Specific immune cell therapy against ovarian cancer in vivo and in vitro

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Key words: 6B11 anti-idiotypic minibody (6B11 mini), ovarian cancer, SKOV3 cells, xenografts, cytokines induced killer cells (CIK), immunocyte therapy, mice

Background and Objective: 6B11 minibody (6B11 mini), an anti-idiotypic vaccine against human ovarian cancer, has been proven to induce specific humoral and cellular immunity against ovarian cancer in vivo and in vitro. This study investigates the safety and efficacy of using 6B11 mini as an antigen to treat ovarian cancer. Methods: After being loaded with purified 6B11 mini, dendritic cells (DCs) were co-cultured with peripheral blood mononucleocytes (PBMNC) and stimulated by various cytokines, including CD3 monoclonal antibody, interleukin-2, interferon-γ, toobtain 6B11 mini-ovarian-cytokine-induced-killer cells (6B11-O-CIK). Tumor-forming ability was determined using soft agar colony-forming assay in vitro and nude mice xenografts in vivo. The acute toxicity of 6B11-OCIK at different doses was observed in BALB/c mice. Cytotoxicity of 6B11-OCIK to different target cells was detected using a 51Cr release test in vitro. The ovarian tumor model was established using severe combined immune deficiency (SCID) mice transplanted with the human ovarian cancer cell line SKOV3. The tumor growth was detected after an injection of 6B11-OCIK into SCID mice. An injection of CIK, PBMNC and physiological saline were used as controls. Results: After a culture period of 14 days in soft agar, SKOV3 cell clones were well formed with a ratio of 50%; while 6B11-OCIK, CIK and PBMNC did not form any clones. All nude mice injected with the human cervical carcinoma cell line Hela (positive control) grew tumors after 14 days. Mice injected with 6B11-OCIK, CIK, PBMNC and normal human fetal lung fibroblast WI-38 cells did not form tumors after 13 weeks. BALB/c mice did not show any abnormal response half an hour after the administration of 6B11-OCIK cells at different doses. Mice were euthanized 13 days after treatments, but no distinct abnormality of the main organs were found. The injected 6B11-OCIK exerted against tumor cells with positive OC166-9, which was related to the limitation of MHC. The tumor weights of SCID mice transplanted with SKOV3 cells were significantly lighter in the 6B11-OCIK treatment group than in the saline group (p = 0.023); while tumor weights were not significantly different between the 6B11-OCIK group with CIK and the PBMNC group (p = 0.540; p = 0.285). Conclusions: The application of 6B11-OCIK in vivo has achieved the safety standard; moreover, 6B11-OCIK has an inhibitory effect on the growth of ovarian cancer cells.

Biotherapy is the latest addition to tumor therapy following surgery, radiotherapy and chemotherapy. Cellular immunity has been identified as playing a major role in anti-tumor immunity, and thus adoptive immune cell infusion has become a powerful tumor therapy. The effector cells of immune cells include: lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TIL), cytokine-induced killer (CIK) cells, cytotoxic T lymphocytes (CTL) and dendritic cells (DC). Both LAK and CIK cells are non-specific immune cells; DC are the most powerful antigen-presenting cells; TIL cells display specificity; however, CTL cells have the most specific killing effect and, therefore, are the best effector cells for adoptive immunotherapy.

In vitro induction and proliferation of CTL cells can be achieved by either directly using tumor antigen immunoreactive peptides or tumor antigen-loaded DCs to stimulate the peripheral blood mononucleocytes (PBMC). Therapy using tumor antigen-loaded DCs to activate CTL cells has been reported to have a promising effect in animal experiments and in clinical research on melanoma and renal cell carcinoma, but little has been reported on ovarian cancer.

