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Expression of 65 000 MW Heat-shock Protein in SCID Mice Infected with *Toxoplasma gondii* after Transplantation of Mouse Fetal Thymus

HIDEYUKI NAGASAWA [1], HAJIME HISAEDA [2], HIROYUKI ISHIKAWA [2], YOICHI MAEKAWA [2], TOHRU SAKAI [2] AND KUNISUKE HIMENO [2]

[1] The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan, [2] Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Tokushima 770, Japan

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ABSTRACT

Heat-shock proteins (HSPs) are evolutionarily highly conserved polypeptides. They are immunodominant antigens in a wide variety of bacteria and parasites. We reported that the induction of 65 000 MW HSP (HSP65) plays an important role in developing protective immunity against infection with *Toxoplasma gondii*. Here, we examined the role of T cells in the expression of HSP65 and in the acquisition of protective immunity in SCID mice transplanted with fetal thymus or liver cells from syngeneic C.B-17 mice and infected with *T. gondii*. When SCID mice lacking mature T and B cells were transplanted with fetal liver cells (FLT-SCID mice), T and B lymphopoiesis were reconstituted to the level of normal mice, whereas only T lymphopoiesis was reconstituted in SCID mice grafted with a fetal thymus (TG-SCID mice). These mice were infected with the Beverley strain bradyzoites of *T. gondii* 7 days after immunization with *Toxoplasma* homogenate. Immunization effect did not occur in untreated SCID mice (UT-SCID mice), all of which died within 2 weeks after infection. HSP65 was not expressed in their macrophages. The survival of TG-SCID mice was significantly prolonged by immunization as compared with that of the immunized UT-SCID mice, and all the immunized FLT-SCID mice acquired complete resistance as well as the immunized C.B-17 mice. HSP65 was expressed in macrophages of TG-SCID mice after immunization, and much higher in those

of FLT-SCID mice. These results indicate that T cells play a crucial role in the expression of HSP65 and in the induction of protective immunity against infection with *T. gondii*.

INTRODUCTION

The role of heat-shock proteins (HSPs) in infection and immunity has been studied in detail. HSPs in parasite infection appear to play important roles in the adaptation of microorganisms (Smejkal et al. 1988; Buchmeier and Heffron 1990). On the other hand, HSP are prominent antigenic proteins that can activate host immune systems (Haregewoin et al. 1989). Among the various HSPs, 65 000 MW (HSP65) has been identified as a target of immune responses by $CD4^+$ T (Ottenhoff et al. 1988), $CD8^+$ T (Koga et al. 1988), and γδ T cells (Haregewoin et al. 1989; Holoshitz et al. 1989; O'Brien et al. 1989).

We reported that HSP65, possessing an epitope located between amino acids 172 and 224 of *Mycobacterium bovis*, is expressed in peritoneal exudate cells (PEC) of mice which have acquired resistance against infection with *T. gondii*, an obligate intracellular parasite (Nagasawa et al. 1992). The level of expression of HSP65 in host PEC closely correlates with the capacity to develop protective immunity against both low-virulent and highly virulent strains of this protozoa. Moreover, we also reported that T cells, especially the γδ T cells, play a crucial role for the expression of HSP65 in BALB/c mice immunized with *Toxoplasma* homogenate (Nagasawa et al. 1994).

SCID mice lack functional T and B cells (Bosma et al. 1983; Custer et al. 1985; Dorshkind et al. 1984), and cannot mount effective cellular and humoral immune responses to foreign antigens. When SCID mice are engrafted with fetal liver cells (designated as FLT-SCID mice) from syngeneic C.B-17 +/+ mice, functional T and B cells are fully reconstituted. On the other hand, when mice are transplanted with a single lobe of fetal thymus (designated as TG-SCID mice), only T cells are reconstituted. Here, we examined the importance of T cells in the development of protective immunity and in the expression of HSP65 in *T. gondii* infection using FLT- and TG-SCID mice.

MATERIALS AND METHODS

Animals

Female BALB/c mice purchased from Japan Shizuoka Laboratory Animal Center (SLC) (Hamamatsu, Japan), and C.B-17 +/+ (C.B-17) and C.B-17 scid/scid (SCID) mice purchased from CLEA Japan Inc. (Tokyo, Japan) were used at 8-10 weeks of age.

