A Thyroid Tumor-Specific Antigen Formed by the Fusion of Two Self Proteins[1]

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Thyroid epithelial cells frequently express one or more members of the rearranged during transfection/papillary thyroid carcinoma (*RET/PTC*) fusion oncogene family during early stages of cancer, and fusion gene transcripts have been found in inflammatory conditions of the thyroid such as the autoimmune disease, Hashimoto's thyroiditis. Because these oncogenes encode chimeric proteins, novel RET/PTC epitopes may be targets of antitumor immune responses. We have been interested in the RET/PTC3 (RP3) fusion protein because this family member is more frequently expressed in radiation-induced and childhood papillary carcinomas than other members of the fusion oncogene family. We hypothesized that the activated kinase of c-RET, in the form of RP3, when expressed in patients with thyroid disease, presents an unusual altered self target for T cell recognition. Interestingly, we find that immunization with mouse RP3 protein can induce a strongly immunogenic response to RP3, although this response is not directed against the peptide comprising the unique fusion region. Rather, the responses are specific for the carboxyl-terminal portion of RP3 that is derived from the self protein c-RET. Furthermore, transplantation of RP3-expressing thyroid tumors into naive mice resulted in leukocytic infiltration, tumor rejection, and induction of RP3-specific T cells. Thus, the somatic fusion of two unrelated self proteins results in the development of a uniquely immunogenic response directed against self epitopes within RP3. These studies may better define the mechanisms controlling the initiation of thyroid-specific immune responses and provide insight into the design of novel molecules for invoking tumor-specific immunity. ***The Journal of Immunology*****, 2003, 170: 861–869.**

While evidence demonstrating the existence of thyroid-specific Ags is pervasive, the evidence that these tissue-specific Ags become targets of antitumor-specific immunity is absent. For example, numerous studies have suggested that thyroglobulin, thyrotropin receptor, and thyroid peroxidase may serve as Ags during thyroid autoimmune disease (1); however, it is unknown whether these Ags represent major targets in anticancer host responses. This is important because targeting tissue-specific Ags in the thyroid may be analogous to the self protein-directed antimelanoma immunotherapies used in patients in which autoimmune depigmentation (vitiligo) can result from targeting such self molecules (2). Similar treatments in thyroid cancer patients may cause autoimmune thyroid disease, the leading cause of hypothyroidism in the U.S. Consequently, we have been interested in determining whether thyroid cancers express tumor-specific targets distinguishable from self that may limit immunization-induced autoimmune disease. Because differentiated thyroid carcinomas originating from the thyroid epithelium frequently express tumor-specific rearranged during transfection/papillary thyroid carcinoma (RET/PTC)[3] fusion proteins (3), we hypothesized that inflammation along with immune responses to these unique Ags during the early stages of thyroid transformation may lead to immune recognition (4) and, by directing immune cells to target RET/PTC proteins, may provide an ideal antitumor therapy. To begin to address such a hypothesis, we set out to measure the nature of RET/PTC immunogenicity.

Papillary thyroid cardcinoma (PTC) is a relatively rare cancer, with an incidence of 3 of 100,000 (5); however, it is the most prevalent endocrine tumor, comprising nearly 80% of all thyroid malignancies (1). Nearly 60% of differentiated thyroid carcinomas of the epithelium, including PTC, is characterized by the frequent expression of one or more members of a family of unique oncogenic fusion proteins resulting from chromosomal inversions or translocations (6, 7). In a majority of PTC, the tyrosine kinase domain (TKD) of the c-RET or tropomyosin receptor kinase proto-oncogenes and the amino-terminal portion of one of several widely expressed genes are juxtaposed to form a hybrid protein with ectopic constitutive tyrosine kinase activity in the cytosol of thyroid epithelial cells (3). The family of 10 fusion genes associated with *c-RET* rearrangement is collectively known as *RET/PTC*. One member of this fusion gene family, *RET/PTC3* (*RP3*), is formed following a paracentric inversion involving *c-RET* and *RFG/ARA70* (8, 9). Of note, *RP3* can be found in thyroid tissue from patients with the chronic thyroid inflammatory disease, Hashimoto's thyroiditis (10, 11). Likewise, somatic rearrangement of *c-RET* has been observed in irradiated human thyroid tissue grafts in SCID mice, supporting the notion that ionizing radiation is an etiologic agent for thyroid cancer (12). Tumor progression to advanced stages (poorly differentiated or undifferentiated thyroid cancer) in humans and in animal models does not correlate with persistent RET/PTC expression (4, 13, 14), a finding inconsistent with other models of multistep carcinogenesis (15).

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[3] Abbreviations used in this paper: RET/PTC, rearranged during transfection/papillary thyroid carcinoma; PTC, papillary thyroid carcinoma; h, human; LNC, lymph node cell; m, mouse; p.t., posttransplant; RP3, RET/PTC3; Tcl1, T cell locus 1; TKD, tyrosine kinase domain.

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In human cancers, mutant proteins involved in cellular transformation harbor structural abnormalities, including rearranged coding regions, internal deletions, point mutations, or viral proteins resulting from insertional mutagenesis. Altered gene products encoding novel fusion oncoproteins may represent candidate tumor-specific Ags capable of eliciting immune recognition and may be ideal targets for tumor immunotherapy because their expression is required for transforming activity. Although published data suggest a role for fusion gene products in human tumor-specific immune responses (16–19), there is no clear understanding of whether fusion proteins represent immunogenic epitopes enabling tumor rejection.

The relationship between lymphocytic infiltrates and thyroid cancer, particularly PTC, in humans is well documented (20, 21). The observation that lymphocytic infiltration of the thyroid coexists with cancer suggests that Ags expressed by tumor cells are responsible for inducing an antitumor immune response (22). We hypothesized that reactivity to transformed thyroid cells or occult tumors may lead to oncoprotein-reactive lymphocyte accumulation in the thyroid. In this study, we have investigated the immunogenic capability of the RP3 fusion protein when presented in the presence of a strong adjuvant or when expressed in preneoplastic thyroid tissue or invasive thyroid tumor grafts to define its role as an antigenic nonself protein and thyroid-specific tumor Ag. Although the RP3 protein represents the fusion of two widely expressed self proteins to which immunological tolerance is most likely induced, we find that fusion confers a unique immunogenic capability upon the RP3 protein. The results of these studies may help to define novel strategies for the immunotherapeutic targeting of oncoproteins at early stages of neoplastic progression.

Materials and Methods

Mice

RP3 transgenic mice (3209 line) have been previously described (23) and have subsequently been backcrossed for >10 generations to endotoxin-resistant *C3H/HeJ* mice using polymorphism selection. Homozygous *RP3* mice were crossed with *C57BL/6-Tpr53* mice and double heterozygotes crossed to generate $RP3^{p53-/-}$ mice (4). Progeny were genotyped using *RP3*- and *p53*-specific primers in a standard DNA amplification reaction (PCR), as previously described (4). Six- to 8-wk-old C57BL/6J-$Prkdc^{scid}$/SzJ (*C57BL/6* SCID) mice (The Jackson Laboratory, Bar Harbor, ME) were used for the growth of tumor tissue and to determine specimen viability. Female *C3H/HeJ* and male *B6C3F1*/J, 6–8 wk old, were obtained from The Jackson Laboratory.

Expression plasmid construction

Human (h) or mouse (m) *RP3*, mouse Ret^{TKD}, or control coding sequence T cell locus 1 (*Tcl1*) were cloned into the pET-29a$^+$ protein expression vector (Novagen, Madison, WI) adjacent to a COOH-terminal hexahistidine tag to facilitate protein purification, and a selectable kanamycin marker to aid in positively selecting transfectants. The *hRP3* gene sequence was amplified from the construct previously described for the generation of *RP3* transgenic mice (23). The amino-terminal gene segment of mouse *Rfg/Ara70* (AF159461) and mouse Ret^{TKD} (AF209436) were amplified using endonuclease restriction site-linked gene-specific primers (*Sal*I-linked *Rfg/Ara70* forward primer, GTC/GAC/GTC/GAC/ATG/AAC/ACA/TCC/CTG/GAA/CAG/AGT/GG; *Bam*HI-linked *Rfg/Ara70* reverse primer, CGG/GAT/CCT/CCT/GAC/TAT/TCT/CTG/AAG/TCT/GTT/TTG/G; *Bam*HI-linked *Ret* forward primer, CTG/GAT/CCA/AAG/TGG/GAA/TTT/CCT/CGG/AAG/AAC; and *Not*I-linked *Ret* reverse primer, GTC/GCG/GCC/GCT/TAG/CTA/TCA/AAT/GTG/TCC/ATT/AAT/TTT/GC). Partial and complete *Bam*HI digestion of *Rfg/Ara70* and *c-RET* genes, respectively, was required for proper ligation of full-length *RP3*. All constructed plasmids were verified by sequence analysis.

