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T Cells from Renal Cell Carcinoma Patients Exhibit an Abnormal Pattern of κB-specific DNA-binding Activity: A Preliminary Report[1]

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ABSTRACT

Recent data suggest that the poor induction of a T-cell response to human renal cell carcinoma (RCC) may be related to alterations in signal transduction pathways. We report that T cells from RCC patients have two alterations in κB motif-specific DNA-binding activity. The first alteration involves the constitutive expression of substantial κB-binding activity in nuclear extracts, which was observed in the electrophoretic mobility shift assay. The magnitude of κB activity in unstimulated patient T cells was similar to that observed in T cells from normal individuals that had been activated *in vitro*. On the basis of Western blotting experiments using antibodies to κB/Rel family proteins, the κB-binding activity constitutively expressed in T cells from RCC patients is composed mostly of the NF-κB1 (p50) subunit. The second abnormality in κB-binding activity in T cells from these patients is that RelA, a member of the Rel homology family which is part of the normal NF-κB complex, was not induced in the nucleus following activation. Western blotting analysis did not detect any RelA in nuclear extracts either before or after stimulation of T cells. The altered κB-binding activity in T cells from RCC patients may impair their capacity to respond normally to various stimuli.

INTRODUCTION

T cells represent an important component of the host immune response to tumors. The activation and potentiation of T-cell immunity is a goal of many forms of cancer immunotherapy including cytokine and adoptive cell treatment (1–10). However, there is evidence documenting that T-cell immunity fails to develop or is suppressed in the tumor bearing host (11–13). Defects in delayed type hypersensitivity (14), cytotoxicity (14, 15), proliferation (14, 15–18), and IL-2[3] production (14) have been reported in cancer patients. In the murine colon tumor model MCA-38, decreased T-cell cytotoxic activity is seen during tumor progression (19). Recent work in this model has documented that tumor-bearing mice are missing TCRζ, as well as the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (20). We have found similar alterations in these signaling elements in T cells from patients with RCC (21). Although changes in these molecules were observed with T cells from both the blood and tumor, tumor-derived lymphocytes were more severely affected. Depressed levels of TCRζ were also observed in tumor-derived T cells from patients with colorectal carcinoma (22). Given the importance of TCRζ, $p56^{lck}$, and $p59^{fyn}$ in T-cell activation (23), defects in their expression may cause alteration in downstream signaling events including the activation and/or nuclear translocation of transcription factors (24).

In this report we determined if alterations in signaling elements of T cells from RCC patients include abnormalities in κB sequence motif-specific binding activity (25). The regulation of many cellular gene products associated with T-lymphocyte activation including cytokines and the IL-2Rα chain involves NF-κB. The κB enhancer-binding proteins are composed of a family of related proteins that include NF-κB1 (p50), RelA (p65), and c-Rel (26–33). These proteins and other members of the κB/Rel family share amino acid sequence homology within a region that is responsible for DNA binding and dimerization (34). In T cells, NF-κB is inducible and is regulated at a posttranslational level (26, 28, 34). Stimulation of T cells with mitogens, phorbol esters, anti-CD3 antibody, and certain cytokines such as IL-2 induces the release of the preformed cytoplasmic NF-κB complex from an inactive complex with an inhibitor, allowing the active form to translocate to the nucleus (26, 34, 35).

The work presented here indicates that T cells from RCC patients have an abnormality in the expression of nuclear NF-κB. This includes the constitutive expression of NF-κB1 and the failure to activate other members of the NF-κB family which are part of the normal complex found in the nucleus after stimulation.

MATERIALS AND METHODS

Isolation of T Cells from Peripheral Blood and Tumor. TIL cells were isolated from surgical specimens from patients with renal cell carcinoma ($n = 13$) participating in protocols at the Cleveland Clinic. All TIL preparations were obtained from the primary tumor. PBLs were obtained from 12 patients 3–5 days after their surgery. Seven of the PBLs had paired TIL samples. TILs were obtained from tumor after a 2-h digestion at 37°C using collagenase type II (3.5 mg/ml; Sigma Chemical Co.) and egg white trypsin inhibitor (1 mg/ml; Sigma) (18, 21). TILs were centrifuged over Ficoll-Hypaque, and T lymphocytes were enriched by passing cells over a nylon wool column followed by negative selection (magnetic cell separation; Miltenyi Biotec Inc., Sunnyvale, CA) using the following microbead-conjugated monoclonal antibodies: anti-CD16 (natural killer cells); anti-CD19 (B cells); and anti-CD14 (macrophages) (21). PBL-derived T cells (RCC patients, $n = 12$; normals, $n = 7$) were obtained following Ficoll-Hypaque density separation and negative selection using the same antibody mixture described for TIL (21). Immunostaining with anti-CD3 antibody after enrichment demonstrated that the T-cell purity was greater than 90%.

