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Diagnosis, transmission and immunology of human Oesophagostomum bifurcum and hookworm infections in Togo

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Chapter 8

Parasite-specific cellular immune responsiveness in humans infected with *Necator americanus* and *Oesophagostomum bifurcum*

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SUMMARY

This study investigated parasite-specific cellular reactivity and Th1- or Th2-like cytokine responses in humans infected with *Necator americanus* and *Oesophagostomum bifurcum*. In patients with mono-infections of *N.americanus, O.bifurcum* larvae were not found in stool cultures during 9 consecutive months of follow-up. In co-infected patients, the level of *O.bifurcum*-specific as well as *N.americanus*-specific IgE was significantly elevated compared to those with *N.americanus* mono-infections. Cellular responses were not strictly dominated by type 1 or type 2 T helper cell reactivity. In co-infected patients cellular hyporesponsiveness to parasite antigens was observed, but the production of TNF-α and IFN-γ was greater. Th2-type cytokines (IL-5 and IL-10) were produced in equal amounts by PBMC from individuals with mono- and co-infections. Such mixed Th1- and Th2-type immune responsiveness associated with persisting gastrointestinal parasitic nematodes may reflect a state of infection at which parasite-induced inflammatory and enteropathogenic responses counteract potentially protective immune responses of the host. Since Th1-type responses are associated with chronic intestinal helminth infection, as suggested by experimental studies in animals, our observations support the idea that helminth co-infection will not only suppress parasite-specific cellular responsiveness but may also direct cytokine production towards a „permissive phenotype“ which favours parasite persistence.

INTRODUCTION

Gastrointestinal infections with roundworm parasites cause significant morbidity and mortality. In the human host, infections tend to be chronic, re-infection rates high, and in addition to their direct pathogenic effects, persisting gastrointestinal worms may predispose for secondary bacterial and protozoan infections. In addition, individuals living in endemic areas are likely to be co-infected with several gastrointestinal nematodes, with small numbers of worms generally being tolerated, and development of symptomatic disease occurring with increasing parasite accumulation. In man, the expression of protective immunity to hookworm remains unclear. Experimental studies in mice have shown that intestinal helminth parasites are highly immunogenic and protective responses against those parasites are critically depending upon CD4+ T cells. Th2-type cytokines including IL-4, IL-5,

IL-9, and IL-13 are required for host protection and cause expulsion of the parasite, while IL-12 and IFN-γ inhibit protective immunity and allow establishment of a chronic infection (Urban *et al.* 1992, Urban *et al.* 1998, Else *et al.* 1993, Finkelman *et al.* 1997, Grencis 1997). This type of protective immunity is in contrast to observations in humans infected with extra intestinal filarial parasites, in whom a dominant Th2-like immunity, i.e. enhanced cellular production of IL-4, IL-5 and IL-10, eosinophilia and augmented IgE and IgG4, represent characteristic traits of parasite persistence and chronic infection (King & Nutman 1992). In northern Togo and Ghana, more than 70% of the rural population is chronically infected with the hookworm *Necator americanus*, and almost 30% is infected with *Oesophagostomum bifurcum* (Pit *et al.* 1999). Until recently human infections with *Oesophagostomum spp.* were considered as rare zoonotic infections and while most *O.bifurcum*-infected individuals remain asymptomatic, in a few patients *O.bifurcum* larvae induce massive granulomatous reactions (nodules) while penetrating the intestinal wall, leading to ulceration and gut perforation (Gigase *et al.*, 1987, Polderman *et al.* 1991, Krepel *et al.* 1994). In *O.bifurcum*- or *N.americanus*-infected humans adult worms may persist for years with little evidence of the development of protective immunity, although epidemiological data support its existence (Woolhouse 1992, Quinnell *et al.* 1990, Maizels *et al.* 1993). In the same population, however, despite intense exposure to the parasite, a small group of individuals will remain free of *O.bifurcum* infection (endemic normals), i.e. they do not excrete eggs in their stools as confirmed by repeated examinations. Therefore, the determination of those anti-parasite host-protective immune responses that limit worm burden and/or fecundity has important pathophysiological as well as epidemiological significance.

