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Regulation of human neutrophil type 3 complement receptor (iC3b receptor) expression during phagocytosis of *Staphylococcus aureus* and *Escherichia coli*

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SUMMARY

Human neutrophils (PMN) express a receptor for iC3b, a cleavage product of C3b. CR3 is an important receptor for phagocytosis of opsonized bacteria and its expression is enhanced by cell activation. We examined PMN CR3 expression during phagocytosis using flow cytometry and a CR3-specific monoclonal antibody. After 30 min phagocytosis of opsonized *S. aureus* and *E. coli*, CR3 expression increased to 151% and 221% of controls, respectively. Unopsonized *S. aureus* had no effect on CR3; however, unopsonized *E. coli* enhanced CR3 expression despite not being phagocytosed. Time–kinetic studies indicated a rapid initial fall in CR3 after addition of bacteria to PMN, followed by enhanced expression within 5–10 min. The initial fall in CR3 probably represented CR3 internalization rather than receptor destruction, as superoxide dismutase, catalase and protease inhibitors had no effect on this. Correlation of CR3 expression with the PMN oxidative response, measured with the intracellular fluorescent probe DCF-DA, demonstrated a dichotomy. Opsonized *S. aureus* and *E. coli* caused an oxidative response from PMN but unopsonized *E. coli*, which caused significant CR3 up-regulation, did not. CR3 up-regulation with unopsonized and opsonized *E. coli* was markedly inhibited by Polymyxin B, suggesting a role for endotoxin. These experiments indicate that CR3 expression can be regulated during phagocytosis, and the mechanisms responsible are distinct from those involved in the oxidative burst. CR3 up-regulation following exposure to bacteria *in vivo* may enhance neutrophil function at sites of infection.

INTRODUCTION

Human phagocytic cells express two major surface receptors for C3b and iC3b, the opsonic fragments of the third component of complement, C3. Complement receptor type 1 (CR1) binds predominantly with C3b, whilst iC3b binds preferentially to the type 3 complement receptor, CR3. We have recently demonstrated that iC3b is the predominant opsonic fragment of C3 present on *Staphylococcus aureus* and *Escherichia coli* following opsonization (Gordon *et al.*, 1988), suggesting that the major interaction with phagocytic cells is mediated by CR3 rather than CR1. Further evidence of the importance of iC3b/CR3 interactions in host defence is provided by our observations that phagocytosis of *Streptococcus pneumoniae* is largely mediated through CR3 (Gordon, Johnson & Hostetter, 1986).

Unstimulated circulating cells express relatively low numbers of complement receptors. Receptor expression can, however, be markedly enhanced by cell activation. Kay, Glass & Salter (1979) first demonstrated enhanced expression of C3b receptors following cell stimulation by leucoattractants. Subsequently increased expression of both CR1 and CR3 has been induced by C5a, N-formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol esters, lymphoblastoid cell supernatants and cell purification procedures (Fearon & Collins, 1983; Berger & Cross, 1984; Berger *et al.*, 1984).

In this report we examine the changes in CR3 expression on human polymorphonuclear leucocytes (PMN) following interaction with unopsonized and opsonized *S. aureus* and *E. coli* in suspension, and correlate alterations in receptor number with phagocytosis and stimulation of oxidative product formation. The results indicate how up-regulation of CR3 occurs, independently of the oxidative burst, during phagocytosis of opsonized bacteria or interaction with unopsonized gram-negative bacteria. This may provide a mechanism by which neutrophil function can be modified at sites of infection.

Abbreviations: CR1, type 1 complement receptor (C3b receptor); CR3, type 3 complement receptor (iC3b receptor); DCF, 2′,7′-dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescin diactetate; DCFH, 2′,7′-dichlorofluorescin; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GHBSS, Hanks' balanced salt solution with 0·1% gelatin; PBS, phosphate-buffered saline; PHS, pooled human serum; PMA, phorbol myristate acetate; PMN, human polymorphonuclear leucocytes; PMSF, phenylmethylsulphonyl fluoride.

