Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice


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In this study, human T cells were provided with a reactivity against the Lewis-Y (LeY) carbohydrate antigen, which is overexpressed on 70% of epithelial-derived tumors, but not normally recognized by T cells. Antitumor reactivity was achieved by transduction of T cells with a gene encoding a cell-surface chimeric receptor composed of single-chain anti-LeY antibody linked to an enhanced cytoplasmic signaling domain made up of CD28 and CD3-ζ. Importantly, the single-chain antibody was humanized to try to reduce potential problems of human anti-mouse antibody responses in patients receiving chimeric receptor-modified T cells in future clinical trials. T cells expressing the chimeric receptor were demonstrated to secrete cytokines and proliferate in response to receptor ligation and lysed LeY+ tumors in vitro. Another aspect of this study was the finding that no activity was observed against normal tissue, as represented by autologous neutrophils that express low levels of LeY. Significantly, systemic delivery of anti-LeY T cells dramatically inhibited established s.c. human ovarian OVCAR-3 tumors (a recognized difficult model to treat) in mice. Finally, we demonstrated that anti-LeY T cells preferentially expanded or accumulated in the tumor compared with control empty vector T cells, thereby providing mechanistic insight into the specific antitumor response. This study supports the use of humanized gene-modified T cells as a potential therapy for LeY+ malignancies.

cancer | immunotherapy | redirected | autoimmunity | trafficking

The specificity and potency of the immune response to infectious agents supports the use of immune components against malignant disease. Indeed, immune elements as diverse as cytokines (1), antibodies (2), vaccines (3), and adoptive cell transfer regimens (4) are beginning to find acceptance in the clinical treatment of cancer. However, clinical trials aimed at harnessing and enhancing the endogenous immune system against most cancers generally result in a very low frequency of durable complete responses (5). Analysis of immune parameters in the majority of patients following immunotherapy has demonstrated the infrequent generation of endogenous T cells with activity against autologous tumor. The low success rate of immunotherapy may therefore be due to a deficiency of tumor-reactive T cells in the immune repertoire or their nonresponsive state, rendering them unable to become activated and expand in response to antigen. In addition, tumor-reactive T cells may not traffic effectively to tumor and may be inhibited by tumor-derived factors such as TGF-β (6).

Recent strategies aimed at providing tumor-reactive T cells include the genetic modification of peripheral blood-derived T cells with receptors that recognize and respond to tumor antigens. Such receptors are generally composed of extracellular domains comprising a single-chain antibody (scFv) specific for tumor antigen, linked to intracellular T cell signaling motifs (7). This genetic modification strategy has the potential to provide tumor-reactive immune cells for a broad range of cancers. Genes can also potentially be used to enable tumor-reactive T cells to expand (8), maintain activity, and possess the ability to traffic to tumor (9) and resist inhibitory signals (10).

A range of chimeric receptor genes exist that encode reactivity against one of various antigens (11–16). However, virtually all of these use murine scFv specific for tumor antigen. Murine scFv can be immunogenic in humans, which may negate recognition of tumor (17). In addition, all currently available chimeric receptors recognize antigen present on a minority of tumors of limited histologic origin. Therefore, we have developed a unique chimeric receptor with a humanized scFv portion (18, 19) that recognizes a carbohydrate antigen, Lewis-Y (LeY), that is overexpressed on a large percentage of tumors of epithelial origin including those originating from colon, breast, prostate, ovary, and lung (20–23). This targeting domain is linked to a combined CD3/CD28 signaling chain to further enhance the efficacy of this receptor.

This study describes the preclinical characterization of T cells bearing a humanized antitumor receptor that specifically reacts against LeY+ tumor cells in vitro, and causes dramatic inhibition of established LeY+ tumor after systemic administration of genetically redirected human T cells in mice. In addition, we demonstrate that normal tissue cells expressing lower amounts of antigen are not harmed by redirected T cells, nor is the antitumor response inhibited by excess numbers of normal LeY+ cells. Finally, this study presents evidence for accumulation of gene-directed tumor-specific human T cells in s.c. tumors in mice after systemic delivery.