Recombinant proteins and peptides

To generate recombinant protein, plasmid constructs were introduced into BLR (DE3) pLysS-competent cells (Novagen, Madison, WI), and protein expression was induced in kanamycin-resistant cells using isopropyl *B*-D-thiogalactopyranoside at a final concentration of 1 mM for 4 h at 37°C. Cells were lysed in denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 8.0), cellular debris were removed by ultracentrifugation at 45,000 rpm for 30 min at 4°C, and hexahistidine affinity-tagged proteins first bound to a Ni-NTA agarose column (Qiagen, Valencia, CA) were then eluted under denaturing conditions using an imidazole gradient. A second round of column chromatography was performed again using a Ni-NTA agarose column to ensure high protein purity. Recombinant proteins were dialyzed against PBS, 0.22 μm filtered, and analyzed for the presence of the 68-kDa rhRP3, the 75-kDa rmRP3, the 45-kDa $rmRet^{TKD}$, or the 14-kDa rTcl1 protein (negative control) on SDS-PAGE by Coomassie blue staining and, in parallel, by Western blot analysis using polyclonal rabbit anti-RET (C-19) and (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Tcl1 Abs, as previously described (23). Proteins were verified to be endotoxin free using the endotoxin neutralizing protein from *Limulus polyphemus* (Sigma-Aldrich, St. Louis, MO). An RP3 peptide (KQTLE NSQEDPKWEF) specific for the fusion breakpoint region was synthesized by the Thomas Jefferson University Peptide Synthesis Facility. This sequence was chosen based on its high MHC-predicted binding coefficient, as measured by an established algorithm (http://syfpeithi.bmiheidelberg.com/scripts/MHCServer.dll/home.htm). Peptide scores >20 were predicted to have significant binding potential.

Viral preparation

Recombinant vaccinia virus was propagated, as previously described (24). The *RP3* construct was cloned into a modified pSC11 plasmid for expression from the vaccinia $P_{7.5}$ promoter. *RP3* gene sequence was introduced into the vaccinia genome by homologous recombination in CV-1 cells, and recombinant viruses were subsequently triple plaque purified in 143B cells in the presence of 5 mg/ml 5-bromo-2′-deoxyuridine (Boehringer Mannheim, Indianapolis, IN). Viral stocks were expanded and titered using 143B $HuTK^-$ cells.

Immunization protocols

Female *C3H/HeJ* mice, *RP3* transgenic mice, or nontransgenic littermates were s.c. immunized in the hind flank with 10 μg rhRP3 or rmRP3, 5 μg $rmRet^{TKD}$, or 10 μg rTCL (control) protein emulsified in CFA (Sigma-Aldrich). This injection was followed 1–2 wk later by a second injection of the same material into the same mice (10 μg of rRP3, 5 μg $rmRet^{TKD}$, or 10 μg rmTcl1 control protein); however, this injection used protein emulsified in IFA (Sigma-Aldrich). For specified experiments, additional controls included injections of PBS in CFA, followed by PBS in IFA (nonimmune mice). One to two weeks following final immunization, lymph node cells (LNC; mandibular, superficial cervical, axillary, lateral axillary, superficial inguinal, mesenteric, and popliteal nodes), spleen cells, and serum were harvested for analysis. For tumor transplant immunization, anesthetized male 6- to 8-wk-old *B6C3F1*/J or *C57BL/6* SCID control mice received 3-mm^3 $RP3^{p53-/-}$ thyroid tumor fragments. Mice were monitored each week for signs of tumor rejection (including reduction in tumor size or stabilization of tumor growth). After 2 mo, LNC and serum were harvested from mice that had rejected tumor fragments.

Lymphoproliferation assays

Single cell suspensions of LNC and/or spleen cells from rRP3-, tumor-, or nonimmunized mice were prepared in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 25 μM 2-ME, 200 U/ml of penicillin, 200 U/ml of streptomycin, 10 mM L-glutamine, and 10% FBS. For T cell proliferation assays, cells were cultured in 96-well plates (2×10^5 cells/well) to which recombinant proteins, RP3-specific peptide, or bacterial control eluates were added at a previously determined optimal concentration of 1 μg/ml. Plates were cultured in a 5% CO_2 humidified incubator for 48 h at 37°C and incubated with 1 μCi of [^{3}H]thymidine/well for additional 18 h. Wells were harvested onto nylon filters, and radioactivity was quantified using scintillant in a gamma counter (Beckman Instruments, Palo Alto, CA). Results are expressed in cpm (mean of quadruple wells).

Enzyme-linked immunosorbent assay

Antisera were collected from rhRP3-immune, $RP3^{p53-/-}$ tumor-immune, and nonimmunized mice by collecting blood from the retroorbital sinus, followed by clotting and centrifugation at 14,000 rpm for 5 min. Serum was removed and stored at 4°C. In ELISA, 250 ng of rhRP3 protein was adsorbed overnight to each well of a 96-well microtiter plate at 4°C. Serial dilutions of sera were added to protein-coated wells and incubated at room temperature for 2 h. Plates were washed and incubated with biotin-conjugated horse anti-mouse Ab (1:1000; Vector Laboratories, Burlingame,

CA), followed by streptavidin-alkaline phosphatase (1:4000; Southern Biotechnology Associates, Birmingham, AL) and subsequently *p*-nitrophenyl phosphate solution. Product detection was performed at 405 nm.

Immunoprecipitation assay

A 1/10 dilution of RP3 immune or nonimmune mouse antisera in immunoprecipitation buffer (2% Triton X-100, 5 mM Tris-HCl (pH 7.5), 10 mM NaCl) was bound to protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) overnight with 10 μg of rhRP3, and washed twice with immunoprecipitation buffer and twice with 10 mM Tris-HCl, pH 7.5. Equivalent amounts of rhRP3 protein in 1× SDS loading buffer was resolved using multiple lanes of a 8% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) for 100 V/h. Membranes were blocked for 1 h in 5% milk-TBST (20 mM Tris-HCl (pH 7.6), 135 mM NaCl, and 1% Tween 20) and cut into individual strips corresponding to the lanes on the original SDS-PAGE gel. Each strip was incubated with a 1/1000 dilution of goat anti-RET (C-19) polyclonal Ab (Santa Cruz Biotechnology) in 5% milk-TBST. After incubation, membranes were treated with HRP-labeled anti-goat IgG (ECL reagent kit; Amersham Pharmacia Biotech) for 30 min at room temperature, according to the manufacturer's protocol. Substrate was added from the ECL reagent kit (Amersham Pharmacia Biotech) and exposed to x-ray film for 1–5 min.

Histological analysis

Thyroid tissues were removed, fixed in 10% Formalin for ≥24 h, and desiccated. Following desiccation, tissue samples were embedded in liquid paraffin and cooled. Paraffin-embedded tissue was cut into 6-μM sections and placed on silanized slides (Fisher Scientific, Pittsburgh, PA). After deparaffinizing, the sections were rehydrated through decreasing concentrations of xylene and alcohol and microwaved for 15 min in 100 mM citrate buffer (pH 6.0). Slides were then stained with H&E, dehydrated, fixed, and mounted.

Statistical analysis

Grouped data were compared and analyzed for statistical significance using an independent samples *t* test (Analyze-it, version 1.65, program add-in for Microsoft Excel; Analyze-It Software, Leeds, U.K.). Groups were considered different when calculated *p* values were less than 0.05.

