AD-A193 791 PHARMACOLOGY OF PERIODONTAL DISEASE(U) UNIVERSITY OF HEALTH SCIENCES/CHICAGO MEDICAL SCHOOL IL DEPT OF PHARMACOLOGY S F HOFF 20 FEB 88 N00014-84-K-0562 1/1

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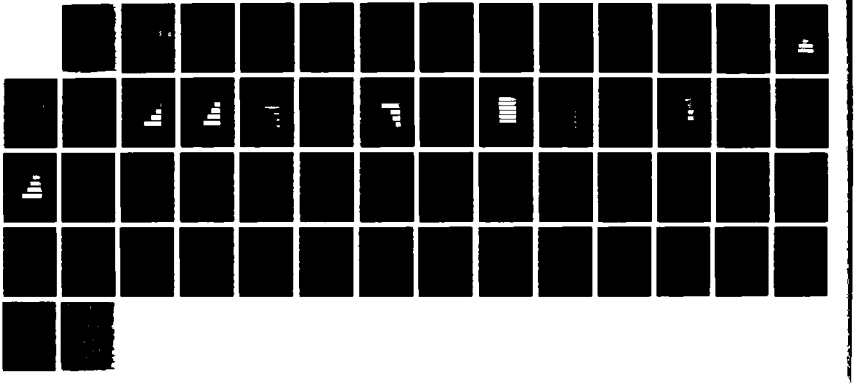

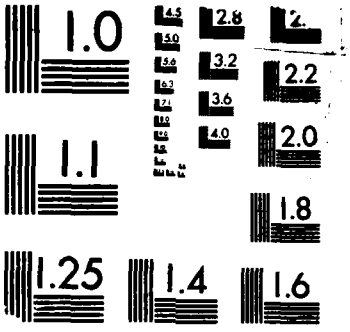

MICROCOPY RESOLUTION TEST CHART

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AD-A193 791

FINAL TECHNICAL REPORT:

PRINCIPAL INVESTIGATOR: STEVEN F. HOFF, Ph.D.
Assistant Professor

SS#: 566-60-0953

TITLE: PHARMACOLOGY OF PERIODONTAL DISEASE

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MAILING ADDRESS: Department of Pharmacology
University of Health Sciences/
The Chicago Medical School
3333 Green Bay Road
North Chicago, IL 60064

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LIST OF TABLES AND FIGURES

SUMMARY OF WORK:

Periodontal disease is a very prevalent disorder, and in the absence of proper oral hygience and dental care, it can progress to a painful inflammatory condition. These conditions require early professional attention, which poses an important problem to all phases of military operations. Professional care is not always readily available during field operations, and an acute episode of periodontal inflammation could decrease the combat efficiency of affected personnel or perhaps become mission abortive, especially during fleet or submarine missions. The latter situation is very costly in terms of time, effort, monies and mission deployments. It becomes imperative to provide early detection of acute periodontal inflammation and methods to prevent further progress of the disease, especially under field or fleet conditions.

There is a wide spectrum of pharmacological agents currently available for intervention in inflammatory and infectious disorders, however the present application of these drugs appear to be less than effective in periodontal disease. We have conducted a focused study of the cellular pharmacology of a number of agents, singly and in combination, which may be useful under field conditions to prevent progresssive periodontal disease. The major objective of this study has been the identification and evaluation of drugs with the greatest potential for preventing advanced periodontal disease under field condtions. The secondary objective of this study was to establish and provide research protocols for future evaluation of drugs potentially useful in pharmacological intervention in periodontal disease.