TG-SCID and FLT-SCID mice

Fetal thymus and liver were obtained from 15-day-old C.B-17 mouse embryos. TG-SCID mice were generated by grafting a single lobe of fetal thymus under the kidney capsule of SCID mice through a dorsal incision, using a 21G needle. Nembutal anesthesia was applied during all operative procedures. FLT-SCID mice were generated as follows. Fetal liver was minced into a finely dispensed cell suspension and $1x10^7$ cells were transplanted into 6-8 week-old SCID mice via the tail vein.

Immunization and infection

Mice were infected with a lethal dose ($5x10^3$ bradyzoites) of the Beverley strain obtained from cysts in the brains of chronically infected mice. To induce protective immunity against infection with a lethal dose of bradyzoites, mice were injected i. p. with 0.2 mg (total protein) of *Toxoplasma* homogenate 7 days before infection. *Toxoplasma* homogenate was prepared from the RH strain tachyzoites by three cycles of freeze-thawing as described earlier (Nagasawa et al. 1991).

Immunoblotting

Protein extracts of adherent PEC homogenates derived from infected mice were mixed with lysate buffer (50 mM Tris-HCl/1% Nonidet P-40/0.1% SDS/1 mM leupeptin/100 mM phenylmethyl sulfonyl fluoride/1 mM pepstatin A/100 mM EDTA/150 mM NaCl). The protein concentration was determined using the BCA (bicinchoninic acid) reagent (Pierce, Rockford, IL). The protein samples were suspended and denatured in sample buffer [0.025 M Tris-HCl, pH 6.8 / 2% SDS / 10% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol/0.002% bromophenol blue] by boiling for 3 min. The protein was separated by SDS/PAGE (12.5% polyacrylamide) at 200V for 30 min. The murine mAb IA10, specific for an epitope located between amino acids 172 and 224 of HSP65 derived from *M. bovis*, was provided by J. DeBruyn (Institute Pasteur de Brabant, Belgium). Proteins were electrophoretically transferred to a PVDF membrane, and mAb binding was assayed using a peroxidase-conjugated secondary antibody as described before (Nagasawa et al. 1992).

Flow cytometry

Peripheral blood lymphocytes (PBL) or non-adherent PEC from TG-SCID and FLT-SCID mice were examined by means of flow cytometry with a single laser FACScan (Becton Dickinson, Mountain View, CA). PBL or PEC were stained with diluted mAbs to analyze the surface phenotype. Anti-CD4 (GK1.5) conjugated to phycoerythrin (PE), anti-B220 (RA4-6A2) conjugated to PE, and anti-CD3 (145-2C11) were purchased from Becton Dickinson. Anti-CD8 (53-6.72), anti-Thy1.2 (30-H12), and anti-TCR γδ were labeled with fluorescein isothiocyanate (FITC). Stained cells were analyzed with gates set to exclude debris, clumps, and dead cells by light scatter gating.

Mitogenic responses

Cells were incubated in RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Australia) supplemented with 20 mM HEPES, 10% fetal bovine serum (Flow Lab. Inc., McLean, VA), 100 U/ml of penicillin, 100 μg/ml streptomycin and $5x10^{-5}$M 2-ME. Spleen cells were cultured at a concentration of $2.5x10^{6}$ cells/ml in 0.1 ml/well in the presence of 2.5 μg/ml Con A (Type IV) (Sigma, St. Louis, MO) or 10 μg/ml LPS (*E. coli* 0111, DIFCO Laboratories, Detroit, MI) for 72 hrs at 37°C in a humidified atmosphere of 5% CO_2 in air, then 0.5 μCi [^{3}H] thymidine was added to each well. Cultures were harvested onto glass-fiber paper 8 hrs later and assayed for radioactivity in a scintillation counter (Aloka, Japan).

