Vaccine Implications of Folate Binding Protein, a Novel Cytotoxic T Lymphocyte-recognized Antigen System in Epithelial Cancers[1]

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ABSTRACT

The immune system can be efficiently stimulated and targeted to specific antigens expressed exclusively or preferentially by experimental cancers. The foremost limitations to extending this vaccine technology to the prevalent epithelial-derived cancers are the lack of: (*a*) identified tumor-associated antigens recognized by cellular immunity; (*b*) antigens expressed on the majority of tumor cells during disease progression; and (*c*) immunogenic CTL epitopes. To date, only HER-2/neu has been shown to be the source of naturally occurring, MHC-restricted, CTL-recognized peptides in epithelial tumors. In this study, we demonstrate that the human high-affinity folate binding protein (FBP), which is a source of antigenic peptides recognized in ovarian cancer, is also recognized in breast cancer. Both immunodominant E39 (FBP, 191–199) and subdominant E41 (FBP, 245–253) epitopes are presented by HLA-A2 in these cancers. These peptides are efficient at amplifying the response of tumor-associated lymphocyte populations in terms of lytic function, enhanced proliferation, and specific IFN-γ release. On a per cell basis, tumor-associated lymphocytes stimulated with the FBP peptides exhibit enhanced cytotoxicity not only against peptide-loaded targets but also against FBP-expressing epithelial tumors of different histologies. Furthermore, FBP peptides induced E39-specific CTLs and E39- and E41-specific IFN-γ and IP-10 secretion in certain healthy donors. The broad distribution of FBP among >90% of ovarian and endometrial carcinomas, as well as 20–50% of breast, lung, colorectal, and renal cell carcinomas, along with pronounced differential overexpression in malignant tissues compared with the extremely limited expression in normal epithelium, suggests the exciting potential of a widely applicable FBP-based vaccine in epithelial cancers.

INTRODUCTION

Anticancer vaccines have taken many forms, from whole tumor cells and viral oncolysates, to most recently, Ag[3]-specific, peptide-based vaccines (1–3). The practical advantage of the former is that the actual CTL-recognized Ags need not be known. The latter approach requires precise knowledge of the tumor Ag recognized by specific antitumor CTLs (3). For vaccination purposes, TAAs may be partially purified, highly purified, or synthetic in nature. The synthetic peptide Ag TAA vaccines (or genes encoding for these Ags) should theoretically offer the best cancer vaccines by delivering the immunogenic Ag(s) capable of inducing an efficient, specific, tumor-protective immunity without interference from irrelevant Ags. The TAA vaccines can be easily and reproducibly manufactured and delivered in high volumes safely.

To extend the advances in vaccine technology to the vast problem of epithelial cancers, CTL-recognized TAAs must be identified first. Such TAAs should also meet the criteria of high and stable expression in tumor cells of a large number of patients during disease progression to allow targeting. To date, only the protein product of the proto-oncogene *HER-2* and the core peptide of MUC-1 have been shown to be a source of CTL-recognized peptides (4–7). The advantage of the HER-2 Ag system over those known for melanoma is that it is expressed in multiple epithelial-derived tumor histologies like breast, ovarian, pancreas, and non-small cell lung carcinomas, making a HER-2 peptide-based vaccine potentially widely applicable (8–12). However, this protein is only overexpressed in 30% of breast and ovarian cancers and less in others (13).

FBP is a membrane-associated glycoprotein originally found as a mAb-defined Ag in placenta and trophoblastic cells but rarely in other normal tissues (14–17). Of interest, this protein has been found in >90% of ovarian and endometrial carcinomas; in 20–50% of breast, colorectal, lung, and renal cell carcinomas; and in multiple other tumor types. When present in cancerous tissue, the level of expression is usually >20-fold normal tissue expression and has been reported to be as high as

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[3] The abbreviations used are: Ag, antigen; TAA, tumor-associated Ag; FBP, folate binding protein; mAb, monoclonal antibody; TAL, tumor-associated lymphocyte; OvTAL, ovarian cancer-associated TAL; BrTAL, breast cancer-associated TAL; HER-2, HER-2/neu; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; IL, interleukin; APC, Ag-presenting cell.

80–90-fold in ovarian carcinomas (18). Although FBP has been investigated extensively as a target of humoral immunity, it has only recently been proposed as a source of CTL-recognized peptides in ovarian cancer (19). To investigate the potential role of FBP as a TAA vaccine, we studied whether these peptides are antigenic for BrTALs and immunogenic in ovarian cancer patients and healthy donors. We investigated whether FBP peptides can amplify CTL-TALs with broad tumor-killing capabilities and activate CTLs from healthy donors.

In this study, we demonstrate that two FBP peptides, E39 (191–199) and F41 (245–253), are recognized by freshly cultured OvTALs and by BrTALs. These peptides correspond to naturally processed Ags on intact tumor cells. Additionally, FBP peptides E39 and E41 are capable of CTL stimulation *in vitro*, resulting in proliferation, peptide-specific cytokine and chemokine release, and enhanced cytotoxicity. E39-stimulated specific CTL-TALs are capable of lysing multiple tumors with different epithelial-derived histologies. Furthermore, E39 could stimulate PBMCs to induce E39-specific CTL activity when presented on DCs from healthy donors. Demonstration of E39 and E41 immunogenicity may be significant for development of TAA vaccines based on FBP.

MATERIALS AND METHODS

TAL Cultures. TALs were isolated from fresh collections of malignant ascites and pleural effusions from four ovarian and two breast cancer patients, respectively, under the approval of the Institutional Review Board. Specimens were processed as described previously (20). Lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma Chemical Co., St. Louis, MO) gradients. Freshly isolated TALs were suspended in RPMI 1640 containing 100 μg/ml L-glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Sigma), 40 μg/ml gentamicin, and 50–100 IU/ml IL-2 (Cetus, Emeryville, CA), cultured at 0.5–1.0 $\times$ 10^6 cells/ml in a humidified incubator at 37°C in 5% CO_2, and maintained at this concentration by the addition of media and IL-2 every 2–3 days, depending on the growth kinetics.

