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Leukotriene B_4 triggers highly characteristic and specific functional responses in neutrophils: studies of stimulus specific mechanisms

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By using human neutrophils we studied the on-off phenomenon for leukotriene B_4 (LTB_4) -induced functional responses compared with fMetLeuPhe (fMLP). LTB_4 induced rapidly appearing and disappearing neutrophil chemiluminescent (CL), superoxide anion formation, aggregatory and membrane depolarizing responses, whereas fMLP responses were slower both in onset and termination. Increases of intracellular calcium concentrations (as reflected by quin2 and fura-2 fluorescence) were of similar magnitude for both stimuli; however, LTB_4 responses were more rapidly terminated and fMLP responses were biphasic. When intracellular calcium fluxes, calmodulin or protein kinase C activities were inhibited by quin2, trifluoperazine, verapamil or 3,4,5-trimethoxybenzoic acid 8-diethylamino)octyl ester (TMB-8), profound changes were noted for chemiluminescent and aggregation kinetics induced by fMLP, whereas kinetics of LTB_4 responses were less affected. When drugs were used to modulate cAMP levels, or to inhibit cyclo- and lipoxygenase metabolites of arachidonic acid, no effects on response kinetics were observed. Cytochalasin B both amplified and delayed responses although chemiluminescent responses to fMLP were amplified more than those to LTB_4. Despite those effects cytochalasin B did not enhance peak fura-2 or quin2 responses to either fMLP or LTB_4. Thus, LTB_4 rapidly initiates functional responses in neutrophils, and stimulus-specific response patterns are already discernable during the mobilization of calcium, and can be modulated by interference with calcium-dependent reactions.

Introduction

Leukotrienes and other lipoxygenase products are potent mediators for functional responses in a great variety of cell types. Leukotriene B_4 (LTB_4) has mostly been associated with activation of inflammatory cell responses such as leukocyte chemotaxis, adhesiveness, aggregation, degranulation and oxidative metabolism [1–6], but it also contracts the smooth muscles of the respiratory tract [7].

Since leukotrienes are not only potent stimuli for these reactions, but also initiate certain functional responses in, e.g., neutrophil granulocytes, much more rapidly than any other known stimuli, it was suggested that they may be more directly involved in the stimulus-response coupling than other agents. Support for this came from studies

Abbreviations: fMLP, formylmethionylleucylphenylalanine; LTB_4, leukotriene B_4; PMA, phorbol myristate acetate; HBSS, Hanks' balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMN, polymorphonuclear leukocytes; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester.

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of neutrophil aggregation [6,8] and oxidative metabolism [3,9], where functional responses occurred within seconds of cell exposure to LTB_4, and were completed after approx. 1–2 min. This characteristic and rapid response pattern was in contrast to what a variety of other stimuli inferred, for example formylated peptides (e.g., fMLP), where peak responses were more delayed [3,6,8,9], and for ionophore A23187 and the tumor promotor, phorbol myristate acetate (PMA), where detectable lag periods before responses have been reported [9–12].

In this paper we describe and analyse the highly stimulus-specific response patterns for leukotrienes and compare them to those produced by formylated peptides and other stimuli. We show that leukotrienes are intimately involved in the stimulus-response coupling in a unique yet rather general manner in that the kinetics of the response is found to be remarkably constant. By employing continuous assays, e.g., chemiluminescence, aggregation, intracellular calcium fluxes and membrane potential changes, where not only initiation of the functional response, but also its termination is possible to follow, it has been feasible to analyse the on-off phenomenon. This has rarely been appreciated in neutrophil physiology studies previously despite the possibilities it offers for assessment of the cellular activation process (cf. Ref. 13). Some of the possible mechanisms in these responses have been explored further, where emphasis has been placed on the role of cyclic nucleotides, calcium fluxes, arachidonate utilization and the cytoskeleton.

Material and Methods

Chemicals. Chemicals were obtained as follows: unless otherwise stated all chemicals were of the highest purity obtainable and were from Sigma Chemical Co. (St. Louis, MO). fMet-Leu-Phe (fMLP) and fNle-Leu-Phe-Nle-Tyr-Leu (fNLPNTL) were from Peninsula Laboratories (San Carlos, CA); A23187, quin2/AM and fura-2/AM were from Calbiochem (La Jolla, CA). Purified human myeloperoxidase was a kind gift from Dr. P. Venge, Upsala, Sweden. The fluorescent dye di-O-C5(3) was from Molecular Probes (Junction City, OR).

Leukotrienes. Natural and synthetic LTB_4 were generous gifts from Drs. C. Malmsten, Karolinska Institute, Stockholm, Sweden and J. Rokach, Merck-Frosst Inc, Dorval, Canada. The ω-oxidized metabolites of LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4 were kindly provided by Dr. J.Å. Lindgren, Karolinska Institute, and had been generated as described previously [3,6,8].

Leukocytes. Heparinized blood from healthy humans was used as a source for neutrophil granulocytes. Pure cell preparations (more than 95%) were obtained by a one-step Percoll centrifugation [6].

Chemiluminescence. Neutrophils ($2.5 \cdot 10^6$ cells/ml) were suspended in HBSS containing luminol (0.17 mM) [3]. Prior to and after addition of the various stimuli, light emission was followed continously in a LKB Luminometer 1250 (Stockholm, Sweden) or a Chronolog Lumiaggregometer (Havertown, PA) as described [3]. Responses are reported with regard to the kinetics of the response (e.g., time until appearance of peak response), as well as to the magnitude of the peak in mV. Since this paper is concerned mainly with response kinetics, figures have been chosen to illustrate that aspect. Because drug effects on kinetics do not necessarily follow the effects on peak magnitudes, text and figures may occasionally appear dissociated. (The possibility that the various reagents used here could interact with components of the chemiluminescence system was addressed by using a cell free system, where cell suspensions were substituted with HBSS and light emission was initiated by adding either xanthine (200 μM) plus xanthine oxidase (0.03 U/ml), H_2O_2 (20 mM), or purified human myeloperoxidase (16 nM).) These systems have been described elsewhere [14]. Apart from the above-mentioned inducers of chemiluminescence, none of the chemicals, used in this study as inhibitors, evoked light emission when added to neutrophils, suspended in luminol and HBSS. At the drug concentrations employed, the pH of the chemiluminescence system remained unchanged.

