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The Effect of Thimerosal on Neutrophil Migration

A Comparison with the Effect on Calcium Mobilization and CD11b Expression

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ABSTRACT. The sulfhydryl-reactive compound thimerosal caused a chemotactic stimulation of neutrophil migration at low concentrations and inhibition of chemoattractant-stimulated chemotaxis at high concentrations. Thiosalicylic acid, an analog of thimerosal devoid of mercury, also stimulated migration at low concentrations and caused inhibition at higher concentrations, though the inhibitory effect was less pronounced than that of thimerosal. These results indicate that the stimulatory effect of thimerosal on migration is due to the thiosalicylic acid moiety of the molecule. In contrast with thimerosal which, especially at higher concentrations than required for optimal stimulation of migration, caused an increase in cytosolic free calcium ($[Ca^{2+}]_i$), thiosalicylic acid had no effect on $[Ca^{2+}]_i$ of the neutrophil. This suggests that the presence of mercury is decisive for the calcium-mobilizing effect, but not for stimulation of migration, and that mobilization of calcium and activation of migration are not related. Thimerosal caused a strong increase of CD11b expression in neutrophils in suspension, especially at inhibitory concentrations, while thiosalicylic acid had no effect on CD11b expression. This could mean (but does not prove) that CD11b expression is more related to the calcium-mobilizing effect of thimerosal than to its stimulation of migration. BIOCHEM PHARMACOL **55**;3: 305–312, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. neutrophil; thimerosal; migration; chemotaxis; calcium; CD11b expression

Migration by neutrophils plays a predominant role in both the anti-microbial and the inflammation-promoting activities of these cells by enabling them to reach the site of infection or inflammation. In spite of extensive research, the molecular basis of the migration process remains largely unknown, with the calcium homeostasis during migration being a matter of particular controversy [1–4]. Most chemoattractants cause an increase in cytosolic free calcium ($[Ca^{2+}]_i$)†, but there is no evidence that the ability to cause an increase in $[Ca^{2+}]_i$ is related to the extent of migration. On the contrary, chemotactic migration is inhibited by a number of agents which cause an increase in $[Ca^{2+}]_i$ [5–9].

Thimerosal is an organomercury compound with sulfhydryl-reactive properties. It is clinically used as a topical antiinfective agent because of its antibacterial and antifungal properties. The substance has a profound effect on calcium homeostasis in a number of cells. While it was originally described as an agent having a specific effect on inositol trisphosphate (IP_3)-sensitive calcium stores, recent studies have shown that in addition to IP_3-sensitive stores, ryanodine-sensitive stores are also affected by thimerosal [10, 11]. The effect of thimerosal is biphasic: at low concentrations it causes cytosolic calcium oscillations in endothelial cells, whereas at high concentrations the oscillations are inhibited and a sustained increase in $[Ca^{2+}]_i$ is observed [12].

Thimerosal causes an increase of $[Ca^{2+}]_i$ in neutrophils [13]. It also causes a strong increase in 5-lipoxygenase metabolites when another activator, such as formyl-methionyl-leucyl-phenylalanine (fMLP), is present [13, 14]. Leukotriene formation depended on the presence of extracellular $[Ca^{2+}]_i$, and it was concluded that the enhancing effect of thimerosal on fMLP-induced leukotriene formation was due to its modulating effect on calcium homeostasis.

Because of the effects of thimerosal on calcium homeostasis and because of sulfur-containing compounds were shown in previous studies to be capable of inducing chemotaxis, we decided to study the effect of thimerosal on neutrophil migration. We set out to determine whether the substance could induce chemotaxis by itself and whether thimerosal could affect chemotactic migration activated by other chemoattractants. In addition, we wished to address the question as to whether the effect on migration was connected with the effect on calcium metabolism. To determine the importance of mercury in the thimerosal molecule, we compared the results of thimerosal with those of thiosalicylic acid, a mercury-less analog of thimerosal (Fig. 1).

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† *Abbreviations:* fMLP, formyl-methionyl-leucyl-phenylalanine; $[Ca^{2+}]_i$, cytosolic free calcium concentration; IL-8, interleukin 8; IP_3, inositol trisphosphate; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; LTB_4: leukotriene B_4.

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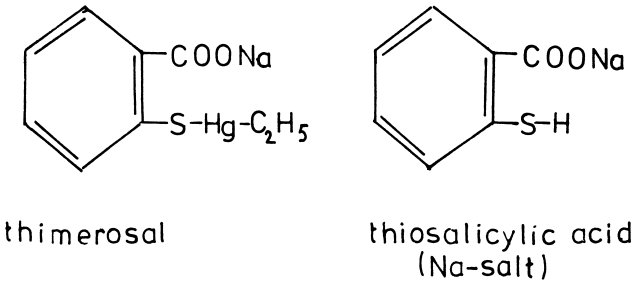

FIG. 1. Formulae of thimerosal and thiosalicylic acid.

