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Effective drug-antibody targeting using a novel monoclonal antibody against the proliferative compartment of mammalian squamous carcinomas*

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Summary. mAb 174H.64, which selectively recognizes an epitope expressed on the proliferating cells of mammalian squamous carcinomas, was covalently coupled to daunomycin (DM) by an acid-sensitive linker and tested for its selective cytotoxicity for squamous carcinomas. A murine lung squamous carcinoma model for chemoimmunotherapy using mAb 174H.64-DM conjugates was developed. This model utilizes the KLN-205 cell line, which metastasizes to the lungs following i.v. injection and shows a pattern of growth similar to those of spontaneous squamous carcinomas, characterized by highly proliferative cells at the periphery of the tumor (reactive with 174H.64) with the keratinized differentiated cells toward the center (not reactive with 174H.64). 174H.64-DM conjugates showed marked and specific cytotoxicity against KLN-205 cells both in vitro and following i.v. injection of the immunoconjugate in mice with established lung metastases. The conjugate was nearly as effective as daunomycin alone when incubated in vitro with KLN-205 cells and much more effective than daunomycin alone in vivo or other control immunoconjugates, which were ineffective. Finally, while the free 174H.64 mAb produced a significantly increased time of survival of mice bearing KLN-205 metastases, a much greater survival was found with mice treated with the 174H.64-DM immunoconjugate, some mice apparently demonstrating long-term survival (>100 days). We conclude that mAb 174H.64 may have potential therapeutic benefit against squamous carcinoma.

Introduction

We have recently developed a novel monoclonal antibody (mAb 174H.64; isotype, IgG_1) that selectively recognizes antigens shared by the basal cells of mammalian stratified squamous epithelium and squamous carcinomas of human, canine, feline and murine origin [15]. Cancers of other histological types did not show reactivity with 174H.64. In well-differentiated squamous carcinoma mAb 174H.64 reacted more strongly with cells in the periphery of the tumor than with cells at the centre of the tumor, suggesting that it preferentially binds to proliferating cells [9, 15]. The antigen detected by this antibody was characterized as two cytoskeletal proteins with approximate molecular masses of 48–50 kDa and 57 kDa [15].

If our hypothesis is correct that 174H.64 binds selectively to the proliferating (stem cell) population then it may be an excellent candidate mAb for therapy of squamous carcinoma. In order to evaluate 174H.64 for this purpose we developed a syngeneic murine animal model, taking advantage of the fact that the antigens detected by the mAb are present on all mammalian squamous carcinomas. KLN-205 is a murine lung squamous carcinoma cell line that metastasizes to the lung following i.v. injection [13]. The resulting lung metastases reacted with mAb 174H.64, demonstrating selective reactivity with the growing cells in the periphery of each tumor [15, 17].

In our previous studies, immunoconjugates were used [3, 4, 5, 19] in which the cytotoxic component was daunomycin (DM) attached to the target-specific conjugant via the acid-sensitive *cis*-aconityl spacer [16]. These daunomycin immunoconjugates showed high specific cytotoxicity on target cells and could be used for purging tumor cells and functional T cells from bone marrow [8]. The present study was designed to test the potential of mAb 174H.64-daunomycin immunoconjugates to target therapy to KLN-205 squamous carcinoma lung tumors.

Materials and methods

Experimental animals. DBA/2 male mice, 8–12 weeks of age, were obtained from the Medical Sciences Animal Centre, University of Alberta.

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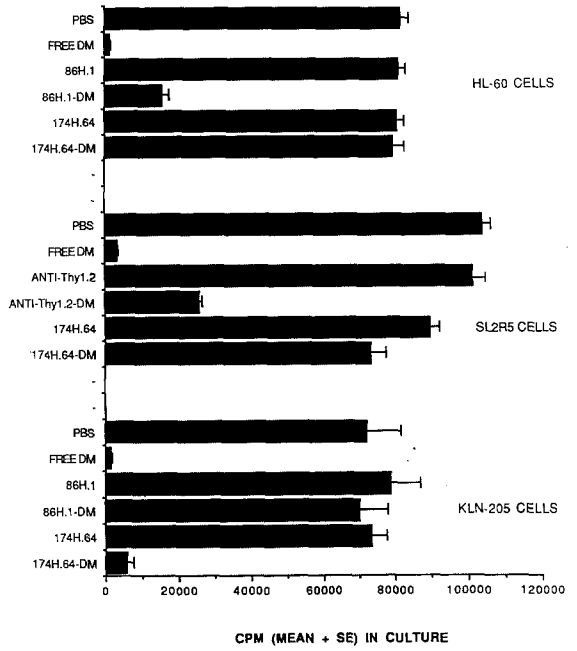

