Allergen from *Fasciola hepatica*; IgE Antibody Production in Mice by Crude Antigen

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Abstract: IgE antibody formation was easily demonstrated from the hosts infected with *Fasciola hepatica*. But, it was known to be difficult that the hosts immunized with antigen extracted from *F. hepatica* produced IgE antibody. IgE antibody, however, was produced relatively easily in the mice immunized with crude antigen of *F. hepatica* in our experiments. Namely, over 2.5mg of challenge antigen per rat was necessary for better reaction of PCA test and male Wistar strain of rat weighing 150g was better as a recipient animal. The best condition for the high titer of IgE antibody production was to immunize mice with 5 times stimulation of 10γ of antigen in 5 days, or with 2 times of 20γ at 20 days interval. Among adjuvants, aluminium hydroxide gel was best for the induction of IgE antibody in mice. ICR mice immunized with 20γ at 20 days interval produced over 2^6 titer of IgE antibody at least one week. The IgE antibody production was compared among four inbred mouse strains, two of their hybrids and six outbred strains. The inbred strains of DBA/2 and BALB/c and their hybrids, (C57BL/6 × DBA/2)F_1 and (BALB/c × DBA/2)F_1 produced the highest titer of IgE antibody. On the other hand, outbred strains of ddY, ddN and ICR produced the low titer of IgE antibody.

INTRODUCTION

Parasites provide the most potent stimulus for immediate type hypersensitivity. Intradermal tests were early used to investigate or to diagnose parasitic infections. In animal fascioliasis, the intradermal test has been used as an important diagnostic tool. Ono *et al.* (1952) studied intradermal test antigen extracted from adult *F. hepatica* and reported that it was very specific for the cattle fascioliasis. The antigen was called as Ono Antigen, and it has been used widely for animal fascioliasis in our country.

On the other hand, Shinoda (1974a) studied the IgE antibody production during the course of the infection of the rabbits experimentally infected with *F. hepatica*. According to his observation, the relatively high titer of IgE antibody was demonstrated

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in all infected rabbits from the 5th week after infection, reached the maximum titer on the 8th week, and maintained untill the 14th week. Shinoda (1974b) also reported the high degree of the potentiated IgE antibody formation for bovine serum albumin induced by the *F. hepatica* infection. In his studies, however, the rabbits injected antigen extracted from *F. hepatica* produced IgE antibody only in low titer. The producing periods were detected shortly and transiently; IgE antibody of all rabbits was terminated at the 3rd week after infection. No potentiation phenomenon of IgE antibody for bovine serum albumin was observed among the rabbits injected crude antigen from *F. hepatica*.

In this paper, attempts were made to determine and to confirm various conditions for IgE antibody production by crude antigen extracted from adult *F. hepatica*.

MATERIALS AND METHODS

Animals: Conventional Wistar rats of both sexes weighing 100 to 300g were used in this study. Conventional Donryu strain of male rats weighing 150g and inbred SD rats of both sexes weighing 150g were also used. These rats were obtained from the Sankyo Lab. Inc., Shizuoka, Japan.

Male mice at the age of 4 to 5 weeks were used throughout the experiment. Outbred mice of ddY, ddN, ICR, C3H/He, C57BL/6 and DBA/2 were obtained from Hokuriku Lab. Inc., Kanazawa and inbred mice of C57BL/6 ($H\text{-}2^b$), BALB/c, DBA/2 ($H\text{-}2^d$) and C3H/He ($H\text{-}2^k$), and their hybrids, (C57BL/6×DBA/2)F_1 and (BALB/c ×DBA/2)F_1, were obtained from the Sankyo Lab. Inc.

Crude extract of *F. hepatica* (CE): CE was made from adult *F. hepatica* obtained from cattle bile ducts in Kanazawa area. Adult worms were lyophilized, homogenized with glass and teflon homogenizers, disrupted by sonication (Insonator, 200M. Kubota, Tokyo) at 140 Watt for 5min and extracted in phosphate buffered saline (PBS) at pH7.2 for 2 days in a refrigerator. The emulsion was centrifuged at 13,000×g for 20min at 0°C. The 13,000×g supernatant was designated as CE.

