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Protein kinase C activation modulates tumour necrosis factor-alpha priming of human neutrophils for zymosan-induced leukotriene B_4 release

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SUMMARY

Neutrophil (PMN) activation by the yeast component zymosan involves the complement receptor type 3 (CD11b/CD18). Recombinant human tumour necrosis factor-alpha (rhTNF-α) augmented the zymosan-stimulated leukotriene B_4 (LTB_4) release from PMN, reaching a fourfold increase at 10^{-9} M. Co-incubation of PMN with 10^{-9} M rhTNF-α and staurosporine resulted in a further dose-dependent increase, which became significantly greater than a purely additive effect at a staurosporine concentration of 10 nM. This synergy was maintained at all doses of staurosporine tested. In addition, doses of phorbol 12-myristate 13-acetate (PMA) that do not activate protein kinase C (PKC) (below 10^{-9} M) also augmented the zymosan-stimulated release of LTB_4. However, doses of PMA above 10^{-9} M progressively inhibited the response to levels below that of zymosan alone. Staurosporine at 50 nM completely prevented, and 10^{-9} M rhTNF-α partially but significantly ($P < 0{\cdot}02$ at 10^{-8} M PMA, $P < 0{\cdot}01$ at 10^{-7} M PMA) reversed, this high-dose PMA inhibition. PKC activation thus opposes the priming effect of rhTNF-α on neutrophils, while PKC inhibition may enhance the ability of rhTNF-α to prime PMN for zymosan activation. The combined effect of rhTNF-α and staurosporine suggests an intracellular synergy rather than simply a direct action due to increased zymosan receptor expression. Thus there appear to be mechanisms whereby the responses of neutrophils may be augmented without activating PKC. Indeed, kinase activation may even exert a degree of feedback control that is antagonized by rhTNF-α treatment.

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a potent mediator of inflammation.[1] *In vivo* it facilitates neutrophil (PMN) extravasation at sites of injury and it has been implicated in the pathogenesis of several disease states, e.g. malaria.[2] Its direct action on neutrophils has been demonstrated in *in vitro* experiments to be limited to a low level of respiratory burst activation, adhesion receptor modulation and secondary lysosomal granule release.[3,4] As a priming agent, however, TNF-α increases the PMN responses to soluble and particulate stimuli, resulting in the augmented release of secondary granule contents, superoxide, lysosomal enzymes and leukotriene B_4.[4 8] In addition, TNF-α enhances myeloperoxidase release from PMN by serum-opsonized fungi in the presence of cytochalasin B[9] and has also been shown to enhance the killing of parasites.[10]

Receptor-coupled activation of PMN causing superoxide generation is linked to the translocation of a calcium- and phospholipid-dependent protein kinase C (PKC) to the cell membrane. Several intracellular proteins are phosphorylated by this kinase[11] following exogenous stimulation with phorbol 12-myristate 13-acetate (PMA), which substitutes for the endogenous PKC ligand, diacylglycerol (DAG). The incubation of isolated PMN with TNF-α also results in the phosphorylation of specific proteins.[12] This phosphorylation, however, is not associated with a translocation of PKC to the cell membrane,[12] nor is it associated with the release of DAG from the hydrolysis of phosphoinositides.[13] In addition, recent work has demonstrated that recombinant human TNF-α alone does not increase intracellular cyclic nucleotide levels or alter calcium concentrations.[4] Finally, and in contrast to a recent report[14] the consensus of evidence demonstrates that TNF does not promote the phopholipase A_2-dependent release of arachidonic acid.[4,13,15,16]

Previous work has linked PMN activation by unopsonized zymosan to the expression of the CR3 receptor (CD11b/CD18),[17,20] and PMN responses to zymosan are synergistically augmented by rhTNF-α.[4] The present study assesses a possible role for PKC in modifying the TNF-α augmentation of the neutrophil response. We have used the PKC activator PMA and the DAG kinase inhibitor R59949[21] as well as staurosporine to examine the capacity of PKC to influence TNF-α augmentation of PMN activation through the CD11b/CD18 receptor complex.