Since the vast majority of tumor antigens are unknown, anti-idiotypic antibodies are often used to replace a tumor antigen vaccine because they can mimic the tumor antigen and are known...
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Materials and Methods

Main materials and reagents. This study has been approved by the Ethics Committee of the People's Hospital, Peking University. Forty BALB/c mice and fifty nude mice were included (female, weight 18 g to 20 g). Twenty-eight severe combined immune deficiency (SCID) mice (SPF class, six to eight weeks old, female, weight 18 g to 20 g) were purchased from the Experimental Animal Division of Peking University Health Science Center (Certificate of Quality No: SCXK Beijing 2002-0001). The human fetal lung fibroblast WI-38 cells are normal human diploid cells confined to a limited number of passages, from ATCC, were purchased from the Shanghai Cell Bank. The cervical cancer HeLa cells were purchased from ATCC, USA and frozen in our laboratory. The human ovarian cancer HOC1A cells (an immortalized human ovarian cancer cell line established by our laboratory, patent No: ZL01130757). The liver cancer HLE cell line was a gift from the Hepatobiliary Surgery Center. The immortalized human ovarian cancer cell line SkOV3 cells were purchased from ATCC, USA and frozen in our laboratory. The human ovarian cancer SkOV3 cell line was a gift from the Shanghai Cell Bank. The cervical cancer HeLa cells were purchased from ATCC, USA and frozen in our laboratory. The human ovarian cancer HOC1A cells (an immortalized human ovarian cancer cell line established by our laboratory, patent No: ZL01130757). The liver cancer HLE cell line was a gift from the Hepatobiliary Surgery Center. The natural killer (NK) cell sensitive strain K562 was frozen and stored in our laboratory on a long-term basis. Cytokines interleukin-2 (IL-2) was purchased from Beijing Ruidi Hetong Medicine Co., Ltd. The mouse anti-human CD3 monoclonal antibody was purchased from Cuba Molecular Biology. The interferon-γ (IFNγ) was purchased from the Shanghai Institute of Biological Products. The interferon-α (IFNα) was purchased from Tianjin Hualida Bioengineering Co., Ltd. The tumor necrosis factor-α (TNFα) was purchased from the Shanghai Saida Biological Pharmaceutical Co., Ltd. The granulocyte macrophage colony-stimulating factor (GM-CSF) was purchased from the Xiamen Tebao Bioengineering Co., Ltd. The interleukin-4 (IL-4) and SCF were purchased from the Academy of Military Medical Sciences and the 6B11 mini was from the Shanghai New Summit Biotechnology Co., Ltd., who is in cooperation with our center.

Methods. Culture of bone marrow dendritic cells (BMDC) and preparation of 6B11-OCIK and CIK. Bone marrow was collected from a HLA-A2+ donor and the cells were kept for CD80/CD86, CD83/CD1a and HLA-DR flow cytometry. Mononuclear cells were isolated and cultured. SCF, TNFα, IL-4 and GM-CSF (1,000 U/mL) were added on day 0. The mixtures were incubated in 5% CO₂ at 37°C. On day 4, about 1/3 volume of serum-free culture medium was added along with the corresponding amounts of TNFα and GM-CSF (1,000 U/mL). According to the total volume, IL-4 (1,000 U/mL) and 6B11 mini (4 μg/mL) were added. The mixtures were then incubated in 5% CO₂ at 37°C. On day 7, further serum-free culture medium was added along with corresponding amounts of TNFα, GM-CSF and IL-4 (1,000 U/mL) and 6B11 mini (4 μg/mL), then IFNγ (1,000 U/mL) according to the total volume. On day 10, the mixtures were checked for signs of colony growth before being further mixed. The cells were kept for CD80/CD86, CD83/CD1a and HLA-DR flow cytometry. On day 12, the mature 6B11 mini-loaded DCs were harvested. PBMCNC (>2 x 10⁹/kg) were collected using the blood cell separator, counted and transferred to disposable plasma separation bags, joined with AIM-V serum-free culture medium, 500 mL, centrifuged at 450 xg for 15 min. The supernatant was then discarded. After repeating the above washing process twice, the cell suspensions were transferred to disposable air-permeable culture bags. Appropriate amount of AIM-V serum-free culture medium was added to reach a cell concentration of 1-2 x 10⁹/mL. BMDC (T/DC: 10/1-100/1) was also added in the 6B11-OCIK group, but not the CIK group. Both groups were joined with IFNγ (2,000 U/mL). On day 13, 6B11 mini (1 μg/mL), interleukin (21,000 U/mL) and mouse anti-human CD3 monoclonal antibody (25 ng/mL) were added into the 6B11-OCIK group culture bags. Everything except 6B11 mini was added to the CIK group culture bags. The mixtures were then incubated in 5% CO₂ at 37°C. On day 16, all cell cultures in the bags were sampled, counted and examined microscopically. The cell proliferation was examined, the serum-free cell culture medium was added to reach a cell concentration of 1 x 10⁶/mL, IL (2,500 U/mL) was then added and the mixtures continued to be cultured. On day 19, all cell cultures in the bags were sampled again, counted and examined microscopically, the serum-free cell culture medium was added to reach a cell concentration of 1 x 10⁶/mL, then IL (2,500 U/mL) was added. All cultures were examined for colonies. On day 20, the cells were plated. On day 21, they were sampled and counted under a microscope with 100% viable cells. The 6B11-OCIK suspensions were transferred using infusion sets to the plasma separation bags, centrifuged and the supernatant was discarded. The normal saline containing IL-2 100 U/mL was added, the mixtures were centrifuged and the supernatant was discarded. After repeating the above washing process twice, IL-2 was added to make up a total of 2.5 x 10⁶ U. The cells were reinfused within six hours. The fresh single-donor PBMCNC collected using the blood separator from the same donor was also reinfused on the same day as a control group for 6B11-OCIK and CIK groups.