Immunization: Mice were immunized 2 to 8 times with 5 to 40γ of CE. Two kinds of adjuvants were used in the immunization; ICR mice were immunized with CE mixed with killed *Bordetella pertussis* organisms of 10^{10} per mouse or aluminium hydroxide gel (alum) of 3mg per mouse.

Passive cutaneous anaphylaxis (PCA) test: PCA test was performed as described (Fujita and Tsukidate, 1981). A volume of 0.1ml of serial diluted sera was injected intradermally into the shaved backs of rats. After 48hr, the rats were challenged with an intravenous injection of 1ml of 1% Evans blue containing 0.625 to 5mg of CE, and were killed 30min later. The diameters of the PCA reaction were measured on the internal surface of the skin. A reaction greater than 5mm was defined as positive.

Indirect hemagglutination (IHA) test: IHA was conducted as described for the purification of allergen from *Dirofilaria immitis* (Fujita, 1975). Sheep red blood cells

were treated with tannic acid (Merk) at 1 : 30,000 for 15min in a water bath at 37°C and the tanned cells were sensitized with antigen (1 : 200 diluted CE) for 15min at room temperature. The IHA test was carried out in 0.6% normal rabbit serum on microtiter plates. The end point of positive reaction was determined as plus 3 defined by Jacobs and Lunde (1957).

RESULTS

(1) PCA reactivity of recipient rats.

(1-a) Influence of challenge antigen dose on PCA reactivity.

In order to know optimal conditions for PCA reaction, challenge antigen dose was determined by using three immunized mice sera with high titer of IgE antibody. As shown in Table 1-a, over 2.5mg of challenge antigen dose per rat was necessary for better reaction of PCA test, when male conventional Wistar strain of rat weighing 150g was used as recipient. Hereafter, PCA test was conducted with challenge antigen dose of 2.5mg per rat.

Table 1. PCA reactivity of recipient rats

Table 1-a. Influence of challenge does on PCA reactivity

challenge antigen does (mg/rat*)	5	2.5	1.25	0.625
PCA titer (mean+S.E.)	$2^{5.2} \pm 2^{0.33}$ **	$2^{5.2} \pm 2^{0.33}$	$2^{4.2} \pm 2^{0.88}$	$2^{3.5} \pm 2^{1.35}$

* Wistar (conv.) ♂ 150g.

** Mean value with three immunized mice sera.

Table 1-b. PCA reactivity according to sex of the recipient rats

recipient rat			Wistar (conv.)	SD (BS.)
PCA titer (mean ± S.E.)	sex	♂	$2^{3.8} \pm 2^{0.33}$*	$2^{1.5} \pm 2^{1.0}$
		♀	$2^{3.8} \pm 2^{0.33}$	$2^{1.0} \pm 2^{1.0}$

* Mean value with three or four immunized mice sera.

Table 1-c. PCA reactivity according to strains of the recipient rats.

species of recipient rat	Wistar (conv. ♂)	SD (BS. ♂)	Donryu (conv. ♂)
PCA titer (mean ± S.E.)	$2^{4.3} \pm 2^{1.02}$ *	$2^{4.1} \pm 2^{1.09}$	$2^{5.0} \pm 2^{0.84}$

* Mean value with six immunized mice sera.

(1-b) PCA reactivity according to sex and age of the recipient rat.

Two kinds of rats, conventional Wistar strain and inbred SD strain, of both sexes were used as recipients for PCA test. About the same degree of reactivity was obtained between sexes in each strain of rats, as shown in Table 1-b. About the same reactivity was also observed among recipient rats weighing 100 to 300g of male conventional Wistar strain, although the PCA titers of these rats were diversed a little from 2^4 to 2^5. From these results, it was determined that the difference of sex and age of recipient rat did not influence on the reactivity of PCA test.

(1-c) PCA reactivity according to strains of the recipient rat.

PCA reactivity was compared among Wistar, SD and Donryu strain of male rats. About the same degree of PCA reactivity was observed in three strains of recipient rats as shown in Table 1-c, although PCA reactivity of Wistar was superior to that of SD rat in the experiment in Table 1-b. We concluded from above results that PCA test was to be conducted with male conventional Wistar strain of rat weighing 150g as recipient, and challenge antigen dose of CE was 2.5mg per rat. This method was employed throughout.

