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A Macrophage-Derived Factor Required by Plasmacytomas for Survival and Proliferation in Vitro

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Plasmacytoma (PCT) cell lines dependent for proliferation and survival on a factor elaborated by the murine macrophage cell line, P388D1, were established in vitro. Adherent peritoneal cells induced by pristane produced 50-fold greater amounts of this activity in vitro than did resident cells. The molecules responsible for plasmacytoma growth were distinct from a number of characterized factors including interleukin-1, -2, and -3, macrophage colony-stimulating factor, B-cell stimulatory factor–1, B-cell growth factor II, epidermal growth factor, transforming growth factor–β, and γ- and β-interferon, none of which were able to support the growth of the factor-dependent PCT cell lines. These results suggest that PCT growth factor may be a novel factor that has not been previously characterized and, further, that its production is associated with the pristane-induced, chronic peritoneal inflammatory response that precedes plasmacytoma formation.

PLASMACYTOMAS (PCT's) ARE INduced in a high percentage of BALB/cAnPt mice after intraperitoneal injection of mineral oil or pristane (*1*). The intraperitoneal oil evokes the formation of a chronic granulomatous tissue on peritoneal surfaces that consists primarily of macrophages and neutrophils. Plasmacytomas arise exclusively in the granulomatous tissue, which suggests that their growth depends on microenvironmental influences provided by the inflammatory cells (*2*). Factors involved in this process in vivo have not been identified; however, Namba and Hanaoka (*3*) reported a 50-kD protein, produced by an adherent phagocytic cell line, which was required by the MOPC 104E PCT cell line for growth in vitro. This factor relieved a block in the G_1 phase of the cell cycle. Also, Metcalf (*4*) demonstrated that cells from several in vivo PCT's proliferated in soft agar if the cultures were supplemented with mouse serum or with normal or mineral oil–stimulated peritoneal cells. Recently, Corbel and Melchers (*5*) reported that supernatant from the P388D1 macrophage cell line supported the growth of several PCT's in vitro. Aarden *et al.* (*6*) described a human monocyte-derived "hybridoma growth factor" that is required for the growth of a murine B-cell hybridoma. To further characterize factors that may be necessary for PCT growth, we have established several new factor-dependent PCT cell lines in vitro. This adaptation requires the addition of supernatant derived from unstimulated rat spleen cells (RSS) or from the macrophage cell line P388D1. We report here the initial characterization of plasmacytoma growth factors (PCT-GF) produced by P388D1 cells that are clearly distinguishable from other previously described growth factors.

The TEPC 2027 and TEPC 1165 PCT's were induced and serially transplanted as ascites tumors in pristane-conditioned BALB/cAnPt mice. Establishment of the T2027tc cell line was accomplished with medium supplemented with 50% RSS produced by culturing 5×10^6 rat spleen cells per milliliter for 24 hours in RPMI 1640 supplemented with 1% fetal calf serum (FCS). A search for more defined sources of PCT-GF activity led us to the P388D1 cell line. When newly confluent monolayers of P388D1 cells (1×10^6 cells per milliliter, 50 ml per 150-cm^2 flask) were cultured for 5 days in fresh RPMI 1640 and 0% or 1% FCS, PCT-GF activity was found in the low-serum supernatant (P388D1 LSSN) (Fig. 1). The T1165tc cell line was established in vitro by using 5 to 10% P388D1 LSSN as the source of PCT-GF activity. The T1165tc and T2027tc primary cultures were started at densities ranging from 1×10^5 to 5×10^5 PCT cells per milliliter in medium

Table 1. PCT-GF activity found in characterized factors and supernatants.