Oligonucleotides Used in Assays. The synthetic oligonucleotide containing a tandem repeat of the consensus sequence for the NF-κB DNA-binding site was obtained from GIBCO-BRL (Gaithersburg, MD). The sequence of the oligonucleotide was 5′-GATCCAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3′. The oligonucleotide corresponding to the κB element from *IL-2Rα* gene was prepared by using an Applied Biosystems oligonucleotide synthesizer (Model 381 A). The sequence of the oligonucleotide was 5′CAACGGCAGGGGAATCTCCCTCTCCTT-3′ (28, 35). Underlined sequences represent the κB motif. In the competition studies an oligonucleotide corresponding to the Oct enhancer element was used (GIBCO-BRL), and the sequence is 5′GATCATGCAAATGATCATGCAAATGATCATGGAAAT-3′. For the preparation of probes in electrophoresis mobility shift assay, radiolabeled double-stranded oligonucleotides were prepared by annealing coding strand template to a complementary 10-base primer and filling in the overhang with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP.

Preparation of Nuclear Extract. Nuclear extracts were prepared by a modified method of Osborn *et al.* (36). Isolated T cells (10^7) were washed

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[3] The abbreviations used are: IL-2, interleukin 2; RCC, renal cell carcinoma; TCRζ, T cell receptor ζ; PBL, peripheral blood lymphocyte; TIL, tumor-infiltrating lymphocyte; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; IFN-γ, γ interferon; IL-2Rα, interleukin 2 receptor α.

twice with cold Hanks' balanced salt solution and the cell pellet was suspended in 40 μl of hypotonic buffer A [10 mM HEPES (pH 7.9)-10 mM KCl-1.5 mM $MgCl_2$-0.1 mM EDTA-1 mM DTT-0.5 mM PMSF-0.1% Nonidet P-40] for 10 min on ice. Nuclei were prepared by microcentrifugation for 10 min at 4°C. The nuclear pellet was suspended in 15 μl of buffer C [20 mM HEPES (pH 7.9)-25% glycerol-0.4 M NaCl-1.5 mM $MgCl_2$-1 mM EDTA-1 mM DTT-0.5 mM PMSF] and incubated for 15 min at 4°C with brief mixing. The mixture was microcentrifuged (16,000 × *g*) for 10 min at 4°C and the supernatant was diluted with 60 μl of modified buffer D [20 mM HEPES (pH 7.9)-50 mM KCl-0.2 mM EDTA-1 mM DTT-1 mM PMSF]. Protein concentration was measured by using the Bradford assay (Bio-Rad, Richmond, CA).

Electrophoretic Mobility Shift Assay. As previously described (37), nuclear extracts (5 μg of protein) were preincubated in a 25-μl total reaction volume containing 20 mM HEPES (pH 7.9), 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 8% glycerol, and 2 μg of poly(dI·dC) (Pharmacia, Piscataway, NJ) for 15 min at 4°C. The reaction mixture was then incubated with the radiolabeled oligonucleotide (2×10^5 cpm) for 20 min at room temperature. The samples were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel with 0.25 × TBE buffer (22.3 mM Tris-22.2 mM boric acid-0.5 mM EDTA). The gels were dried and analyzed by autoradiography.

Western Blotting. The antibodies used in these experiments were polyclonal rabbit anti-mouse antibodies to NF-κB1 (p50) and RelA (p65) (kindly provided by Dr. Ghosh, National Cancer Institute, Frederick, MD). Normal rabbit serum (2 μg/μl) (The Jackson Laboratory, West Grove, PA) was used as a control.

T cells were lysed by incubation in lysis buffer for 10 min on ice. Equal amounts (10 μg) of nuclear protein from cell lysates were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. The membranes were washed with Tris-buffered saline-Tween (0.1%) with 5% nonfat dried milk overnight at 4°C or 2 h at room temperature and then blotted for 1–2 h with the appropriate antibody. The membranes were washed with Tris-buffered saline-Tween and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Amersham, Arlington Heights, IL). Thereafter, membranes were developed with enhanced chemiluminescence reagents (DuPont New England Nuclear, Boston, MA) and exposed to film.