Ig production

Mouse Ig was quantified by means of a sandwich ELISA. Polystyrene microtiter plates (Costar, Cambridge, MA) were coated with the capture reagent containing 20 μg of goat anti-mouse Ig (IgG, IgM and IgA; Cappel Laboratories, Malvern, PA) in carbonate coating buffer and incubated at 4°C overnight. The plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with 1% BSA in PBS-Tween for 30 min at room temperature. After washing the plates with PBS-Tween, serum samples 100-fold diluted with PBS-Tween containing 0.01% BSA were added, and incubated on the plates for 2 hrs at room temperature. The plates were rinsed thoroughly, then 50 μl of a 1:1,000 dilution of the alkaline phosphatase-conjugated goat anti-mouse IgG antibody or anti-mouse IgM antibody (Sigma) in carbonate buffer was added to each well. The plates were incubated for another 2 hrs at room temperature, washed, and the substrate, paranitrophosphate (Sigma), was added to each well. Chromatophore development was detected using an MTP-32 microplatereader (Colona Electronics, Ibaragi, Japan) in 405 nm.

RESULTS

Reconstitution of lymphopoiesis in SCID mice grafted with fetal thymus or fetal liver cells

SCID mice were transplanted with fetal thymus or fetal liver cells from 15-day-old C.B-17 mouse embryos. Six weeks later, the development of lymphocytes in the PBL of those mice was examined by staining with either a combination of anti-CD4 and anti-CD8 or a combination of anti-Thy1.2 and anti-B220 (Fig. 1). The percentages of $CD4^+$ and $CD8^+$ T cells in either TG-SCID or FLT-SCID mice were comparable with those in PBL of normal BALB/c mice. The analysis of Thy1.2/B220 profiles showed, however, that B cells did not differentiate in TG-SCID mice in contrast to FLT-SCID and BALB/c mice.

Mitogenic responses and Ig production by TG-SCID and FLT-SCID mice

The functions of the reconstituted lymphocytes of TG-SCID and FLT-SCID

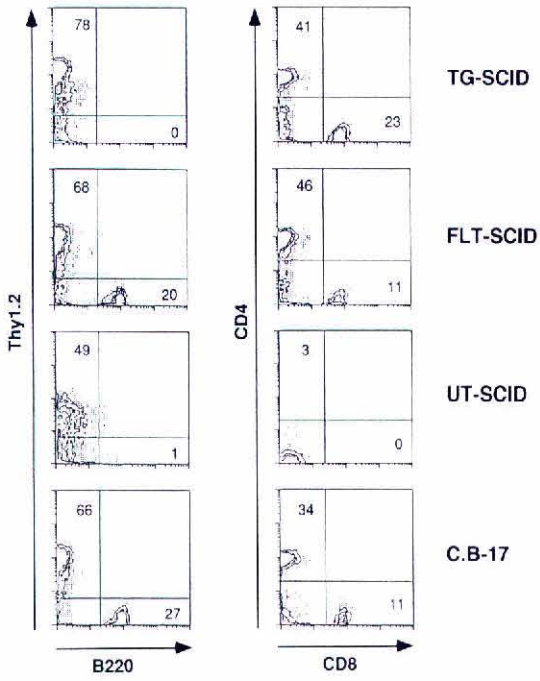

Fig. 1 Two-color flow cytometry in PBL from TG-SCID and FLT-SCID mice 1.5 months after transplantation. PBL from TG-SCID, FLT-SCID, UT-SCID, and C.B-17 mice were stained with a combination of anti-CD8 and anti-CD4 or a combination of anti-Thy1.2 and anti-B220, respectively. Solid lines indicate the fraction of each subpopulation and the percentage of each fraction is indicated.

mice were examined. As shown in Table 1, spleen cells from both TG-SCID and FLT-SCID mice responded to Con A as well as those from normal C.B-17 mice, whereas an LPS response was scarcely seen in those from TG-SCID mice. To further evaluate the state of the B cell reconstitution, we measured serum Ig level using an ELISA. The level of circulating IgG and IgM in both TG-SCID and SCID mice was as low as the background, whereas the level of these Igs in FLT-SCID mice was comparable with those of normal C.B-17 mice. These data indicate that only T cells were reconstituted in TG-SCID mice, whereas T and B cells appeared in FLT-SCID mice.

Table 1. Mitogenic responses and Ig level in SCID mice grafted with fetal thymus or liver cells.