Factor or supernatant	Amount tested per milliliter	PCT-GF activity (U/ml)	
		T1165tc	T2027tc
Pure factors			
IL-1, human	0.5 to 5,000 U	0	0
rIL-2, human	0.3 to 1,000 U	0	0
IL-3, mouse	0.3 to 350 U	0	0
BSF-1, mouse	0.1 to 560 U	0	0
EGF, mouse	6 pg to 20 ng	0	0
TGF-β, human, with 2.5 ng of EGF	6 pg to 20 ng	0	0
γ-IFN, mouse	0.1 to 500 U	0	
β-IFN, mouse	0.1 to 1,000 U	0	
C3b, human	5 pg to 5 μg	0	0
Partially purified factors			
BCGF-II, mouse	0.1 to 50 U	0	0
Macrophage-CSF, mouse	1 to 10,000 U	0	0
Thymosin F5, bovine	5 pg to 500 μg	0	0
Supernatants			
P388D1 LSSN		100	100
Resident APC-SN, BALB/cAnPt		<0.1	<0.1
Pristane-induced APC-SN, BALB/cAnPt		22	9
Other reagents			
Con A	5 pg to 50 μg	0	0
LPS	5 pg to 50 μg	0	0

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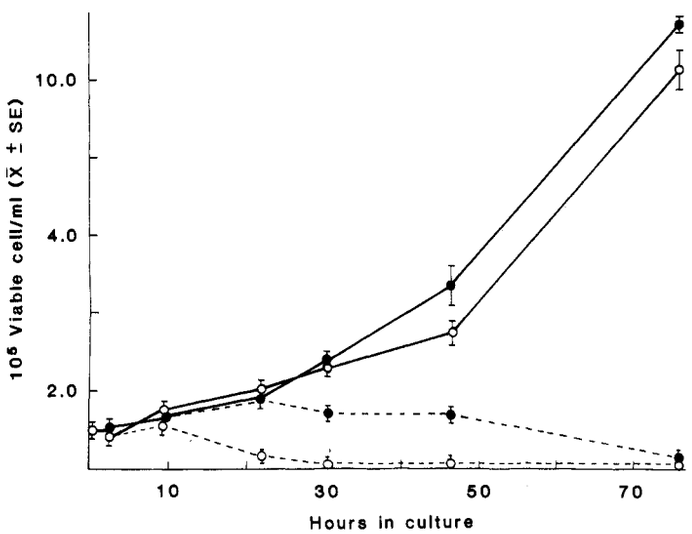
(*7*) supplemented with RSS or P388D1 LSSN. In addition to the PCT cells, these cultures contained numerous adherent cells. During the initial culture period large cell losses occurred, but colonies of proliferating cells were usually observed. Furthermore, the PCT cells failed to proliferate when the cells were gently resuspended and transferred to new wells in medium containing PCT-GF, but they continued to grow in the primary culture wells. This suggested that initially there were at least two requirements for adaptation of the in vivo tumor line to primary culture: the PCT-GF and the adherent cells that were present in the primary cultures. After 2 to 3 weeks, however, a proliferating population emerged that required only the PCT-GF for growth. The T1165tc and T2027tc cell lines have been cloned and serially subcultured for 18 months and still require PCT-GF for proliferation and survival. T1165tc secretes immunoglobulin A [IgA(κ)] myeloma protein whereas T2027tc is an immunoglobulin G_1 [IgG_1(κ)] producer. Giemsa-stained cells from log-phase cultures revealed that all cells possessed the distinctive morphological characteristics of PCT cells, indicating that the proliferating cells are in fact PCT cells. In addition, these cells grow as plasma cell tumors when transferred back into BALB/cAnPt mice.

Figure 1 shows that, in the absence of the P388D1 LSSN, both cell lines failed to proliferate and more than 99% of the T1165tc and T2027tc cells were dead within 24 and 72 hours, respectively. However, in the presence of the P388D1 LSSN, the number of cells increased logarithmically. Additional studies have shown that the activity in the P388D1 supernatant relieves a block in the G_1 phase of the cell cycle (*8*).

Using the P388D1 LSSN we have established and characterized four additional PCT cell lines. One (T1198tc) is totally dependent on P388D1 LSSN for survival and proliferation. Two cell lines (LPC1tc and M315tc) grow very slowly in the absence of P388D1 LSSN, but in its presence respond with a twofold increase in [^{3}H]thymidine incorporation per day. The fourth cell line (M460Dtc) is totally independent of P388D1 LSSN and grows equally well in its presence or absence.

