

Dendritic Cell-Mediated Cross-Presentation of Antigens Derived from Colon Carcinoma Cells Exposed to a Highly Cytotoxic Multidrug Regimen with Gemcitabine, Oxaliplatin, 5-Fluorouracil, and Leucovorin, Elicits a Powerful Human Antigen-Specific CTL Response with Antitumor Activity in Vitro¹

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Gemcitabine, oxaliplatin, leucovorin, and 5-fluorouracil (GOLF) is a novel multidrug regimen inducing high levels of necrosis and apoptosis in colon carcinoma cells. This regimen is also able to promote a process of Ag remodeling including up-regulation of immunotherapy targets like carcinoembryonic Ag (CEA), thymidylate synthase (TS). We have conducted a preclinical study aimed to investigate whether these drug-induced modifications would also enhance colon cancer cell immunogenicity. Several CTL lines were thus generated by in vitro stimulating human HLA-A(*)02.01⁺ PBMCs, from normal donors and colon cancer patients, with autologous dendritic cells cross-primed with cell lysates of colon cancer cells untreated, irradiated, or previously exposed to different drug treatments including the GOLF regimen. Class I HLA-restricted cytolytic activity of these CTL lines was tested against colon cancer cells and CEA and TS gene transfected target cells. These experiments revealed that CTLs sensitized with GOLF-treated cancer cells were much more effective than those sensitized with the untreated colon carcinoma cells or those exposed to the other treatments. CTL lines sensitized against the GOLF-treated colon cancer cells, also expressed a greater percentage of T-lymphocyte precursors able to recognize TS- and CEA-derived peptides. These results suggest that GOLF regimen is a powerful antitumor and immunomodulating regimen that can make the tumor cells a suitable means to induce an Ag-specific CTL response. These results suggest that a rationale combination of GOLF chemotherapy with cytokine-based immunotherapy could generate a chemotherapy-modulated Ag-specific T-lymphocyte response in cancer patients able to destroy the residual disease survived to the cytotoxic drugs. *The Journal of Immunology*, 2005, 175: 820–828.

In the hope to improve the efficacy of anti-cancer treatments, attempts have been made to combine cancer vaccines with biologic agents or cytotoxic drugs and to test new strategies aimed at inducing a simultaneous multi-antigen specific immune response (1–5). In previous studies, we have tested the activity in

vitro against human colon cancer cells of a chemotherapy regimen with multiple cytotoxic drugs, such as gemcitabine (GEM),³ oxaliplatin (OXA), leucovorin (LV), and 5-fluorouracil (5-FU) (i.e., GOLF regimen). The results showed that GOLF induced far greater antitumor activity (superadditive) than those obtainable with all of the other possible combinations of the four drugs (data not shown), including the OXA + LV + 5-FU (OLF), which is currently considered as the standard treatment for metastatic colorectal cancer (6). The GOLF regimen compared with the above mentioned combinations showed the unique ability to kill colon cancer cells by inducing either necrosis and apoptosis, a feature that was not shared by OLF (Fig. 1). The GOLF combination has also been tested in clinical trials in advanced colon cancer patients in whom it has been shown to have a good safety profile and highly significant antitumor activity (7).

The favorable antitumor activity of GOLF regimen has been explained on the grounds that GEM is able to modify the pharmacokinetics and pharmacodynamics of either 5-FU and OXA (Refs.

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Received for publication June 1, 2004. Accepted for publication April 15, 2005.

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¹ This work was supported by a grant from "Istituto Superiore di Sanità" no. 1A2/F7 (Research Unit, to E.B.).

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³ Abbreviations used in this paper: GEM, gemcitabine; OXA, oxaliplatin; LV, leucovorin; 5-FU, 5-fluorouracil; GOLF, GEM, OXA, LV, 5-FU; DC, dendritic cell; IRI, irinotecan; CEA, carcinoembryonic Ag; TS, thymidylate synthase; LAK, lymphokine-activated killer.

8 and 9 and unpublished results). The addition of GEM to the FOLFOX could also have an immunological interest insofar as Nowak et al. (10–12) have recently shown its ability to enhance tumor Ag cross-presentation in mouse models giving rise to an *in vivo* efficient antitumor immune response.

The aim of the present study was to investigate whether the GOLF regimen could induce molecular and structural changes in human colon cancer cells leading to a substantial increase of tumor cell immunogenicity, capable of making them a suitable means to prime a tumor-specific CTL response. To this end, we generated and characterized human CTL lines *in vitro* by stimulating HLA-A(*):02.01⁺ PBMCs, collected from normal donors and colon cancer patients, with low dose IL-2 and autologous Ag-loaded dendritic cells (DCs). CTL lines were obtained from PBMCs cocultured with DC loaded with Ags derived from a mix of two different colon cancer cell lines untreated or previously exposed to GOLF or other control treatments.

Materials and Methods

Cell cultures and human mononuclear cells

WiDr, SW-1463, and HT29 colon carcinoma cell lines were purchased from American Type Culture Collection. CIR-A2 cell line (13) was provided by Dr. Jeffrey Schlom (Experimental Oncology Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD). All of the tumor cell lines were cultured as previously described (3, 4). PBMCs for the *in vitro* generation of CTL lines (see below) were obtained by Ficoll-Hypaque gradient centrifugation of heparinized blood collected from normal donors or cancer patients carrying HLA-A(*):02.01 haplotype.

Peptide synthesis

Known CEA [(CEAP)-1 (IQNDTGFYT), (CEAP)-2 (LLSVTRNDV)], and TS [TS-1 (AVSEHQLLH), TS-2 (FLHLLIAEIH), and TS-3 (TSTTSLELD)] peptide epitopes with HLA-A(*):02.01 binding motifs were synthesized and characterized as previously described (3, 4). Another CEA-derived epitope peptide [(CAP)-1 (YLSGANLNL)] (14) was kindly provided by Dr. Jeffrey Schlom. The TS-1, TS-2, TS-3, CEAP-1, and CEAP-2 peptides were selected because of their high HLA-A(*):02.01⁺ binding score predicted according to the Parker's algorithm (15).