	Mitogenic responses (S. I.*)		Serum Ig concentration (μg/ml)	
	Con A	LPS	IgM	IgG
TG-SCID	33.2 ± 7.6	1.6 ± 0.2	35.8 ± 6.9	96.6 ± 7.3
FLT-SCID	12.5 ± 2.7	5.2 ± 0.4	>500.0	1511.6 ± 77.2
UT-SCID	1.5 ± 0.3	1.5 ± 0.4	<10	<10
C.B-17	20.5 ± 5.9	10.9 ± 3.6	>500.0	912.2 ± 7.3

* S.I., stimulation index.
# Each value is the mean ± SE of four or five independent experiments performed in triplicate.

The induction of protective immunity in TG-SCID and FLT-SCID mice

We reported that T cells play an important role in protective immunity against infection with *T. gondii* (Nagasawa et al. 1991). This was also demonstrated in this study using SCID mice. We evaluated the protective function of T cells in TG-SCID and FLT-SCID mice against *Toxoplasma* infection. The mice were infected with 5x 10^3 of bradyzoites 7 days after immunization with *Toxoplasma* homogenate. As shown in Fig. 2, all FLT-SCID and BALB/c mice, controlled the infection with bradyzoites after the immunization. The survival of TG-SCID mice was significantly prolonged by immunization compared with UT-SCID mice, which died within 2 weeks after infection. However, TG-SCID mice did not acquire total resistance to the infection compared with immunized FLT-SCID and BALB/c mice.

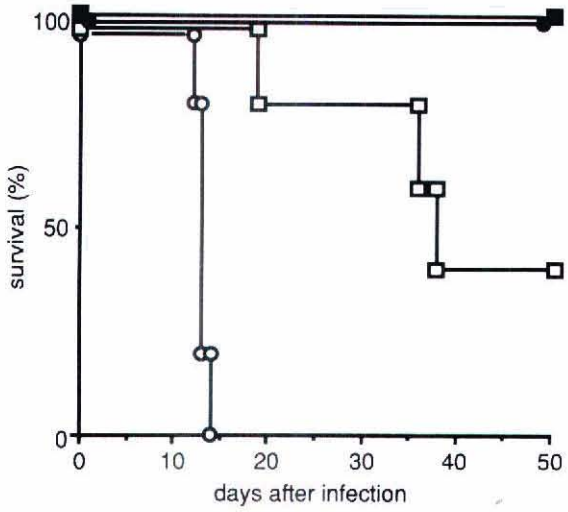

Fig. 2 Effect of immunization on resistance to infection with *Toxoplasma*. TG-SCID (open square), FLT-SCID (closed square), UT-SCID (open circle) and C.B-17 mice (closed circle) were infected with $5x10^3$ bradyzoites of the Beverley strain 7 days after immunization with 0.2 mg of *Toxoplasma* homogenate as described in materials and methods.

Expression of HSP65 in macrophages of TG-SCID and FLT-SCID mice that acquired resistance against infection with the Beverley strain

TG-SCID or FLT-SCID mice were immunized and challenged with $5x10^3$ of bradyzoites 2 months after transplantation as described in Materials and Methods. Seven days later, the expression of HSP65 in host macrophages were examined. Figure 3 shows that HSP65 was significantly expressed in macrophages of TG-SCID mice, and much higher in those of FLT-SCID mice. HSP65 expression was undetectable in macrophages of UT-SCID mice that had been infected 7 days after immunization. These results were confirmed by two additional experiments. We reported that γδ T cells play an important role in HSP65 expression after immunization with *Toxoplasma* homogenate (Nagasawa et al. 1994). Figure 4 shows γδ T cells in the peritoneal cavity of both TG-SCID and FLT-SCID mice. The number of γδ T cells were increased after infection.

