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Chemo-immunotherapy of colorectal carcinoma: preclinical rationale and clinical experience

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Summary Advanced colorectal cancer is a common disease with an high mortality rate. For four decades, pharmacological treatment of the advanced disease was based on the use of 5-fluorouracil alone or in combination with biomodulators such as folinic acid and intereferon alpha. In the last 5 years, response to therapy has been considerably ameliorated thanks to the discovery of new drugs such as oxaliplatin and CPT-11. These agents, in combination with 5-fluorouracil, according to various schedules of treatment, have reached a significant improvement of palliation, response rate and survival. Immunotherapy is an uprising modality of treatment for human cancer including colorectal carcinoma. Its rationale is based on the knowledge that tumour cells are genetically unstable and produce molecular structures which allow their recognition and destruction by the immune-surveillance system. Therefore, humoral as well as cellular compartments of the immune system can be utilized according to a "passive" strategy (e.g. monoclonal antibody administration and adoptive immunotherapy) or an "active" approach, by using different modalities of vaccine therapy. In this context, monoclonal antibodies (mAbs) and cancer vaccines are being tested for the treatment of advanced colorectal cancer. Due to their genetic instability and extraordinary adaptative potential, tumour cells may acquire resistance to the immune effectors and mAbs exactly as they do for cytotoxic drugs. To improve the results of both immunological and chemical modality of cancer treatment, an increasing number of authors is starting to combine chemo and immunotherapy in the attempt to circumvent the limitations of both strategies.

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This report tries to review the possible rationale of the chemo-immunotherapy combination, illustrating preliminary results of preclinical and clinical studies.

Key words: immunotherapy · chemo-immunotherapy · colorectal carcinoma

Conventional treatment of colorectal carcinoma

Colorectal carcinoma is the second most frequent cause of cancer-related deaths in Western countries. It affects about one million people every year throughout the world, 500,000 of whom die as a result of its complications. Although the disease is diagnosed in a stage formally classified as loco-regional phase (stage II–III) in 70–80% of cases, many of these patients are already affected by micrometastases. This means that, even after radical surgery, more than one-third of patients experiences tumor recurrence that leads to a worsening in their clinical condition and to a highly compromised prognostic picture [1].

During the last 15 years, a large number of controlled clinical trials have attempted to reduce relapse rate by using post-surgical (adjuvant) chemotherapy to eradicate the minimal residual disease. The majority of these studies used 5-fluorouracil (5-FU), alone or in combination with bio-modulators such as folinic acid (FA) or levamisole, in patients with stage II–III colorectal carcinoma. The results have clearly demonstrated that the adjuvant chemotherapy leads to a statistically significant 33% reduction in mortality only in the high-risk populations of patients with stage III disease, also showing that 5-FU/FA combination for six consecutive months is the best drug treatment choice in the adjuvant setting [2–6]. The mean 5-year survival of stage II patients was in fact 75%, whereas that of stage III patients was 25–50%, depending on the number of lymph nodes involved. The results of more recent studies suggest that adjuvant chemotherapy may also be useful as a means of extending the survival of stage II patients with negative prognostic factors at the time of diagnosis such as: (a) T4 stage; (b) intestinal obstruction or perforation; (c) a poorly differentiated neoplasm; (d) vascular, lymphatic or neuronal invasion; (e) microscopic lymph node invasion [2–10].

Until a few years ago, 5-FU (alone or in combination with bio-modulators) was the only recognised systemic palliative treatment for patients with metastases, whose mean survival rarely reached 12 months even in the most optimistic clinical trials [2–6]. However, the efficacy of the treatment of metastatic colorectal carcinoma has significantly improved since the advent of poly-chemotherapeutic regimens containing one of the latest third-generation cytotoxic drugs [2–9]. Both the CPT-11 topoisomerase inhibitor and the platinum derivative oxaliplatin have been tested along with 5-FU (in bolus or continuous infusions, or in oral pro-drug form). In a large number of different administration schedules, these drug combinations have been found to have a good toxicological profile and extraordinary anti-tumour activity. The patients undergoing poly-chemotherapy showed a high objective response rate (35–50%) and a real increase in survival [2–10]. According to a recent study sponsored by the National Cancer Institute, the combination of oxaliplatin with 5-FU and FA administered according to the regimen proposed by de Gramont et al. (FOLFOX-4) proved to have the best toxicological and efficacy profile. Therefore, this regimen is now the first-line reference treatment for metastatic colorectal carcinoma [9]. In any case, although the efficacy of these combinations has been confirmed in a large number of clinical trials, the prognosis of metastatic colorectal carcinoma is still poor. In fact, a mean patient survival is of less than 20 months, with very few patients with a single metastasis removed by radical surgery (metastasectomy), alive after 5-year [2–9]. These observations justify the continuous search for new drugs and, above all, new therapeutic strategies capable of overcoming drug resistance of tumor cells.

Immunotherapy and immune responses

It has long been known that the human body is equipped with an immune-surveillance system that possesses a number of different tasks, including that of host defence against overgrowth of transformed cells. To this end, the immune system makes use of a humoral component (antibody production) and a cellular component which may be antigen specific (cytotoxic T lymphocytes and delayed hypersensitivity), or antigen-independent natural immunity (natural killer cells and macrophages) [11].

Because of their genetic instability, tumour cells undergo gradual genotypic and phenotypic alterations during disease progression. This biochemical pattern, that is specifically associated with malignant phenotype, allows neoplastic cells to survive in the hostile environment of the host organism, and resist both chemo- and radiotherapies. However, this occurrence that has been always considered an adverse event, could offer unexpected opportunities. In fact, it is possible that altered protein structures generated spontaneously or under treatment with antitumor agents, would behave as actual neo-antigens. If this is the case, these altered molecules could represent ideal targets for cancer vaccine-activated antigen specific cytotoxic effector cells of host's immune system [12].

In line with this hypothesis, it must be pointed out that tumour cells synthesise fetal antigens such as carcino-embryonal antigen (CEA) [13] and mutated functional structures such as p53 and K-ras. In addition, malignant cells

could simply overexpress functional structures, such as growth factor receptors (EGFR), adhesion molecules (EP-CAM-17.A), enzymes (including thymidylate synthases) and/or the proteins responsible for pleiotropic drug resistance (PgP, MRPs and LRP), whose levels are extremely low in their normal counterparts [12, 13]. Under such conditions, these molecular structures can be considered actual tumour-associated antigens (TAAs) insofar as they are mainly or exclusively produced by tumour cells and recognised as non-self by the immune system [14].

The first attempts to treat colon carcinoma by means of immunotherapy were made in the early 1980s by infusing monoclonal antibodies against tumour antigens [15–24]. Moreover, non-specific stimulation of host's immune system was performed with levamisole, *Bacillum Calmette Guerine* (BCG) or inflammatory cytokines such as interferons and IL-2 with dismaying results [24].

More recently, novel and much more promising approaches have been introduced in passive and active immuno-therapies of colorectal carcinoma, as illustrated in the next paragraphs concerning a brief historical overview of these therapeutic modalities.

Passive immunotherapy

Immunotherapy is based on the host administration of monoclonal antibodies (mAbs) against membrane antigens expressed by tumour cells. These mAbs are lethal for target cells by means of complement activation and/or antibody-dependent cellular cytotoxicity (ADCC). Additionally, they may have a direct anti-tumor effect by binding to, and consequently functionally inhibiting, membrane receptors that are important for the proliferation and survival of tumour cells.