6B11-OCIK acute toxicity assay. Forty SPF Class BALB/c mice were divided into four groups, 10 in each group. Different doses of 6B11-OCIK cells were infused intravenously: low-dose (2 x 10⁶/g), medium-dose (5 x 10⁶/g) and high-dose (2 x 10⁶/g) corresponding to 1, 2.5 and 10 times of the dosages used in human treatment (a mean weight of 20 g was used in this study). The fresh single-donor mononuclear cells were infused as a control group. The mice were weighed with standard digital scales using milligram accuracy on the day before the infusion, and one, six, eight and twelve days after the infusion at the same time of day to avoid the bias caused...
by their diet and other daily activities. All animals were observed 30 minutes after the infusion for any significant abnormal reactions (toxic reactions, allergic reactions, local skin irritations, abnormal behaviors or death). They were further observed for 13 consecutive days for any unusual reactions or deaths. The mice were sacrificed 13 days after the infusion for anatomic observation of their hearts, livers, spleens, lungs, kidneys, brain, muscles, skin and other important organs for any abnormalities.

6B11-OCIK soft agar colony-forming assay. Equal amounts of 1.2% agarose kept at 50°C and 2x RPMI1640 medium kept at 37°C were mixed, transferred onto horizontally placed sterile plates, cooled to room temperature and incubated in CO₂ as the bottom agar plate. The 6B11-OCIK cells, CIK cells, fresh single-donor PBMNC and SKOV3 cells were resuspended with RPMI 1640 culture medium and pipetted to form single-cell suspensions. Viable cells were counted using 0.04% trypan blue and the cell viability was calculated. Appropriate amounts of culture medium were then added to reach a cell concentration of average 5 x 10⁶/mL in all three types of cells. Two mL of 0.7% agarose kept at 50°C and 2 mL of 2x RPMI-1640 culture medium kept at 37°C were mixed in sterile test tubes, and 0.3 mL of SKOV3 cell suspensions (concentration adjusted) were added when the temperature cooled down to about 40°C. The mixture was transferred onto the bottom agar plate forming a double-layer agar. The same method was applied to the other three types of cells. Two plates were prepared for each type of cell. Once the top agar became solidified, all plates were then incubated in 5% CO₂ at 37°C, and were examined microscopically on day eight and day 14.

In vivo tumorigenicity of 6B11-OCIK in nude mice. Fifty nude mice were divided into five groups, 10 mice in each group. The 6B11-OCIK, CIK, cells and fresh single-donor PBMNC end-product cells were inoculated subcutaneously with 5 x 10⁷ cells/bur well/mean cpm of maximum release well) x 100%

Rate of target cell ⁵¹Cr natural release = (mean cpm of natural release well/mean cpm of maximum release well) x 100%