RESULTS

T-cell Nuclear Extracts from RCC Patients Display Altered κB-specific Binding Activity. We determined whether the defects in T-cell-signaling elements from RCC patients involved abnormal κB-specific DNA-binding activity. To address this question, T cells were cultured in medium alone or with one of the following stimuli; IL-2; cross-linked anti-CD3 antibody; or both. After 4 h of incubation nuclear extract was isolated since kinetic studies from our laboratory and others (35) have shown that maximinal levels of NF-κB are detectable in the nucleus at this time. Nuclear extracts from tumor and peripheral blood derived T cells were reacted with a [^{32}P]dATP-radiolabeled oligonucleotide containing tandem repeats of the κB recognition sequence. The DNA-protein complexes were separated from free oligonucleotide by nondenaturing acrylamide gel electrophoresis. The nucleoprotein complex obtained with T cells from RCC patients was compared to that from T cells of healthy volunteers.

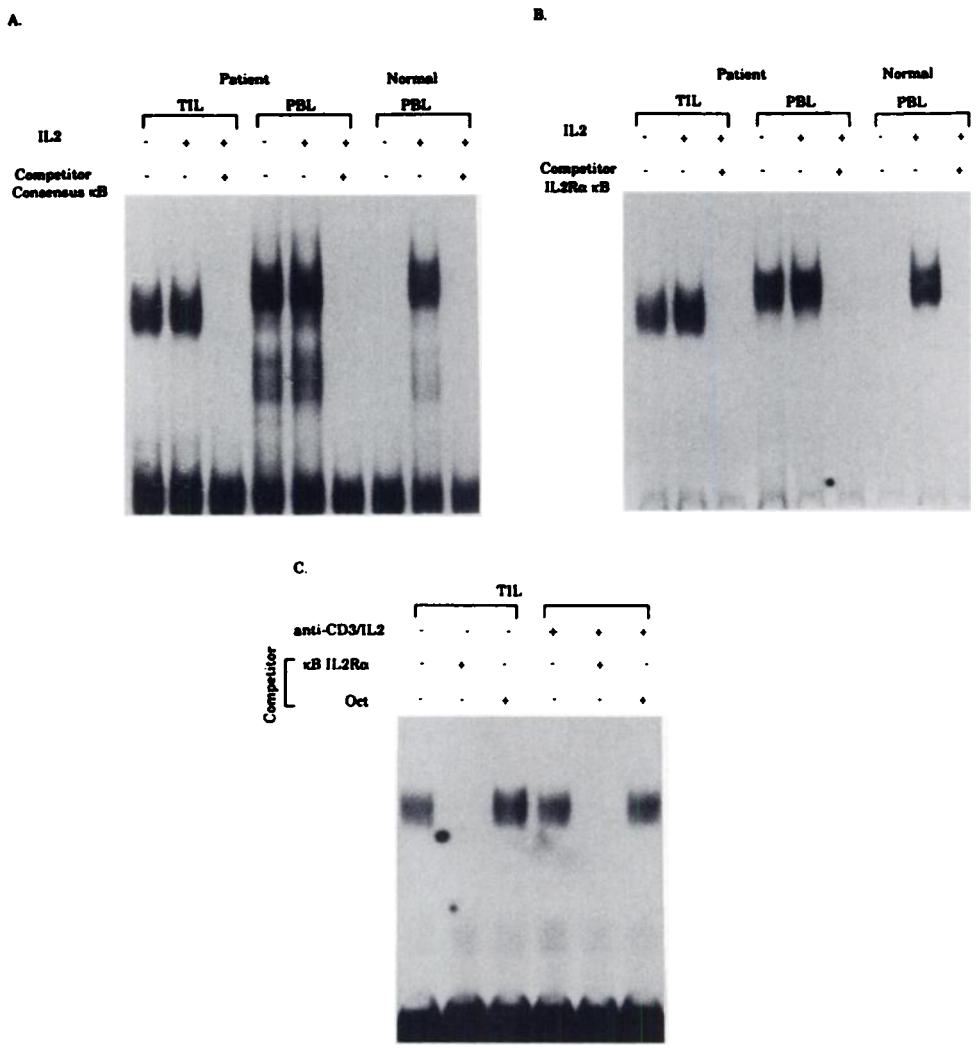

Fig. 1. T cells from RCC patients have altered κB-binding activity. T cells from RCC patients (blood and tumor) and from normal individuals were cultured with medium alone for 4 h or with different stimuli: IL-2 (1000 units/ml) (*A* and *B*) or IL-2 (1000 units/ml) plus anti-CD3 (*C*). Nuclear extracts were isolated and electrophoresis mobility shift assays were performed with the κB consensus oligonucleotide (*A*) and the oligonucleotide that corresponds to the κB sequence of the *IL-2Rα* gene (*B* and *C*). Unlabeled oligonucleotides were added at 100-fold excess to the labeled probe as a competitor. +, the stimulation of T cells and/or the addition of competitor to the EMSA.

While IL-2 or cross-linked anti-CD3 antibody treatment resulted in activation of κB-binding activity in PBL from normal volunteers, T cells derived from the blood or tumor of RCC patients constitutively express κB-binding activity (Fig. 1). Unstimulated T cells from patients contained a DNA-protein complex which was comparable in magnitude to that observed with activated T cells from normal individuals that were stimulated *in vitro* (Fig. 1). The DNA-protein complex from tumor-derived T cells had a slightly faster mobility than the complexes from autologous peripheral blood T cells and from activated T cells of normal individuals. Stimulating patient T cells with IL-2 or anti-CD3 did not result in any increase in the intensity of the DNA-protein complex. We then determined whether the DNA-protein complex detected in unstimulated T cells from RCC patients was also observed, using a κB sequence probe from the *IL-2Rα* gene. A complex similar to that seen with the κB consensus sequence was also observed, indicating that the difference was not related to the specific motif used as a probe (Fig. 1, *B* and *C*). The sequence specificity of the binding activity was demonstrated by performing competition studies with 100-fold excess unlabeled oligonucleotides corresponding to the κB sequence or an unrelated sequence [Oct (Fig. 1) or CRE (not shown)]. These alterations were observed in TIL and PBL from 13 and 12 renal patients, respectively. Data from four experiments are presented in Fig. 2, where each experiment represents nuclear extract derived from a different patient and normal healthy volunteer.