Up to date, little is known about the parasite-specific cellular responsiveness in humans chronically infected with *O.bifurcum* and *N.americanus,* and the factors and mechanisms which support protective immunity or which contribute to parasite persistence and enteropathy remain to be investigated. The aim of this study was to analyse the extend to which parasite-specific cellular reactivity in humans with mono- or co-infections with *O.bifurcum* and *N.americanus,* was dominated by Th1- or Th2-type

immune responses, and whether helminth co-infections may lead to biased or exhausted immunocompetence in patients.

MATERIAL AND METHODS

Study population and classification of patient groups

This investigation was conducted in northern Togo (West Africa) where *O.bifurcum* and *N.americanus* infections are highly endemic (Polderman *et al.* 1991). Patients originated from a rural village in northern Togo, close to the Ghanaian border (*O.bifurcum* prevalence 63%, hookworm prevalence 75%, n=184). In individuals with a *N.americanus* mono-infection (n=20) *O.bifurcum* larvae were not found in stool cultures during 9 consecutive months of observation. Informed consent was obtained from all patients before parasitological examination and blood withdrawal.

Parasitological and serological examination and classification of patient groups

Infections with *O.bifurcum* and *N.americanus* were detected by stool cultures as previously described (Polderman *et al.* 1991, Krepel *et al.* 1995). In order to distinguish between *N.americanus* and *O.bifurcum* infections, three grams of stool from all participants were cultured for seven days in the moist environment of a petri-dish. The infective L3 larvae were collected, identified as *O.bifurcum* or *N.americanus* and counted. Infection intensity was indicated as the number of L3 larvae recovered from these cultures.

For the determination of *O.bifurcum*- or *N.americanus*-specific IgE in patients, sera were preabsorbed with protein G (Pharmacia, Uppsala, Sweden) as previously described by Quinnell *et al.* 1995. Briefly, diluted sera (1:4) in 0.035M PBS (pH 7.8) were incubated on a rotor with an equal volume of Protein G at 4°C overnight. Thereafter, samples were centrifuged for 15 min and supernatants collected. Microtitration plates (Maxisorb, Nunc) were coated with *O. bifurcum*- and *N.americanus*-specific antigen at 5 µg protein/ml in 0.1M sodium carbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with PBS containing 1% bovine serum albumin for 1 h at 37°C, then washed with 0.035M PBS and preabsorbed sera (final dilution 1:40) was added in duplicates and incubated at 4°C overnight. After washing (as above), anti-human IgE mouse monoclonal antibody (Sigma) was used, followed

by AP-conjugated rabbit anti-mouse antibody (Sigma) (1 h at 37°C), and after incubation with p-nitrophenyl phosphate (pNPP) for 1 h at room temperature absorbance was read at 405 nm. To correct for assay variation, results were expressed as ratios between the absorbance values of samples and defined control sera.

Preparation of Oesophagostomum bifurcum and hookworm antigens

Following treatment of patients with pyrantel pamoate and purgation, adult worms of *O.bifurcum* and *N.americanus* were isolated as described by Polderman *et al.* 1991. Isolated adult worms were extensively washed in PBS, transferred into a Ten-Broek tissue grinder and then extensively homogenised on ice. The homogenate was then sonicated twice (30% intensity) for 3 min on ice, centrifuged at 16000g for 30 min at 4°C. The supernatants were collected, then sterile filtered (0.22µm) and the protein concentration determined with the BCA protein assay (Pierce). The protein concentration of the PBS-soluble *O.bifurcum*-antigens (OesAg) was 4.8mg/ml; the *N.americanus*-antigens (NecAg) contained 1.1mg/ml protein.

Isolation of peripheral blood mononuclear cells (PBMC) and cell culture experiments