MATERIALS AND METHODS

Isolation of Human Neutrophils

Neutrophils were isolated from the buffy coat of blood of healthy donors. The buffy coat was diluted with a fourfold volume of heparinized medium and layered on top of Ficoll-amidotrizoate ($d = 1.077$). After centrifugation the pellet was resuspended, and starch was added to sediment erythrocytes. After sedimentation, the neutrophil-containing supernatant was collected and centrifuged. The remaining erythrocytes were removed by hypotonic hemolysis and the neutrophils suspended in medium. The cells consisted of more than 95% neutrophils and were more than 99% viable, as determined by Trypan blue exclusion. As a second control for cell integrity, lactate dehydrogenase (LDH) release was measured. The LDH release was always less than 3% (percentage of maximal enzyme release, obtained by treating the cells with Triton X-100). None of the experimental procedures affected viability. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5% (w/v) bovine serum albumin and 20 mM Hepes buffer pH 7.3. Unless otherwise stated, the medium was supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ during the experiments. The final cell suspension during the experiments contained 3×10^6 neutrophils per milliliter.

Neutrophil Migration

Cell migration was measured with the Boyden chamber technique [15], as described by Boyden [16], and modified by Zigmond and Hirsch [17]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter with a pore size of 3 μm. Medium supplemented with 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.5% (w/v) bovine serum albumin was present in both the upper and lower compartment, unless otherwise indicated. Neutrophils were placed in the upper compartment of the chamber, followed by incubation for 40 min at 37°. After migration, the filters were fixed and stained and the distance travelled into the filter in micrometers was determined according to the leading front technique [17]. Chemotactic assays were carried out in duplicate and the migration distance of the neutrophils determined at five different filter sites.

Determination of Intracellular Calcium

Neutrophils (1×10^7 cells per mL) were incubated with 1 μM Fura-2/AM for 30 min at 37°, in the presence of 1 mM Ca^{2+}. After washing, the cells were resuspended in medium and used at a concentration of 3×10^6 cells per mL. Fura-2 fluorescence was measured in a Perkin Elmer LS50B fluorescence spectrophotometer (Perkin Elmer Ltd), equipped with a thermostated cuvette compartment and a mixing device. Fluorescence (emission wavelength 510 nm) was recorded at two excitation wavelengths (340 nm and 380 nm), and the data were used to calculate the concentrations of cytosolic free calcium. Traces given are representative for three different experiments.

Flow Cytometric Analysis of CD11b Expression

Neutrophils (3×10^6 per mL) were incubated for 10 min with the indicated compounds at 37°. After stimulation, a sample of 67 μL cell suspension was mixed with 25 μL Mab (FITC-labeled monoclonal antibody against CD11b, diluted 1:16) and 8 μL buffer, and incubated for 30 min on ice in the dark. After dilution with 4 mL medium, the mixture was centrifuged, and the pellet suspended in 0.5 mL paraformaldehyde (1% in 0.9% NaCl). The suspension was mixed and placed in the dark at 4° for 30 min. Subsequently the suspension was centrifuged and washed twice with medium, after which the cells were resuspended in 0.5 mL medium. From this suspension, 10,000 cells were analyzed with a flow cytometer (FACScan, Beckton Dickinson) within 24 hr. The values given are those of the mean fluorescence intensity, which correlate directly with CD11b antigen density. The values given were corrected for autofluorescence and non-antigen-specific antibody binding by measuring both cells which were not treated with antibody and cells which were treated with mouse lgG.

Statistical Analysis

All mean values for the chemotactic assays are arithmetical means ± SEM of four experiments. Whenever random migration or activated migration was considerably different for different cell batches, values were expressed as percentage of control. Significances were calculated with Student's *t*-test; a value of $P < 0.05$ was considered as statistically significant.

RESULTS

Migration

Thimerosal caused an increase in neutrophil migration (in the absence of other chemotactic agents). The increase occurred both when thimerosal was present in the lower compartment only, and when thimerosal was present in both compartments. When thimerosal was present in the lower compartment only, the increase in migration was more pronounced and occurred at a higher concentration