(2) IgE antibody production of ICR mice immunized with CE.

(2-a) Influence of adjuvants on IgE antibody production.

IgE antibody titer in ICR mice was compared between two kinds of adjuvants, *B. pertussis* and alum. Mice were immunized with 2 times of 25γ of CE for 5 days apart, with 5 times of 10γ in 5 days or with 8 times of 6γ in 8 days mixed with either of adjuvants. PCA titer at 14 days after primary antigen stimulation became higher in all the mice immunized with alum than the mice accepted with *B. pertussis* as adjuvant. On the other hand, IHA titer was higher in the mice with *B. pertussis*, as shown in Table 2. Hereafter, we used alum as adjuvant for IgE antibody production in mice.

Table 2. IgE antibody production in ICR mice immunized with CE from *F. hepatica* mixed with *B. pertussis* or aluminium hydroxide gel as adjuvant

immunized with		reciprocal of IHA titer	reciprocal of PCA titer
25γ × 2	B.P.*	$2^{3.2} \pm 2^{0.47}$***	$2^{0.4} \pm 2^{0.36}$
	Alum**	$2^{1.7} \pm 2^{0.24}$	$2^{2.3} \pm 2^{0.71}$
10γ × 5	B.P.	$2^{4.0} \pm 2^{0.44}$	$2^{2.8} \pm 2^{1.44}$
	Alum	$2^{1.2} \pm 2^{0.13}$	$2^{3.9} \pm 2^{0.43}$
6γ × 8	B.P.	$2^{4.2} \pm 2^{0.57}$	$2^{0.8} \pm 2^{0.33}$
	Alum	$2^{1.2} \pm 2^{0.22}$	$2^{1.8} \pm 2^{0.42}$

* Killed *Brodetella pertussis* organisms (10^{10}/mouse).

** $Al(OH)_3$ gel (3mg/mouse).

*** Mean with six or seven immunized mice sera of 14 days after primary.

(2-b) IgE antibody production according to the times of antigen stimulation.

The production of PCA and IHA antibody was studied in the mice immunized with various times of antigen stimulation of CE, with results in Fig. 1. Three groups of mice were immunized with 2 times of 25γ in 5 days, with 5 times of 10γ in 5 days or with 8 times of 6γ in 8 days. These mice were bled 14 days after primary antigen stimulation, and PCA and IHA activity was determined. PCA titer became highest in the mice accepted with 5 times of 10γ in 5 days. IHA titer was highest in the mice with 25$\gamma \times 2$, but it did not change so much according to the times of antigen stimulation.

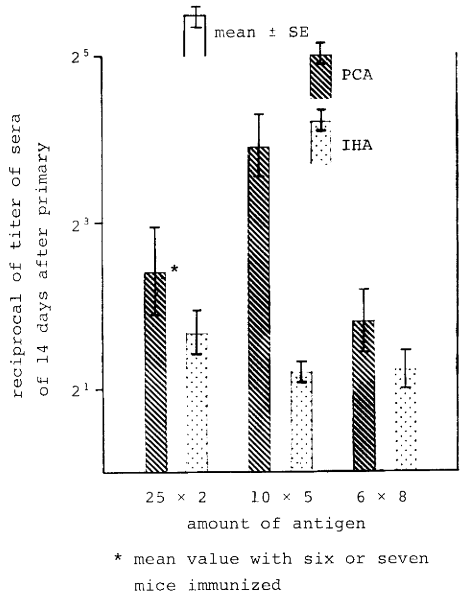

Fig. 1. PCA and IHA antibody titer in mice according to the times of antigen stimulation.

(2-c) IgE antibody production according to the day of booster after primary immunization

IgE antibody production is known to be produced effectively by the secondary antigen stimulation, but the detail IgE antibody production in case of the immunization with parasite antigen is not clearly understood. Therefore, twice repeated stimulations with 5γ or 10γ or CE at various intervals were given six or seven mice per group. Mice were bled 5 days after booster immunization, and PCA activity was assayed. As shown in Fig. 2, in the case of 5$\gamma \times 2$ immunization, twice repeated stimulations at 10 days or 20 days interval produced relatively high titer of IgE antibody in mice, whereas, in the case of 10$\gamma \times 2$, 20 days interval stimulated IgE antibody production strongest.