Materials and Methods

Cells. MCF-7 (breast), PANC-89 (pancreas), OVCAR-3 (ovarian), HT29 (colon), MDA-MB-231 (breast), T47D (breast), A431 (epidermoid), and Hey (ovarian) are LeY+ expressing tumor cell lines (all from American Type Culture Collection, Manassas, VA). COLO 205 (colon) and MDA-MB-435 (breast) are LeY−negative tumor cell lines. All tumor cell lines were cultured in complete medium composed of RPMI medium 1640, 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 2 mM glutamine (all from Invitrogen). T cells were cultured in the same medium with the addition of 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 5 × 10−5 M 2-mercaptoethanol (Invitrogen), and 600 units/ml human recombinant IL-2 (National Cancer Institute, Frederick, MD). OVCAR-3 cells were initially passaged twice s.c. in NOD-scid mice to establish a line that reproducibly formed s.c. tumor upon subsequent injection into mice.

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Abbreviations: LeY, Lewis-Y; MFI, median fluorescence intensity.

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Chimeric Receptor Construction and Retroviral Vector Production. DNA encoding the anti-LeY chimeric receptor was generated by using standard molecular biology techniques (see Supporting Text, which is published as supporting information on the PNAS web site) using a scFv (19) generated from the humanized monoclonal antibody Hu3S193 (18). A schematic representation, nucleic acid and protein sequence of the construct is presented in Fig. 6, which is published as supporting information on the PNAS web site.

T Cell Isolation and Transduction. Human peripheral blood mononuclear cells (PBMCs) were isolated from normal donor buffy coats and transduced by using retroviral supernatant as described (24). Briefly, PBMCs were stimulated with anti-human CD3 (OKT3 30 ng/ml, Ortho Biotech) and IL-2 (600 units/ml), followed 3 and 4 days later by incubation with supernatant from the PG13 retroviral producer cell line containing polybrene (8 μg/ml, Sigma-Aldrich) and centrifugation for 1 h at 1,000 × g. Transduced T cells were then selected by culture in G418 (Geneticin, Invitrogen) for 5 days.

Antibodies and Flow Cytometry. Expression of the chimeric receptor was determined by staining with a mouse monoclonal antibody specific for the idiotype of the scFv, LMH-3 IgG1 anti-3S193 idiotype (25) [supplied by Ludwig Institute for Cancer Research (LICR)], followed by phycoerythrin (PE)-conjugated anti-mouse Ig F(ab’2) (Chemicon). Detection of the LeY antigen was performed by using a humanized monoclonal IgG1, clone Hu3S193 (LICR), followed by FITC-conjugated sheep anti-human Ig F(ab’2) fragment (Silenus/Chemicon). Controls consisted of secondary antibody alone. Cell surface phenotyping of transduced cells was determined by direct staining with PE-conjugated anti-human CD8 and PE-Cy5-conjugated anti-human CD4 (Dako Cytomation). Determined by direct staining with PE-conjugated anti-human CD8 (26). Briefly, IFN-γ secretion was determined by using ELISA of supernatants from overnight coculture of 1 × 10⁶ T cells and 5 × 10⁵ tumor cells per ml. Cytotoxicity was assessed by using a 4-h ⁵¹Cr-release assay. Proliferation was determined in response to culture with plastic-immobilized anti-3S193 idiotype for 3 days and in the presence of 0.5 μCi of tritiated thymidine for the final 16 h (1 Ci = 37 Gbq).

Assays of Reactivity Against Neutrophils. Leukocytes (fresh or cryopreserved) autologous to T cell donors were enriched for neutrophils by passage over ficoll/hyphaque followed by red blood cell lysis of the pellet using lysis buffer (0.15 M NH₄Cl with 1.0 M KHCO₃ and 0.1 mM Na₂EDTA at pH 7.2–7.4). Neutrophils were then used as target cells in cytokine release assays or loaded with ⁵¹Cr (hot cells) and used in cytotoxicity assays as described above. In experiments where neutrophils were used in the same well as MCF-7 cells, 2 × 10⁶ unlabeled (cold) neutrophils were added with 2 × 10⁶ labeled MCF-7 cells.