Superoxide anion production. Superoxide anion production was analyzed by the cytochrome *c* reduction method [3,15] at 37 °C with continuous stirring of neutrophils treated with 5 μg/ml cytochalasin B. The concentration of cytochrome *c*

was 50 μM. Absorbance was read at 549 nm and superoxide ion production was calculated as nmol reduced cytochrome *c* using an absorption coefficient of 21 $mM^{-1} \cdot cm^{-1}$.

Aggregation. Measurements were made in a standard platelet aggregometer (Model 300 BD, Payton Associates, Buffalo, NY) [6,8]). To a siliconized cuvette, containing a stir bar revolving at 900 rpm, 0.45 ml of a neutrophil suspension containing $1.15 \cdot 10^7$ cells/ml was added. After warming the cells at 37° C for 2 min, 50 μl of the stimulus was added. To provide the necessary amplification for a well-defined aggregation wave the aggregometer/recorder system was calibrated with a neutrophil suspension diluted 50% (v/v) with HBSS. The resulting changes in light transmission were recorded as ΔT. The results are expressed as the maximal light transmission (peak value), time to peak value, degree of disaggregation 1 min after peak value, or ΔT at 3 min if no disaggregation occurred. That ΔT reflects PMN aggregation has been demonstrated previously [16].

Enzyme release. For lysozyme determinations neutrophils ($1.0 \cdot 10^7$ cells/ml) were incubated with indicated stimuli at 37° C for 60 min. The stimulated net release of lysozyme is expressed as percent total cellular lysozyme content, determined after lysis of neutrophils with 0.2% Triton X-100 [17].

Intracellular Ca^{2+}. Concentrations were calculated from the change of quin2 or fura-2 fluorescence [13,18]. Neutrophils ($5 \cdot 10^6$ cells/ml) in HBSS supplemented with 20 mM Hepes (pH 7.4) were incubated at 37° C with 10 μM quin2/AM or 0.5 μM fura-2/AM for 30 min. Loaded cells were washed twice, reconstituted in HBSS (with Ca^{2+} at 1.27 mM) with 20 mM Hepes and stored on ice until use. Cells were then warmed at 37° C with continuous stirring of the cell suspension. The excitation wavelength was set at 340 nm and emission at 490 nm for quin2 and at 510 nm for fura-2. In addition, fura-2/AM-loaded cells were also excited at 380 nm. Since those responses were always mirror images of recordings obtained at 340 nm they are not reported. After a stable baseline had been established, stimulus was added and emitted light was recorded until return to baseline. The system was controlled by addition of EGTA, Tris buffer, Triton X-100 and $CaCl_2$ as described [15], calculations of calcium concentrations were performed according to Ref. 18. For experiments concerning buffering of intracellular Ca^{2+}, neutrophils were loaded with 2.5–5 μM quin2/AM according to the same protocol.

Membrane potential. The cyanine dye di-O-C5(3) was added to the cells at a final concentration of 20 nM [15]. Excitation and emission wavelengths were 460 nM and 510 nM. Measurements were made in a spectrofluorometer, at 37° C and with continuous stirring of the cell suspensions.

Cell viability. Cell viability was assessed by exclusion of trypan/blue.

Statistical calculations. These were performed using a Student's *t*-test of paired samples, and $P < 0.02$ was chosen as the level of significance.

Results

Characterization of stimulus-specific response patterns

LTB_4 and its ω-oxidized metabolites 20-OH-LTB_4 and 20-COOH-LTB_4 induced a rapidly occurring and rapidly disappearing chemiluminescence response in neutrophils where peaks occurred after 7 s and responses were terminated within 1 min (Fig. 1) [3]. The kinetics of the response was not modified by changes of luminol concentration between 170 and 1.7 μM or if neutrophil concentrations were varied between 0.7 and $5 \cdot 10^6$ PMN/ml (although the amplitude was directly proportional to cell concentration; data not shown). LTC_4 (0.1–1 μM) did not evoke chemiluminescence.

In contrast to the rapid but short-lived chemiluminescent response to all B_4 leukotrienes, formyl peptides (fMLP and fNLPNTL, 0.01–1 μM) generated significantly slower responses, with peaks after 45–60 s and a return to the base line after 2 min (Fig. 1).

Neutrophils, once exposed to LTB_4, did not react to a second LTB_4 administration (within 2–5 min) unless a concentration of at least 10-fold higher was used (data not shown). The kinetics of such a second response were similar to that of the first. If, instead, fMLP was subsequently added after the first LTB_4 stimulation, a kinetically normal fMLP response ensued, where peak values for the fMLP response were enhanced (cf. Ref. 19).