Heparinized blood was collected from patients, and PBMC were isolated within 36 hours by Ficoll-Paque (Pharmacia) density gradient centrifugation. Cell culture experiments were conducted as previously described by Soboslay *et al.* (1994). Briefly, PBMC were adjusted to 1x 10^7/ml in RPMI (Gibco, Grand Island, NY) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin and 0,25 µg/ml amphotericin B; they were then used immediately to stimulate cytokine secretion, or cryopreserved for proliferation assays. For proliferation assays, cells were thawed and seeded at 1x 10^5 cells/well in sterile round-bottomed 96-well microtitre plates (Costar, Cambridge, MA). Cells were suspended in RPMI (as above) containing 10% FCS, and kept in 5% CO_2 at 37°C and saturated humidity. For mitogenic stimulation with phytohaemagglutinin (PHA; 1:100; Gibco) and for antigenic stimulation with OesAg (24 µg/ml), NecAg (30 µg/ml) and streptolysin-O (SL-O; 1:50; Difco), cultures were maintained for 3 or 5 days respectively. For the last 18 hr, 1 µCi of [^{3}H]thymidine was added;

cells were then harvested on glass fibre filters (Skatron) and the incorporated radioactivity determined by scintillation spectroscopy (Beta Plate; LKB-Pharmacia). Data are indicated as mean values of triplicate cultures in c.p.m. minus baseline stimulation.

Determination of cytokine production

Freshly isolated PBMC were cultured, in 5% CO_2 at 37 °C, at a concentration of $3{,}7 \times 10^6$ cells/ml in RPMI (as above) supplemented with 1% heat-inactivated FCS, in the presence of *O.bifurcum*-derived antigen, *N.americanus*-derived antigen, PHA or streptolysine-O at the same concentrations as used for proliferation assays. Cell culture supernatants were collected after 48hr and stored in liquid nitrogen. Cytokine secretion by stimulated PBMC was quantified by sandwich ELISA using cytokine specific monoclonal and polyclonal antibodies for interleukin-2(IL-2), IL-4, IL-5, IL-10 (Pharmingen) as described by Soboslay *et al.* 1994. Interferon-γ (IFN-γ) and Tumour Necrosis Factor-α (TNF-α) was quantified by ELISA (Holland Biotechnology) as recommended by the manufacturer.

Statistical data analysis

Results are indicated as mean values (mean ± SEM of different groups). Mean values of patient groups were compared using Mann-Whitney non-parametric test.

RESULTS

Parasitological and serological data on study population

Demographic data for the study population are shown in Table 1. Investigations of patients' parasite-specific serological reactivity showed that *O.bifurcum*- and *N.americanus*- specific IgG and IgG subclasses were strongly reactive, but no differences were observed between the study groups (data not shown). Parasite-specific determination of IgE, however, clearly distinguished between co-(*O.bifurcum* and *N.americanus*) and mono-(*N.americanus*) infected individuals. In co-infected patients the level of OesAg-specific as well as NecAg-specific IgE was significantly elevated compared to individuals mono-infected with hookworm. In mono-infected patients, with potential exposure to *O.bifurcum,* OesAg-specific IgE responses were lower ($P<0.01$) than in co-infected patients, but significantly higher ($p<0.02$) than in hookworm-infected patients from

O.bifurcum-free areas (data not shown).

Table 1: Parasitological and serological data on study groups. Patients originated from a village where 63% of the population was infected with O. bifurcum and 75% with N. americanus.

	O.bifurcum and *N.americanus* infected	*N.americanus* infected (endemic)
Patients	**26**	**20**
Male/Female	10/16	8/12
Median age (range)	35 (12-61)	12 (10-40)
O.bifurcum L3 per 3g stool	10	0
Median (range)	(1-116)	
N.americanus L3 per 3 g stool	25	**30**
Median (range)	(1 -258)	(1-238)
IgE – reactivity to OesAg		0.23 ± 0.05
(OD ± SEM)	0.41 ± 0.06**	
IgE – reactivity to NecAg		0.53 ± 0.05
(OD ± SEM)	0.76 ± 0.07	

*(** p = 0.021)*

Cellular reactivity in mono-and co-infected individuals

PBMC from patients were stimulated with mitogens (PHA, ConA) as well as *O.bifurcum*- (OesAg), *N.americanus*- (NecAg) and *Streptococcus pyogenes*-specific (SL-O) antigens. Cellular responses of PBMC to the mitogen PHA were similar in the patient groups. Cellular responses to bacterial SL-O were lowest in co-infected patients when compared to mono-infected patients (Table 2). Similarly, cellular reactivity to OesAg was lowest in *O.bifurcum* and *N.americanus* co-infected patients (Table 2). In response to NecAg, cellular reactivity was low, both in co- (*O.b.* and *N.a.*) as well as in mono- (*N.a.*) infected patients (Table 2).