Generation of DCs and CTL cultures

PBMCs were obtained by means of Ficoll-Hypaque gradient separation of buffy coats or blood samples collected from HLA-A(*):02.01-typed healthy donors and colon cancer patients (16). The DCs used for the *in vitro* T cell stimulation were generated by culturing PBMCs in presence of 50 ng/ml GM-CSF and 0.5 ng/ml IL-4 (both purchased from R&D Systems) 7 days as described by other authors (17, 18). To estimate DC enrichment before CTL stimulation, the cultures were examined for the expression of CD11a, CD11c, HLA-I(A,B,C), CD80, CD83, and CD86 markers.

These cells were used to stimulate autologous PBMCs used for CTL line generation as described in previous studies (3, 4, 19) with the only exception that DCs used for CTL stimulation were not pulsed with epitope peptides but loaded with tumor cell lysates. Before being used as APC for CTL stimulation, DCs were routinely evaluated for the immune phenotype and had to show at least a 90% purity level.

To *in vitro* generate CTL lines, different samples of irradiated autologous DCs were loaded (exposed for four hours), respectively, in complete AIM-V medium containing human AB serum with: 1) a lysate of untreated tumor cells; 2) lysate of previously irradiated (30,000 rads) tumor cells; and 3) lysate of tumor cells that had undergone different chemotherapy treatments (see below) before being added to the lymphocyte cultures at a final DC/CTL ratio of 1:5.

The haplotype of the healthy donor AF81 was A2, A23, B18, B35, Cw3, Cw4, DR 51/2/3, DR51/2/3, while that of the other one MD80 was A2, A3, B18, B8, Cw2, Cw, DR 51/2/3, DR51/2/3, DQ, DQ.

Drug treatment of cancer target cells

The following drugs were used in this study: VP-16 (Pierce Pharma), GEM (Eli Lilly), OXA (Sanofi-Synthelabo), LV (Lederle), 5-FU (Roche), and irinotecan (IRI; Aventis Pharma). The most effective conditions to obtain the maximal cytotoxic and proapoptotic effects of the drug combination were extrapolated from previous experiments (data not shown). Actually,

before being sonicated in serum-free AIM-V and loaded onto DCs, WiDr and HT29 colon carcinoma cells were, respectively, treated as follows. Subconfluent cells were trypsinized, counted, and seeded in 24-multiwell plates (TPP) at a final dilution of 10⁵ cells/well in 1 ml of complete medium. The medium was harvested after 24 h of incubation at 37°C and 5% CO₂. Subsequently, 1 ml of fresh medium was added to the tumor cells used in group A (control), group B (to be irradiated), whereas 1 ml of fresh medium containing VP-16 at final concentrations of 100 µg/ml (for group C), or GEM at final concentrations of 50 µg/ml (for group D, (GEM), F (GEM + IRI + FUFA, designated as GILF) and H (GOLF)), or IRI at final concentrations of 100 µg/ml (for group E (IRI + FUFA, designated as IFL)), or OXA at final concentrations of 10⁻⁵ M/ml (group G, OLF) was added to the tumor cells. After 30 min of incubation for groups D, F, and H, and 4 h of incubation for groups C, E, and G, the medium was replaced with 1 ml of fresh medium (for groups A, B, C, and D) or medium containing 10⁻⁴ MLV (for groups E (IFL), F, G (OLF), and H). After a further 30 min of incubation, the medium was withdrawn, and replaced with 1 ml of fresh medium (for groups A–D) or fresh medium containing 5-FU at final concentrations of 10⁻³ M (for groups E–H). After 24 h, 1 ml of fresh medium (for groups A–D), or fresh medium containing 100 µg/ml IRI (group F, GILF) or OXA at the concentration of 10⁻⁵ M (for group H) was added to the wells. After 4 h, the medium was withdrawn, and fresh medium containing no drugs was added to the samples in the groups A–D, whereas medium containing 10⁻³ M 5-FU was added to the samples in groups E–H. Cells in group B received the same treatment of the controls with the only exception that they were gamma-irradiated with 30,000 rads 48 h before being harvested, washed, sonicated, and used for CTL stimulation.

The cells were subsequently incubated at 37°C and 5% CO₂ for 24 h before being detached, washed three times with DPBS, counted, and concentrated at the final concentration of 10⁷ cells/ml serum-free AIM V medium. The WiDr and HT29 cells, either untreated (group A), irradiated (group B), or treated with chemotherapy (groups C–H), were mixed at 1:1 ratio and sonicated (ultrasound wave at 42 KHz, Branson 5510 Ultrasound baths, VWR International) for 5 s in 1 ml, 50 µl of which was first filtered (centrifugal filter devices ultra free-MC 0.45 µm; Millipore) and then added to 10⁶ DCs for CTL stimulation.

Transfection of WiDr target cells with HLA-A(*):02.01 gene

To use colon cancer cells as targets of class I HLA-restricted CTLs, HLA-A(*):02.01 expression of WiDr cells was transiently augmented by transfection with plasmid carrying the HLA-A(*):02.01 gene sequence, as previously described (4). HLA-A(*):02.01 expression was evaluated before any experiments on target cells by indirect flow cytometry using the A2.69 mAb.

Transfection of other target cells

To use CIR-A2 as possible targets of CEA- and TS-specific/HLA-A(*):02.01-restricted CTL subsets, they were transfected with a plasmid (pCDNA3; Invitrogen Life Technologies) expressing the target (CEA or TS) gene sequence using the above described procedure (4). Also in this case, Ag expression was evaluated on target cells before any experiment by indirect flow cytometry by using, respectively, the TS-106 and COL-1 mAbs. Untransfected CIR-A2 cells expressed low levels of TS (25–30%) and no level of CEA, although after gene transfection, their expression was augmented to 50–55% and 45–50%, respectively.

Cytotoxic assays

⁵¹Chromium release assays were performed as described in previous studies (3, 4, 19). HLA-A(*):02.01 molecule expression on WiDr target cell membrane as well as TS and CEA expression in CIR-A2 target cells was induced or increased by gene transfection before each experiment (4). Specific lysis was calculated as follows:

% specific lysis =

$$\frac{\text{observed release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined from the wells to which 100 µl of medium were added instead of effector cells. Total releasable radioactivity was obtained after treating the target with 2.5% Triton X-100. The tumor targets' characteristics are expressed in the Table I.