In vivo therapeutic effect of 6B11-OCIK in subcutaneous tumor bearing SCID mice. The SCID mice were inoculated subcutaneously on the right side of their back with SKOV3 cells after extended culture, 100 μL containing a total of 1 x 10⁷ cells per mouse and tumor growth was closely observed. When the tumor grew to the size of about 0.5 cm, the SCID mice were divided into four balanced groups according their tumor sizes. Their ears were snipped and included in different experimental groups to receive cell infusions. Due to individual difference, a tumor in one mouse was too big and was therefore excluded in order to avoid interference with the results. The control group had six mice inoculated with normal saline. We injected 100 μL of 6B11-OCIK, CIK and fresh donor PBMCN containing 4 x 10⁶ cells intravenously via the tail veins; while the control group was given 100 μL of normal saline. All tumors were observed and their sizes were measured. The experiment terminated when the tumors grew to about 1 cm before possible rupture. The tumors were then excised, weighed and photographed.

Statistical analysis. The variance analysis and multiple comparisons were applied. Please see software name and version, etc.

Results

Determination of DC maturation. As DC matured, they were observed microscopically to have the following changes: initial cell adherence and clustering as induced by cytokines, then 3–5 days later, changed from adherence to semi-suspension then to suspension. The cells became swollen and developed burr-like protrusions with various lengths emerging from the cell membrane. The expressions of cell surface co-stimulatory molecules (CD80, CD86), MHC-II molecules (HLA-DR) and DC symbolic molecules (CD1a, CD83) were examined using flow cytometry (FCM). The mature DCs that we cultured had high expression rates of CD80 at 65%, CD86 at 86.2% and HLA-DR at 93.7%, increased expression rate of CD83 at 21.4% and CD1a at 2.2%, which were consistent with the surface molecules’ expression patterns of mature DCs (Fig. 1).

6B11-OCIK acute toxicity assay. No obvious abnormal reactions were observed within 30 minutes after infusion in all
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Experimental and control groups. All mice presented healthy fur and good spirits. In the following 13 days, no obvious abnormal reactions were observed in any groups. All mice maintained healthy fur, good spirit, normal diet and weight gain. The average weight gain of the 10 mice in different groups was 0.99 g in the $2 \times 10^6$/g group, 0.62 g in the $5 \times 10^5$/g group, 0.548 g in the $2 \times 10^5$/g group and 0.550 g in the PBMNC control group. The weight changes in all groups are shown in Figure 2. All mice were euthanized 13 days after infusion for anatomical observation. No significant abnormalities were found in their hearts, livers, spleens, lungs, kidneys, brain, muscles, skin and other major organs.

6B11-OCIK soft agar colony-forming assay. After being cultured for eight days, the culture dishes were examined under inverted microscopes for colony formation and we found that the positive control SKOV3 started to develop small colonies, while the 6B11-OCIK, CIK and fresh single-donor PBMNC did not. By day 14, we found well-formed SKOV3 cell colonies with a colony formation rate of 50%, while the 6B11-OCIK, CIK and fresh single-donor PBMNC showed no colony formation.

In vivo tumorgenicity in nude mice. Three to seven days after inoculation, we noted unabsorbed white spots at the injection sites in the 6B11-OCIK, CIK, fresh single-donor PBMNC and WI-38 groups, but some mice in the HeLa group started to develop small nodules. By day 14, no abnormalities were seen at the injection sites in the 6B11-OCIK, CIK, fresh single-donor PBMNC and WI-38 groups while all mice in the positive control HeLa group developed tumors. All mice continued to be observed for one week, then half of them were euthanized. No tumors formed in the 6B11-OCIK, CIK, fresh single-donor PBMNC and WI-38 groups while all mice in the positive control HeLa group developed tumors. No significant organ metastases were noted during anatomical observation in all groups. The remaining five mice in each group continued to be observed for 10 more weeks, during which, the 6B11-OCIK, CIK, WI-38 group and fresh single-donor PBMNC groups remained free of tumors.

Figure 1. Expression of CD molecules on mature dendritic cells.