Tumor Targets. The SKOV3 ovarian carcinoma cell line was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression (SKOV3.A2) as described (21). The SKBr3 breast cancer line was similarly transfected with the *HLA-A2* gene (by Drs. M. Disis and M. Cheever, University of Washington, Seattle, WA) but expresses moderate levels of HLA-A2 (SKBr3.A2) when compared with SKOV3.A2. Both lines were maintained in RPMI 1640 with 10% FCS and 250 μg/ml G418 (Sigma). SW480 is an established, well-characterized HLA-$A2^+$ colon cancer cell line, and PAN-1 is a HLA-$A2^+$ pancreatic cancer cell line. SKBr3.A2, SKOV3.A2, and SW480 express HER-2 with decreasing levels in this order. SKOV3.A2, SW480, and Panc-1 have been shown to express FBP (17, 19).

Phenotype Analysis. The HLA-A2 status of the TALs and tumor lines was determined by indirect staining with anti-HLA-A2 mAbs, BB 7.2 and MA 2.1 (American Type Culture Collection), followed by goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA), and analyzed on a Coulter Epics C Flow Cytometer (Coulter Electronics, Hileah, FL). HER-2 expression was tested similarly using the Ab2 mAb (Oncogene Science, Manhasset, NY). FBP expression was analyzed using the MOv18 mAb generously donated by Centocor (Malvern, PA).

Synthetic Peptides. The FBP sequence was interrogated for potential HLA-A2-binding nonamers using the known binding motifs for this molecule (22). Five peptides were selected for synthesis based on the presence of leucine, isoleucine, or valine in the dominant anchor positions, P2 and P9, and their potential to form amphiphilic helices (19). Peptides were prepared by the Synthetic Antigen Laboratory of University of Texas M. D. Anderson Cancer Center. Identity and purity of final materials were established by amino acid analysis and analytical reverse phase-high performance liquid chromatography. All peptides used in this study were between 92 and 95% pure. The symbols, position, and sequence of the peptides used in this study are as follows: E37 (FBP, 25–33) RIAWARTEL; E38 (FBP, 112–120) NLGPWIQQV; E39 (FBP, 191–199) EIWTHSTKV; E40 (FBP, 247–255) SLALMLLWL; and E41 (FBP, 245–253) LLSLALMLL. All of these peptides are low to moderate binders, except E38, which is a high-affinity binder to HLA-A2. The low-affinity HLA-A2 binding peptide E71 (HER-2, 798–807) QLMPYGCLL and the CTL epitope E75 (HER-2, 369–377) were used as specificity controls (5).

Cytotoxicity Assays. Cytotoxicity was determined by standard chromium-release assays as described previously (20). Briefly, tumor targets were labeled with 100–150 μCi of sodium chromate (^{51}Cr; Amersham, Arlington Heights, IL) for 1.5 h at 37°C, washed twice, and plated at 2000–2500 cells/well in 100 μl in 96-well, V-bottomed plates (Costar, Cambridge, MA). Effectors were added at designated E:T ratios in 100 μl/well. After 5–20 h of incubation, 100 μl of culture supernatant were collected, and ^{51}Cr release was measured on a γ-counter (Gamma 5500B; Beckman, Fullerton, CA). All determinations were done in triplicate or tetraplicate. Results are expressed as percentage of specific lysis as determined by:

$$\%\ \text{specific lysis} = \frac{\text{Experimental mean cpm} - \text{spontaneous mean cpm}}{\text{Maximum mean cpm} - \text{spontaneous mean cpm}} \times 100$$

For peptide-pulsed cytotoxicity assays, the T2 line (generously donated by P. Creswell, Yale University, New Haven, CT) was used as target. T2 is a human T-cell/B-cell fusion product containing an Ag-processing defect in the transporter-associated proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides that can be effectively displaced by exogenous HLA-A2-binding peptides (23). The T2 cells were labeled with ^{51}Cr as above, washed, and then incubated with peptide for 1.5 h at 37°C prior to standard cytotoxicity assays. To increase the sensitivity of detection at low E:T ratios by uncloned effectors, 20-h CTL assays were used in parallel with the 5-h assays. T2 without peptide (T2-NP) was also used as a control. For cold target inhibition assays, unlabeled T2 were incubated with peptide for 1.5 h and then added to standard cytotoxicity assays with chromium-labeled tumor targets and effectors. The cold:hot target ratio was 15:1.

CTL Induction Experiments. Freshly cultured TALs were plated at 1×10^6 cells/ml in 24-well culture plates (Costar) in RPMI 1640/10% FCS without IL-2. T2 cells were irradiated with 100 Gy (Cesium source), washed, and incubated with saturating concentrations of individual FBP peptides for 1.5 h prior to being added to six parallel TAL cultures at a 10–15:1 responder:stimulator ratio. After 48 h, 50 IU/ml of IL-2 were added. Medium and IL-2 were then added every 2–3 days as needed. Parallel control cultures were established with T2-NP in the exact same manner. After 1 week in culture, cells were counted, and the proliferation index was calculated as a ratio of peptide-stimulated culture cell number to the control cultures stimulated with T2-NP. This approach was preferred over DNA synthesis (thymidine incorporation) because it also indicates the viability of the cells after stimulation.

For stimulation with E39 presented on DCs, PBMCs were collected from three healthy HLA-$A2^+$ donors. DCs were generated by the CD14 method, *i.e.*, from plastic adherent PBMCs after culture in 1000 units/ml granulocyte/macrophage-colony stimulating factor and 500 units/ml of IL-4. DCs generated by this method express the CD13 marker, high levels of MHC-I, MHC-II, CD86, and CD-54, moderate levels of CD40, and low levels of CD80 (24, 25). This phenotype is consistent with immature DCs, which can uptake large amounts of Ags. DCs were pulsed with E39 at 50 μg/ml in serum-free medium, followed by tumor necrosis factor α (50 units/ml). DC-E39 were used as stimulators for autologous plastic nonadherent PBMCs at a ratio of 1:25 (stimulator:responder). IL-2 was added 24 h later in all cultures at 60 IU/ml. Cytotoxicity assays were performed as above at 1 week from the last stimulation.