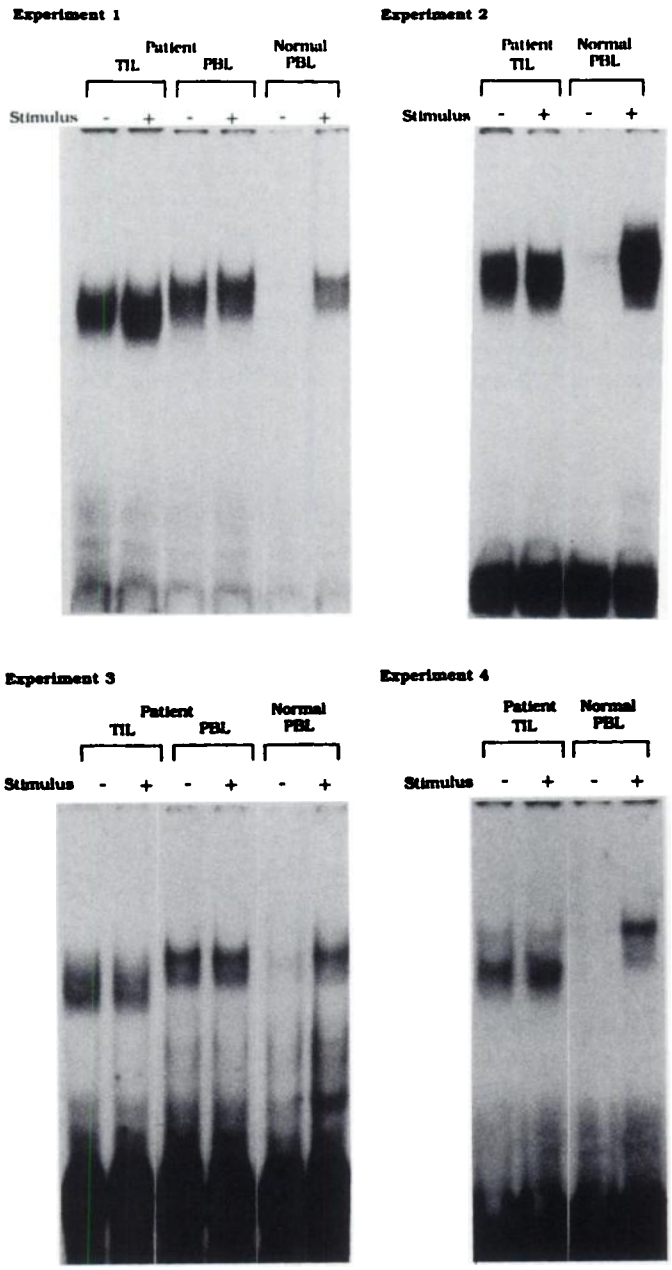

Fig. 2. Altered κB-binding activity is a consistent finding in TIL and PBL from RCC patients. Results from four experiments are presented where each experiment represents electrophoresis mobility shift assays performed on nuclear extracts from different patients and normal volunteers. In experiments 1, 3, and 4, T cells were stimulated with cross-linked anti-CD3, whereas in experiment 2, T cells were stimulated with IL-2 (1000 units/ml). In all experiments nuclear extract was obtained after 4 h of stimulation. Nuclear extract from T cells cultured in medium alone was used to define the level of κB-binding activity of unstimulated T cells. The oligonucleotide used in these experiments contains the κB sequence of the *IL-2Rα* gene. +, stimulation of T cells; −, cells cultured in medium alone.

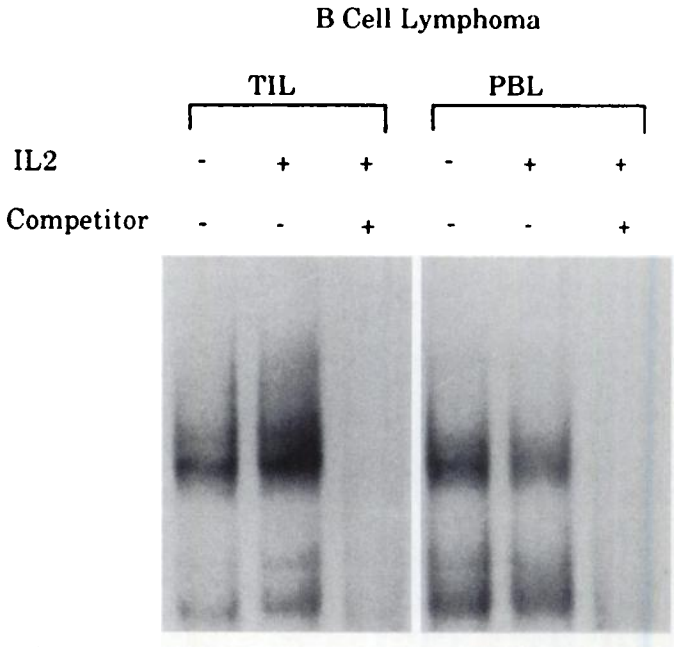

Fig. 3. Altered κB-binding activity is seen in T cells from B-cell lymphoma patients. T cells were isolated from the peripheral blood and involved lymph nodes of a patient with non-Hodgkin's B-cell lymphoma (diffuse large noncleaved cell) by negative selection using antibodies to CD19 (B cells), CD56 (natural killers) and CD14 (macrophages). T cells were cultured in medium alone or stimulated with IL-2 (1000 units/ml) for 4 h. Thereafter, electrophoresis mobility shift assays were performed with nuclear extracts and the radiolabeled oligonucleotide corresponding to the κB sequence of the *IL-2Rα* gene. Where indicated, 100-fold excess of unlabeled oligonucleotide was added as a competitor for the radiolabeled probe. Similar results were obtained with nodal T cells derived from two additional B-cell lymphoma patients (data not shown). +, indicates stimulation of T cells and/or the addition of competitor to EMSA.

The possibility that nuclear κB-binding activity in unstimulated T cells from RCC patients was the result of cell isolation or culturing procedures was also examined. A comparison was made between the level of κB sequence-binding activity in freshly isolated T cells to that in T cells that had been incubated in medium alone for 4 h. Comparable levels of NF-κB complexes were detected from both T cell populations, suggesting that short-term culture did not account for the presence of κB-binding activity in unstimulated T cells from RCC patients (data not shown). Moreover, the enzyme solution used to obtain T cells from the tumor did not appear to induce NF-κB nuclear translocation since nuclear κB-binding activity was not detected after enzymatic treatment of peripheral blood T cells obtained from normal individuals (data not shown).