Results

RP3 is a nonself protein that stimulates class II-restricted Ag-specific T cell and serological responses

RP3 is a somatically activated, unique protein that consists of two fused self proteins that may encode immunogenic determinants after intracellular Ag processing. To determine the immunogenicity of the RP3 fusion protein, naive *C3H/HeJ* female mice were immunized with small amounts (10 μg) of purified rhRP3 protein in CFA. Two weeks following immunization, LNC were harvested from rhRP3-immunized and mock-immunized mice and cultured with rhRP3 or control proteins to assess T cell proliferative responses in vitro. LNC from naive mice immunized with rhRP3 proliferated in response to rhRP3, but not to an unrelated control protein purified using an identical purification methodology (Fig. 1*A*). Flow cytometric analysis identified the predominant proliferating cell population as $CD4^+$ (not shown). Consistent with these data, cell proliferation was significantly inhibited using anti-class II $I\text{-}A^k$ or $I\text{-}E^k$ Ab, but not with an isotype control antiserum (Fig. 1*B*). Because the novel amino acid sequence of the hRP3 fusion region represents a unique nonself amino acid sequence, a synthetic 15-mer peptide corresponding to this region and selected based on its MHC-binding potential was used for in vitro stimulation. Interestingly, lymphocytes from rhRP3-immunized mice responded only mildly to this synthetic peptide, suggesting that sequences other than the unique fusion region may constitute the natural immunodominant epitopes of RP3 (Fig. 1*A*). In contrast to rhRP3-immunized mice, LNC from control *C3H/HeJ* female mice failed to proliferate significantly above background in response to either rhRP3 protein or to the fusion region peptide (not shown).

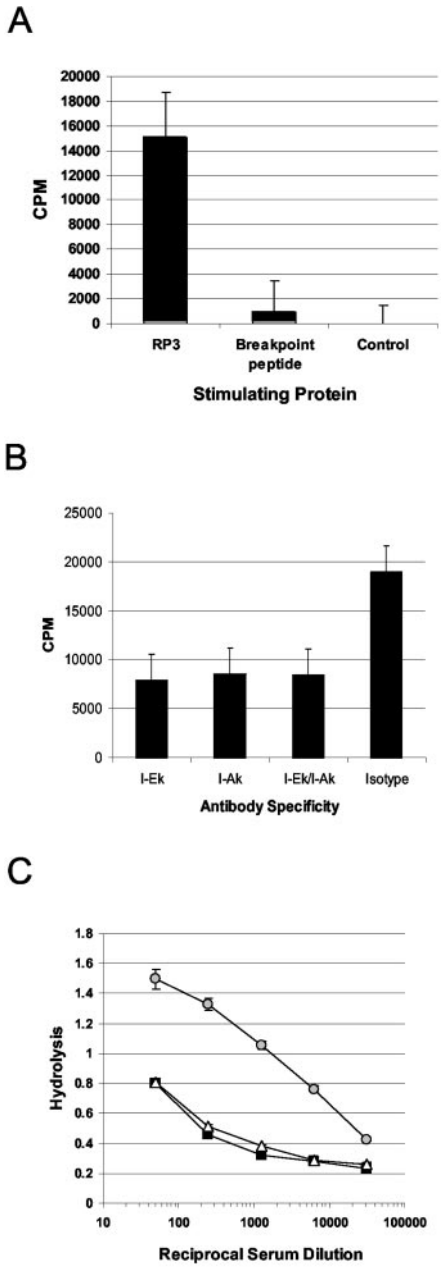

FIGURE 1. RP3-specific lymphoproliferation in wild-type mice. Wild-type *C3H/HeJ* female mice were immunized s.c. twice with 10 μg rhRP3 and adjuvant. One week following final injection, LNC and serum were collected. *A*, LNC from rhRP3-immunized mice show an in vitro recall proliferative response to rhRP3, but not to an unrelated protein rTcl1 purified using the same methodology, or a synthetic peptide corresponding to the RP3 fusion region. RP3 stimulation is significantly different from controls ($p < 0.02$), whereas response to the RP3 breakpoint peptide is not. *B*, Inhibition of rhRP3-immune LNC proliferation with Abs to MHC class II $I\text{-}E^k$ or $I\text{-}A^k$, but not with an unrelated isotype control Ab. Ab inhibition for each group (specificity) is significantly different from isotype control ($p < 0.001$). *C*, Serum from rhRP3-immunized mice (gray circles) shows reactivity to rhRP3 protein using ELISA. $RP3^{p53-/-}$ tumor-immunized (■) or nonimmunized (△) mice failed to produce specific Ab reactivity. Data are representative of three independent experiments. Statistical significance was determined by an independent samples *t* test (Analyze-it).

The induction of $CD4^+$ T cell responses specific for RP3 suggested that B cell responses might also be stimulated in immunized mice following exogenous protein immunization. To ascertain this, serum was collected from rhRP3- and control-immunized mice,

and the development of specific Ab responses was measured using ELISA. Although Ab reactivity to rhRP3 protein was not observed in sera samples from control-immunized mice, reactivity to rhRP3 was appreciable in sera from rhRP3-immunized mice (Fig. 1*C*). Additionally, immunoprecipitation results confirmed the specificity of rhRP3-immune sera by precipitation of the 68-kDa rhRP3 protein, in contrast to that of serum from control-immunized naive mice, which lacked rhRP3 reactivity (not shown). Taken together, these data demonstrate that RP3 is immunogenic, eliciting class II-mediated $CD4^+$ LNC proliferation and Ab reactivity following conventional adjuvant-based immunization schemes using small amounts of recombinant protein.

LNC proliferation and cross-reactivity following immunization with mRP3 protein

Because rhRP3 protein immunization of mice induced reactivity to whole RP3 fusion protein, but not fusion region peptide, the possibility existed that the observed RP3 reactivity was a product of a xenogenic response. Although the amino acid sequence of hRP3 and its respective counterparts expressed in mouse are 92% homologous, the immunogenic properties of hRP3 protein in mice may result from the remaining 8% amino acid differences. To address this concern, a *mRP3* fusion gene was engineered because such fusions are not known to exist in mouse thyroid tissue. Using specific PCR primers, the amino-terminal portion of the mouse *Rfg/Ara70* gene and the TKD of the c-RET proto-oncogene were amplified from adult *C3H/HeJ* spleen and salivary gland cDNAs, respectively. Subsequently, the two products were juxtaposed and cloned, in frame, into a bacterial expression vector. Because the c-RET tyrosine kinase domain (Ret^{TKD}) of both mouse and human forms of RP3 contain the highest scoring-predicted immunogenic epitopes, the gene sequence identical with the nonmutated Ret^{TKD} segment of the *mRP3* fusion gene was also cloned using the same vector system. rmRP3 and $rmRet^{TKD}$ proteins were produced and purified, and their expression was confirmed by Western blot analysis using a human-mouse cross-reactive Ret^{TKD}-specific Ab (Fig. 2*A*).

To measure the immunogenicity of the mRP3 protein, naive *C3H/HeJ* mice were immunized with rmRP3 protein, and 1 wk following final injection in vitro proliferation assays were performed on harvested LNC. Unlike control-immunized LNC that failed to proliferate in response to either protein, lymphocytes from rmRP3-immunized mice proliferated strongly and specifically to rmRP3 (Fig. 2*B*) and rhRP3. Similar results were obtained in reciprocal experiments in which rhRP3-immune LNC were stimulated with rmRP3 (Fig. 2*B*). These data show that mRP3 and hRP3 proteins are equally immunogenic and that xenogenicity cannot exclusively account for the observed anti-RP3 T and B cell reactivity, suggesting a unique immunological characteristic of the RP3 fusion protein.