Fig. 1. Specific inhibition of in vitro proliferation of three cancer cell lines by different immunoconjugates. A sample of 2×10^4 cells was added to each well and allowed to grow in RPMI-1640 medium with 10% fetal calf serum for 2 days. On the 3rd day the KLN-205, SL2R5 and HL60-2 cell lines were incubated on ice for 2 h with one of the immunoconjugates of mAb-DM, or one of the mAb alone or with free daunomycin (*DM*). The concentration of free daunomycin was 60 μg/ml, and that used in the immunoconjugates was at a concentration of 6.5 μg/ml; all mAb were used at 200 μg/ml. After washing three times and incubating at 37° C for another 24 h, cell cultures were pulsed for 4 h at 0.5 μCi/well [^{3}H]thymidine to measure cell proliferation. *PBS*, phosphate-buffered saline

Cell lines. A murine squamous carcinoma line, KLN-205 [13], a murine leukemia cell line, SL2R5 [18] and a human myeloid leukemia line, HL60-2 [10] were obtained from the American Type Culture Collection, Rockville, Md. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum.

Preparation of immunoconjugates. Immunoconjugates were prepared as described [5, 19] according to the method of Shen and Ryser [16]. mAb were obtained from ascites fluids, which were subjected to protein precipitation with 40% $(NH_4)_2SO_4$ followed by dialysis against phosphate-buffered saline (140 mM NaCl, 2.65 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) at 4° C for 24 h. This protein fraction was further purified by affinity chromatography on a protein-A-Sepharose column (Pharmacia). Daunomycin and *cis*-aconitic anhydride were purchased from Sigma Chemical Co., St. Louis, Mo. Purified monoclonal antibody 174H.64 was obtained from Biomira Inc., Edmonton, Canada. mAb anti-Thy1.2 was obtained from NEN and mAb 86H.1, which reacts against human myeloid cells [12] including HL-60 cells, was made in our laboratories.

Treatment of cells with cytotoxic immunoconjugates or other agents. Cells in phosphate-buffered saline were treated with the immunoconjugates or other agents for 2.5 h on ice. Thereafter the cells were washed three times and injected intravenously into recipient mice, or incubated for 24 h at 37° C after which [^{3}H]thymidine incorporation by the cells was measured.

[^{3}H]Thymidine incorporation. Cell cultures were pulsed for 4 h with 0.5 μCi/well [^{3}H]thymidine (specific activity 2.0 μCi/mmol) (NEN). Cells were harvested onto glass fibers with a Titertek semi-automated multiple-sample collector (Flow Laboratories, Mississauga, Ontario). Thymidine incorporation was determined by liquid scintillation spectrometry.

Squamous carcinoma animal model. The animal model has been described previously [15], using KLN-205 cells [13].

^{125}I-dUrd uptake. Mice were injected with 2 μCi/each of ^{125}I-dUrd (Edmonton Radiopharmaceutical Centre, Edmonton, Alberta, Canada) and 4 h later the lungs were removed, cut into small pieces, washed with 10% trichloroacetic acid three times for 3 days and assessed for radioactivity content using a gamma counter.

Statistical analysis. Most data were assessed by Student's *t*-test. Survival data were assessed by a modified Wilcoxon test.