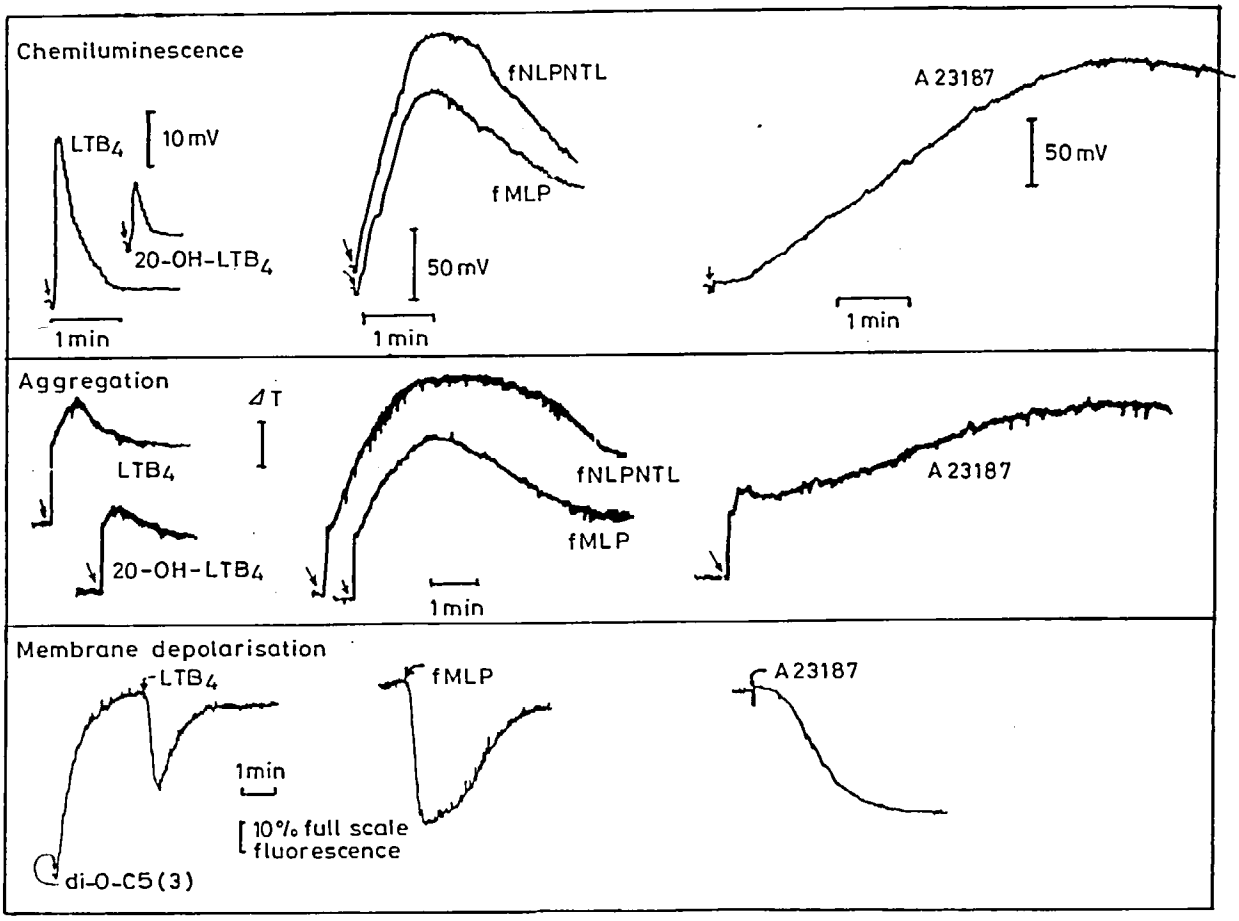

Fig. 1. Neutrophil chemiluminescence (upper panel), aggregation (middle panel) and membrane depolarization (assessed by di-O-C5(3); lower panel), in response to leukotrienes, formyl peptides and the calcium ionophore A23187.

Likewise, if fMLP was the first stimulus and was subsequently followed by a second fMLP addition, that second addition required a concentration at least 10-fold that of the first to elicit a response, whereas substituting the second fMLP addition with LTB_4, resulted in a kinetically normal LTB_4 response (data not shown).

A23187 elicited an even more protracted responses (Fig. 1). With appropriate amplification of the photomultiplier, no lag periods before the cells responded could be observed. However, with the amplification set at a level suitable for recording the peak response, lag phases were often recorded which may last 30 s to 2 min.

Assessment of superoxide ion formation with these agents showed response kinetic responses similar to those observed for chemiluminescence (Fig. 2). Also, aggregation exhibited a clear kinetic similarity to chemiluminescence and superoxide ion generation (Fig. 1). Although time-points for maximal aggregation differed from those of chemiluminescence, the kinetics of responses elicited by different stimuli were similar (Fig. 1). Thus, leukotrienes caused a rapid aggregation and disaggregation, whereas the formylpeptides required a 3 to 5-times more time to produce a maximal response. Moreover, the disaggregation was slower. Finally, A23187 generated a slow and continuous aggregation that persisted for several minutes, and was irreversible. LTC_4 (at 0.1–1 μM) did not induce aggregation.

As noted for chemiluminescence, homologous desensitization was observed for fMLP and LTB_4, which could partly be overcome by increasing

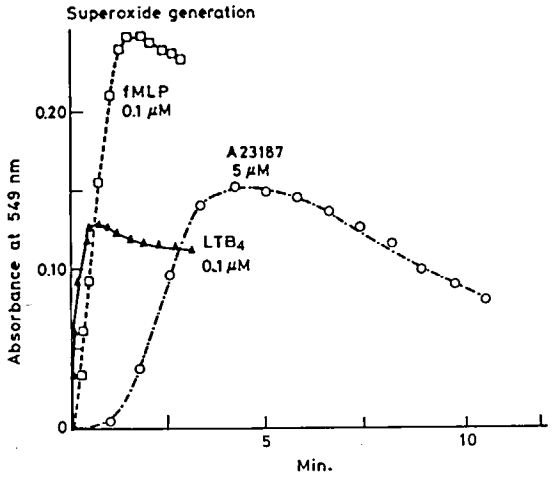

Fig. 2. Neutrophil superoxide generation responses to to LTB_4, fMLP and A23187. The graph represents one experiment (of many) where cells from one donor were used for all three stimuli.

stimulus concentrations at reexposure. Again, the stimulus-specific response kinetics were preserved for the second response [8].

Similar stimulus-specific response kinetics were also noted for changes of the membrane potential of human neutrophils (Fig. 1).