The 20-year history of passive immunotherapeutic approaches to colon carcinoma began with the use of murine antibodies that rapidly led to the formation of human anti-mouse immunoglobulin antibody response (HAMA). These HAMA were then responsible of interfering severely with subsequent administration of mouse mAbs.

Despite this, the 17-1A murine mAbs against the epithelial cellular-adhesion molecule (Ep-CAM) adhesion molecule (Edrecolomab/Panorex®) has been successfully used in the adjuvant treatment of colorectal carcinoma. Various studies have demonstrated that edrecolomab reduces the risk of relapse and increases patient survival at the price of an extremely low level of toxicity [25]. The cytotoxic mechanism of this mAb is still unknown, although it is suspected that it is mediated by ADCC along with activation of an anti-idiotypic network. One particularly interesting observation in these patients was that edrecolomab is capable of preventing remote metastases but does not protect against the appearance of loco-regional relapses. This suggested that edrecolomab may bind and opsonize only circulating tumour cells, thus facilitating phagocytosis in activated macrophages, granulocytes and, probably, dendritic cells. Two large international trials explored the role of edrecolomab in stage III colorectal cancer. The first study (157-001) was conducted on 1839 patients in North and South America adopting the Mayo Clinic treatment schedule with or without edrecolomab [26]. The second trial (157-002) was conducted on 2761 patients in Europe, New Zeeland, South Africa and Asia and explored the same schedule of chemotherapy, with or without edrecolomab, but with a control arm treated with the monoclonal antibody only [15]. In the first study a modest survival benefit was registered by the addition of edrecolomab, without differences in DFS [26]. In the 157-002 study no additional benefit in term of disease free survival (DFS) and overall survival (OS) was seen in edrecolomab/LV/FU arm. Moreover, OS in the arm treated with edrecolomab alone was significantly lower than that found in the chemotherapy arm [15].

Several hypotheses were considered to explain the conflicting results. It is recognised that the level of expression of target antigen on individual tumor cells, the antibody affinity and the presence of a minimal threshold number of antibodies bound to the target cells, which was dependent on antibody affinity, were of major importance for effective lysis through ADCC [16]. In colon carcinoma, Ep-CAM expression is found in a high proportion of cases, but the extent of expression varies substantially in intensity and homogeneity [17]. The activity of edrecolomab is higher against cells expressing higher amounts of Ep-CAM [17]. Probably, the low affinity of edrecolomab together with the high variability in Ep-CAM expression in colon cancer may partially explain the conflicting results reported [18]. Alternatively, the different outcomes in these studies may reflect a different patient population. Actually, the studies were conducted in different geographic regions, and population differences in regulation of immune responses may influence overall results [18]. Furthermore, differences in OS between the two studies are probably related to the effect of an imbalance in treatment for recurrence disease (post-study treatment effect) [19]. Another phase III study is conducting in North America and Europe (157-003) with the aim to compare the overall survival rates for patients treated with edrecolomab *vs* surgery alone after curative surgery for stage II colon cancer [20]. The results are expected in the near future.

Cetuximab (C225) is another mAb, currently being tested in colorectal carcinoma. It is a humanised chimeric G1 immunoglobulin recognizing the extracellular binding domain of the epidermal growth factor (EGF) receptor (EGFR/HERB-1). This receptor is a trans-membrane glycoprotein that is involved in signalling pathways affecting cellular growth, differentiation, proliferation and apoptosis [21]. EGFR/HERB-1 is overexpressed on the plasma mem-

brane of numerous epithelial neoplasms, including colorectal cancers. Furthermore, EGFR/HERB-1 seems to have an important role in the pathogenesis of CRC and its expression appears to be associated with poor survival and increased risk of invasion and metastasis [22]. It has been demonstrated that cetuximab inhibits the growth of CRC cell lines both in vitro and in vivo [23]. Binding of cetuximab to EGFR/HERB-1 inhibits the activation of receptor-associated intracellular thyrosine-kinase activity and consequently the proliferation of neoplastic cells. It is believed that the cellular mechanism of action of cetuximab is related to the inhibition of cell cycle progression, increased apoptosis, inhibition of angiogenesis, and a possibly amplification of the antineoplastic cytotoxic effects of chemotherapy [24].

Moreover, this antibody was found to enhance the antitumor effect of irinotecan in CRC cell lines in vitro and in mice xenograft models [27, 28]. In Phase I/II studies, cetuximab showed a good toxicity profile since the most frequent side effects were limited to skin rash, asthenia, fever, nausea, elevation in aminotranferases. Anaphylactoid or anaphylactic reactions occurred in about 2% of patients. The optimal biologic dose was determined by saturation of antibody clearance, and confirmed by blocking EGFR activation and downstream signalling in biopsy specimens from patients. This dose is reached with 400 mg/m^2 (initial "loading dose") followed by a weekly maintenance dose of 250 mg/m^2 [29]. In 120 patients with metastatic colorectal cancer (CRC) pretreated with irinotecan-containing regimens, cetuximab in combination with irinotecan showed a response rate and a disease control rate of 19% and 46%, respectively [30]. Three different small phase I/II studies reported preliminary but highly encouraging results with a response rate between 48% and 74%, and a disease control rate between 90% and 95% [31–33]. In the BOND trial, 329 patients affected by metastatic CRC overexpressing EGFR and with progressive disease after irinotecan-based chemotherapy, were randomised to receive either cetuximab plus irinotecan at the same dose and schedule on which they had been progressing or cetuximab as a single agent [34]. The results of this trial demonstrated a significant superiority of the combination schedule in terms of response rate (23% *vs* 11%), disease control (56% vs 32%), and median time to progression (TTP) (4.1 *vs* 1.5 months). In this study the median overall survival curves (8.6 *vs* 6.9 months) demonstrated only a trend in favour of combination schedule, without a real statistical significance. It was probably because OS was a secondary endpoint and the study was underpowered for it; furthermore cross-over was accepted. Both response and survival were higher in patients who presented skin rash, but there was no correlation between the percentage of EGFR-expressing cells or staining intensity and outcome. All these data indicate that cetuximab is able to overcome irinotecan resistance. Furthermore, the combination of cetuximab and the FOLFOX-4 schedule is currently being evaluated in the first-line setting and adjuvant chemo-immunotherapy studies using monoclonal antibodies are under investigation [35].

In recent years, another class of mAbs directed against components of tumor-associated neo-angiogenesis [36] process entered the antitumor armamentarium. Among them, the humanized mAb bevacizumab (Avastin^{TM}) has been utilized in clinical trials on the basis of its suppressive activity on vascular endothelial growth factor (VEGF) [37]. Bevacizumab is a humanized variant of a murine anti-human monoclonal antibody that in preclinical study showed the ability to block VEGF. The rational bases of the use of this antibody stem from the biological role of VEGF, a diffusible glycoprotein produced by normal and neoplastic cells, which is a key agent for tumor associated neo-angiogenesis [36]. Bevacizumab has shown the ability to inhibit the growth of human tumor xenografts and has been tested alone or combined with different cytotoxic drugs in several Phase I–II clinical trials in colorectal carcinoma patients [38]. The results of these trials have been conflicting and sometime disappointing [39]. Nevertheless, a small trial involving patients who had received no treatment for advanced colorectal carcinoma, showed that the addition of bevacizumab to 5-FU + leucovorin (FL) increased the response rate from 17 to 40% [40]. As expected, this finding promoted the clinical experimentation of the antibody in combination with cytotoxic drugs. Recently, Hurwitz and colleagues [41] conducted a large multicenter randomized trial which involved 813 patients with metastatic colorectal carcinoma with no prior chemotherapy, aimed to compare the effects of combination of bevacizumab with irinotecan (I) + 5-fluorouracil (F) + leucovorin (L) (IFL) chemotherapy with placebo + IFL. The authors reported in the chemoimmunotherapy arm a significant increase in response rate (44.8 *vs* 34.8%, $P = 0.004$), median progression free survival (10.6 vs 6.2 months, $P = 0.001$) and above all survival (20.3 *vs* 15.6 months, $P < 0.001$). All of the patients who progressed were allowed to switch the treatment to FOLFOX and continue the treatment with the mAb. Toxic effects of bevacizumab + IFL were more pronounced respect to those of chemotherapy alone. However, they were mainly limited to grade 3 hypertension (11% of patients) and increased thrombophilia risk. The results of this study demonstrated the clear superiority of the combined chemoimmunotherapy treatment over chemotherapy alone.

