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Differential Role of Fas/Fas Ligand Interactions in Cytolysis of Primary and Metastatic Colon Carcinoma Cell Lines by Human Antigen-Specific CD8⁺ CTL

Elke S. Bergmann-Leitner and Scott I. Abrams¹

We have previously identified mutated *ras* peptides reflecting the glycine to valine substitution at position 12 as HLA-A2-restricted, CD8⁺ CTL neo-epitopes. CTL lines produced against these peptide epitopes lysed the HLA-A2⁺ Ag-bearing SW480 primary colon adenocarcinoma cell line, although IFN- γ treatment of the targets was necessary to achieve efficient cytotoxicity. Here, we compared the lytic phenotype of the SW480 cell line to its metastatic derivative, SW620, as an in vitro paradigm to further characterize the nature of a HLA class I-restricted, Ag-specific CTL response against neoplastic cell lines of primary and metastatic origin. Although both colon carcinoma cell lines were lysed by these Ag-specific CTL following IFN- γ pretreatment, the mechanisms of lysis were distinct, which reflected differential levels of sensitivity to the Fas pathway. Whereas IFN- γ pretreatment rendered SW480 cells sensitive to both Fas-dependent and -independent (perforin) pathways, SW620 cells displayed lytic susceptibility to Fas-independent mechanisms only. Moreover, pretreatment of SW480 cells with the anti-colon cancer agent, 5-fluorouracil (5-FU), led to enhanced Fas and ICAM-1 expression and triggered Ag-specific CTL-mediated lysis via Fas- and perforin-based pathways. In contrast, these phenotypic and functional responses were not observed with SW620 cells. Overall, these data suggested that 1) IFN- γ and 5-FU may enhance the lytic sensitivity of responsive colon carcinoma cells to immune effector mechanisms, including Fas-induced lysis; 2) the malignant phenotype may associate with resistance to Fas-mediated lysis in response to Ag-specific T cell attack; and 3) if Ag-specific CTL possess diverse lytic capabilities, this may overcome, to some extent, the potential "escape" of Fas-resistant carcinoma cells. *The Journal of Immunology*, 2000, 164: 4941–4954.

Major histocompatibility complex-restricted CD8⁺ CTL and CD4⁺ T cells have been shown to play crucial roles in host defense against human malignancies and, thus, have become important considerations in the design of cancer vaccine-based clinical trials (1–5). However, in a number of experimental systems, it is also becoming clearer that neoplastic cells may evade cell-mediated immune responses at multiple levels of the effector/target interaction (reviewed in Refs. 6–8), which, consequently, may impact the metastatic process. Tumor escape mechanisms may include down-regulation of TCR-MHC/Ag recognition and engagement, cell-cell adhesion, costimulation activities, and aspects related to tumor-induced immune suppression of T cell proliferation and T cell effector functions within the host-tumor microenvironment.

The Fas/Fas ligand (FasL)² system has been characterized as an essential process for the maintenance and support of immune privilege and immune homeostasis of peripheral lymphoid interactions under both normal and pathologic conditions (9–11). Additionally, Fas/FasL interactions that occur between immune effector cells

and susceptible Ag-bearing target cells have been portrayed as potentially important components of T lymphocyte-mediated cytotoxicity (9, 12, 13). However, the disengagement or loss of the Fas death pathway as a potentially major antitumor determinant, for example, may allow immune escape and proliferation of a selective cohort of malignant cells, which, consequently, may facilitate metastatic development. The notion that loss of sensitivity to Fas-mediated apoptosis may play an important role in the progression of malignant behavior is supported by the observation that cancerous cells, including hematologic and nonhematologic solid tumors that display resistance to chemotherapeutic agents, may concomitantly exhibit resistance to functional Fas expression (14–16).

During antitumor immune interactions, a potentially important cellular component of functional Fas ligation on Ag-bearing targets may be MHC-restricted, tumor-specific CD8⁺ CTL, although their involvement as relevant players under those conditions in human carcinoma (in vitro or in vivo) remains largely uncharacterized. To study this issue, we have developed an in vitro model system consisting of HLA-A2-restricted CD8⁺ CTL specific for *ras* oncogene determinants (17, 18). We demonstrated that such CTL, established from both normal (18) and carcinoma-bearing (17) individuals, can lyse an HLA-A2⁺ primary colon adenocarcinoma cell line (SW480) naturally bearing endogenous Ag. However, this was achieved following IFN- γ treatment of the target cells, which appeared to be essential for modulation of their lytic susceptibility. The role of IFN- γ in modulating the lytic outcome introduced the notion that during neoplastic progression, (colon) carcinoma cells may evade T cell-mediated immunity, not only at the levels of Ag recognition and cell-cell adhesion, but also at the level of Ag-specific T cell attack, perhaps involving alterations in their responsiveness to Fas/FasL-induced lysis. In this study, we examined and compared the nature and spectrum of the CTL lytic

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² Abbreviations used in this paper: FasL, Fas ligand; CMA, concanamycin A; 5-FU, 5-fluorouracil; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; sFasL, soluble FasL; TDLN, tumor-draining lymph node.

mechanisms operative against the SW480 and SW620 colon adenocarcinoma cell lines. The latter of these lines represented a lymph node-derived metastatic lesion established previously from the same patient (19). Thus, this paradigm allowed us to investigate, at least initially in an *in vitro* model system, an Ag-specific cellular immune response against human colon carcinoma cells of primary and metastatic origin; to focus on potential fundamental differences in their response toward Ag-specific CD8⁺ CTL attack; and to determine the correlation of that response with their characterized malignant phenotype.

Overall, we found that following IFN- γ treatment both tumor cell lines were lysed by these Ag-specific CD8⁺ CTL, but that the mechanisms of lysis were distinct at the level of involvement of the Fas death pathway. While the SW480 cell line displayed an IFN- γ -inducible, Fas-sensitive phenotype, the SW620 cell line maintained a Fas-resistant phenotype, which could not be reversed by IFN- γ treatment. These observations revealed a potentially important link between the malignant or metastatic phenotype with responsiveness to Fas-mediated killing by human MHC class I-restricted, Ag-specific CD8⁺ CTL. Moreover, the finding that lysis of IFN- γ -treated SW620 tumor cells proceeded alternatively through a perforin-based effector mechanism suggested that triggering of Fas-independent killing by such MHC-restricted, Ag-specific CD8⁺ CTL may circumvent, at least to some magnitude, the potential "escape" of Fas-resistant colon carcinoma cells.

Materials and Methods

Production of HLA-A2-restricted, anti-ras Val12 epitope-specific CD8⁺ CTL lines

CD8⁺ CTL lines specific for mutated *ras* Val12 epitopes were generated as described (17, 18). CD8⁺ T cell lines were restimulated weekly with irradiated, autologous EBV-transformed B cells as APC, previously pulsed with peptide (5 μ g/ml; Multiple Peptide Systems, San Diego, CA; >97% purity by HPLC) and β_2 -microglobulin (0.5 μ g/ml; Intergen, Purchase, NY) in the presence of recombinant human IL-2 (10 U/ml; Cetus, Emeryville, CA). The *ras* 4–12(Val12) peptide epitope sequence is YKLVVVGAV, while that of *ras* 5–14(Val12) is KLVVVGAVG.

Cytotoxicity assay

Cytotoxicity was analyzed by ⁵¹Cr release assays in the absence and presence of neutralizing mAb or concanamycin A (CMA) (Sigma, St. Louis, MO) as an inhibitor of perforin-mediated lysis (20, 21). Briefly, target cells were radiolabeled with 200 μ Ci of Na₂⁵¹Cr O₄ (Amersham, Arlington Heights, IL) for 60 min at 37°C. In some experiments, ⁵¹Cr release was compared with [¹²⁵I]IUdR release, as a measurement of nuclear damage. To that end, target cells were radiolabeled with 20 μ Ci of 5-[¹²⁵I]IUdR iodo-21-deoxyuridine (Amersham) for 3 h at 37°C, as described (22). Effectors and targets (1 \times 10⁴/well) were incubated in 96-well, U-bottomed plates (Costar, Cambridge, MA) for 12 h at various ratios in the absence and presence of neutralizing mAb reactive with HLA-A2 (clone BB7.2 from the American Type Culture Collection (ATCC), Manassas, VA), Fas (CD95, clone ZB4; Immunotech, Westbrook, ME), FasL (CD95L, clone NOK-1; PharMingen, San Diego, CA), ICAM-1 (CD54, clone 84H10; Serotec, Raleigh, NC), or nonpolymorphic epitopes of the $\alpha\beta$ -TCR (clone BMA031; Immunotech). Isotype-matched control Ab included UPC-10 (IgG2a) and MOPC-21 (IgG1) (both from Cappel, West Chester, PA) or anti-carcinoembryonic Ag (CEA-specific clone Col-5 (IgG2b), as described in Ref. 18). Ab were used as affinity purified (10 μ g/ml), except BB7.2 and isotype-matched Col-5, which were used as ascites (1:10 dilution). In the case of CMA, CTL were preincubated at 37°C for 2 h with the compound (10 μ M) before adding radiolabeled target cells, as described (20, 21) to inhibit granule-mediated lysis (12, 23). After incubation, supernatants were collected using a supernatant collection system (Skatron, Sterling, VA), and radioactivity was measured in a gamma counter. In the case of [¹²⁵I]IUdR release, cultures were treated with 0.3% Triton X-100 before collection, as described (22). Cytotoxic activity was defined as percent specific release of ⁵¹Cr or ¹²⁵IUdR and determined by the equation: [(experimental_{cpm} - spontaneous_{cpm})/(maximum_{cpm} - spontaneous_{cpm})] \times 100. Effector cell preparations included: anti-*ras* 4–12(Val12) CD8⁺ CTL line (18), anti-*ras* 5–14(Val12) CD8⁺ CTL line (17), and HLA-A2-restricted CD8⁺ CTL lines reactive with immunodominant peptide

epitopes of influenza virus (Matrix_{58–66}) (24) or MART-1 (MART-1_{27–35}) (25).

Target cells

The HLA-A2⁺, *ras* Val12⁺ SW480 (CCL-228) and SW620 (CCL-227) colon adenocarcinoma cell lines (19, 26) (both from ATCC) were used as targets and, where indicated, were treated for 18–24 h with recombinant human IFN- γ (sp. act. 2.4 \times 10⁷ U/mg; 250 U/ml) (Biogen Research, Cambridge, MA) or 5-fluorouracil (5-FU; Sigma) before radiolabeling and testing. As described in the ATCC product sheets, the SW480 and SW620 cell lines were acquired shortly after their establishment and have been cryopreserved since then. All cell lines used in these experiments, including tumor cell lines, were mycoplasma-negative as determined by PCR analysis using the *Mycoplasma* detection kit from ATCC. An enzyme-based immunoassay was used for the detection of the Val12 determinant, as described (17, 18). Briefly, tumor cell lysates were produced by extraction in nonionic detergent. The protein concentration of the nuclei-free material was determined, and aliquots were stored at -70°C until analysis. A pan-*ras* mAb (clone RAS10; IgG2a isotype) (Oncogene, Cambridge, MA) was used as the capture Ab, and an anti-*ras* Val12 mAb (clone DWP; IgG2b isotype) (Oncogene) was used as the detection Ab in combination with an affinity-purified, goat anti-mouse IgG2b-specific Ab conjugated to HRP (Southern Biotechnology Associates, Birmingham, AL). After the addition of the substrate, *o*-phenylenediamine dihydrochloride (Sigma), and hydrogen peroxide, the OD was measured at 490 nm.