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MATERIALS AND METHODS

Reagents

All chemicals were analar grade from BDH (Poole, U.K.) unless stated otherwise. Recombinant human tumour necrosis factor-alpha (rhTNF-α) was a kind gift from Dr G. R. Adolf (Ernst-Boehringer Institute, Vienna, Austria) (6×10^7 U/mg). It was 95% pure by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with no detectable endotoxin content and was stored diluted to 1 μg/ml in phosphate-buffered saline pH 7·3 (PBS) at −70° until used. PMA (Sigma, Poole, U.K.), staurosporine (Novabiochem, Nottingham, U.K.) and R59949 (Janssen Life Sciences Products, Grove, U.K.) were dissolved in dimethylsulphoxide (DMSO) and stored at −70°. Zymosan A particles (Sigma) were boiled in sterile 0·15 M NaCl, washed and sonicated as previously described.[4] This unopsonized zymosan was stored at 4° until used. All cell incubations for LTB_4 release were carried out in a Tyrode buffer (136·7 mM NaCl, 12 mM $NaHCO_3$, 5 mM $MgCl_2$, 5 mM glucose, 1·36 mM $CaCl_2$, 0·27 mM KCl, 0·4 mM NaH_2PO_4), pH 7·3, containing 0·1% gelatin (Difco, East Molesey, U.K.) (Tyrode's gelatin, TG). Cell incubations for FACS analyses and vitamin B_{12} binding protein release were performed in a Krebs–Ringer Phosphate buffer (12·7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 0·7 mM $CaCl_2$, 11 mM dextrose, pH 7·4) (KRPG).

[^{125}I]TNF-α, 463 Ci/mmol (Amersham, Bucks, U.K.), silicone fluid DC 550 (BDH) and light mineral oil M3516 (Sigma) were used for the TNF-α binding experiments.

Neutrophil isolation

Human neutrophils were isolated from the peripheral blood of healthy volunteers by dextran sedimentation and Ficoll–Hypaque density centrifugation and plated as monolayers in 35-mm dishes (Nunc Gibco, Uxbridge, U.K.) as previously described.[4] The purity of the neutrophil preparation was >95% by morphology and was essentially platelet free. Preliminary experiments demonstrated no significant differences in the number of adherent cells following pretreatment with any of the agents used in these studies.

CR3 expression

PMN were suspended in PBS containing 2% (w/v) bovine serum albumin (BSA; Miles, ICN Biomedicals, High Wycombe, Bucks, U.K.) at $2{\cdot}5 \times 10^6$/ml and treated with the soluble ligand for various times at 37°. The cells were spun down at 11,000 *g* for 15 sec, resuspended in 50 μl of PBS/BSA and put on ice immediately. The cells were incubated with 50 μl of a 1:2000 dilution of monoclonal antibody to CD11b (Serotec, Oxford, U.K.) on ice for 30 min, followed by 50 μl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Miles) diluted 1:30 (v/v) in PBS/BSA as previously described.[4] Fluorescence was analysed using a FACS 440 (Becton-Dickinson, Oxford, U.K.), and the data expressed as the percentage of cells demonstrating an increase in fluorescence above background.

[^{125}I]TNF-α binding to PMN

PMN (2×10^6) in Eppendorf tubes (Sarstedt) were incubated in 400 μl of Tyrode's buffer, alone or containing 50 nM staurosporine, for 30 min at 37°. The tubes were transferred to ice, and aliquots of rhTNF-α, 10^{-10} to 10^{-8} M in 50 μl of Tyrode's, were added. This was followed immediately by the addition of a further 50 μl of buffer containing ~10^5 c.p.m. [^{125}I]TNF-α. Non-specific binding was assessed using 2 μg of rhTNF-α. After 2 hr on ice, 2×200-μl aliquots were layered onto 150 μl of a mixture of four parts silicone fluid plus one part light mineral oil (to give a density of 1·02 gm/ml) in cold 400-μl Eppendorf tubes. Tubes were spun at 12,000 *g* for 1 min to separate bound and free tracer; the tubes were sliced through and the pellets counted for 1 min in a gamma-counter (Kontron, Watford, Herts, U.K.).