To dissect the components of RP3 immunogenicity, we engineered a self protein that is identical with the c-RET portion of the mRP3 molecule ($rmRet^{TKD}$). This region also contains a predicted immunogenic self epitope, but lacks the unique fusion region segment of RP3. We next compared the immunogenicity of the RP3 fusion protein to Ret^{TKD}. Interestingly, rmRP3-immune LNC were capable of recognizing the $rmRet^{TKD}$ protein, as measured by lymphocyte proliferation. In addition, proliferation of both rmRP3 and rhRP3-immune LNC occurred in $rmRet^{TKD}$-stimulated cultures, although to a lesser extent than when these cultures were stimulated with whole mRP3 or hRP3 protein (Fig. 2*B*). Neither immune nor nonimmune lymphocytes proliferated well to control recombinant protein. These data indicated that an immunogenic self epitope(s) is encoded within the TKD portion of the RP3 molecule and that the unique breakpoint-specific amino acid sequence may not be essential for anti-RP3 immunity.

Tolerance to the Ret^{TKD} self protein

The above experiments demonstrate the immunogenicity of the mouse Ret^{TKD} when presented as a portion of the fused RP3 protein. Because the Ret^{TKD} is a self protein to which mice should be tolerant, we investigated whether immunization under conditions that would distinguish self from nonself proteins by injecting mice with this nonfused domain of mRP3 is capable of evoking an immune response. Naive mice were immunized with either whole rmRP3 or $rmRet^{TKD}$ protein in adjuvant; LNC were harvested and cultured; and in vitro proliferation assays performed. As observed previously, LNC from rmRP3 fusion protein-immunized mice responded strongly to both rmRP3 and rhRP3 stimulation and to a lesser extent to $rmRet^{TKD}$ protein (Figs. 2 and 3). In contrast, LNC from $rmRet^{TKD}$-immunized mice failed to proliferate in response

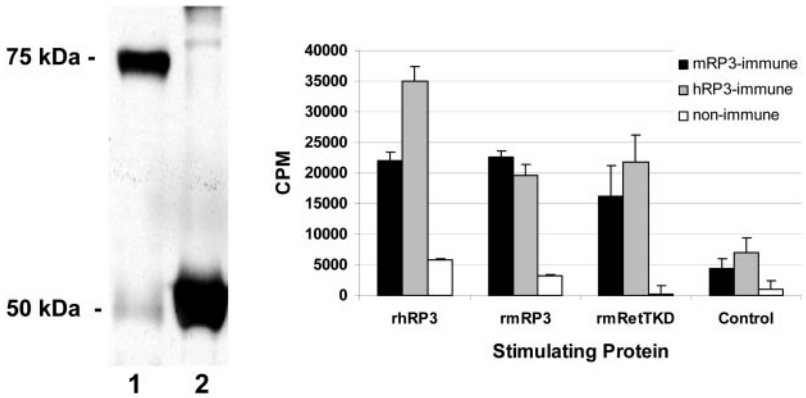

FIGURE 2. Cross-reactivity between mRP3 and hRP3 protein with dominant epitopes in Ret^{TKD}. *Left panel*, Bacterially produced rmRP3 proteins were purified under denaturing conditions, dialyzed against PBS, and analyzed for the presence of the 75-kDa rmRP3 (*lane 1*) and the 45-kDa rmRet TKD (*lane 2*) by Western blot analysis using polyclonal rabbit anti-RET Abs. *Right panel*, LNC harvested from naive *C3H/HeJ* female mice immunized s.c. twice with 10 μg rmRP3 and adjuvant (filled bars), 10 μg rhRP3 and adjuvant (gray bars), or PBS and adjuvant (open bars) were assayed in vitro for specific proliferation in response to 1 μg/ml rmRP3, rhRP3, $rmRet^{TKD}$, or control bacterial eluate (containing unrelated proteins) 1 wk after final immunization. The amount of proliferation between rhRP3- and rmRP3-primed immune cells restimulated with rmRP3 or $rmRet^{TKD}$ was the same; however, hRP3-primed cells responded better to hRP3 than mRP3 ($p < 0.001$). rhRP3-immune cells (but not rmRP3-immune cells) proliferated more strongly in vitro with rhRP3 than rmRP3 ($p < 0.001$). Primary responses (nonimmune cells) were detected against rhRP3 or rmRP3, but not against $rmRet^{TKD}$ ($p < 0.01$). Data are representative of three independent experiments. Statistical significance was determined by an independent samples *t* test (Analyze-it).

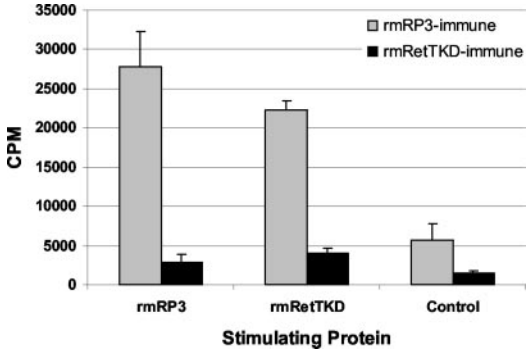

FIGURE 3. Self tolerance to a nonfused portion of rmRP3 is preserved. Naive *C3H/HeJ* mice were immunized twice with either 10 μg rmRP3 (gray bars) or 5 μg rmRet^{TKD} protein (filled bars), both in adjuvant. One week after final injection, LNC were harvested and assayed for in vitro proliferation in response to stimulation with 1 μg/ml rmRP3, 1 μg/ml rmRet^{TKD}, or 1 μg/ml control bacterial eluate (unrelated proteins). Data are representative of two independent experiments. Mice immunized with rmRet^{TKD} domain did not respond compared with controls, whereas both hRP3 and mRP3 were able to restimulate mRP3-immune lymphocytes ($p < 0.001$). rmRP3-immune cells responded strongly to rmRP3 and rmRet^{TKD} in contrast to rmRet^{TKD}-immune cells, which did not significantly respond to these same Ags ($p < 0.0001$). Statistical significance was determined by an independent samples *t* test (Analyze-it).

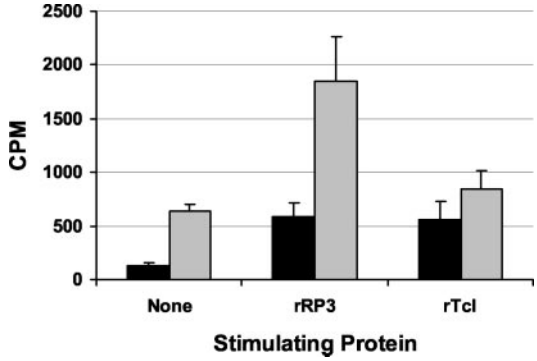

FIGURE 4. T cell tolerance in *RP3* transgenic mice. LNC were harvested from *RP3* transgenic mice (filled bars) or nontransgenic littermates (gray bars) 1 wk following a second s.c. immunization with 10 μg rhRP3 protein in adjuvant. Cultured LNC were stimulated in vitro with 1 μg/ml rhRP3, the same concentration of an unrelated control protein (rTcl1), or with PBS alone (none), and assayed for proliferation. Proliferation of RP3-primed LNC from RP3 transgenic mice was not significantly different from controls. In contrast, cultured LNC proliferated significantly more to hRP3 protein from RP3-primed nontransgenic littermates than from unrelated protein-primed controls or from RP3 transgenic mice ($p < 0.01$). Data are representative of three independent experiments. Statistical significance was determined by an independent samples *t* test (Analyze-it).

to stimulation with rmRP3, or the immunizing protein, rmRet^{TKD} (Fig. 3). LNC from neither immunized mice proliferated when stimulated with a protein control. Given these results, we predicted that only when the RP3 fusion protein was synthesized as a self protein would tolerance be observed.

Tolerance to RP3 fusion protein in transgenic mice

RP3 is the product of two fused self proteins to which T cell tolerance has most likely been induced; however, the above experiments demonstrate LNC and serological reactivity to the novel fusion protein occurs in immunized mice. Unlike wild-type mice, *RP3* transgenic mice express the *hRP3* fusion gene as a self protein exclusively in the thyroid epithelium (23) by virtue of a bovine thyroglobulin promoter that is known to be active at an early stage of glandular development. To determine whether thyroid-specific expression of RP3 confers T cell tolerance to whole RP3 protein, adult transgenic mice (>4 mo of age) were immunized with rhRP3 protein, and 2 wk later LNC were harvested. Compared with nontransgenic mice immunized with rhRP3 protein, T cells from immunized *RP3* transgenic mice failed to proliferate in response to rhRP3 protein (Fig. 4). LNC from both rhRP3-immunized *RP3* and nontransgenic mice failed to proliferate to an unrelated control protein. Although RP3 mice are reported to develop mild leukocytic infiltrates in their thyroid (25, 26), thyroid glands resected from immunized and nonimmunized *RP3* transgenic mice showed no difference in the degress of infiltration, as determined by histological analysis (not shown).