In some contrast with the above, fura-2 and quin2 fluorescence increments, reflecting changes of intracellular calcium ion concentration in neutrophils, exhibited a less pronounced, yet discrete differences between fMLP and LTB_4 (Fig. 3A). In the quin2 system peak, LTB_4 responses occurred slightly earlier (at 1.5 min) than fMLP responses (2.2 min). This was not observed in the fura-2 assay, where peak responses occurred much faster than in the quin2 system, but at the same time for both stimuli. Another difference (disclosed by both systems) was that fMLP responses did not return to the baseline as fast as those when cells had been

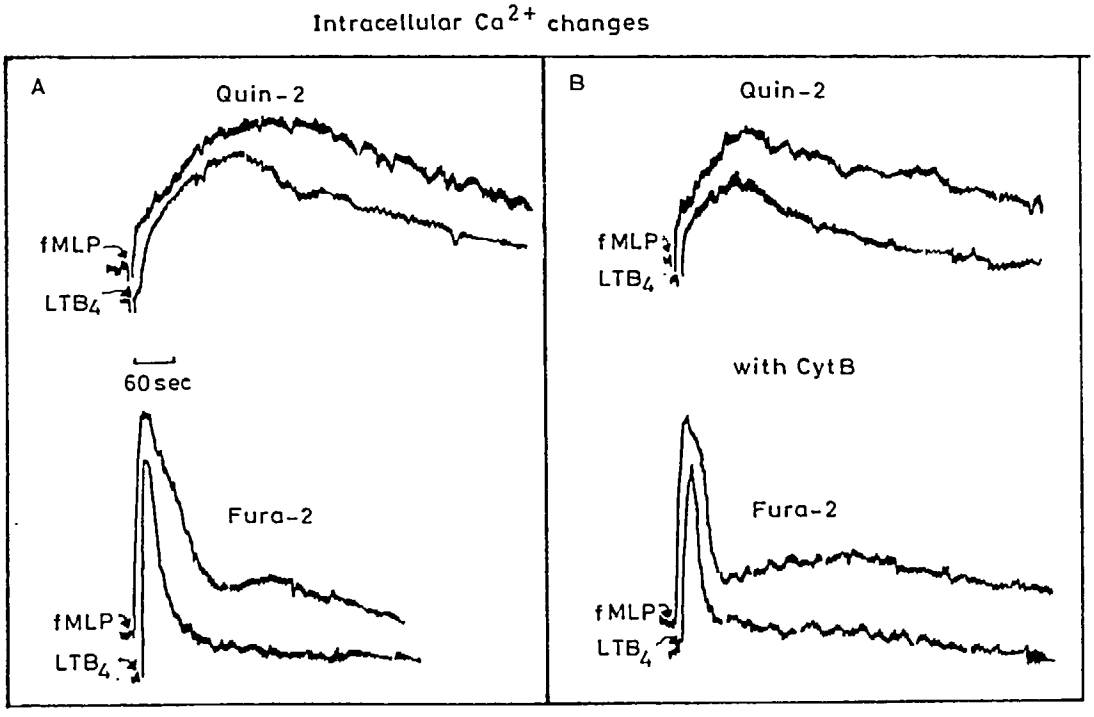

Fig. 3. (A) Changes of intracellular calcium concentrations (assessed by quin2 or fura-2 fluorescence) in neutrophils in response to LTB_4 and fMLP (both at 0.1 μM). The baseline fura-2 fluorescence corresponds to 81 ± 4 nM ($n = 4$) and the peaks correspond to 492 ± 19 nM ($n = 5$) for fMLP and 451 ± 34 nM Ca^{2+} for LTB_4. In the quin2 system, Ca^{2+} concentrations 5 min after stimulation corresponded to 370 nM for fMLP and to 250 nM for LTB_4. (B) Quin2 and fura-2 responses when cells had been treated with 5 μg/ml of cytochalasin B 3 min prior to addition of fMLP or LTB_4 (at 0.1 μM). The tracings shown here are from one out of three separate experiments with similar results. In each experiment, neutrophils from one donor were tested for all conditions depicted in the figure.

activated with LTB_4 (Fig. 3A). Also, a second smaller fura-2 fluorescence peak, occurring after approx. 3–5 min was noted consistently for fMLP but not for LTB_4 (Fig. 3A). The magnitudes of the first and major peak in both assays were, however, identical for equimolar concentrations of LTB_4 and fMLP (cf. Ref. 13).

Effect of modulation of cAMP concentrations

We then investigated whether a concomitant rise of intracellular cAMP was associated with the rapid decline of the LTB_4 response (cf. Ref. 14). By treating neutrophils with the phosphodiesterase inhibitor theophylline (at 0.1 mM for 30 min) or with dibuturyl cyclic AMP (at 1 mM for 30 min), the magnitude of peak chemiluminescence responses were significantly enhanced to $138 \pm 17\%$ of controls or reduced to $56 \pm 18\%$, respectively ($n = 3$); however, no effect on LTB_4 response kinetics could be found (Fig. 4A). A significant inhibition of LTB_4 chemiluminescence peak heights was observed when forskolin, a stimulator of adenylate cyclase [20], was used (Table I).