Flow cytometry

Cell surface marker analysis. Untreated, IFN- γ -pretreated or 5-FU-pretreated tumor cells were analyzed by flow cytometry using commercially available mAb reactive with HLA-A2 (One Lambda, Canoga, CA), Fas (clone DX-2; PharMingen), ICAM-1 (clone 84H10; Serotec), LFA-3 (clone BRIC-5; Serotec), or isotype-matched Ab (MOPC-21 and UPC-10). Affinity-purified, FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD) was used as a second-step reagent. Data were expressed as the percentage of specific positive cells and mean fluorescence intensity (MFI) of the gated positive cells. Although anti-Fas clone DX-2 was employed in all flow cytometric analyses illustrated here, similar patterns were observed with anti-Fas clones ZB4 and CH-11 (Immunotech).

Effector/target binding assay. The influence of IFN- γ on effector/target conjugate formation was measured by a flow cytometric-based technique. SW480 or SW620 tumor cells (with or without IFN- γ pretreatment) were prelabeled with a red fluorescent marker (PKH26; 2 μ M/10⁶ cells) (Sigma), while the effector cells were prelabeled with a green fluorescent marker (BCECF; 10 nM/10⁶ cells) (Molecular Probes, Eugene, OR) (as described by the manufacturers). PKH26-prelabeled targets (1 \times 10⁵) were mixed with BCECF-prelabeled CTL (2 \times 10⁵) (in a total volume of 0.2 ml culture medium) in U-bottomed, 12 \times 75 mm polypropylene tubes and gently centrifuged (450 rpm for 2 min) to facilitate cell-cell contact. Cell suspensions were then incubated for 60 min at 37°C in the absence and presence of the indicated Ab. Afterward, cultures were gently resuspended by pipetting and transferred to an appropriate vessel for immediate analysis by flow cytometry for dual expression of BCECF⁺ PKH26⁺ events within the PKH26⁺ gated population. Control preparations included tumor cells 1) incubated in the absence of effectors; 2) incubated with irrelevant effectors (i.e., MART-1 CTL); and 3) mixed with relevant effectors (*ras* CTL), but analyzed immediately without prior incubation. The data were expressed as the mean \pm SEM of triplicate tubes/group.

Induction of FasL expression on CTL

Up-regulation of cell-surface FasL expression on resting CTL (i.e., 5–7 days after *in vitro* culture) was accomplished by stimulation (of 1 \times 10⁶/well) for 2 h at 37°C in 24-well plates (Costar) with either immobilized anti-CD3 mAb (clone HIT3a from PharMingen, 1 μ g/well) or coculture with IFN- γ -pretreated SW480 or SW620 cells (2 \times 10⁵/well). In the case of activation by Ag-bearing carcinoma cells, SW480 or SW620 cells were first plated at a concentration of 2 \times 10⁵ cells/well and then allowed to adhere to wells in the absence or presence of IFN- γ after an overnight incubation at 37°C. Monolayers were gently washed to remove loosely bound or nonadherent tumor cells before the addition of CTL. After TCR activation, CTL were recovered by pipetting. An aliquot was then examined for cell-surface expression of FasL (FITC-conjugated clone NOK-1) by two-color flow cytometry with PE-labeled anti-CD8 mAb (PharMingen), while another aliquot was employed in CTL assays. Greater than 97% of the recovered cells from the CTL-tumor cell cocultures were CD8⁺ T lymphocytes, as determined by flow cytometry.

TUNEL assay

Apoptotic cell death or DNA fragmentation was analyzed by the TUNEL assay, as described (27, 28). Briefly, after incubation at 37°C for 24 h in the absence or presence of CH-11 (29) or an isotype (IgM) control Ab (MOPC-104E; Cappel), tumor cells were washed in saline, fixed with 1% paraformaldehyde (30 min at 4°C), then resuspended and maintained in 70% ethanol for at least 2 h at -20°C. Cells were then washed in saline, aliquoted, and incubated for 30 min at 37°C in 50 μ l of a cacodylate reaction buffer/sample (consisting of 0.2 M potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 2.5 mM cobalt chloride, 0.25 mg/ml BSA, and 0.5 nM biotin-16-dUTP) with or without 100 U/ml TdT (reagents from Boehringer Mannheim, Indianapolis, IN). Afterward, cells were washed with saline and resuspended in 100 μ l of a saline sodium-citrate buffer/sample (supplemented with 5% nonfat dry milk and 0.1% Triton X-100) and streptavidin-FITC (2.5 μ g/ml), followed by incubation for 30 min at room temperature in the dark. Cells were washed again in saline and analyzed immediately by flow cytometry for quantitation of TUNEL⁺ cells.

Tumorigenic assays

Boyden chamber. Cellular penetration through a type IV collagen-precoated membrane filter in a Boyden chamber format was employed as an *in vitro* assay of tumor chemoinvasion, as described (30). Cell lines were retrieved from culture, washed, and, in the case of the anchorage-dependent cell lines (SW480, SW620, nontumorigenic BALB/3T3 fibroblasts from the ATCC, CCL163), reincubated in a culture flask at a subconfluent density for 6 h to promote adherence. Suspension cell lines (Jurkat, EBV-B cells autologous with the anti-*ras* 4-12(Val12) CTL line) were incubated in a parallel set of flasks. Afterward, serum-containing medium was removed by aspiration from monolayers (for adherent cells) or by centrifugation (for suspension cells) and replaced with serum-free medium. After an overnight incubation period under these serum-free conditions, the cells were recovered, washed, and resuspended at 4×10^5 cells/ml in serum-free medium for preparation and use in the Boyden chamber. Despite the absence of serum during this period, viability of all cell lines tested was >95%. Serum-containing culture medium (29 μ l) was employed as a chemoattractant and placed in wells of the lower chamber, which was then covered with a polycarbonate membrane filter (10 μ m pore size, 25 \times 80 mm; Neuroprobe, Gaithersburg, MD) precoated with type IV collagen (25 μ g in 1 ml PBS for 60 min at 37°C) as an extracellular matrix. The various cell lines (2×10^4 in 50 μ l of serum-free medium) were then added to wells of the top chamber (six replicates/sample). The chamber was covered with Parafilm and incubated at 37°C for 16–20 h. After incubation, the filter was collected and stained immediately with Diff-Quik (Dade Behring, Newark, DE). The cells on the upper surface were wiped away with a paper towel to allow the cells that penetrated the opposing surface of the membrane filter to be readily distinguished. The filter was air-dried on a microscope slide, and the cells were counted.

Tumor growth in athymic mice. Female athymic (*nu/nu*) mice on a BALB/c background (National Cancer Institute/Frederick Cancer Research Animal Facility, Frederick, MD), ≥ 6 wk of age, were inoculated s.c. in their right flank with either SW480 or SW620 tumor cells (3×10^6 or 10×10^6 cells/mouse in 0.1 ml HBSS, where indicated). Tumor growth was measured every 2–3 days for up to 30 days by digital caliper in two dimensions, and the volumes were calculated according to the formula of (width² \times length)/2, as described (31). Additionally, the superficial inguinal lymph node cells draining the tumor site were analyzed for tumor cells as a surrogate evaluation of metastatic activity *in vivo*. After the experiment was completed, tumor-draining lymph nodes (TDLN) and contralateral non-TDLN were isolated, and single cell suspensions were prepared from each mouse separately. Individual cell preparations were then stained by indirect immunofluorescence and analyzed by flow cytometry for cell-surface expression of DF3 (MUC-1) (32). Briefly, lymph node cells were treated with a mouse IgG1 anti-human DF3 mAb (kindly provided by Dr. D. Kufe, Dana-Farber Cancer Institute, Boston, MA) in the presence of “Fc Block” (rat anti-mouse CD16/32, clone 2.4G2; PharMingen), followed by a second incubation with PE-conjugated goat anti-mouse IgG1 (Kirkegaard & Perry), as described above. MOPC-21 (IgG1) was used as an isotype control primary Ab.

In vitro selection of Fas-insensitive SW480 cells

SW480 cells were plated at 5×10^5 /T25 flask and allowed to adhere overnight. IFN- γ (250 U/ml) was added the next day, followed 24 h later by CH-11 (1 μ g/ml). When the flasks achieved confluency, cells were recovered and recultured under these same conditions for two additional cycles of IFN- γ plus CH-11. The cells (termed SW480.sel) were then maintained and propagated in the absence of IFN- γ /CH-11 and, after three

further passages, were used in the experiments. SW480 cells derived from culture in the presence of IFN- γ , CH-11, and ZB4 (10 μ g/ml) (termed SW480.sel/ZB4) were included as a control for specificity of CH-11-mediated effects.

Statistical analysis

Where indicated, the data were expressed as the arithmetic mean \pm SEM of three or more independent experiments. Control (or untreated) groups were compared with experimental groups using a two-sided, paired *t* test, with values of *p* < 0.05 considered statistically significant. In the case of the lymph node dissemination assay, experimental groups were compared using a two-sided, unpaired *t* test, with values of *p* < 0.05 considered statistically significant.

Results

In vitro and *in vivo* characteristics of the SW480 and SW620 colon adenocarcinoma cell lines

We first compared the SW480 and SW620 cell lines for potential similarities and differences in several tumorigenic properties. These initial studies also served to substantiate the original description of their malignant nature: i.e., the SW620 cell line, established from a metastatic lesion, would possess enhanced malignant characteristics compared with the SW480 cell line, established from the primary site (19). To that end, we measured 1) chemoinvasive abilities *in vitro* by Boyden chamber (Fig. 1A) and 2) tumor growth with regional lymph node dissemination *in vivo* in an athymic mouse xenograft model (Fig. 1, B and C).

To evaluate chemotactic/invasive potential (i.e., chemoinvasion), SW480 and SW620 cells were compared for their ability to migrate through and penetrate a type IV collagen-coated membrane in a Boyden chamber format (Fig. 1A). The results indicated that both SW480 (*p* < 0.007) and SW620 (*p* < 0.0006) cells displayed a significant ability to penetrate the membrane, when compared with the BALB/3T3 fibroblast cell line as a negative control. However, SW620 cells expressed a higher, statistically significant (*p* < 0.0008) response when compared with SW480 cells. The observation that the Jurkat T cell leukemia line and an EBV-B cell line poorly penetrated the membrane also suggested that migration through the matrix was not a nonspecific consequence under these assay conditions. The magnitude of the responses seen here was consistent with the range of activities reported with other nonmalignant and malignant cell populations (33). It has been proposed that matrix metalloproteinases (MMP)-2 (gelatinase A) and MMP-9 (gelatinase B) play important roles in tumor cell-mediated disruption and invasion of the extracellular matrix (34, 35). In fact, MMP-2 and MMP-9 production has been associated with heightened malignant or metastatic potential in a diversity of human tumors, including colorectal carcinomas (36, 37). Therefore, we examined whether the SW480 and SW620 cell lines differed in their ability to express those activities, as determined by gelatin zymography (34, 35). Analysis of serum-free culture supernatants from SW480 and SW620 cells for MMP-2 and MMP-9 revealed tumor cell-associated quantitative differences. In comparison with SW480 cells, SW620 cells expressed enhanced levels of both gelatinase activities (data not shown).