Tumor Treatment in Vivo. Groups of six or seven NOD-scid mice (Walter and Eliza Hall Institute, Melbourne) were irradiated at 2.5 Gy and then injected s.c. on the abdomen with 5 × 10⁶ OVCAR-3 cells in 200 μl of PBS on day 0. In two experiments, mice received 1 × 10⁷ adoptively transferred anti-LeY T cells i.v. on each of days 0, 1, 2, and 5 after tumor inoculation. In another experiment, T cells were administered on days 7, 8, 9, and 12. Nontreated mice and those receiving control T cells transduced with empty vector (in equal numbers as those mice receiving anti-LeY T cells and on the same days) served as controls in these experiments. Tumor size was measured by using calipers and presented as the product of perpendicular diameters.

Cell Tracking and Immunohistochemistry. In a time course in vivo experiment, irradiated NOD-scid mice were injected s.c. with 5 × 10⁶ OVCAR-3 tumor cells followed by i.v. injection of either 1 × 10⁷ anti-LeY T cells or empty vector T cells on each of days 7, 8, 9, and 11 after tumor inoculation. Two different donors were used to derive the transduced T cells. Two mice (one that had received LeY-specific T cells and one that received control T cells) from each donor were culled on days 10, 12, 14, 17, and 29, and various tissues were collected and processed as follows to produce a single cell suspension: spleens were crushed in ACK lysis buffer and washed with PBS, blood from cardiac puncture was treated with ACK lysis buffer and washed in PBS, and tumor and lungs were minced finely and digested in an enzyme mix composed of RPMI medium 1640 plus DNase 1 (Sigma-Aldrich), hyaluronidase V (Sigma-Aldrich), and collagenase IV (Worthington) for 2 h at 37°C on a shaker, before filtering through a 70-μm sieve and washed in PBS. Flow cytometric analysis was performed for the presence of human T cells by using the anti-human CD45-FITC antibody (BD Biosciences Pharmingen) to determine the level of human T cells as a percentage of total number of tissue cells.

Immunohistochemistry was performed on 5-μm frozen sections (see Supporting Text). Sections were stained with FITC-conjugated mouse anti-human CD45 (BD Biosciences Pharmingen) and examined by using a confocal microscope. Sections were also stained with propidium iodide (50 ng/ml) to detect cell nuclei.

Results

LeY Antigen Expression on a Range of Tumors. In this study, we determined the specificity and activity of anti-LeY T cells against epithelial tumors. Therefore, a panel of 24 carcinoma cell lines from various tissue origins were examined for LeY expression. Half (n = 12) of these epithelial cell lines expressed LeY on their surface (Fig. 1 a–l), including those of breast, ovarian, epidermoid, pancreatic, and colorectal origin. This finding was consistent with previous data in the literature (20–23). However, expression levels varied widely from low (median fluorescence intensity, MFI < 100) to medium (MFI of 100–200) and high (MFI > 200). Two representative LeY-negative tumor lines, COLO 205 (colon) and MDA-MB-435 (breast), were used as negative controls in subsequent T cell function assays.

Transduced T Cells Express Chimeric Anti-LeY. T cells were transduced with the anti-LeY chimeric gene as described in Materials and Methods, and demonstrated to express anti-LeY receptor after selection in G418 (Fig. 1 m and n). Approximately 50% of T cells expressed low to moderate levels of receptor. The composition of T cell subsets varied with individual PBMC donors but all cultures were a mixture of CD4⁺ (20–49%) and CD8⁺ (44–74%) cells (data not shown).

Anti-LeY T Cells Secrete IFN-γ in Response to LeY⁺ Tumor Cells. Cytokine secretion by T cells in response to antigen is important in the activation and maintenance of an immune response toward antigen, and we therefore investigated the ability of transduced T cells to secrete IFN-γ as an indicator of T cell response against LeY⁺ targets. IFN-γ secretion was specifically detected in response to >70% of LeY⁺ lines (Fig. 2). However, the level of IFN-γ varied widely, with high amounts being secreted in response to OVCAR-3 and MCF-7 cells, moderate levels against PANC-89 and T47D, and lower levels against the remaining tumor lines. There appeared to be an association between LeY antigen expression and T cell reactivity, with higher levels of target antigen producing a greater IFN-γ response from T cells.