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MATERIALS AND METHODS

Polymorphonuclear leucocytes

Human polymorphonuclear leucocytes (PMN) were prepared from heparinized blood of healthy donors by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). PMN were washed in calcium- and magnesium-free Hanks' balanced salt solution (Gibco, Grand Island, NY) with 0·1% gelatin (GHBSS) and finally resuspended in GHBSS with calcium and magnesium. All steps following dextran sedimentation of erythrocytes were performed at 4° to prevent alterations in receptor expression during purification. Purity and viability, the latter assessed by exclusion of trypan blue, were >96%. Assays were commenced within 1 hr of purification, and the cells were finally washed immediately before use.

Bacteria

S. aureus Wood 46 (NCTC 5263), a protein A-negative strain, and a K antigen-positive serum-resistant, clinical isolate of *E. coli* 9009, were used for these experiments. K antigen was detected by erythrocyte agglutination inhibition activity of saline extracts (Glynn & Howard, 1970). Cultures were maintained on blood agar plates at 4° and subcultured monthly. Bacteria were grown overnight in Mueller–Hinton broth (MH) (Difco, Detroit, MI), then washed and resuspended in phosphate-buffered saline (PBS) to the desired concentration, determined by a spectrophotometric method.

Serum and opsonization

A serum pool (PHS) was prepared from a group of healthy donors and stored in small aliquots at −70°. Two × 10^8 bacteria were opsonized for 30 min at 37° in 400 μl of 20% PHS for receptor assays or 10% PHS for phagocytic assays, then washed and resuspended in 1 ml GHBSS.

Unopsonized bacteria were incubated in an identical fashion in GHBSS. Bacteria were washed again in GHBSS just prior to addition to PMN.

Phagocytic uptake assay

Bacteria were grown overnight in 10 ml MH broth containing 10 μl of [^{3}H]thymidine (specific activity 0·92 TBq/mmol; CEA, Siren, France). Quantification of the uptake of opsonized and unopsonized [^{3}H]labelled bacteria after 30 min phagocytosis was performed as described elsewhere (Verhoef, Peterson & Quie, 1977). Briefly, 2 × 10^6 PMN and 1 × 10^7 radiolabelled bacteria were incubated with shaking for 30 min to allow phagocytosis to occur. Phaocytosis was halted by the addition of 3 ml ice-cold PBS, and cell-associated and non-cell-associated bacteria were separated by three washes and differential centrifugation steps at 160 *g* for 10 min at 4°. Liquid scintillation counting of the pellet, as a proportion of the total bacteria added, was used as a measure of the percentage phagocytosis.

Phagocytosis and receptor expression assay

Phagocytosis was allowed to proceed for the desired time, as above, and further changes in receptor expression were then halted by the addition of 1 ml ice-cold PBS. PMN were sedimented by centrifugation at 160 *g* for 5 min at 4° and resuspended in PBS-0·02 M azide to a concentration of 1 × 10^7/ml. Receptor expression was quantified by flow cytometry using the murine monoclonal antibody OKM10, specific for the ligand binding site of CR3 (Wright *et al.*, 1983), kindly provided by Dr G. Goldstein (Ortho Research Laboratory, Raritan, NJ). Briefly, 50 μl of PMN were incubated with 50 μl of OKM10 in PBS-azide (final concentration 2·5 μg/ml) for 30 min on ice. Cells were washed twice with PBS-azide and resuspended in a 1:40 dilution of fluorescein isothiocyonate-conjugated goat anti-mouse IgG (Silenus, Melbourne VI) and kept on ice for a further 30 min. After two further washes, cells were resuspended in PBS-azide and flow cytometric analysis was performed on a FACS IV cell sorter (Becton-Dickinson, Mountain View, CA). Controls were included in which the primary antibody was omitted or replaced by an irrelevant monoclonal antibody, TIB 92, reactive with I-A^k (ATCC, Rockville, MD).

PMN oxidative response

The production of oxygen metabolites by PMN following stimulation by opsonized and unopsonized bacteria or FMLP was assessed by the formation of intracellular 2′,7′-dichlorofluorescin (DCF) by flow cytometry, as described by Bass *et al.* (1983). One × 10^7 PMN in calcium- and magnesium-free GHBSS was loaded by incubation with 2·5 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Eastman Kodak, Rochester, NY) for 15 min at 37°, then washed and resuspended in 1 ml GHBSS. Aliquots of 100 μl of PMN were then incubated with 5 × 10^6 bacteria for 30 min at 37° with shaking. The reaction was stopped by addition of 200 μl ice-cold PBS, and intracellular fluorescence was determined by flow cytometry.

