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Analysis of effector T cells against the murine syngeneic tumor MethA in mice orally administered antitumor polysaccharide SPR-901

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Abstract. The growth of MethA tumor was significantly inhibited by oral administration of the α-glucan SPR-901 in BALB/c (+/+) mice but not in nude mice. Mice treated orally with SPR-901 exhibited an augmentation of antigen-specific resistance against rechallenge with the tumor cells. The tumor-neutralizing activity of regional lymph node cells from MethA-bearing mice against the tumor was augmented by oral administration of SPR-901. The tumor-neutralizing activity of lymph node cells from SPR-901-treated mice mainly appeared in $Lyt2^+$ cells. Furthermore, lymphokine-activated killer activity of these cells was enhanced by administration of SPR-901. The antitumor effect of SPR-901 was abrogated in mice depleted of either $L3T4^+$ or $Lyt2^+$ cells, and in cyclosporin-A-treated mice. These results suggest that $Lyt2^+$ cells are important effector cells in MethA-bearing mice orally administered SPR-901 and that functional exertion of both $Lyt2^+$ and $L3T4^+$ T cells is necessary for the antitumor effect of orally administered SPR-901 in vivo.

Key words: Polysaccharide SPR-901 – Antitumor effect – Oral administration – T cell effector

Introduction

There have been numerous reports describing the antitumor effect of β-glucans isolated from various natural sources that are composed mainly of β1,3-glucosidic linkages [10, 25, 31]. The antitumor effect of these polysaccharides seems to be attributable to a restoration or augmentation of the immunological responsiveness specific to the tumor, involving activation of various immunocompetent cells, such as neutrophils, macrophages, T cells and natural killer/lymphokine-activated killer (NK/LAK) cells [4–6, 25, 26].

SPR-901 (RBS), an α-glucan composed mainly of α1,6-glucosidic, linkages exhibited a potent antitumor effect against murine syngeneic tumors and an antibacterial-infection effect following either intraperitoneal (i.p.) or oral administration [11, 27]. Furthermore, it has already been shown that oral administration of SPR-901 enhances the accessory functions of macrophages, such as cytokine production and antigen presentation [28], and interleukin-2 (IL-2) production of spleen cells by concanavalin A stimulation [29]. However, the details of the mechanisms of the antitumor effect of SPR-901 remain obscure.

It has been elucidated that the immunological responses to tumors require a variety of cell populations. Among the various effector cells, T cells appear to play a central role in the antitumor immune responses [19]. The methylcholanthrene-induced fibrosarcoma, MethA, is commonly used for studying antitumor immune mechanisms. Macrophages and T cells are thought to be important effectors against MethA [9, 15, 16].

In the present study, to elucidate the mechanisms of the antitumor effect of SPR-901, we examined the role of T cells in tumor growth suppression by oral administration of SPR-901 using the BALB/c syngeneic tumor MethA. We have found that both $L3T4^+$ and $Lyt2^+$ T cells are necessary for the induction of an antitumor effect of orally administered SPR-901 although $Lyt2^+$ cells are the final effector cells in antitumor immunity. The implications of the mechanisms of the antitumor effect of SPR-901 are discussed.

Materials and methods

Animals. Female mice of an inbred BALB/c strain were obtained from Charles River Breeding Laboratory (Atsugi, Japan) and used at 6–12 weeks of age.

Tumors. MethA, methylcholanthrene-induced fibrosarcoma of BALB/c origin, was maintained in vivo using syngeneic BALB/c mice as ascitic