The results of this study were so impressive that they recalled the media attention and promoted a controversial registration by the Food and Drug Administration of bevacizumab. The antibody was indicated in the treatment of metastatic CRC in combination with the two currently recommended treatment schedules, i.e. FOLFIRI and FOLFOX. This is a very controversial point for several reasons: (a) the

authors of the trial did not produce any direct or indirect demonstration of biological effects in vivo of bevacizumab; (b) it was hypothesized that the mAb could increase tumor vessel permeability to the drugs (i.e. 5-FU, FA, and irinotecan), thus enhancing their bioavailability at the tumor site. Nowadays, the FL schedule has been substituted by the two new treatment regimens named FOLFIRI and FOLFOX, where 5-FU is given as continuous infusion instead of short-term or bolus infusion. The two treatment schedules have shown different pharmacokinetics which leads to reduced side effects and improved anti-tumor activity [42].

Other anti-EGFR antibodies, such as ABX-EGF, EMD 72000, h-R3, bi-specific antibodies, such as M 26.1, MDX-447, and H 22-EGF, showed a good safety profile and are now under early clinical investigation [29].

As the number of monoclonal antibodies under clinical development keeps growing, it is becoming evident that it will be far more complex to demonstrate clinical benefit with these agents than with conventional cytotoxic drugs. There are several obstacles to the use of molecular-targeting agents in clinical trials. They include patient's selection, identification of appropriate biologically active doses from phase I studies, treatment schedules, optimal combinations with chemotherapy, radiotherapy or other molecular targeted therapies. Another difficulty in translating preclinical results to clinical setting is the limited predictive value of preclinical models, so that the dose and schedule tested may be sub-optimal and bulky disease may not be responsive. In this context, cetuximab may be considered like as a paradigm. Preliminary data revealed a relationship between intratumor EGFR expression and drug efficacy. However, several other studies failed to demonstrate this assumption, because other factors, i.e. activation of downstream signaling pathways, presence of other activating growth factors and/or EGFR mutations, could influence target cell response. In this regard, the development and validation of immunohistochemical methods measuring the activation of EGFR-pathways with phosphorylation-specific antibodies could be relevant [29]. Besides, dose selection has been based on toxicity as well as pharmacokinetic parameters, such as plasma concentrations above a biologically relevant level or saturation of clearance, which suggests a complete occupancy of drug-binding sites. Preclinical studies showed a linear relationship between inhibition of the receptor mediated signaling pathways and antitumor activity [29]. To determine the biologically relevant dose, tumor could be sampled with biopsy before and after treatment, but generally it is very difficult to obtain sequential tumor tissues specimens. With the aim to overcome this problem, investigators have used normal skin to develop pharmacodynamic surrogate markers of EGFR inhibition. This approach has two relevant limits: there is not necessarily a similar relationship between EGFR inhibition in epidermis and cancer tissues, and the downstream effects of EGFR inhibition could be molecularly different in the tumor [43]. Furthermore, it is important to establish the activity of these cytostatic compounds in phase II/III trials, also including functional imaging techniques, such as MRI and PET, and/or surrogate biomarkers, such as EGFR inhibition in the skin, which are all under investigation.

Active immunotherapy

Specific active immunotherapy or vaccine-therapy is founded on Jensen's principle that the administration of particular biological agents (vaccines) stimulates an immune response in the host. This antigen-elicited immunity may be non-specific, or specifically directed against particular molecular structure(s) [target antigen(s)],

The hypothesis underlying active immunotherapy in cancer treatment is that an efficient immune response against neoplastic cells cannot be initiated by malignant cells alone. In fact, the response must be properly strengthened by one of the various vaccination strategies currently available, so that target cells could be recognised and killed by effector lymphocytes (cytotoxic T lymphocytes). Thereafter, the consequent release of antigens from dying tumour cells, allows the anti-tumour immune reaction to become sufficiently self-sustaining and self-potentiating as to provide prolonged systemic anti-tumour protection.

Unlike antibodies, which recognise epitopic segments inside whole antigenic molecules, activated effector lymphocytes and their precursors use membrane receptors (T cell receptors: TCRs) to recognise antigenic structures, such as small peptide epitopes. These peptides, generated from proteasome-dependent intracellular proteolysis, are bound to class I (8–9 aminoacids recognized mainly by CD8+ effector lymphocytes) or to class II (higher and more variable number of aminoacids, mainly recognized by CD4+ effector lymphocytes) molecules of the major histocompatibility complex (MHC) present on target cell membrane and antigen-presenting cells [44, 46]. The surfaces of the majority of human cells express a number of peptide epitopes that are bound to human MHC (i.e. HLA) molecules. In case of malignant cells, HLA-bound non-self peptides derived from "tumor-associated antigens" (TAA), can be recognised by activated antigen-specific cytotoxic effector lymphocytes. This can be demonstrated even though neoplastic cells are incapable per se to initiate *de novo* immune responses.

Once the TCR has bound the antigen-specific epitope presented by MHC, an intracellular signal activates the cytotoxic capacity of lymphocytes. However, this biochemical event is not sufficient to start immunocompetent cell proliferation required for clonal expansion of antigen-specific lymphocytes. In order to activate adequate lymphocyte proliferation processes, a correct TCR/epitope/HLA interaction is not

sufficient. Actually, a correct lymphocyte/target cell interaction must take place between co-accessory molecules [B7.1 (CD80), B7.2 (CD86), CD40, LFA, ICAM)] present on stimulator cell membrane, and specific counter-receptors (CD28, CD40L, LFA, etc.) expressed on lymphocyte membranes. In the absence of this latter interaction, immune lymphocytes enter apoptosis, which leads to the progressive exhaustion of the specific clone and consequent antigen-selective immuno-depression [47]. Tumour cells do not express co-accessory molecules and are therefore incapable of initiating or amplifying an antigen-specific immune response. However, co-accessory molecules are present in the heterogeneous class of so-called professional antigen-presenting cells (APCs), which are the only cells capable of initiating an antigen-specific immune response. These include dendritic cells (DCs), B lymphocytes and macrophages, and are present in lymph nodes, medullary and peripheral blood, in dermis and sub-mucosa [48–50]. APCs have the unique capacity of up-taking, incorporating and processing antigens released by virus-infected cells or tumour cells, and correctly presenting the HLA/epitope complex to precursors of effector lymphocytes [47–49].