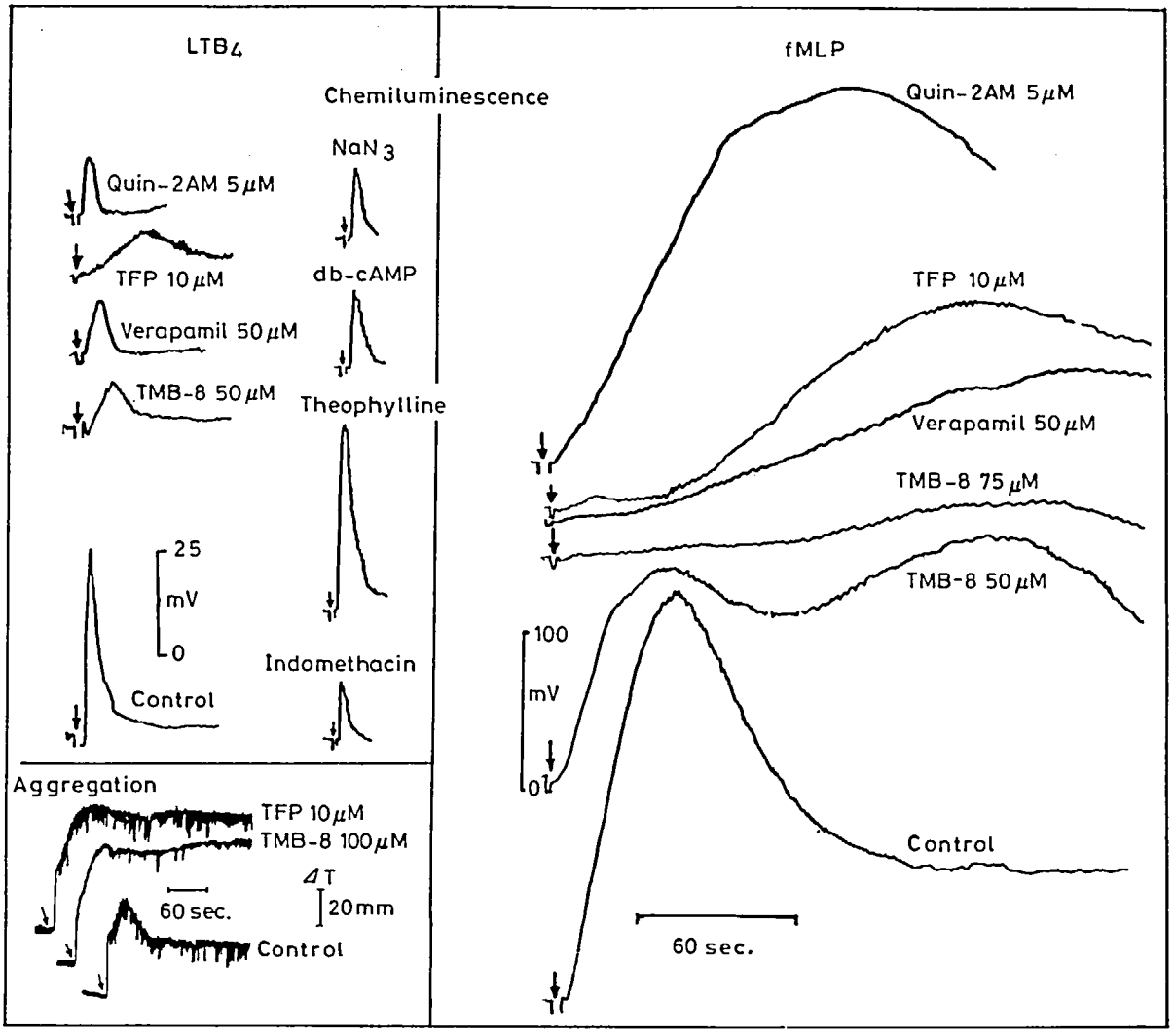

Fig. 4. Effects of various pharmacological agents on chemiluminescent and aggregatory responses to LTB_4 and fMLP in neutrophils. db-cAMP, dibuturyl cyclic AMP; TFP, trifluoperazine.

TABLE I

EFFECT OF VARIOUS PHARMACOLOGIC AGENTS ON CHEMILUMINESCENCE IN NEUTROPHILS

If not otherwise stated, neutrophils were treated with the various agents for 15 min at 37 °C. Ethanol, the solvent for indomethacin and acetyl salicylic acid, never exceeded 0.1% in final concentration. Results are given as calculated IC_{50} values.

Agent	IC_{50} (μM)		
	LTB_4	fMLP	*n*
Indomethacin	10	3 *	6
Acetylsalicylic acid	50	30	3
Nordihydroguaiaretic acid	0.8	0.8	2–9
Forskolin	10	n.d.	3
Trifluoperazine	8	8	5
TMB-8	50	75 [b]	4
Verapamil	100	600 *	4
Colchicine [a]	10	n.d.	3

[a] Colchicine was added to cells kept on ice, the suspensions were subsequently warmed to 37 °C for 30 min.

[b] Because of appearance of a second peak (cf. text) only the IC_{50} value for the first peak is given here.

* $P < 0.02$ for differences between fMLP and LTB_4.

Dependence on divalent cations and their fluxes

The requirement of extracellular Ca^{2+} was assessed by suspending neutrophils in HBSS without Ca^{2+}, Mg^{2+} (after Percoll separation) and comparing their responses to cells resuspended in regular HBSS. It was found that LTB_4 and fMLP chemiluminescence response kinetics were identical although peak magnitudes were markedly reduced (data not shown). Similar results have been obtained for the aggregation assay [8].

The dependence on Ca^{2+}-fluxes was also tested using drugs which interfere with Ca^{2+}. When intracellular Ca^{2+} was buffered with 5 μM quin2 [21] the magnitude of the chemiluminescence response was significantly reduced for LTB_4 (to $39 \pm 18\%$ of controls; $n = 4$) and less for fMLP ($81 \pm 17\%$; n.s.; $n = 3$). Contrasting effects were noted for peak response kinetics, with a slight nonsignificant prolongation being observed for LTB_4 and a pronounced effect for fMLP responses (i.e., a doubling of the time to peak (Fig. 4)).

TMB-8, a suggested inhibitor of, for example, intracellular calcium fluxes [11,12,22,23], was also used. LTB_4 peak responses were only slightly delayed (from a mean of 7 s to 12 s at 50 μM; n.s.; $n = 4$; Fig. 4), whereas peak heights were significantly reduced (Table I). The inhibition could partly be ascribed to an effect unassociated with cation fluxes, since superoxide ion-induced chemiluminescence was inhibited in the cell free system (Table II). In contrast to the modest effect on LTB_4 response kinetics, fMLP chemiluminescence was significantly modified. When TMB-8 was used at 25–60 μM ($n = 5$), enhancements of the characteristic single-peaked chemiluminescent response were noted. With higher concentrations, kinetics changed so that a second peak appeared, reaching its maximum after 3–4 min. At a concentration of 60 μM TMB-8, the first peak disappeared completely and only the second peak was present. At even higher TMB-8 concentrations (above 70–100 μM), the second peak also disappeared gradually and no responses were observed at concentrations higher than 100 μM (Fig. 4).

TABLE II

EFFECT OF VARIOUS PHARMACOLOGIC AGENTS ON CHEMILUMINESCENCE INDUCED IN A CELL-FREE SYSTEM

Light emission was commenced by addition of the stimuli as described in Materials and Methods and in Ref. 13. + denotes that no inhibition, but stimulation of chemiluminescence was noted. n.d. = not done.