Table I. Characteristics of the target cells

Target Cells	HLA-A(*)02.01	Haplotype	Source	Purpose of Use
WiDr	Neg	A24;A32;B15;B18	Colon carcinoma	Negative control
A2-WiDR	55 (\pm 12) %			CTL-sensitive targets
pC-WiDr	Neg			Negative control
SW-1463	65 (\pm 8) %	A(*)02.01; -; -; -	Colon carcinoma	CTL sensitive targets
LNCAP	35 (\pm 6.5) %	A(*)02.01; -; -; -	Prostate carcinoma	Negative control
CIR-A2	90 (\pm 2.5) %	A(*)02.01; -; -; -	Lymphoblastoid cells	Negative control
CEA-CIR-A2	89 (\pm 1.5) %			CTL targets (CEA ⁺)
TS-CIR-A2	92 (\pm 6.4) %			CTL targets (TS ⁺)
pC-CIR-A2	92 (\pm 2.7) %			Negative control

A2-WiDr, WiDr cells transfected with the HLA-A(*)02.01 gene; pC-WiDr, WiDr cells transfected with the plasmid (pC3) backbone; TS-CIR-A2, CIR-A2 cells transfected with the TS gene; CEA-CIR-A2, CIR-A2 cells transfected with the CEA gene; pC-CIR-A2, CIR-A2 cells transfected with the plasmid backbone. HLA-A(*)02.01, CEA, and TS expression were measured by using indirect fluorescence flow cytometry by, respectively, using: A2.69, COL-1, and TS-105 mAbs. Parental CIR-A2 cells expressed low levels of TS (25–30%) and no level of CEA, whereas after the specific gene transfection, their expression was augmented to 50–55% and 45–50%, respectively.

Specific inhibition of HLA-restricted CTL activity by anti-HLA mAbs

A2.69 (anti-HLA-A(*)02.01 mAb; One Lambda) was used to suppress specifically HLA-A(*)02.01-restricted cell-mediated immunity, whereas UPC-10 mAb (HLA-unrelated mAb; Cappel/Organon Technique), which does not react with human colon cancer cells, was used as a negative control. Abs were incubated with target cells for 1 h before the cytotoxic assay.

Cell extraction and immunoblotting

Protein extraction and immunoblotting (20) were performed by using COL-1 (14 μ g/ml) (21), TS-106 (10 μ g/ml), and an anti-HSP-90 mAb (12 μ g/ml) (Calbiochem). The bands were visualized using the Protoblot (Promega) color development system, as described by the manufacturer.

Flow cytometry

The procedure for single-color flow cytometric analysis has been previously described (21). Conjugated mAbs were all purchased from BD Biosciences, whereas W6/32, (anti-HLA class I), A2.69 (anti-HLA-A(*)02.01), COL-1 (anti CEA mAb), TS-106 (anti-TS mAb), and MOPC-21 were, respectively, purchased by Scra, Sussex, England, One Lambda, and Cappel/Organon Tecknica). Samples were analyzed by using a Becton Dickinson FACScan equipped with a blue laser with an excitation level of 15 nW at 488 nm.

Statistical considerations

The between-mean differences were statistically analyzed using StatView statistical software (Abacus Concepts). The results were expressed as the mean \pm SD of four determinations made in three different experiments, and the differences determined using the two-tailed Student's *t* test for paired samples. Values of $p < 5\%$ were considered statistically significant. Differences in cytolytic effects produced by effector CTLs were evaluated taking into account the percentage of specific cytotoxicity at all effector/target cell ratios. Therefore *p* values were calculated using covariance analysis performed on the regression of the percentage of specific ⁵¹Cr-release over the logarithm of the number of effector cells/well. All data relative to cell-mediated cytotoxicity are expressed in terms of mean LU₅₀/10⁶ values without conventional SE or SD of the mean. Actually no statistical analysis can be performed using these parameters, which are not suitable for covariance analysis of regression lines.

Results

Effects of GOLF regimen on colon cancer cells in vitro

The multidrug GOLF regimen was found to possess high antitumor activity in colon cancer patients (7) and potent cytotoxic and proapoptotic activity in colon cancer cells in vitro (Fig. 1). This antitumor activity appeared to be significantly greater if compared with that of each single combination of GEM + OXA (GEMOX), GEM + 5-FU, and also of OLF (data not shown). These findings have been supported by the results of three recent clinical trials showing the powerful antitumor activity of the GOLF regimen in patients with metastatic colorectal and gastric cancer (Ref. 7 and manuscript submitted for publication).