Various methods can now be used to initiate an *in vivo* immune response, some of which are currently being tested in patients with colorectal carcinoma. The strategically simplest method is the parenteral administration of irradiated (autologous or allogenic) tumour cells, especially if they are administered together with immuno-adjuvants such as BCG, which attract a large number of APCs to the injection site [51].

The anti-tumour activity of the re-injection of autologous tumour cells dissolved in OncoVAX^{TM} (a preparation containing tumour cells + BCG), and the toxicity of the vaccination, has already been studied in patients with colorectal carcinoma by various authors. These studies have shown that such injections have limited activity in controlling metastatic disease, but have led to much more interesting results in the context of adjuvant treatment [52]. Randomised trials (some carried out in the 1980s) demonstrated an advantage in terms of 5-year disease-free survival in stage II-III patients given multiple vaccinations of irradiated autologous tumour cells + BCG after surgery, in comparison with survival of patients treated with surgery alone [52].

Unfortunately, a multi-centre trial sponsored the Eastern Co-operative Oncology Group (ECOG) failed to confirm these data and found no difference between its vaccine and support therapy groups, thus inducing a certain scepticism towards this therapeutic approach [35]. However, a subsequent quality control study of the reagents used in the ECOG trial showed that a reagent used in about 12% of the patients failed to pass the quality controls: 15% of these were in the experimental arm and showed no signs of specific immunisation as revealed by means of delayed skin hypersensitivity reaction testing (DTH) [52]. Finally, a new meta-analysis of four prospective randomised trials (immunotherapy *vs* control) involving 723 patients in stage II–III after surgery definitively demonstrated a significant advantage in terms of recurrence-free intervals and recurrence-free survival (and, in one study, also overall survival) in stage II patients, who showed a clear delayed hypersensitivity reaction after treatment (DTH >5 mm) [53].

The main lesson learned from these studies is that, unlike chemotherapy (which acts directly on neoplastic cells), specific active immunotherapy provides anti-tumour effects mediated by the host's immune system. Consequently, before evaluating the clinical response, it is necessary to produce clear and detailed data concerning the characterisation of the immune response specifically induced by the vaccine at both cellular and humoral level.

Another point confirmed by these studies is that immunotherapy seems to work best under conditions of *minimal disease*: i.e. when the highly immunosuppressive tumour mass has been reduced to a minimum and can be more easily eliminated by an immune reaction. This strategy can certainly take advantage of the new genetic engineering techniques that can induce tumour cells used for sensitization to produce cytokines and co-accessory molecules [54, 55]. The same techniques are also utilized for preparing new vaccine generations utilizing virosomes or immunogenic viral infection [56, 57].

A number of studies have already shown that, in order to obtain an immune response, the antigens contained in, or released by, tumour cells must be incorporated and processed by APCs. These "professional" cells committed for antigen presentation are capable of presenting peptide epitopes or CD1-restricted non-peptide lipid antigens [58] to lymphocyte precursors and thus initiating an immune reaction. This process is known as *cross-priming* and is mainly mediated by the DCs present in peripheral blood [59].

The discovery of this mechanism has induced many researchers to change their approach by replacing the administration of lysed or irradiated tumour cells with the administration of *ex vivo* cultured autologous DCs loaded with tumour antigens. In this case, the source of antigenic material is represented by tumor-associated proteins, viral or complementary DNA, m-RNA, heat shock proteins, or whole tumour cell lysates [60].

Heat shock proteins (HSPs) are a heterogeneous family of proteins that share the common characteristic of being expressed in situations of cell stress (such as that induced by heat). The most immunogenic are known as gp96, hsp70, hsp90 and calreticulin [61]. The HSPs in tumour cells are loaded with epitopic peptides. If dying or decaying tumour cells release them, the HSPs can be incorporated into DCs by means of a receptor-mediated mechanism, and thus give rise to an immune response [62]. The advantage of adminis-

tering HSPs therefore resides in the fact that these antigenic peptides are directly delivered to the DCs, thus allowing poly-epitopic and poly-antigenic immunisation [60].

Immunotherapy for advanced colorectal carcinoma

An even more recent immunotherapeutic approach is related to the generation of antitumor vaccines against specific tumour-related or tumour-specific antigens. As mentioned above, it is now widely accepted that lymphocytes recognise antigens bound to HLA molecules on the membrane of target cells. However, this binding is not random but determined by specific amino acid sequences in the epitopic peptides known as *amino acid consensus motifs*. Many of the sequences specific for the more common HLA haplotypes have already been published [63]. It is therefore possible to identify and predict the number of epitopic sequences present in a specific antigen with a known amino acid sequence [64], and this has allowed the use of DC exposed to these epitopes *ex vivo* in order to stimulate a mono-antigenic lymphocyte response with both *in vitro* and *in vivo* anti-tumour activity [65, 66].

Various immunotherapeutic approaches to colorectal carcinoma are currently being tested in clinical trials using CEA-derived immunogenic peptides with or without DCs [67–71]. Preliminary results have shown that it is possible to induce an antigen-specific cellular or humoral response, even if it has not yet been demonstrated that successful immunisation leads to a clinical benefit or an advantage in terms of survival.

In line with this approach, a number of experimental protocols have been developed for the *ex vivo* generation of antigen-specific cytotoxic T-lymphocyte (CTL) cell lines directed against the most common tumor-associated antigens (TAAs, e.g.CEA, PSA, PTH-rP, etc). These CTL lines, that can be produced from the peripheral blood mononuclear cells (PBMCs) of both normal and cancer patients, can be reinfused in patients with immuno-therapeutic intent [44, 64, 72–74].

The possible clinical applications of chemo-immunotherapy

As in the case of pharmacological treatments, one of the main causes of the failure of immunotherapy is the appearance of effector-resistant tumour cells. Tumour cells can escape a vaccine-activated immune system because of their reduced expression of the MHC/peptide complex or of a defect in antigen processing due to the secretion of APC suppressive factors [75, 76]. Another possibility is that, even if they are recognised by the immune system, tumour cells can resist the cytotoxic attack by effector lymphocytes [75, 76]. Like drug and radio-resistance, the appearance of immuno-resistant neoplastic cells depends on their heterogeneity and the tumour burden [75, 76].

Various preclinical and clinical studies are attempting to circumvent this problem by combining immunotherapy with cytoreductive strategies (chemotherapy, radiotherapy and surgery). The rationale underlying this combination is based on the fact that, in addition to rapid debulking, chemotherapy also cause phenotypic alterations in tumour cells that make them more susceptible to the cytotoxic activity of effector cells. This phenomenon can be due, at least in part, to changes of the antigenic/immunogenic profile of malignant cells (i.e. "drug-induced antigen remodelling", DIAR). This includes changes of the expression of antigens already present in cancer cells (see below), or induction of novel antigenic specificities, as demonstrated in the case of triazene compounds [77]. A number of empirically designed trials involving patients with colorectal, kidney, pancreas and liver cancers have already been carried out with the aim of evaluating cytotoxic treatment combined with biological agents and cytokines (e.g. IL-2 and IFN-α). However, the results of these studies appear to be heterogeneous and scarcely reproducible in terms of objective responses and clinical benefit [78–81].

Over the last ten years, a number of researchers have examined the possibility of using drugs and biological agents (Table 1) in order to increase at least the *in vitro* susceptibility of tumour cells to the cytotoxic effectors of the immune system [i.e. CTL, tumor infiltrating lymphocytes (TIL) or lymphokine-activated killer (LAK) cells] by means of various mechanisms of action [82].