Vitamin B_{12} binding protein release

The secondary granule marker vitamin B_{12} binding protein (B_{12} BP) was measured by a competitive binding assay as previously described.[4] Briefly, supernatants from PMN activated in suspension were incubated with [^{57}Co]cyanocobalamin (Amersham) and unbound tracer was removed by addition of charcoal (Norit SX1). Bound tracer was counted in a gamma-counter (Kontron) and the percentage release calculated using sonicated cells for total B_{12} BP content. A small background release of B_{12} BP (<10%) was observed as a result of manipulation of the cells.[22]

Leukotriene B_4 release

Neutrophil monolayers washed in prewarmed TG were preincubated at 37° with either rhTNF-α or PMA for 10 min, inhibitor for 20 min or both. The supernatant was decanted and zymosan particles ($1{\cdot}4 \times 10^8$/ml) added for a further 25 min. LTB_4 release was measured in the supernatants by radioimmunoassay as previously described.[4] Briefly, standard (kindly supplied by Dr B. Spur, Institut Henri Beaufour, Paris, France) or sample was incubated with LTB_4 antibody (Merck Frosst, Dorval, Canada) and [^{3}H]LTB_4 (Amersham) overnight at 37°. Unbound tracer was removed with charcoal (Norit SX1), coated with dextran (T70, Pharmacia, Milton Keynes, Bucks, U.K.), and bound tracer was counted in a beta-counter (Pharmacia-LKB).

Statistics

Previous studies have demonstrated greater than 10-fold differences in PMN LTB_4 generation between donors. For this reason the SEM of results from a series of experiments are usually very large. This variability necessitates the use of paired statistical analysis to allow comparison of values within individual experiments. The two-tailed paired Wilcoxon or Student's *t*-test were used to compare groups of data expressed as the mean ± SEM of results from groups of experiments using PMN from different donors.

RESULTS

TNF priming in the presence of PKC inhibition

rhTNF-α augmented the unopsonized zymosan-triggered release of LTB_4 in a dose-dependent manner, reaching a maximum fourfold increase at a dose of 10^{-10} M ($P<0{\cdot}05$, $n=6$). This increase was dependent on the time of preincubation with rhTNF-α, the optimum time being 10 min. rhTNF-α alone did not stimulate LTB_4 generation.

Preincubation of PMN with staurosporine alone at doses of 10–100 nM resulted in a maximum 50% increase in the release of LTB_4 from zymosan-stimulated PMN ($P<0{\cdot}05$, $n=7$) at a staurosporine dose of 50 nM.

Table 1. Effect of TNF and staurosporine on zymosan-stimulated LTB_4 release

Staurosporine (nM)	LTB_4 release (ng/10^6 PMN) Expected* (additive)	Observed†	Significance‡ level
10	0·77±0·26	1·03±0·21	$P<0.05$
25	0·64±0·45	0·93±0·22	$P<0.05$
50	0·99±0·33	1·38±0·42	$P<0.05$
100	1·03±0·24	1·57±0·21	$P<0.02$

* The expected release (assuming an additive effect of staurosporine and rhTNF-α co-incubation) was calculated from the sum of the value for 10^{-9} M rhTNF-α pretreatment with each individual dose of staurosporine in each of seven independent experiments.

† The observed zymosan-induced LTB_4 release following PMN co-incubation with doses of staurosporine and 10^{-9} M rhTNF-α.

‡ The level of significance using the Wilcoxon matched-pairs test, between each of the observed and the expected values.