Our original study proposed using a set of anatomical and biochemical approaches for the quantitative evaluation of drugs in periodontal disease using the rice rat model system. After reevaluating the literature and discussions with the research group at the Naval Dental Research Institute, we decided to ~~modify the work by using~~ isolated human polymorphonuclear leukocytes (PMN) as our model system for drug evaluation. This was done for several reasons. (1) The rice rat does NOT accurately parallel the human disease state. (2) The rice rat model involves very complex tissues, containing many different cell types, which would confound the quantitative evaluation of drug activities. (3) In humans, the PMN is the primary cell type responding to bacterial invasion and plaque-accummulation associated with periodontal disease. Also, they may cause the subsequent progress to a chronic inflammatory state with destruction of supporting periodontal tissues. (4) Because the PMNs are so important in the periodontal inflammatory process, the isolated human PMN provide an excellent simplified model for the study of drug actions.

In order to accomplish our objectives, we established five different assays to evaluate drug effects on PMN functions. A brief description of each assay is given below, with a detailed protocol and chemical/solutions list provided in the methods section of this report.

CHEMOTAXIS ASSAY:

An important aspect of the inflammatory process is the ability of the PMNs to migrate to a diseased site. The effects of various anti-inflammatory and antimicrobial drugs can be studied using this assay to monitor cell motility and chemotaxis (ie. the ability to migrate toward a stimulating chemical). Briefly, the PMNs are placed in a central cell of an agrarose-filled petri dish and allowed to migrate between the agarose layer and the bottom of the dish. One of five wells surrounding the cells contains a solution of FMLP (N-formyl-methionyl-leucyl-phenylalanine), which is a potent chemotactic stimulant. PMNs can be incubated in the presence or absence of various drugs, and after two hours the cells are quickly fixed and stained. Two parameters of chemotaxis, distance traveled and area of cell migration, have been evaluated on a computerized image analyzer.

DEGRANULATION ASSAYS:

During an inflammatory response, PMNs are stimulated to release several types of specific granules, which contain different hydrolytic enzymes and other proteins. Under stimulated conditions we assayed for (1) lysozyme, (2) beta glucuronidase, and (3) lactoferrin. In this way we were able to differentiate the types of granules released under various conditions, including drug treatment.

SUPEROXIDE ASSAY:

Superoxide production is one of the primary mechanisms by which PMNs destroy bacteria. Excessive production of superoxide radicals may also cause damage to normal healthy tissues.

In addition to the above 5 assays, we included an assay for the release of LACTATE DEHYDROGENASE, which provides an accurate assessment of cell viability.

DRUG EVALUATION:

In determining which drugs were to be evaluated, we first conducted a thourough literature search and found that several non-steroidal anti-inflammatory agents (NSAID) and antimicrobials had been evaluated by the above methods (TABLE I. From the literature). We then chose several compounds from different groups of NSAIDs (Tolmetin, Meclofenamate) and antimicrobials (Chlorhexidine, Amoxicillin and Doxycycline). After completing our study of these compounds, we were able to decide which combinations of a NSAID and antimicrobial might demonstrate the most effective actions on PMN functions. In this case we were trying to avoid antagonistic effects between the two drugs, and perhaps demonstrate some synergism, which could be very beneficial under therapeutic conditions. The table below provides a summary of the effects we found in the literature and in our studies. A complete description of our results follows this Table.

TABLE I. SUMMARY OF DRUG EFFECTS

DRUG:	CHEMOTAXIS	ENZYME RELEASE			SUPEROXIDE PRODUCTION
		BETA-GLUC	LYSOZYME	LACTOFERRIN	
TOLMETIN	I	D	D	-	D
MECLOFENAMATE	D	D	D	D	D
CHLORHEXIDINE	D	ne	ne	ne	I
DOXYCYCLINE	D*	ne	ne	ne	D
AMOXICILLIN	ne	ne	ne	ne	ne
		LYSOSOMAL ENZYME RELEASE			
(From the literature)					
INDOMETHACIN	D	Decreased			D
NAPROXEN	D	Decreased			NR
IBUPROFEN	D	Decreased			D
ASPIRIN	D	Decreased			D
CHLORTETRACYCLINE	D	Decreased			D
CLINDAMYCIN	±	Decreased			D
DRUG COMBINATIONS:					
MECLOFENAMATE + CHLORHEXIDINE	D[a]	-	-	-	D[a]
MECHLOFENAMATE + DOXYCYCLINE	D[b]	-	-	-	D[a]