For example, our group has reported that briostatin 1 (a biological agent derived from the *bungula neritina* sea sponge) can sensitise breast and colon cancer cells to the cytotoxic effect of LAK cells by modulating the expression of the adhesion molecules involved in target effector adhesion [83]. It has also been demonstrated that the calcium depletion induced by the calcium antagonist verapamil can sensitise the same tumour cells to the cytotoxic effect of LAK cells *in vitro* by potentiating the lymphocyte-activated cytotoxic hit [84]. The findings of this last study led to the design of a phase I clinical trial in which verapamil was administered in combination with IL-2 (subcutaneously and intravenously) to patients with various neoplasms including metastatic colorectal carcinoma. The results of this investigation provided evidence that the treatment afforded noticeable effects in terms of clinical benefit and limited toxicity [85].

Other more recently published studies have demonstrated the possibility of using sub-lethal doses of cytotoxic drugs in order to increase the sensitivity of tumour cells to the lethal effects of TILs, of non-HLA-restricted LAK cells, as well as of antigen-dependent effector CTL. A first study showed that cisplatin and VP16 made prostate carcinoma cells more sensitive to the lytic effects induced by CTLs,

Table 1. History of The Pharmacological Treatment of Advanced Colorectal Cancer

Drug	Biomodulator or Drug
Monochemotherapy era (1950–1999): (Thymidylate synthase inhibition)	
5-fluorouracil	Alone
	Levamisole
	Intereferon alpha
	Folinic acid
Raltitrexed	
Poly-chemotherapy era (2000–2004):	
Bolus 5-fluorouracil (IFL)	Folinic acid, CPT-11
Infusional 5-fluorouracil (FOLFIRI)	Folinic acid, CPT-11
Oral fluoropyrimidines (XELIRI)	
5-fluorouracil (FOLFOX)	Folinic acid, Oxaliplatin
Oral fluoropyrimidines (XELOX)	
Passive immunotherapy (1999–2002):	
Edrecolomab (anti Ep-CAM moAb)	
Cetuximab (anti EGF receptor moAb)	
Bevacizumab	
Active specific immunotherapy (1995–2004):	
CEA directed vaccines	
Mucine directed vaccines	
Co-accessory molecules based vaccine	
Autologous cancer cell vaccination	
Chemoimmunotherapy (2002–2005):	
CPT-11 and 5-fluorouracil/folinic acid	Cetuximab
CPT-11 and 5-fluorouracil/folinic acid	Bevacizumab
Gemcitabine, oxaliplatin, 5-fluorouracil,	Granulocyte
Folinic acid	Macrophage Colony Stimulating Factor (GM-CSF)
	Interleukin 2

TILs and LAK cells [84]. A second study described similar results in colon carcinoma cells that become more sensitive to antigen-specific CTLs after a pharmacological pretreatment 5-FU, CPT-11 and cisplatin [with or without interferon-alpha (INFα)] [85]. The results of both studies suggested that these drugs might be able to amplify the intracellular death signal (FAS/FASL, TRAIL- BCL2/BAX-pathway) activated in the tumor target cells by the killing machinery (cytokines, FASL, perphorins extracellular ATP, etc) used by the effector lymphocytes.

A further example of 5-FU-induced immunosensitisation comes from an *in vitro* model of colon and breast cancer in which both CEA and thymidilate synthase (TS) were selected as CTL target antigens. In this model, 5-FU-based chemotherapy simultaneously increased the expression of both antigens and the sensitivity of the tumour cells to the cytotoxic effects of the CEA- and TS-specific lymphocytes. This immunosensitising effect was certainly related to the increased antigen expression insofar as the cytotoxicity was restricted by HLA molecules and inhibited in antigen competition assays [88, 89]. This finding was particularly important because TS is the enzymatic target of 5-FU (the basic drug for the treatment of colon carcinoma), and its increased expression in tumour cells leads to resistance to chemotherapy. One of these studies also demonstrated that activated lymphocytes were resistant to 5-FU, which is much more toxic for target immunocompetent cells during the proliferation phase of clonal expansion [88]. These results justify the use of combined chemo- and immunotherapy in the treatment of colon carcinoma.

It has been hypothesised that the immune system cannot eradicate tumours since compromised APCs are not capable of presenting TAA epitopes to CTL precursors, thus suggesting that TAAs may not be immunogenic *in vivo* [49, 57, 89]. However, it is necessary to point out that, in order to activate an antigen-specific immune response, any immunotherapeutic agent or approach must take into consideration the role of APCs (particularly DCs) [47–49, 57, 90]. Tumour tissues contain necrotic and apoptotic cells for a number of different reasons [89, 90] especially after the exposure to an effective chemotherapy treatment. The material of decaying cells and the apoptotic bodies contain antigens and CTL epitopes. This material can give rise to an antigen specific immune-response only if : (a) it is released into lymphatic vessels and the blood stream, and (b) it is subsequently incorporated into APCs like DCs. These cells are in fact, able to process these antigens, and present the derived epitopes to the CTL precursors.

A rationale use of cytokines able to increase the number and the activity of peripheral DCs in vivo after polychemotherapeutic treatment, could give rise to an antigen-specific immune reaction with anti-tumour activity. This mechanism would generate a sort of tumour-specific self-vaccination with the antigens released by drug-damaged cancer cells [47–49, 57, 90].

On the basis of such precise immunobiological and pharmacological factors, it has therefore been hypothesised that the combination of "traditional" chemotherapy with immunotherapy may be worth exploring in order to improve the results of both. We have recently designed a phase II trial to test anti-tumour and immunological activity and toxicity of a chemo-immunotherapy with gemcitabine, oxaliplatin, leucovorin and fluorouracil (GOLF) followed by subcutaneous GM-CSF and IL-2 in patients with advanced colorectal carcinoma. Preliminary studies have shown that the GOLF chemotherapy regimen is well tolerated and active in the treatment of colon carcinoma. It also induces phenotypical alterations in tumour cells, with the appearance and/or overexpression of TAAs that can be recognised by the immune system. The results of various preclinical studies demonstrate that it can sensitise tumour cells to the cytolytic effect

of cytotoxic T-lymphocytes by: (a) increasing the expression of TAAs such as CEA and TS; (b) interfering with the anti-apoptotic defence mechanisms of tumour cells. It is also capable of inducing processes of programmed cell death in tumour tissues, with the consequent formation of apoptotic bodies. These bodies are incorporated by APCs such DCs, monocytes, macrophages and B-lymphocytes, and activate immunological processes similar to those elicited by the use of the latest techniques of anti-cancer vaccination. Sequential combination with GM-CSF (molgramostim) and IL-2 has been proposed in order to potentiate chemotherapy on the bases of DIAR strategy. It is well known that GM-CSF plays a role in activating DCs and macrophages, and that IL-2 is involved in the stimulation of both T- and B-lymphocytes [93, 94]. Furthermore, the results of a recent study show that the sequential combination of GM-CSF and IL-2 is active and well tolerated in cancer patients, even with advanced disease, including colon carcinoma. Moreover, the biological activity of this cytokine combination seems to be an ideal support for vaccine-therapy protocols. In fact, after only one treatment cycle, the peripheral blood of the patients showed: (a) an increased concentration and function of activated DCs and monocytes; (b) an increased level of memory-T lymphocytes and CD4 cells with a cytotoxic phenotype (TH1) [95].

