Cancer immunology and colorectal cancer recurrence

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1. ABSTRACT

The recurrence of a cancer – local or distant (metastasis) – is manifested by the persistence of cancer cells in the organism after the ablation of the primary lesion, an ineffective anticancer immune response, and by the activity of biological/immunological factors that can stimulate and sustain its development. This review focuses on colorectal carcinoma and discusses some aspects of cancer immunology regarding cancer development and its recurrence. It is addressed also to the clinician to provide new insights helpful for designing better therapeutic strategies and patient’s follow up. Therapeutic approaches used during and after surgical treatments, found capable of modulating immunity (differently affecting disease outcome), will also be described.

2. INTRODUCTION

Standard surgical procedures for the treatment of colorectal carcinoma permit a satisfactory control of local recurrence by the en-bloc resection of the efferent vascular territory and lymphatic drainage, including lymph nodes, together with the intestinal tract affected by the cancer. However, according to the degree of cancer invasiveness, tumor cells can residue in the body after the ablation of primary cancer. The spread of cancer cells can be simultaneous to progressive invasion and expansion of cancer within the wall of the bowel. Various studies have demonstrated that a prognostic factor for colorectal cancer recurrence is the degree of vascular and lymphatic vessel microinvasion that is essential for spreading the cancer
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cells (1-4). However, such findings need to be associated with cancer progression after evading the immune control.

3. APPROACHES FOR THE PREVENTION OF COLORECTAL CANCER RECURRENCE AND RELATED IMMUNOLOGICAL ASPECTS

At present, adjuvant treatments by chemotherapy (for both colon and rectal cancers) or radiation therapy (for rectal cancers, often in association with chemotherapy) are recommended in presumed postoperative minimal residual disease (rectum) and distant cancer spread (colon and rectum) cases. This recommendation is due to the characteristics of local extension and invasion by primary neoplasms in their development (clear indication for stage III cancers, still controversial for stage II) (5).

Systemic treatments are employed to clear the organism from single cancer cell spreads and micrometastases. Micrometastases can occur also as a consequence of tumor cell spreading during surgery. However, they will not necessarily cause progressing lesions, because, in some cases, cells can enter in a non-replicative state. This non-replicative state is described as “tumor dormancy”: a condition that is still under study regarding its mechanism. It is proposed that it may depend from cell characteristics (angiogenic and non-angiogenic type), immunity (adaptive immunity cell activity, IFN-gamma), and microenvironmental factors able to interact with cancer-cell surface molecules, activating stress through the p38 pathway (6-9). Tumor dormancy is a phenomenon described in both experimental and clinical studies. It appears to be linked to inhibition of pro-angiogenic factors (in some cases especially effective when the primary tumor is still present) and explains the dynamics by which the tumor recurrence arise. Depending on the original characteristics of the metastatic cells, resection of the primary tumor is also suggested enabling the reactivation of the proliferation process at various degrees, a paradoxical effect (9, 10).

The above observations stress the importance of therapeutic approaches during post-surgical periods. At this stage the absence of possible primary tumor inhibitory effect, immune suppression induced by surgical trauma, and the release of reparative process factors with pro-angiogenic effects (HIF-1, vascular endothelial growth factor-VEGF, MMPs, arginine, polyamines, EGF, etc) may stimulate persistent tumor microfoci to develop. Under this aspect, recently introduced therapeutic agent VEGF-directed antibody bevacizumab finds a rationale for treatment. The choice of a laparoscopic approach, when possible, versus open surgery appears also favorable to maintain a more efficient immune function (11-15).

The role of peri-operative transfusions (especially allogenic) in colorectal cancer patient prognosis regarding the recurrence of cancer is still controversial. It was suggested that alterations in the patient’s antitumor response might be induced by allogenic blood components. Some authors have found correlations with a shorter time of cancer recurrence while others have not. However, all authors found a lower survival time and higher peri-operative mortality in patients that needed more than three blood transfusions, but the correlation was considered more linked to other concomitant factors (performance status, underlying illnesses of the patients) than a direct immunological effect of the transfusion components on cancer outcome (16-19).