(3) Time course of IgE antibody production of mice.

Time course of PCA and IHA antibody production was examined with four groups of the mice with twice repeated immunization of 5γ, 10γ, 20γ or 40γ of CE at 20 days interval. The mice immunized with primary injection produced IgE antibody transiently at 12 days after injection, and the antibody activity disappeared rapidly. But, higher and longer IgE antibody titers were observed in the mice accepted with booster injection. Among the mice immunized, the mice with 20$\gamma \times 2$ or 40$\gamma \times 2$ showed relatively better IgE antibody production against CE. On the other hand, almost no or only poor IHA antibody production was observed in these mice on this condition, as shown in Fig. 3.

(4) IgE antibody production elicited by CE in different strains of mice.

Preliminary experiments confirmed that a booster immunization on day 20 gave a

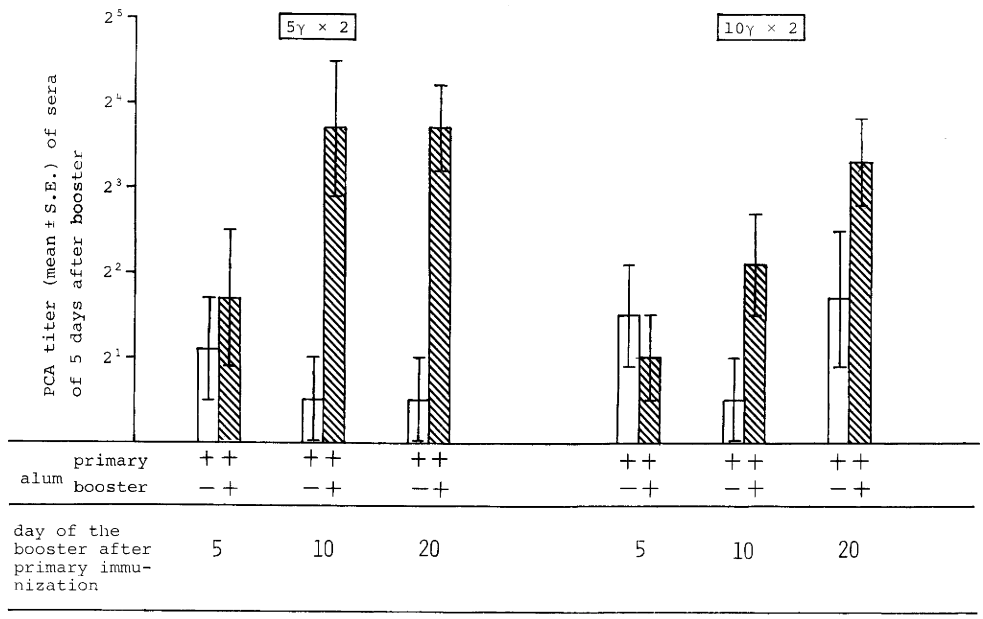

Fig. 2. IgE antibody production in ICR mice according to the day of booster after primary immunization.