Statistics

Differences between means were compared by the Student's *t*-test with $P<0.05$ considered significant.

RESULTS

Opsonic requirements for phagocytosis

The opsonic requirements for phagocytosis of *S. aureus* and *E. coli* by human PMN, as assessed by the uptake of radiolabelled bacteria, are shown in Table 1. The presence of serum was required for phagocytosis of both bacteria ($P<0.001$). No significant phagocytosis of unopsonized bacteria, preincubated in GHBSS, was detected.

Table 1. Serum requirement for phagocytosis of *S. aureus* and *E. coli*

	Phagocytosis (%)	
Opsonin	*S. aureus* ($n=6$)	*E. coli* ($n=6$)
GHBSS	4·5 ± 1·6	2·67 ± 1·2
PHS 10%	53 ± 5·3	55·7 ± 6·8
	$P<0.001$*	$P<0.001$

* Student's *t*-test.

Phagocytosis was determined by uptake of radiolabelled bacteria, as described in the Materials & Methods.

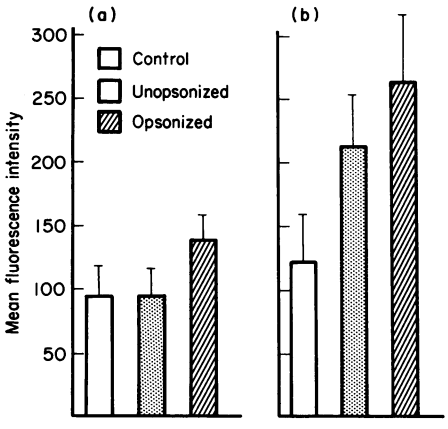

Figure 1. Pooled results from six and eight experiments, respectively, for (a) *S. aureus* and (b) *E. coli* showing effect on PMN mean fluorescence intensity staining with OKM10, directed against CR3, of incubation with bacteria for 30 min. Incubation with opsonized *S. aureus*, unopsonized *E. coli* and opsonized *E. coli* caused a significant increase in CR3 expression ($P=0{\cdot}005$, $P<0{\cdot}001$ and $P<0{\cdot}001$, respectively), with the increase with opsonized *E. coli* exceeding unopsonized ($P<0{\cdot}001$).

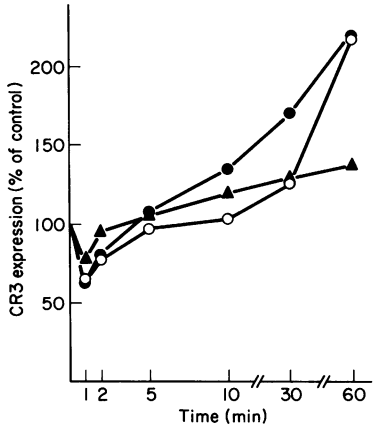

Figure 2. Kinetics of changes in PMN CR3 expression after stimulation with unopsonized *E. coli* (○) and opsonized *S. aureus* (▲) and *E. coli* (●). Figure shown is a representative experiment of three performed, in which mean fluorescence intensity of 5000 cells is expressed as percentage of control unstimulated cells.

Enhanced expression of CR3 after 30 min phagocytosis

PMN were added to opsonized and unopsonized *S. aureus* and *E. coli* and phagocytosis was allowed to proceed for 30 min. The reaction was stopped by the addition of ice-cold PBS, and receptor expression was determined by flow cytometry. The pooled results from six and eight experiments, respectively, for *S. aureus* and *E. coli* are presented in Fig. 1, which shows the changes in PMN mean fluorescence intensity after PMN interaction with unopsonized and opsonized bacteria. Opsonized, but not unopsonized *S. aureus*, increased CR3 expression to $151\pm25\%$ of control after 30 min ($P=0{\cdot}005$). A different pattern of increased expression was detected when PMN were added to *E. coli*. Unopsonized *E. coli*, which are not phagocytosed, nevertheless induced significant up-regulation of PMN CR3, and opsonized *E. coli* increased this further. CR3 expression on PMN incubated with unopsonized and opsonized *E. coli* increased to $178\pm32\%$ and $221\pm42\%$ of CR3 expression on control unstimulated cells, respectively ($P<0{\cdot}001$ for both). After 30 min the increase in CR3 expression for PMN exposed to opsonized *E. coli* was significantly greater than for unopsonized bacteria ($P<0{\cdot}001$).