*Table 2: Cellular reactivity to mitogens (PHA, ConA), bacterial antigen streptolysin-O (SL-O), O.bifurcum-specific (OesoAg) and N.americanus-derived antigens (NecAg) (cpmx1000 ± SEM) of PBMC from doubly and singly N.americanus and O.bifurcum-infected patients. Values on cellular reactivity are shown as net proliferation in cpm±SEM of triplicate cell cultures from which baseline responses, i.e. no antigen added, have been subtracted. ** ($p < 0.05$)*

Stimulation	*O.bifurcum* and *N.americanus*-infected (n=26)	*N.americanus*-infected (n=18)
PHA (5µg/ml)	55921 ± 5940	69403 ± 10790
ConA (5µg/ml)	45329 ± 6539	78061 ± 12760
NecAg (30µg/ml)	240 ± 141	36 ± 23**
OesoAg (24µg/ml)	704 ± 230	2094 ± 719**
SL-O (1:50)	10543 ± 3049	16622 ± 5948

*Table 3: Production of Th1-type (IL-2, IFN-γ, TNF-α) and Th2-type (IL-4, IL-5, IL-10) cytokines (pg/ml ±SEM) by PBMC from O.bifurcum and N.americanus-infected patients in response to bacterial antigen (SL-O, 1:50), O.bifurcum-specific (OesoAg, 48 μg/ml) and N.americanus-derived antigens (NecAg, 11 μg/ml). (** p<0.01, * p<0.05). Values on cytokine production in response to bacterial and and helminth antigens are shown as net production from which spontaneous background cytokine secretion (i.e. no antigen control) has been subtracted.*

		Patients	
Cytokine	Stimulation	*O.bifurcum* and *N.americanus*-infected (n=26)	*N.americanus*-infected (n=20)
IL-2	SL-O	2612 ± 349	1664±247
	NecAg	13±11	<5
	OesoAg	14±5	15±15
IFN-γ	SL-O	2608±97	2532±194
	NecAg	729±117**	137±51
	OesoAg	274±72**	31±12
TNF-α	SL-O	5716±746	6166±630
	NecAg	7246±1508*	2943±954
	OesoAg	6476±912	4433±723
IL-4	SL-O	454±64**	112±34
	NecAg	28±14*	<5
	OesoAg	27±11	9±6
IL-5	SL-O	5537±666**	3334±544
	NecAg	142±52	60±43
	OesoAg	515±168	581±1208
IL-10	SL-O	3547±249**	1668±296
	NecAg	1163±194	1187±305
	OesoAg	1559±208	1463±297

Cytokine production by PBMC in mono-and co-infected patients

Th1-type cytokines

Substantial amounts of several Th1-type cytokines were secreted by PBMC from *N.americanus* and *O.bifurcum* co-infected patients. In response to both OesAg and NecAg, peripheral blood cells from co-infected individuals secreted more IFN-γ and TNF-α than PBMC from those with *N.americanus* mono-infections (Table 3). Cellular production of IL-2 in response to OesAg and NecAg remained low (max. 30 pg/ml), and no significant differences were observed between patient groups. However, in response to PHA (not shown) and bacterial SL-O substantial amounts of IL-2 were secreted, with higher concentration being produced by PBMC from co-infected individuals.

Th2-type cytokines

O.bifurcum- and *N.americanus*-derived antigens stimulated low level cellular production of IL-4 in both patient groups (Table 3). IL-5 and IL-10 were induced in equal amounts by OesAg and NecAg in PBMC from those with mono- and co-infections, but in response to bacterial SL-O significantly more IL-5 and IL-10 was produced by PBMC from co-infected patients. The amount of IL-5 and IL-10 induced by the mitogen PHA (data not shown) was lower in mono-infected patients.

DISCUSSION

Experimental studies in ruminants and laboratory rodents infected with gastrointestinal nematodes have indicated that Th2-type cytokine responses were critically important for expulsion of the parasite (Finkelman *et al.* 1991, Ishikawa 1998, Urban *et al.* 1998). In the human host detailed investigations on cytokine responses and protective immunity are confounded by concurrent helminth infections as frequently observed in populations where gastrointestinal helminths are endemic. Such co-infections and the aggregation of several parasites may predispose for inappropriate immune responsiveness, or else deviate or even exhaust immunocompetence of the human host.