Next, we examined tumorigenicity in athymic mice in a xenograft model. The results indicated that both tumor cell lines, when compared with each other at either a higher or lower inoculum, proliferated similarly and progressively over the course of the experiments (Fig. 1B), which verified their tumorigenicity in this *in vivo* setting. In addition, we analyzed the inguinal TDLN cells for the presence of tumor cells as a surrogate assessment of metastatic ability *in vivo* (Fig. 1C). TDLN cells, as well as the contralateral lymph node cells that did not drain the tumor site (non-TDLN), were examined for cell-surface expression of DF3 (MUC-1) (32),

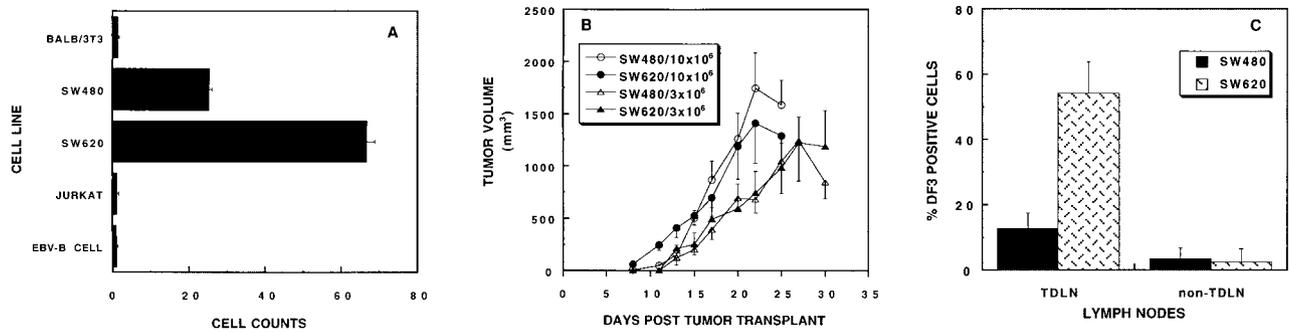


FIGURE 1. Comparison of SW480 to SW620 cells for differences in tumorigenic properties. *A*, Cellular penetration in a Boyden chamber as an *in vitro* assay of chemoinvasion, as described in *Materials and Methods*. After incubation, the cells that penetrated the membrane filter were enumerated under the microscope. Data were expressed as the mean \pm SEM of five separate experiments. *B*, Tumor growth in athymic mice as a measure of tumorigenicity. Athymic mice were inoculated with either SW480 or SW620 tumor cells (10×10^6 or 3×10^6 cells/mouse in experiments 1 and 2, respectively). Data were expressed as the mean \pm SEM of five and four mice/group at each time point in experiments 1 and 2, respectively. *C*, Tumor cell migration to draining lymph nodes as a surrogate assessment of metastatic ability. At the end of experiment 2 (by day 30), TDLN and the contralateral non-TDLN were analyzed by flow cytometry for cell-surface expression of DF3. Staining with MOPC-21 as an isotype-matched primary Ab (range, 3–11%) was subtracted from the values shown. Data were expressed as the mean \pm SEM of four mice/tumor-bearing group. Similar patterns were observed by intracellular staining of human cytokeratin, which distinguishes normal from malignant cells (not shown).

a tumor-associated Ag found on colon adenocarcinomas and other human carcinomas (4). In control experiments, nearly 100% of both SW480 and SW620 populations were found to express DF3, whereas $<5\%$ of the lymph node cells from normal (non-tumor-bearing) athymic mice were reactive with anti-DF3 mAb.

Despite the fact that tumor volume was comparable between both tumor-bearing groups at the time of analysis (Fig. 1*B*), the results indicated that a significantly ($p < 0.01$) higher percentage of DF3⁺ cells were found within TDLN of mice bearing SW620 cells compared with those of SW480 cells (Fig. 1*C*). In contrast, the DF3⁺ cells from the non-TDLN of mice bearing SW480 cells were similar to those bearing SW620 cells, but lower than those from the corresponding TDLN, particularly for the SW620 group ($p < 0.006$). The observation that the percentage of DF3⁺ cells from TDLN of SW480-bearing mice was higher, albeit not significantly ($p = 0.209$), than those from their non-TDLN counterparts suggested the possibility for weak tumor cell migration to regional

lymph nodes. Colony formation assays *in vitro* substantiated the patterns observed by flow cytometry (data not shown). These results affirmed not only the tumorigenicity of SW480 and SW620 cells *in vivo* (in athymic mice), but also potential disparities in their metastatic capacity, as determined using regional lymph node dissemination as a surrogate endpoint.

Role of Fas in CTL-mediated lysis of SW480 and SW620 colon adenocarcinoma cell lines

We compared the lytic sensitivity of SW480 and SW620 cell lines, before and after treatment with IFN- γ , to CD8⁺ CTL restricted by HLA-A2 and specific for the mutant *ras* epitope sequence 4–12 containing Val at position 12 (Fig. 2*A*). In the absence of IFN- γ pretreatment, both tumor cell lines were weakly susceptible to Ag-specific CTL, although it appeared that SW620 cells displayed a

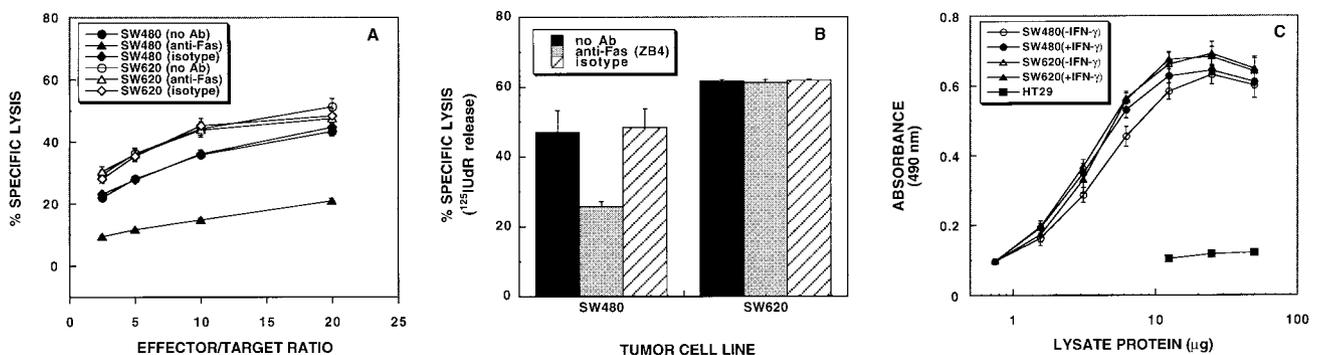


FIGURE 2. Fas-dependent and Fas-independent pathways in CTL-mediated lysis of IFN- γ -pretreated SW480 and SW620 cells. *A*, CTL-mediated lysis of IFN- γ -pretreated SW480 or SW620 cells was measured by ^{51}Cr release at multiple E:T ratios (in a 12-h assay; spontaneous release of ^{51}Cr for all targets was $\leq 13\%$). Assays were also conducted in the absence and presence of anti-Fas mAb, clone ZB4 ($10 \mu\text{g/ml}$). Because of the size or overlap of symbols, error bars may be partly masked. Percent specific lysis of untreated SW480 cells at E:T ratios of 20:1 and 5:1, 8 ± 4 and 4 ± 1 ; for untreated SW620 cells, 22 ± 3 and 13 ± 1 . *B*, IFN- γ -pretreated SW480 or SW620 cells were radiolabeled with [^{125}I]IUdR and tested against anti-*ras* CTL (E:T ratio, 10:1; 12 h assay) to determine the extent of nuclear damage. Assays were also conducted in the absence and presence of Ab, as in *A*. Data in *A* and *B* were expressed as the mean \pm SEM of triplicate cultures and are representative of five and four separate experiments, respectively. *C*, Effect of IFN- γ pretreatment on expression of the mutant *ras* Val12 determinant in SW480 and SW620 cells. Cell lysates were collected from tumor cells, as shown, and then assayed in titrating amounts in a mutant *ras* Val12-specific ELISA. Data were expressed as the mean OD (490 nm) \pm SEM of five separate experiments.

Table I. Phenotypic comparison of SW480 to SW620 colon carcinoma cells before and after IFN- γ pretreatment^a

Tumor Cell Line	IFN- γ Pretreatment	mAb Directed Against % Positive Cells (MFI)			
		HLA-A2	ICAM-1	LFA-3	Fas
SW480	Untreated	72 \pm 8 (68 \pm 7)	39 \pm 6 (42 \pm 6)	96 \pm 0.4 (114 \pm 9)	13 \pm 4 (30 \pm 2)
	Pretreated	97 \pm 1 (544 \pm 32)	98 \pm 1 (289 \pm 35)	96 \pm 0.4 (90 \pm 9)	65 \pm 4 (41 \pm 3)
SW620	Untreated	96 \pm 1 (100 \pm 10)	53 \pm 8 (35 \pm 5)	97 \pm 0.4 (92 \pm 5)	2 \pm 0.4 (18 \pm 2)
	Pretreated	97 \pm 0.4 (499 \pm 23)	95 \pm 1 (214 \pm 26)	96 \pm 1 (70 \pm 3)	15 \pm 4 (20 \pm 1)

^a Tumor cells, either untreated or pretreated with IFN- γ , were evaluated by flow cytometry for cell-surface expression of the indicated molecule. Data were expressed as the mean \pm SEM of the percentage positive cells and MFI (in parentheses) of 8 (for HLA-A2, ICAM-1), 4 (for LFA-3), and 12 (for Fas) separate experiments. Staining with the appropriate isotype control Ab, which ranged from 2 to 10%, was subtracted from the values shown above. Data in bold type indicate statistically significant responses, based on comparison with the corresponding untreated preparations. For SW480 cells: HLA-A2: percentage positive cells, $p < 0.02$; MFI, $p < 0.0001$; ICAM-1: percentage positive cells, $p < 0.0001$; MFI, $p < 0.0001$; Fas: percentage positive cells, $p < 0.0001$. For SW620 cells: HLA-A2: MFI, $p < 0.0001$; ICAM-1: percentage positive cells, $p < 0.001$; MFI, $p < 0.001$; Fas: percentage positive cells, $p < 0.005$.

higher degree of lytic sensitivity (Fig. 2A). Following IFN- γ pretreatment, our results indicated that these anti-*ras* 4–12(Val12) epitope-specific CTL lysed both SW480 and SW620 cell lines with comparable lytic efficiency at multiple E:T ratios (Fig. 2A). CTL-mediated lysis of IFN- γ -pretreated SW480 and SW620 tumor cells was effector cell-specific, as determined by using HLA-A2-restricted, CD8⁺ CTL lines specific for epitopes of unrelated tumor (melanoma/MART-1) or viral (influenza) antigenic systems. Under these assay conditions, lytic activity expressed by these control CTL lines was observed only in the presence of their appropriate exogenous peptide (data not shown).

Although the overall magnitude of lysis between the two tumor cell lines was similar, the mechanisms leading to cell death appeared to be different. Using neutralizing anti-Fas mAb, lysis of IFN- γ -pretreated SW480 cells, but not SW620 cells, was effectively inhibited, thereby supporting the presence and absence of a Fas-dependent component, respectively (Fig. 2A). The inhibition observed against SW480 cells with anti-Fas mAb was specific, because isotype-matched Ab failed to block the lytic response (Fig. 2A). The observations that anti-Fas mAb (at an optimal dose of 10 μ g/ml, based on inhibition of Ag-specific CTL-mediated lysis of IFN- γ -pretreated SW480 or SW620 cells in mAb titration experiments) failed to block lysis of SW620 cells and incompletely blocked lysis of SW480 cells suggested that cytotoxicity also proceeded through Fas-independent mechanisms—a premise that is characterized further in subsequent experiments. We also examined whether CTL-mediated lysis of IFN- γ -pretreated SW480 and SW620 cells was associated with the induction of a target nuclear lesion and whether that process was Fas dependent. To that end, we measured lytic activity against IFN- γ -pretreated SW480 and SW620 cells, which had been prelabeled with ¹²⁵IUdR as an isotopic marker for potential damage to the nuclear compartment (Fig. 2B). Our findings revealed similar lytic patterns, as well as a similar degree of lytic inhibition by anti-Fas mAb, to that observed by ⁵¹Cr release (Fig. 2, B vs A). These data demonstrated the capacity of these anti-*ras* 4–12(Val12)-specific CD8⁺ CTL to recognize two colon adenocarcinoma cell lines endogenously expressing antigenic *ras* epitopes, and the likelihood that lysis (as determined by either ⁵¹Cr or ¹²⁵IUdR release) of these two targets appeared to involve, at least in part, distinct lytic mechanisms.