Results

Inhibition of in vitro proliferation of KLN-205 cells by 174H.64-DM

We have previously described the selective reactivity of mAb 174H.64 with all mammalian squamous carcinomas tested including the KLN-205 murine lung squamous carcinoma cell line [15]. To investigate the specific cytotoxicity of the immunoconjugate, the KLN-205, SL2R5 and HL60-2 cell lines were treated on ice for 2 h with mAb 174H.64-DM, 86H.1-DM, anti-Thy1.2-DM, free daunomycin, 174H.64, anti-Thy1.2 or 86H.1 and, following washing, the cells were subsequently cultured for another 24 h. Figure 1 demonstrates the specificity of cell killing activity of the different antibodies in comparison with daunomycin alone. While the HL-60 cells and SL2R5 cells were each inhibited by their respective mAb, only KLN-205 cells were inhibited by the 174H.64-DM immunoconjugate. It is noteworthy that the inhibition in vitro of the proliferation of KLN-205 cells by the 174H.64-DM immunoconjugate was nearly as effective as that of daunomycin alone.

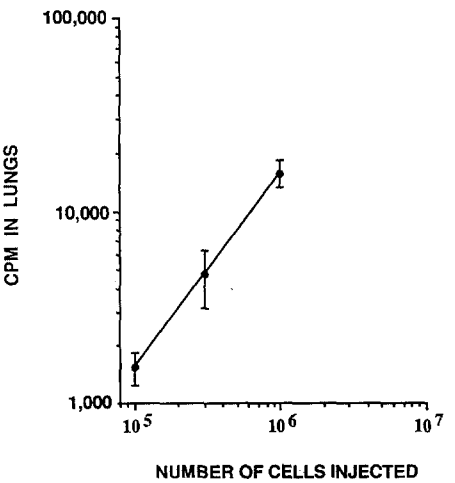

Fig. 2. Dose/response curve of KLN-205 cell proliferation in the lungs of DBA/2 mice. Different groups of DBA/2 mice were injected i. v. with 10^5, 3×10^5 or 10^6 KLN-205 cells. On the 23rd day after injection, cell proliferation in the lungs was measured by assessing ^{125}I-dUrd uptake

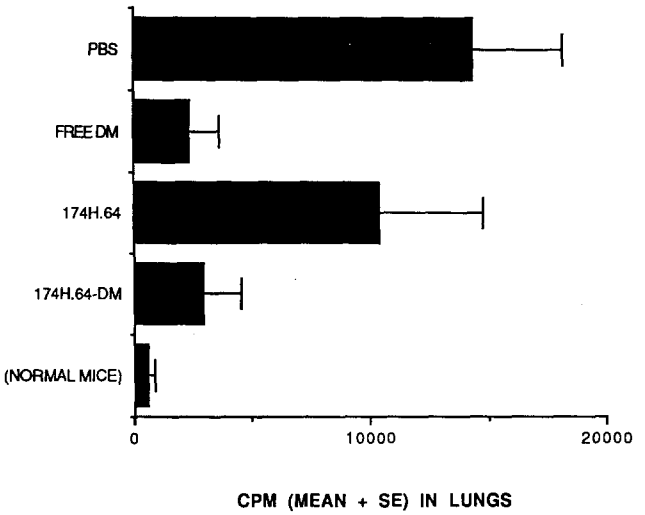

Fig. 3. In vivo growth inhibition of KLN-205 cells following in vitro 174H.64-DM treatment. KLN-205 cells were incubated on ice for 2.5 h with either culture medium, mAb 174H.64 (200 μg/ml), 60 μg/ml daunomycin or 200 μg/ml 174H.64-DM (containing 6.5 μg/ml of daunomycin) and were then washed and injected i.v. into DBA/2 mice. On the 23rd day after injection, tumor growth was measured by assessing ^{125}I-dUrd uptake in the lungs. Each group comprised 18 mice