In conclusion, the combination of chemo- and immunotherapy may have a dual function. Chemotherapy can initially cause cytoreduction, over-expression (DIAR) and release of TAAs, formation of apoptotic bodies, and sensitisation of tumour cells to the cytolytic activity of effector lymphocytes. Subsequent treatment with cytokines (e.g. with GM-CSF and IL-2) could stimulate antigen presentation, and expand tumor-specific CTL clones, thus originating and supporting an immune response with potential anti-tumour activity. Our GOLF + GM-CSF + IL-2 protocol can be considered a good example of this type of approach. In fact, aims of the trial were: (a) to evaluate the treatment's anti-tumour activity in terms of the percentage of responses and time to disease progression; (b) to evaluate its toxicity on the basis of the onset of adverse events; and (c) to evaluate its immunobiological activity. The preliminary results in the first 15 patients pointed out that this regimen provides considerable anti-tumour activity (i.e. a 70% objective response rate) with a time to progression of more than eight months. Furthermore, the responding patients showed self-immunisation against CEA, TS and colon carcinoma cell lysates, demonstrated by means of proliferative responses of patient's lymphocytes challenged with these tumor antigens. Finally, seven patients with an HLA-A2 haplotype showed an increase in lymphocyte precursors specific for known epitopes of CEA and TS with high receptor affinity [96].

On the basis of these considerations, we believe that combined chemo- and immunotherapy certainly warrants further investigation as a means of maximising the anti-tumour effect of both treatment modalities.

References

1. Ferlay J, Bray F, Pisani P, Parkin DM (2001) Cancer incidence, mortality and prevalence worldwide. In: GLOBOCAN 2000 version 1.0 IARC CancerBase No. 5. IARC Press, Lyon
2. Grem JL (1996) 5-Fluoropyrimidines. In: Chabner BA, Longo DL (eds) Cancer chemotherapy and biotherapy principles and practice, 2nd edn. Lippincot William & Wilkins, Philadelphia, pp 149–163
3. Francini G, Petrioli R, Lorenzini L, Mancini S, Armenio S, Tanzini G, Marsili S, Aquino A, Marzocca G, Civitelli S, Mariani L, De Sando D, Lorenzi M (1994) Folinic acid and 5-fluorouracil as adjuvant chemotherapy in colon cancer. Gastroenterology 106:899–906
4. O'Connell MJ, Laurie JA, Kahn M, Fitzgibbons RJ Jr, Erlichman C, Shepherd L, Moertel CG, Kocha WI, Pazdur R, Wieand HS, Rubin J, Vukov AM, Donohue JH, Krook JE, Figueredo A (1998) Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. J Clin Oncol 16:295–300
5. Skibber JM, Minsky BD, Hoff PM (2001) Cancer of the Colon. In: De Vita V, Hellman S, Rosenberg SA (eds) Cancer principles and practice of oncology, 6th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1216–1271
6. Von Hoff DD (1998) Promising new agents for treatment of patients with colorectal cancer. Semin Oncol 25:47–52
7. Salts LB, Locker PK, Pirotta N, Eltring GL, Miller LL (1999) Weekly irinotecan (CPT-11), leucovorin (LV), and Fluorouracil (FU) is superior to daily x 5 LV/FU in patients with previously untreated metastatic colorectal cancer. ("Abstract"). Proc Am Soc Clin Oncol 18:898
8. Fischel JL, Etienne MC, Formento P, Milano G (1998) Search for the optimal schedule for the oxaliplatin/5-fluorouracil association modulated or not by folinic acid: preclinical data. Clin Cancer Res 4:2529–2535
9. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR (2004) A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 22:23–30
10. Gill S, Loprinzi CL, Sargent DJ, Thomé SD, Alberts SR, Haller DG, Benedetti J, Francini G, Shepherd LE, Seitz JF, Labianca R, Chen W, Cha SS, Heldebrant MP, Goldberg RM (2004) Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: who benefits and by how much? J Clin Oncol 22:1797–1806
11. Rosenberg SA (1992) Karnofsky Memorial Lecture. The immunotherapy and gene therapy of cancer. J Clin Oncol 10:180–199
12. Wang RF, Rosenberg SA (1999) Human tumor antigens for cancer vaccine development. Immonol Rev 170:85–100
13. Thompson JA, Grunert F, Zimmermann W (1991) Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. J Clin Lab Anal 5:344–366
14. Evans TR, Kaye SB (1999) Vaccine therapy for cancer—Fact or fiction? Q J M 92:299–307
15. Swartzberg L (2001) Clinical experience with edrecolomab: a monoclonal antibody therapy for colorectal carcinoma. Crit Rev Oncol Hematol 40:17–24
16. Fields AL, Keller AM, Schwartzberg L, Bernard S, Pazdur R, Kardinal C, Cohen J, Schultz J, Eisenberg P, Barber L, Edmundson

