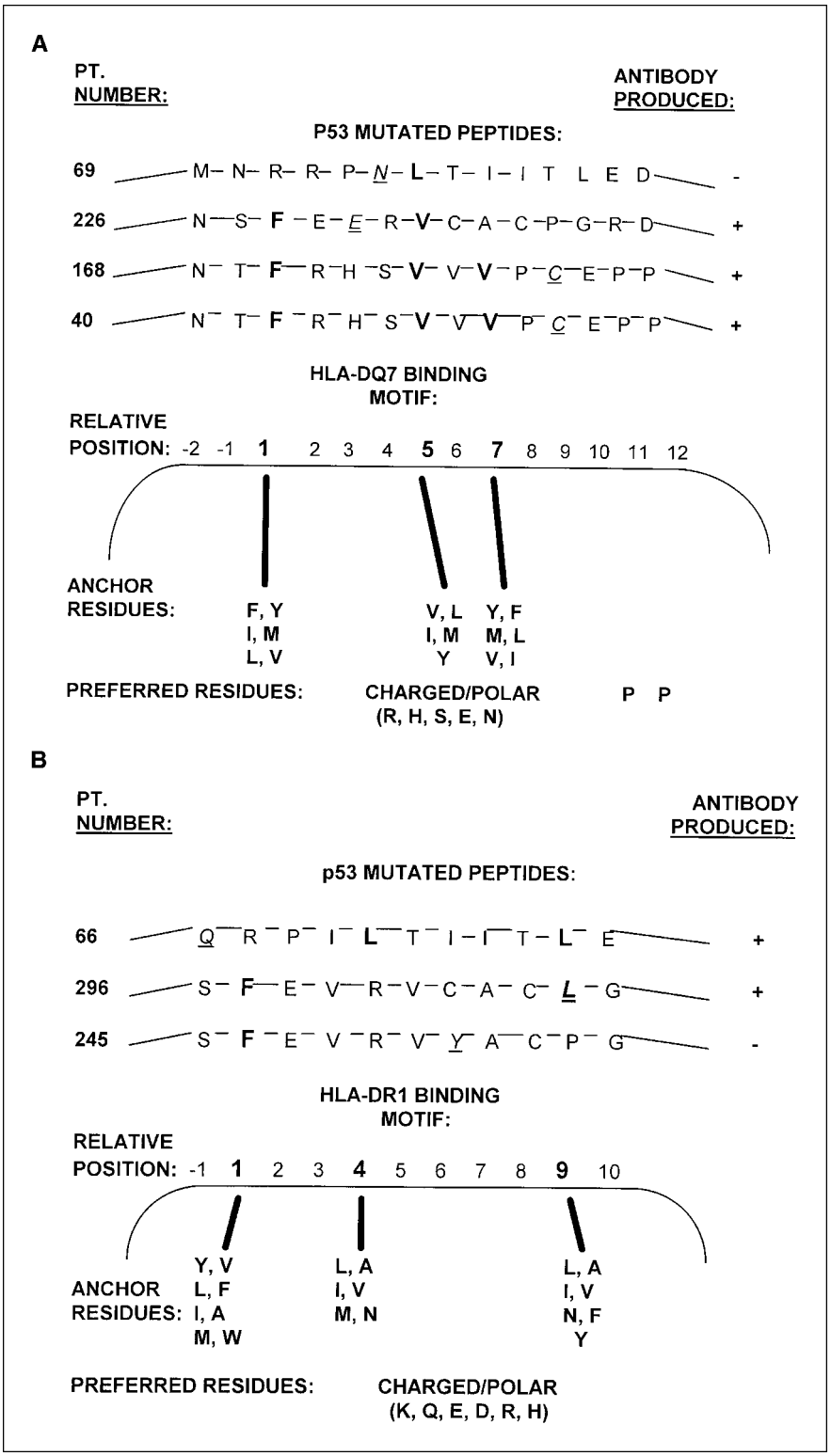

Fig. 3. *A,* HLA-DQ7 (DQB1*0301) binding motif (1) and p53 mutant peptide sequences. The mutated amino acid residues are in italics and underlined. Amino acids that match predicted anchor residues are in bold letters. If the peptides were predicted to bind two or more anchor residues, antibody production was predicted. *B,* HLA-DR1 (DRB1*0101) binding motif and p53 mutant peptide sequences. The mutated amino acid residues are in italics and underlined. Amino acids that match predicted anchor residues are in bold letters. All patients had the DRB1*0101-DQB1*0501 haplotype. In patients wherein more than one anchor residue was predicted to bind, antibody production was seen (patients 66 and 296). Conversely, p53 serum antibodies were not seen in the patient where less than two anchor residues bound the motif (patient 245).

a lack of Th1 bias often seen using DC-based IVS procedures (33). The lower limit of the assay may reflect insufficient sensitivity to detect cytokines produced by a low number of cells, such as the IL-5 secretion T cells portrayed in Fig. 4B. The quantity of IL-6 and IL-8 secreted by the CD4+ T cells derived from the three healthy donors were not consistent with either the antigen-presenting cell used, mature DC or PBMC, or the peptide stimulation, wild-type or mutant p53 peptides. Stimulations were done using either autologous DC or PBMC, with similar results, to rule out that alterations in cytokine secretion were associated with the particular induction conditions used.