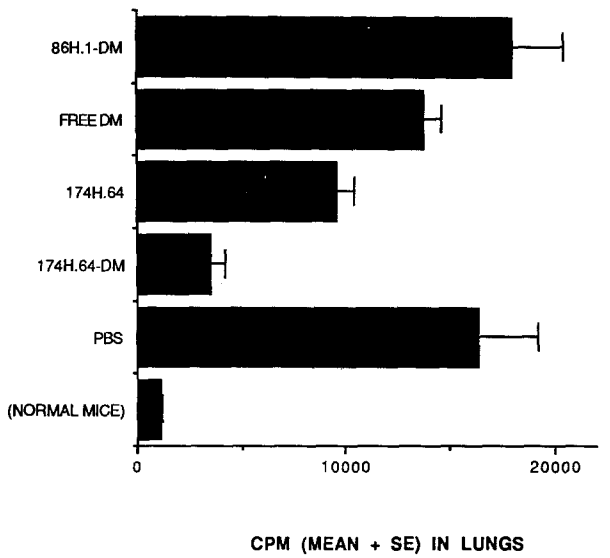

Fig. 4. Growth inhibition of lung metastatic KLN-205 cells following i.v. injection of 174H.64-DM. 10^6 KLN-205 cells were injected i.v. into normal DBA/2 mice. After 3 days various groups were injected intravenously every second day for 10 days (five times total) with either 1 ml PBS (n = 9), 300 μg 174H.64-DM (containing 10 μg daunomycin) (n = 12), 300 μg 174H.64 (n = 7), 100 μg daunomycin (n = 7) or 300 μg 86H.1-DM (containing 10 μg daunomycin (n = 9). On the 23rd day after injection, tumor growth was measured by assessing ^{125}I-dUrd uptake

In vitro treatment of KLN-205 cells with 174H.64-DM inhibits tumor cell proliferation in the lungs of i.v. injected mice

For this experiment we employed the ^{125}I-dUrd uptake assay as a measure of KLN-205 tumor cell proliferation in the lungs. We first established that the uptake of ^{125}I-dUrd in the lungs is directly proportional to the number of i.v. injected KLN-205 cells at least over the range of 10^5–10^6 cells (Fig. 2).

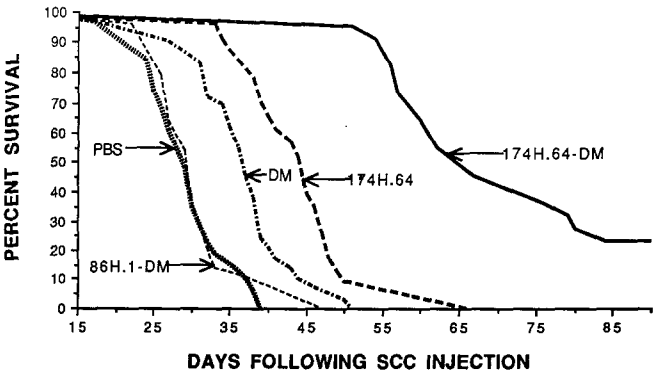

Fig. 5. Therapeutic effect of 174H.64-DM on the survival of mice bearing KLN-205 squamous carcinomas. Three days after 1×10^6 KLN-205 cells had been injected i.v. into normal DBA/2 mice, the latter were injected intravenously every second day for 10 days (five times total) with either (A) 1 ml PBS, (B) 300 μg mAb 86H.1-DM (containing 10 μg daunomycin), (C) 100 μg DM, (D) 300 μg 174H.64, or (E) 300 μg 174H.64-DM (containing 10 μg daunomycin). Groups A and B do not differ significantly from each other. Groups C, D and E showed significantly greater survival than groups A and B (P <0.001). Significantly greater survival also occurred in the following order E>D>C (P <0.001)

KLN-205 cells were then treated on ice for 2.5 h with 174H.64-DM immunoconjugate or with various controls, including phosphate-buffered saline, 174H.64 alone or free daunomycin. Thereafter cells were washed three times and injected into DBA/2 mice (10^6 cells/mouse). On the 23rd day after injection, tumor growth in the lung was monitored using ^{125}I-dUrd uptake. Results in Fig. 3 show that the 174H.64-DM pretreatment significantly reduced the growth of lung tumors by approximately 85% and was as effective as incubation of the cells with daunomycin alone (Fig. 3).

mAb 174H.64-DM injected i.v. inhibits the growth of established lung metastatic KLN-205 cells in vivo

The purpose of this experiment was to investigate the specific cytotoxicity of 174H.64-DM in vivo. A sample of 10^6 KLN-205 cells was injected i.v. into DBA/2 mice. After 3 days 174H.64-DM was injected i.v. every second day for 10 days (five injections). On the 23rd day after injection of the tumor cells, tumor growth was measured by assessing ^{125}I-dUrd uptake in the lungs. The results in Fig. 4 show that 174H.64-DM significantly (P <0.001) reduced ^{125}I-dUrd uptake by about 80% while 86H.1-DM had no significant effect. Daunomycin alone had a slight, but insignificant effect but 174H.64 alone reduced the tumor cell proliferation by approximately 42% (P <0.001).