Cytokine Assays. Peptide-stimulated, parallel-cultured TALs were replated at 1×10^6 cells/ml after 1 week and restimulated in the same fashion as described above. Supernatants from the parallel cultures were harvested at 24 and 48 h prior to the addition of IL-2 and stored at −20°C until analyzed. Supernatants from DC-E39-stimulated PBMCs were also collected at 24 and 48 h. Peptide-specific cytokine release from the TALs was measured in 50 μl for IFN-γ and IL-4 at the two time points using ELISA kits (BioSource, Camarillo, CA) with a sensitivity of 4 pg/ml according to the manufacturer's instructions. Results are given as pg/ml produced by 1×10^6 cells.

IP-10 ELISA. The ability of cells to secrete IP-10 in response to FBP peptides was determined by culturing PBMCs and collecting supernatants at corresponding times. The levels of IP-10 secreted were determined using a modified sandwich ELISA (R & D Systems, Minneapolis, MN). A flat-bottomed, 96-well microtiter plate was coated with 100 μl/well of monoclonal anti-human IP-10 (2 μg/ml in PBS, pH 7.2) for 24 h at room temperature. The plate was subsequently washed with PBS (pH 7.4), 0.05% Tween 20 and then blocked with 3% ovalbumin, 5% sucrose, and 0.05 NaN_3. IP-10 standards were made from recombinant human IP-10 in a solution consisting of Tris-buffered saline (TBS; pH 7.3), 0.05% Tween 20, and 0.1% BSA using serial dilutions. One hundred μl/well of the standards and the cell supernatants were plated in duplicate and left at room temperature for 2 h. After washing the plate three times, 100 μl/well of biotinylated monoclonal anti-human IP-10 [100 ng/ml in TBS (pH 7.3), 0.1% BSA] was added, followed after washing by 100 μl/well of streptavidin-peroxidase conjugate. Chromogen substrate, 100 μl/well, consisted of DMSO and H_2SO_4. Plates were read at 450 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Richmond, CA). Standard dilutions of IP-10 ranged from 4000 to 15.6 pg/ml. This method consistently detected IP-10 concentrations >31.25 pg/ml in a linear fashion.

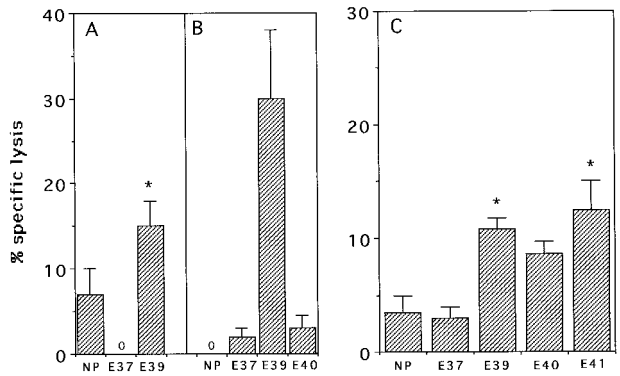

Fig. 1 Freshly cultured OvTALs and BrTALs recognize FBP peptides. *A*, HLA-$A2^+$ BrTAL-1 isolated from a pleural effusion, cultured in IL-2 without specific stimulation, was tested for recognition of FBP peptides E37, E39, and E40 (*B*). The experiments shown in Fig. 1*A* were performed using the same culture as effectors at 8 days from each other. Peptides were used at 25 μg/ml in *A* and 50 μg/ml in *B*. E:T ratios were 20:1 and 30:1, respectively. *C*, OvTALs were tested for recognition of FBP peptides (E37-E41) or no peptide (T2-NP) as a negative control. The 5-h ^{51}Cr-release assays were performed in triplicate at an E:T ratio of 20:1 and repeated two to four times for each effector. Pooled data with four OvTAL populations in 12 independent assays demonstrate E39 to be the most consistently recognized FBP peptide. E40 and E41 were recognized by some TALs ($n = 2$) but not others. The results are expressed as percentage of specific lysis (*, $P < 0.05$ *versus* NP); *bars*, SE.

RESULTS

Ovarian and Breast Cancer-associated Lymphocytes Recognize FBP Peptides. The isolated TALs from breast cancer patients were cultured in medium containing IL-2 without restimulation with autologous tumor. Cytotoxicity assays using peptide-loaded T2 cells as targets were performed with the TALs within 7–14 days of culture initiation to limit the *in vitro* expansion of irrelevant clones. One of two BrTALs, designated BrTAL-1, recognized E39. The results in Fig. 1*A* show specific recognition by BrTAL-1 of FBP peptide E39 compared with another FBP peptide, E37, or control T2 cells, which were not pulsed with peptide (T2-NP). The presence of CTLs with this epitope specificity was confirmed by retesting the E39 recognition 8 days later (Fig. 1*B*) at higher E:T ratio (30:1) and higher Ag concentration (50 μg/ml). These results indicated that the E39 specificity is present *in vivo* among the breast-associated CTLs and stable within the first 3 weeks of culture.

OvTALs were also screened for FBP peptide reactivity. Four of four OvTALs recognized FBP peptides. We found that among the FBP peptides, E39 was recognized most consistently by the four OvTALs. Fig. 1*C* shows the pooled results of experiments using as effectors fresh OvTAL cultures. E37, a low affinity binder, and E38, a high affinity binder, were not