The co-incubation of PMN with staurosporine and 10^{-9} M rhTNF-α resulted in a further augmentation of LTB_4 release. Detailed analysis of the preincubation of PMN with staurosporine and TNF indicated that there was a difference between the purely additive effect of staurosporine and TNF, as calculated separately, and the observed augmentation when both agents were present together, thus demonstrating a synergistic increase in LTB_4 release in response to zymosan. This synergy became significantly greater than the predicted additive effect of TNF and staurosporine at 10 nM ($P<0.05$, $n=7$, Wilcoxon test) and remained significant at all doses of staurosporine tested (Table 1).

TNF priming in the presence of diacylglycerol kinase inhibition

The diacylglycerol kinase inhibitor R59949 over the range 0·6–10 μM did not affect the zymosan-induced LTB_4 release or its augmentation by rhTNF-α in three independent experiments (data not shown).

CR3 expression in the presence of PKC inhibition

There was a small increase in the expression of CR3 on PMN after 30 min incubation with staurosporine, reaching significance at 100 nM ($P<0.05$, $n=4$, t-test). Co-incubation of PMN with rhTNF-α (10^{-9} M) and staurosporine resulted in a dose-dependent but additive augmentation of CR3 expression at the higher concentrations (Fig. 1).

TNF binding with staurosporine

Preincubation of PMN with 50 nM staurosporine did not significantly alter the binding of radiolabelled TNF to PMN (Fig. 2).

Secondary granule release in the presence of PKC inhibition

PMN in suspension incubated with staurosporine caused a dose-dependent increase in B_{12} BP release above unstimulated

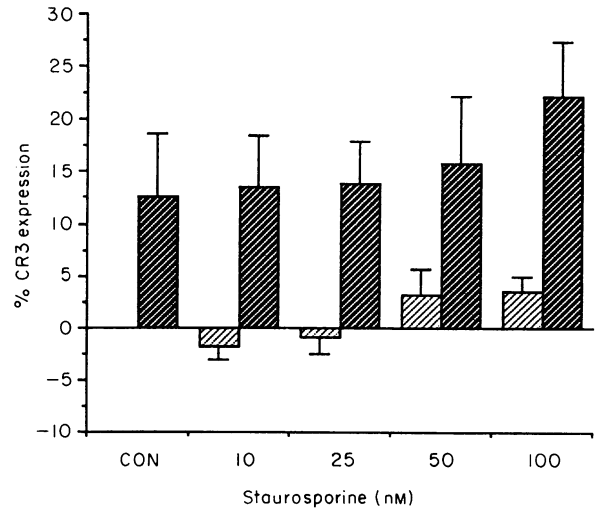

Figure 1. CR3 expression on isolated PMN following incubation with doses of staurosporine in KRPG for 20 min followed by KRPG alone (▨) or containing 10^{-9} M rhTNF-α (▨) for 10 min at 37°. Data represent mean±SEM for cells from four different donors.

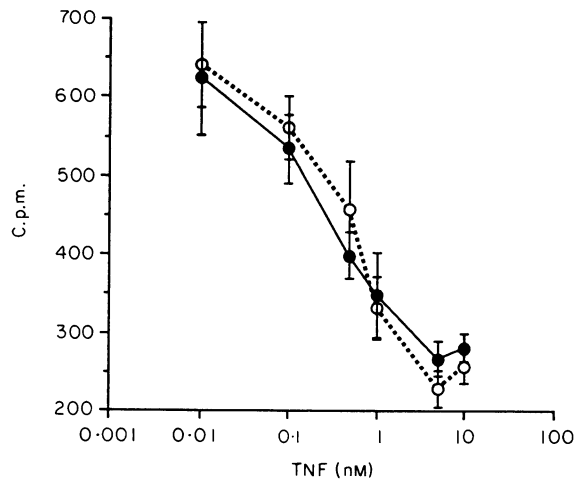

Figure 2. Binding of [^{125}I]TNF-α to PMN, with (●) or without (○) pretreatment with 50 nM staurosporine for 20 min. Data represent mean±SEM for three separate experiments.

levels to a maximum of twofold greater at 100 nM staurosporine ($P<0.02$, $n=4$, t-test). Co-incubation of PMN with 10^{-9} M rhTNF-α and staurosporine resulted in an additive increase in release (Fig. 3). The use of adherent PMN as opposed to PMN in suspension produced identical results (data not shown).