Chemotherapies (as well as radiation therapy) can affect immune responses. The evidence of immune suppressive effects by standard chemotherapies (e.g. increase of sIL-2R and regulatory T-cells, and a decrease of cytotoxic T-cells) is encouraging the design of new therapeutic approaches (continuous infusion, metronomic – continuous low dosage - treatments, etc.) (20). A working immune system is necessary to assist the effectiveness of anticancer treatments (21). Numerous studies are demonstrating the immunomodulatory activity of many commonly used anticancer chemotherapeutics when administered at lower dosages than in suggested existing protocols. For example, 5-fluourouracil resulted in the increase of the cell mediated immunity when administered at low dosages, especially by continuous pump infusion (22-25).

Immunity and cancer continuously interplay affecting each other. This is made clearer by developments in immunobiology permitting to rethink the general mechanisms through which immunity is challenged allowing cancer to grow. These immunologic concepts are referred to primary cancer evolution but in part can also be applied to the recurrence of cancer. For a better understanding, the present view of cancer-immunity interplay during cancer development will be summarized followed by its interpretation to cancer recurrence process.

4. GENERALITIES IN CANCER IMMUNOBIOLOGY

Cancer is a biologically multifaced pathology. It can be described as an articulated process that derives its characteristics from an active interplay between the transformed (cancer) cells and normal cells of the host. As this interplay evolves, cancer may or may not develop. This fundamentally depends on the host’s immune competence and its capability of mounting an effective immune response against the tumor cells.

The sequence of carcinogenic events is described by the term transformation. This manifests in progressive modifications of gene expression (26, 27), alterations of metabolic pathway activities (e.g. glycosylation, MHC molecule synthesis, etc.), and changes in cell phenotypic characteristics (e.g. reduced or absent expression of MHC class I molecules, neo-expression of aberrant carbohydrates structures, tumor-specific antigens – TSA, and tumor associated antigens – TAA, on cell surface) (28-31). The expression of abnormal or new molecules with variable immunogenicity (e.g. stress proteins, MUC1, HLA-E) and the loss/alteration of self-recognition markers (MHC class I molecules) can elicit immune responses (31-33).

The cancer cells, the cells of the host’s tissues, the host’s immune cells and related molecular products,
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together with the stromal structures, generate the so-called tumor microenvironment (35-39). This microenvironment is progressively created by a developing tumor which can influence the responsiveness of the immune cells, inhibiting the antitumor immune response allowing cancer cell survival, progression, and spread in the host’s organism (35, 40, 41).

When the cells of the immune system fail to eliminate all tumor cells from the organism, tumors with reduced immunogenicity may emerge and escape recognition and destruction by reshaping their immune-phenotype. Interferon gamma, paradoxically, plays a part in inducing and assisting this reshape (28). Combination of host-protective and tumor-promoting functions of the immune system during tumor development has been described as cancer immunoediting and immune sculpturing (43, 44). According to Dunn and collaborators, this dynamic process is composed of three phases: elimination, equilibrium, and escape. Elimination is the result of resumed immune activities by cancer immunosurveillance. Equilibrium is the phase during which antitumor immune response balances the cancer growth and, more importantly, can contribute to reshaping of tumor immunological phenotype (inhibited expression of the targeted antigen by cancer cells or selection of cancer clones not expressing the targeted antigen). Finally, escape indicates the final outgrowth of tumors that have evaded immunological restraints during the equilibrium phase. During these three phases the major events are represented by: invasive expansion of the resistant tumor clones and increase of cancer cell heterogeneity; generation of hypoxic conditions and neoangiogenesis induction; immune response and tumor escape. These three aspects are valid both for primary and recurrent cancers.

The metastatic cells are heterogeneous, with lost adhesiveness to the other cells (mobile and invasive phenotype), with expression of superficial molecules allowing the adhesion to the endothelia and transmigration inside specific tissues. These last properties are also related to alterations in glycosylation. The aberrant glyco-structures exhibited on the cancer cell surface can produce changes in the immunogenicity and in the capability to adhere to other cells with impact on immune recognition and microenvironmental interactions (45-49). Aberrant glycosylation was also found to be induced by hypoxia, a quite frequent condition in the growing cancer tissue and in metastatic clones. Colon cancer cells cultivated under hypoxic conditions presented increased expression of selectin ligands, the sialyl Lewis x and sialyl Lewis a, at the cell surface, leading to increased adhesion of cancer cells to endothelial E-selectin. These findings once again point out the critical influence of tumor microenvironment in conditioning cancer evolution (50-54).