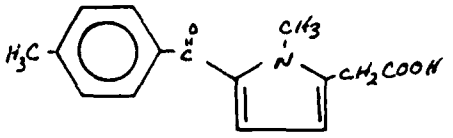
I=increased; D=decreased; ne=no effect; NR=not reported
±=conflicting reports; -=not done; * = from the literature

[a]=not significantly different from mechlofenamate alone

[b]=significantly different from mechlofenamate or doxycycline alone (ie. Synergistic effect)

RESULTS:

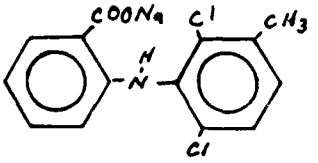
TOLMETIN



Tolmetin is structurally unique among anti-inflammatory compounds, and was included in this study for that reason. It is more potent than aspirin and less potent than indomethacin, with respect to anti-inflammatory activity.

This non-steroidal anti-inflammatory drug (NSAID) has proven to be unusual in one way from other NSAIDs in that it significantly stimulates rather than inhibits PMN chemotaxis (Figure 1) (ANOVA $F=10.828$, df 3, $p< .01$). The Tukey-HSD procedure for multiple ranges shows this effect to be significant from control ($p< .05$) at the 50 ug/ml drug concentration. This is very clearly a dose-dependent process, which we have demonstrated occurs at drug concentrations found under therapeutic conditions. In addition Tolmetin causes a significant dose-dependent reduction in the release of beta-glucuronidase (dot filled bars) and lysozyme (hatched bars) from azurophilic and specific granules respectively (ANOVA $p<.01$ for both enzymes) (Figure 2). The Tukey HSD procedure specifies that the effect is significant ($p<.05$) at the 100 ug/ml drug concentration. Cell death does not significantly increase with larger doses as indicated by the steady lactate dehydrogenase (LDH) levels (solid bars in Figure 2). The production of superoxide radials is also significantly inhibited by Tolmetin (Figure 3) (ANOVA $F=22.191$, df3, $p<.01$), with the effect significant at all drug concentrations (Tukey-HSD, $p<.05$). Our work on Tolmetin was presented at the 1986 FASEB meeting in St. Louis (see attached abstract).

MECLOFENAMATE



Meclofenamate is a member of the fenamates, which are a group of aspirin-like drugs. This NSAID is somewhat different from Tolmetin and appears to be much more potent in our assays. For example, Meclofenamate causes a significant dose-dependent reduction in chemotaxis by PMNs (Figure 4) (ANOVA, $F=133.979$, df4, $p<.0001$), with complete inhibition at 20 ug/ml. The Tukey-HSD