Figure 2. Weight variation of BALB/c mice after injection of 6B11-OCIK.
In vitro killing effect of 6B11-OCI on target cells. The killing effect of 6B11-OCI on from two volunteers on various target cells were examined using ⁵¹Cr release test. The single-factor analysis and multiple comparisons showed that under the effector-to-target cell ratio of 20:1, the killing effect of 6B11-OCI on the HOC1A group (OC166-9, HLA-A²⁺) was statistically significantly different from that on the HLE (OC166-9, HLA-A²⁺) and K562 groups (p = 0.045; p = 0.031), but no significant difference from the SKOV3 group (OC166-9, HLA-A²) (p = 0.110). There was no significant difference among the SKOV3, HLE and K562 groups. Under the effector-to-target cell ratios of 10:1 or 5:1, there was no significant difference in the killing effect of 6B11-OCI among all groups, but the killing effect of 6B11-OCI on HOC1A cells was higher than those on SKOV3, HLE and K562 cells, see Figure 3. These results indicated that 20:1 was the effective effector-to-target cell ratio for 6B11-OCI in vitro, that 6B11-OCI had a specific killing effect on tumor cells and was associated with MHC restriction.

In vivo therapeutic effect of 6B11-OCI on subcutaneous tumor-bearing SCID mice. To avoid rupture, the experiment was terminated when the tumors grew to about 1 cm in size. The tumors were then excised and weighed on an analytical scale. The mean tumor weights in the 6B11-OCI group, CIK, fresh single-donor PBMC and normal saline groups were respectively 0.484 ± 0.133 g, 0.576 ± 0.301 g, 0.646 ± 0.115 g and 0.858 ± 0.456 g. The tumor weight of the 6B11-OCI group had a statistically significant difference from that of the normal saline control group (p = 0.023), but no difference from the CIK or fresh single-donor PBMC groups (p = 0.540; p = 0.285).

**Discussions**

Wang et al.² treated patients with early liver cancers using autologous CIK and found that CIK cells had significantly higher tumor inhibitory rate than LAK cells. Shi et al.³ reinfused autologous CIK cells to 13 cases of early and progressive hepatocellular carcinoma. The researchers found that CIK cells effectively improved the patients' cellular immunity and, in addition, found no side-effects during clinical observation. Jiang et al.⁴ divided 57 cases of advanced gastric cancer patients into two groups. One group was treated with chemotherapy and autologous CIK and the other group with chemotherapy only. The results showed that during the first 14–21 days of the treatment, the plasma tumor markers in the former group decreased significantly; their immune function was enhanced; their quality of life improved and their survival time had been prolonged. The Cell Therapy Center in the People's Hospital, Peking University has obtained the approval from the SFDA and has started autologous CIK treatment for leukemia. The phase I/II clinical trials with a total of 100 cases has confirmed that somatic cell therapy is safe and effective, with a clinical complete remission rate of 70% compared to 20% in the control group (internal data). Meng Fan-Dong et al.⁵ collected the peripheral blood mononuclear cells of five post-operative ovarian cancer patients, induced them into CIK with various cytokines and reinfused them into the patients. They found that following the CIK reinfusion, their peripheral blood CD3⁺, CD3⁺/CD8⁺, CD3⁺/CD56⁺ lymphocyte subsets significantly increased indicating that the CIK treatment can improve patients cellular immunity.

The above are successful examples for CIK treatment. Nevertheless, CIK treatment is still a non-specific treatment, while CTL cells, the most specific killing cells, are the best effector cells for adoptive immunotherapy. CTL induction requires tumor-associated antigens but few ovarian cancer tumor-associated antigens are currently known. In ovarian cancer DC vaccine studies, the in vitro DC-sensitizing methods include HER2/neu, MUC1 polypeptide, tumor cell lysates pulsed DC sensitization⁶,⁷ and the fusion of dendritic cells to ovarian cancer cells.⁸ These methods can all induce a specific anti-tumor immune response. The phase I clinical trial of in vitro HER2/neu and MUC1 peptide DC sensitization for ovarian cancer treatment also demonstrated good results.⁹ These methods have advantages and disadvantages in clinical application: a single antigen-induced immune response has a limited capacity, so it cannot induce the maximum anti-tumor immune response. For the autologous tumor cell freeze-thaw lysates method, it is difficult to determine the optimal stimulating dosage. Furthermore, there also exist normal auto-antigens which may induce autoimmune diseases and it may be difficult to collect the tumor cells in some patients. The above issues have restricted their clinical application.