Agent	(% inhibition)		
	xanthine plus xanthine oxidase	H_2O_2	myeloperoxidase
Trifluoperazine (300 μM)	69±2 * (5)	9±5 (2)	40±5 * (2)
TMB-8 (100 μM)	36±4 * (5)	+4±8 (2)	5±5 (2)
Quin2/AM (1 μM) [a]	0 (3)	24±7 * (5)	n.d.
Indomethacin (10 μM)	10±8 (4)	+10±8 (3)	n.d.
Acetylsalicylic acid (50 μM)	+45±9 * (2)	+35±0 * (2)	1±7 (3)
Nordihydroguaiaretic acid (8 μM)	47±9 * (3)	35±3 * (2)	n.d.
NaN_3 (4 μM)	+107±5 * (3)	1±3 (2)	100 * (2)

* $P < 0.02$ vs. control.

[a] As quin2 concentrations within the cell were unknown.

Trifluoperazine, which may act as an inhibitor of calmodulin-dependent, cGMP-stimulated protein kinase as well as protein kinase C [11,12,24], significantly decreased magnitudes and increased time to appearance of peaks, but similarly for both fMLP and LTB_4 (Table I, Fig. 4). As noted with TMB-8, trifluoperazine also acted as a scavenger for superoxide ions (Table II).

Verapamil, blocking inter-alia transmembrane calcium fluxes [11,25,26], delayed the appearance of chemiluminescence peaks for fMLP significantly more than for LTB_4 (250% and 110% of controls, respectively, for the IC_{50} concentrations) (Fig. 4). The magnitude of the fMLP response was less influenced by verapamil than that of LTB_4 (Table I).

TMB-8 and trifluoperazine also changed the aggregation responses. When neutrophils were treated with high concentrations of TMB-8 (above 100 μM) or trifluoperazine (above 10 μM), response kinetics were profoundly altered. The characteristic early peak and rapid disaggregation induced by LTB_4 was abolished, and, instead, LTB_4 induced a continuous aggregation response, not followed by any disaggregation (Fig. 4). At lower concentrations, peak values and response kinetics were identical to those of untreated neutrophils.

Interventions with arachidonic acid utilization

In order to determine whether the rapid and transient responses to LTB_4 were due to formation of either stimulatory or inhibitory cyclooxygenase products, neutrophils were treated with indomethacin (10–0.1 μM) or acetylsalicylic acid (5–0.05 mM) for 15 min and subsequently exposed to LTB_4 or fMLP. Although the magnitude of the peak chemiluminescence response was decreased by these high acetylsalicyclic acid and indomethacin concentrations (Table I), response kinetics remained unchanged (Fig. 4). Acetylsalicyclic acid and indomethacin did not inhibit the chemiluminescence generated in the cell-free system (Table II). Similar indomethacin effects on LTB_4-induced aggregation have been reported previously [8].

The possibility that neutrophil responses were dependent on a patent lipoxygenase pathway was tested by treating human neutrophils with the lipoxygenase inhibitor nordihydroguaiaretic acid [27]. This depressed the magnitude of chemiluminescence peaks equally for both LTB_4 and fMLP (Table I), but did not change response kinetics (data not shown). However, it also inhibited chemiluminescence in the cell free system (Table II) indicating a scavanger effect.

Inhibition of metabolism of leukotrienes

We next tested whether the rapid decline of the LTB_4 response might be due to its further transformation to biologically less active metabolites, e.g., by action of the myeloperoxidase-H_2O_2-halide system [28]. After adding sodium azide (to inhibit extracellular myeloperoxidase) (Fig. 4), or catalase, superoxidase dismutase and mannitol (to inhibit H_2O_2, O_2- and $OH^{\cdot}$, respectively), chemiluminescence response kinetics remained unchanged (data not shown), whereas the magnitude of peak values were reduced (Table II) as reported [3,13]. Sodium azide may have inhibited chemiluminescence by interference with myeloperoxidase (Table II). Also, aggregation responses were unaffected by these drugs [8].

Dependence on the cytoskeleton

The dependence on the cytoskeleton was assessed by inhibiting microtubules with colchicine (10–0.1 μM) and microfilaments with cytochalasin B (0.1–5 μg/ml). With cytochalasin B, enhancement of chemiluminescence was noted, being maximal at 0.5–1.0 μg/ml. Such treatment was associated with a distinct change of both response kinetics and peak magnitudes. Gradually, with increasing cytochalasin B concentrations, the magnitude of the peak response to LTB_4 and fMLP was significantly amplified, for LTB_4 4-fold and for fMLP 10-fold. LTB_4 responses also aquired a dual-peaked appearance (Fig. 5). Colchicine treatment did not modify the LTB_4-response kinetics, however, it did reduce the peak values (Table I) and the release of lysozyme by 20%.

The ablation of all stimulus-specific aggregation characteristics by cytochalasin B have been reported elsewhere [8]. The findings of those studies were largely parallel to those reported here for chemiluminescence, including a change from a single-peaked aggregation response to LTB_4 to a dual-peaked one, when cytochalasin was used at concentrations above 0.5 μg/ml. Cytochalasin B

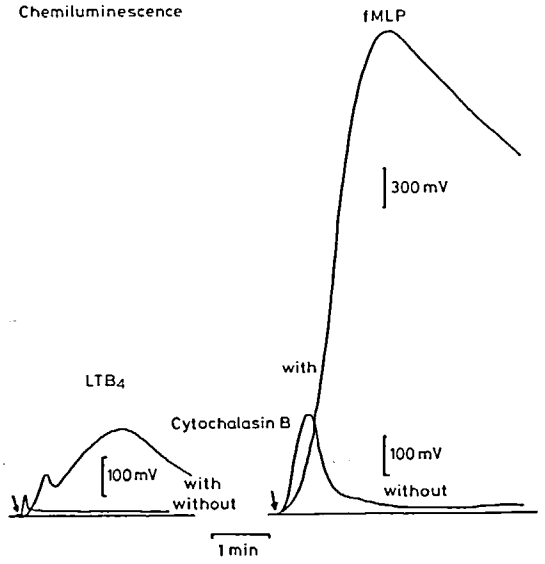

Fig. 5. Chemiluminescence curves of neutrophils in response to 100 nM LTB_4 or fMLP when neutrophils had been treated with 5 μg cytochalasin B/ml 1 min prior to the addition of the stimuli. LTB_4 chemiluminescence increased from 53 ± 9 mV to 226 ± 15 mV (for the second and larger peak) and fMLP from 317 ± 28 mV to 4771 ± 229 mV ($n = 8$–9).

did not enhance fura-2 or quin2 fluorescence increments induced by fMLP or LTB_4 (Fig. 3B).