Kinetics of CR3 expression

The expression of PMN CR3 following exposure to opsonized *S. aureus*, and unopsonized and opsonized *E. coli* for different time intervals, is shown in Fig. 2, which is a representative experiment of three performed. The striking finding is that for all three stimuli there was an early reduction in PMN CR3 expression (by 30–40%) within 1–2 min of addition of PMN to bacteria. After 5 min, CR3 expression had returned to original levels, and thereafter continued to increase with further incubation. The greater increase in CR3 observed with opsonized compared with unopsonized *E. coli* at 30 min was no longer apparent after 60 min incubation.

The observed early reduction in CR3 following stimulation could reflect receptor internalization, or receptor inactivation or destruction by neutrophil proteolytic enzymes or oxygen radicals generated during cell activation (Bender, Van Epps & Chenoweth, 1987). To exclude the latter possibility assays were performed in the presence of either 1 mM phenylmethylsulphonyl fluoride (PMSF) or 50 μg/ml superoxide dismutase and 100 μg/ml catalase (all Sigma, St Louis, MO). Neither affected the pattern of CR3 expression, indicating that the early loss of receptors was most likely caused by receptor internalization.

Lack of correlation between receptor expression and oxidative product formation

The alteration in CR3 expression following PMN exposure to unopsonized and opsonized bacteria was not necessarily associated with phagocytosis—in particular unopsonized *E. coli* were not phagocytized but stimulated a marked increase in CR3 numbers. To examine whether CR3 changes were coupled to other intracellular events the PMN oxidative product formation in response to unopsonized and opsonized bacteria was measured. PMN were loaded with 2′,7′-dichlorofluorescin (DCFH) by preincubation with dichlorofluorescin diacetate. Following the PMN oxidative burst DCFH is converted to the fluorescent compound 2′,7′-dichlorofluorescein, which is trapped intracellularly and quantified by flow cytometry (Bass *et al.*, 1983). Figure 3 shows the oxidative product formation in response to different stimuli. No oxidative response was detected when PMN and unopsonized *S. aureus* or *E. coli* were incubated in suspension. However, opsonized bacteria, which were phagocytosed, did induce an oxidative response and for *E. coli* this was comparable to that seen when PMN were stimulated with 5×10^{-7} M FMLP (Sigma). Thus the intracellular processes involved in stimulation of CR3 up-regulation and the oxidative burst are not associated.

Effect of FMLP and Polymyxin B on *E. coli*-induced CR3 up-regulation

The ability of unopsonized *E. coli* to induce PMN CR3 up-regulation without phagocytosis suggested that some bound or soluble component of the cell of gram-negative bacteria acti-

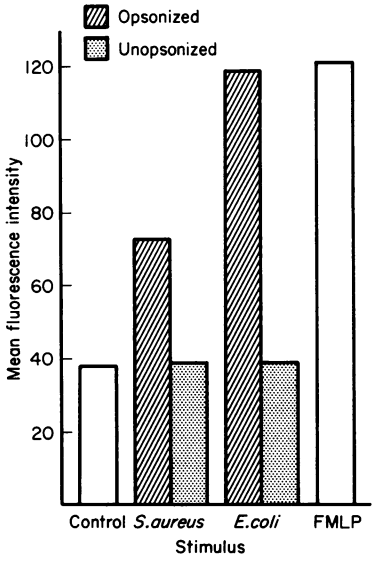

Figure 3. Effect of unopsonized and opsonized *S. aureus* and *E. coli* on the PMN oxidative response, as measured by intracellular formation of 2′,7′-dichlorofluorescein. Shown is a representative experiment of three performed, expressing the mean fluorescence intensity of 5000 cells. The response to 5×10^{-7} M FMLP is shown for comparison.