S, Wissel P (2002) Edrecolomab (17-1A antibody) in combination with 5-Fluorouracil based chemotherapy in the adjuvant treatment of stage II colon cancer: results of a randomized North America phase III study. Proc Am Soc Clin Oncol 21:128a(abstract 508)
17. Punt CJ, Nagy A, Douillard JY, Figer A, Skovsgaard T, Monson J, Barone C, Fountzilas G, Riess H, Moylan E, Jones D, Dethling J, Colman J, Coward L, MacGregor S: Edrecolomab alone or in combination with fluorouracil and folinic acid in the adjuvant treatment of stage III colon cancer: a randomised study. Lancet 31;360(9334):671–677
18. Velders MP, van Rhijn CM, Oskam E, Fleuren GJ, Warnaar SO, Litvinov SV (1998) The impact of antigen density and antibody affinity on antibody-dependent cellular cytotoxicity: relevance for immunotherapy of carcinomas. Br J Cancer 78:478–483
19. Pantel K, Braun S, Passlick B, Schlimok G (1996) Minimal residual epithelial cancer: diagnostic approaches and prognostic relevance. Prog Histochem Cytochem. 30(3):1–60
20. Veronese ML, O'Dwyer PJ (2004) Monoclonal antibodies in the treatment of colorectal cancer. Eur J Cancer 40:1292–1301
21. Alliot C, Spizzo G, Punt CJ (2003) Edrecolomab in the adjuvant treatment of colorectal carcinoma. Lancet 361:82–83
22. Haller DG: Update of clinical trials with edrecolomab (2001) a monoclonal antibody therapy for colorectal cancer. Semin Oncol 28(1 Suppl 1):25–30
23. Baselga J (2002) Why the epidermal growth factor receptor? The rationale for cancer therapy. Oncologist 7(Suppl 4):2–8
24. Hemming AW, Davis NL, Kluftinger A, Robinson B, Quenville NF, Liseman B, LeRiche J (1992) Prognostic markers of colorectal cancer: an evaluation of DNA content, epidermal growth factor receptor, and Ki-67. J Surg Oncol 51:147–152
25. Liu B, Fang M, Schmidt M, Lu Y, Mendelsohn J, Fan Z (2000) Induction of apoptosis and activation of the caspase cascade by anti-EGF receptor monoclonal antibodies in DiFi human colon cancer cell lines do not involve the c-Jun N-terminal kinase activity. Br J Cancer 82:1991–1999
26. Mendelson J (2001) The epidermal growth factor receptors: a target for cancer therapy. Endocr Relat Cancer 8:3–9
27. Ciardiello F, Bianco R, Damiano V, De Lorenzo S, Pepe S, De Placido S, Fan Z, Mendelsohn J, Bianco AR, Tortora G (1999) Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin Cancer Res 5:909–916
28. Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ (2002) Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. Cancer Res 8:994–1003
29. Mendelson J, Baselga J (2003) Status of epidermal growth factor receptor antagonists in biology and treatment of cancer. J Clin Oncol 21:2787–2799
30. Saltz L, Rubin M, Hochster H, Tchekmeydian NS, Waksal H, Needle A, LoBuglio A (2001) Cetuximab (IMC-C225) plus irinotecan (CPT-11) ia active in CPT-11 refractory colorectal cancver (CRC) that expresses epidermal growth factor receptor (EGFR). Proc Am Soc Clin Oncol 20:3a(Abstr 7)
31. Lutz MP, Schoffski P, Folprecht G, Haag C, Seufferlein T, Beutel G, Marschner JP, Mueser M, Harstrick A, KöHne H (2001) A phase I/II study of cetuximab (C225) plus irinotecan (CPT-11) and 24 h infusional 5 F U/folinic acid (FA) in the treatment of metastatic colorectal cancer (MCRC) expressing the epidermal growth factor receptor (EGFR). Ann Oncol 13(Suppl 5):73(abstract 265PD)
32. Rosenberg AH, Loehrer PJ, Needle MN, Waksal H, Hollywood E, Ramos L, Saltz LB (2002) Erbitux (IMC-C225) plus weekly irinotecan (CPT-11), fluorouracil (5FU) and leucovorin (LV) in colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFr). Proc Am Soc Clin Oncol 21:135a (Abstr 536)
33. Van Laethem JL, Raoul JL, Mitry E, Brezault C, Husseini F, Cals L, Vedovato JC, Mueser MM, Rougier P (2003) Cetuximab (C225) in combination with bi-weekly irinotecan (CPT-11), infusional 5-fluorouracil (5-FU) and folinic acid (FA) in patients (pts) with metastatic colorectal cancer (CRC) expressing the epidermal growth factor receptor (EGFR). Preliminary safety and efficacy results. Proc Am Soc Clin Oncol 22:264 (Abstr 1058)
34. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 351:337–345
35. van den Eertwegh AJM, Baars A, Pinedo HM (2001) Adjuvant treatment of colorectal cancer: toward tumor-specific immunotherapies. Cancer Metastasis Rev 20:101–108
36. Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186
37. Kabbinavar F, Schulz J, McCleod M, Patel T, Hamm J, Hecht J, Perrou B, Griffing S, Nelson B, Novotny W (2004) Bevacizumab, a monoclonal antibody to vascular endothelial growth factor, prolongs progression-free survival in first line colorectal cancer in subjects who are not suitable candidates for first-line CPT-11. Proc Am Soc Clin Oncol 22:249(abstract 3516)
38. Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400
39. Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E (2003) Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 21:60–65
40. Fernando NH, Hurwitz HI (2004) Targeted therapy of colorectal cancer: clinical experience with bevacizumab. Oncologist 9(Suppl 1):11–18
41. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron Ari, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. New Engl J Med 350:2335–2342
42. Meyerhardt JA, Mayer RJ (2005) Systemic therapy for colorectal cancer. New Engl J Med 352:476–487
43. Baselga J (2003) Skin as surrogate tissue for pharmacodynamic end points: is it deep enough? Clin Cancer Res 9:2389–2390
44. Jung S, Schluesener HJ (1991) Human T lymphocytes recognize a peptide of single point-mutated oncogenic ras proteins. J Exp Med 173:273–276
45. Anderson KS, Alexander J, Wei M, Cresswell P (1993) Intracellular transport of class I MHC molecules in antigen processing mutant cell lines. J Immunol 151:3407–3419
46. Melero I, Nathan B, Lieping C (1997) Costimulation, tolerance and ignorance of cytolytic T lymphocytes in immune responses to tumor antigens. Life Sci 60:2035–2041
47. Grabbe S, Beissert S, Schwarz T, Granstein RD (1995) Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy? Immunol Today 16:117–121
48. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Ann Rev Immunol 9:271–296
49. Bell D, Young JW, Banchereau J (1999) Dendritic cells. Adv Immunol 72:255–324
50. Hanna MG, Peters LC (1978) Specific immunotherapy of estab-