PKC activation modulates zymosan-stimulated LTB_4 release

PMA at increasing doses from 10^{-11} M to 10^{-6} M had a bidirectional effect on the zymosan-stimulated LTB_4 release from PMN. Maximal augmentation of the LTB_4 release occurred at 10^{-9} M PMA ($P<0.002$). Higher doses progressively inhibited the response to levels below that for zymosan alone (Fig. 4). Using paired t-tests the data show that, at PMA doses

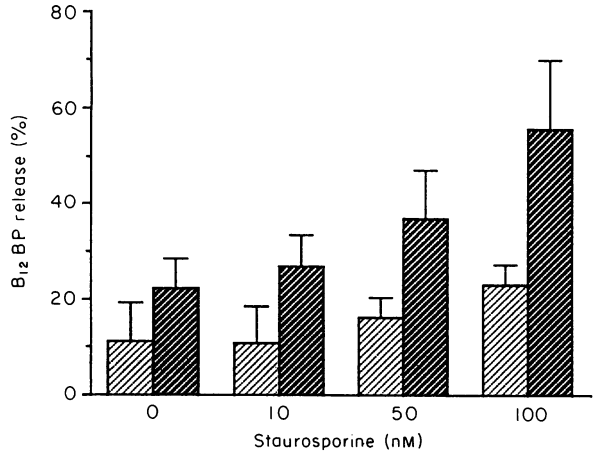

Figure 3. Vitamin B_{12} binding protein release from PMN in suspension incubated with doses of staurosporine in KRPG for 20 min followed by KRPG alone (▨) or containing 10^{-9} M rhTNF-α (▨) for 10 min at 37°. Data represent mean ± SEM for cells from four different donors.

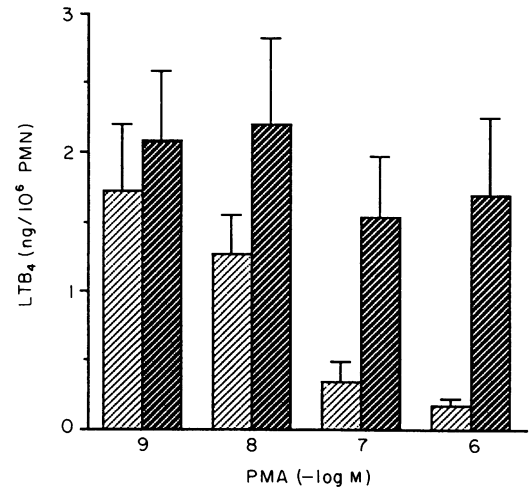

Figure 5. LTB_4 release from PMN monolayers preincubated at 37° for 20 min in TG alone (▨) or containing 50 nM staurosporine (▨) before treatment with doses of PMA for a further 10 min. A total of $1{\cdot}4 \times 10^8$ particles of zymosan were then added for a further 25 min. Data represent mean ± SEM for cells from seven different donors.