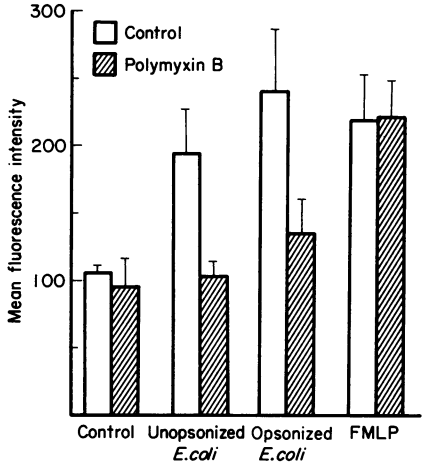

Figure 4. Effect of presence of 2 μg/ml Polymyxin B on CR3 up-regulation stimulated by unopsonized and opsonized *E. coli* ($n=4$). Polymyxin B significantly reduced the up-regulation induced by both bacterial stimuli ($P<0.01$) but had no significant effect on control CR3 expression ($P>0.5$), nor on FMLP-induced stimulation.

vates PMN. *E. coli* produce FMLP as their major peptide neutrophil chemotactic factor (Marasco *et al.*, 1984), and the possibility that this was responsible for CR3 up-regulation was first considered by stimulating PMN with bacteria alone, FMLP alone or a combination or both. The response to 5×10^{-7} M FMLP plus unopsonized or opsonized *E. coli* was approximately additive to that observed with FMLP or bacteria alone, suggesting that interaction with different cell-surface receptors was responsible (data not shown). The role of endotoxin as a mediator of *E. coli*-induced CR3 up-regulation was next investigated by performing assays in the presence of 2 μg/ml Polymyxin B (Sigma), which binds to the lipid A portion of bacterial lipopolysaccharides (Morrison & Jacobs, 1976). Figure 4 illustrates the effect of Polymyxin B on CR3 up-regulation. Polymyxin B substantially reduced CR3 expression induced by opsonized *E. coli* ($P<0.01$) and almost totally blocked the response to unopsonized *E. coli* ($P<0.01$), indicating that endotoxin played a crucial role in the PMN response to gram-negative bacteria. The inhibition by Polymyxin B was not due to a non-specific effect on neutrophil function as it had no effect on increased CR3 expression induced by FMLP.

DISCUSSION

We have examined the changes in CR3 expression following exposure of PMN to unopsonized and opsonized *S. aureus* and *E. coli*. After 30 min phagocytosis of opsonized *S. aureus* PMN membrane CR3 expression increased by approximately 50%. CR3 up-regulation following PMN exposure to *S. aureus* was dependent upon phagocytosis, as unopsonized *S. aureus*, which are not phagocytosed, had no effect on CR3 numbers. Exposure of PMN to *E. coli* led to a different pattern of CR3 response. Incubation of PMN with unopsonized, non-ingested *E. coli* for 30 min resulted in a marked increase in CR3 expression, whilst even greater CR3 expression was stimulated by opsonized *E. coli*. The changes observed with opsonized bacteria are particularly striking when it is considered that large amounts of cell membrane are internalized during phagocytosis (Petty, Hafeman & McConnell, 1981).

The kinetic studies indicate that the rise in CR3 was preceeded by a rapid, transient fall of 30–40% in surface CR3 expression (Fig. 2). Within 5 min baseline expression of CR3 had returned and then a time-dependent increase in receptors was detected. Patrone *et al.* (1983) have suggested that loss of complement receptors may result from damaging effects of oxygen metabolites on the receptor structures. This does not appear to be the explanation for our findings as the presence of catalase, superoxide dismutase or the protease inhibitor PMSF had no effect on receptor kinetic changes. Furthermore, the changes in CR3 expression were detected with stimuli which did not produce a detectable oxidative response. More probably, the early reduction in receptors results from membrane internalization. The subsequent increase in CR3 surface expression could then result from a redistribution of intracellular pools of receptor, which are known to exist in association with specific granules (O'Shea *et al.*, 1985). Receptor numbers could also be maintained in part by recycling of internalized CR3 in a manner similar to that recently described for CR1, which recycles via a prelysosomal, predegradative compartment (Malbran *et al.*, 1988). Final CR3 expression would thus reflect a balance between receptor internalization, recycling and receptor redistribution from intracellular pools.