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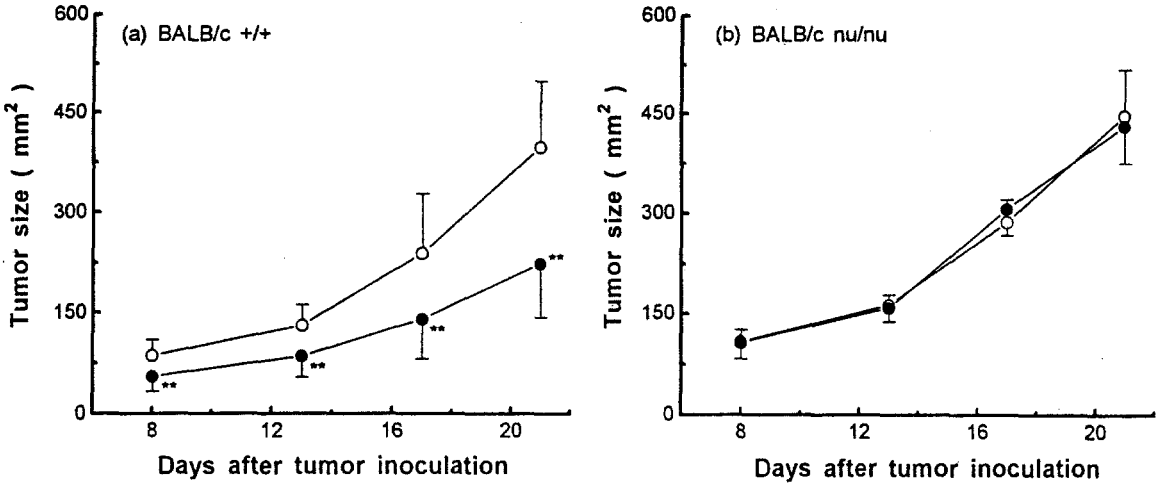

Fig. 1a, b. Effect of SPR-901 on MethA tumor growth. Mice were inoculated i.d. with 6×10^4 MethA cells on day 0 and orally administered SPR-901 (●) or water (○) from days 1 to 10. Each *symbol* and *vertical line* represents the mean of 10 mice and the SD. ** P <0.01 compared with the water-treated group by the Mann-Whitney test

cells. YAC-1, P815 and colon 26 were maintained in culture in vitro. It has been reported that MethA and colon 26 tumors are antigenically distinct and immunologically not cross-reactive with each other [21].

Preparation and administration of SPR-901. SPR-901 was synthesized from sucrose using dextran sucrase prepared from *Leuconostoc mesenteroides* sbsp. dextranicum [11]. It was dissolved in distilled water and orally administered to mice by an intubation needle. As controls, mice received distilled water instead of the sample solution.

Preparation of lymph node cells. Mice were sacrificed by cutting the carotid artery. The regional (axillary) lymph node cells were removed and mechanically dissociated into a single-cell suspension with a slide glass in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete RPMI-1640 medium). The cell suspension was passed through a stainless-steel mesh and washed twice with the medium.

Evaluation of the antitumor effect. BALB/c mice were intradermally (i.d.) inoculated with 6×10^4 viable MethA cells in the right flank on day 0 and orally administered SPR-901 at 30 mg kg^{-1} day^{-1} on days 1–10. When the growth of the rechallenged tumor was measured, mice were inoculated i.d. with 10^7 MethA cells or 5×10^4 colon 26 cells on the other side of the primary tumor inoculation on day 11. The growth of tumor cells was measured as the product of the longest and shortest diameters of the tumor on the indicated days. The percentage of growth inhibition was determined according to the formula: inhibition ratio (%) = 100× (tumor $\text{growth}_{\text{water}}$ – tumor $\text{growths}_{\text{PR-901}}$)/ tumor $\text{growth}_{\text{water}}$. For the Winn assay (tumor neutralization assay), lymph node cells were mixed with 2×10^5 MethA cells in Hanks' balanced salt solutions at a 5:1 or 10:1 lymph node cell to tumor cell ratio. Mice were inoculated i.d. with the mixture in a volume of 0.1 ml. Several days after the inoculation of the mixture, tumor growth was measured.

Treatment of lymphocytes with monoclonal antibody (mAb) and complement in vitro. Anti-L3T4 mAb and anti-Lyt2 mAb were prepared from culture medium of the hybridoma cell lines RL172/4 and 83-12.5, respectively. Lymphocytes were adjusted to a concentration of 10^7 cells/ml in complete RPMI-1640 medium and treated for 45 min at 37°C with anti-L3T4 or anti-Lyt2 antibody (final concentration 1:5). The cells were then centrifuged, washed twice with the medium and then resuspended in the medium containing guinea pig serum at a final dilution of 1/30. The cell suspension was incubated for 30 min at 37°C and washed three times with complete RPMI-1640 medium.