Phenotypic and functional differences in Fas expression by SW480 and SW620 colon adenocarcinoma cell lines

Phenotypic analysis indicated that IFN- γ pretreatment of both SW480 and SW620 cell lines led to enhanced cell-surface expression of HLA-A2, ICAM-1, and Fas molecules, as determined by changes in the percentage of positive cells and/or MFI as a relative estimation of ligand density (Table I). Furthermore, although both cell lines expressed comparable amounts of HLA-A2 and ICAM-1 molecules, the proportion of SW480 cells positive for Fas expression after IFN- γ pretreatment was >4-fold higher compared with SW620 cells, which is consistent with a more dominant role of Fas in lysis of IFN- γ -pretreated SW480 cells. Additionally, SW480 cells were compared with SW620 cells for expression of the mutant *ras* Val12 determinant, before and after treatment with IFN- γ (Fig. 2C). The results indicated that lysates isolated from both tumor cell lines, when compared with each other in a titratable fashion, expressed equivalent amounts of the mutant *ras* Val12 determinant. Furthermore, treatment with IFN- γ under these conditions did not appreciably alter mutant *ras* Val12 epitope expression when the cell lines were compared with each other or to the respective untreated preparations (Fig. 2C). In contrast, no specific immune reactivity was observed using lysates from the HT-29 colon carcinoma cell line, which was employed as a negative control because it contains the wild-type Gly residue at position 12 (26). These observations suggested that IFN- γ -induced modulation of their lytic phenotype did not appear to correlate with *ras* Val12 epitope production, as determined qualitatively by this ELISA.

Functional Fas expression was analyzed in response to agonistic anti-Fas mAb (clone CH-11), recombinant human soluble FasL (sFasL), or preactivated FasL-bearing (melanoma/MART-1-reactive) CTL as stimuli for Fas engagement (Figs. 3 and 4). Pretreatment of SW480 cells with IFN- γ enhanced or restored their sensitivity to Fas relative to the untreated SW480 cells, as determined by their lytic susceptibility to CH-11 (Fig. 3, A and C) or sFasL (Fig. 3B) in a dose-dependent fashion, as well as to anti-CD3 mAb preactivated FasL-expressing CTL (Fig. 3C). Jurkat cells were employed as a Fas-sensitive positive control and were readily lysed under these conditions (Fig. 3). Additional controls included an isotype-matched Ab for CH-11, which was unable to induce lysis of Jurkat or IFN- γ -pretreated SW480 cells. In contrast to what was observed with SW480 cells, pretreatment of SW620 cells with

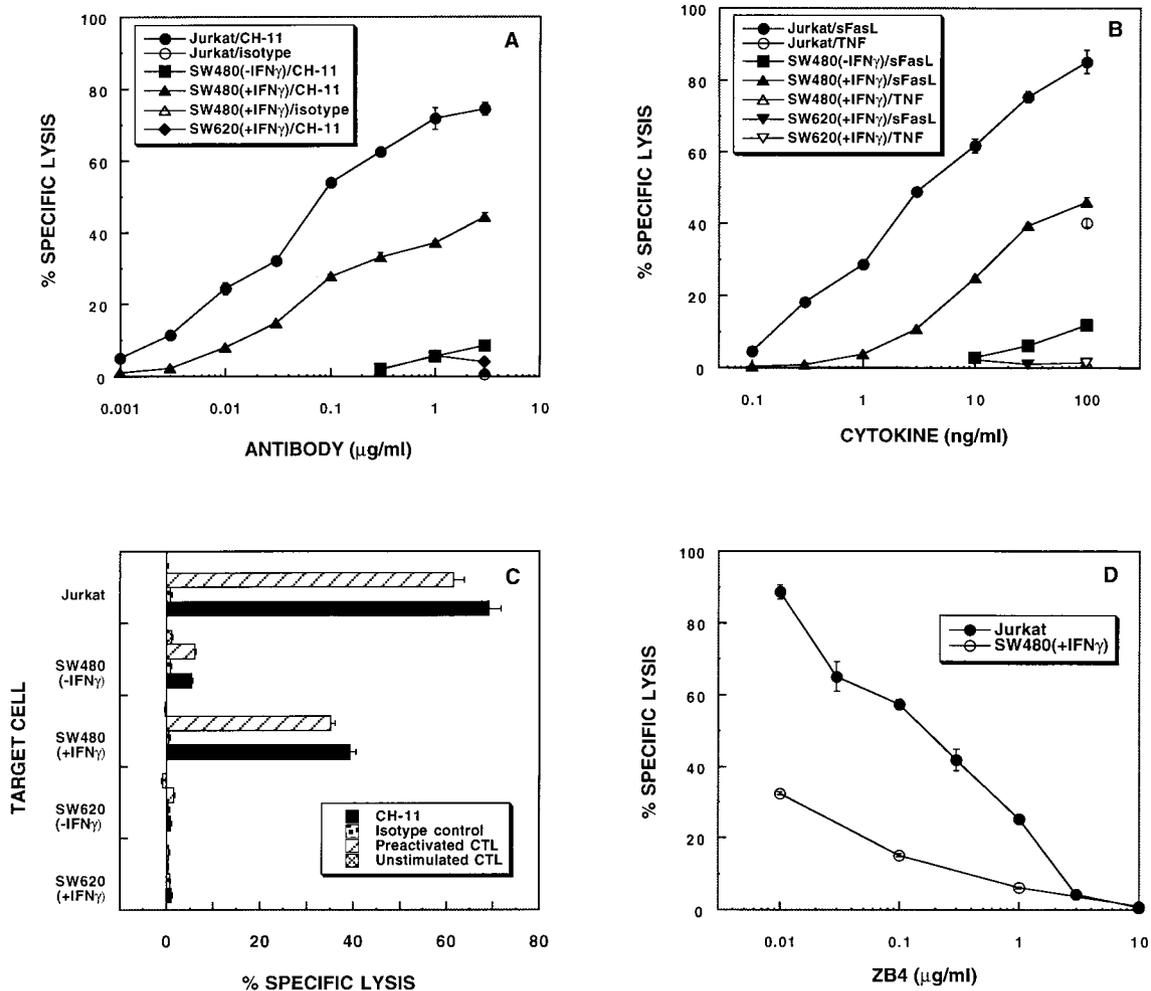


FIGURE 3. Comparison of SW480 to SW620 cells for functional Fas expression. *A*, Tumor cells, either untreated ($-IFN-\gamma$) or pretreated with $IFN-\gamma$ ($+IFN-\gamma$), were incubated with CH-11 or an isotype-matched Ab (at the highest concentration tested, $3 \mu\text{g/ml}$). Cell death was measured by ^{51}Cr release (18 h assay). *B*, In parallel, these same tumor cell lines were incubated with sFasL or $TNF-\alpha$ (at the highest dose tested, 100 ng/ml ; $\sim 1000 \text{ U/ml}$). The extent of CH-11 (*A*)- or sFasL (*B*)-induced lysis of SW620 cells was the same with or without $IFN-\gamma$ pretreatment. Also, in *A* and *B*, Jurkat cells served both as a Fas- and TNF -sensitive positive control. *C*, Anti-MART-1₂₇₋₃₅-specific CTL were incubated either in the absence ("unstimulated") or presence ("pre-activated") of immobilized anti-CD3 mAb. Afterward, one aliquot was analyzed for FasL expression by flow cytometry, while a second aliquot was used as effectors in ^{51}Cr release assays against the indicated target cells (E:T ratio, 10:1). CH-11 or isotype ($1 \mu\text{g/ml}$) used as in *A*. *D*, In additional control experiments, clone ZB4 was tested at multiple concentrations for potency, based on its ability to inhibit CH-11 ($1 \mu\text{g/ml}$)-mediated lysis of Jurkat and $IFN-\gamma$ -pretreated SW480 cells. Percent specific lysis of Jurkat and $IFN-\gamma$ -pretreated SW480 cells with CH-11, but without ZB4, 94 ± 5 and 48 ± 2 , respectively. Data in all panels were expressed as the mean \pm SEM of triplicate cultures and are representative of four separate experiments for *A*–*C* and two separate experiments for *D*.

$IFN-\gamma$ did not render them Fas sensitive (Fig. 3), despite the increase in the proportion of cells positive for Fas receptor expression (see Table I). Furthermore, the relative contribution of the Fas pathway in Ag-specific CTL-mediated lysis of these two targets (Fig. 2) correlated with their responsiveness to Fas-mediated cytotoxicity using CH-11-, sFasL-, or FasL-expressing effectors (Fig. 3). In contrast to Jurkat cells, which were also lytically sensitive to $TNF-\alpha$ (Fig. 3*B*) or $TNF-\beta$ (data not shown), both SW480 and SW620 cells, whether untreated or pretreated with $IFN-\gamma$, remained lytically resistant to those cytokines. The potency of ZB4 was confirmed, based on its ability to inhibit CH-11-mediated lysis of Jurkat cells and $IFN-\gamma$ -pretreated SW480 cells in a dose-dependent fashion (Fig. 3*D*). Moreover, similar to what was observed by ^{51}Cr release (Fig. 3, *A* and *C*), pretreatment of SW480 cells with $IFN-\gamma$, but not SW620 cells, led to enhanced Fas-mediated apoptosis in response to CH-11, as determined by TUNEL staining (Fig. 4, *A* and *B*). Jurkat cells were included as a Fas-sensitive

control and to verify specificity of CH-11-mediated apoptosis in this assay format (Fig. 4*C*).

Effector mechanisms involved in CTL-mediated lysis of SW480 and SW620 colon adenocarcinoma cell lines

Next, we examined the role of TCR-MHC/peptide and Ag-independent adhesion events in anti-*ras* Val12 CTL-mediated lysis of $IFN-\gamma$ -pretreated SW480 and SW620 cells using mAb directed against those cell-surface interactions (Fig. 5). We showed that mAb directed against HLA-A2, ICAM-1, and nonpolymorphic epitopes of the $\alpha\beta$ -TCR, but not LFA-3 (data not shown), strongly abrogated lysis of both targets (Fig. 5*A*), indicating that $IFN-\gamma$ -induced modulation of the lytic phenotype (of both targets) involved engagement of both Ag-specific (TCR-MHC/peptide) and Ag-independent (ICAM-1) signals. These experiments also affirmed that CTL-mediated lysis of both $IFN-\gamma$ -pretreated targets was HLA-A2 restricted. The observations that pretreatment of both

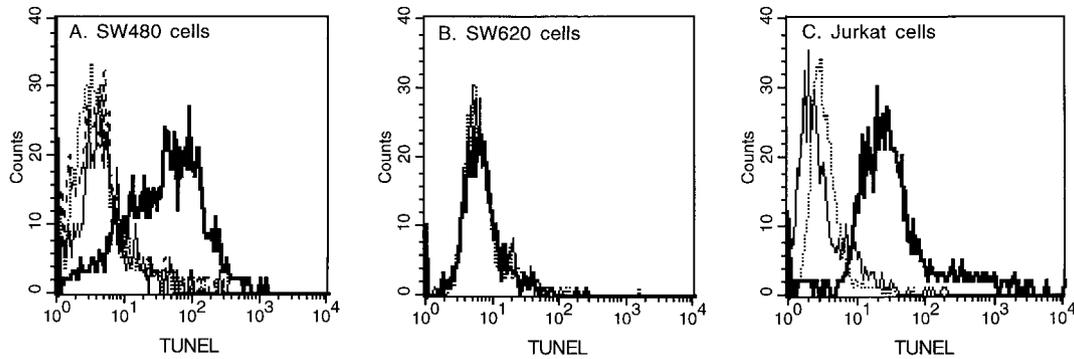


FIGURE 4. Apoptotic cell death induced by agonistic anti-Fas mAb. IFN- γ -pretreated SW480 (A) or SW620 (B) cells were incubated without (dashed line) or with (solid thick line) CH-11 (1 μ g/ml). Solid thin line represents untreated tumor cells incubated with CH-11. (C) Jurkat cells were incubated without (solid thin line) or with CH-11 (solid thick line). After a 24-h incubation, all tumor cell populations were analyzed for apoptotic death by the TUNEL assay. Dotted line in all panels denotes tumor cells (Jurkat or IFN- γ -pretreated SW480 or SW620 cells) cultured with CH-11 but analyzed by the TUNEL assay without the enzyme. Data are representative of four separate experiments.