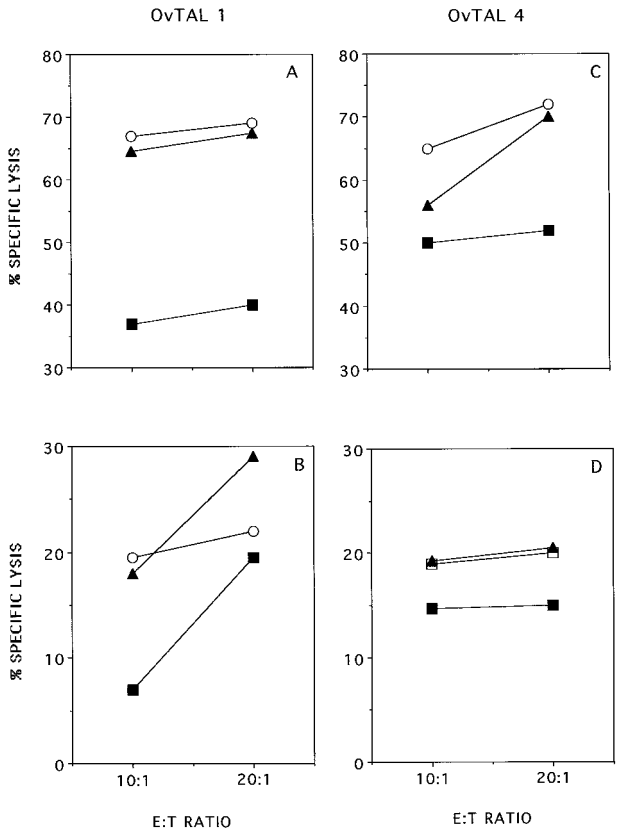

Fig. 2 E39 (FBP, 191–199) reconstitutes an epitope corresponding to a naturally processed and presented Ag in ovarian cancer. Cold target inhibition assays were performed with OvTAL-1 (*A* and *B*) and OvTAL-4 (*C* and *D*). T2 loaded with E37 (negative control peptide; ▲), E39 (■), or no peptide NP (○, □) were tested at a cold:hot ratio of 15:1 for inhibition of the recognition of the ovarian cancer cell line SKOv3.A2 by OvTALs at E:T ratios of 10 and 20:1 in 5-h (*B* and *D*) and 20-h (*A* and *C*) assays. Results are expressed as percentage of specific lysis.

significantly recognized in these assays and, therefore, served as internal specificity controls. E41 was highly recognized by two of four OvTALs tested and one of two BrTALs. The recognition of these peptides by freshly cultured, *in vitro* unstimulated TALs documents the presence of precursor CTLs specific for these epitopes *in vivo*, suggesting *in vivo* priming to these Ags. E39 (FBP, 191–199) appears to be an immunodominant CTL epitope. The overlapping peptides E40 (FBP 247–255) and E41 (FBP 245–253) form a subdominant CTL epitope, because both induced CTL recognition. E41 was better recognized than E40, and its level of recognition was comparable with E39 (19).

FBP Peptide E39 Is a Naturally Processed Ag. To determine whether E39 reconstitutes CTL epitopes that are presented on ovarian tumor cells, cold target inhibition assays were performed. T2 pulsed with E39 (T2–39) were used to block the recognition of TAL populations for the ovarian cancer cell line, SKOV3.A2. T2–39, but not T2–37 or T2-NP, effectively inhibited the tumor lysis by OvTAL-1 (Fig. 2, *A* and *B*) and OvTAL-4 (Fig. 2, *C* and *D*) in 5 h (Fig. 2, *B* and *D*) and 20 h (Fig. 2, *A* and *C*) cytotoxicity assays ($P < 0.05$). The inhibitory effects of T2/E39 increased over time, between 5 and 20 h, suggesting that the recognition of the epitope formed by E39 was not transient or nonspecific. These experiments were repeated, and the results were confirmed (data not shown). These findings suggest that the CTLs specific for the E39 epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, these data demonstrate that FBP-derived peptides are naturally processed Ags. The fact that the levels of lysis were low at 5 h but significantly increased over time suggested that: (*a*) FBP-specific CTLs may be present at low frequency in TALs; and/or (*b*) FBP-specific CTLs represent memory effectors that require restimulation for high expression of CTL function (26).

Induction of Proliferation and Specific IFN-γ Release by FBP Peptides E39 and E41. The results (Figs. 1 and 2) indicate the presence of FBP-specific CTLs in TALs that express low levels of recognition of tumor Ags. This raised the question of whether CTL functions can be amplified. To address this question, we used T2 cells as APCs because they can be exogenously loaded with peptides. Except for low concentrations of IL-2 added 48 h after stimulation, no other costimulatory agents or CTLs supporting cytokines were used to assess the sensitivity of these CTL-TALs to peptide stimulation. Short-term cultured TALs were split into parallel cultures and stimulated with irradiated T2 loaded with either E37, or E39, or E41, or NP as a control. E41 was chosen over E40 as the subdominant epitope to study because of its better recognition by TALs. E40 and E41 differ by only two terminal residues. Because E41 is recognized more frequently and better than E40, we hypothesized that E41 forms a more closely related epitope recognized by TALs than E40. Because T2 are allogeneic to the responders, a certain level of allospecific proliferation and cytokine secretion to T2-NP was expected. For this reason, T2E-37 was used as an additional internal specificity peptide control. One week later, cells in these cultures were counted, and proliferation indexes were determined as compared with the T2-NP- and T2E-37-stimulated cultures. Fig. 3*A* shows the effects of E39 and E41 stimulation on OvTAL-1 and OvTAL-2 numbers. Both peptides induced enhanced TAL proliferation over the level observed with T2-NP in both populations ($P < 0.05$); E39 was superior to E41 in OvTAL-1, whereas E41 was better than E39 in inducing OvTAL-2 proliferation. This suggested differences in precursor frequency and state of activation between the responders in these cultures.

To determine whether FBP peptide stimulation induces cytokine production, we determined the levels of IFN-γ and IL-4. E39 and E41 induced specific IFN-γ release in peptide-stimulated OvTAL. Both E39- and E41-stimulated OvTAL-2 demonstrated peptide-specific IFN-γ production at 24 h (not shown), and the IFN-γ release increased at 48 h (Fig. 3*B*). Similar results were obtained with OvTAL-1. No IL-4 was produced in response to these peptides in either TAL culture (Fig. 3*B*). These findings suggest that the FBP peptides have the ability to activate IFN-γ secretion, which may be relevant for the cytokine-mediated effector pathway. These results also show that IFN-γ production does not parallel OvTAL proliferation. E39 was a weaker stimulator of OvTAL-2 proliferation than E41; however, the IFN-γ levels induced by each peptide were similar.