An efficient anticancer immune response (both against primary and recurrent cancers) requires cooperation of innate and adaptive immunity. Natural killer (NK) cells, dendritic cells (DC) and cytotoxic T lymphocytes (CTL) are essential for the elimination of cancer cells. Both NK and CTL – by close contact with a target (i.e. cancer cell) through the so-called “immunological synapse” – release enzymes causing death of the target cell (direct cytotoxicity). These enzymes cause lysis (perforins) and apoptosis (granzymes – serine protease) of aggressed cells. When the activity of cytotoxic cells (i.e. NK, CTL) is inhibited, cancer growth can progress. This is well documented in both experimental and clinical studies (55-57).

5. INFLAMMATION AND ANTICANCER IMMUNITY

Anticancer immune response is an articulated network that is continuously modulated by a dynamic tumor microenvironment. In this microenvironment, the immune cell subpopulations play a double-edged role in the cancer network, paradoxically assisting tumor escape (61-65). Reframing the problem from a microenvironmental prospective, early modifications (induced by initial cancer cells either primary clone or micrometastasis) appear to activate responses to maintain local homeostasis in the tissue. Release of active molecules like IL-1beta, TNF-alpha, type I IFNs, stress proteins (like Heat Shock Proteins – HSPs), as well as products of local metabolism (like breakdown products of hyaluronan), can trigger and attract innate immunity cells (macrophages, neutrophils) that surround and infiltrate the initial tumor mass and establish an inflammatory reaction (66). Dendritic cells, as well as NK cells and CTLs, will be activated. The initial response results in an acute inflammatory process (34, 65). If the acute inflammation and NK attack is not sufficiently effective against the tumor, the increasing mass of tumor cells and host cells releasing products will maintain the inflammatory response: the inflammation can become chronic inducing paradoxical modulation of an anticancer immune response (67,68).

The concept of inflammation as a possible pro-carcinogenetic factor was firstly proposed by Virchow in 1863, and re-proposed by Balkwill and Mantovani in 2001 (69). Virchow observed that a “lymphoreticular infiltrate” (indicating presence of inflammation) was detectable in tumors and suggested that chronic inflammation may assist in tumor development. Recent data has supported the role of pro-inflammatory cytokines, chemokines, other inflammatory molecules (e.g. leukotriens, nitric oxide –NO, prostaglandins) and cellular infiltrates as effectors of chronic inflammation in the tumor microenvironment, promoting tumor development. In this regard, is helpful the definition of H.F. Dvorak (1986) about the continuous tissue remodeling of a cancer: wounds, wounds that do not heal (70). In fact, by his definition, we can state that: 1) the healing of a wound resulting in restitutio ad integrum is due to mechanisms that allow a self-limiting process of tissue remodeling guided by a balanced inflammatory response; and 2) the tumor evolution is the result of subversion of physiological regulation mechanisms in which the progressively altered inflammatory network of tumor microenvironment paradoxically assists in cancer progression.

New evidence in cellular and molecular cancer immunobiology supports the role of inflammation in tumor development (67, 68).
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First, the NFkappaB – an important transcription factor in inflammatory response and tumor cells – was found to regulate the expression of genes that encode for important proteins for the control of stress response, maintenance of intercellular communications, regulation of cellular replication and apoptosis (71, 72). The importance of pro-inflammatory molecules to tumor promoters was shown in experimental models of cancer metastases in the mouse (by injection of CT26 colon cancer cells or by spontaneously metastatic 4T1 breast cancer cells). Injection of lipopolysaccharide (LPS) increased the metastases, an effect that was inhibited by blocking NFkappaB. The mediator of LPS-induced inflammation was TNF-alpha. (73). NFkappaB results to be a key component linking inflammatory cell activity and tumor progression by anti-apoptotic gene transcription of cancer cells. In addition, we can suggest the described LPS-assisted tumor growth as a component of the pro-carcinogenic environment activation by microbial agents, as in the colon (74, 75).