Measurement of lymphokine-activated killer (LAK) activity. Lymph node cells (10^7 cells/ml) were cultured for 4 days at 37°C in complete RPMI-1640 medium supplemented with 30 U/ml IL-2 (Genzyme, Mass., USA); the cultured cells were then recovered by centrifugation and washed with the medium. Target cells were incubated with 3.7 MBq $Na^{51}CrO_3$ (Amersham, Tokyo, Japan) for 60 min at 37°C in complete RPMI-1640 medium and washed with the medium. The cultured lymph node cells (10^6) and the ^{51}Cr-labeled target cells (10^4) were incubated for 4 h at 37°C in 0.2 ml medium using a 96-well microplate. After incubation, the cells were centrifuged and the radioactivity in 100 μl supernatant was counted using a gamma counter. The percentage lysis was determined according to the formula: lysis (%) = 100× (experimental release – spontaneous release)/ (maximal release – spontaneous release).

Deletion of L3T4$^+$ and Lyt2$^+$ cells in vivo. The following rat anti-(mouse Ig) mAb were used for in vivo depletion in the ascites form: IgG2b anti-L3T4 (GK1.5) and IgM anti-Lyt2 (83-12.5). Aliquots of 0.3 ml ascitic fluid (1/10 dilution) were injected i.p. starting the day before tumor inoculation. The mAb was injected every other day for 12 days.

Statistics. The statistical significance of the data was determined by Student's t-test or the Mann-Whitney test.

Results

Antitumor effect of oral administration of SPR-901

We first compared the effect of SPR-901 on the MethA tumor growth between euthymic BALB/c and athymic BALB/c nude mice to discover whether T cells participated in the expression of the antitumor effect of SPR-901. Oral administration of SPR-901 sinificantly inhibited the tumor growth in BALB/c (+/+) mice but not in BALB/c (*nu/nu*) mice (Fig. 1). It would thus appear that T cells play essential roles in the antitumor effect of SPR-901.

Next, to determine the contribution of the MethA-specific immune response to the antitumor resistance in SPR-901-treated mice, MethA-bearing mice were rechallenged i.d. with 10^7 cells of MethA or 5×10^4 cells of colon 26 tumor after SPR-901 administration. The growth of the

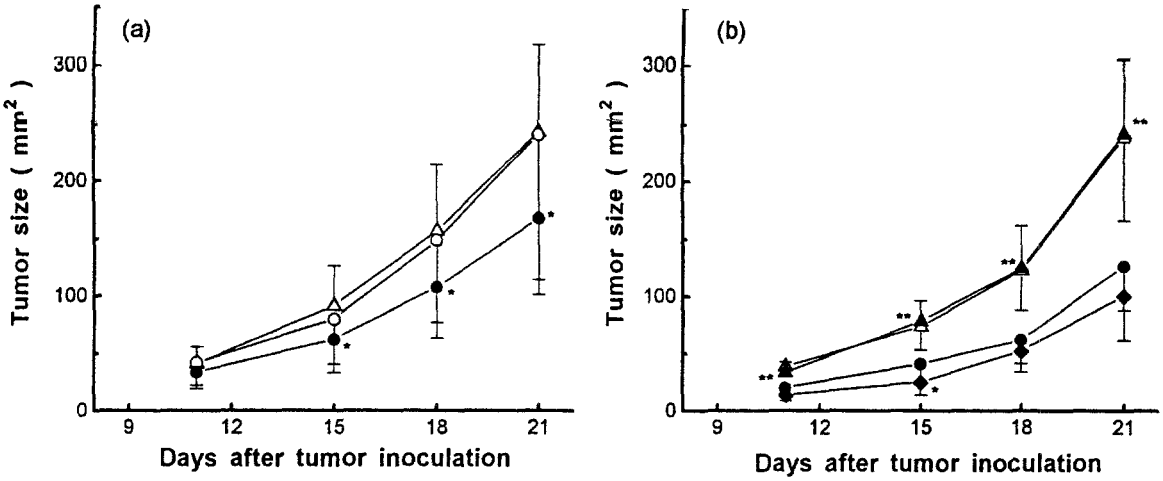

Fig. 2a, b. Effect of SPR-901 on tumor-neutralizing activity of regional lymph node cells. **a** MethA tumor only (△), whole lymph node cells from mice administered SPR-901 (●) or water ○); **b** MethA tumor only (△), lymph node cells treated with anti-L3T4 mAb and complement (◆), anti-Lyt2 mAb and complement (▲), or complement only (●), from mice administered SPR-901. Each *symbol* and *vertical line* represents of the mean of 8–10 mice and the SD. * $P < 0.05$, ** $P < 0.01$ compared with the water-treated group (**a**) or the group treated with complement only (**b**) by the Mann-Whitney test