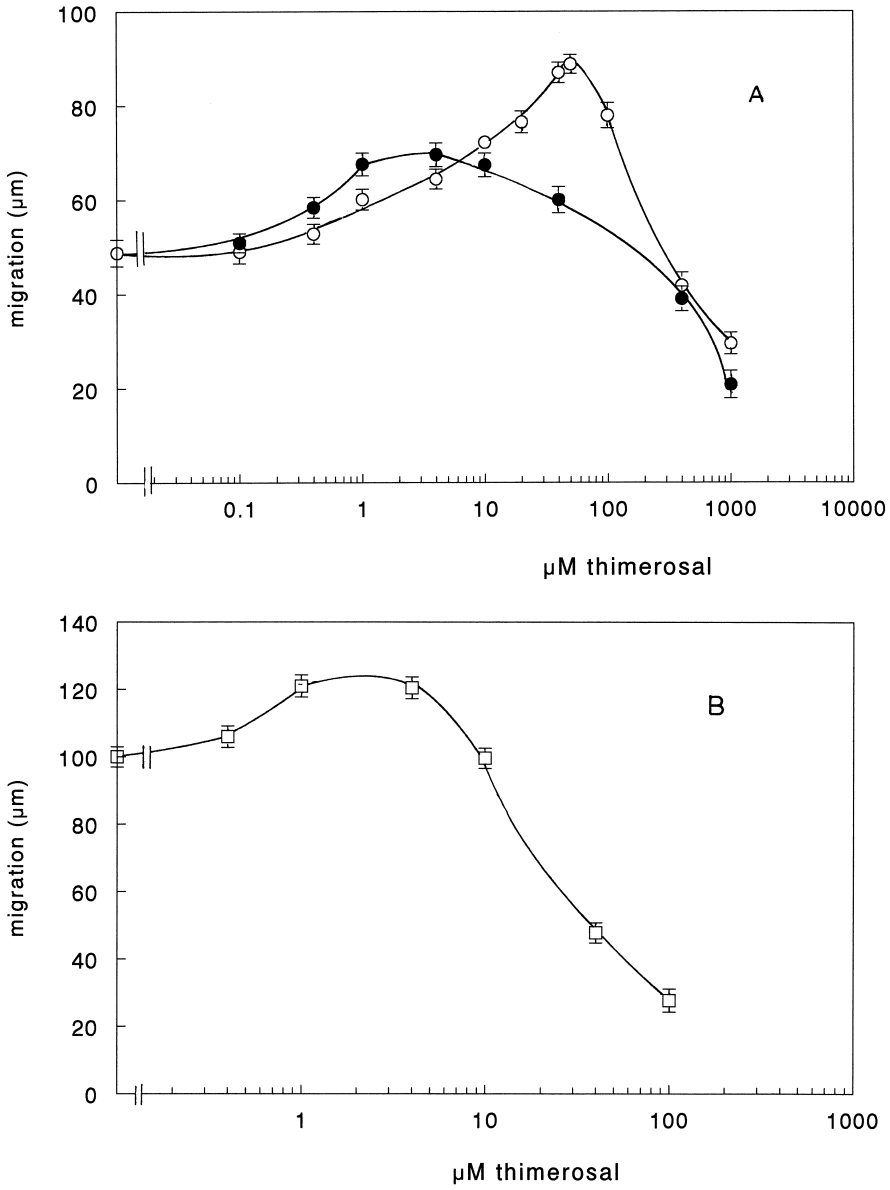

FIG. 2. The effect of thimerosal on random migration (A) and on fMLP-activated chemotaxis (B). For the effect of thimerosal on random migration (A), the drug was present in both compartments of the Boyden chamber (——●——), or in the lower compartment only (——○——). For the effect on fMLP-activated chemotaxis, cells were preincubated with the indicated concentration of thimerosal for 15 min and subsequently placed in the upper compartment of the Boyden chamber. A: values for concentrations higher than 4 μM (—○—) or 1 μM (—●—) are significantly different from control ($P < 0.05$); B: values for 1 and 4 μM, and for 40 and 100 μM, are significantly different from control ($P < 0.05$).

than when thimerosal was present in both compartments (Fig. 2A). Chemotactic migration stimulated by fMLP was enhanced by thimerosal at low concentrations, but inhibited at high concentrations (Fig. 2B). Preincubation of neutrophils with 50 μM thimerosal had little effect on random migration, but inhibited fMLP- or interleukin 8 (lL-8)-induced chemotaxis so strongly that migration was significantly less than random migration (Table 1). The inhibitory effect of thimerosal was not restricted to receptor-mediated activators. Phorbol myristate acetate (PMA)-activated migration (10^{-10} g/mL PMA in the lower compartment) was equally inhibited: migration was reduced from 80.7 ± 2.3 μm in the absence of thimerosal to 36.6 ± 2.1 μm after preincubation with 50 μM thimerosal, indicating that the observed inhibitory effect was not due to interference with surface receptors.