Second, effector cells of the anticancer immune response (and their products) demonstrated a double-faceted activity which depended on their interplay with progressively organized tumor microenvironment. The effector cells could either elicit antitumor responses or help tumor development and immune escape. An example is illustrated by macrophages. The M1 type macrophages can delete cancer cells, produce IL-12 with activation of cytotoxic lymphocytes (induction of IFN-gamma production) and Th1 CD4+ cells; however, in response to the microenvironment, macrophages can also inhibit cytotoxic cells by accumulation of nitric oxide (NO) and assume a suppressive phenotype (M2) (68). Myeloid cells and macrophages release free radicals. Reactive oxygen intermediates – ROI (hydroxyl radical - OH•, superoxide -O2•) and reactive nitrogen intermediates – RNI (nitric oxide - NO•, and peroxynitrite - ONOO-) are, under normal conditions, important defenses against microbes. They are synthesized by the host’s enzymes such as myeloperoxidase, NADPH oxidase, and nitric oxide, under the regulation of inflammatory signaling pathways. In case of chronic inflammation, the activity of ROI and RNI can produce negative effects on host’s cells by oxidative damage and nitration of DNA bases which increases the risk of DNA mutations. In a tumor microenvironment, they can sustain genetic instability and further mutational events in transformed cells. They can also damage immune cells responsible for anticancer response (76, 64). This mechanism can stimulate the emergence of non-homogeneous cell clones, reducing the effectiveness of specific treatments (e.g. vaccination), as well as allowing the establishment of resistant clones of cancer cells resulting in recurrences.

Persistence of local inflammation produced by solid tumors can lead to activation of a systemic anti-inflammatory response which interferes with the local anti-tumor immune response (65). To understand how this apparent paradox is possible, a reference to a description of the interplay between tumor and immunity is given:

During early tumor mass development, the primary transformed cells (or the metastatic cells) start deregulated replications. This process induces expression of stress molecules and local delivery of “danger signals” (mainly pro-inflammatory cytokines) in the tissue stressed by the tumor clone growth (34, 38). As a consequence, innate immunity cells are elicited in the local environment. If immune surveillance works, then NK cells will kill the transformed cells by recognizing stress molecules on their surfaces (e.g. by NKG2D receptor) and produce IFN-gamma, important for adaptive immunity Th1 response. They will also produce TNF-alpha useful for activating phagocytes. The neutrophils, the M1 macrophages and dendritic cells can phagocytose dead cells. The NK cell cytotoxicity is triggered by down-regulated or altered expression of MHC class I molecules (ligands for the inhibitory Ly49 receptors – mouse, or KIRs – human) in transformed cells, and by changes in glycosylation of superficial structures (sensed by lectin-like activation receptors), as well as by tumor expression of stress molecules (MICA/B in the human, Rae-1 in the rat, and H60 in the mouse). However, shedding of soluble MICA molecule can prevent this activity by inducing internalization of the receptor, therefore, lacking its superficial expression (77-84).

The DC-NK cell cross-talk will permit the priming and maturation of DC; maturation that will be completed with the migration into lymph nodes (80). Here they will present on their superficial membrane tumor peptides in a complex with MHC molecules for triggering adaptive immunity cells. On one hand, the MHC class I – peptide complex will prime and activate specific CD8+ cytotoxic T cell (CTL) response (IFN-gamma, perforin and granzyme/granulysine production); on the other hand, the MHC class II-peptide complex will prime and activate CD4+ T helper cells in a Th1 polarized mode (IL-2 and IFN-gamma production) sustaining the cytotoxic cell activity. However, as above described, transformed cells can escape from elimination and cancer can progress (85-87). In the Th1/Th2 model of immune response physiological balance, the Th1 cells sustain the anticancer cytotoxic activity, and the Th2 cells sustain the B cell maturation as well as down-regulate Th1 functions for reconstitution of homeostasis inside the damaged tissue.