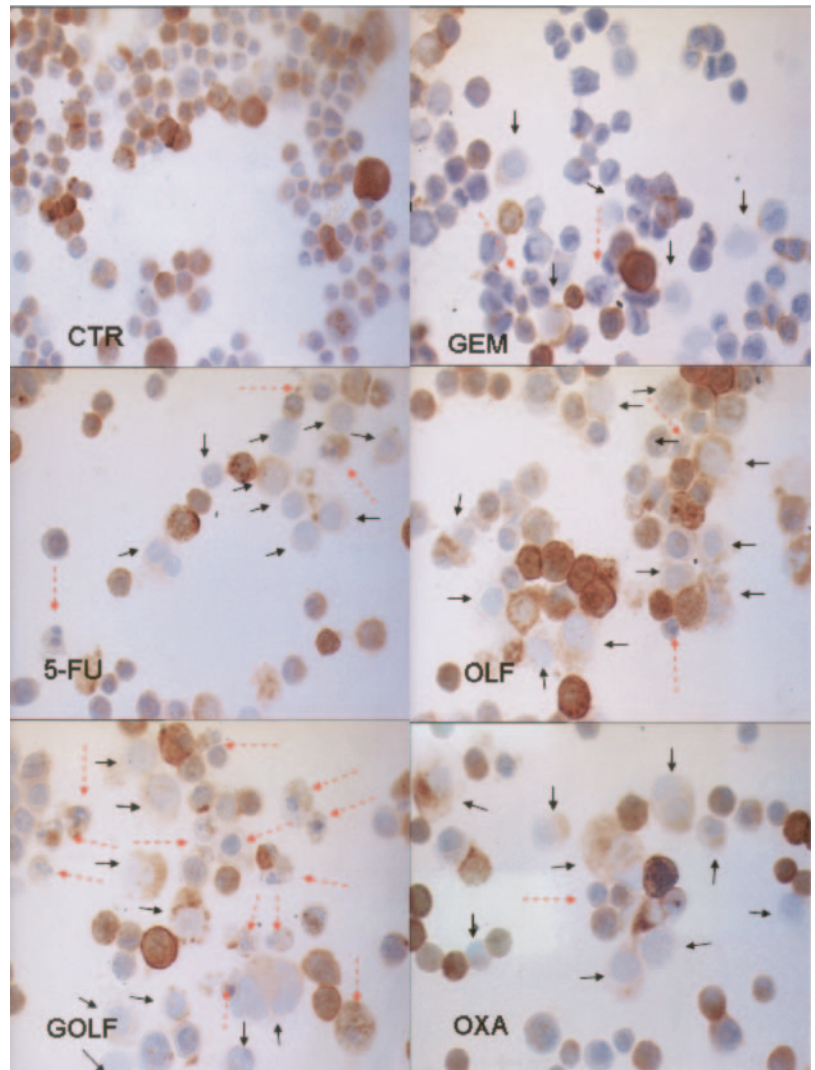
In previous studies, we have shown that 5-FU up-regulates CEA and TS expression in breast and colon cancer cells (MDA-MB-231, WiDr, HT-29, and SW-1463), increasing their susceptibility to the cytolytic activity of CEA- and TS-specific CTL lines (3, 4). TS is particularly important because it is the main target enzyme inhibited by 5-FU metabolites (22) and represents the major source of thymidylate in human cells, which is indispensable for DNA replication (23–25). TS is a mRNA-binding protein capable of self-regulation depending on the levels of cofactors and substrates, and its expression is under strict control of proteins regulating the cell cycle. It is expressed only during the S phase of cell cycle in normal cells, but can be constitutively expressed in cancer cells (23–25). TS over-expression or mutation is associated with the occurrence of tumor cell resistance to 5-FU and to a number of other antimetabolites (26). It is also of main interest the observation that abnormal TS patterns is predictive for poor prognosis in patients with several gastro-enteric malignancies (27).

In the present study, we found that the GOLF combination, compared with 5-FU, OXA, GEM and OLF, was the only regimen able of inducing either high levels of necrosis and apoptosis (Fig. 1) still retaining an augmented expression of TS and CEA (Figs. 1 and 2). The GOLF combination also showed the ability to maintain a high expression of heat shock proteins like the HSP-90 (Fig. 2, C and D). It must be pointed out that HSP-90, a molecular chaperone involved in intracellular protein maturation and Ag processing (28), could theoretically enhance the release of Ag peptide epitopes indispensable for inducing a multi-antigen-specific CTL response.

In vitro GOLF treatment enhances the immunogenicity of human colon cancer cells. We investigated whether autologous DCs loaded with Ags derived from necrotic and apoptotic tumor cells previously exposed to the GOLF combination could be used to give rise to a multi-antigenic/multi-epitopic CTL response with antitumor activity in vitro. We studied the functional and Ag-specific antitumor activity of CTL lines, generated in vitro by stimulating human HLA-A(*)02.01⁺ PBMCs from normal donors and colon cancer patients, with IL-2 and autologous Ag-loaded DCs. In particular, we compared the cytotoxic activity of CTLs generated with DCs loaded with lysates of untreated colon cancer cells (i.e., CCR-CTLs), with that of CTLs generated with DC loaded with lysates of the same colon cancer cell lines previously exposed to the GOLF regimen (i.e., GOLF-CTLs). No significant difference in immune phenotype (CD3, CD56, CD4, CD8) was detected between the two groups of lymphocytes (data not shown).

To test the antitumor activity of these CTL lines, ⁵¹Cr-release assays were performed by using SW-1463 and HLA-A(*)02.01

FIGURE 1. A pathology study performed on WiDr cells exposed to different drug treatments (immunostaining for CEA expression). GOLF regimen kills WiDr cells by inducing either necrosis and apoptosis (original magnification, $\times 450$). Long red dashed arrows indicates the apoptotic cells; the short black arrows show the necrotic cells. CTR, untreated control WiDr cells; GEM, gemcitabine-treated cells; 5-FU, 5-FU-treated cells; OXA, oxaliplatin-treated cells; OLF, OLF multidrug-treated cells; GOLF, GOLF multidrug-treated cells.



gene transfected WiDR (A2-WiDr) colon carcinoma cells as targets. SW-1463, a colon carcinoma cell line not used for CTL in vitro sensitization, was chosen as a target for its HLA-A(*02:01 haplotype and for the high expression of target Ags such as CEA and TS. Conversely, WiDR cells were considered a good target because they were previously used for CTL in vitro sensitization. These cells were negative for the expression of HLA-A(*02:01 molecule, and for this reason, they needed to be transfected with HLA-A(*02:01 gene before being used as targets in the CTL assays. Untransfected WiDr, WiDr transfected with the plasmid backbone and the HLA-A(*02:01⁺ prostate carcinoma LNCap cells were used as negative controls.

The results of a representative experiment performed with PBMCs of a normal healthy donor (AF81), showed that that GOLF-CTLs elicited greater HLA-A(*02:01-restricted cytolytic activity than CCR-CTLs against HLA-A(*02:01-transfected WiDr and SW-1463 colon carcinoma target cells (Fig. 3). In all cases, anti-HLA-A(*02:01, A2.69 and anti-class I (A,B,C) HLA, W6.32, mAbs (data not shown in figure), but not UPC-10 mAb (non-reacting negative control) severely reduced the cell-mediated cytotoxicity, thus confirming the HLA-A(*02:01 restriction of effector CTLs. Similar results were also obtained by using CTL lines generated by using the same method, from HLA-A(*02:01⁺ PBMCs derived from colon cancer patients (Table II). To verify whether the enhanced cytolytic activity of healthy donor- and colon cancer

patient-derived GOLF-CTLs is specific for colon carcinoma targets, the cytotoxic activity of these CTL lines was also tested against LNCaP cells, which were supposed to express a completely different antigenic pattern. All of the CTL lines showed a minimal cytotoxic activity against these targets but no difference was observed between CRC- and GOLF-CTL groups (Fig. 3 and Table II).