Survival of mice receiving 174H.64-DM therapy

The above experiment was repeated but instead of sacrificing the mice to monitor the growth of KLN-205 cells in the lungs, the mice were simply monitored for survival.

The results in Fig. 5 show that 174H.64-DM-treated mice survived significantly longer ($P < 0.001$) than untreated or antibody-treated mice. 174H.64 alone also improved the survival of tumor-bearing mice ($P < 0.001$).

Discussion

The present study was designed to test the hypothesis that a mAb directed against the proliferative (stem) cell compartment of squamous carcinomas has therapeutic potential. We have used a novel mAb, 174H.64, which appears to detect a unique epitope on cytoskeletal proteins that may serve as a marker for the stem-cell population in normal stratified squamous epithelia and squamous carcinomas [15]. mAb 174H.64 reacts with all squamous carcinomas tested regardless of histological origin [15] as well as with all squamous carcinomas of all mammals tested, including those of humans (>62 tested), dogs (10 tested), cattle (40 tested) and cats (2 tested). In the present study we took advantage of its reactivity with a murine metastatic lung squamous carcinoma, KLN-205, to develop an animal model for chemoimmunotherapy with a daunomycin conjugate of mAb 174H.64 (174H.64-DM). Previous studies using the KLN-205 cell line [13, 17] have shown that the tumor periphery of the lung metastatic nodules is composed of highly proliferative cells, which are the progenitors of the more differentiated cells in the central portion of the tumor. We have shown that mAb 174H.64 selectively stains the peripheral layer of KLN-205 lung tumors as well as human and bovine squamous carcinoma [15]. This provided the rationale for using the KLN-205 model to attempt to target therapy selectively to the "stem" cells of the tumor as it might be expected that the internal, more differentiated cells are no longer capable of neoplastic proliferation.

We chose daunomycin as the cytotoxic component of the immunoconjugate because of our extensive experience with this drug [3, 4, 5, 8, 19]. We have shown that an immunoconjugate of anti-Thy1.2 mAb and daunomycin possesses high specific cytotoxicity for target cells and could be used for the specific purging of Thy1.2$^+$ tumor cells and murine functional T cells from the bone marrow [8]. In the present report, we have shown that an immunoconjugate of mAb 174H.64 with daunomycin also produces marked, specific inhibition of the growth of KLN-205 murine squamous carcinoma both in vitro and in vivo when compared to irrelevant conjugates, free monoclonal antibodies, or free daunomycin.

Our first experiments were designed to demonstrate the specificity of the immunoconjugate killing in vitro. For this we conducted a criss-cross experiment with three cell lines and three immunoconjugates which, on the basis of immunofluorescent and/or immunoenzyme testing, should be specific for their specific cell lines. Exquisite specificity and excellent killing were noted in each case: 86H.1-DM killed only HL-60 cells, anti-Thy1.2-DM killed only SL2-R5 cells and 174H.64 killed only KLN-205 cells. Interestingly, the killing of KLN-205 cells by the 174H.64 immunoconjugate was nearly as effective as the free daunomycin added to the culture.

An extension of the in vitro experiment was then performed whereby the KLN-205 cells were incubated in vitro with the immunoconjugate, free antibody or daunomycin, washed, injected i.v., and the metastatic growth in the lungs monitored. In this case a marked reduction of the metastatic growth of the KLN-205 cells in the lungs was achieved by preincubation with the 174H.64-DM conjugate. Again the reduction achieved with the immunoconjugate was about the same as that achieved with free daunomycin. In vitro incubation with the unconjugated mAb 174H.64 caused a slight but statistically non-significant reduction in growth of KLN-205 cells in the lungs.