Thiosalicylic acid, an analog of thimerosal devoid of mercury, also stimulated neutrophil migration (at lower concentrations than thimerosal), and at high concentrations inhibited fMLP-activated neutrophil chemotaxis (Fig. 3). The decrease in migration per concentration unit was considerably less for thiosalicylic acid than for thimerosal.

TABLE 1. The effect of divalent cation-complexing agents on inhibition of fMLP- or lL-8-stimulated migration by thimerosal

	Migration (μM)	
	−	+ thimerosal
A: fMLP		
Ca^{2+}, Mg^{2+}	99.8 ± 1.9	46.6 ± 2.3
Ca^{2+} (no Mg^{2+})	96.8 ± 2.2	43.8 ± 2.0
EGTA, Mg^{2+}	94.9 ± 2.0	42.9 ± 2.3
EDTA	89.9 ± 2.2	87.0 ± 1.8
B: lL-8		
Ca^{2+}, Mg^{2+}	107.6 ± 1.6	32.7 ± 1.9
Ca^{2+} (no Mg^{2+})	102.8 ± 2.1	28.6 ± 2.1
EDTA, Mg^{2+}*	102.3 ± 2.2	26.7 ± 2.0
EDTA	99.7 ± 2.0	96.0 ± 1.9

Cells were preincubated with 50 μM thimerosal for 15 min at 37° and subsequently placed in the upper compartment of the Boyden chamber. In the lower compartment either 10^{-9} M fMLP or 4×10^{-9} M lL-8 was present. Random migration in the presence of Ca^{2+} and Mg^{2+} was 50.8 ± 1.9 μm (for the difference in random migration in the presence of EGTA etc., see Fig. 4). The concentration (if present) of Ca^{2+}, Mg^{2+}, EGTA or EDTA was 1 mM.

Divalent Cations

The activation of migration by thimerosal was not dependent on extracellular Ca^{2+}. The increase in migration was approximately the same in the presence of Ca^{2+} or Mg^{2+} alone. Traces of Ca^{2+} were not required, because in the presence of EGTA (with Mg^{2+}) the increase continued to be observed (Fig. 4). In the presence of EGTA alone, without added Mg^{2+}, only a slight decrease in stimulation was observed; under these conditions micromolar Mg^{2+} might still be present. However, stimulation of migration was considerably less in the presence of EDTA, i.e., in the absence of both Ca^{2+} and Mg^{2+} (Fig. 4). For inhibition of fMLP-activated chemotaxis by thimerosal, the difference was even more pronounced: while inhibition by thimerosal occurred both in the presence of Ca^{2+} or EGTA, inhibition was absent when EDTA was present (Table 1). In contrast, stimulation of migration by thiosalicylic acid was only slightly impaired in the presence of EDTA, and inhibition of fMLP-activated migration was not affected by the presence of EDTA (Table 2).

Thimerosal caused a slow increase in cytosolic free calcium concentration, as measured with the Fura-technique. The increase was most pronounced at higher concentrations of thimerosal than those required for stimulation of migration (Fig. 5A). In contrast with thimerosal, thiosalicylic acid had no effect on cytosolic free calcium at the concentrations studied (0.1–500 μM; some results are shown in Fig. 5B). Preincubation of neutrophils with thimerosal abolished the enhancement of cytosolic free calcium induced by 10 nM fMLP (Fig. 5C), while pretreatment of neutrophils with thiosalicylic acid had little effect on the fMLP-induced increase in cytosolic calcium.

CD11b Expression

Thimerosal caused a strong upregulation of CD11b expression in neutrophils. The effect occurred in a concentration-dependent manner; exposure of cells to both 10 μM and 50 μM thimerosal gave an enhancement, but the higher concentration was more effective (Fig. 6). As a comparison, the upregulation of CD11b expression induced by 10^{-9} and 10^{-6} M fMLP is shown. CD11b expression induced by fMLP was further enhanced by 50 μM thimerosal (results not shown). At a concentration of 1 μM (the optimal concentration for chemotaxis when applied in the lower compartment), thiosalicylic acid did not give a significant change in CD11b expression as compared with control cells; there was also no effect at a higher concentration (10 or 100 μM) of thiosalicylic acid.