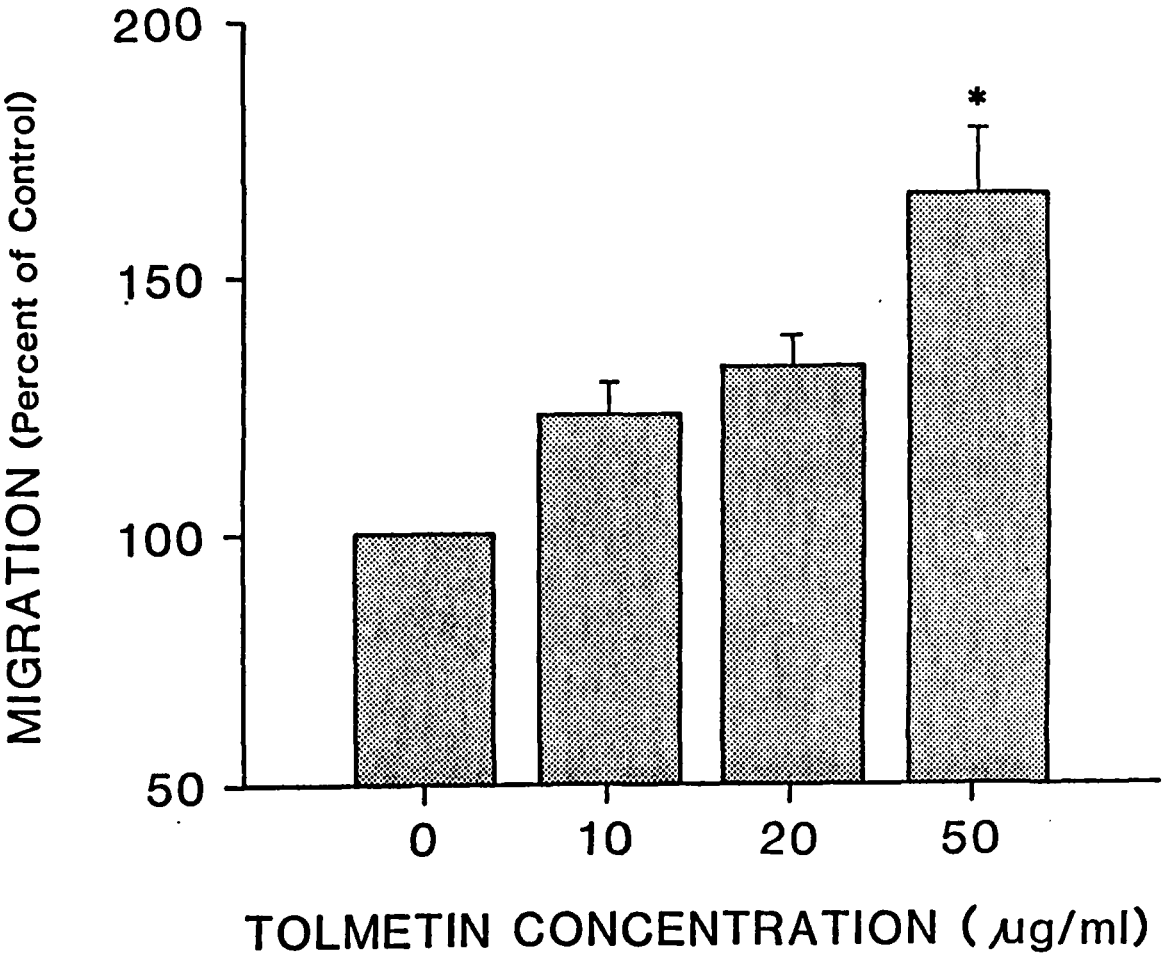


FIGURE 1. Tolmetin-Chemotaxis

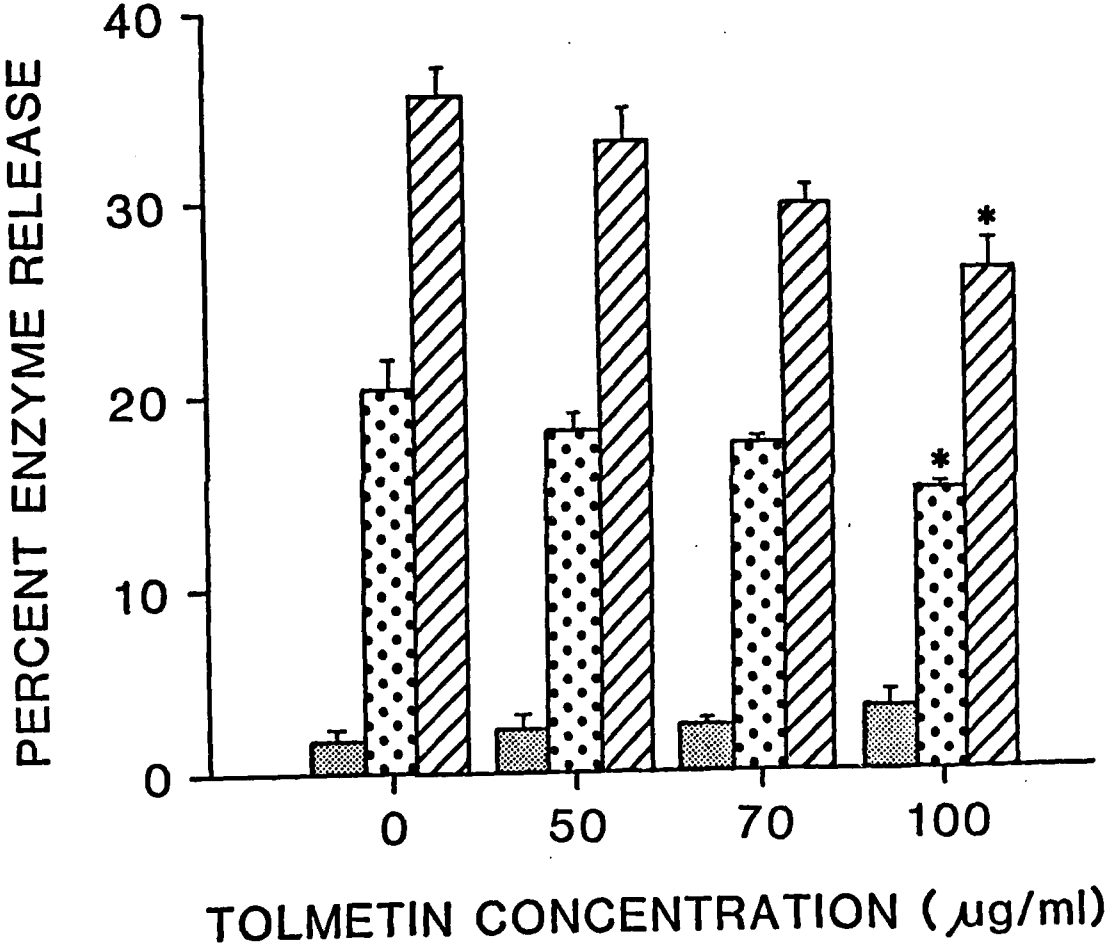


FIGURE 2: Tolmetin-Degranulation

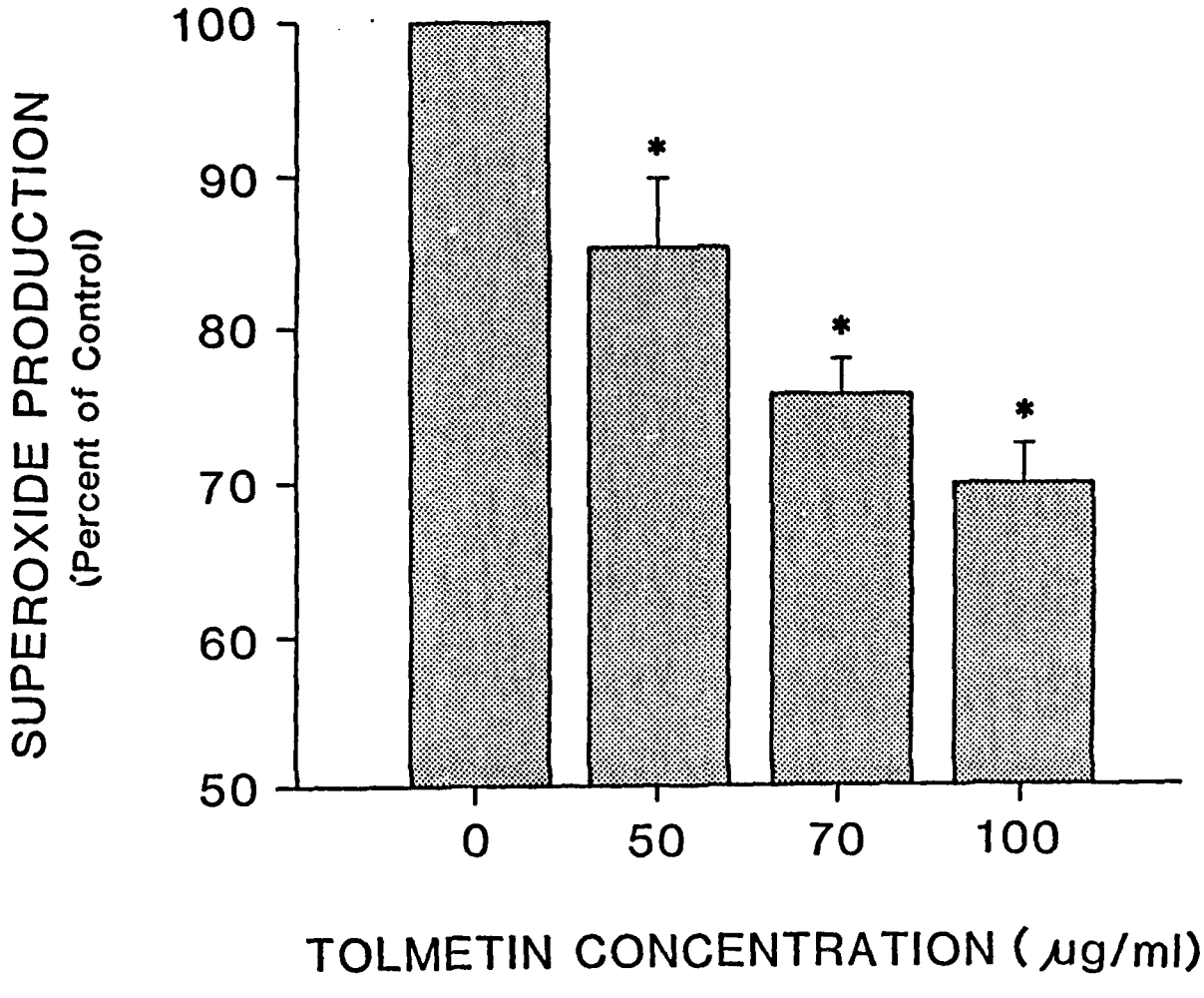
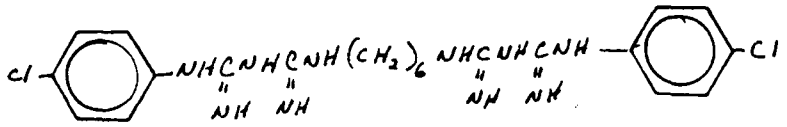


FIGURE 3: Tolmetin-Superoxide

procedure reveals that the drug effect is significant at the 5, 10 and 20 ug/ml concentrations ($p<.05$). Meclofenamate also causes a pronounced reduction in the release of beta-glucuronidase (ANOVA $F=29.293$, df3, $p<.0001$), lysozyme (ANOVA $F=38.622$, df2, $p<.0001$), (Figure 5) and lactoferrin (ANOVA $F=16.3922$, df3, $p<.05$) (Figure 6), which is significant at all three drug concentrations (Tukey-HSD, $p<.05$). Meclofenamate does not cause any significant increase in cell