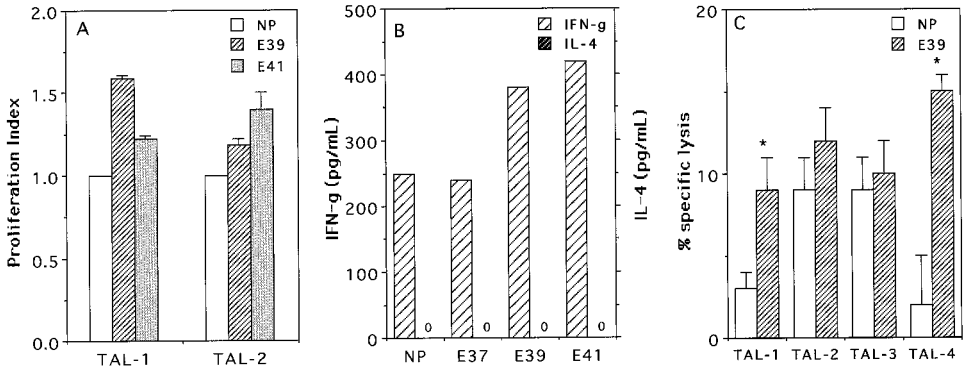

Fig. 3 Stimulation with FBP peptides results in enhanced OvTAL proliferation and IFN-γ release and cytotoxicity. *A*, OvTAL-1 and OvTAL-2 respond differently to E39 and E41. OvTALs were cultured in parallel and stimulated with irradiated T2 loaded with E39, E41, or no peptide (*NP*). Cell counts were performed after 1 week. Results are expressed as proliferation index. *B*, OvTAL-2 demonstrate peptide-specific IFN-γ release. Parallel cultures of OvTAL-2 stimulated with T2 loaded with E37, E39, E41, and NP were replated at 1×10^6 cells/ml in 1 ml and restimulated with T2 and the corresponding peptides E39, E41, E37, or T2-NP. IFN-γ and IL-4 were measured 48 h later. This result is representative of replicated experiments. *C*, enhanced cytotoxicity after FBP peptide stimulation. OvTALs 1–4 were stimulated in parallel with irradiated T2 loaded with E39 or NP. Recognition of E39 was assessed in standard CTL assays after 1 week at an E:T of 10:1. Results are expressed as percentage of specific lysis (∗, $P < 0.05$ *versus* NP); *bars*, SE.

Enhanced Cytotoxicity of FBP Peptide-stimulated TALs. One week after primary E39 stimulation, the TAL cultures were evaluated for specific Ag recognition. We used lower E:T ratios (5:1) than in the assays with fresh TALs on the rationale that if there was an increase in specific CTLs compared with the original levels, elicitation of specific recognition should be detected when fewer effectors were used. Two of four TAL populations (OvTAL-1 and OvTAL-4) revealed an increase in lytic activity, on a per cell basis, for E39 compared with parallel cultures stimulated with T2-NP (Fig. 3*C*), whereas OvTAL-3 was resistant. These results show individual related differences in the responsiveness of OvTAL to E39 and E41 when presented on T2 cells. These results indicate a requirement for the relevant peptide for CTL-TAL expansion and expression of the lytic function. OvTAL-2 stimulated with E39 showed weak specificity for E39 (≤25% increase over controls). E41-stimulated OvTAL-2 also exhibited marginal specificity to E41 (38% increase over control T2-E37; data not shown). In terms of amplification of CTL activity, OvTAL-2 and OvTAL-3 were considered nonresponders. OvTAL-2 responded to E39 and E41 with similar high levels of IFN-γ, preferential proliferation to E41 *versus* E39, but poor activation of CTL activity. Because under conditions of allogeneic stimulation, which are expected to amplify an alloreactive response, restimulation of TALs with the self-peptide E39 succeeded in amplifying a CTL response to the tumor, E39 is immunogenic.

Epithelial Tumor Lysis by FBP Peptide-stimulated TALs. In separate experiments, OvTAL-1 and OvTAL-2 stimulated twice by FBP peptide-loaded T2 over 2 weeks were evaluated for recognition of multiple tumor targets at an even lower E:T ratio of (2.5:1) compared with the previous experiments. To establish whether E39 stimulation enhances recognition of SKOV3.A2 (HER-2^{hi}, FBP^{+}), we used as effector OvTAL-1 stimulated with T2-E39 and T2-E41, or with T2-NP and T2-E37 as controls. The results (Fig. 4*A*) show that T2-E39 stimulated OvTAL-1 recognized SKOV3.A2 at significantly higher levels than control (T2-NP/E37)-stimulated OvTAL-1. Of interest, the levels of SKOV3.A2 lysis observed with 1×10^4 effectors in this assay were similar with the levels observed with 8×10^4 unstimulated effectors of the same line (Fig. 2), indicating that E39 stimulation lead to an enrichment in E39-specific effectors. The recognition of FBP-expressing epithelial tumors was verified by using as targets lines expressing different levels of HER-2. OvTAL-1 lysed SKOV3.A2, SW480, and the pancreatic line PAN-1 significantly better than SKBR3.A2 (Fig. 4*B*).

Lysis of these targets appeared not to be dependent on the levels of HER-2. SKOV3.A2 expresses significantly higher levels of HER-2 than SW480 and PAN-1. However, all three lines were recognized at similar levels by OvTAL-1. SKBR3.A2 expresses similar HLA-A2 levels as PAN-1 and SW480. The HLA-A2 levels in these targets are lower than of SKOV3.A2. SKBR3.A2 expresses higher levels of HER-2 than SKOV3.A2. SKBR3.A2 was lysed only marginally by E39-stimulated OvTAL-1. Because all of these tumors express HER-2, one might suggest that this Ag system is being recognized, although one would expect less HER-2 recognition of the lower HER-2-expressing tumor SW480 and higher recognition of SKBR3.A2. This was not seen in these experiments. In Western blotting using MOV18 mAb, we detected very low levels of FBP in SKBR3.A2 cells, compared with SKOV3.A2. Thus, the increase in lysis of SKOV3.A2 *versus* SKBR3.A2 by E39-stimulated OvTAL-1 is supportive of the hypothesis of recognition of E39 or of structurally similar epitope rather than of HER-2.