Table 1. Effect of SPR-901 on rechallenged tumor growth

Group	Tumor size (mm^2)	
	MethA	Colon 26
Water	194.5±75.6	54.6±11.7
SPR-901	143.1±19.8*	50.3±18.2
Control[a]	205.9±48.9	51.9±13.8

Mice were inoculated i.d. with MethA on day 0 and administered SPR-901 p.o. on days 1–10. The mice were inoculated i.d. with MethA or colon 26 (rechallenged tumor) on the other side on day 11. The rechallenged tumor size was measured 13 days (for MethA), and 16 days (for colon 26) after the inoculation. Each group consisted of 5–15 mice. Results are means ±SD

* $P < 0.05$ compared with water-treated group by the Mann-Whitney test

[a] Mice not inoculated with primary MethA tumor and without any treatment

rechallenged MethA tumor was significantly suppressed in SPR-901-treated mice. The growth of colon 26, which is not antigenically related to MethA, however, was not affected in these mice (Table 1). These results indicated that expression of the antitumor effect of SPR-901 required the participation of T cells and that oral administration of SPR-901 augmented an antigen-specific immune response against MethA.

Tumor-neutralizing activity of lymph node cells from SPR-901-treated mice

To determine the phenotype of T cells mediating tumor growth suppression in SPR-901-treated mice, we attempted a Winn assay using the regional lymph node cells. A suppressive effect on tumor growth was observed when MethA cells plus lymph node cells from mice treated with SPR-901 were injected, but not when the mixture contained lymph node cells from control mice (Fig. 2a). To elucidate further the phenotypes of T cell subsets in the lymph node cells possessing growth-suppressive activity conferred by SPR-901 administration in the Winn assay, the lymph node cells were treated with anti-L3T4 or anti-Lyt2 antibody and complement before this assay. The treatment of these cells with anti-Lyt2 mAb and complement significantly abolished the tumor-neutralizing effect, but the anti-L3T4 antibody and complement did not affect the tumor-growth-suppressive activity (Fig. 2b). These results suggested that tumor-growth-suppressive activity of regional lymph node cells from SPR-901-treated MethA-bearing mice mostly depends on the $Lyt2^+$ T cell subset.

LAK activity of the regional lymph node cells from SPR-901-treated MethA-bearing mice

Cytotoxic activities of the regional lymph node cells cultured with IL-2 (LAK activity) were measured. Augmented cyotoxic activities of these cells against YAC-1 and P815 cells were observed in SPR-901-treated mice (Fig. 3a). Augmentation of the activities by oral administration with SPR-901 was also shown in tumor-rechallenged mice (Fig. 3b).

Antitumor effect of SPR-901 in mice depleted of $L3T4^+$ or $Lyt2^+$ cells

To determine the participation of T cell subsets in the induction of the antitumor effect of SPR-901 in vivo, we examined the antitumor activity against MethA in mice depleted of either $L3T4^+$ or $Lyt2^+$ cells. Anti-L3T4 antibody or anti-Lyt2 antibody injection into mice almost completely abrogated the antitumor effect of oral admin-