death under our experimental conditions (ANOVA, $F=2.875$, df3, $p>.1$) (Figure 5). Superoxide production is also markedly inhibited (Figure 7) (ANOVA $F=12.707$, df3, $p<.005$), and this is significant at the 20 and 50 ug/ml drug concentrations (Tukey-HSD, $p<.05$).

CHLORHEXIDINE



Chlorhexidine is a very potent antibacterial, which is applied topically. As with most NSAIDs, this antibacterial agent also caused a significant dose-dependent reduction in chemotaxis (Figure 8) (ANOVA $F=24.519$, df3, $p<.0002$), which was significant at the 15 and 25 ug/ml drug concentrations (Tukey-HSD, $p<.05$). Chlorhexidine did NOT alter the stimulated release of beta-glucuronidase (ANOVA $F=0.157$, df3, $p>.9$), lysozyme (ANOVA $F=0.073$, df3, $p>.9$) (Figure 9), or lactoferrin (ANOVA $F=0.2066$, df3, $p>.8$) (Figure 10), eventhough a significant increase (ANOVA $F=6.510$, df3, $p<.02$) in the amount of cell death occured at the 100ug/ml drug concentration (Tukey-HSD, $p<.05$) (Figure 9). An interesting effect was observed, where a significant increase (ANOVA $F=9.532$, df3, $p<.01$) in the production of superoxide radicals occured in the presence of drug concentrations non-lethal to the PMNs (50ug/ml, Tukey-HSD, $p<.05$) (Figure 11). It should be noted that some variation in drug effects were observed between different lot numbers of chlorhexidine. At this point it is not clear if these variations are caused by inadequate quality control or deterioration with increasing shelf aging.