Our investigation of humans with *N.americanus* and *O.bifurcum* mono- or co-infections showed that expression of cellular immunity in patients was not exclusively dominated by type 1 or type 2 T helper cell responses, PBMC from both patient groups produced TNF-α, Th1-like IFN-γ and IL-2 but also the Th2-like

cytokines IL-4, IL-5 and IL-10. Cellular responsiveness to *O.bifurcum*-derived antigens were statistically lower in co-infected patients, while interestingly, in those doubly infected cases, much more IFN-g and TNF-a were induced by NecAg and OesoAg than in those individuals with a single hookworm infection. Such pronounced parasite-specific cellular production of TNF-α and IFN-γ in co-infected patients indicates that *N.americanus* and *O.bifurcum* stimulate not only systemic inflammatory but in all likelihood enteropathogenic responses as well. TNF-α has been shown to cause significant intestinal pathology, which was enhanced by IFN-γ (Garside *et al.* 1993, Sartor 1994), and in synergy TNF-α and IFN-γ may cause more severe intestinal damage. The capacity of *Necator*- and *Oesophagostomum*-derived antigens to induce inflammatory and Th1-type cytokines may contribute to those enteropathies which manifest as villus atrophy and crypt hyperplasia, as typically observed with intestinal nematode infections (Miller 1979, Prociv 1997, Coutinho *et al.* 1996). Thus, elevated production of TNF-α and Th1-like IFN-γ in co-infected patients may reflect more severe intestinal inflammation and pathology caused by concurrent infection with *N.americanus* and *O.bifurcum*. Interestingly, levels of IL-4 as being produced spontaneously as well as in response to SL-O and NecAg were statistically higher in co-infected cases, but also the net production of IFN-g in response to NecAg and OesoAg were elevated ($p<0.05$) in those doubly infected, while spontaneous IFN-g production by PBMC was similar in both groups. For IL-2, only low levels were secreted, both spontaneously as well as in response to NecAg and OesoAg. Such low-level production of Il-2 by PBMC in response to helminth antigens has been reported previously (Soboslay et al. 1992, 1994, and 1999) and this may be due to the very nature of IL-2. IL-2 is an autocrine growth factor and the values as determined in this study may simply reflect the use of the cytokine as it is produced.

In our study, PBMC from mono- and co-infected patients secreted equivalent amounts of IL-5 and IL-10. *N.americanus*-derived antigens, and to a much greater extent *O.bifurcum* antigens, induced cellular production of IL-5 which will activate and induce migration of eosinophils. Activated eosinophils may

then participate in cellular cytotoxic defence mechanisms, which may operate against developmental stages of the parasite, preventing parasite invasion, migration and maturation. But eosinophil immigration into intestinal tissues, their activation and degranulation may also result in local tissue damage, in severe cases leading to ulceration and bleeding (Miller 1979, White *et al.* 1986, Prociv & Croese 1990, Walker *et al.* 1995). In oesophagostomiasis patients, such activated eosinophils may then contribute to enteropathy, i.e. massive granulomatous reactions (nodules) of the intestinal wall as observed in patients with overt clinical disease (Polderman & Blotkamp 1995).

The tissue-dwelling stages of *N.americanus* and *O.bifurcum* are likely to provoke an intense immunological response (Ogilvie *et al.* 1978, Caroll & Grove 1986, Polderman *et al.* 1993, Polderman & Blotkamp 1995, Prociv 1997), but in the human host *N.americanus* infection was found to induce parasite antigen-specific cellular responsiveness in some cases only - cellular unresponsiveness and low levels of total IgE in infected individuals were attributed to the very low level of hookworm infection (Maxwell *et al.* 1987). However, pronounced lymphocyte proliferation was observed with a higher infection dose of *N.americanus* in man (Taylor & Turton 1976). In our study, cells from hookworm infected individuals proliferated to some extent in response to O.bifurcum-specific antigens, and their PBMC secreted several cytokines in response to O.bifurcum antigens as well. We attribute such responsiveness to antigenic cross-reactivity between hookworm and *O.bifurcum* which has been found previously (Polderman et al. 1993) and our recent investigations have confirmed that *O.bifurcum* infection will induce IgG4 and IgE which cross reacted with hookworm antigens (*Pit et al.* unpublished observation).