During cancer establishment and progression inside the tissue/organ of origin, cancer expansion resulting in pressure on host tissue, stroma and surrounding cells induces a permanent stimulation sustaining local inflammatory reaction and delivery of immunologically active molecules (cytokines, chemo-attractants, antigens etc.) within the intercellular matrix. At the same time, tumor cells deliver into the microenvironment cytokines and growth factors which modulate immunity contributing to remodeling of tissue architecture (33, 35, 37, 40). This phenomenon can interfere with an appropriate anticancer immune response, by transition from a locally acute response to a chronic inflammation, mainly involving macrophages and stimulating fibrocytes. In this context, macrophages can assume a different polarization (M2) by becoming important tumor supporters in cancer microenvironment, collaborating in impairing cytotoxic cell activities by delivering high concentrations of oxidation molecules (i.e. nitric oxide, free oxygen radicals, peroxide).
and releasing other molecules (i.e. IFN-alpha, TNF-alpha, IL-1beta, TGF-beta, IL-10, PGE2) helpful to cancer establishment. IL-1beta can also be produced by cancer cells; it increases invasiveness of cancer and its metastatic spread (68,87-89). When these conditions occur, the initial antitumor cytotoxic response (sustained by CD4+ Th1 cells) shifts to a tolerant-suppressive response for the intervention of CD4+ Th2 cells, CD4+CD25+Foxp3+ T regulatory (Treg) lymphocytes, myeloid-derived suppressor cells (MDSCs) releasing IL-10, IL-6, IL-4 and TGF-beta into the microenvironment. Through this process cancer progression is aided (56,64,85).

During the state of full malignancy (i.e. tissue invasion and metastatic spread) tumor cells, immune cells and stromal/endothelial cells actively release Fas-L and deliver pro-inflammatory factors and immunosuppressive cytokines as well as pro-angiogenic factors and extracellular matrix modifiers (e.g. IL-1beta, IL-4, IL-6, IL-10, TNF-alpha, HIF-1, VEGF, MMPs, hyaluronidases) into the tumor microenvironment. This leads to local changes in tissue structures and in immunity that can extend to systemic levels predisposing the establishment of additional tumor diffusion and metastasis. The above factors can also interfere with the normal metabolism of the organism (90-95).

The signal transducer and activator of transcription 3 (STAT3) represents another link between cancer proliferation and survival, and cancer influence on immune cells (inhibition). STAT3 results constitutively activated cancer cells and this activation can be propagated to immune cells by tumor STAT3-regulated factors, which include VEGF and IL-10 (39,96,97). VEGF is one of the important molecules able to "awake" dormant tumor cells by creating a pro-angiogenic environment in tissue niches were they can migrate both in the organ in which the cancer arose and in distant metastatic sites (9,10). These factors mediate a cross-talk between cancer and immune cells with immunosuppressive effect as well as possible collaboration to maintain chronic inflammation that is able to sustain the maturation of CD4+CD25+Foxp3+ Treg lymphocytes and IL-17 producing cells, as suggested by recent data (98).

Metalloproteinases help the mobilization and migration of cells, and IL-1beta collaborates in the progression of the tumor and induces adhesion molecule expression (e.g. Intercellular Adhesion Molecule-1, ICAM-1) that helps in metastatic spread to adhere to endothelia and localize in the tissues. These molecules are originated both by inflammatory and cancer cells (35,36,38). VEGF, IL1-beta, TGF-beta and IL-17 levels should be considered in postoperative follow-ups for monitoring possible risk of cancer recurrence.

6. TUMOR ESCAPE AND CONDITIONS FOR RECURRENCE

Immune ignorance has to be taken into consideration not only during initial stages of tumor development but also during its evolution; in particular as it is biasing immune control of recurrence (99). In the initial steps of transformed cell clone formation resulting in primary cancer, the possibility of defective interaction of cancer cells with immune cells can be generated by the limited number of mutated cells, low degree of phenotypic changes vs. the original self-phenotype, and possible absence of co-receptor ligands enabling triggering of recognition complex on immune cells. Consequently, these factors may not lead to a direct and effective contact with immune effector cells (an essential condition for the recognition process). Because the transformed-cell/immune-cell interactions are initially elicited by the delivery of danger signals, it is also suggested that tumor cells can lack danger signal production (100). In post-operative recurrences impediments to cancer recognition may be due to: anatomical barriers (e.g. scar tissue, fibrosis); immune editing caused by previous therapies and immune responses with changes or limited amount of expressed antigens; conditions that limit the migration to and maturation in the lymph nodes of naïve T-cells or naïve DCs after interaction with tumor cells (e.g. in post-operative sites after lymphadenectomy and subversion of the regular lymphatic drainage) (101, 102). Vascular modifications produced after surgery may also allow (with progressive reparative processes and fibrosis) sites of ischemia and hypoxia able to stimulate HIF-1 production, induction of VEGF expression, and biologically significant collagen accumulation (52).