To measure the level of PCT-GF present in various preparations of supernatants and purified factors, a [^{3}H]thymidine microproliferation assay was established with 24-hour (T1165tc) and 72-hour (T2027tc) end points for the assays. Both cell lines responded in a dose-dependent manner to the activity present in the P388D1 LSSN with measurable activity at concentrations of less than 0.1% (Fig. 2). We define a unit of activity as the amount that elicits 50% of the maximal [^{3}H]thymidine incorporation by T1165tc under standard assay conditions (Fig. 2). The standard P388D1 LSSN prepared as described from dense monolayers with 0% or 1% FCS (legend to Fig. 1) contained 100 U of PCT-GF per milliliter. The supernatant from proliferating populations of P388D1 cells (10% FCS) exhibited little or no PCT-GF activity.

Since the pristane-induced oil granuloma is necessary for the induction and transplantation of early PCT's, we examined the ability of resident (normal control) and pristane-induced adherent peritoneal cells to produce the PCT-GF activity in vitro under the same low serum conditions used for



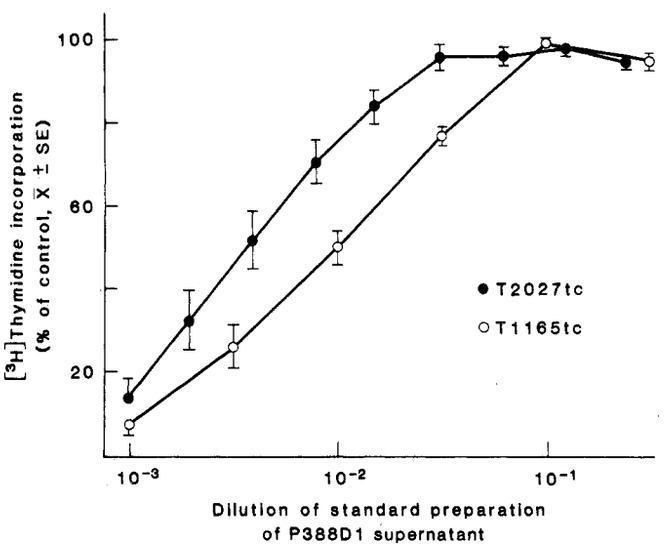


Fig. 1 (left). Growth of T2027tc (●) and T1165tc (○) PCT cell lines in the presence and absence of P388D1 low serum supernatant (P388D1 LSSN). Stock cell cultures were grown in culture medium (*7*) supplemented with 10% P388D1 LSSN and harvested during the log phase of growth for all experiments. The cells were washed twice and resuspended at 1×10^5 cells per milliliter in culture medium (*7*) containing a 1:8 dilution of a standard preparation of P388D1 LSSN (solid lines) or in control medium (broken lines). The plasmacytoma cells were added in 1.5-ml volumes to individual wells of 24 well plates and incubated at 37°C in a humid atmosphere of 5% CO_2. At the indicated times, 100-μl samples were taken and the number of viable cells per milliliter was determined by trypan blue exclusion. Results are expressed as the mean ± SE of triplicate cultures. P388D1 LSSN containing PCT-GF activity was produced as follows. P388D1 cells were initially grown to confluence (1×10^6 cells per milliliter) as adherent monolayers in RPMI 1640 supplemented with 10% FCS and antibiotics. At this time the spent medium was replaced with fresh RPMI 1640 supplemented with antibiotics and 0 or 1% FCS (50 ml per 150-cm^2 flask), and the cultures were incubated for an additional 144 hours. The supernatants were pooled, centrifuged, and sterile-filtered. The standard lot of P388D1 LSSN was dialyzed against 50 volumes of RPMI 1640, and 1-ml samples were stored at −20°C for use in all assays. The T2027tc, T1165tc, and P388D1 cell lines used in these experiments were free of mycoplasma contamination. Fig. 2 (right). Response of T1165tc and T2027tc PCT cells to increasing dilutions of P388D1 LSSN. Dilutions of standard P388D1 LSSN were made in culture medium and added to triplicate wells of 96-well plates in 0.1-ml volumes. T1165tc and T2027tc cells were prepared as described in Fig. 1, resuspended in culture medium at 1×10^5 cells per milliliter, and added in 0.1-ml volumes to all wells. The cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours (T1165tc) or 72 hours (T2027tc). Each well was then pulsed for 2 hours with 0.5 μCi of [^{3}H]thymidine (specific activity, 5 mCi/mmol), harvested over glass fiber filters and counted in a liquid scintillation counter. Results are expressed as a percent of the maximum [^{3}H]thymidine incorporation obtained with the standard P388D1 LSSN and represent the mean ± SE of five experiments. Maximum and background radioactivities, respectively, were 40,625 ± 1257 and 442 ± 98 count/min for T2027tc and 9739 ± 1015 and 392 ± 77 count/min for T1165tc.