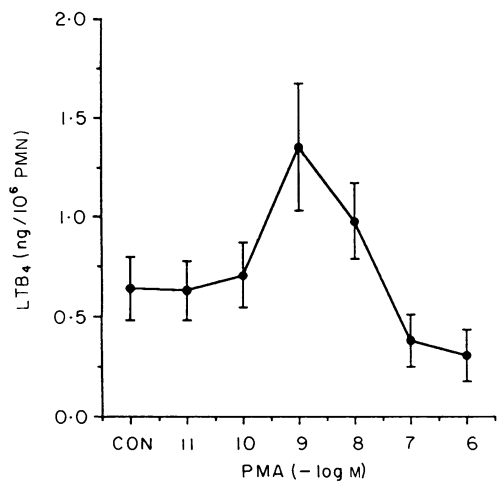

Figure 4. LTB_4 release from PMN monolayers pretreated in TG alone (CON) or containing doses of PMA for 10 min at 37° followed by $1{\cdot}4 \times 10^8$ particles of zymosan for a further 25 min. Data represent mean ± SEM for cells from 13 different donors.

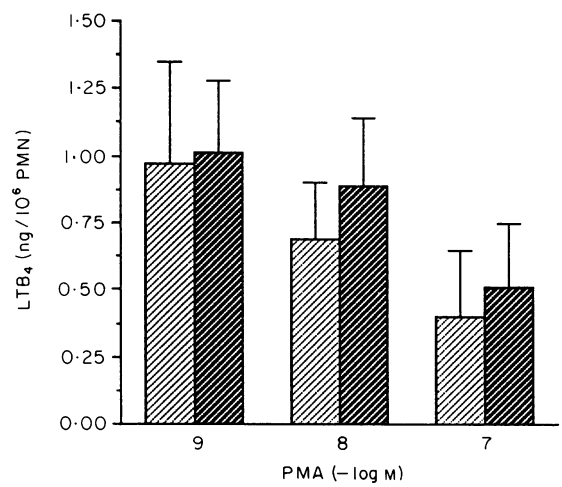

Figure 6. LTB_4 release from PMN monolayers preincubated at 37° for 10 min in TG alone (▨) or containing 10^{-9} M TNF (▨) before treatment with doses of PMA for a further 10 min. A total of $1{\cdot}4 \times 10^8$ particles of zymosan were then added for a further 25 min. Data represent mean ± SEM for cells from six different donors.

of 10^{-8} to 10^{-6} M, LTB_4 generation was significantly inhibited compared with the maximal augmentation seen at 10^{-9} M. Staurosporine (50 nM) reversed this inhibition (10^{-8} to 10^{-6} M) so that the maximal augmentation of LTB_4 generation seen at 10^{-9} M PMA was sustained (Fig. 5). Furthermore, rhTNF-α (10^{-9} M) partially, but significantly, reversed this inhibition ($P < 0{\cdot}02$ at 10^{-8} M, $P < 0{\cdot}01$ at 10^{-7} M, t-test) (Fig. 6).

DISCUSSION

The unopsonized zymosan-induced release of LTB_4 was enhanced in the present study by preincubation of PMN with staurosporine. The augmentation of zymosan-induced LTB_4 release by TNF has been documented previously. However, pretreatment of PMN with both staurosporine and TNF resulted in an increase in LTB_4 generation in response to zymosan that was significantly greater than the sum of the individual effects. Since staurosporine increased CR3 expression but not TNF binding, its effect on the unprimed zymosan response may be through an increase in CR3 receptor number. This is supported by the observation that staurosporine increases the phagocytosis of zymosan by PMN (data not shown). However, the observed capacity of staurosporine to augment TNF priming in a synergistic manner suggests the involvement of an additional intracellular mechanism. Staurosporine is an extremely potent PKV inhibitor,[23] but is by no means specific, having a significant effect on a variety of kinases.[24,25] Preliminary experiments confirmed that staurospor-

ine inhibited the PMA-stimulated release of superoxide from PMN at doses above 10 nM. In addition, the inhibitory effect of high doses of PMA on zymosan-stimulated LTB_4 was reversed in a dose-dependent way by staurosporine. Thus, while other cellular effects of staurosporine cannot be excluded, it is evident that under the experimental conditions employed in this study PKC was indeed inhibited by staurosporine.