We next examined whether there was an association between CR3 up-regulation and another intracellular event that can follow membrane perturbation—the oxidative burst, as previous stimuli enhancing expression, such as FMLP or PMA, also induced an oxidative response. This was measured by quantification of the intracellular conversion of 2′,7′-dichlorofluorescin to fluorescent 2′,7′-dichlorofluorescein. We observed a dichotomy between stimulation of receptor expression and oxidative product formation. Only opsonized bacteria, which were phagocytosed, stimulated a PMN oxidative response, whereas unopsonized *E. coli*, which increased

CR3, did not. This demonstrates that the processes involved in control and mediation of each of these events are distinct and dissociable.

Several groups of ligand are known to bind to CR3. Ross *et al.* (1985) first suggested two distinct binding sites in CR3—one for iC3b, and an epitope with lectin-like activity which binds unopsonized yeast and zymosan. Subsequently binding of bacterial lipopolysaccharide to CR3 was demonstrated (Wright & Jong, 1986). CR3 binds iC3b (and fibrinogen) by recognizing an Arg-Gly-Asp (RGD) or related peptide sequence (Wright *et al.*, 1987, 1988). These observations provide an explanation for our findings. Firstly, the effects on CR3 induced by opsonized bacteria suggest that changes occur secondary to signals generated by binding of opsonins with their receptors on PMN. Up-regulation of CR3 could itself be regulated by iC3b–CR3 interaction. This is likely since we have demonstrated that iC3b is the major opsonic fragment on both bacteria (Gordon *et al.*, 1988), although lower estimates of C3b to iC3b conversions (16–28%) have been made by others (Newman & Mikus, 1985). In addition, it is also possible that changes in CR3 occur subsequent to IgG–Fc receptor or C3b–CR1 interactions. Secondly, the role of endotoxin, which is known to bind to macrophages through CR3 (Wright & Jong, 1986) was investigated by examining the effect of Polymyxin B on CR3 up-regulation by unopsonized and opsonized *E. coli*. When assays were performed in the presence of 2 μg/ml Polymyxin B a substantial inhibition of CR3 increase to opsonized bacteria occurred, whilst for unopsonized *E. coli* CR3 up-regulation was almost totally inhibited. The inhibition was not due to Polymyxin B effects on PMN, as no reduction of the response to FMLP was observed. The possibility that FMLP or related products were produced by *E. coli* and then interacted with PMN FMLP receptors was also considered. However, when PMN were incubated with both FMLP and unopsonized *E. coli*, a much greater increase was observed over either stimulus alone, suggesting that the stimuli were interacting with separate receptors. The observation that unopsonized bacteria did not stimulate an oxidative burst yet FMLP does provides further evidence against bacterial interaction with FMLP receptors.

The *in vivo* significance of our findings is not known, but these results suggest that PMN receptors may be up-regulated at sites of infection. There are two potential physiological advantages of this for the host. Firstly, it is tempting to speculate that this would facilitate attachment and ingestion of opsonized bacteria. However, there may not be a direct relationship between CR3 expression and function. Recent reports have failed to demonstrate an association between increased surface CR3 expression and enhanced neutrophil adherence or aggregation (Vedder & Harlan, 1988; Philips *et al.*, 1988). Instead changes in CR3 configuration may be more important than absolute changes in numbers. Detmers *et al.* (1987) suggest that receptor aggregation into a clustered configuration is necessary for iC3b binding and signalling phagocytosis. They noted that although both PMA and FMLP enhanced CR3 expression, only PMA induced receptor aggregation and functional changes. Secondly, C3 receptors play an important role in regulation of intracellular killing (Leijh, Van Zwet & Van Furth, 1984; Hart *et al.*, 1986). CR3 up-regulation during phagocytosis may 'prime' neutrophils to ensure optimal intracellular killing or, as recently described for *Schistosoma* (Moqbel *et al.*, 1987), increase complement-dependent cytotoxicity.

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