Hypoxia leads to glycolysis and acidic pH in the tumor microenvironment and stimulation to produce HIF-1 and subsequent expression of VEGF. VEGF can play a dual role as a pro-angiogenic factor (allowing dormant tumor cells to rescue their proliferative potential) and an immune suppressive agent (103, 104). Immature dendritic cells can help the tumor to be tolerated by deficits in the MHC-peptide complex – TCR complex interaction in the absence of necessary co-receptors. In fact, immune tolerance occurs when antigen presentation is weak, leading to immune recognition without immune activation. Immune tolerance appears to be more frequent during advanced tumor stages helping cancer dissemination and recurrence after ablation of the primary cancer (105). Reduced antigenicity of TAA is another important factor leading to immune ignorance/tolerance. Additional alterations in the production of tumor processed proteins (e.g. secondary to impaired function of the proteasome system) and altered structure in MHC molecule complexes can reduce the sensitivity to MHC-I dependent cytotoxic T-cell responses (106).

As cited in previous paragraphs, tumor cells can release a wide range of immunologically active molecules (as cytokines, chemokines, Fas-L) and condition the immune response in the cancer microenvironment, and in advanced stages, in the organism as well. For example tumor-derived Fas-ligand, binding to the Fas receptor on immune cells, promotes apoptosis of immune cells (107).

Cytokines (especially IL-4, IL-6, IL-10, TGF-beta) originating from both cancer and immune cells, permits the creation of a local environment favorable to shift CD4+ helpers from a Th1 to a Th2 response, and
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atraction of Treg lymphocytes (CD4+CD25+) that can complete their maturation inside the cancer by expressing Foxp3+ (90, 108,109). The importance of these cells in cancer is demonstrated in various studies where the depletion or block of Treg cells (e.g. by metronomic cyclophosphamide administration) improved the efficacy of immunotherapies (e.g. increased competence of dendritic cell-based tumor vaccines) (110). Studies regarding the maturation of Treg cells showed that activated NK cells can prevent the CD28-mediated conversion of naïve Treg (CD4+CD25-) to activated (CD4+CD25+Foxp3+) cells. Receptor signaling appears to be required for the homeostasis of Treg cells in their TGF-beta-dependent conversion from CD4+CD25- to CD4+CD25+ (111,112). Monitoring appearance or variations of Treg lymphocyte levels should be helpful for cancer recurrence risk assessment in postoperative follow-ups.

7. CONCLUSIONS AND PERSPECTIVES

Understanding cancer immunobiology has considerably improved in recent years and new information is continuously available. The knowledge of tumor dormancy and the importance of inflammation and its factors playing a role in stimulating systemic regulatory responses permits treatment and possibly prevention of recurrence of cancer by immunological means (i.e. by: preoperative and post-operative immune evaluations and monitoring patients; accuracy of histological evaluation at resection sites and in lymph nodes to evidence tumor microfoci and angiogenic factor expressions; type and degree of immune infiltrates inside tumors; evaluation of circulating MDSCs and Foxp3+ Treg cells; metronomic and infusion-pump delivered therapy vs. high dose and bolus chemotherapy; anti-angiogenic and anti-oxidant treatments; laparoscopic approaches; depletion of negative regulators of the immunity – Treg; etc.). Introducing immune tests in clinical practices could significantly add to cancer staging and provision to patients’ prognosis. In this regard, favorable attempts are made (113-115). The planning of adjuvant therapies in early post-operative period according to the biological characteristics of the tumor altogether the immunological characteristic of the patient can add a contribute to reduce the risk of recurrence.

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**Abbreviations:** CEA: carcino-embryonic antigen CTL: cytotoxic T lymphocytes, DC: dendritic cell, DNA:
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