DISCUSSION

Thimerosal has some effects on the neutrophil: it modifies migration and causes an increase in cytosolic free calcium as well as an upregulation of CD11b expression. The migration-changing properties of thimerosal are primarily confined to the thiosalicylic acid moiety of the molecule, because thiosalicylic acid itself is able to modulate migration. Though the Hg-C_2H_5 group might influence the effect on migration by causing a shift in the concentration required for activation, it is certainly not needed for an effect on migration. This also accounts, at least partly, for the inhibitory effect of higher concentrations of these compounds on chemotactic peptide-activated chemotaxis. In this regard, thimerosal and thiosalicylic acid resemble a number of other sulfur-containing agents, mostly antirheumatic drugs, all of which cause a cGMP-mediated increase in neutrophil migration at low concentrations as well as an inhibition at high concentrations [see 20 for a review]. It is tempting to speculate that at least part of the stimulatory effect and inhibitory effect of thimerosal and thiosalicylic acid is due to a comparable cGMP-mediated action. In addition, the presence of the mercury group might cause an additional inhibitory effect, because the decrease in migration per concentration unit was considerably less for thiosalicylic acid than with thimerosal. This is not exceptional, because thimerosal is a sulfhydryl reagent due to the mercury, and it is known that sulfhydryl reagents inhibit neutrophil migration [18, 19].

The activating and inhibitory concentrations of thimerosal on migration are quite similar, and the inhibitory effect sharply increases over a small concentration range. The sharp increase in inhibition with the increase in thimerosal concentration accounts for the fact that the maximal stimulating concentration (50 μM) is inhibitory for fMLP-activated chemotaxis. The application of a concentration of 50 μM in the lower compartment further implies that the concentration which is sensed by the migrating cell in a concentration gradient is considerably lower. The sharp decline in migration at higher thimerosal concentrations

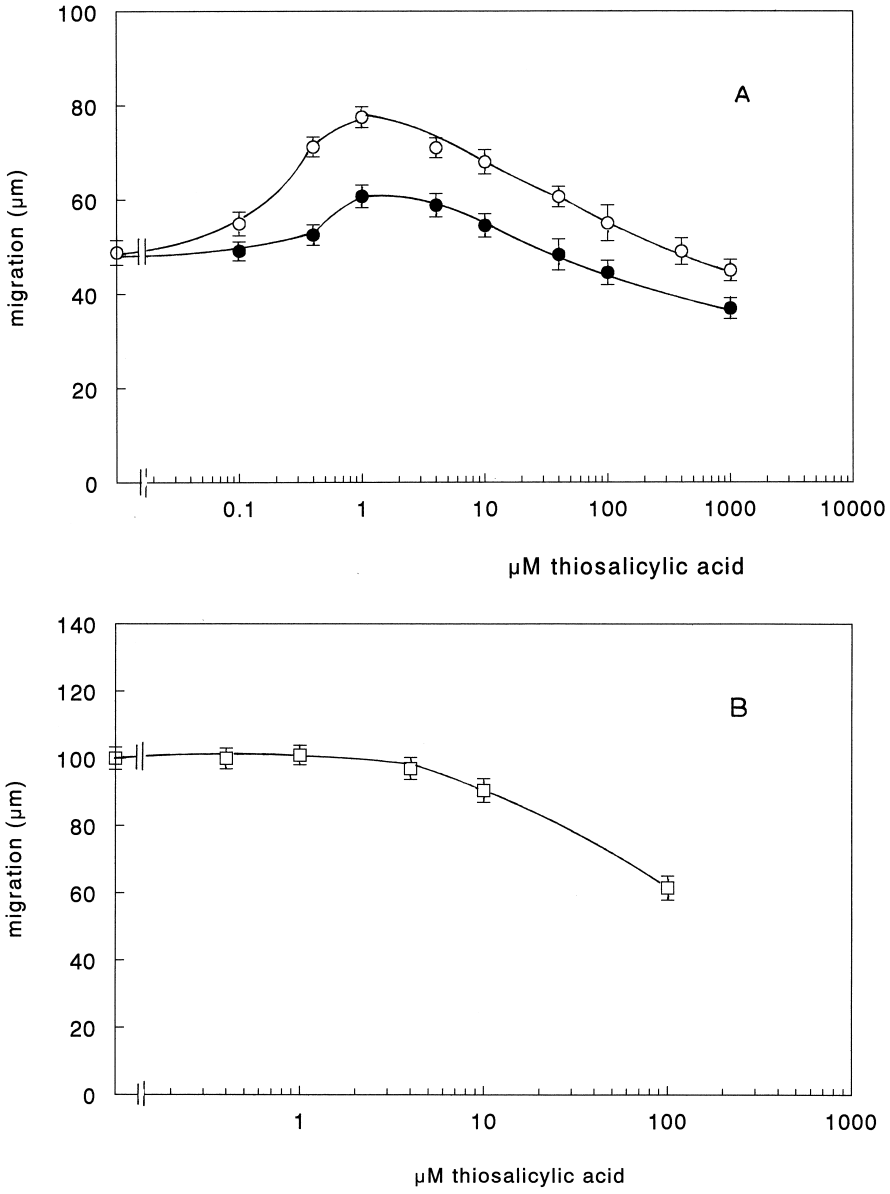

FIG. 3. The effect of thiosalicylic acid on random migration (A) and on fMLP-activated chemotaxis (B). For the effect of thiosalicylic acid on random migration (A), the compound was present in both compartments of the Boyden chamber (——●——), or in the lower compartment only (——○——). For the effect on fMLP-activated chemotaxis, cells were preincubated with the indicated concentration of thiosalicylic acid for 15 min and subsequently placed in the upper compartment of the Boyden chamber. A: values for concentrations between 0.4 µM and 40 µM (—○—), or for 4, 10, and 1,000 µM (—●—) are significantly different from control ($P < 0.05$); B: values for 10 and 100 µM are significantly different from control ($P < 0.05$).