P388D1 LSSN production. Pristane-induced adherent peritoneal cell supernatant (APC-SN) exhibited more than 50 times the PCT-GF activity that resident APC-SN did (Fig. 3). The slope of the dose-response curve indicated the possible presence of inhibitory substances in APC supernatants. Dialysis of supernatants removed the inhibition present in the pristane-induced APC-SN but did not improve the efficacy of the resident APC-SN (Fig. 3). Furthermore, no inhibition of PCT-GF activity was observed when the same dilutions of the dialyzed APC-SN's were assayed in the presence of a limiting amount of PCT-GF (1 U/ml, P388D1 LSSN). Thus, the absence of PCT-GF activity in the resident APC-SN was not due to the presence of inhibitors but was the result of little or no PCT-GF production. PCT-GF activity in three different preparations of pristane-induced APC-SN ranged from 10 to 22 U/ml, whereas resident APC-SN's always exhibited less than 0.1 U/ml. Similar results were obtained with the T2027tc cell line. It is likely that the source of the PCT-GF activity is the macrophage population; however, the presence of contaminating cell types (such as neutrophils) as potential sources has not been ruled out. The identity and specificity of the inhibitory activity is not known.

A number of monocyte- and lymphocyte-elaborated factors have been identified that may affect the differentiation and proliferation of lymphoid and other cells of hematopoietic origin. We therefore examined several well-characterized interleukins and other factors for the ability to support the growth of the factor-dependent PCT cell lines (Table 1). These included purified human IL-1, recombinant human IL-2, purified murine IL-3, purified murine B-cell stimulatory factor–1 (BSF-1), partially purified murine B-cell growth factor II (BCGF-II), purified murine epidermal growth factor (EGF), purified human transforming growth factor–β (TGF-β), purified murine γ- and β-interferon, partially purified murine macrophage colony-stimulating factor (CSF), and thymosin fraction 5 (F5), a partially purified preparation of bovine thymic hormones (*9*). All of the tested factors of human origin are functional in the corresponding murine systems (*10*). None of the characterized factors mentioned above expressed any PCT-GF activity when assayed over wide concentration ranges, suggesting that PCT-GF is distinct from these molecules (Table 1). We also tested purified human C3b (*9*), the major cleavage product of the third component of complement, which, when bound to Sepharose, has been shown to participate in the promotion of murine B-cell replication in vitro (*11*). Soluble human C3b (5 μg/ml to 50 pg/ml) did not possess any PCT-GF activity (Table 1), nor was it able to inhibit PCT-GF activity when assayed in the presence of limiting amounts of PCT-GF (1 U/ml, P388D1 LSSN). IL-1 was of particular interest because the P388D1 cell line produces high concentrations of this lymphokine (80 U/ml, P388D1 LSSN) (*12, 13*). The PCT-GF activity was easily separated from IL-1 activity on the basis of isoelectric points by using chromatofocus chromatography, indicating that these two factors are distinct entities (Fig. 4). PCT-GF activity was found in two closely eluting peaks of *p*H 6.4 and 6.2, whereas IL-1 eluted at *p*H 5.1 and 4.8 which agrees with previously reported *p*I values for IL-1 (*14*). Preliminary sizing experiments suggest a molecular size of approximately 25 kD, which also differentiates this molecule from the 50-kD factor described by Namba and Hanaoka (*3*). Furthermore, neither lipopolysaccharide (LPS) or concanavalin A (Con A), potent B and T lymphocyte mitogens, could substitute for or synergize with PCT-GF. Additional studies have shown that partially purified preparations of PCT-