The next two experiments were designed to test directly the in vivo therapeutic potential of the 174H.64-DM immunoconjugate. Immunotherapy was started 3 days following the i.v. injection of 10^6 KLN-205 cells, at a time when the lung metastatic cells should have been well established [13, 17]. Five therapeutic injections of 174H.64-DM were given i.v. every second day for 10 days. Growth of the KLN-205 cells in the lungs was monitored at day 23 by using the ^{125}-IdUrd uptake assay and survival was monitored for >100 days. The two estimates of the therapeutic potential of the various compounds gave very similar results. Mice injected with mAb 86H.1-DM showed no therapeutic benefit and showed similar tumor growth and survival to those injected with phosphate-buffered saline. Daunomycin alone gave a slight reduction of growth and a slight increase in survival. An even greater therapeutic benefit was achieved with unconjugated mAb 174H.64. However, by far the best therapy was achieved following i.v. injection with the 174H.64-DM immunoconjugate where an >80% reduction in tumor cell growth in the lungs and a prolonged survival were achieved. Greatly prolonged survival was achieved in 20% (5 mice) of the mice that survived >100 days. The therapeutic benefit of the 174H.64 immunoconjugate is even more impressive if one considers that the therapeutic regimen used in the present experiments may not be an optimal one.

The efficacy of 174H.64-DM probably depends on the specificity of its binding to the target cell surface and the accumulation of the drug inside the target cells [1, 7, 14]. Pimm and coworkers [14] have studied human tumors growing in nude mice injected with daunomycin immunoconjugates. They found that the tumors showed localization of both the drug and antibody and at the time of analysis (3 days after injection), the tumor levels of daunomycin were over 100 times those seen in mice injected with free daunomycin. Dillman et al. have recently demonstrated the accumulation [^{3}H]daunorubicin in lysosomes and nuclei of tumor cells after incubation with a conjugate containing [^{3}H]daunorubicin linked to monoclonal antibody via an acid-labile *cis*-aconityl spacer [7]. Thus the mechanism of the selective antitumor effect of the immunoconjugate appears to involve endocytosis of the antigen-bound immunoconjugate followed by pH-dependent release of the cytotoxic component into the lysosome, resulting in the recovery of its full pharmacological activity. Those results are consistent with our observations that at the same daunomycin concentration the immunoconjugate 174H.64-DM showed a marked suppression of the growth of KLN-205 cells in vivo while free daunomycin did not,

although the free daunomycin showed the same or better cytotoxic potency as the immunoconjugate following in vitro incubation.

Gallego and coworkers [11] investigated four different linkage groups for coupling daunomycin to antibody. They found that conjugates made with a *cis*-aconityl linkage, which was the same one used in the present work, displayed the greatest selective cytotoxicity against tumor cells. Our results appear to substantiate the efficacy of daunomycin immunoconjugate made with *cis*-aconityl linkage groups.

Unconjugated mAb 174H.64 alone showed a significant antitumor effect in vivo (Figs. 4, 5), while no such effect could be observed on tumor cell growth in vitro (Fig. 1). This in vivo efficacy of the antibody may be due to antibody-dependent cellular cytotoxicity or other cytolytic mechanisms mediated by the immune system including complement-mediated cell lysis [6]. Such antitumor effects elicited by the antibody part of the immunoconjugate may be beneficial in patients with an intact immune system.

mAb 174H.64 appears to detect a novel epitope expressed on certain cytoskeletal proteins found only in the stem cell populations of normal and neoplastic stratified squamous epithelium [15]. The present experiments as well as our previous observations [15] suggest that the epitope detected by mAb 174H.64 is also expressed on the surface of the KLN-205 cells. Other cell-surface determinants have been reported for some cytoskeletal proteins [2, 20]. As the 174H.64 epitope may also be present on the surface of the basal cells of stratified squamous epithelium [15] some skin toxicity might have been expected in the present experiments. However, we have never seen any evidence of any skin lesions nor any other obvious gross pathology in over 100 mice that have received multiple injections of either the free 174H.64 or the 174H.64-DM conjugate including our long-term (>100 day) survivors.

On the basis of our studies in mice we conclude that mAb 174H.64 may have promising therapeutic potential in patients with squamous cell carcinoma. In our preliminary radioimmunoimaging studies with radiolabeled 174H.64 excellent in vivo localization of this mAb in human squamous cancer was observed (McEwan, MacLean, Sykes and Noujaim, unpublished results). Further studies in human cancer patients are underway to evaluate further the potential of mAb 174H.64 for in vivo diagnosis and therapy of human squamous cancers.

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