SW480 and SW620 cells with IFN- γ led to enhanced HLA-A2 and ICAM-1 expression, and that mAb directed against those molecules also inhibited lysis suggested the possibility that IFN- γ may have influenced conjugate formation. To that end, we examined the effect of IFN- γ pretreatment on effector/target binding by two-color flow cytometry (Table II). Following IFN- γ pretreatment, there was a substantial increase in the frequency of double-positive cell complexes, as a measurement of binding or adhesion capacity between CTL and SW480 or SW620 tumor targets. Such binding activity was effector cell specific, because the frequency of double-positive cell complexes formed between anti-MART-1 CTL and IFN- γ -pretreated SW480 or SW620 tumor targets was similar to the control preparations (Table II) and did not rise compared with those formed with anti-*ras* CTL. Anti-ICAM-1 mAb, but not an isotype control, abrogated the formation of double-positive cell complexes (Table II), which was consistent with its ability to inhibit lytic activity (Fig. 5A), further supporting the involvement of

ICAM-1-based interactions in effector/target binding and the lytic process.

Moreover, the use of anti-Fas mAb and CMA, a compound previously reported to potently inhibit perforin-based cytotoxicity (20, 21), helped to elucidate further the nature of the cytolytic pathway(s) used against the primary and metastasis-derived tumor cell lines. The results indicated that anti-Fas mAb inhibited lysis of IFN- γ -pretreated SW480 cells by >50%, with a marginal effect observed against IFN- γ -pretreated SW620 cells that appeared similar to the isotype Ab control (Figs. 2 and 5B). Nontoxic doses of CMA inhibited lysis of IFN- γ -pretreated SW480 cells by nearly 50%, while it blocked essentially the entire lytic response against IFN- γ -pretreated SW620 cells (Fig. 5). The lytic inhibition patterns observed against both targets using anti-Fas mAb were similar to those observed with anti-FasL mAb (Fig. 5B), providing further evidence for the presence or absence of functional Fas/FasL interactions in the mechanism of lysis of IFN- γ -pretreated

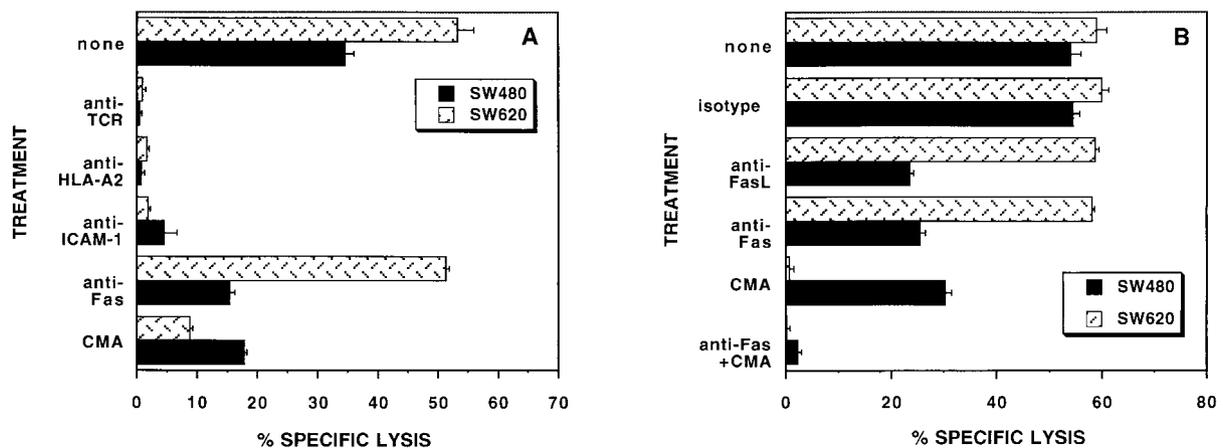


FIGURE 5. Ag-specific CTL-mediated lysis of IFN- γ -pretreated SW480 and SW620 cells involves distinct effector mechanisms. A, Cytotoxicity against IFN- γ -pretreated tumor cells (E:T ratio, 10:1) was assayed in the presence of the indicated blocking mAb. Isotype-matched Ab had no significant inhibitory effect (not shown). Assays were also conducted in the presence of CMA (10 μ M). B, In a separate experiment, cytotoxicity against IFN- γ -pretreated tumor cells was assayed in the presence of anti-FasL mAb, anti-Fas mAb, CMA, or a combination of both anti-Fas mAb and CMA. Ab, 10 μ g/ml; CMA (10 μ M). Data in A and B were expressed as the mean \pm SEM of triplicate cultures and are representative of four separate experiments. CMA (at an optimal inhibitory dose of 10 μ M, based on titration experiments) did not alter viability of either cell population, as determined by trypan blue dye exclusion and spontaneous release of 51 Cr from radiolabeled targets. The inhibitory effect was at the level of the effector cell, because pretreatment of the CTL, but not of the target cells, resulted in reduction of lysis (not shown).

Table II. Effect of IFN- γ pretreatment of SW480 and SW620 colon carcinoma cells on effector cell-target cell conjugate formation

Tumor Cell Line	IFN- γ (+/-) ^a	CTL ^b	Ab ^c	% Conjugates ^d
SW480	-	<i>ras</i>	None	4.4 \pm 0.03
	-	MART-1	None	4.9 \pm 0.03
SW480	+	<i>ras</i>	None	35.4 \pm 0.6
	+	<i>ras</i>	Anti-ICAM-1	4.5 \pm 0.2
	+	<i>ras</i>	Isotype	35.2 \pm 1.8
	+	MART-1	None	4.2 \pm 0.1
SW620	-	<i>ras</i>	None	5.7 \pm 0.3
	-	MART-1	None	2.9 \pm 0.9
SW620	+	<i>ras</i>	None	30.6 \pm 1.4
	+	<i>ras</i>	Anti-ICAM-1	5.2 \pm 0.8
	+	<i>ras</i>	Isotype	38.1 \pm 1.4
	+	MART-1	None	3.9 \pm 0.5

^a Tumor cells, untreated (-) or pretreated (+) with IFN- γ , as described in Table I and Fig. 2, were then labeled with a red fluorescent marker (PKH26).

^b Anti-*ras* 4-12(Val12) CTL or anti-MART-1₂₇₋₃₅ CTL were pre-labeled with a green fluorescent marker (BCECF).

^c Anti-ICAM-1 or isotype control (MOPC-21) (10 μ g/ml) were included in the assay.

^d After incubation, cell suspensions (E:T ratio, 2:1) were immediately analyzed by flow cytometry for dual expression of BCECF⁺ PKH26⁺ events within the PKH26⁺ population. Additional controls included tumor cells 1) incubated in the absence of effectors; and 2) mixed with relevant effectors (*ras* CTL), but analyzed immediately without prior incubation. Under those conditions, two-color staining was <4%. The data were expressed as the mean \pm SEM of triplicate tubes/group and are representative of three separate experiments.

SW480 and SW620 cells, respectively. Lastly, the combination of anti-Fas mAb plus CMA completely abolished lysis of IFN- γ -pretreated SW480 cells, whereas CMA alone was sufficient to abrogate lysis of IFN- γ -pretreated SW620 cells (Fig. 5B).

To determine whether the nature and spectrum of anti-carcinoma effector mechanisms mediated by these anti-*ras* 4-12(Val12)-specific CTL were either unique to this particular line or more representative in scope of the antitumor CTL response, we examined the lytic properties of a second, independently isolated, HLA-A2-restricted, anti-*ras* CD8⁺ CTL line. This particular CTL line was previously established from a patient with metastatic duodenal carcinoma in a mutant *ras* peptide-based phase I clinical trial, whose primary tumor contained the Val12 mutation (17). This CTL line displayed specificity for a closely related epitope (i.e., *ras* sequence 5-14 with Val at position 12) and exhibited efficient lytic activity against IFN- γ -pretreated, but not untreated, SW480 cells (17). Using this anti-*ras* 5-14(Val12)-specific CTL line, we then

explored the role of Fas-dependent and Fas-independent mechanisms in lysis of IFN- γ -pretreated SW480 and SW620 cells using anti-Fas mAb and CMA (Fig. 6), as described above (Figs. 2 and 5). Thus, based on the relative contributions of anti-Fas mAb and CMA toward inhibition of cytotoxicity by the anti-*ras* 5-14(Val12)-specific CTL, we found that lysis of IFN- γ -pretreated SW480 cells involved both Fas- and perforin-based components, whereas lysis of IFN- γ -pretreated SW620 cells predominantly involved a perforin-based mechanism (Fig. 6).

The observations that both anti-*ras* CTL lines resembled each other in lytic ability supported the notion that the nature of the anti-carcinoma CTL response may not necessarily reflect unique functional properties of the effector cells; instead, the response may suggest rather unique, intrinsic characteristics of the target cells that ultimately determine their susceptibility to the available range of immune effector mechanisms. To support that concept, and to exclude the possibility that the inability of both anti-*ras*

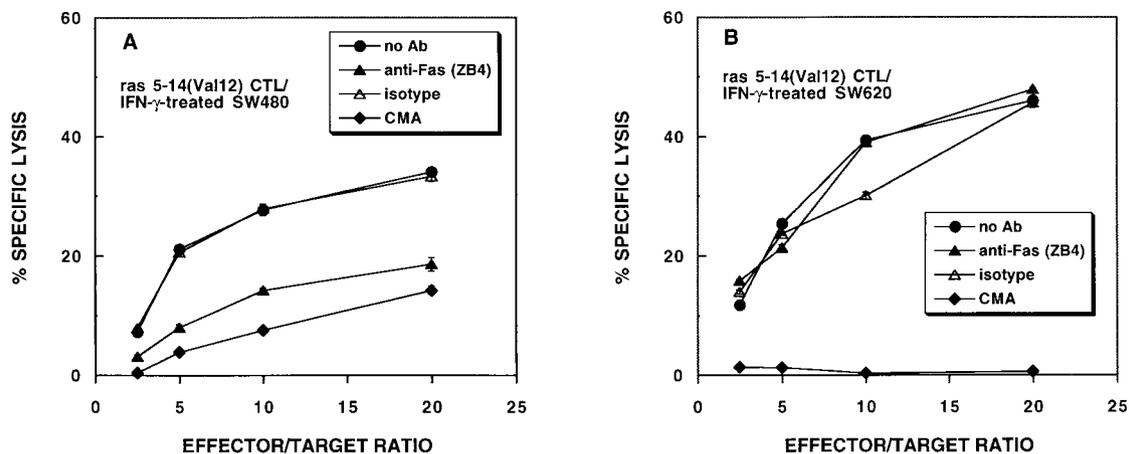


FIGURE 6. IFN- γ -pretreated SW480 and SW620 cells were lysed by a second, independently isolated, HLA-A2-restricted, anti-*ras* Val12-specific CD8⁺ CTL line. Using a second anti-*ras* Val12-specific CD8⁺ CTL line, the nature and spectrum of cytotoxic mechanisms used against Ag-bearing, IFN- γ -pretreated SW480 and SW620 cells was determined as in Figs. 2 and 5. ⁵¹Cr release assays were conducted in the absence ("no Ab") and presence of ZB4 or an isotype control, or CMA to determine the role of Fas-dependent and Fas-independent pathways in Ag-specific tumor cell killing of SW480 (A) or SW620 (B) tumor cells. Data in both panels were expressed as the mean \pm SEM of triplicate cultures and are representative of four separate experiments.