may also be the cause of the maximum chemotactic migration being different from that for chemokinetic migration. For agents where the decrease in migration occurs over a larger concentration range, the optimal concentration for chemotaxis and chemokinesis is mostly the same. This applies not only to thiosalicylic acid, but also to a number of sulfur-containing agents which we have studied in recent years [20].

Hatzelmann et al. [13] found that thimerosal enhanced leukotriene formation in the presence of fMLP. Because some leukotrienes are chemotactic, this might provide a clue as to the induction of the chemotactic effect of thimerosal. However, there are several reasons why this is unlikely. Neither thimerosal nor fMLP alone caused an increase in leukotriene metabolites: this occurred only when both were present. However, thimerosal alone had a stimulating effect on migration. The release of leukotriene B_4 (LTB_4) induced by fMLP and thimerosal required extracellular calcium. This indicates that neither the stimulating nor the inhibitory effect of thimerosal is mediated by LTB_4, because the effects of thimerosal on migration still occur in the absence of extracellular calcium. Furthermore, the concentration of thimerosal which caused maximal stimulation of leukotriene formation in the presence of fMLP was inhibitory for fMLP-activated migration in the chemotaxis assay. That the enhancing effect of migration

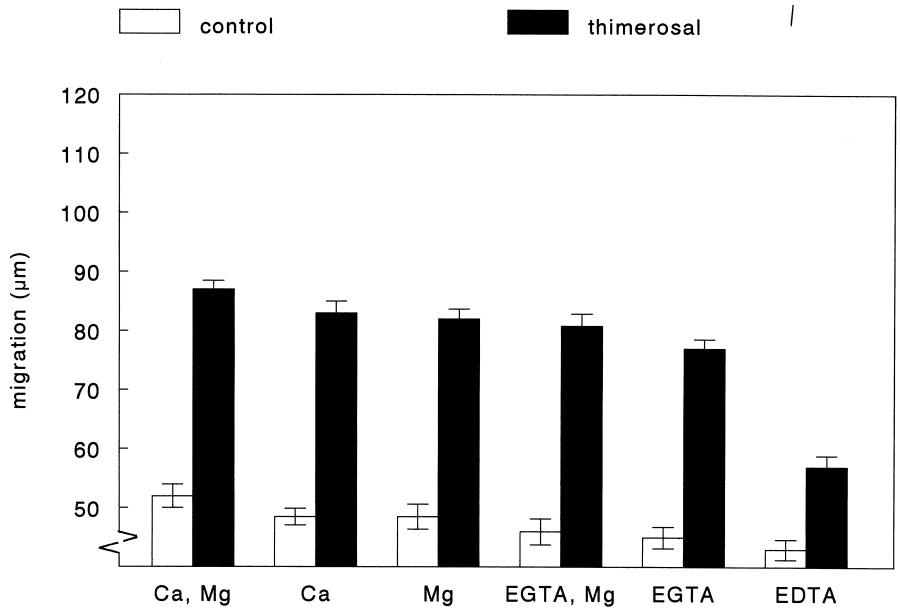

FIG. 4. The effect of calcium and magnesium on stimulation of migration by thimerosal. Open bars: control, no thimerosal; closed bars: migration stimulated by 50 μM thimerosal in the lower compartment. Ca: 1 mM Ca^{2+}; Mg: 1 mM Mg^{2+}; EGTA: 1 mM; EDTA: 1 mM. All values obtained with thimerosal are significantly different ($P < 0.01$) from control.

by thimerosal is due to leukotriene formation is thus highly unlikely.

The ability of thimerosal to induce an enhancement of $[Ca^{2+}]_i$ is related to the presence of Hg in the molecule, because thiosalicylic acid does not have the ability to change $[Ca^{2+}]_i$. The latter observation further implies that the effect on migration is not related to the effect on $[Ca^{2+}]_i$. This view is supported by the finding that there was no relation between the concentration of thimerosal which gives maximal stimulation of migration and that which gives the strongest increase in $[Ca^{2+}]_i$. Thimerosal causes an abolition of the fMLP-activated change in $[Ca^{2+}]_i$. This is probably not the only cause of inhibition of fMLP-activated chemotaxis by thimerosal, because thiosalicilic acid also inhibits fMLP- or lL-8-activated migration but has no effect on changes in $[Ca^{2+}]_i$ induced by either fMLP or lL-8.