In the present study rhTNF-α activates PMN for two direct responses: secondary granule release and increased CR3 expression. The latter may be a consequence of the former, since a store of CR3 has been demonstrated in PMN secondary granule membranes.[26,27] Staurosporine additively increased the B_{12} BP (secondary granule) release and CR3 expression induced by TNF, indicating that these responses to TNF are not directly mediated by PKC, and suggesting that PKC inhibition may enhance the PMN response to TNF. Thus background kinase activity appears to antagonize the TNF response.

The activation of PKC is reported to inhibit receptor-coupled stimulation of PMN.[28] This may occur through the phosphorylation of G protein subunits,[29] the uncoupling of activated G protein from phospholipase C (PLC),[30,31] the phosphorylation of specific receptors[32] or the shedding of receptors from the cell surface.[33] A bidirectional dose effect of PMA has been described.[34] The mechanism of the low-dose effect is unkown. There is, however, a threshold at 10^{-9} M PMA, above which PKC activity,[35] superoxide release and degranulation become measurable. In addition, TNF-α has been shown to enhance PMA-induced hydrogen peroxide production at PMA concentrations around 10^{-9} and 10^{-8} M.[36] The present study demonstrates that 10^{-8} to 10^{-6} M PMA inhibited zymosan-induced LTB_4 release, thus suggesting a role for PKC in limiting PMN activation by zymosan. Staurosporine (at doses inhibitory to PKC activity) abolished this effect. In addition, 10^{-9} M TNF partly antagonized this PMA effect, and this antagonism was statistically significant for six separate experiments.

Inhibition of DAG kinase with the specific inhibitor R59949 raises intracellular DAG levels and results in a PKC-dependent increase in superoxide generation by PMN treated with the chemotactic stimulus formyl-methionyl-leucyl-phenylalanine (FMLP).[21] Since in the present study neither the zymosan-induced LTB_4 release nor the TNF-augmented response was affected by the DAG kinase inhibitor R59949 at concentrations that augmented FMLP-induced superoxide release, it is assumed that neither rhTNF-α nor zymosan released DAG. These results indicate that neither ligand activates PKC. Indeed the activation of PMN by TNF has previously been shown to be independent of a direct PKC translocation.[12] Interestingly, the results of the present study suggest that PKC mobilization and activation may in fact have directly antagonistic effects on the mechanism of TNF priming, as well as the zymosan–PMN interaction.

The data presented here suggest that the observed modulation of TNF priming by PKC may occur at the level of the CR3 receptor and may also involve post-receptor events. Thus, activation of PKC with PMA may inhibit the TNF augmentation of the zymosan-induced LTB_4 response by an effect on CR3 receptor expression, while inhibition of PKC may augment intracellular events triggered by TNF. Recent studies[4,13] have demonstrated that the mechanism of TNF priming is independent of a phosphatidylinositol-specific phospholipase C activity and of a rise in intracellular calcium, while neither CR3 nor the TNF receptor is structurally related to the family of G protein-controlled receptors.[37–39] Moreover, the cytoplasmic domain of the TNF receptor has no homology with any known kinases or their substrates, in particular PKC and tyrosine kinases.[39] Whether TNF priming results in the hydrolysis of other membrane phospholipids such as phosphatidylcholine (PC) by a PC-specific phospholipase C or D is not known. Alternatively, TNF priming may affect the levels of cyclic AMP within the PMN synergistically when the CR3 receptor is occupied.[40]

The data provided here demonstrate that the interaction of PMN with zymosan, an event augmented by TNF, is sensitive to the level of PKC activation. The increased responses seen in the presence of staurosporine may be related to the endogenous PKC activity or to the activity of other kinases sensitive to staurosporine. This suggests that in PMN there exists a degree of feedback control of stimulation which may be dependent on background PKC activity and confirms that mechanisms exist whereby the responses of PMN may be augmented without activating PKC.

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