Anti-LeY T Cells Lyse LeY⁺ Tumor Cells. The ability of T cells, in particular CD8⁺, to directly lyse specific target cells is an important function that is necessary for efficient immune activity against infectious disease and tumors. Therefore, we determined
the ability of T cells bearing the anti-LeY receptor to lyse various target cells in a 4-h 51Cr-release assay. Transduction with the anti-LeY chimeric receptor enabled T cells to lyse LeY+ tumor cells (Fig. 7, which is published as supporting information on the PNAS web site). The LeY high-expressing tumor MCF-7 was lysed to a greater degree (83% at 40:1 effector/target ratio) than the medium-expressing tumor PAN-89 (57% at the same ratio, once empty vector T cell nonspecific lysis is deducted) (Fig. 7a). The LeY low-expressing cell lines A431 and HT29 were lysed to a lesser degree (33% and 23%, respectively); however, the LeY-negative line COLO-205 was not lysed. Specificity of the interaction was confirmed by the lack of lysis of LeY+ cells by control T cells transduced with empty vector (Fig. 7b).

**Proliferation of Anti-LeY T Cells in Response to Receptor Ligation.**

Proliferation of T cells in response to antigen is important for an effective immune response and has been demonstrated for T cells expressing other antitumor chimeric receptors. Therefore, we investigated the ability of the anti-LeY chimeric receptor to endow T cells with LeY-specific proliferative capacity. Anti-LeY-transduced T cells were demonstrated to specifically proliferate upon incubation with immobilized anti-idiotype antibody (Fig. 8, which is published as supporting information on the PNAS web site). Proliferation in response to anti-idiotype depended on chimeric anti-LeY expression, because no proliferation was observed in response to incubation with an irrelevant antibody (anti-c-myc).

**Anti-LeY T Cells Are Not Reactive with LeY Autologous Neutrophils.**

The potential for autoimmunity is a concern in the use of T cells reactive with TAA that have some normal tissue distribution. Because neutrophils express low levels of LeY antigen (MFI of 32, Fig. 3a), equivalent to some other normal tissues, and are readily available from T cell donors, they were used to determine whether anti-LeY T cells could respond against normal tissue. In contrast, MCF-7 tumor cells express high levels of LeY antigen (Fig. 3b). Anti-LeY T cells secreted IFN-γ in response to LeY+ tumor cells (MCF-7) but not in response to the same number of autologous neutrophils (Fig. 3c), suggesting that tumor cells could be targeted and normal tissue may be unaffected. In addition, neutrophils were not lysed by anti-LeY T cells whereas LeY+ tumor cells were lysed (Fig. 3d), again suggesting that T cells armed with the chimeric anti-LeY receptor could discriminate between normal tissue and some tumor cells. Although neutrophils were not targeted by anti-LeY T cells, it remained possible that neutrophils may interfere with T cell reactivity against tumor cells by competing for receptors. To determine whether this might occur, 51Cr-labeled tumor cells were incubated with T cells in the presence of a 10-fold excess of unlabeled neutrophils. No inhibition of tumor cell lysis was seen under these conditions (Fig. 3d), thereby indicating that lower-level expression of LeY on neutrophils did not impact on antitumor activity of T cells. Control T cells did not lyse neutrophils or LeY+ tumor targets (Fig. 3e).

**Systemically Delivered Anti-LeY T Cells Inhibit Tumor Growth in Mice.**

Having demonstrated antitumor activity of anti-LeY T cells in vitro, we next wanted to determine their ability to impact on tumor
requirement for the anti-LeY chimeric receptor on T cells for tumor cell lysis was demonstrated with anti-LeY T cells against media (311 pg/ml) lower than those secreted by empty T cells against neutrophils (464 pg/ml). After 4–5 weeks in mice receiving anti-LeY T cells, tumors regressed in 17 of 19 (89%) of mice achieving total remission of tumor (over 90% of mice) (Fig. 4a). Tumors regressed over a period of 35–40 days in response to four i.v. injections of anti-LeY T cells (circles). Tumors grew progressively in nontreated mice (triangles) and in mice receiving control T cells transduced with empty vector (squares). Tumor inhibition experiments were performed three times with similar results. Anti-LeY T cells inhibited tumor growth to a significantly greater extent when compared to control T cells (two-tailed P value = 0.002, Mann–Whitney test).