Preneoplastic thyroid tissue grafts expressing RP3 are not rejected in naive mice

Because injection of purified rRP3 indicated that exogenous exposure of the fusion protein can evoke a strong T cell proliferative response, we next assessed whether RP3-expressing thyroid tissue is also capable of evoking responses in naive mice following organ transplantation. To measure acute immune responses against RP3-expressing preneoplastic thyroid in vivo, thyroid lobes harvested from 4-mo-old *RP3* transgenic mice were s.c. transplanted into haplocompatible *B6C3F1* and SCID control mice. Thyroid tissue from donor mice at 4 mo of age is composed primarily of small RP3-expressing follicles containing primary and secondary hyperplasia (23). Three weeks posttransplant (p.t.), 10 of 10 thyroid grafts remained intact (Fig. 5) and functional, as determined by thyroglobulin expression and follicular morphology (not shown). These transplants were free of immune cell infiltration in both *B6C3F1* recipients and immunodeficient SCID recipient mice, as determined by histopathological analysis.

Thyroiditis in established transgenic thyroid grafts following infection with RP3, but not wild-type, vaccinia virus

To measure the ability to induce antithyroid immunity mediated by anti-RP3 responses, naive haplocompatible and SCID mice were

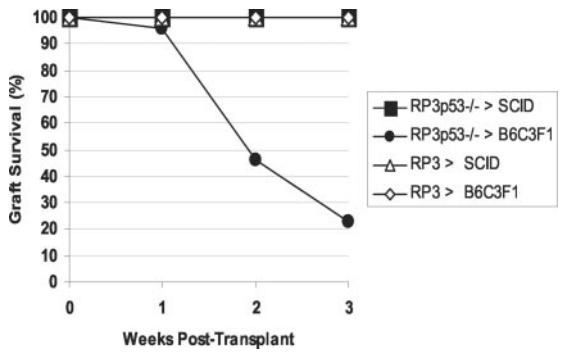

FIGURE 5. Immunological rejection of RP3-expressing thyroid tumors, but not preneoplastic thyroid grafts in haplocompatible mouse recipients. Naive *B6C3F1* and control *C57BL/6* SCID mice were s.c. transplanted with a thyroid tumor fragment (3 mm^3) harvested from a 6-mo-old *RP3*$^{p53-/-}$ transgenic mouse or a single thyroid lobe harvested from a 4-mo-old *RP3* transgenic mouse and monitored for graft survival. One hundred percent graft survival was noted when *RP3*$^{p53-/-}$ tumors were transplanted into SCID mice (filled square; 10 of 10) and, likewise, when *RP3* thyroid lobes were transplanted into either *B6C3F1* (♦; 10 of 10) or *C57BL/6* SCID (△; 10 of 10) mice. In contrast, *RP3*$^{p53-/-}$ tumors transplanted into haplocompatible mice (●) were rejected in 77% (21 of 27) of the recipients 3 wk following transplant. Data are representative of three independent experiments.

s.c. transplanted with a single *RP3*-expressing transgenic thyroid lobe and monitored for graft rejection for 5 mo. All grafts in recipient mice formed stable and measurable masses (10 of 10), suggesting a lack of complete tissue rejection and resolution (Fig. 5). To determine whether peripheral T cells are capable of recognizing the graft when stimulated under inflammatory conditions, recipient mice were infected i.v. with 10^7 PFU recombinant vaccinia virus encoding RP3, 10^7 PFU wild-type vaccinia virus, or PBS at 5 mo p.t. Mice were monitored weekly for rejection of established transplants. After 2 mo, minimal reductions in graft size were noted from recombinant virus-infected mice ($-7.79 +/- 2.21\%$) compared with wild-type virus-infected mice ($+3.17 +/- 3.45\%$). To determine whether thyroid transplants were rejected and to measure the degree of leukocyte involvement following immunization, the transplanted *RP3* thyroid lobes were resected, embedded, sectioned, and analyzed histopathologically. Examination of transplanted *RP3* thyroid lobes from RP3 vaccinia virus-infected mice (Fig. 6) revealed thyroiditis with infiltration of neutrophils, monocytes, and lymphocytes in 25–50% of the transplant (Fig. 6*C*), whereas transplants from PBS and wild-type vaccinia virus-infected mice remained free of leukocytic infiltration (Fig. 6, *A* and *B*). Extensive lymphocytic infiltration of the follicles caused significant organ disruption, although noninfiltrated regions of the thyroid transplant remained normal in morphology (Fig. 6*C*). Despite widespread thyroiditis, complete destruction and resolution of *RP3* thyroid grafts were not observed following single immunization with either recombinant or wild-type virus. The sequential order of thyroid transplant and immunization was not critical because similar results were observed in naive mice vaccinated with recombinant vaccinia virus 2 wk before a transgenic thyroid transplants (not shown).

Rejection of rapidly growing RP3-expressing thyroid tumors in naive mice

Because thyroids from 4-mo-old *RP3* transgenic mice are slow growing, rarely metastasize, and remain encapsulated (23), it was possible that thyroid organ transplants, in contrast to vaccinia virus-infected tissues, were incapable of eliciting a sufficiently strong inflammatory signal for the initiation of an appreciable graft rejection response. Consequently, to derive a strong inflammatory signal from thyroid grafts, we used tumor fragments expressing RP3 protein. *RP3* mice that have been crossed to *p53* null mutants ($RP3^{p53-/-}$) develop large, fast-growing, poorly differentiated thyroid carcinomas (4). Although *RP3* expression is eventually lost in these advanced tumors, thyroid tumors from 6-mo-old $RP3^{p53-/-}$ mice still express uniformly detectable levels of RP3, as previously demonstrated (4). To address the possibility that the inflammatory properties of malignant growth may enhance the induction of antithyroid responses in vivo, 3-mm^3 thyroid tumor fragments from 6-mo-old $RP3^{p53-/-}$ mice were transplanted s.c. into haplocompatible recipient mice and monitored weekly for tumor outgrowth. Whereas 100% of the $RP3^{p53-/-}$ thyroid tumor fragments transplanted into SCID mice grew rapidly, only

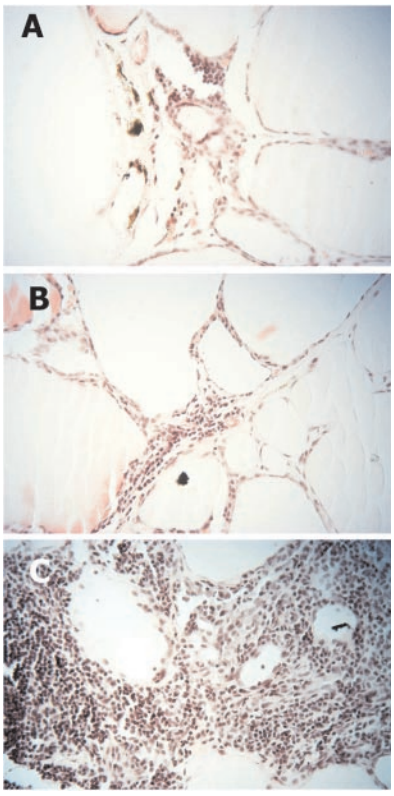

FIGURE 6. Thyroiditis in preneoplastic *RP3* thyroid grafts following RP3 sensitization. *RP3* thyroid grafts transplanted s.c. were viable in naive *B6C3F1* recipient mice 5 mo following transplant. Following this 5-mo period, thyroid graft recipient mice received a single injection of PBS (*A*), 10^7 PFU wild-type vaccinia virus (*B*), or 10^7 PFU RP3 vaccinia virus (*C*). Two months following infection, thyroid grafts were resected, paraffin embedded, sectioned, and H&E stained, and the degree of leukocytic infiltration was examined by histological analysis. Magnification ×25. Representative of two experiments.