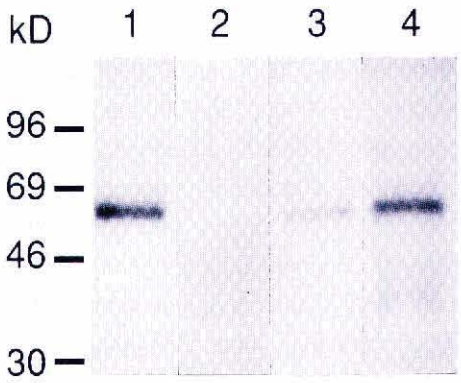

Fig. 3 Electroblots of peritoneal macrophages from reconstituted SCID mice after infection with *Toxoplasma*. Lysates of peritoneal macrophages were harvested from C.B-17 (lane 1), UT-SCID (lane 2), TG-SCID (lane 3) and FLT-SCID mice (lane 4) infected with $5x10^3$ bradyzoites of the Beverley strain 7 days after immunization with 0.2 mg of *Toxoplasma* homogenate as described. Each lane was loaded with 10 μg of protein.

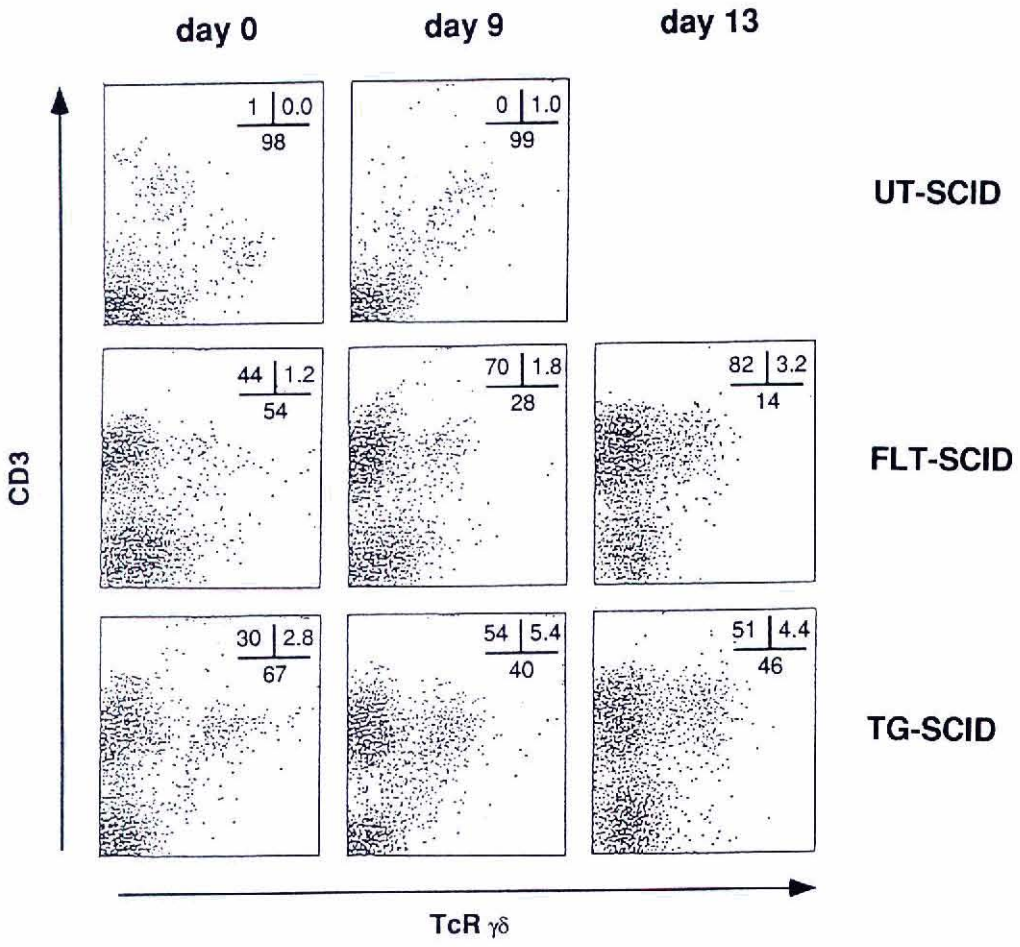

Fig. 4 Two-color flow cytometry in non-adherent PEC from TG-SCID and FLT-SCID mice. PEC from TG-SCID, FLT-SCID and UT-SCID mice 0, 9 and 13 days after infection with *Toxoplasma* bradyzoites were stained with a combination of anti-CD3 and anti-TcR γδ. The percentage of each subpopulation is indicated.