Anti-idiotypic antibodies can mimic tumor antigens and have the following advantages: easy preparation, beneficial for vaccine standardization, high purity of protein, easy to be modified at gene level, no potential risk of carrying tumor virus and cancer genes like original tumor antigens (such as tumor protein, antigen polypeptide, tumor antigen extracts, whole tumor cells or apoptotic tumor cells). In 2003, Chwatterjee et al.¹⁰ loaded mice DCs with the anti-idiotypic antibody 3H1 which is similar
to the carcinoembryonic antigen (CEA) and immunized C57BL/6 mice via subcutaneous injection once every other week a total of three times. The results suggested that the anti-idiotypic antibody-loaded DCs could induce similar antigen-specific humoral and cellular immunity to the protein antigens.

Prepared by our laboratory, 6B11 mini is a partially humanized anti-idiotypic antibody, and has obtained a national invention patent (Patent No: ZL01130756.0). Our previous studies showed that using 6B11 mini to immune BALB/c mice, the specific Ab3 could be induced without any adjuvant. It served as a vaccine for immunotherapy for immuno-reconstructed SCID mice with human lymphocytes bearing human ovarian cancers in their ascites. This treatment has extended the survival time for more than 15 days in mice.

Based on our previous work, this study established the production protocol of 6B11-OCIK by using 6B11 mini-loaded DCs co-activated with normal human peripheral blood lymphocytes using various cytokines, and examined the safety and effectiveness of 6B11-OCIK in the treatment of ovarian cancer. This has opened up new avenues for the immunotherapy for small residual focal or recurrent ovarian cancers that express OC166-9.

Autologous lymphocyte infusion, in theory, does not cause any toxicity. However, given the fact that the collected lymphocytes are cultured in vitro for more than 20 days, they are exposed to multiple environmental factors such as culture materials, culture medium and cytokines. For safety reasons, it is crucial to establish an acute toxicity test. Our results showed that although xenograft rejection could not completely be ruled out, and that infusing the end product (human 6B11-OCIK) did not cause significant toxicity to the mice. The mice maintained normal growth, healthy hair, good spirit and weight gain during observation. Therefore 6B11-OCIK does not cause significant acute toxicity on mice.

As we know, normal human peripheral blood lymphocytes can not survive in vitro for long. In our study, the final product 6B11-OCIK human peripheral blood lymphocytes were obtained after being treated with various cytokines and co-cultured with 6B11-loaded DCs. In theory, it should not have any tumorigenicity in vitro, but more research needs to be carried out to ensure human safety. To this end, we designed and performed the in vitro soft agar colony-forming assay. The results showed that 6B11-OCIK does not have tumorigenicity in vitro.

In vivo animal studies will be required to determine the in vivo tumorigenicity of 6B11-OCIK. In our study, we used high-grade cervical cancer HeLa cells as a positive control, and the commonly recognized human normal diploid cells WI-38 as a negative control. The HeLa cells, with their strong in vivo proliferating ability, formed subcutaneous tumors, while no tumors developed in the normal human diploid cells WI-38 group, indicating satisfactory system reliability. We also found no tumors developed even in the high 6B11-OCIK concentration group, indicating that 6B11-OCIK does not have in vivo tumorigenicity in nude mice.

In our study, the 6B11-OCIK group was established based on the conventional CIK culture loaded with 6B11 mini; so in addition to CIK’s non-specific killing effect, it should also produce ovarian cancer-specific killing CTL cells. The in vitro killing experiment showed that 6B11-OCIK has higher killing effect on OC166-9 antigen positive and HLA-A2+ HOC1A cells than that on SKOV3, HLE and K562 cells, indicating that 6B11-OCIK has a specific tumor-killing effect and is associated with MHC restriction. The in vivo tumor inhibitory experiment demonstrated that the mean tumor weights in the 6B11-OCIK, CIK and fresh single-donor PBMCN groups had statistically significant differences compared with the normal saline control group, indicating that 6B11-OCIK has a good in vivo tumor inhibitory effect in animals. Although there was no difference found among the 6B11-OCIK, CIK and fresh single-donor PBMCN groups, we still noted a better tumor inhibitory effect in the 6B11-OCIK group than that in the CIK group. This difference may become statistically significant with repeated reinfusion or in animals with normal immune status.

Nevertheless, due to the complexity of the immune system, the therapeutic effect of 6B11-OCIK will ultimately need to be verified by clinical trials.

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References