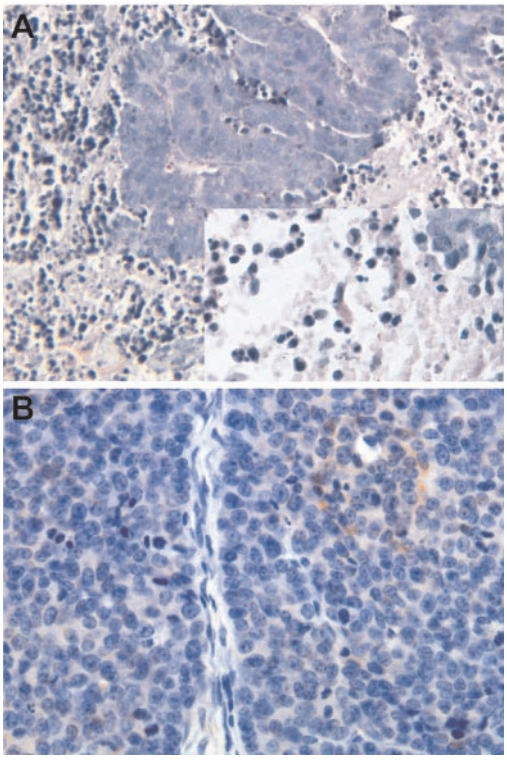

FIGURE 7. Leukocytic infiltration of $RP3^{p53-/-}$ tumor grafts in syngeneic mice. Resected tumor tissue fragments were evaluated for immune infiltration 1 mo p.t. by histological analysis. *A*, Leukocytic infiltration primarily composed of polymorphonuclear neutrophils, monocytes, and lymphocytes was evident within remaining thyroid tumor tissues resected from immunocompetent *B6C3F1* mice. Magnification ×40. *Inset*, Higher magnification of leukocytes infiltrating the tumor tissue shown in *A*. Magnification ×60. *B*, Leukocytic infiltrates were not evident in tumors resected from control immunodeficient *C57BL/6* SCID mice. Magnification ×40. Representative of four experiments.

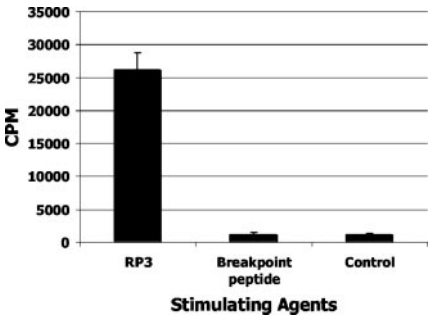

FIGURE 8. RP3-specific proliferation of LNC from tumor-rejecting mice in vitro. Two months p.t., LNC from *B6C3F1* mice that had rejected *RP3*$^{p53-/-}$ thyroid tumor grafts were harvested and cultured with rhRP3 protein (1 μg/ml), an unrelated protein rTcl1 (1 μg/ml), or an RP3 breakpoint peptide (1 μg/ml). LNC proliferation was specific for hRP3 and significantly greater than responses to the breakpoint peptide or control (rTcl1) protein ($p < 0.00001$). Breakpoint peptide responses were no different from controls ($p = 0.81$). Data are representative of three independent experiments. Statistical significance was determined by an independent samples *t* test (Analyze-it).

23% of tumor fragments transplanted into *B6C3F1* recipients grew progressively, as assessed at 3 wk p.t. (Fig. 5). Histopathological analysis was performed on the remaining thyroid transplants resected from *B6C3F1* recipients at 1 mo p.t. Compared with tumors grown in SCID mice, tumor resected from *B6C3F1* mice was infiltrated with numerous polymorphonuclear neutrophils and lymphocytes (Fig. 7).

RP3-specific T cell, but not B cell, responses in tumor-rejecting mice

Leukocyte involvement suggested Ag-specific T cell responses may be elicited during *RP3* tumor rejection. To investigate this, 2 mo following tumor transplant, *B6C3F1* recipient mice were sacrificed and autopsied to verify the complete rejection of *RP3*$^{p53-/-}$ thyroid tumor. Complete rejection was confirmed by analysis of the transplant site following specimen embedding, fixation, and H&E staining. Furthermore, if RP3 is a dominant tumor Ag in tumor-rejected recipients, a robust lymphoproliferative response should be detectable by standard proliferation assay. To measure T cell responses to RP3 in these mice, regional and distal lymph nodes were harvested from tumor-rejecting mice, cultured, and assayed in vitro for a proliferative response to serial dilutions of rhRP3 protein. *RP3*$^{p53-/-}$ tumor-sensitized LNC proliferated strongly to rhRP3 protein, but not to unrelated control protein stimulation (Fig. 8). Because RP3 is composed of two widely expressed self proteins, RFG/ARA70 and c-RET, the only unique portion of RP3 in the tumor tissue is the fusion breakpoint region. Therefore, to measure fusion region-specific responses, cultured tumor-rejecting LNC were stimulated with the fusion region-specific peptide in vitro. Similar to rRP3-immune lymphocytes, LNC from tumor-rejecting mice did not proliferate to the fusion region peptide, suggesting that natural processing of this epitope from tumors does not evoke a thyroid-specific immune response. LNC from nonimmunized *B6C3F1* mice failed to respond to rRP3, an RP3 fusion region peptide, or an unrelated protein (not shown). To measure the humoral response to RP3 during thyroid tumor rejection, serum was collected from tumor-rejecting mice and assayed for Ag specificity by ELISA. In contrast to the serological reactivity detected in rRP3-immunized mice, Ab responses specific to rhRP3 protein were not detected in serum samples from *RP3*$^{p53-/-}$ tumor-rejecting *B6C3F1* mice, suggesting that Ab reactivity is not required for tumor rejection (Fig. 1*C*).

Discussion

In this study, we investigated the immunogenicity of RET/PTC3 (RP3), a novel thyroid tumor-specific fusion protein, frequently expressed in human thyroid cancers and in thyroid epithelial cells of patients afflicted with Hashimoto's thyroiditis. Because no mouse homologue exists to study the role of RP3 in disease, we synthesized a mRP3 protein and studied its immunogenicity in normal and *RP3* transgenic mice. Unlike other transgenic models (27, 28) that have used nonphysiological surrogate tumor Ags, RP3 is a somatically activated oncoprotein, formed following DNA damage, and expressed in an exclusive tissue-specific manner in human thyroid epithelium. Because RP3 represents a unique protein, we hypothesized that it would behave as a tumor Ag in naive mice and a self protein in *RP3* trangenic mice, and thus assessed its capacity to elicit a response under immunization conditions that would distinguish self from nonself proteins.

Results obtained from proliferation assays demonstrate the specific T cell immunogenicity of RP3 fusion protein when presented by APCs. We predicted that immunity generated against a protein derived from the fusion of two widely expressed self proteins, such as RFG/ARA70 and c-RET, would be directed against the unique fusion region segment of the chimeric molecule because tolerance to the flanking regions would likely occur following T cell development. On the contrary, we found little reactivity to the fusion breakpoint peptide predicted to bind I-E^k, suggesting that a nonbreakpoint region of the fusion protein encoded the dominant immunogenic epitope(s). Interestingly, robust T cell responses to the mRet^{TKD} were observed following RP3 immunization consistent with the location of an immunogenic epitope in this region, as predicted by MHC-binding algorithms. Tolerance in normal mice was observed, however, when the self protein parts of RP3 were used as immunogens. These data indicate that an autoimmune response against proteins may occur by a process that increases or alters epitope production following the fusion of two unrelated proteins.