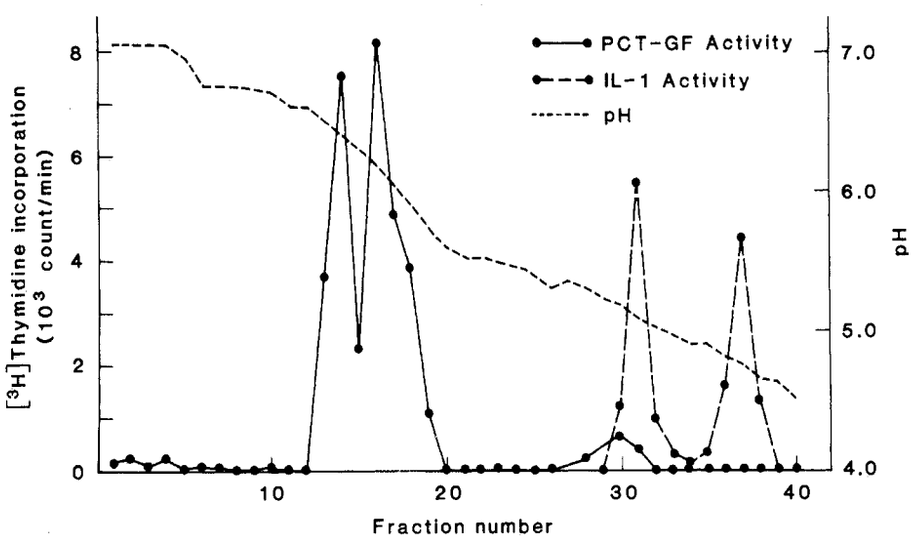


Fig. 3. Production of PCT-GF activity by normal and pristane-induced adherent peritoneal cells (APC's). The APC's were prepared from untreated BALB/cAnPt mice or those which had received 0.5 ml of pristane intraperitoneally 30 days previously. The mice were killed and their peritoneal cavities flushed with 10 ml of cold RPMI 1640 containing antibiotics and 1% FCS. The number of macrophages and monocytes was determined from total and differential cell counts. The cells were seeded into 24-well culture dishes at 1×10^6 macrophage-monocytes per well and allowed to adhere for 2 hours at 37°C. Each culture was gently flushed to remove nonadherent cells and then incubated at 37°C in 1 ml of RPMI 1640 with 1% FCS. Although equal numbers of macrophage-monocytes were used in each culture, the pristane-induced peritoneal cell populations also contained substantially elevated numbers of neutrophils (30% compared with 2% in normal mice), which were also adherent. After 5 days the supernatants were collected and centrifuged, and samples were dialyzed against 50 volumes of RPMI 1640. All supernatants were stored at −20°C. Three replicate supernatants per group were prepared independently from APC's from separate animals. Increasing dilutions of nondialyzed (●) and dialyzed (○) supernatants prepared from the normal (solid lines) and pristane-primed (broken lines) APC's were assayed in triplicate for PCT-GF concentrations through the use of the T1165tc cell line (legend to Fig. 2). The results are presented with standard P388D1 LSSN (■) as a positive control (P388D1 LSSN dialyzed versus RPMI 1640; legend to Fig. 1). Maximum and background radioactivities, respectively, were 5050 ± 156 and 211 ± 13 count/min.

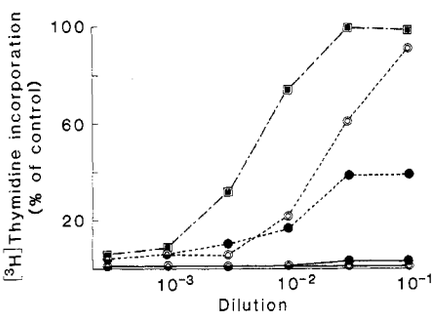


Fig. 4. Separation of PCT-GF and IL-1 activities in P388D1 LSSN. A sample of P388D1 LSSN was exhaustively dialyzed against 60% saturated ammonium sulfate. The precipitate was centrifuged at 8000*g* for 30 minutes, resuspended in H_2O, and dialyzed into 0.025*M* imidazole-HCl, *p*H 7.4. This material was applied to a 1 × 30 cm chromatofocusing column (Pharmacia PBE74) and chromatographed over a range of *p*H 7 to *p*H 4 with a 1:10 dilution of Polybuffer 74, *p*H 4.0. Fractions (5 ml) were assayed in duplicate at various dilutions for IL-1 activity in a thymocyte proliferation assay with thymocytes from C3H/HeJ mice (*13*) and for PCT-GF activity with T2027tc cells (legend to Fig. 2). Backgrounds of 910 and 204 count/min were subtracted from the IL-1 and PCT-GF assays, respectively.