To verify whether tumor recognition is dependent on stimulating Ags, we tested the same targets for lysis by the weak responder OvTAL-2 restimulated with E39 and E41 or with E37 as control. Results in Fig. 4*C* show that E39 and E41 were weak stimulators of OvTAL-2 cytotoxicity for tumors compared with OvTAL-1. However, OVTAL-2 stimulation with E41 increased recognition of SKOV3.A2, although marginally over the recognition by E37-stimulated OvTAL-2. In contrast, E39 stimulation

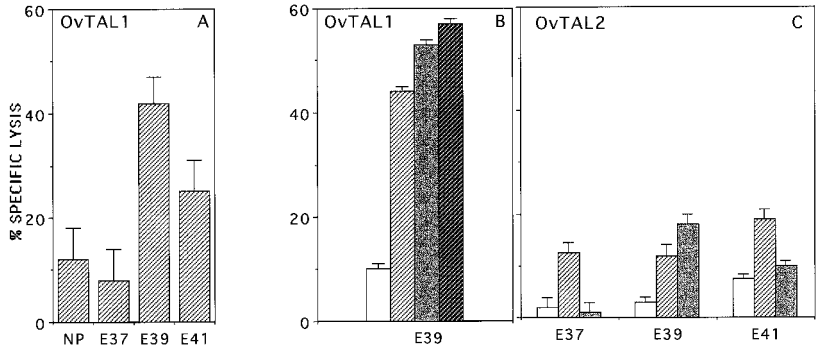

Fig. 4 FBP peptide-stimulated OvTALs recognize tumors of different histologies. *A*, OvTAL-1 was stimulated in parallel with irradiated T2 loaded with E37, E39, E41, or NP twice at weekly intervals and tested at the same E:T ratio (2.5:1) for lysis of SKOV3.A2. *B*, E39-stimulated OvTAL-1 recognize FBP-expressing tumors of different histologies: breast SKBr3.A2 (HLA-A2^{low}, HER-2/neu^{high}, FBP$^{\pm}$), □; ovarian SKOv3.A2 (HLA-A2^{high}, HER-2/neu^{high}, FBP^{+}), ▨; colon SW480 (HLA-A2^{low}, HER-2/neu^{low}, FBP^{+}), ■; and pancreas PAN-1 (HLA-A2^{low}, HER-2/neu^{high}, FBP^{+}), ■. *C*, OvTAL-2 stimulated twice at weekly intervals with irradiated T2 loaded with E37, E39, or E41 were tested for recognition of tumor targets expressing FBP. Results are expressed as percentage of specific lysis; *bars*, SE. All TAL-1 cultures were tested in standard 20-h cytotoxicity assays at an E:T ratio of 2.5:1.

increased recognition of SW480 compared with stimulation with E41 or E37. These results support the possibility of differences in epitope presentation by these tumors.

Stimulation of PBMCs from Healthy Donors with DC-E39 Induces E39-specific CTL Activity IFN-γ and IP-10 Production. The results presented above demonstrated that E39 and E41 can stimulate effector functions in OvTAL. However, for preventative vaccination approaches, the ability of a tumor peptide to stimulate CTLs from peripheral T cells of healthy donors is an important factor in considering its immunogenicity. To address whether E39 can activate CTL activity from healthy donor PBMCs, we used an autologous system. In this system, the autologous PBMC-derived DCs were used as APCs. The results of primary stimulation with E39 pulsed DCs (DC-E39) are shown in Fig. 5. We used three different donors to ensure that the results are relevant. The HER-2 peptide E71 was used as specificity control, because it binds HLA-A2 with very low affinity, lower than E39, and it is not recognized by HER-2-specific CTL-TALs (5). Its presence in the assay aimed to balance the exogenous E39 concentration delivered to T2 cells. The results of primary stimulation show an individual-dependent pattern of lytic responses to activation by E39. Donor 1 responded with weak E39-specific CTL activity, donor 2 responded with high levels of E39-specific CTL activity, whereas donor 3 showed no response to E39 in a 5-h CTL assay. The presence of activated E39-specific CTLs was confirmed in donor 3 by retesting the CTL lysis in a 20-h assay. These results indicate that E39 precursors are present in the peripheral blood of healthy donors, and in a fraction of donors, they can be rapidly activated by peptide to exhibit E39-specific lysis.

To determine whether DC peptide stimulation can induce IFN-γ, PBMCs from donor 1 were stimulated in a separate experiment with DC-E39 and DC-E41. DC-NP and DC-E75 (HER-2 peptide) were used as negative and positive controls, respectively. The results (Fig. 6) show that E41 was more potent than E39 in induction of IFN-γ from T cells of this donor within 48 h after primary stimulation. IFN-γ levels secreted in response to E41 were similar to levels induced by E75. The weak IFN-γ

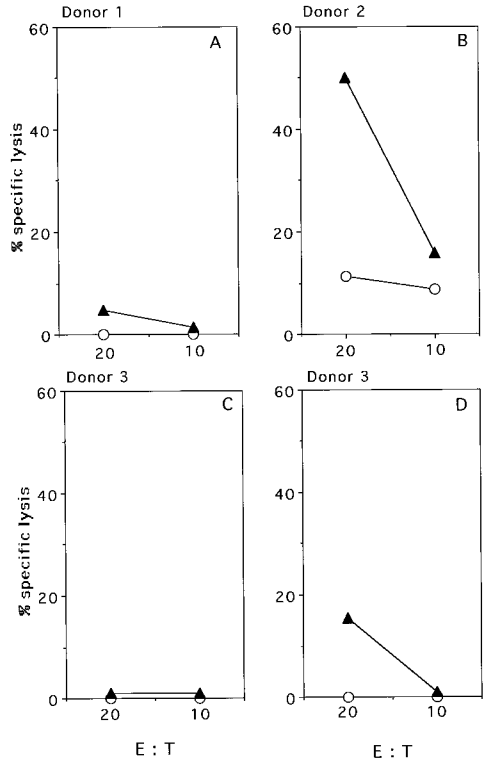

Fig. 5 E39 recognition by primary E39-stimulated PBMCs from three healthy donors. Autologous DCs were used as APCs. T2 cells were pulsed with E39 (▲) or control E71 (○) peptide at 25 μg/ml. Results are from 5-h (*A–C*) and 20-h (*D*) CTL assays. Experimental conditions are as described in "Materials and Methods."

stimulatory ability of E39 was confirmed with another donor (data not shown). IFN-γ production in response to E39 and E41 was not detected within the first 6 h of peptide stimulation, suggesting that activated E39/E41-specific CTLs are not present in this healthy donor.