Discussion

Head and neck cancer patients are capable of generating anti-p53 antibodies because 16% (7 of 43) of the patients had reactive sera. The antibodies detected in the sera recognized both mutated and wild-type p53 proteins in accordance with previous studies (19, 34, 35). As was found with other tumor types, not all head and neck cancer patients with a p53 gene mutation had detectable antibody to the oncoprotein. Because immunoprecipitation detected one reactive serum whereas Western blot assays discovered seven sera with antibody present, the Western blot technique seemed to be much more sensitive. There was a relatively high incidence of anti-p53 antibodies detected in this patient population when compared with other studies, such as lung cancer patients (13%; ref. 19) or breast cancer patients (9%; ref. 36), although 23% of patients with colorectal carcinoma had serum p53 antibodies (37). In other studies of head and neck cancer patients from 18% to 44% of the patients had serum p53 antibodies (38, 39). Nearly half (7 of 15, 47%) of our patients with tumors harboring a p53 mutation had an immunologic response to both mutant and wild-type p53 protein. This finding implies that tolerance was abrogated in these patients.

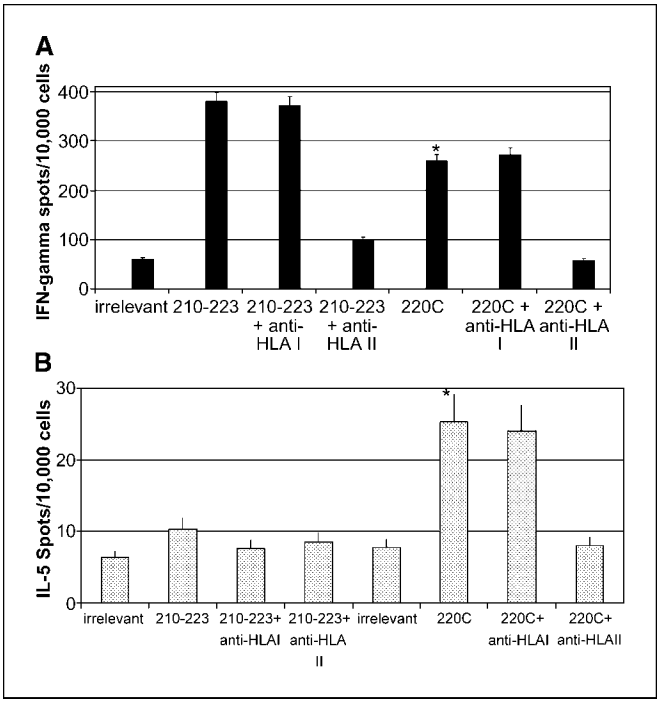

Fig. 4. IFN-γ and IL-5 ELISPOT assays to measure $CD4^+$ T-cell responses in healthy HLA-$DQ7^+$ donor PBMC after stimulation with wild-type $p53_{210\text{-}223}$ or mutant $p53_{220C}$ peptides. *A,* after 1 wk of IVS using autologous, mature DC pulsed with the wild-type $p53_{210\text{-}223}$ or $p53_{220C}$ peptide, respectively. HLA-$DQ7^+$ healthy $CD4^+$ T cells were assayed using IFN-γ ELISPOT assays. Stimulator cells in these assays were autologous, mature day 7 DC loaded with the same wild-type $p53_{210\text{-}223}$ or $p53_{220C}$ peptides. Pretreatment with either HLA-A, HLA-B, HLA-C – specific mAb W6/32 (30), HLA-DR – specific mAb (L243), or HLA-DR, HLA-DP, HLA-DQ antigen – specific mAb (LGII-612.14) or without any mAb was done to block the recognition of $CD4^+$ T cells as described in Materials and Methods. Columns, mean spots; bars, SD. A representative of experiments repeated at least twice with four separate HLA-$DQ7^+$ healthy donor PBMC ($P < 0.05$). *B,* after 1 wk of IVS using autologous, mature DC pulsed with the wild-type $p53_{210\text{-}223}$ or $p53_{220C}$ peptide, respectively. HLA-$DQ7^+$ healthy $CD4^+$ T cells were assayed using IL-5 ELISPOT assays. Stimulator cells in these assays were autologous, mature day 7 DC loaded with the same wild-type $p53_{210\text{-}223}$ or $p53_{220C}$ peptides. Pretreatment with either HLA-A, HLA-B, HLA-C – specific mAb W6/32 (30), HLA-DR – specific mAb, (L243) or HLA-DR, HLA-DP, HLA-DQ antigen – specific mAb (LGII-612.14) or without any mAb was done to block the recognition of $CD4^+$ T cells as described in Materials and Methods. Columns, mean spots; bars, SD. A representative of experiments repeated twice with four separate HLA-$DQ7^+$ healthy donor PBMC ($P < 0.05$).