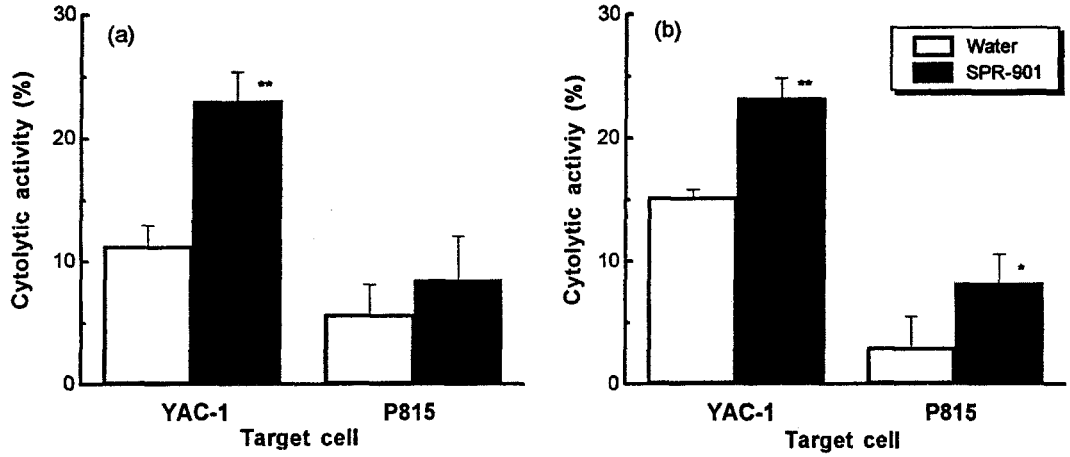

Fig. 3a, b. Effect of SPR-901 on lymphokine-activated killer activity of regional lymph node cells. **a** Lymph node cells from primary MethA-bearing mice; **b** lymph node cells from the tumor-rechallenged mice. Each *bar* and *vertical line* represents the mean of 5 mice and the SD. * $P < 0.05$, ** $P < 0.01$ compared with the water-treated group by Student's *t*-test

Table 2. Effect of SPR-901 on MethA tumor growth in mice treated with anti-L3T4 or anti Lyt2 mAb

Group	Tumor size (mm^2) following treatment with:		
	Saline	Anti-L3T4	Anti-Lyt-2
Water	562.5 ± 101.0	460.6 ± 68.6	555.0 ± 102.1
SPR-901	354.4 ± 66.3** (37.0)	406.7 ± 103.9 (11.7)	558.9 ± 146.8 (–0.7)

Mice were inoculated i.d. with 6×10^4 cells of MethA tumor on day 0, and administered SPR-901 or water on days 1–10. The treatment with mAb is described in materials and methods. The tumor size was measured on day 21. The value in parentheses represents the percentage of tumor growth inhibition. Results show means ± SD
** $P < 0.01$ compared with water-treated group by the Mann-Whitney test

Table 3. Effect of SPR-901 on MethA tumor growth in mice treated with cyclosporin A (CsA)

Group	Tumor size (mm^2) following treatment with:	
	Saline	CsA
Water	337.0 ± 136.6	255.9 ± 45.1
SPR-901	212.4 ± 124.0* (37.0)	242.0 ± 135.3 (5.4)

Mice were inoculated i.d. with 6×10^4 cells of MethA tumor on day 0 and orally administered SPR-901 or water on days 1–10. The tumor size was measured on day 21. The values in parentheses represents the percentage of tumor growth inhibition. Results show means ± SD
* $P < 0.05$ compared with water-treated group by the Mann-Whitney test

istration of SPR-901 (Table 2). These results suggested that both L3T4+ and Lyt2+ T cells are required for induction of an antitumor effect by SPR-901 in vivo.

Disappearance of antitumor effect of SPR-901 in cyclosporin-A(CsA)-treated mice

The antitumor effect of SPR-901 was examined in CsA-treated mice. CsA is well known as an immunosuppressive agent involving inhibition of IL-2 production from activated T cells [14]. Mice were inoculated i.d. with MethA cells on day 0 and injcted i.v. with CsA (2.5 mg/kg, Sandoz, Basel, Switzerland) on days 1, 4, 7 and 10. In these mice, the antitumor effect of SPR-901 disappeared (Table 3).

Discussion

In the present study, we obtained evidence that oral administration of SPR-901 augmented T-cell-mediated immune responses against MethA, causing tumor growth suppression.

A variety of cell populations are included in the immune responses to tumors. Among these effectors, T cells appear to play a central role in the antitumor immune responses [19]. Because no antitumor effect of SPR-901 was shown in athymic nude mice and antitumor resistance against the second-challenge tumor, the so-called concomitant immunity mentioned by Fisher et al. [7], was augmented by SPR-901 in a tumor-antigen-specific manner in euthymic mice; there was no doubt that the antitumor effect of SPR-901 was mediated by functional T cells.