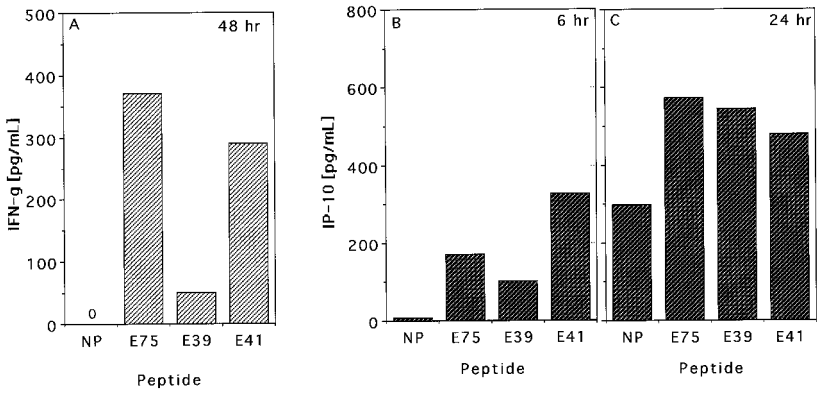

Fig. 6 *A,* E39 and E41 induced IFN-γ from isolated $CD8^+$ cells of healthy donors. Peptides were presented by autologous CD14-derived DCs for 48 h in the absence of IL-2 or IL-12. E75 at the same concentration was used as positive control. *B* and *C,* E39 and E41 at 50 μg/ml rapidly induce IP-10 from isolated $CD8^+$ cells (*B*) or isolated PBMCs from distinct healthy donors. Results indicate pg/ml, 10^6 cells. HER-2 peptide E75 was used as a positive control.

Because different $CD8^+$ functions are sensitive to different levels of Ags, we determined in parallel from the same culture supernatants the presence of the antiangiogenic chemokine IP-10. IP-10 was detected within 6 h of stimulation from this donor in response to both E39 and E41 (Fig. 6*B*). Induction of IP-10 by E39 and E41 was confirmed with a second donor (Fig. 6*C*). There were differences in the levels of response to E39 and E41 between these two donors, suggesting that factors independent of the peptide sequence control the immunogenicity of FBP epitopes. These results indicate that E39 and E41 are immunogenic and can activate distinct effector functions in PBMCs from healthy donors, a property that makes FBP valuable for cancer vaccine development.

DISCUSSION

In this study, we found that freshly cultured ovarian and breast TALs, not previously subjected to Ag stimulation (anti-CD3, autologous tumor) *in vitro,* recognize FBP peptides, and most consistently E39 (FBP, 191–199). Cold target inhibition studies demonstrated that the antigenic peptides E39 and E41 are naturally expressed epitopes on ovarian cancer cells. Together, these data prove that FBP functions as a TAA recognized by CTL-mediated immunity in these cancers. Both immunodominant and subdominant epitopes have been described for the HER-2 protein (4, 5). This concept also holds for FBP, with E39 serving as an immunodominant peptide with the most consistent and highest level of recognition among consecutive TAL populations. E41 appears to be a subdominant epitope with high levels of recognition among some ovarian and breast TAL cultures but not others. These observations are important for understanding the T-cell repertoire responding to a processed tumor (self) Ag. Identification of two CTL epitopes on FBP may proffer a selective advantage for its use for cancer vaccination studies because FBP is expressed in outbred populations. In other systems of immunological disease under $CD8^+$ cell control, shifts in epitope dominance have been described recently and may be favorable or unfavorable predictors of disease outcome (27). Therefore, strategies to enhance immunity to tumors may need to be adjusted based on the presence of individual epitopes (27).

Analysis of FBP peptide immunogenicity indicated that E39 has the ability to activate both major CTL effector functions from TALs, *i.e.,* cytolysis and cytokine (IFN-γ) secretion. The stimulatory effects on cytolysis were evident in two of four OvTALs tested. E39 and E41 peptide-stimulated CTLs were capable of lysing FBP-expressing tumors of different histologies, *i.e.,* ovarian, colon, and pancreas. These tumors have been shown by different groups using biochemical, immunohistochemical, and binding approaches to express FBP (16). E39-stimulated OvTALs not only could recognized allogeneic HLA-$A2^+$ ovarian cancer cells but also lysed other FBP-expressing HLA-$A2^+$ epithelial colon and pancreatic cells. A common feature of E39- and E41-specific TALs was that they induced low levels of lysis in short term (5-h) assay but high levels of lysis in long 20-h assays. We do not know at this time whether this reflects the low density of E39/E41-specific CTLs in TALs or high-density, FBP-specific CTLs that lyse targets using predominantly the Fas-FasL instead of the perforin pathway (28).

In developing an immune response to cancer, the critical test of vaccination strategy is the ability of the Ag to activate primary responses by naïve/resting effectors. This requires significantly stronger signaling by Ags and costimulation than activation of memory responses or restimulation of established effector CTL clones (*i.e.,* from CTL-TALs; Ref. 29). Primary *in vitro* stimulation of T cells from PBMCs of healthy donors with autologous DCs pulsed with E39 resulted in induction of specific recognition of E39 in cytolytic assays, in addition, because it stimulated both IFN-γ and IP-10 secretion. The latter was more sensitive than IFN-γ secretion to FBP. Because IP-10 and other non ELR-CXC chemokines have been implicated in angiostasis and tumor regression (30), our results indicate that FBP may bear a significant immunogenic potential. The fact that IP-10 was detected at a time (6 h) when IFN-γ in culture was not detectable deserves further consideration. Another possibility is that low levels of IFN-γ were absorbed or consumed. Another possibility is that IP-10 induction in $CD8^+$ cells may not require IFN-γ, because it was demonstrated that IFN-γ is not absolutely necessary for *in vivo* IP-10 expression (31). Furthermore, stimulation of OvTALs with DC-E39 resulted in rapid amplification of OvTAL specificity for E39 compared with

control DC-NP stimulation.[4] These results indicate that E39 is immunogenic, *in vitro,* in peptide form when DCs are used as APCs, and activation induced by E39 and E41 may condition the environment for type 1 cytokine response by attracting $CD4^+$ cells of Th1 phenotype (32). This response may protect from Th2 cytokine-induced tolerance.