CTL ability in recognizing CEA and TS Ags

Since the GOLF regimen treatment enhances CEA and TS expression in colon cancer cells, we investigated whether the CTL lines sensitized against GOLF-treated colon cancer cells, were able to lyse the HLA-A(*02:01⁺ CIR-A2 target cells induced to express TS or CEA after transfection with TS or CEA plasmid (designated as pTS and pCEA). CIR-A2 targets, untreated or transfected with the plasmid backbone (pCDNA3) were not lysed by CCR- or GOLF-CTLs. In contrast, both types of CTLs killed CIR-A2 targets transfected with TS. In this case, GOLF-CTLs showed a much higher cytolytic activity than that induced by CCR-CTLs (Fig. 4). Furthermore, GOLF-CTLs but not CCR-CTLs were capable of lysing CIR-A2 cells transfected with pCEA (Fig. 4). Taken together, these results suggest that the GOLF-CTLs contained subsets of HLA-A(*02:01-restricted effectors T cells, which were more efficiently able to recognize TS and CEA peptide epitopes.



FIGURE 2. Immunoblot analysis of TS, CEA, and HSP-90 in colon cancer cells exposed to the GOLF regimen. The cell homogenates underwent SDS-PAGE. *A*, Immunoblotting with mAb TS-106, which recognizes two bands of 36 kDa (free TS) and 38.5 kDa (TS of the ternary complex) and with mAb COL-1 for CEA detection of untreated, 5-FU-, and GOLF-treated WiDr cells. *B*, Immunoblotting performed with an anti-HSP-90 mAb on WiDr and HT-29 colon carcinoma cell lines. Immunoblot analysis of HSP-90 expressed by WiDr (lane 2), WiDr GOLF-treated (lane 3), HT-9 (lane 4), and HT-9 GOLF-treated (lane 5). The same amount of cell protein (20 μ g) was loaded in each lane. A positive control is represented by WiDr shocked at 42°C for 30 min, before loading on the SDS-PAGE (lane 1). Blots were detected with an anti-HSP-90 mAb (Calbiochem). Protein m.w. standards are shown on the right (kDa).

For this purpose, we tested the ability of CCR-CTL and GOLF-CTL lines to recognize CIR-A2 target cells pulsed with known epitopes from CEA (CEAP-1, CEAP-2, and CAP-1) and TS (TS-1, TS-2, and TS-3) with HLA-A(*)02.01 binding motifs. The results, illustrated in Fig. 5, show that both CTL lines were able to lyse

CIR-A2 target cells pulsed with each one of these peptides. As expected, the GOLF-CTLs showed greater ability in recognizing some of these peptides (i.e., all tested TS peptides, CEAP-1, and CAP-1) (Fig. 5) but were not able to lyse CIR-A2 target cells pulsed with the two negative control peptide epitopes, PTR-2 and PTR-4, derived from PTH-rP (30) a tumor Ag that is not expressed in colon cancer cells.

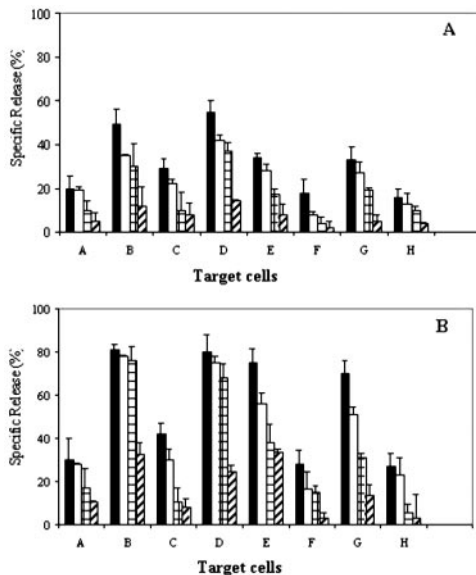


FIGURE 3. Cytolytic activity of CCR-CTLs (*A*) and GOLF-CTLs (*B*) derived from a healthy donor (AF81) against colon cancer cells. CTL activity was tested in ^{51}Cr -release assays against: colon carcinoma WiDr cells (*A*); WiDr transfected with HLA-A(*)02.01 gene (A2-WiDr) (*B*); A2-WiDr in presence of a blocking anti-HLA-A(*)02.01 A2.69 mAb (*C*); A2-WiDr in presence of a non-reacting UPC-19 mAb (*D*); (HLA-A02.01⁺) colon carcinoma SW-1463 cells (*E*); SW-1463 in presence of a blocking anti-HLA-A(*)02.01 A2.69 mAb (*F*); SW-1463 in presence of a non-reacting UPC-19 mAb (*G*). Prostate carcinoma LNCap cells (*H*) were used as a negative control. The results are expressed as the percentage of specific lysis at different E/T ratios (25/1 (■); 12.5/1 (□), 6.25/1 (▤), 1:1 (▥)) (mean values and SDs of triplicate determinations in individual experiments). Significantly different values ($p < 0.05$, two-tailed *t* test) were observed comparing the results from CCR-CTLs vs GOLF-CTLs against HLA-A(*)02.01 gene-transfected WiDr cells, and CTL assay against HLA-A(*)02.01 gene-transfected WiDr cells in presence or absence of A2.69 mAb. Similar results were also obtained by using CTL lines generated from five different HLA-A(*)02.01 normal donors (data not shown in figure) and colon cancer patients against the same targets in the same conditions.