DISCUSSION

We reported that the expression of HSP65 in host macrophages closely correlates with the protection against *Toxoplasma* infection (Nagasawa et al. 1992). That is, HSP65 is expressed in the PEC of mice infected with a low-virulence strain (Beverley strain) of *T. gondii*, as determined by electroblotting using a mAb specific for microbial HSP65. This HSP, however, is not expressed when the mice are infected with the highly virulent RH strain of *T. gondii*. Furthermore, HSP65 is expressed at high levels in macrophages of hosts that have acquired resistance against infection with a lethal dose of bradyzoites of the Beverley strain by immunization with *Toxoplasma* homogenate or against the highly virulent RH strain of *T. gondii* by vaccination with a sublethal dose of live Beverley strain bradyzoites.

The mechanisms of protective immunity against *T. gondii* infection have been analyzed by many authors. Most have suggested that cell-mediated immunity is the major defense mechanism of the host against this infection (Nagasawa 1984; Krahenbuhl and Remington 1982) and humoral immune responses confer only partial protection (Krahenbuhl et al. 1972; Johnson et al. 1983; Sharma et al. 1984; Pavia 1986). As demonstrated in this study, HSP65 expression was induced in peritoneal macrophages of BALB/c mice infected with a low-virulence strain of *Toxoplasma* (Beverley strain) but not in those of either nude or SCID mice. Moreover, in transplantation studies using SCID mice, we found that TG-SCID and FLT-SCID mice acquired resistance against infection with *Toxoplasma* parasites when they had been immunized with *Toxoplasma* homogenate, and HSP65 was expressed in their peritoneal macrophages (Fig. 3). Thus, T cells appear to play an important role in the HSP65 expression after *Toxoplasma* infection, as well as in the acquisition of protective immunity.

Although TG-SCID mice survived longer than UT-SCID mice after *T. gondii* infection, resistance was not complete, in contrast to FLT-SCID or C.B-17 mice. Further, the expression level of HSP65 was low in TG-SCID mice compared with that in FLT-SCID or C.B-17 mice. Thus, the protective potential and the HSP65 expression level seem to be closely correlated as reported (Nagasawa et al. 1992). Understanding the cause of these differences between TG-SCID and FLT-SCID may be valuable in clarifying the mechanisms of protection and expression of HSP65 in *Toxoplasma* infection. One explanation for these differences may be that T and B cells are fully reconstituted in FLT-SCID mice, while only T cells are present in TG-SCID mice. B cells may be required as APC to induce efficient protective immunity or specific antibodies against *Toxoplasma* may partly participate in protection by mediating ADCC or complement-dependent lysis, although transfer of a specific antibody did not confer any protective potential upon nude and SCID

mice (data not shown). Another explanation for these differences between TG-SCID and FLT-SCID may be that reconstitution of γδ T cells is different in quantity and quality between these two groups of reconstituted SCID mice. If the differentiation of γδ T cells is more incomplete in TG-SCID, than in FLT-SCID mice, this may explain why TG-SCID mice did not acquire the complete resistance and strong expression of HSP65 against *T. gondii* infection. A subset of γδ T cells recognizes HSP65 (Haregewoin et al. 1989; Holoshitz et al. 1989; O'Brien et al. 1989). Furthermore, γδ T cells increase rapidly in the blood of patients acutely infected with *T. gondii* (De Paoli et al. 1992; Scalise et al. 1992). This T cell subset is thought to represent a first line of defense against infection (Young and Elliott 1989, Hiromatsu et al. 1992). We found that γδ T cells play an important role in protective immunity (submitted for publication) and the expression of HSP65 in mice acquired resistance against *Toxoplasma* infection (Nagasawa et al. 1994). Thus, the distinct development of T cells, especially the γδ T cells, in these two groups of reconstituted SCID mice could be reflected as differences in protective immunity and HSP expression. This motion is still under investigation.

In conclusion, transplantation studies using SCID mice confirmed and extended our findings that T cells play a crucial role in acquiring resistance against *Toxoplasma* infection and in HSP65 expression in the peritoneal macrophages of mice infected with *T. gondii*. Furthermore, the induction of HSP65 in host macrophages is closely correlated with protection against *Toxoplasma* infection.

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