**More LeY-Specific T Cells than Control T Cells Accumulate in Tumors.** To determine the extent of trafficking of LeY-specific T cells to tumor and other tissues, a time course in vivo experiment was performed. Irradiated NOD-scid mice were injected s.c. with 5 × 10^6 OVCAR-3 cells, and mice received four i.v. injections of either anti-LeY T cells or empty vector T cells on each of days 7, 8, 9, and 11 after tumor inoculation. Two different human donors were used to generate the transduced T cells. Mice were culled on days 10, 12, 14, 17, and 29, and various tissues were collected. Tumor tissues were frozen, sectioned, and stained with human anti-CD45 Ab. This antibody detects human cells of hematopoietic origin, and thus any cells detected could only have been derived from the adoptively transferred cells that were entirely T cells in culture before transfer.

Human T cells were detected in tumors of mice treated with anti-LeY T cells (Fig. 9, which is published as supporting information on the PNAS web site). Most T cells were in small scattered groups (Fig. 9b), although occasional larger groups were detected (Fig. 9a). Tumors from mice treated with empty vector T cells (Fig. 9c) showed low numbers of T cells (most usually none) in the sections examined. No CD45^+ cells were seen in tumor sections of mice given no T cells (Fig. 9d).

To further investigate trafficking and to more accurately quantify T cells in tumors, tissues from the time course experiment were also examined for the presence of human T cells by flow cytometry after staining for human CD45. A significantly greater percentage of cells were detected in tumors of mice treated with these mice showed that the human anti-LeY T cells did not persist (data not shown) and were not present at the time of relapse.
LeY-specific T cells compared with those treated with empty vector T cells ($P = 0.007$, Mann–Whitney, nonparametric test) (Fig. 5 a and b). This increase in localization of specific T cells was consistently observed at multiple time points for T cells from either of two donors. In spleen, blood, and lungs (Fig. 5 c–h), the percentage of CD45$^+$ cells were similar in mice from both donors, but CD45$^+$ cells were generally higher in tissues from mice given empty vector T cells compared with those from mice given LeY-specific T cells. High percentages of CD45$^+$ cells were detected in the spleen, blood, and lungs on day 10, decreased to low levels by day 17 and were barely detectable on day 29.

Discussion

Genes encoding antitumor chimeric receptors have great potential for the generation of tumor-reactive T cells for the immunotherapy of cancer (27). Recently, an enhanced receptor gene format that includes both activation and costimulatory domains within a single molecule has been demonstrated to produce increased antitumor effects in vitro and in vivo (12). Importantly, we have demonstrated improved antitumor activity against tumor in mice by using this receptor format specific for the breast cancer-associated antigen, erbB-2. Although these earlier studies served as proof-of-principle, the chimeric receptor contained mouse components including the mouse scFv specific for erbB-2, making it less suitable for use in a clinical trial.

The current study demonstrates the ability of a chimeric T cell receptor containing a humanized single chain antibody to redirect T cell reactivity against LeY$^+$ tumor cells. Considering a clinical application, it is important that the chimeric receptor is nonimmunogenic in humans, because an antireceptor response would likely inhibit the function of transduced T cells or result in their destruction. The intact humanized monoclonal IgG from which the anti-LeY scFv was derived has been demonstrated to be nonimmunogenic in a clinical trial in humans (28), where no antibody specific for the anti-LeY antibody was observed in any patient participating in the trial. However, this does not ensure that an antichimeric receptor response will not occur in a clinical trial because novel linker and junction regions exist in the chimera. Nevertheless, demonstrated lack of response against the bulk of the construct is encouraging, and only use in a clinical trial will prove conclusively whether it is nonimmunogenic.

The chimeric receptor characterized in this study represents a unique reagent with the ability to target tumors of diverse histologic origin. The receptor is specific for LeY, a fucosylated carbohydrate moiety present on various cell surface proteins and lipids (29). Although LeY function remains poorly defined, its expression can be associated with increased metastasis and poorer prognosis (30, 31). It is an advantage to address molecular targets that are associated with increased malignancy. LeY is expressed on 60–90% of epithelial cancers, including those of breast, pancreas, ovary, colon, gastric, and lung cancer (20, 22, 23, 32).