T cells can now be divided into two mutually exclusive subsets, L3T4+ Lyt2– (L3T4+ cells) and L3T4– Lyt2+ (Lyt2+ cells), which are restricted to class II and class I major histocompatibility complex MHC molecules, respectively. There are numerous reports defining the nature of T cell subsets responsible for tumor rejection or inhibition [2, 8, 12, 17, 20, 23, 31]. Recently, Fuyama et al. reported that both L3T4+ and Lyt2+ cells had tumor-neutralizing activity and were responsible for the antitumor immune resistance in MethA-bearing mice [9]. Our observations suggest that, as far as tumor-neutralizing activities are concerned, SPR-901 treatment in tumor-bearing mice might augment the activity of Lyt2+ cells rather than that of L3T4+ cells in vivo. It has been reported that Lyt2 antigen is expressed not only on cytotoxic T cells (CTL) but also on some T-LAK or T-LAK precursor cells [1]. LAK activity of lymph node cells from MethA-bearing mice was enhanced by oral ad-

ministration of SPR-901. The participation of these LAK cells as one type of effector cell in the antitumor effect of SPR-901 can not be ruled out. CTL against MethA could not be induced from these cells after in vitro culturing with mitomycin-C-treated MethA cells (data not shown). As pointed out by Fuyama et al., if CTL are involved in the rejection of the MethA tumor, these CTL may be different from the classical CTL that can be detected by in vitro culturing with mitomycin-C-treated MethA cells [9]. Further experiments are needed to characterize in more detail the Lyt2$^+$ cells possessing tumor-neutralizing activity.

Several reports have indicated that systemic administration of mAb of certain isotypes is effective in the selective depletion of the functional T cell population in vivo [3, 18]. We therefore chose this approach to analyze T cell phenotypes associated with the antitumor effect of SPR-901. Interestingly, although the enhancement of tumor-neutralizing activity of lymphocytes by oral administration of SPR-901 was mainly shown in Lyt2$^+$ cells rather than L3T4$^+$ cells, the depletion of Lyt2$^+$ cells as well as L3T4$^+$ cells in vivo abrogated the antitumor effect of SPR-901. Some investigators have reported that Lyt1$^+$ and/or L3T4$^+$ Th cells play important roles in the generation of CTL [2, 8, 12, 13]. Furthermore, Shu et al. demonstrated that the induction of Lyt2$^+$ CTL was dependent on the helper function of L3T4$^+$ T cells via the secretion of IL-2 [2, 8]. The antitumor effect of SPR-901 was abrogated in mice treated with cyclosporin A. Therefore, it is likely that the antitumor effect of SPR-901 administered orally is mediated by Th (L3T4$^+$) cells functioning as inducers, for example by secretion of IL-2, resulting in the augmentation of tumor-killing activities of Lyt2$^+$ effector cells. This is also supported by our previous studies showing that IL-2 production by splenocytes following stimulation with concanavalin A was augmented by oral administration of SPR-901 not only in normal mice but also in tumor-bearing mice [28, 29].

We previously showed that oral administration of SPR-901 augmented the accessory function of macrophages, such as antigen-presenting activity and cytokine production [27]. Recent studies from several laboratories have demonstrated that Th cells to tumor antigens are activated by the antigens processed by antigen-presenting cells, causing CTL induction and cytokine production in vitro [12, 13] and tumor rejection immunity in vivo [22]. It is likely that oral administration of SPR-901 may activate or restore macrophage/Th cell interactions and their cytokine production, causing enhancement of Lyt2$^+$ killer cell activities. It is known that macrophages can act not only as accessory cells but also as effector cells in antitumor immune responses [15, 16]. Further studies are necessary to clarify the mechanisms for the antitumor effect of SPR-901.

In conclusion, oral administration of SPR-901 produced a marked antitumor effect against the murine syngeneic tumor, MethA, through the augmentation of T cell immunity. Both Lyt-2$^+$ cells and L3T4$^+$ cells seem to be necessary for the antitumor effect following oral administration of SPR-901 in vivo, as effector cells and as helper cells respectively.

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