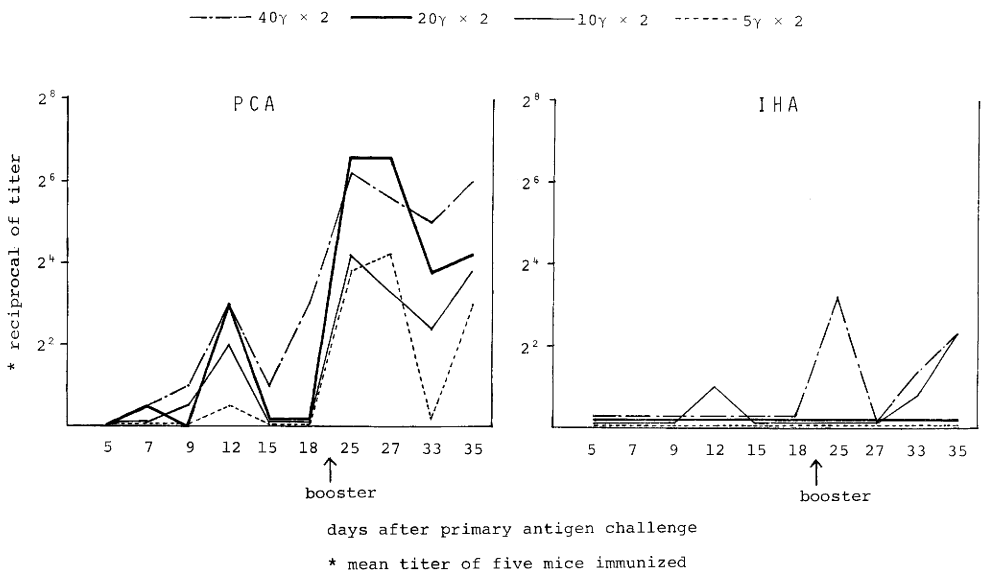

Fig. 3. Time course of PCA and IHA antibody production in the ICR mice sequential challenged with 2 times of CE for 20 days interval.

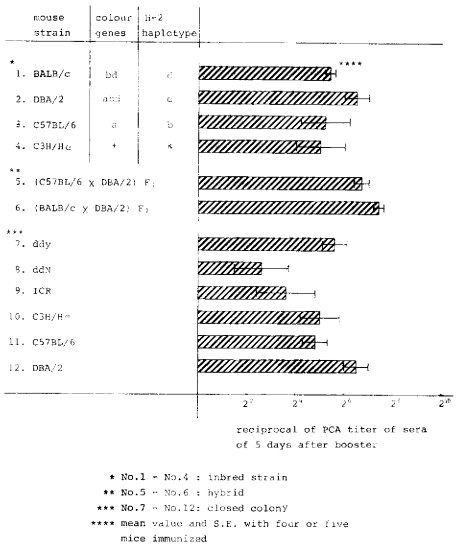

Fig. 4. PCA titers of different strains of mice immunized with 20γ of CE and boosted at 20 days.

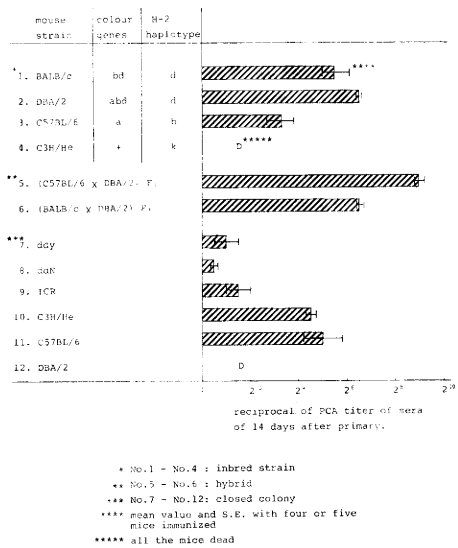

Fig. 5. PCA titer of different strains of mice accepted with fifth repeated antigen stimulation of 10γ of CE in 5 days.

better IgE antibody response in ICR mice. Four inbred mouse strains, BALB/c, DBA/2 C57BL/6 and C3H/He, two of their hybrids, (C57BL/6×DBA/2)F_1 and (BALB/c×DBA/2)F_1, and six outbred strains, ddY, ddN, ICR, C3H/He, C57BL/6 and DBA/2, were immunized with 20γ of CE and boosted at 20 days. Their serum PCA titers were assayed at 5 days after booster injection. Among mice tested, little differences in IgE production could be observed, as shown in Fig. 4.

Then, IgE antibody production was compared among the mice groups accepted with fifth repeated antigen stimmulation of 10γ of CE in 5 days. The mice sera were taken at 9 days after last immunization, and PCA activity was assayed. In this case, PCA response was different conspicuously dependent on mice strains. The inbred strains of DBA/2 and BALB/c and their hybrids, (C57BL/6×DBA/2)F_1 and (BALB/c×DBA/2)F_1, produced heigher PCA titers. On the other hand, outbred strains of ddY, ddN and ICR produced lower PCA titers, as shown in Fig. 5.