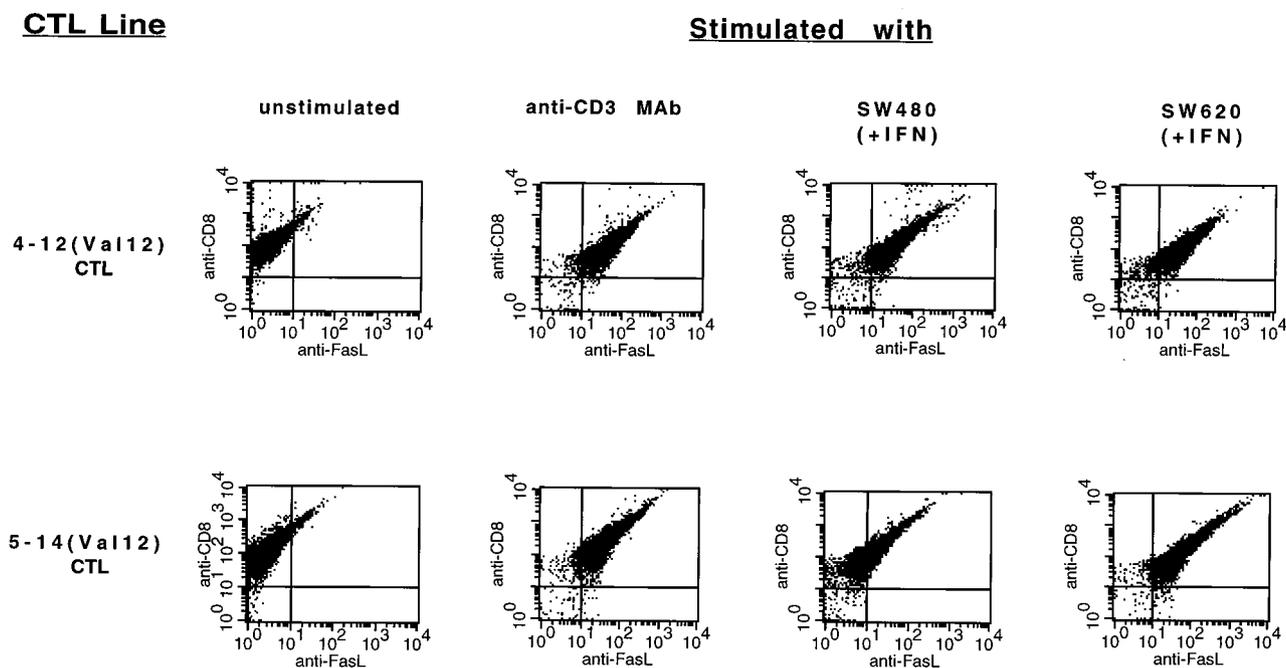


FIGURE 7. Induction of FasL expression on anti-*ras* Val12 CD8⁺ CTL lines after TCR stimulation. Anti-*ras* 4–12(Val12)-specific CTL and anti-*ras* 5–14(Val12)-specific CTL were stimulated *in vitro* with immobilized anti-CD3 mAb or adherent IFN- γ -pretreated SW480 or SW620 cells. Parallel CTL cultures incubated in the absence of anti-CD3 mAb or tumor cells were characterized as unstimulated. Nonadherent cell suspensions were then analyzed for cell-surface expression of both CD8 and FasL markers. Data are representative of three separate experiments.

Val12-specific CTL lines to lyse IFN- γ -pretreated SW620 cells via Fas/FasL interactions was due to the failure of these targets to trigger FasL up-regulation, we examined these CTL effectors for FasL expression before and after interaction with Ag-bearing tumor cells or anti-CD3 mAb as a positive control stimulus (Fig. 7). The results indicated that resting CTL expressed low levels of FasL before any TCR activation. In contrast, following stimulation of these CTL lines with either immobilized anti-CD3 mAb or IFN- γ -pretreated, Ag-bearing SW480 or SW620 tumor cells, we observed a substantial increase in the proportion of CD8⁺ FasL⁺ cells recovered from (co)culture (Fig. 7). FasL expression by both preactivated CTL lines was functional, as determined by lysis of Jurkat cells that was inhibitable by neutralizing anti-Fas mAb (data not shown). These findings not only provided phenotypic evidence for FasL up-regulation by these CTL lines in response to Ag-specific stimulation, but also indicated that the inability of IFN- γ -pretreated SW620 cells to be killed, in part, via a Fas-dependent pathway was not due to the failure of these targets to trigger functional FasL expression on the effector cell surface.

Effect of 5-FU on Fas expression and CTL-mediated lysis of SW480 and SW620 colon adenocarcinoma cell lines

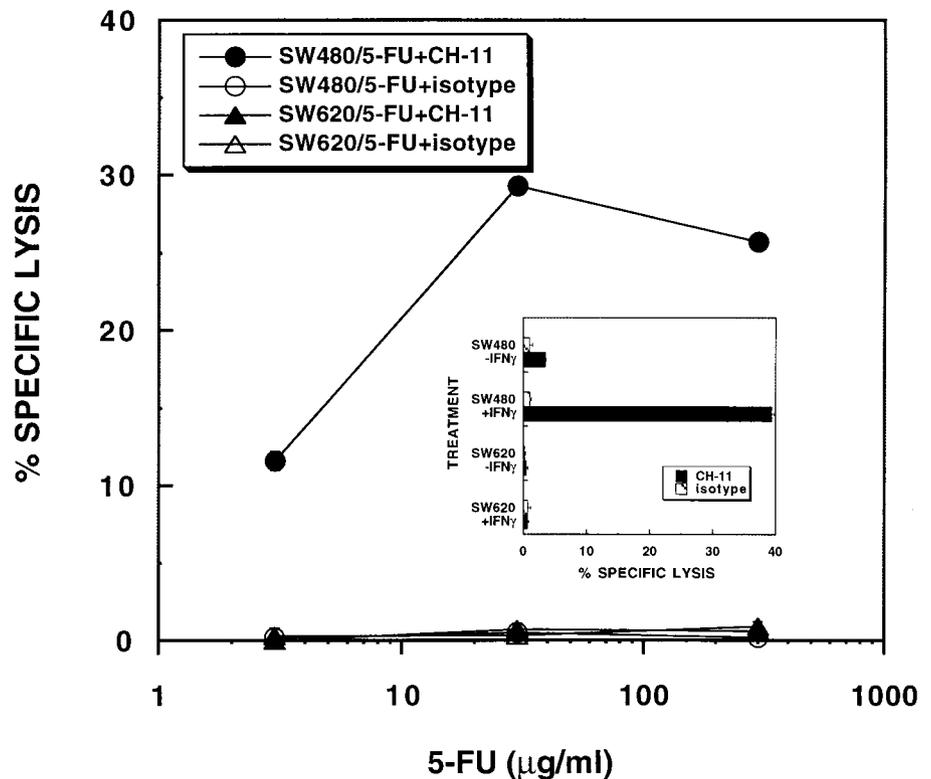
It has been proposed that certain anticancer chemotherapeutic agents, such as 5-FU, doxorubicin or cisplatin, may influence cell surface and/or functional Fas expression on susceptible tumor cells and that the increased sensitivity to Fas engagement may account, in part, for the cytotoxic activity of those drugs (38–40). Interestingly, the acquisition of tumor cell resistance to such chemotherapeutic agents has also been associated with the onset of resistance to Fas-mediated apoptosis (14–16), which may play an integral role in tumor progression from primary to metastatic disease. The observations that SW480 and SW620 cell lines differed markedly in cell-surface expression of Fas receptors and/or functional responsiveness to Fas engagement introduced the hypothesis that

chemotherapy-induced modulation of the Fas death phenotype in susceptible tumor cells may subsequently render those cells susceptible to Ag-specific CTL attack involving that pathway.

First, we showed that incubation of SW480 cells with 5-FU enhanced the sensitivity of these cells in a dose-dependent fashion to Fas-mediated cytotoxicity in response to coculture with CH-11 (Fig. 8). In the absence of CH-11 or in the presence of an isotype control Ab, no demonstrable lysis was observed. The effect of CH-11 on IFN- γ -pretreated SW480 was included as a positive control for comparison (Fig. 8, insert). These observations revealed that the functional effect achieved with 5-FU at the highest dose tested was >65% of that attained with IFN- γ -pretreated SW480 cells. In contrast to what was observed with SW480 cells, SW620 cells under these same treatment conditions remained resistant to the cytotoxic effects of CH-11 (Fig. 8).

The observation that only SW480 cells became sensitive to CH-11 in the presence of 5-FU implied potential up-regulation of cell-surface Fas receptors. Therefore, we examined cell-surface expression of Fas, as well as HLA-A2 and ICAM-1. We found that 5-FU treatment of SW480 cells at both 3 μ g/ml ($18 \pm 6\%$; $p < 0.03$) and 30 μ g/ml ($16 \pm 4\%$; $p < 0.007$) significantly augmented the percentage of cells expressing Fas receptors relative to the untreated controls ($8 \pm 1\%$; in five separate experiments). Additionally, 5-FU did not increase the percentage of SW480 cells positive for HLA-A2, but did so significantly for ICAM-1 at both 3 μ g/ml ($67 \pm 6\%$; $p < 0.002$) and 30 μ g/ml ($63 \pm 14\%$; $p < 0.02$) relative to the untreated controls ($38 \pm 8\%$; in five separate experiments). The increase in ICAM-1 (particularly MFI) and Fas expression on SW480 cells was not as dramatic as the effect induced by IFN- γ (refer to Table I, for example). In contrast to what was observed with SW480 cells, 5-FU at either 3 μ g/ml ($0 \pm 2\%$) or 30 μ g/ml ($0 \pm 1\%$) did not augment cell-surface expression of Fas receptors on SW620 cells ($3 \pm 2\%$). Also, 5-FU at either dose had no demonstrable enhancing effect on HLA-A2 expression and

FIGURE 8. Effect of 5-FU treatment on susceptibility of SW480 and SW620 cells to Fas-mediated lysis by agonistic anti-Fas mAb. SW480 and SW620 cells were pretreated with 5-FU at multiple concentrations and recultured with CH-11 or an isotype control Ab (MOPC-104E) (each at 1 $\mu\text{g}/\text{ml}$). Untreated ($-\text{IFN-}\gamma$) and $\text{IFN-}\gamma$ -pretreated tumor cells (in the absence of 5-FU pretreatment) were examined at the same time for comparison (see insert). Cell death was determined by ^{51}Cr release assays (18 h assay). 5-FU pretreatment alone, at the concentrations shown, did not alter SW480 or SW620 cellular viability based on trypan blue dye exclusion and spontaneous release of ^{51}Cr relative to the untreated controls. Data were expressed as the mean \pm SEM of triplicate cultures and are representative of four separate experiments.



only a marginal effect at 3 $\mu\text{g}/\text{ml}$ ($43 \pm 5\%$) and 30 $\mu\text{g}/\text{ml}$ ($45 \pm 11\%$) on ICAM-1 expression by SW620 cells ($40 \pm 11\%$).