However, how might the formation of a fusion protein lead to such changes in Ag processing? Presumably, folding of the c-RET kinase domain is not altered by fusion to RFG/ARA70, given the preservation of its activity. Thus, one might expect similar handling of this domain by the Ag-processing machinery in the context of both c-RET and RP3, and therefore tolerance to any c-RET-derived epitopes generated by processing of RP3. There are several potential explanations for why this is not the case. One factor to consider is the change in subcellular location, from the inner leaflet of the plasma membrane (c-RET) to the cytosol (RP3). This may substantially alter the Ag-processing pathways that are available. For example, both endosomal/lysosomal proteases and the cytosolically located proteasome have been implicated in the processing of cytosolic Ags for class II-restricted presentation (29). Perhaps c-RET is targeted principally to the endocytic compartment, whereas RP3 is targeted to the proteasome, with the epitopes that are generated in each case being distinct. This scenario presumes endogenous presentation of both proteins (presentation by the cell that expresses the protein), but it may also be that most presentation of c-RET and RP3 requires transfer of the proteins to professional APCs (cross-priming). In this case, both proteins would most likely be presented through the classic endocytic pathway, a possibility that is currently being tested. Even so, variable processing of the carboxyl-terminal domain of RP3 is still a strong possibility, as it has been shown that binding of Ab to an Ag, analogous to fusion of the carboxyl-terminal region of c-RET with the amino-terminal region of RFG/ARA70, significantly alters the efficiency with which particular epitopes are generated (30). Finally,

it should be noted that lysosomal proteases cathepsin B, D, and L are up-regulated in thyroid cancers and in thyroid tissue from patients with autoimmune disease (31, 32). Thus, it may be a change in the processing machinery rather than a change in the substrate that is responsible for generation of the transformation-associated epitope. Whichever of these mechanisms proves correct, the formation of RP3 predisposes thyroid epithelium to overcome tolerance through the recognition of self epitopes. Future studies will help to resolve the processing mechanism specific for the RP3 fusion protein.

Even though the fusion of two self proteins is an important feature of the immunogenicity of RP3, the malignant state of the RP3-expressing tissue determined whether thyroid-specific immunity could be induced. For example, although direct immunization led to RP3-specific immunity, premalignant thyroidal expression of RP3 alone was not sufficient for the unprimed induction of immunity against thyroid tissue grafts expressing RP3. Although the immunogenic properties of thyroid grafts are different from other organs such as skin (33), the lack of immune response to RP3-expressing thyroid grafts may be due to insufficient proinflammatory mediator production at the time of priming. Indeed, even strongly immunogenic viral Ags have a tolerizing effect when expressed in skin grafts without coincident inflammation and are immunogenic only following a localized inflammatory reaction (34, 35). Furthermore, tissue grafts encoding the E7 tumor Ag of human papillomavirus type 16 are rejected more readily when Ag priming occurs in the presence of an LPS-induced inflammatory reaction (36). Consistent with this idea, we find high level expression of a number of key proinflammatory mediators by developed thyroid carcinomas, but fewer and lower amounts of these mediators in preneoplastic thyroid lesions of *RP3* transgenic mice.[4] Early neoplastic lesions may thus represent an immunological crossroad between tolerance to self and immunity to tumor, with direction being determined by the extent and amount of cytokines produced from stressed or transformed cells. In addition to these inflammatory mediators, the state of newly grafted tissue, tumor fragments vs single thyroid lobes excised en bloc, may also influence the effector response because increased cellular apoptosis and necrosis may facilitate Ag uptake and cross-presentation by professional APCs, resulting in thyroid tissue rejection. Even though, in some cases, antitumor Ab may facilitate Ag processing for tumor rejection, humoral responses were not detected in RP3 tumor-rejecting mice, a finding not inconsistent with other tumor systems (37). Even though these data support a T cell-mediated rejection mechanism, they do not explain the reasons for the lack of humoral responses. In addition, thyroid cells expressing RP3 secrete a number of inflammatory cytokines (J. Russell, M. Santoro, and J. L. Rothstein, manuscript in preparation) that may bias immune responses toward T cell immunity; however, future studies will be necessary to understand the strength of this response in vivo.

Previous data have shown frequent expression of *RP3* fusion gene at very early stages of cancer (38) and in thyroid tissue of patients with thyroid autoimmune disease, suggesting that this expression may underlie the coexistence of neoplasia (10, 11) and autoimmunity. Studying the early stages of RP3 expression in mouse thyroid tissue will help to evaluate the inflammatory conditions within the organ observed at very early stages following thyroid transformation, but before carcinoma and/or autoimmune disease occurs. This is important because the time frame between cancer initiation and detection can be many years, over which time the immunological landscape of the transformed thyroid may change. This is more apparent when considering the studies presented in this work, which may provide the basis for understanding the widespread effects of how oncogene-altered epithelial cells may evoke immune responses. RP3 expression in thyroid tissue may provide both a strong antigenic and chemotactic stimulus for monocytes and lymphocytes to infiltrate oncogene-expressing tissue in patients with disease-prone MHC backgrounds. Accordingly, this suggests that to evoke an effective immune response against oncogene-expressing tissue, an alteration in the target tissue may be necessary. This change appears following the transition of thyroid tissue from normal to neoplastic, possibly mimicking the proinflammatory effects brought on by immunizing the host with adjuvants or with highly immunogenic viruses. Thus, the expression of RP3 in thyroid tissue of patients with neoplastic thyroid disease supports a model of autoimmunity whereby organ immunogenicity is a consequence of oncogenic transformation, resulting in the development of chronic inflammation.

[4] J. P. Russell, R. M. Melillo, M.D. Castellone, M. Santoro, and J. L. Rothstein. The thyroid tumor antigen and tyrosine kinase oncoprotein, RET/PTC3, induces the secretion of proinflammatory cytokines. *Submitted for publication.*

References

1. Becker, K. L. 1995. *Principles and Practices of Endocrinology and Metabolism*. J. B. Lippincott Company, Philadelphia, PA.
2. Rosenberg, S. A., and D. E. White. 1996. Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. *J. Immunother. Emphasis Tumor Immunol. 19:81.*
3. Bongarzone, I., M. A. Pierotti, N. Monzini, P. Mondellini, G. Manenti, R. Donghi, S. Pilotti, M. Grieco, M. Santoro, A. Fusco, et al. 1989. High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. *Oncogene 4:1457.*
4. Powell, D. J., Jr., J. P. Russell, G. Li, B. A. Kuo, V. Fidanza, K. Huebner, and J. L. Rothstein. 2001. Altered gene expression in immunogenic poorly differentiated thyroid carcinomas from RET/PTC3p53$^{-/-}$ mice. *Oncogene 20:3235.*
5. Ries, L. A. G., M. P. Eisner, C. L. Kosary, B. F. Hankey, B. A. Miller, L. Clegg, and B. K. Edwards, eds. *SEER Cancer Statistics Review, 1973–1999*, National Cancer Institute. Bethesda, MD.
6. Rabes, H. M., E. P. Demidchik, J. D. Sidorow, E. Lengfelder, C. Beimfohr, D. Hoelzel, and S. Klugbauer. 2000. Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-Chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. *Clin. Cancer Res. 6:1093.*
7. Kroll, T. G., P. Sarraf, L. Pecciarini, C. J. Chen, E. Mueller, B. M. Spiegelman, and J. A. Fletcher. 2000. PAX8-PPARγ1 fusion in oncogene human thyroid carcinoma. *Science 289:1357.*
8. Santoro, M., N. A. Dathan, M. T. Berlingieri, I. Bongarzone, C. Paulin, M. Grieco, M. A. Pierotti, G. Vecchio, and A. Fusco. 1994. Molecular characterization of RET/PTC3; a novel rearranged version of the RETproto-oncogene in a human thyroid papillary carcinoma. *Oncogene 9:509.*
9. Gao, T., K. Brantley, E. Bolu, and M. J. McPhaul. 1999. RFG (ARA70, ELE1) interacts with the human androgen receptor in a ligand-dependent fashion, but functions only weakly as a coactivator in cotransfection assays. *Mol. Endocrinol. 13:1645.*
10. Wirtschafter, A., R. Schmidt, D. Rosen, N. Kundu, M. Santoro, A. Fusco, H. Multhaupt, J. P. Atkins, M. R. Rosen, W. M. Keane, and J. L. Rothstein. 1997. Expression of the RET/PTC fusion gene as a marker for papillary carcinoma in Hashimoto's thyroiditis. *Laryngoscope 107:95.*
11. Sheils, O. M., J. O'Eary, V. Uhlmann, K. Lattich, and E. C. Sweeney. 2000. *ret*/PTC-1 activation in Hashimoto thyroiditis. *Int. J. Surg. Pathol. 8:185.*
12. Mizuno, T., S. Kyoizumi, T. Suzuki, K. S. Iwamoto, and T. Seyama. 1997. Continued expression of a tissue specific activated oncogene in the early steps of radiation-induced human thyroid carcinogenesis. *Oncogene 15:1455.*
13. Santoro, M., M. Papotti, G. Chiappetta, G. Garcia-Rostan, M. Volante, C. Johnson, R. L. Camp, F. Pentimalli, C. Monaco, A. Herrero, et al. 2002. RET activation and clinicopathologic features in poorly differentiated thyroid tumors. *J. Clin. Endocrinol. Metab. 87:370.*
14. Tallini, G., M. Santoro, M. Helie, F. Carlomagno, G. Salvatore, G. Chiappetta, M. L. Carcangiu, and A. Fusco. 1998. RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. *Clin. Cancer Res. 4:287.*
15. Vogelstein, B., E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. Smits, and J. L. Bos. 1988. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med. 319:525.*
16. Chen, W., D. J. Peace, D. K. Rovira, S. G. You, and M. A. Cheever. 1992. T-cell immunity to the joining region of p210BCR-ABL protein. *Proc. Natl. Acad. Sci. USA 89:1468.*
17. Bocchia, M., T. Korontsvit, Q. Xu, S. Mackinnon, S. Y. Yang, A. Sette, and D. A. Scheinberg. 1996. Specific human cellular immunity to *bcr-abl* oncogene-derived peptides. *Blood 87:3587.*
18. Ten Bosch, G. J., A. C. Toornvliet, T. Friede, C. J. Melief, and O. C. Leeksma. 1995. Recognition of peptides corresponding to the joining region of p210BCR-ABL protein by human T cells. *Leukemia 9:1344.*
19. Pawelec, G., H. Max, T. Halder, O. Bruserud, A. Merl, P. da Silva, and H. Kalbacher. 1996. BCR/ABL leukemia oncogene fusion peptides selectively