In contrast with E39, E41 activated IFN-γ production in both PBMCs and TALs, but its stimulatory effects on cytolysis were weak. It should be noted that both E39 and E41 stimulated TAL proliferation. This suggests that E39 and E41 may have distinct effects in activation of different effector functions. The weak IFN-γ response induced by E39 compared with E41 may indicate that memory E39-specific CTLs are present with low frequency in healthy donors (26), but also that E41 has a stronger activating ability of APC/T cells for IFN-γ production. The reasons for the different effects of E39 and E41 in activation of CTL effector functions are under investigation. E41 binding affinity to HLA-A2 is only slightly lower than E39. Furthermore, OvTALs recognized E39 and E41 at similar levels, but E39 specificity was detected more frequently in four of four *versus* two of four TALs, respectively. It is possible that the resistance of certain TALs to stimulation by E41 (and sometimes by E39) reflects the presence in these patients of functionally silenced CTL clones. Thus, E41-specific CTLs may be tolerized more frequently or more extensively. Such CTLs are currently described in studies with conventional Ags, whereas attempts are being made to devise approaches to reactivate their silenced functions (27, 29). The facts that the functional silencing of these clones is attributed to either Ag stimulation in the absence of costimulation (29) or lack of epitope-specific helper $CD4^+$ cells (33), or cytokine (IL-4, IL-10, and transforming growth factor β)-induced tolerance (34) may be relevant to the FBP system.

One of the most promising aspects of epithelial cancer vaccine development is that epithelial tumors share common CTL-recognized epitopes, indicating that a TAA-specific vaccine may be widely applicable. Our results show that E39-stimulated OvTALs lyse ovarian, colon, and pancreatic tumor lines. Because FBP is overexpressed in 90% of ovarian and 50–70% of breast cancers, these results further illustrate the clinical potential for targeting a widely expressed tumor Ag like FBP.

FBP was originally identified independently by three lines of investigation (reviewed in Ref. 19) as: (*a*) the LK26 Ag (16); (*b*) the Ag recognized by MOv18 and MOv19 mAbs (32); and (*c*) the high-affinity FBP from placenta and KB carcinoma cell lines (17). The true extent of FBP expression is still unknown because the mAbs currently used require more complex techniques than simple immunofluorescence staining to detect FBP (18). Because of some intrinsic differences between the LK26, MOv18, and MOv19 mAbs, different levels of FBP expression on some tumors have been reported. There is general agreement that only low levels of FBP expression exist in some normal tissues such as choroid plexus, lung, thyroid, kidney, and sweat glands.

High levels of FBP are preferentially expressed in a wide range of cancerous tissue (18). The reason for this differential expression is unknown, but one hypothesis involves the upregulation of the folate receptor to compensate for the loss of the alternative folate processing pathway involving the *tetrahydrofolate* reductase gene, which is frequently deleted in cancer cells (35, 36). The highest levels of expression of FBP have been found in ovarian carcinomas, with >90% of all ovarian carcinomas expressing elevated levels of this protein. The levels of overexpression have been shown to be >20 fold that of normal tissue routinely and reported to be as high as 80–90-fold in one study (18, 19). The extent of FBP expression in other tumors is probably underestimated, as alluded to above; however, multiple tumor types have been shown to overexpress the LK26/FBP Ag, including 20–50% of colorectal, breast, lung, and renal cell carcinomas, as well as many other tumor types (19).

Soluble peptide vaccines with a single peptide CTL epitope and immunoadjuvant are currently in trials for both melanoma (37) and the E75, HER-2 peptide (38). The complete response data are not yet available. Early results indicate that in the use of tumor peptide E75 as immunogen with incomplete Freund or granulocyte/macrophage-colony stimulating factor as adjuvant may not lead to tumor-specific CTL and clinical responses (38, 39). Because FBP peptides are recognized by freshly isolated OvTALs that are activated *in vivo* and lyse autologous tumors, development of approaches for *in vitro* and *in vivo* activation of CTLs by those peptides becomes a key issue. Immunogenicity for CTL induction is dependent on the Ag sequence and the delivery system to APCs. Although some conventional or viral Ag (*e.g.,* influenza) can induce CTLs by helper-independent, costimulation-independent mechanisms (33), the majority of known tumor peptides require costimulatory signals (from APC molecules or cytokines; Ref. 40) or Ag modification to induce CTL differentiation (29). The fact that E39 can activate both TALs and PBMCs suggests that E39 is a potentially immunogenic epitope for pCTL activation.

We are currently investigating the dominant and subdominant epitopes from both HER-2/neu (E75, GP2, and C85) and FBP (E39 and E41) in parallel to determine the immunogenicity of each of these epitopes to induce primary CTLs with tumor killing ability and the requirement for proinflammatory cytokines, such as IL-12, in activating APCs or reversing tolerance. We are also investigating the possibility of immune-gene therapy for these types of epitopes by delivering the minimal CTL epitope together with the endoplasmic reticulum translocation sequence using vaccinia vectors to the APCs and proteasome inhibitors to enhance targeting of the epitope precursor protein to the MHC-I processing and presentation pathway.

As effective delivery systems are developed, the key to a successful epithelial cancer vaccine still depends on the discovery of CTL-recognized TAAs to be targeted with the vaccine. In conclusion, FBP serves as an endogenous source of CTL-recognized and CTL-stimulatory epitopes, which are naturally expressed on a large number of epithelial tumors. Because FBP is overexpressed with high frequency, FBP peptides E39 and E41 may serve as the basis of a widely applicable epithelial cancer vaccine. Clinically, these findings would support the development of a polyspecific vaccine composed of dominant and subdominant peptides from either a single or multiple TAAs.

[4] D-K. Kim and C. G. Ioannides, manuscript in preparation.

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Clinical Cancer Research

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