DISCUSSION

In order to get the purified allergen for diagnosis or for investigation purposes of fascioliasis, preliminary experiments were conducted, and the conditions for better production of IgE antibody were studied in the mice immunized with crude antigen extracted from adult *F. hepatica*. According to the paper written by Shinoda (1974a),

IgE production was very poor and transiently in the hosts immunized with *F. hepatica* crude antigen, whereas the infection of *F. hepatica* induced relatively high titer of IgE antibody. The *Nippostrongylus brasiliensis* infection also induced high titer of IgE antibody in the hosts, but the immunization with *N. brasiliensis* did not induce any IgE antibody (Ogilvie, 1967; Wilson and Block, 1968). Sumi (1971) reported that the rabbits immunized with *Dirofilaria immitis* antigen mixed with adjuvant produced almost no IgE antibody, but when live *D. immitis* worms were transplanted into pleural cavity of rabbit, the host produced IgE antibody with very ease. Thus, parasitic infection was said to be necessary for IgE antibody production (Mota, 1964). In this study, however, we demonstrated relatively high titer of IgE antibody in the mice immunized with crude antigen of adult *F. hepatica*.

Certainly, it was a little difficult for the mice immunized with parasite crude antigen to produce the IgE antibody with high titer and longer period. But, if we selected the dose of antigen, the times and interval of antigen stimulation, and mouse strain properly, we could induce mice IgE antibody very easily. The system to induce IgE antibody in mice by using the extracted parasite antigen was though to be very useful for the further study of parasite antigen.

Andrews and Meister (1978) studied the differences in susceptibility to infection with *F. hepatica* among mouse strains. Mice of thirty one different strains were intraperitoneally infected with 24hr-old flukes taken from the donor mice which had been infected orally with approximately 500 metacercaria. The mortality rate was determined from day 20 to 39 after infection. The inbred strains of mice showed a resistance lower than that found in outbred. Among inbred strains, the mortality rate of AJ mice was lowest (46%), these of AKR/A and C57BL/10 were shown as lower values (59 and 60%), these of DBA/2 and BALB/c were higher (70 and 73%) and these of C3H/Tif and C57BL/6 were highest (83 and 84%). The difference between mortality rates for inbred strains of AJ mice and C57BL/6 mice was highly significant, although their infection rates were almost 100%. However, interestingly, these six cross-breeds of which one parent was of BALB/c strain showed the highest degree of resistance to the *F. hepatica* infection, and their mortality rates were only 17 to 31%.

In the present study, the IgE antibody production was compared among different strains of outbred, inbred and their hybrid mice immunized with crude antigen from *F. hepatica*. However, no relationship could be observed between the resistance to *F. hepatica* infection and the IgE antibody production in these different strains of mice.

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肝蛭のアレルゲン

系統間マウスの粗抗原に対する IgE 抗体産生能とその産生条件

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寄生蠕虫感染は宿主内に特異的な IgE 抗体を誘導するばかりでなく，虫に無関係な不特定多数の抗原に対する IgE 抗体の産生をも増強（potentiation）する．しかし，蠕虫由来の抗原で免疫すると，これらの IgE 抗体の産生は難しいとされてきた．われわれは肝蛭のアレルゲンの性質を知るために肝蛭粗抗原の免疫活性を調べたが，適当な条件下で粗抗原で免疫すると，マウスは比較的高タイターの IgE 抗体を産生した．すなわち，PCA 反応の条件としては，ウイスター系雄性（150g）のラットを用い，チャレンジ抗原量は2.5mg 以上とすること，また IgE 抗体産生にはマウスを粗抗原10γ で5日間5回免疫する方法か，20γ を20日間隔で2回免疫する方法が良いこと，アジュバントとしては水酸化アルミニウムゲルが優れていることなどが明らかにされた．ICR マウスを20γ，20日間隔2回免疫すると，2^6以上の IgE 抗体価を少なくとも1週間

以上持続した．また，inbred strain 4種，hybrids 2種，closed colony 6種のマウスについて，IgE 抗体産生能を比較検討した．inbred では DBA/2系が最もよく反応し，次いで BALB/c 系の順であった．hybrids のなかでは，この C57BL/6 と DBA/2 とのF_1マウスが最もよく反応し，両親より高い IgE 抗体を産出した．しかし closed colony の dd 系や ICR 系のマウスの IgE 抗体産生能は低かった．なお，この IgE 抗体産生能と肝蛭の感染に対するマウスの抵抗性との間には，何ら有意な関係は見出せなかった．

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