Discussion

Regardless of whether chemiluminescence, aggregation and membrane depolarization were followed after stimulation with leukotrienes of the LTB_4 family, kinetically similar responses were observed.

The similarity became more apparent when functional responses to other classes of stimulants were compared. The formyl peptides, which are supposed to engage receptors of one (or of very similar) kind(s), albeit distinctly different from that for LTB_4 [29,30], generated responses in which not only the peaks appeared later but also termination of chemiluminescence and membrane potential changes, as well as disaggregation, occurred more slowly. Despite differences in absolute time to peak reactions, which are related to the actual functional response, relative proportions between leukotriene and formyl peptide responses were preserved. This suggests that the surface receptor-mediated stimulus-response coupling may be uniform for one class of stimuli. A difference was also observed for intracellular calcium responses to LTB_4 and fMLP. Although onset of the rise of Ca^{2+} concentration after addition of LTB_4 and fMLP was rather similar, when assessed by fura-2 fluorescence, onset of the LTB_4 responses were faster in the quin2 system. In both assays fMLP responses persisted longer than the LTB_4 responses (cf. Ref. 31), and in the fura system fMLP responses assumed a dual-peaked appearance (Fig. 3). The mechanism for the second peak of the fMLP response is unclear, but may point to the contribution of additional activation steps [26]. It is, thus, possible that subsequent stimulus-specific chemiluminescent and aggregation response patterns are already formed at the level of activation of the phospholipase C (since formation of inositol triphosphate isomers also differ between fMLP and LTB_4) [13]. They may also depend on phenomena occurring after the initial mobilization of intracellular calcium (cf. Ref. 26). Nonetheless, the rapid responses to leukotrienes suggest that these substances have more direct access to the response coupling than do formyl peptides, which may need endogenous LTB_4 formation for full expression [32].

It has been suggested that LTB_4-induced increments in neutrophil adhesiveness (as well as lung strip contraction [7]) are due to formation of a cyclooxygenase product, conceivably thromboxane, since indomethacin treatment was reported to inhibit those responses [33]. It is, however, unclear whether cyclooxygenase products are involved in neutrophil responses, since adhesion to plastics, aggregation and chemotaxis are neither inhibited by such treatment (Refs. 1, 8 and Lindström et al., unpublished observations), or moderately inhibited (such as demonstrated here for chemiluminescence). Nonetheless, kinetic features of the responses were not changed, indicating that the stimulus-response coupling was not affected in a qualitative way. Also, it is at present unclear whether inhibition of peak response magnitude conferred by indomethacin depends on an action on cyclooxygenation or whether other phenomena are of significance, e.g., decrements of binding of ligands (fMLP) to their receptors or by in-

fluencing membrane events linked to the activation of adenylate cyclase [34].

Another unique feature for leukotriene-triggered responses is the rapid termination of the response, irrespective of whether chemiluminescence, membrane potential, or aggregation is tested. From the present results it appears not to be due to the formation of an inhibitory cyclooxygenase product, e.g., prostacyclin [35,36]. The results of repeated challenges with LTB_4 and with the lipoxygenase inhibitor nordihydroguaiaretic acid argues against the possibility that neutrophils were desensitized by an ensuing endogenous leukotriene formation. Moreover, the fast termination of LTB_4-elicited chemiluminescence was probably not due to rapid elevations of cAMP [37], or metabolism of added LTB_4 [28], since none of the drugs acting on these systems conferred changes of response kinetics [13,38]. It is, at present, unclear how the faster termination of the LTB_4-induced calcium response relates to the kinetics of the other functional responses. More research is also needed to evaluate the significance of the second wave of fluorescence in fura-2 loaded neutrophils stimulated by fMLP.

Absence of extracellular calcium did not modify neutrophil response kinetics (although peak heights were reduced). However, quite marked changes were observed for neutrophil chemiluminescence and aggregation when putative inhibitors of (a) intracellular calcium fluxes (quin2) [21] and TMB-8 [11,12,22,23]) or (b) calmodulin-dependent kinase and protein kinase C (trifluoperazine [11,12,24]) or on even more complex calcium-dependent reactions (verapamil [11,25,26]) were used. Although the exact mechanism for drug effects remains obscure appearance of peak chemiluminescence was delayed and disaggregation was abolished by all drugs.

Thus, we would suggest that interference with calcium-associated events (or other effects) conferred by these four drugs hamper the on-off phenomenon. More research is needed to clarify the mechanisms by which and the reason why fMLP response kinetics were more prolonged than LTB_4, and why LTB_4 peaks were more susceptible to inhibition than fMLP. That the magnitude of the peak responses were reduced for chemiluminescence and enhanced for aggregation, might partly be explained by an inhibitory action of TMB-8 and trifluoperazine on light production in the luminol system [3]. As shown by the experiments with cell-free luminescent systems scavenging of light by a drug may impair detection of other cellular effects of that particular drug. It also emphasizes the need for careful evaluation of chemical interactions in a luminescent system [14].

Cytochalasin B treatment markedly changes aggregation and chemiluminescence responses, enhancing peak magnitudes as well as ablating all stimulus-specific responses [8]. Since there is, as yet, no explanation for the sharp, single-peaked leukotriene chemiluminescent and aggregation responses were transformed into a broad-shouldered dual-peaked response by cytochalasin B, further work is needed. But this clearly points to an effect on mechanisms intimately involved in the stimulus-response coupling, yet not clearly associated with changes in the calcium fluxes (Fig. 3b). It may be related to effects on the cytoskeleton or translocation of protein kinase C [31,39].