Activity of CTLs generated with different anti-cancer treatments

Although GOLF is a very active combination, it must be pointed out that FOLFOX (in the present in vitro study designated as OLF) and IFL (irinotecan + 5-FU + LV) are currently recommended as the standard treatments for colorectal cancer (6). We have thus investigated whether the immunomodulating activity of GOLF regimen was also shared by these highly cytotoxic combinations, and in particular whether GEM was also able to modify the effects of IFL as it does with the OLF regimen. Thus, we generated de novo CTL lines from two HLA-A(*)02.01⁺ donors (MD80 and the above studied AF81) by stimulating their PBMCs with autologous DCs loaded with the mixed lysates of the WiDr and HT-29 colon cancer cells pretreated with: 1) GEM; 2) IFL; 3) GEM + IFL (GILF); 4) OLF; and 5) GOLF. To evaluate whether the immunostimulating activity of GOLF was chemotherapy-specific, we provided three possible controls, by generating CTL lines with the lysates of the same colon cancer cells: 1) untreated (CCR); 2) exposed to gamma-irradiation with 30,000 Rads; or 3) exposed to a drug such as the topoisomerase II inhibitor etoposide (VP-16), which is ineffective on colon cancer cells. Cytofluorimetric annexin tests, cytometric cell counts, and the colorimetric MTT assays were performed to evaluate the proapoptotic and antitumor activity of these drugs and drug combination on WiDr and HT-29 colon cancer cells. Once again we found that the GOLF regimen exerted the greatest antitumor activity (in terms of induction of apoptosis and necrosis) (Fig. 1 and data not shown). No significant effect was conversely observed when the cells were exposed to VP-16 and GEM alone. Prevalently, irradiation killed by inducing apoptosis, whereas OLF, IFL, and GILF seemed to exert their antitumor activity by inducing a completely different mechanism of cell death (necrosis).

After four in vitro stimulations, all of the CTL lines were evaluated for immune phenotype, which was not different among the different lines and was similar to that observed in the previous experiments. The CTLs were then compared in terms of cytotoxic

Table II. Cytolytic activity of CCR- and GOLF-CTLs derived from colon cancer patients^a

PBMCs' Donors	CCR-CTLs vs A2-WiDr cells		GOLF-CTLs vs A2-WiDr cells		CCR-CTLs vs LNCap cells		GOLF-CTLs vs LNCap cells	
	E/T Ratio, 25/1	E/T Ratio, 12.5/1	E/T Ratio, 25/1	E/T Ratio, 12.5/1	E/T Ratio, 25/1	E/T Ratio, 12.5/1	E/T Ratio, 25/1	E/T Ratio, 12.5/1
SM	54 (5.1)	40 (2.2)	95 (2.1)	73 (1.7)	29 (7.3)	19 (4.2)	35 (5.1)	24 (1.7)
RI	17 (4.3)	22 (0.9)	37 (7.2)	34 (2.2)	19 (2.3)	17 (4.9)	20 (5.2)	24 (3.2)
DA	25 (5.4)	20 (1.3)	43 (1.2)	36 (1.8)	29 (3.4)	27 (2.3)	31 (1.8)	26 (3.2)
BR	35 (13.6)	23 (0.8)	82 (4.5)	58 (10.2)	30 (6.4)	23 (2.8)	37 (2.7)	35 (7.3)
PA	67 (9.3)	45 (1.1)	82 (7.5)	59 (7.8)	23 (7.5)	15 (2.1)	26 (3.5)	19 (2.8)
MC	58 (6.4)	27 (10.2)	83 (3.2)	40 (8.9)	31 (3.4)	23 (8.2)	38 (6.2)	29 (3.9)

^a CTL lines derived from the PBMCs of six different HLA-A(*02:01)⁺ colorectal carcinoma patients (SM, RI, DA, BR, PA, and MC) in vitro sensitized against untreated (CCR/CTLs) and GOLF-treated colon cancer cells (GOLF/CTLs) were tested in ⁵¹Cr-release assays against colon carcinoma WiDr target cells transfected with HLA-A(*02:01 gene (A2-WiDr). HLA-A(*02:01)⁺ prostate carcinoma LNCap cells were used as a negative control. The results are expressed as the percentage of specific lysis at 25/1 and 12.5/1 effector: target ratios \pm SD. Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing the results from CCR-CTLs vs GOLF-CTLs against A2-WiDr cells and not for CCR-CTLs vs GOLF-CTLs against LNCap cells.

activity against colon carcinoma cells and TS- and CEA-transfected CIR-A2 target cells. The results of this new set of experiments showed that the GOLF-CTLs elicit the greatest cytolytic activity against the A2-WiDr (Fig. 6A) as well as against TS and CEA gene-transfected CIR-A2 targets (Fig. 7). In contrast, the cytolytic activity of all the other groups of CTLs against these targets was not different from that elicited by the CRC-CTLs (immunized against the untreated colon carcinoma cells) (Figs. 6 and 7). No significant difference among all of the groups was conversely observed when their cytolytic activity was tested against untransfected WiDr (Fig. 6B). These results suggest that the immunomodulating activity of GOLF is regimen specific and is not shared with the above-mentioned treatments even in the presence of GEM. Also in these experiments, the enhanced cytotoxic activity of GOLF-CTLs appears to be related with a better ability of these CTLs to recognize Ags such as CEA and TS.

Discussion

It is widely believed that immune resistance, like drug and radio resistance, may depend on the degree of cancer cell heterogeneity and thus on tumor burden. Various strategies have been investigated in the attempt to overcome this type of resistance. One possibility is to reduce tumor burden by combining the immunotherapy with radiotherapy and/or chemotherapy insofar as this could

lead to significant debulking and simultaneously affect the phenotype of tumor cells, thus making them more susceptible to the vaccine-activated effectors.

In an attempt to avoid the occurrence of immune resistance, a number of empirically designed clinical studies on different malignancies have investigated the possibility of combining biological agents and/or cytokines (e.g., IL-2 and IFN- α) with cytotoxic drugs. These trials provided heterogeneous results in terms of clinical response and survival (29–35). We and others have previously

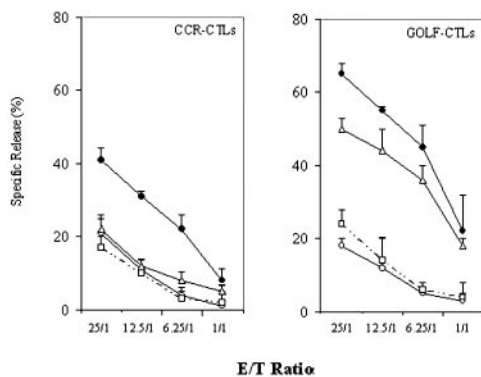


FIGURE 4. Cytotoxic ability of CCR-CTLs and GOLF-CTLs derived from the PBMCs of an A(*02:01)⁺ healthy donor (AF81) to lyse in ⁵¹Cr-release assays, HLA-A(*02:01)⁺ CIR-A2 target cells transfected with TS or CEA. The results are expressed as the percentage of specific lysis at different E/T ratios (mean values and SDs of triplicate determinations in individual experiments). Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing results from CCR-CTLs vs GOLF-CTLs against TS or CEA gene-transfected CIR-A2, the corresponding value obtained using the same cell line and CIR-A2 target cells transfected or not with plasmid backbone.