lished visceral micrometastases by BCG-tumor cell vaccine alone or as an adjunct to surgery. Cancer 42:2613–2625
51. Baars A, Buter J, Pinedo HM (2002) Making use of the primary tumor. BioEssay 25:79–86
52. Vermorken JB, Claessen AM, van Tinteren H, Gall HE, Ezinga R, Meijer S, Scheper RJ, Meijer CJ, Bloemena E, Ransom JH, Hanna MG Jr, Pinedo HM (1999) Active specific immunotherapy for stage II and stage III human colon cancer: a randomized trial. Lancet 353:345–350
53. Parmiani G, Colombo MP, Melani C, Arienti F (1997) Cytokine gene trasduction in the immunotherapy of cancer. Adv Pharmacol 40:259–307
54. Parmiani G, Rodolfo M, Melani C (2000) Immunological gene therapy with ex vivo gene modified tumor cells: a critique and a reappraisal. Hum Gene Ther 11:1269–1275
55. Waelti ER, Gluck R (1998) Delivery to cancer cells of antisense L-myc oligonucleotides incorporated in fusogenic, cationic-lipid-recostitued influenza-virus envelopes (cationic virosomes). Int J Cancer 77:728–733
56. Aarts WM, Schlom J, Hodge JW (2002) Vector based vaccine/cytokine combination therapy to enhance induction of immune response to a self- antigen and anti-tumor activity. Cancer Res 62:5770–5777
57. Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM (2001) Dendritic cells acquire antigens from live cells for cross-presentation to CTL. J Immunol 166:3717–3723
58. Brigl M, Brenner MB (2004) CD1 antigen presentation and T cell function. Ann Rev Immunol 22:817–890
59. Nestle FO (2000) Dendritic cell vaccination for cancer therapy. Oncogene 19:6673–6679
60. Srivastava PK, Menoret A, Basu S, Binder RJ, McQUade KL (1998) Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 8:657–665
61. Basu S, Binder RJ, Ramalingam T, Srivastava PK (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70 and calreticulin. Immunity 14:303–313
62. Falk K, Rötzschke O, Stevanovic S, Rammensee HG (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290–296
63. Parker KC, Bednarek MA, Coligan JE (1994) Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 152:163–175
64. Robbins PF, Kawakami Y (1996) Human tumor antigens recognized by T cells. Curr Opin Immunol 8:628–636
65. Tsang KY, Zaremba S, Nieroda CA, Zhu MZ, Hamilton JM, Schlom J (1995) Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer Inst 87:982–990
66. Marshall JL, Hoyer RJ, Toomey MA, Faraguna K, Chang P, Richmond E, Pedicano JE, Gehan E, Peck RA, Arlen P, Tsang KY, Schlom J (2000) Phase I study in advanced cancer patients of a diversified prime boost vaccination protocol using recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. J Clin Oncol 18:3964–3973
67. Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, Schlom J, Ryback ME, Lyerly HK (1999) A phase I study of active immunotherapy with carcinoembryonic antigen peptide(CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignacies expressing carcinoembryonic antigen. Clin Cancer Res 5:1331–1338
68. Kantor J, Irvine K, Abrams S, Kaufman H, DiPietro J, Schlom J (1992a) Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen vaccinia virus vaccine. J Natl Cancer Inst 84:1084–1091
69. Kantor J, Irvine K, Abrams S Snoy P, Olsen R, Greiner J, Kaufman H, Eggensperger D, Schlom J (1992b) Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a nonhuman primate. Cancer Res 52:6917–6925
70. Foon KA, John WJ, Chakraborty M, Serratt A, Garrison J, Flett M, Bhattacharya-Chatterjee M (1997) Clinical and immune responses in advanced colorectal cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen. Clin Cancer Res 3:1267–1276
71. Kass E, Schlom J, Thompson J, Guadagni F, Graziano P, Greiner JW (1999) Induction of protective host immunity to carcinoembryonic antigen (CEA), a self antigen in CEA transgenic mice, by immunizing with a recombinant vaccinia-CEA virus. Cancer Res 59:676–683
72. Correale P, Walmsley K, Nieroda C, Zaremba S, Zhu M, Schlom J, Tsang KY (1997) In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer Inst 89:293–300
73. Houbiers JG, Nijman HW, van der Burg SH, Drijfhout JW, Kenemens P, van de Velde CJ, Brand M, Momberg F, Kast WM, Melief CJM (1993) In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. Eur J Immunol 23:2072–2077
74. Francini G, Scardino A, Kosmatopoulos K, Lemonnier KA, Capoccia G, Sabatino M, Pozzessere D, Petrioli R, Lozzi L, Neri P, Fanetti G, Cusi MG, Correale P (2002) High Affinity HLA-A(*)02.01 peptides from parathyroid-hormone related protein generate in vitro and in vivo antitumor CTL response without autoimmune side effects. J Immunol 169:4840–4849
75. Dalerba P, Maccalli C, Casati C, Castelli C, Parmiani G (2002) Immunology and immunotherapy of colorectal cancer. Crit Rev Oncol Hematol 46:33–57, 2003
76. Zinkernagel RM (2001) Immunity against solid tumors. Int J Cancer 93:1–5
77. Graziani G, Faraoni I, Grohmann U, Bianchi R, Binaglia L, Margison GP, Watson AJ, Orlando L, Bonmassar E, D'Atri S (1995) O6-alkylguanine-DNA alkyltransferase attenuates triazene-induced cytotoxicity and tumor cell immunogenicity in murine L1210 leukemia. Cancer Res 55:6231–6236
78. Heinzer H, Huland E, Huland H (2001) Systemic chemotherapy and chemoimmunotherapy for metastatic renal cell cancer. World J Urol 19:111–119
79. Elias L, Binder M, Mangalik A, Clark D, Morrinson B, Altobelli KK, Smith A (1999) Pilot trial of infusional 5-fluorouracil, interleukin-2, and subcutaneous interferon α for advanced renal cell carcinoma. Am J Clin Oncol 22:156–161
80. Lauro S, Bordin F, Trasatti L, Lanzetta G, Della Rocca C, Frati L (1999) Concurrent chemoimmunotherapy in metastatic clear cell sarcoma: a case report. Tumori 85:512–514
81. Lau WY, Leung TWT, Lai BS, Liew CT, Ho SKJW, Yu SCH, Tang AMY (2001) Preoperative systemic chemoimmunotherapy and sequential resection of unresectable hepatocellular carcinoma. Ann Surg 233:236–241
82. D'Atri S, Marini S, Tentori L, Tricarico M, Fuggetta MP, Bonmassar E (1991) Drug-mediated increase of susceptibility of human lung cancer to NK or LAK effector cells. Immunopharmacology 21:199–210
83. Correale P, Caraglia M, Fabrocini A, Guardasi R, Pepe S, Patella V, Marone G, Pinto A, Bianco AR, Tagliaferro P (1995) Bryostatin 1 enhances lymphokine activated killer sensitivity and modulates the beta 1 integrin profile of cultured human tumor cells. Anticancer Drugs 6:285–290
84. Correale P, Tagliaferri P, Celio L, Genuav G, Montagnani S, Bianco AR (1991) Verapamil upregulates sensitivity of human

colon and breast cancer cells to LAK-cytotoxicity in vitro. Eur J Cancer 27:1393–1395
85. Tagliaferri P, Correale P, Mottola M, De Simone G, Montesarchio V, Matano E, Rea A, Morabito A, Famiani M, Ciardiello F, Tortora G, Caraglia M, Barile C, Palmieri G, Bianco AR (1996) High-dose recombinant interleukin-2/Verapamil combination in advanced cancer. Eur J Cancer 32:1436–1437
86. Frost P, Ng CP, Belldegrun A, Bonavida B (1997) Immunosensitization of prostate carcinoma cell lines for lymphocyte (CTL, TIL, LAK)-mediated apoptosis via the fas-fas-ligand pathway of cytotoxicity. Cell Immunol 180:70–83
87. Bergmann-Leitner ES, Abrams SI (2001) Treatment of human colon carcinoma cell lines with anti-neoplastic agents enhances their lytic sensitivity to antigen-specific CD8+ cytotoxic T lymphocytes. Cancer Immunol Immunother 50:445–455
88. Correale P, Aquino A, Pellegrini M, Micheli L, Cusi MG, Nencini C, Petrioli R, Prete SP, De Vecchis L, Turriziani M, Giorgi G, Bonmassar E, Francini G (2003) Treatment of colon and breast carcinoma cells with 5-fluorouracil enhances expression of carcinoembryonic antigen and susceptibility to HLA-A(∗)02.01 restricted, CEA-peptide-specific cytotoxic T cells in vitro. Int J Cancer Apr 20;104(4):437–445
89. Correale P, Del Vecchio MT, Di Genova G, Savellini GG, La Placa M, Terrosi C, Vestri M, Urso R, Lemonnier F, Aquino A, Bonmassar E, Giorgi G, Francini G, Cusi MG (2005) 5-fluorouracil-based chemotherapy enhances the antitumor activity of a thymidylate synthase-directed polyepitopic peptide vaccine. J Natl Cancer Inst 97:1437–1445
90. Bellone M (2000) Apoptosis, cross-presentation, and the fate of the antigen specific immune response. Apoptosis 5:307–314
91. Onier N, Hilpert S, Arnould L, Saint-Giorgio V, Davies JG, Jeannin JF, Jeannin JF (1999) Cure of colon cancer metastasis in rats with the new lipid A OM 174. Apoptosis of tumor cells and immunization of rats. Clin Exp Metastasis 17:299–306
92. Belhocine T, Steinmetz N, Hustinx R, Bartsch P, Jerusalem G, Seidel L, Rigo P, Green A (2002) Increased uptake of the apoptosis-imaging agent (99 m)Tc recombinant human Annexin V in human tumors after one course of chemotherapy as a predictor of tumor response and patient prognosis. Clin Cancer Res 8:2766–2774
93. Markowicz S, Engleman EG (1990) Granulocyte-macrophage colony stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. J Clin Invest 85:955–961
94. Morgan DA, Ruscetti FW, Gallo R (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193:1007–1008
95. Correale P, Campoccia G, Tsang KY, Micheli L, Cusi MG, Sabatino M, Bruni G, Sestini S, Petrioli R, Pozzessere D, Marsili S, Fanetti G, Giorgi G, Francini G (2001) Recruitment of dendritic cells and enhanced antigen-specific immune reactivity in cancer patients treated with hrGM-CSF (molgramostim) and hr IL-2: results from a Phase Ib Clinical Trial. Eur J Cancer 37:892–902
96. Correale P, Cusi MG, Tsang KY, Del Vecchio MT, Marsili S, Placa ML, Intrivici C, Aquino A, Micheli L, Nencini C, Ferrari F, Giorgi G, Bonmassar E, Francini G (2005) Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. J Clin Oncol 23:8950–8958