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References

1 Palmblad, J., Malmsten, C., Uden, A.-M., Rådmark, O., Engstedt, L. and Samuelsson, B. (1981) Blood 58, 658–661.
2 Dahlen, S.-E., Björk, J., Hedqvist, P., Arfors, K.E., Hammarström, S., Lindgren, J.A. and Samuelsson, B. (1981) Proc. Natl. Acad. Sci. USA 78, 3887–3891.
3 Palmblad, J., Gyllenhammar, H., Lindgren, J. and Malmsten, C.L. (1984) J. Immunol. 132, 3041–3045.
4 Hafström, I., Palmblad, J., Malmsten, C.L., Rådmark, O. and Samuelsson, B. (1981) FEBS Lett. 130, 146–148.

5 Ford-Hutchinson, A.W., Bray, M.A., Doing, M.v., Shipley, M.E. and Smith, J.H. (1980) Nature 286, 264–265.
6 Ringertz, B., Palmblad, J., Rådmark, O. and Malmsten, C.L. (1982) FEBS Lett. 147, 180–182.
7 Dahlen, S.-E., Hedqvist, P., Westlund, P., Granström, E., Hammarström, S., Lindgren, J. and Rådmark, O. (1983) Acta Physiol. Scand. 118, 393–403.
8 Ringertz, B., Palmblad, J. and Lindgren, J. (1985) J. Lab. Clin. Med. 106, 132–140.
9 Serhan, C.N., Broekman, J., Korchak, H.M., Marcus, A.J. and Weissmann, G. (1982) Biochem. Biophys. Res. Commun. 107, 951–958.
10 Williams, A.J. and Cole, P.J. (1981) Immunology 43, 847–858.
11 Smolen, J. (1984) J. Lab. Clin. Med. 104, 1–10.
12 Korchak, H., Rutherford, L.E. and Weissmann, G. (1984) J. Biol. Chem. 259, 4070–4075.
13 Lew, P.D., Monod, A., Waldfogel, F.A. and Pozzan, T. (1987) Eur. J. Biochem. 162, 161–168.
14 Gyllenhammar, H. (1987) J. Immunol. Methods 97, 209–213.
15 Metcalf, J.A., Gallin, J.I., Neuseef, W.M. and Root, R.K. (1986) Laboratory Manual of Neutrophil Function Raven Press, New York.
16 Ringertz, B. (1986) Acta Pathol. Microbiol. Immunol. Scand. (Section C) 94, 1–9.
17 Hafström, I., Palmblad, J., Malmsten, C., Rådmark, O. and Samuelsson, B. (1981) FEBS Lett. 130, 146–148.
18 Pollock, W.K. and Rink, T.J. (1986) Biochem. Biophys. Res. Commun. 139, 308–314.
19 Gay, J.C., Beckman, J.K., Brash, A.R., Oates, J.A. and Lukens, J.N. (1984) Blood 64, 780–785.
20 Takenawa, T., Ishitoya, J. and Nagai, Y. (1986) J. Biol. Chem. 261, 1092–1098.
21 Lew, P.D., Wollheim, C.B., Waldvogel, F.A. and Pozzan, T. (1984) J. Cell. Biol. 99, 1212–1220.
22 Christiansen, N.O., Larsen, C.S. and Juhl, H. (1986) Biochim. Biophys. Acta 882, 57–62.
23 Verghese, M.W., Fox, K., McPhail, L.C. and Snyderman, R. (1985) J. Biol. Chem. 260, 6769–6775.
24 Curnutte, J.T., Badney, J.A., Robinson, J.M., Karnovsky, M.J. and Karnovsky, M.L. (1984) J. Biol. Chem. 259, 11851–11857.
25 Della Bianca, V., Grzeskowiak, M., Togni, P.D., Cassatella, M. and Rossi, F. (1985) Biochim. Biophys. Acta 845, 223–236.
26 Andersson, T., Dahlgren, C., Pozzan, T., Stendahl, O. and Lew, P.D. (1987) Mol. Pharmacol. 30, 437–443.
27 Salari, H., Braquet, P. and Borgeat, P. (1984) Prostaglandin Leukotr. Med. 13, 53–60.
28 Henderson, W.R. and Klebanoff, S.J. (1983) J. Biol. Chem. 258, 13522–13527.
29 Goldman, D.W. and Goetzl, E.J. (1984) J. Exp. Med. 159, 1027–1041.
30 Snyderman, R. and Pike, M.C. (1984) Annu. Rev. Immunol. 2, 257–281.
31 Omann, G.M., Traynor, A.E., Harris, A.L. and Sklar, L.A. (1987) J. Immunol. 138, 2626–2632.
32 Gyllenhammar, H., Ringertz, B., Hafström, I., Palmblad, J. and Borgeat, P. (1988) Lipids 23, 89–95.
33 Goetzl, E.J., Brindley, L.L. and Goldman, D.W. (1983) Immunology 50, 35–41.
34 Abramson, S., Korchak, H., Ludevig, R., Edelson, H., Haines, K., Levin, R.I., Herman, R., Rider, L., Kimmel, S. and Weissmann, G. (1985) Proc. Natl. Acad. Sci. USA 82, 7227–7231.
35 Puustinen, T. and Uotila, P. (1984) Prostaglandin Leukotr. Med. 14, 161–167.
36 Boxer, L.A., Allen, J.M., Schmidt, M., Yoder, M. and Baehner, R.L. (1980) J. Lab. Clin. Med. 95, 672–678.
37 Claesson, H.E. and Feinmark, S.J. (1984) Biochim. Biophys. Acta 804, 52–57.
38 Cheung, K., Archibald, A.C. and Robinson, M.F. (1983) J. Immunol. 130, 2324–2328.
39 Pike, M.C., Jakoi, L., McPhail, L.C. and Snyderman, R. (1986) Blood 67, 909–913.