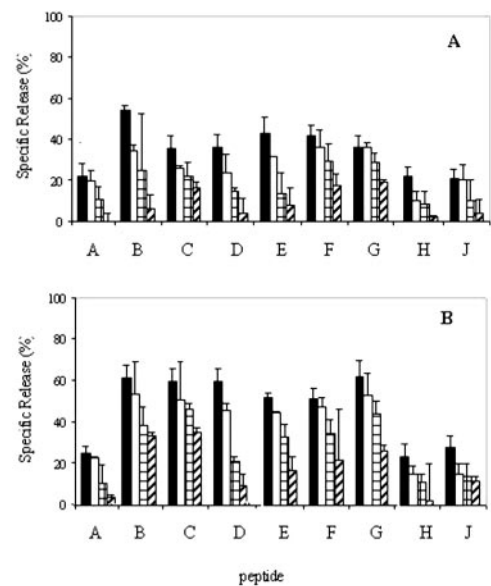


FIGURE 5. Cytotoxic activity of CCR-CTLs (A) and GOLF-CTLs (B) derived from the PBMCs of an HLA-A(*02:01)⁺ healthy donor (AF81) against peptide-pulsed CIR-A2 target cells. Target cells were pulsed with different known peptides with HLA-A(*02:01 binding motifs derived from TS (TS-1 (B), TS-2 (C), TS-3 (D)); CEA (CEAP-1 (E), CEAP-2 (F), CAP-1 (G)); and PTH-rP (PTR-2 (H) and PTR-4 (J)) 1 h before the cytotoxic assay. Unpulsed CIR-A2 (A) and CIR-A2 pulsed with PTR-2 and PTR-4 peptide derived from an Ag not expressed in colon cancer cells were used as a negative control. The results are expressed as specific release (%) at different E/T ratios (25:1 (■), 12.5:1 (□), 6.25:1 (▤), 1:1 (▨)). Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing results from GOLF-CTLs vs CRC-CTLs tested against TS-1, TS-2, TS-3, and CAP-1-pulsed CIR-A2 target cells. Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing the co-respective results obtained from each one of the two lines (GOLF-CTLs or CRC-CTLs) when tested against TS and CEA peptide-pulsed CIR-A2 vs those obtained when the same lines were tested against the controls (unpulsed CIR-A2 or CIR-A2 pulsed with PTR-2 and -4 peptide).

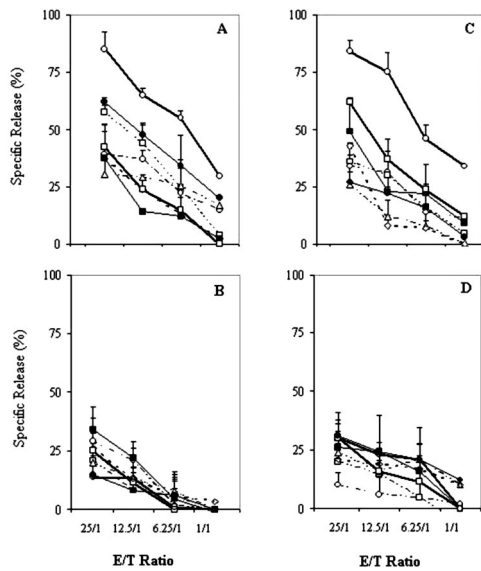


FIGURE 6. Cytolytic activity of CTL lines derived from the PBMCs of two healthy donors MD80 (A and B) and AF81 (C and D) sensitized against colon cancer cells exposed to different treatments (medium (—○—), 30,000 Rads (—□—), VP-16 (△), GEM (◇); IFL (■), GILF (□), OLF (◆); GOLF (—○—)) against HLA-A(*)02:01 transfected WiDr target cells (A2-WiDr) (A and C). Untransfected WiDr cells were used as a negative control (B and D). The results are expressed as specific release (%) at different E/T ratios. Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing results from GOLF-CTLs vs any other CTL line when tested against HLA-A(*)02:01-transfected WiDr target cells.

described the ability of cytotoxic drugs such as triazenes, 5-FU, VP-16, CPT-11, to sensitize tumor cells to the cytolytic activity of Ag-specific CTLs (1–4, 36). We first used this approach many years ago in a different tumor model, showing that the *in vivo* treatment of tumor-bearing mice with triazene compounds led to the appearance of novel transplantation Ag(s) in neoplastic cells provoking a more efficient cell-mediated tumor rejection (37). More recently, we found that the *in vitro* treatment of various colon and breast carcinoma cell lines with sublethal doses of 5-FU is followed by a significant increase in the expression of CEA (3) and TS (4), and a consequent immune sensitization to cytotoxic activity of class I HLA matching specific CTL lines. Other possibilities of overcoming the adaptive response of tumor cells and the consequent occurrence of Ag-specific immune resistance have been explored. They include the simultaneous immunization of cancer patients against multiple Ags by using as a vaccination means autologous irradiated cancer cells (37) or tumor cells induced to express inflammatory cytokines and coaccessory molecules by using the genetic engineering (38, 39), viral constructs (40), or heat shock proteins extracted by cancer cells and containing multiple Ag-derived peptides (41, 42). The rationale underlying this approach resides on the knowledge that dying tumor cells release Ags that are incorporated and processed by circulating APCs and presented to CTL precursors (3–5, 37). Similarly, several heat shock proteins released or extracted by tumor cells can deliver multiple tumor cell Ag epitopes directly to DCs expressing the specific receptors (43). In this context, Basu et al. (44) have shown that the occurrence of necrosis is indispensable to obtain a sufficient release of heat shock proteins (including the HSP-90, HSP-96, and HSP-70), which in turn deliver a partial maturation signal into the DCs. Therefore, the results of the latter study could suggest that the immune adjuvant property of GOLF regimen is dependent on its ability to induce in the tumor cells either necrosis