The somewhat low parasite-specific cellular reactivity to *N.americanus*- as well as to *O.bifurcum*-derived antigens in the patients of our study could be due to a low level infection, but also, parasite persistence as well as chronic co-infection may have deviated or suppressed cellular responsiveness. Similarly, cellular hyporesponsiveness to parasite antigen is a characteristic trait in patients with patent filarial infection (King *et al.* 1992, Sartono *et al.*, 1995) and schistosomiasis (Grogan *et al.*,

1996). In onchocerciasis patients co-infected with HIV 1 (Sentongo *et al.* 1998) or *Mansonella perstans* (Soboslay *et al.* 1997), parasite-specific cellular responses and cytokine production were deficient as compared to those with a single infection. From these observations it was concluded that chronic helminth infections as well as helminth-virus co-infections suppress and gradually exhaust the parasite-specific immunocompetence of patients. One mechanism by which cellular unresponsiveness to helminth antigens could be mediated is the spontaneous secretion and high level production of IL-10. Overproduction of IL-10 will down-regulate cytokine production in general and depress parasite-specific cellular reactivity in filariasis patients (Mahanty *et al.* 1995). In addition, IL-10 will inhibit MHC class II expression on antigen presenting cells (de Waal Malefyt *et al.* 1991) and suppress both type 1 and type 2 T helper cell activation (Del Prete *et al.* 1993). Therefore, our observations support the idea that chronic helminth co-infection will not only suppress parasite-specific cellular responsiveness but also deviate cytokine production towards a „permissive phenotype" which will favour parasite persistence.

In humans with chronic helminth infections there is little direct evidence that Th2-like immune responses are protective even though immunoepidemiological data support the existence and development of host protective immunity (Woolhouse 1992, Quinnell *et al.* 1995, Pritchard *et al.* 1995). The decreasing prevalence of infection with increasing age and an aggregated distribution of *O.bifurcum* and *N.americanus* in humans (DSS Pit, unpublished observation) - as also observed e.g. with schistosomiasis and other helminth parasites (Hagan *et al.* 1991, Maizels *et al.* 1993, Woolhouse 1998) - support the idea that acquired immunity could be responsible for the pattern of infection in endemic populations. Previous investigations on immunity to *N.americanus* found a significant negative correlation between total IgE and parasite weight and fecundity suggesting protective immune effector mechanisms against *N.americanus*, which reflect Th2 cell activation (Pritchard *et al.* 1995). In our study, parasite-specific IgE was highest in co-infected individuals, which may simply reflect the parasite load - high parasite-specific IgE may mediate protective ADCC but may also induce immediate hypersensi-

tivity reactions. In parallel, elevated total non-specific IgE may compete with specific IgE - such polyvalent IgE may reduce the risk of inappropriate anaphylactic responses and at the same protect the parasite from IgE-mediated ADCC reactions (Pritchard 1993). Thus, parasite-specific IgE and concurrently elevated total non-specific IgE during chronic helminth infection have been considered either as host- or as parasite-protective, respectively. This suggests a balanced parasite-host co-existence with chronic hookworm infections, during which the immune system may have a controlling influence, but the evolution of a potentially protective immune response is prevented by the worm's survival strategies (Pritchard 1995).

From our observations we conclude that with chronic hookworm and *O.bifurcum* infections cellular responses are induced which are not strictly dominated by type 1 or type 2 T helper cell reactivity. However, in co-infected patient's cellular hyporesponsiveness to parasite antigens together with a greater production of TNF-α and IFN-γ was observed - with Th2-type cytokines being equally present in mono- and co-infected individuals. Such a mixed type of immunity may reflect a state of infection in which inflammatory enteropathogenic responses counteract potentially protective immune responses of the host. To distinguish anti-parasite and host-protective responses from parasite-mediated enteropathogenic reactions might help to define intervention strategies which inhibit intestinal inflammation and promote immunological mechanisms which operate to reject gastrointestinal nematodes.

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