We also determined whether T cells derived from a different type of tumor would have similar alterations in κB-binding activity. For these experiments, T cells were isolated from involved lymph nodes

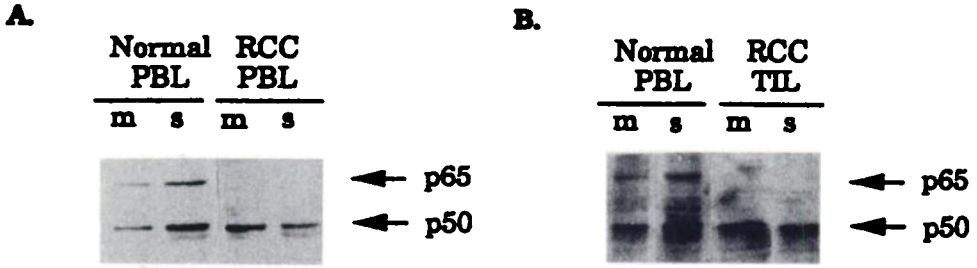

Fig. 4. T cells from RCC patients fail to express RelA (p65) in the nucleus after stimulation *in vitro*. (*A*) T cells from the peripheral blood of a RCC patient and a healthy donor were incubated *in vitro* for 4 h in either medium alone (*m*) or with the stimulus (*s*), IL-2 (1000 units/ml) plus cross-linked anti-CD3 antibody. Thereafter, cells were lysed and equivalent amounts of nuclear protein (15 μg) were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Western blotting was then performed using anti-bodies to NF-κB1 (p50) and RelA (p65) as described in "Materials and Methods." (*B*) Similar results were obtained with TIL from another RCC patient. For comparison are results from T cells isolated from a healthy donor (different than the one presented in *A*)

of patients with non-Hodgkin's B cell lymphomas ($n = 4$) by negative selection. Substantial κB-binding activity was observed in nuclear extract from nonmalignant T cells derived from the B cell lymphoma patients. Stimulation with IL-2 or anti-CD3 antibody did not increase the magnitude of the complex detected by electrophoresis mobility shift assay (Fig. 3, representative data). These results demonstrate that altered κB-binding activity is present in another tumor type. Whether abnormal κB-binding activity is a common feature of T cells from patients with cancer will require analysis of T cells from multiple histological types of tumors.

Characterization of the κB-specific Binding Proteins in T Cells of RCC Patients. Western blotting was performed to identify which of the κB-specific binding proteins are present in nuclear extracts of T cells from RCC patients before and after stimulation. To detect NF-κB1 and RelA in nuclei, T cells were cultured in medium alone or with cross-linked anti-CD3/IL2 (stimulus) for 4 h. Both NF-κB1 and RelA were present in nuclear extracts from T cells of healthy individuals, and their levels increased significantly after *in vitro* stimulation (Fig. 4). In contrast, T cells from the peripheral blood and tumor of RCC patients expressed abundant NF-κB1 in the absence of *in vitro* stimulation (Fig. 4, *A* and *B, Lanes m*) and this level did not increase after T-cell activation (Fig. 4, *A* and *B, Lanes s*). We found no expression of RelA in T-cell nuclear extract of RCC patients before and after activation by Western blotting. However, Western analysis of cytoplasmic extract from the same samples demonstrated that RelA was present but not translocating to the nucleus after stimulation (data not shown). Similar results were observed with T cells from the peripheral blood of 4 other RCC patients (data not shown).

DISCUSSION

The results presented here show that T cells from patients with renal cell carcinoma have alterations in κB-specific DNA-binding activity as compared to T cells from the peripheral blood of normal individuals. Substantial κB-binding activity is detected in these cells in the absence of stimulation and this complex is composed mostly of NF-κB1 (p50). Stimulation with either IL-2 or anti-CD3 antibody did not induce nuclear expression of RelA (p65). Therefore, in patient T cells there is no induction of a normal NF-κB complex (p50/p65) after *in vitro* stimulation.