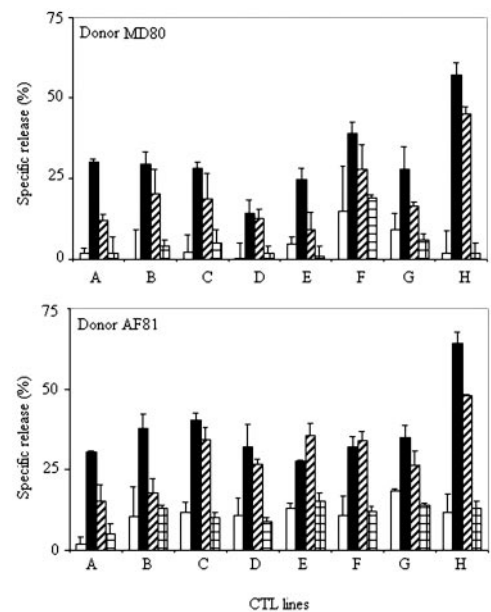


FIGURE 7. Cytolytic activity of CTL lines derived from the PBMCs of two healthy donors (MD80 and AF81) sensitized against colon cancer cells pre-exposed to different treatments (medium (A); 30,000 Rads (B); VP-16 (C); GEM (D); IFL (E), GILF (F), OFL (G), GOLF (H)) against TS (■) and CEA (▨) transfected CIR-A2; untreated CIR-A2 (□) and CIR-A2 transfected with the plasmid backbone (▤) target cells. The results are expressed as specific release (%) at an E/T ratio of 12.5/1. Significantly different values ($p < 0.05$, two-tailed t test) were observed comparing results from GOLF-CTLs vs any other CTL line when tested against TS or CEA gene-transfected CIR-A2.

and apoptosis a property which is not shared by the other treatments investigated in the present study. This particular GOLF-induced multiple modality of killing may, in fact, provide a stronger danger signal to either DCs and lymphocytes precursors giving rise to a more efficient CTL response.

Interesting information in this field also derived from the results of a more recent study investigating the immune-modulating effects of several antitumor agents like lymphokine-activated killer (LAK) cells, gamma-radiation, and 5-FU on the tumor uptake by HLA-matched DCs, and DC presentation of tumor Ags to autologous T lymphocytes.

The antitumor agents were chosen for their ability to induce completely different kinds of cell death in the same gastric cancer cells. An analogy to our work is that they found completely distinct patterns of class I MHC cross-presentation to CTL precursors of tumor-released Ags, when gastric carcinoma cells had been previously exposed to each one of the above-described agents. Even though LAK cells and radiation were the best inducers of apoptotic death, the highest rate of tumor uptake by monocyte-derived (GM-CSF driven) DCs was associated with 5-FU, followed by radiation. They also showed that DCs that had taken up 5-FU- or LAK-treated tumors, also up-modulated IL-12 production and presented tumor-associated Ag with increased efficiency, as shown by class I MHC-restricted IFN- γ release and cytotoxic responses by autologous lymphocytes (45).

These authors subsequently investigated the effects of apoptotic cell death in the same model induced by different chemotherapeutic agents on tumor phagocytosis by DCs and presentation to CTLs showing that the products of early apoptosis cannot efficiently cross-activate MHC class I-restricted antitumor lymphocytes even in the presence of DC-maturing factors. Conversely, secondary necrosis was found associated with robust T cell response. By

using the annexin-V-FITC (Ann-V) and propidium iodide (PI) staining cytofluorometric assay they were able to distinguish the occurrence of early apoptotic (Ann-V⁺/PI⁻) induced by cisplatin, by the late (after a 24 h) apoptotic/secondary necrotic (Ann-V⁺/PI⁺) death prevalently induced by the two anthracycline derivatives, epirubicin and doxorubicin. In this context they finally found the two anthracyclines able to increase tumor expression of HSP-70 and uptake of tumor cell components by DCs, whereas cisplatin treatment had no effect on HSP-70 and was associated with poor tumor uptake by DCs (46). The results of these studies seem to support our hypothesis that the contemporary induction of necrosis and apoptosis in cancer cells may be important for the generation of a more efficient Ag-specific immune response. The activation of intracellular pathways, which lead to necrosis and/or apoptosis, may in fact affect target Ag expression, the activation of danger signals (like HSP modulation), the production of immunomodulating (stimulating or suppressive) substances, which may significantly influence the efficiency of either Ag presentation and immune response.

Various empirical trials have investigated this possibility in colon carcinoma patients, some of which have obtained convincing results in terms of immunological and antitumor activity, especially when the immunological reagents have been used under minimal disease conditions (46–49)

In the present study, we have investigated the possibility of using a 5-FU-based multidrug treatment schedule that induces high levels of cell death induced by apoptosis and necrosis, Ag modulation, and maintaining a high HSP-90 expression in colon cancer cells. Moreover, under opportune immunological conditions, this drug combination can also be used to prime a multi-tumor-associated Ag-specific immune reaction in vitro. Our results, in fact, suggest the possibility of generating more effective multiantigenic CTLs in vitro by stimulating donor PBMCs with autologous DCs loaded with GOLF-treated tumor cell lysates. In comparison with those generated with Ags released by untreated colon carcinoma cells, these lymphocytes had greater killing ability against class I HLA-matching colon cancer cells and CIR-A2-expressing CEA and TS.

We hypothesize that chemotherapy enhances the expression and release of specific Ags and Ag peptide-loaded heat shock proteins in dying and apoptotic tumor cells. It follows that the uptake of these immunogenic molecules by DCs may allow the recognition of cryptic Ag epitopes by effector precursors, leading to a more effective antitumor activity.

In conclusion, our findings demonstrate the in vitro ability of GOLF multidrug chemotherapy to modulate the phenotype of tumor cells making them more immunogenic and provide a possible model for the design of clinical trials of chemoimmunotherapy of colorectal cancer.

Disclosures

The authors have no financial conflict of interest.

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