Because we found that 5-FU sensitized SW480 cells to Fas-mediated lysis, which was also correlated, in part, with the up-regulation of Fas receptors, we then examined the susceptibility of these 5-FU-pretreated targets to Ag-specific CTL through that pathway (Fig. 9). Accordingly, SW480 and SW620 cells were pretreated with 5-FU at two concentrations, based on an optimal and suboptimal response to CH-11 (Fig. 8). The results indicated that 5-FU-pretreated SW480 cells became sensitive to CTL-mediated lysis, which was apparent at the higher dose but still detectable at the lower dose relative to the untreated cells. The inclusion of ZB4 in the lytic assay resulted in $\geq 50\%$ inhibition of lysis (Fig. 9B), confirming a functional role of Fas/FasL interactions in the mech-

anism of tumor cell killing induced by 5-FU pretreatment. An isotype-matched control Ab failed to inhibit lysis. The observation that CMA could block lysis also by $\geq 50\%$ suggested that the remainder of the CTL lytic response was mediated by a perforin-based mechanism (Fig. 9B). In contrast to what was observed with 5-FU-pretreated SW480 cells, pretreatment of SW620 cells with 5-FU (at either dose) did not further sensitize them to CTL-mediated lysis relative to the untreated cells (Fig. 9C). However, $\text{IFN-}\gamma$ pretreatment of SW620 cells enhanced their susceptibility to CTL-mediated lysis. Thus, 5-FU-pretreated SW480 cells displayed heightened susceptibility to CTL-mediated lysis as compared with 5-FU-pretreated SW620 cells (Fig. 9, A vs C at 30 $\mu\text{g}/\text{ml}$). Lastly, lysis of 5-FU-pretreated SW480 or SW620 cells by anti-*ras* Val12 CTL was shown to be effector cell specific, because an irrelevant

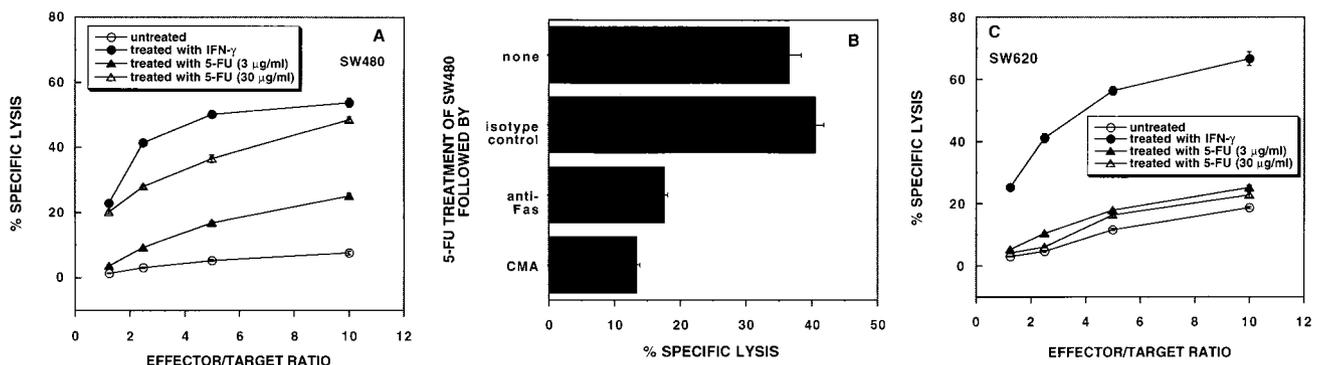


FIGURE 9. 5-FU sensitizes SW480, but not SW620, to Fas-mediated cytotoxicity in response to Ag-specific CTL. **A**, SW480 cells were pretreated with $\text{IFN-}\gamma$ or 5-FU as in Fig. 8. After treatment, tumor cells were used as targets in ^{51}Cr release assays, and lytic activity was determined using anti-*ras* 4-12(Val12)-specific CTL at multiple E:T cell ratios. **B**, In a separate experiment, the nature of the CTL effector mechanisms against 5-FU-pretreated (30 $\mu\text{g}/\text{ml}$) SW480 cells was determined (E:T ratio, 10:1) in the absence ("none") and presence of ZB4 or an isotype control (MOPC-21) (each at 10 $\mu\text{g}/\text{ml}$) or CMA (10 μM). **C**, Same as in A, except targets were SW620 tumor cells. Data in all panels were expressed as the mean \pm SEM of triplicate cultures and are representative of four separate experiments.

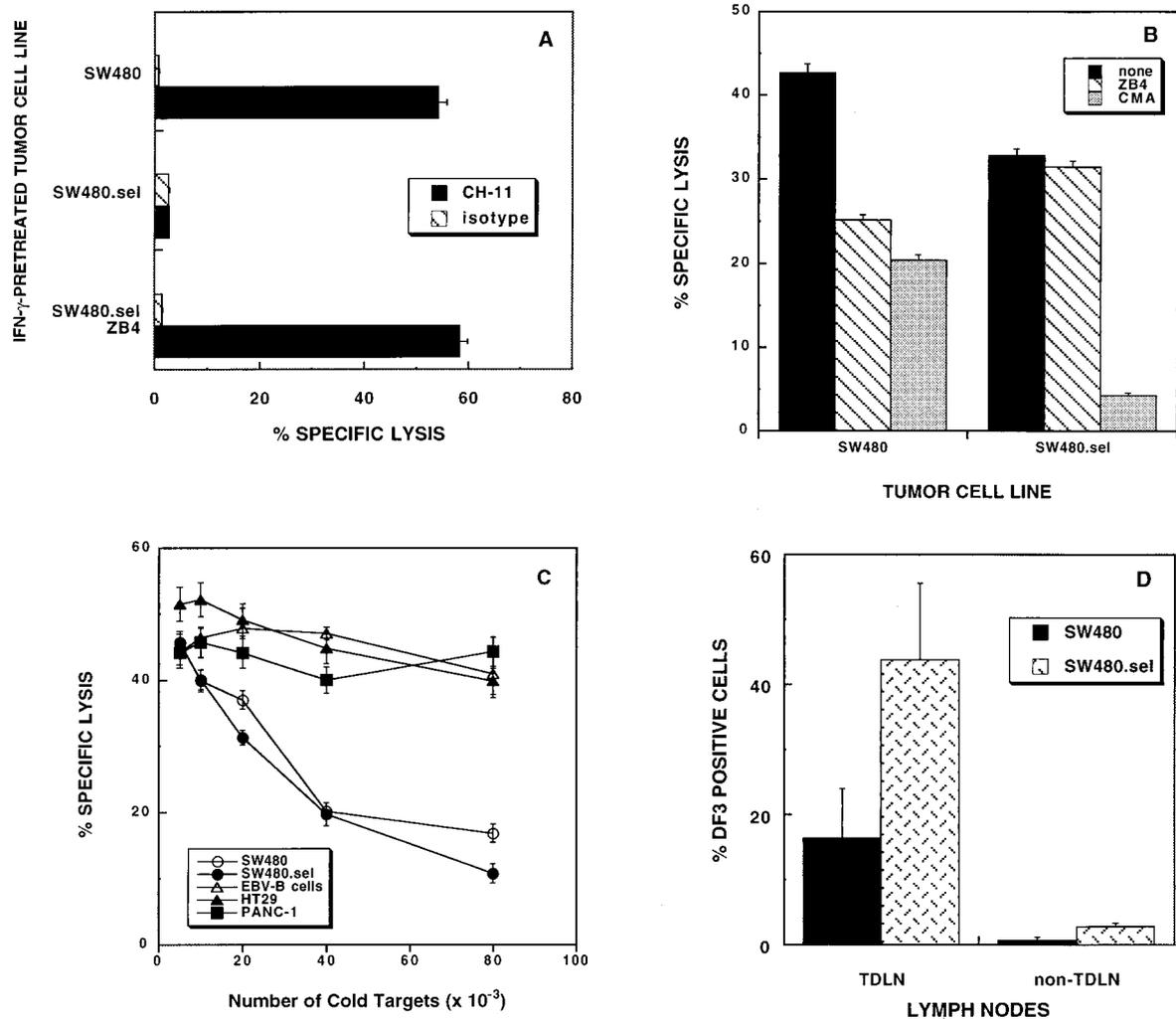


FIGURE 10. Isolation of Fas-insensitive SW480 cells with enhanced metastatic ability. *A*, SW480.sel were tested for functional Fas activity, as in Fig. 3A, using IFN- γ -pretreated cells (with CH-11 or MOPC-104E isotype, each at 1 μ g/ml). SW480.sel/ZB4 cells, derived in the presence of IFN- γ , CH-11, and ZB4, were included as a control for specificity of CH-11-mediated effects. *B*, Anti-*ras* CTL-mediated lysis of IFN- γ -pretreated SW480 or SW480.sel cells (at E:T ratio of 10:1), as determined in Fig. 5. *C*, SW480.sel cells retain their Ag recognition capability, as determined by cold target inhibition. Anti-*ras* 4–12(Val12) CTL was assayed against 51 Cr-labeled (“hot”) IFN- γ -pretreated SW480 targets (effector:hot target ratio, 10:1) in the presence of various unlabeled (“cold”) IFN- γ -pretreated competitor targets (shown in the legend box) at multiple cold target densities. CTL were preincubated with cold targets for 30 min before adding hot targets (assay length, 12 h). Percent specific lysis in the absence of cold target competitor, 50 ± 1 . Data in A–C were expressed as the mean \pm SEM of triplicate cultures and are representative of at least four separate experiments. *D*, Tumor cell dissemination to draining lymph nodes, as determined in Fig. 1C. Athymic mice received 5×10^6 cells/mouse s.c. Tumor growth by SW480 and SW480.sel cells at the time of analysis averaged 1345 ± 334 and 1424 ± 262 mm 3 , respectively. TDLN and non-TDLN from each mouse were analyzed for DF3 cell-surface expression. Data were expressed as the mean \pm SEM of five mice/tumor-bearing group and are representative of two separate experiments.

CTL line (with specificity for an influenza viral epitope) failed to lyse these targets unless the appropriate exogenous peptide was added (data not shown). The observation that the viral epitope-specific CTL line could only lyse these SW480 targets in the presence of their respective exogenous peptide also precluded the possibility that 5-FU treatment rendered them more amenable to nonspecific lysis.

Correlation between Fas insensitivity and metastatic phenotype

To explore the potential interplay between the acquisition of Fas insensitivity and metastatic phenotype, we have taken the following approach: 1) to culture the primary tumor cell line, SW480, with CH-11 (and IFN- γ) to select for the outgrowth of Fas-insensitive tumor cells; and 2) to then explore the potential correlation between Fas insensitivity and metastatic phenotype, based on lymph node metastasis (described in Fig. 1C).

After three consecutive *in vitro* cell passages with IFN- γ and CH-11 to sensitize the responsive SW480 cells to Fas-mediated lysis, the resulting cell population was propagated in the absence of IFN- γ /CH-11 and then tested for functional Fas activity. SW480 cells cultured under those conditions (termed SW480.sel) were insensitive to Fas-mediated lysis induced by CH-11, as compared with the parental (unselected) SW480 population (Fig. 10A). As an additional control for the *in vitro* selection process, we established a SW480 population that had been cultured in parallel, but with IFN- γ , CH-11, and ZB4 (termed SW480.sel/ZB4) to specifically neutralize the functional impact of CH-11. SW480.sel/ZB4 cells, in contrast, were sensitive to Fas-mediated lysis triggered by CH-11, indicating that the loss of Fas sensitivity associated with SW480.sel cells was likely due to selective pressure against Fas engagement and consequent depletion of Fas-sensitive cells, rather than to Fas-independent factors. Phenotypic analysis of SW480.sel

cells for HLA-A2 and ICAM-1, pre- and posttreatment with IFN- γ , revealed a pattern of expression similar to that of SW480 cells, whereas analysis of Fas receptor on SW480.sel cells revealed a pattern of expression similar to that of SW620 cells (data not shown).