Immunodominant epitopes derived from the p53 protein, the result of conformational changes caused by missense mutations, also do not seem to account for the antibody response against p53. Lubin et al. (40) and Schlichtholz et al. (41) found that immunodominant epitopes were localized predominantly in the amino terminus and, to a lesser extent, in the carboxy terminus. Using our Western blot technique with p53 fusion proteins (of evolutionarily conserved regions), no immunodominant epitopes were found to correlate with antibody production. Because almost all (six of seven) patients producing anti-p53 antibodies had either DQB1*0301 or DQB1*0501 alleles, we postulated that the peptide's amino acid structure and ability to bind to HLA class II binding grooves or motifs may be implicated in the anti-p53 response.

To test our hypothesis, the published class II allele-specific HLA-DQ7 (DQA1*0501-DQB1*0301) binding motif (32) was matched to the mutant p53 peptide sequences to determine if the peptides were ligands for the binding motif. Typically, peptides of varying lengths, often 12 to 24 residues, bind class II molecules and, therefore, binding motifs exhibit open ends, such that portions of entire proteins could bind if appropriate anchor residues were exposed (42). Therefore, the best possible fit of the mutated p53 amino acid sequence into the class II binding motifs was attempted. Four patients had the DQB1*0301 allele, and HLA-DR alleles associated with the HLA-DQ7 molecule. Three other patients possessed the DRB1*0101-DQB1*0501 haplotype of the HLA-DR1 molecule. The peptide sequences (14-mers and 11-mers) from patients that produced antibodies fit the motif extremely well; both the mandatory anchor residues and the preferred charged/polar and proline residues were present in the mutant p53 peptides. The patients that did not generate anti-p53 antibodies had mutant peptide sequences that did not fit the class II binding motifs. Only one of the p53 mutations resulted in the creation of a new anchor residue. In a vast majority of the cases (six of seven), the wild-type p53 peptide fit the motif, and mutation was nearby. In the case of HLA-DQ7 binding, the mutation was within two amino acid residues away from a potential anchor-binding residue. This suggested that the mutation was creating a neoantigen that could be presented by the class II complexes.

The competitive binding assay to the DQ7 molecule further suggests that p53 oncoprotein binding to certain class II molecules determines antibody production. The lack of binding of the DQ7 molecule by the mutated peptide of patients 226 shows that predicting which peptides will bind may depend upon other factors including the peptide's tertiary structure *in vivo*. In this particular case, the mutation caused a glutamic acid residue (charged) to be encoded instead of a glycine residue (small) which could result in steric hindrance of binding. Whereas this study is suggestive of binding of p53 oncoproteins to specific class II molecules as the determining factor in anti-p53 antibody production, the actual *in vivo* process involving conformation changes may be more complex.

Mutated and wild-type p53 molecules are also targets for class I–restricted peptide-induced CTL (2, 7). Less is known about the role of class II presentation of oncoproteins. The

effect of stimulation of healthy, HLA-DQ7$^+$ healthy donor PBMC was modeled *in vitro* using a mutant, HLA-DQ7–binding, mutant p53 peptide, and induced a Th2-biased cytokine profile. This peptide ($p53_{220C}$) was identified in two of our head and neck squamous cell carcinoma patients with anti-p53 serum antibodies and was found to bind to HLA-DQ7 molecules with 10-fold higher affinity than the wild type peptide ($p53_{210\text{-}223}$).

We observed that the enhanced binding of the mutant $p53_{220C}$ peptide was associated with lower IFN-γ and higher IL-5 secretion by PBMC stimulated from healthy HLA-DQ7$^+$ donors, whose T cells were stimulated with the mutant $p53_{220C}$ peptide. This is consistent with a skewing toward the Th_2 population of CD4 T cells.' Further experiments are necessary outside the scope of the studies reported here to clarify more subtle effects on T-cell phenotypic subsets. We conclude that a form of immune escape may be exerted on antitumor effector T cells by tumors presenting mutated p53 peptides. This mechanism has been suggested in tumor bearing renal cell carcinoma or melanoma patients, which could be reversed after tumor removal (12). Because a missense mutation in p53 is among the most common alterations in human cancer, these finding have important implications for strategies to reverse immunosuppressive effects of tumor burden, such as cancer vaccines.

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