The presence of substantial κB-binding activity in nuclear extracts from T cells of RCC patients without prior stimulation is distinct from what is normally observed with T cells from healthy individuals where little or no binding activity is detected in the absence of stimulation (35, 38). The constitutive expression of κB-binding activity in T cells from the peripheral blood and tumors of these patients suggests that the abnormal expression of κB activity represents a generalized event which affects much of the host T-cell population. We also noticed in the majority of experiments that the κB-binding complex isolated from TIL migrated faster than the complex from PBL of RCC patients. What accounts for the difference in migration rate is not known since NF-κB1 is the predominant DNA-binding protein isolated from both sources of T cells (Fig. 4 and data not shown).

Previous studies have reported NF-κB1 expression in resting lymphocytes and in certain T cell lines (35, 39, 40). NF-κB1 in the nucleus of T cells may translate into suppression rather than induction of gene expression. In a nontransformed $CD4^+$ T-cell clone the expression of NF-κB1 homodimers in the nucleus was associated with suppression of IL-2 transcription (40). Following antigen stimulation the induction of IL-2 gene expression correlated with a reduction in NF-κB1 homodimers and a concomitant increase in the NF-κB1/RelA heterodimer (40). In addition, overexpression of NF-κB1 in T cells has been shown to repress IL-2 promoter activity (40). Whether the increase in nuclear NF-κB1 suppresses gene expression following antigen stimulation remains to be determined.

Our findings demonstrate that at least one member of the Rel homology family was not inducible in T cells from the RCC patients. Unlike activated T cells from normal individuals, T cells from RCC patients had little or no detectable RelA (p65) in their nuclear extracts after stimulation. Whether other members of the κB/Rel family proteins such as c-Rel (41) are expressed in the nucleus of T cells from RCC patients after *in vitro* stimulation remains to be determined.

The mechanism(s) responsible for the inability to induce κB-binding activity is not known but may relate to defects in upstream signaling elements (34). The release of NF-κB from IκBα in the cytoplasm and the translocation of κB-binding activity into the nucleus may involve the phosphorylation of IκBα (34, 42). *In vitro* studies demonstrate that the phosphorylation of IκBα is mediated by PKC (34, 42). Moreover, protein tyrosine kinases associated with the TCR/CD3 complex are linked to PKC activation in that they are responsible for the phosphorylation of phospholipase C-γ1 (23, 43). The activation of phospholipase C-γ1 causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the formation of diacyglycerol which in turn activates PKC (43). We have demonstrated that T cells from RCC patients are missing TCRζ, $p56^{lck}$, and $p59^{fyn}$ (21).[4] The absence of these signaling elements in T cells may prevent normal activation and the phosphorylation of IκBα, thus inhibiting the translocation of p50/p65 complex into the nucleus. This possibility is supported by Western blotting experiments where we have demonstrated that while RelA does not translocate to the nucleus following activation of patient T cells, it is present in the cytoplasm along with IκBα (data not shown).

[4] Finke *et al.*, manuscript in preparation.

The constitutive expression of high levels of NF-κB1 and failure to induce other members of the κB/Rel family of proteins following the stimulation of T cells from RCC patients may have a negative impact on T-cell function. We reported previously that TIL from patients with RCC have a proliferative defect when compared to peripheral blood T cells (18). However, the proliferative defect of TIL may not be related to the altered expression of κB-binding activity since the inducibility of κB-binding activity is reversible after *in vitro* culture, whereas the defect in proliferation is not (18).[5] Moreover, the proliferative defect is present in TIL but not PBL, whereas T cells from both sources display altered κB-binding activity. It may be that the altered κB-binding activity is related to depressed production of cytokines. Recently, it was reported that splenic T cells from mice bearing the renal cell carcinoma line RENCA have altered expression of the κB/Rel family of proteins, which correlated with reduced expression of IFN-γ mRNA (44). The abnormal κB-binding activity in TIL from RCC patients is consistent with no or infrequent expression of cytokine mRNA (IL-2 or IFN-γ) (45) or IL-2Rα mRNA (18), which is usually associated with T-cell activation. However, when activated *in vitro*, TIL from RCC patients are capable of producing IL-2 and IFN-γ, although the level of production may be low relative to T cells from normal individuals (46).[6] Additional studies to measure the rate of IFN-γ (and IL-2) gene transcription between T cells from normal individuals and RCC patients are needed to determine whether the alterations in κB-binding activity are related to impaired cytokine production.

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