TABLE 2. The effect of EDTA on stimulation of random migration and inhibition of fMLP-activated chemotaxis by thiosalicylic acid

	Migration (μm)	
	−	+ thiosalicylic acid
A: Stimulation of migration		
Ca^{2+}, Mg^{2+}	51.0 ± 1.8	76.3 ± 2.0
EDTA	43.7 ± 2.2	63.6 ± 1.9
B: Inhibition of fMLP-activated migration		
Ca^{2+}, Mg^{2+}	93.6 ± 1.9	54.6 ± 2.0
EDTA	83.7 ± 1.8	46.3 ± 2.1

(A): Thiosalicylic acid (1 μM) was absent (−) or present (+) in the lower compartment of the Boyden chamber. As the activating effect of thiosalicylic acid was studied, no fMLP was involved. (B) Cells were preincubated without (−) or with (+) 100 μM thiosalicylic acid for 15 min at 37°, and then placed in the upper compartment of the Boyden chamber (thiosalicylic acid remained present in the upper compartment during chemotaxis). In situation B, 10^{-9} M fMLP was present in the lower compartment. The concentration (where present) of Ca^{2+}, Mg^{2+}, or EDTA was 1 mM.

A change in the concentration of cytosolic free calcium in the neutrophil is not absolutely required for chemotactic migration, because fMLP-activated migration is possible in the absence of extracellular calcium and without a change in cytosolic free calcium [4]. On the other hand, removing all cellular calcium, or blocking calcium channels of intracellular stores with verapamil, impairs migration. Calcium plays a role in actin polymerization, and remodelling is essential for neutrophil migration. An increase in $[Ca_i]$, although not necessary for stimulus-induced actin polymerization, plays a role in filament severing. Furthermore, agents which cause a high level of $[Ca_i]$ such as ionophores, and cytochalasins which interfere with filament formation, are inhibitory for neutrophil migration [21]. It is therefore quite possible that thimerosal-induced calcium mobilization is partly responsible for the inhibition of fMLP-activated migration.

The results obtained—an enhancement of $[Ca^{2+}]_i$ by the sulfhydryl-reactive thimerosal, and no effect by thiosalicylic acid—support the view that the increase in $[Ca^{2+}]_i$ is due to a reaction of thimerosal via the mercury group, with critical sulfhydryl groups in structures that regulate calcium stores. These sulfhydryl groups have been found to be present in sarcoplasmic reticulum [22]. It thus seems likely that such sulfhydryl groups are also present on the calciosomes, which are endoplasmic reticulum-like structures which function in the neutrophil as calcium stores. It seems conceivable that the strong inhibition of fMLP- or lL-8-