LeY can also be expressed at low levels on some normal tissues including mucosa of esophagus, stomach, and large and small intestine, some exocrine cells of the pancreas, some epithelial cells of gall bladder, ciliated epithelium of trachea and bronchus (33–36), neutrophils (37), hair follicles (38), high endothelial venules of peripheral lymph nodes (39), and one report of CD34$^+$ hematopoietic stem cells (40) (which we could not confirm using our antibody, data not shown). The lower expression on many normal tissues, and its frequent restriction to luminal surfaces, may enable
specific targeting of T cells against tumor tissue without undue toxicity against normal tissues. This possibility is supported by our investigations using autologous neutrophils (representative normal tissue) expressing lower levels of LeY, which were not affected by anti-LeY antibodies. In addition, in the presence of hU3193 antibody in patients, no effect on neutrophil count was observed (28). Although encouraging, the lack of antibody localization to normal tissue does not definitively mean that LeY-specific T cells will not localize, because T cells may have an enhanced ability to cross endothelial barriers in some cases. The only way to answer the question of whether T cells will penetrate and affect normal tissues bearing a carbohydrate antigen is in a clinical trial.

The lack of activity of anti-LeY-directed T cells against neutrophils may partly be due to their lower expression of LeY. This finding is supported by the overall correlation between LeY expression level on tumor cells and the degree of antitumor T cell activity. Correlation between antigen expression and T cell activity has been observed by using T cells armed with other chimeric receptors (41). We consider the tendency of T cells to react with LeY-high tumor cells to be an advantage because reactivity to normal tissues may otherwise be an advantage because reactivity to normal tissues may represent a test of therapy more appropriately. To determine the effectiveness of anti-LeY T cells in vivo, we chose to deliver the T cells systemically against s.c. tumor. This type of model is generally accepted to represent a test of therapy more appropriately and that involves trafficking of tumor in a proportion of mice.

Analysis using flow cytometry and immunohistochemistry of tumor tissue showed that LeY-specific T cells trafficked to tumor and accumulated within tumor to a greater extent than empty vector T cells. Although this observation gives some insight into possible mechanistic contributions, besides specificity, that may be important in tumor inhibition, it is not entirely clear why tumor-specific T cells were found in larger numbers in tumor. Two possible explanations spring to mind: first, specific T cells may reach tumor in small numbers similar to control T cells, but may receive a proliferative signal upon encounter with antigen of the chimeric receptor bearing both CD28 and CD3ζ signaling components. Alternatively, initial small numbers of specific T cells entering tumor may promote an inflammatory environment that may lead to further infiltration of T cells.

This study presents unique data with respect to several aspects. First, direct observation of human T cells were shown to target and kill tumors expressing a carbohydrate antigen, LeY, which is not normally recognized by T cells, thus increasing the range of tumor-associated targets of various cancers available for treating by adoptive immunotherapy. Second, the chimeric receptor expressed by the T cells was humanized to reduce the chance of human anti-mouse antibody (HAMA). This chimeric receptor will be used in the clinic, and it was important to establish in vitro and in vivo characterization of its function. Third, no activity was observed with LeY-specific T cells against normal tissue, as represented by autologous neutrophils that express low levels of LeY. The issue of autoimmunity with use of chimeric receptors has not been previously addressed experimentally and yet is repeatedly mentioned as a possible problem of adoptive immunotherapy; this study attempts to address this issue with gene-redirected T cells. Fourth, significant tumor regression of an established s.c. tumor occurred after treatment of mice with LeY-specific T cells. The s.c. tumor model is regarded as a difficult model to treat because it involves trafficking to, and penetration of, solid tumor by T cells as well as killing of the tumor. Finally, trafficking of human T cells to tumor and other tissues was demonstrated by both flow cytometry and immunohistochemistry. Anti-LeY T cells appeared to preferentially expand or accumulate in the tumor compared with empty vector T cells.

The functional characterization of the chimeric receptor described in this study supports the use of adoptively transfused transduced T cells in patients with any of a wide range of malignancies that express high levels of LeY.

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