Next, we examined whether SW480.sel cells retained their sensitivity to Ag-specific CTL and whether that lytic process was Fas independent. The data indicated that SW480.sel cells were lysed by anti-*ras* CTL; albeit, to a lesser extent than that observed with the parental SW480 cells (Fig. 10B). Lytic activity was Fas independent, because CMA, but not ZB4, efficiently blocked cytotoxicity, consistent with the Fas-insensitive phenotype determined by engagement with CH-11 (Fig. 10A). The lower level of lysis seen against SW480.sel cells was unlikely due to a reduction or loss of Ag recognition, because these cells were just as efficient as the parental SW480 cells in cold target inhibition (Fig. 10C) and effector/target binding assays (data not shown). Both SW480 and SW480.sel cells displayed a comparable dose-response profile in their ability to inhibit lysis of radiolabeled parental SW480 cells. The specificity of the cold target inhibition response was determined using cold targets that 1) lacked expression of both HLA-A2 and a *ras* codon 12 mutation (HT-29 colon carcinoma); 2) expressed HLA-A2, but lacked expression of a *ras* codon 12 mutation (autologous EBV-B cells); and 3) expressed both HLA-A2 and a *ras* codon 12 mutation, but the mutation encoded an inappropriate amino acid substitution of Gly to Asp (PANC-1 pancreatic carcinoma, Ref. 41) (Fig. 10C).

Lastly, we examined whether SW480.sel cells, when compared with SW480 cells, exhibited enhanced metastatic behavior in vivo, as determined by lymph node migration in an animal model (as described in Fig. 1C). Despite the fact that s.c. tumor volume was comparable between both tumor-bearing groups at the time of analysis (Fig. 10D), the results indicated that a significantly ($p < 0.03$) higher percentage of DF3⁺ cells were found within TDLN of mice bearing SW480.sel cells, compared with those of SW480 cells (Fig. 10D). In contrast, the DF3⁺ cells from the non-TDLN of mice from both tumor-bearing groups were similar and substantially lower than those from the corresponding TDLN. Overall, these findings indicated that SW480 cells selected for functional Fas resistance in vitro displayed enhanced ability to disseminate to draining lymph nodes, as compared with the parental SW480 cells and the SW620 metastatic cell line (see Fig. 1C).

Discussion

The resistance of solid tumors (e.g., carcinomas) to one or more immune effector mechanisms, such as cell death through the Fas pathway, may confer a selective survival advantage that could contribute to tumor escape and the metastatic process. In this study, we have begun to explore and characterize in an in vitro model system the nature and spectrum of Ag-specific CD8⁺ CTL effector mechanisms involved in cytotoxicity of human colon carcinoma cells focusing on potential disparities in Fas-mediated killing between tumor cell lines of primary and metastatic origin. We demonstrated that following IFN- γ pretreatment, while both the SW480 and SW620 cell lines were lysed by *ras* oncogene-specific CD8⁺ CTL, the lytic mechanisms were dissimilar at the level of tumor-cell sensitivity to the Fas death pathway. The SW480 cell line exhibited an IFN- γ -inducible, Fas-responsive phenotype, while the SW620 cell line sustained a functionally Fas-unresponsive phenotype that could not be circumvented by IFN- γ treatment under these conditions or even following a 3-day culture period with IFN- γ (data not shown; up to 1,000 U/ml \pm TNF- α (42) as a potential modulator of Fas expression).

The inability of IFN- γ to endow a Fas-sensitive phenotype in SW620 cells was not likely due to a general or global defect in their responsiveness to IFN- γ , because these cells efficiently up-regulated cell-surface expression of HLA-A2 and ICAM-1 molecules and, to a lesser extent, Fas receptors as a consequence of IFN- γ exposure (Table I). Furthermore, the inability of IFN- γ -pretreated SW620 cells to undergo Fas-mediated lysis was demonstrated and confirmed using two independently produced anti-*ras* Val12-specific CD8⁺ CTL lines (Fig. 6). These observations are consistent with the hypothesis that the malignant or metastatic phenotype characterized for the SW620 cell line (19) and supported here (Fig. 1) was associated, at least in part, with resistance to Fas-mediated lysis, measured both in response to Ag-specific T cell attack and Fas pathway stimuli. It is important to point out that although SW480 cells exhibited enhanced sensitivity to Fas-mediated lysis by anti-Fas triggering and Ag-specific CD8⁺ CTL, this Fas-responsive phenotype was not characteristic of the entire tumor cell population. This observation is consistent with the notion that cells comprising the primary tumor lesion may be heterogeneous in functional Fas expression.

Although IFN- γ appeared unable to enhance or restore functional Fas expression in SW620 cells, it was important for modulation of Ag-specific CTL-mediated lysis. Under these conditions, the lytic phenotype of SW620 cells was correlated with enhanced effector/target binding (Table II), and expression and functional participation of HLA-A2 and ICAM-1 molecules (Fig. 5), which were likely necessary for further promoting or strengthening the effector/target interaction as a prerequisite for efficient T cell activation and execution of perforin-mediated lysis. The finding that lysis of IFN- γ -treated SW620 tumor cells proceeded alternatively through a perforin-based effector mechanism suggested that triggering of Fas-independent lysis by such MHC-restricted, Ag-specific CD8⁺ CTL may bypass, at least to some extent, the Fas-resistant phenotype of certain colon carcinoma populations.

Similar to what was observed with IFN- γ , treatment of SW480 cells, but not SW620 cells, with 5-FU led to enhanced functional Fas expression (Figs. 8 and 9). Moreover, these findings demonstrated for the first time the ability of a chemotherapeutic agent to sensitize a solid tumor cell line (i.e., primary colon adenocarcinoma) to MHC class I-restricted, Ag-specific CD8⁺ CTL attack, which lends support to the concept of chemoimmunotherapy. Ag-specific CTL activity against 5-FU-pretreated SW480 cells was associated with a small, but significant, increment in the percentage of cells expressing Fas receptors, as well as a significant increase in the percentage of cells positive for ICAM-1 expression. Conversely, the failure of 5-FU to further sensitize SW620 cells to Ag-specific, CTL-mediated lysis was correlated with the inability of 5-FU to significantly augment the percentage of those cells positive for ICAM-1 expression. Although additional studies are necessary, these findings implicate important functional roles of Fas, ICAM-1, and, perhaps, yet unidentified receptor/ligand interactions in the mechanism of action of 5-FU on modulation of the lytic phenotype. In preliminary studies, we also examined whether the coincubation of IFN- γ and 5-FU would further enhance Fas expression by SW480 or SW620 cells and found no additional enhancement above that observed with IFN- γ alone, as determined by flow cytometry (data not shown).

Support of the notion that the Fas death phenotype may have correlated not only with Fas receptor expression but also potentially with downstream postreceptor events was provided by preliminary findings in our laboratory regarding the regulation of intracellular levels of caspase-3. For example, following IFN- γ pretreatment, we observed an increase in pro-caspase-3 protein expression in SW480 cells that was not found in SW620 cells. The

increase closely paralleled the cells' relative sensitivity to functional Fas activity. Although the precise molecular and biochemical mechanisms by which IFN- γ sensitizes responsive human cell types to Fas activation remain to be fully understood and may vary from cell system to cell system, Ossina et al. (43) reported in a human colon carcinoma cell line (HT-29) the induction of several apoptosis-related genes including selected members of the caspase (e.g., *Ice*, *CPP32*, *FLICE*) and *bcl-2* (e.g., *bak*) families. Similarly, Keane et al. (44) reported in several human breast carcinoma cell lines the up-regulation of certain caspases (e.g., *Ice* subfamily).

In an effort to potentially link the acquisition of Fas insensitivity and metastatic phenotype, we have sought to culture the primary tumor cell line, SW480, with IFN- γ and CH-11 to select for the outgrowth of Fas-insensitive SW480 tumor cells and subsequently analyze the association between Fas insensitivity and metastatic phenotype based on the lymph node metastasis assay (Fig. 10). Overall, our findings indicated that SW480 cells selected for functional Fas resistance in vitro exhibited enhanced ability to disseminate to draining lymph nodes, as compared with the parental SW480 cells and the SW620 metastatic cell line (Figs. 1 and 10). Thus, these experiments support a potential interplay between Fas responsiveness and metastatic phenotype, as determined by this experimental approach. Ongoing studies are aimed at further understanding the potential mechanistic link between these two functional outcomes, and to determine whether the loss of Fas sensitivity directly accounts for the enhanced metastatic ability or represents one or more intrinsic characteristic features that parallel the development of a more malignant or metastatic phenotype.

In the tumor microenvironment, IFN- γ and other potentially relevant cytokines may be provided endogenously by immune system interactions, such as by CD4⁺ T cells (3, 5), following interaction with MHC class II⁺ APC presenting exogenous Ag. Ag-specific CD8⁺ CTL may then lyse cytokine-modified tumor cells through Fas-dependent and/or Fas-independent pathways, depending upon the intrinsic susceptibility of the tumor population to one or more immune effector mechanisms. In addition to cell contact-dependent immune interactions occurring between Ag-specific CD8⁺ CTL and their tumor targets, cell contact-independent interactions involving the secretion of sFasL following Ag-specific immune stimulation, for example, may play important roles in the regulation of the anti-neoplastic response. In normal human T lymphocytes following cellular activation, it has been reported that FasL may be released as a soluble product that maintains functional activity (45, 46). These cell contact-independent lytic mechanisms may represent biologically significant pathways for the elimination of Ag-negative tumor cells. However, the ability of cancerous cells within primary or metastatic lesions to resist Fas-mediated apoptosis in response to engagement with FasL-bearing effector cells or sFasL may constitute a novel mechanism of tumor escape influenced by Ag-independent immune interactions or cell-derived soluble products. In support of that hypothesis, we used recombinant sFasL as a surrogate source of physiologically produced sFasL, as well as preactivated FasL-bearing CTL, and found evidence for tumor-specific differences in their functional response toward FasL-mediated cytotoxicity (Fig. 3).

Although our studies here focused on the design of an in vitro system to analyze the roles of IFN- γ , 5-FU, and Fas in human Ag-specific CTL-mediated lysis of colon carcinoma cells, the results may have implications for fresh or autologous tumor isolates, which warrants further investigation, and may provide insights into the processes of both tumor immunity and tumor escape for at least a potential subset or fraction of malignancies. For example: 1) down-regulation of cell-surface Fas receptors and/or elements of Fas-mediated signaling may represent unique tumor escape mech-

anisms against Ag-specific T cell attack; 2) IFN- γ and certain anticancer agents, such as 5-FU, may help to enhance or restore a Fas-sensitive phenotype in certain colon carcinoma cells (i.e., SW480), supporting a potentially important role of IFN- γ (or those cytokines that induce endogenous IFN- γ production) or chemotherapeutics in active- and/or passive-based immunotherapies; 3) the inability of IFN- γ or 5-FU to sensitize SW620 cells to Fas-mediated apoptosis or Ag-specific CTL attack suggests that such "loss-of-function" may be an important adaptation in the evolution of malignant potential; and 4) IFN- γ may act to promote efficient CTL/target interactions leading to enhanced T cell activation and triggering of granule (perforin/granzyme)-mediated lysis of metastatic carcinoma cells that might otherwise remain resistant or refractory to Fas-mediated apoptosis (i.e., SW620).

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