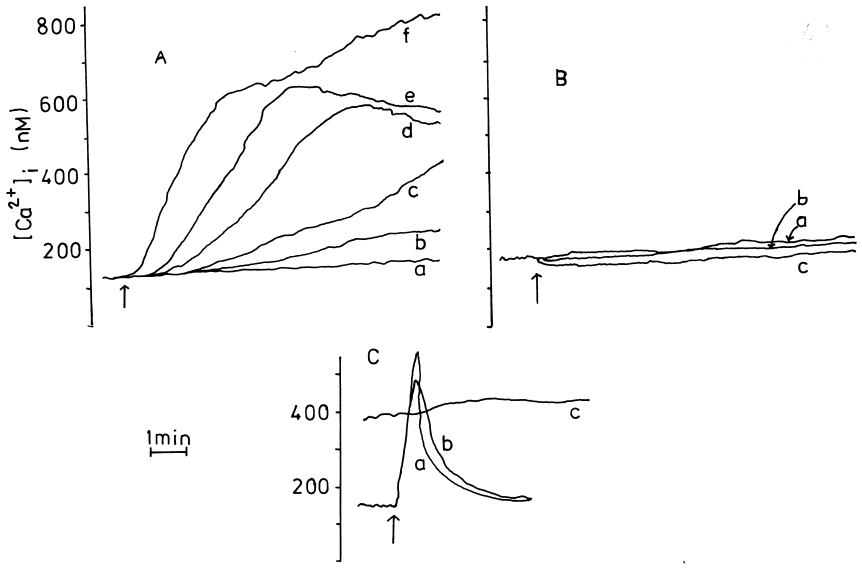

FIG. 5. The effect of thimerosal on $[Ca^{2+}]_i$ and on fMLP-induced changes of $[Ca^{2+}]_i$. A: effect of increasing concentrations of thimerosal on $[Ca^{2+}]_i$: a: control; b: 20 μM; c: 50 μM; d: 100 μM; e: 200 μM and f: 500 μM thimerosal. B: effect of increasing concentrations of thiosalicylic acid on $[Ca^{2+}]_i$: a: 1 μM; b: 10 μM; c: 200 μM thiosalicylic acid. C: the effect of preincubation without (a), with 100 μM thiosalicylic acid (b) or with 50 μM (c) thimerosal for 15 min at 37°, on the enhancement of $[Ca^{2+}]_i$ induced by 10 nM fMLP. The arrow indicates the time of addition of thimerosal (A), thiosalicylic acid (B), or fMLP (C).

induced chemotactic migration that occurs with high thimerosal concentrations is at least partly due to reaction with sulfhydryl groups, either the same or possibly other than those of the calciosomes. It is known that sulfhydryl-reactive agents strongly interfere with chemotaxis [18, 19]. It seems likely that by blocking these sulfhydryl groups, calcium channels are opened, as suggested by Abramson and Salama [22]. The other part of the inhibition at high concentrations of thimerosal resembles that of thiosalicylic acid and that of other sulfur-containing compounds [20, for review]; no adequate explanation for this effect has yet been made, but cGMP metabolism might very well be implicated.

Thimerosal caused an upregulation of CD11b expression both at chemotactic concentrations (for a concentration of 50 μM in the lower compartment, this optimum was between 0 and 50 μM, depending on the location of the cell in the filter) and inhibitory concentrations (50 μM and more). Neither chemotactic concentrations nor inhibitory concentrations of thiosalicylic acid had an effect on CD11b

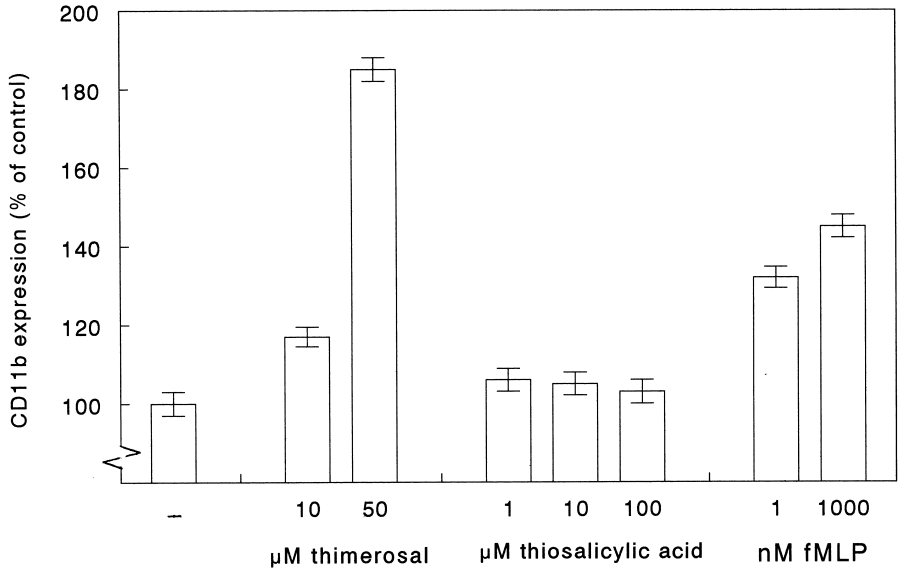

FIG. 6. The effect of thimerosal and thiosalicylic acid on CD11b expression. As a comparison the effect of fMLP is included. Cells were exposed to the indicated concentrations of agents for 10 min at 37°, after which the levels of CD11b were measured as described in "Materials and Methods" and expressed as a percentage of control (cells not treated with activator). Values given are the means ± SEM of three experiments. *$P < 001$; **$P < 0.001$ (as compared with control cells).

expression. This suggests that the upregulation of CD11b expression of neutrophils in suspension is primarily an indication for the calcium-mobilizing ability and inhibitory effect of thimerosal, rather than for its stimulating effect on migration. A correct comparison between chemotaxis and CD11b upregulation is difficult: in the test system of the Boyden chamber, cells are adherent and present in a concentration gradient, while CD11b upregulation is measured in suspension. It cannot be excluded that part of the lack of correlation between the effect on CD11b and on migration is due to this difference. On the other hand, a high expression of CD11b at high concentrations of thimerosal could explain the stronger inhibitory effect of thimerosal as compared with thiosalicylic acid. For migration, a certain degree of adhesion via adhesion molecules is required, but if the adhesion is too strong, the cells will no longer be able to displace themselves. The exact relation between CD11b expression and migration therefore remains to be determined.

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