GF do not exhibit interferon activity (*15*).

Our results indicate that the intraperitoneal administration of pristane to BALB/cAnPt mice results in the stimulation of cells capable of producing PCT-GF in vitro. This agrees with the observations of Metcalf (*4*) and suggests that PCT-GF levels in the oil granuloma may be substantially elevated, providing an environment in which early tumor cells can proliferate. The results presented here provide strong evidence that PCT-GF is distinct from IL-1, IL-2, IL-3, BSF-1, BCGF-II, macrophage-CSF, EGF, TGF-β, and γ- and β-interferons.

The identification of a factor that supports PCT growth in vitro presents the intriguing possibility that this factor is involved in the establishment and maintenance of PCT's in vivo and may thus be important in the tumorigenic process. Because there are other known and probably unknown factors present in the P388D1 supernatant, any evaluation of the possible role of PCT-GF in normal lymphocyte proliferation or differentiation will require purified preparations of this material. Experiments to elucidate the structure and function of this factor will thus be important to our understanding of normal and malignant cell growth.

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The 35-Nucleotide Spliced Leader Sequence Is Common to All Trypanosome Messenger RNA's

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In Trypanosomatidae the messenger RNA's (mRNA's) that code for the variant surface glycoproteins (VSG's), tubulins, calmodulin, and at least a subset of other proteins contain a common 35-nucleotide leader sequence at their 5′ ends. Hybrid-arrested in vitro translation has been used to show that all mRNA's in both African and South American trypanosomes contain this 35-nucleotide sequence. Oligonucleotides complementary to this sequence blocked translation of all trypanosome mRNA's in a rabbit reticulocyte lysate system, but did not inhibit translation of mRNA's from other organisms lacking this sequence. An oligonucleotide complementary to the VSG mRNA downstream from the spliced leader sequence arrested only VSG synthesis. Thus, the 35-nucleotide leader sequence is a general feature of all trypanosome mRNA's. The high specificity of oligonucleotides complementary to the spliced leader for their target sequence suggests that analogues permeable to the cell membrane may be useful in the treatment of trypanosomal infections.

AMONG THE MANY UNUSUAL AND interesting features of trypanosomes is the fact that at least some of their messenger RNA's have a common 35-nucleotide leader sequence at their 5′ nontranslated ends (*1–10*). This sequence was first recognized in the complementary DNA's (cDNA's) for the VSG's, the surface coat proteins of African trypanosomes whose sequential expression enables the parasite to evade the host's immune system (*1*). This same sequence was subsequently found at the 5′ ends of the calmodulin and tubulin cDNA's of African trypanosomes (*5, 6*), and in some cDNA's of *Trypanosoma cruzi*, the South American trypanosome that does not undergo antigenic variation (*11*). The sequence has been termed the miniexon (*2*) or spliced leader sequence (*12*), and is encoded within a repeated DNA sequence in the genome. In the African trypanosome *T. brucei*, the best studied case, the spliced leader is transcribed from a 1.35-kb DNA sequence that is present in about 200 copies, most of which occur within tandem arrays (*13*). The initial transcript from these repeats is a 140-nucleotide RNA that contains the spliced leader (*14*) and a cap structure (*15*) at its 5′ end. Two models have been proposed for the transfer of the 35-nucleotide leader sequence to the ends of the mRNA's (*14*), a primer mechanism and a bimolecular splicing mechanism. Neither model has been proven nor is the biological function of the leader sequence known.

Tandem DNA repeats containing the coding region for a highly homologous sequence occur in other members of Trypanosomatidae, for example, *Leptomonas collosoma* (*16*), suggesting that this may be a general phenomenon for this family of protozoans. However, not all RNA's of these organisms have this sequence; it is not found in structural RNA's such as the large ribosomal RNA's and 5*S* ribosomal RNA, or in their

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