bind to certain HLA-DR alleles and can be recognized by T cells found at low frequency in the repertoire of normal donors. *Blood 88:2118.*
20. Matsubayashi, S., K. Kawai, Y. Matsumoto, T. Mukuta, T. Morita, K. Hirai, F. Matsuzuka, K. Kakudoh, K. Kuma, and H. Tamai. 1995. The correlation between papillary thyroid carcinoma and lymphocytic infiltration in the thyroid gland. *J. Clin. Endocrinol. Metab. 80:3421.*
21. Dailey, M. E., S. Lindsay, and R. Skahen. 1955. Relation of thyroid neoplasms to Hashimoto disease of the thyroid. *Arch. Surg. 70:291.*
22. Segal, K., M. Ben-Bassat, A. Avraham, G. Har-El, and J. Sidi. 1985. Hashimoto's thyroiditis and carcinoma of the thyroid gland. *Int. Surg. 70:205.*
23. Powell, D. J., Jr., J. Russell, K. Nibu, G. Li, E. Rhee, M. Liao, M. Goldstein, W. M. Keane, M. Santoro, A. Fusco, and J. L. Rothstein. 1998. The RET/PTC3 oncogene: metastatic solid-type papillary carcinomas in murine thyroids. *Cancer Res. 58:5523.*
24. Eisenlohr, L. C., J. W. Yewdell, and J. R. Bennink. 1992. Flanking sequences influence the presentation of an endogenously synthesized peptide to cytotoxic T lymphocytes. *J. Exp. Med. 175:481.*
25. Jhiang, S. M., J. E. Sagartz, Q. Tong, J. Parker-Thornburg, C. C. Capen, J. Y. Cho, S. Xing, and C. Ledent. 1996. Targeted expression of the *ret*/PTC1 oncogene induces papillary thyroid carcinomas. *Endocrinology 137:375.*
26. Santoro, M., G. Chiappetta, A. Cerrato, D. Salvatore, L. Zhang, G. Manzo, A. Picone, G. Portella, G. Santelli, G. Vecchio, and A. Fusco. 1996. Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. *Oncogene 12:1821.*
27. Akkaraju, S., W. Y. Ho, D. Leong, K. Canaan, M. M. Davis, and C. C. Goodnow. 1997. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity 7:255.*
28. Gerard, C. M., N. Baudson, K. Kraemer, C. Ledent, D. Pardoll, and C. Bruck. 2001. Recombinant human papillomavirus type 16 E7 protein as a model antigen to study the vaccine potential in control and E7 transgenic mice. *Clin. Cancer Res. 7:838s.*
29. Mukherjee, P., A. Dani, S, Bhatia, N. Singh, A. Y. Rudensky, A. George, V. Bal, S. Mayor, and S. Rath. 2001. Efficient presentation of both cytosolic and endogenous transmembrane protein antigens on MHC class II is dependent on cytoplasmic proteolysis. *J. Immunol. 167:2632.*
30. Watts, C., A. Antoniou, B. Manoury, E. W. Hewitt, L. M. McKay, L. Grayson, N. F. Fairweather, P. Emsley, N. Isaacs, and P. D. Simitsek. 1998. Modulation by epitope-specific antibodies of class II MHC-restricted presentation of the tetanus toxin antigen. *Immunol. Rev. 164:11.*
31. Metaye, T., J. L. Kraimps, J. M. Goujon, B. Fernandez, N. Quellard, P. Ingrand, J. Barbier, and F. Begon. 1997. Expression, localization, and thyrotropin regulation of cathepsin D in human thyroid tissues. *J. Clin. Endocrinol. Metab. 82:3383.*
32. Shuja, S., and M. J. Murnane. 1996. Marked increases in cathepsin B and L activities distinguish papillary carcinoma of the thyroid from normal thyroid or thyroid with non-neoplastic disease. *Int. J. Cancer 66:420.*
33. Isakov, N., and S. Segal. 1983. Immunogenicity of the mutated H-2Kbm1 antigen(s): test of thyroid graft rejection between B6.C-H-2bm1 and C57BL/6 mice following reciprocal immunization with normal versus malignant cells. *Immunobiology 165:485.*
34. Frazer, I. H., D. M. Leippe, L. A. Dunn, A. Liem, R. W. Tindle, G. J. Fernando, W. C. Phelps, and P. F. Lambert. 1995. Immunological responses in human papillomavirus 16 E6/E7-transgenic mice to E7 protein correlate with the presence of skin disease. *Cancer Res. 55:2635.*
35. Doan, T., K. A. Herd, P. F. Lambert, G. J. Fernando, M. D. Street, and R. W. Tindle. 2000. Peripheral tolerance to human papillomavirus E7 oncoprotein occurs by cross-tolerization, is largely Th-2-independent, and is broken by dendritic cell immunization. *Cancer Res. 60:2810.*
36. Frazer, I. H., R. De Kluyver, G. R. Leggatt, H. Y. Guo, L. Dunn, O. White, C. Harris, A. Liem, and P. Lambert. 2001. Tolerance or immunity to a tumor antigen expressed in somatic cells can be determined by systemic proinflammatory signals at the time of first antigen exposure. *J. Immunol. 167:6180.*
37. Koeppen, H. K., S. Singh, H. J. Stauss, B. H. Park, D. A. Rowley, and H. Schreiber. 1993. CD4-positive and B lymphocytes in transplantation immunity. I. Promotion of tumor allograft rejection through elimination of CD4-positive lymphocytes. *Transplantation 55:1349.*
38. Fusco, A., G. Chiappetta, P. Hui, G. Garcia-Rostan, L. Golden, B. K. Kinder, D. A. Dillon, A. Giuliano, A. M. Cirafici, M. Santoro, et al. 2002. Assessment of RET/PTC oncogene activation and clonality in thyroid nodules with incomplete morphological evidence of papillary carcinoma: a search for the early precursors of papillary cancer. *Am. J. Pathol. 160:2157.*