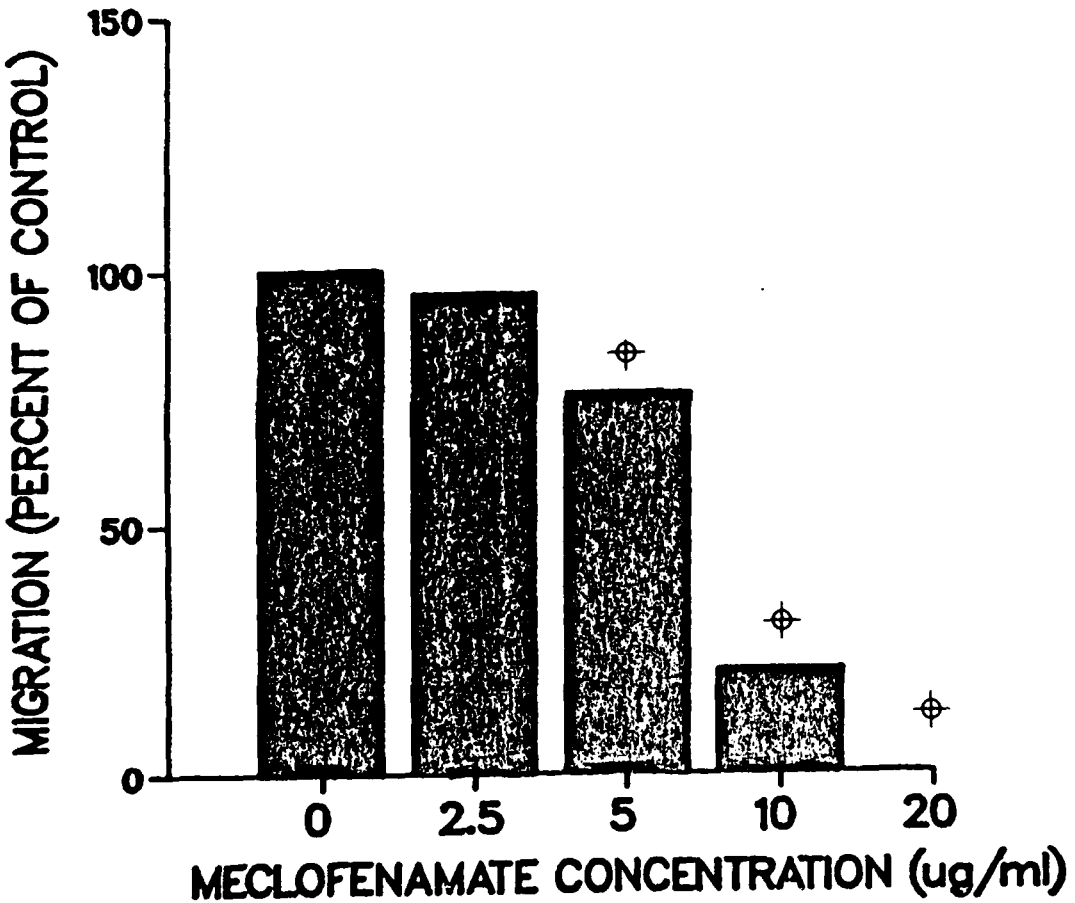


FIGURE 4: Meclofenamate-Chemotaxis

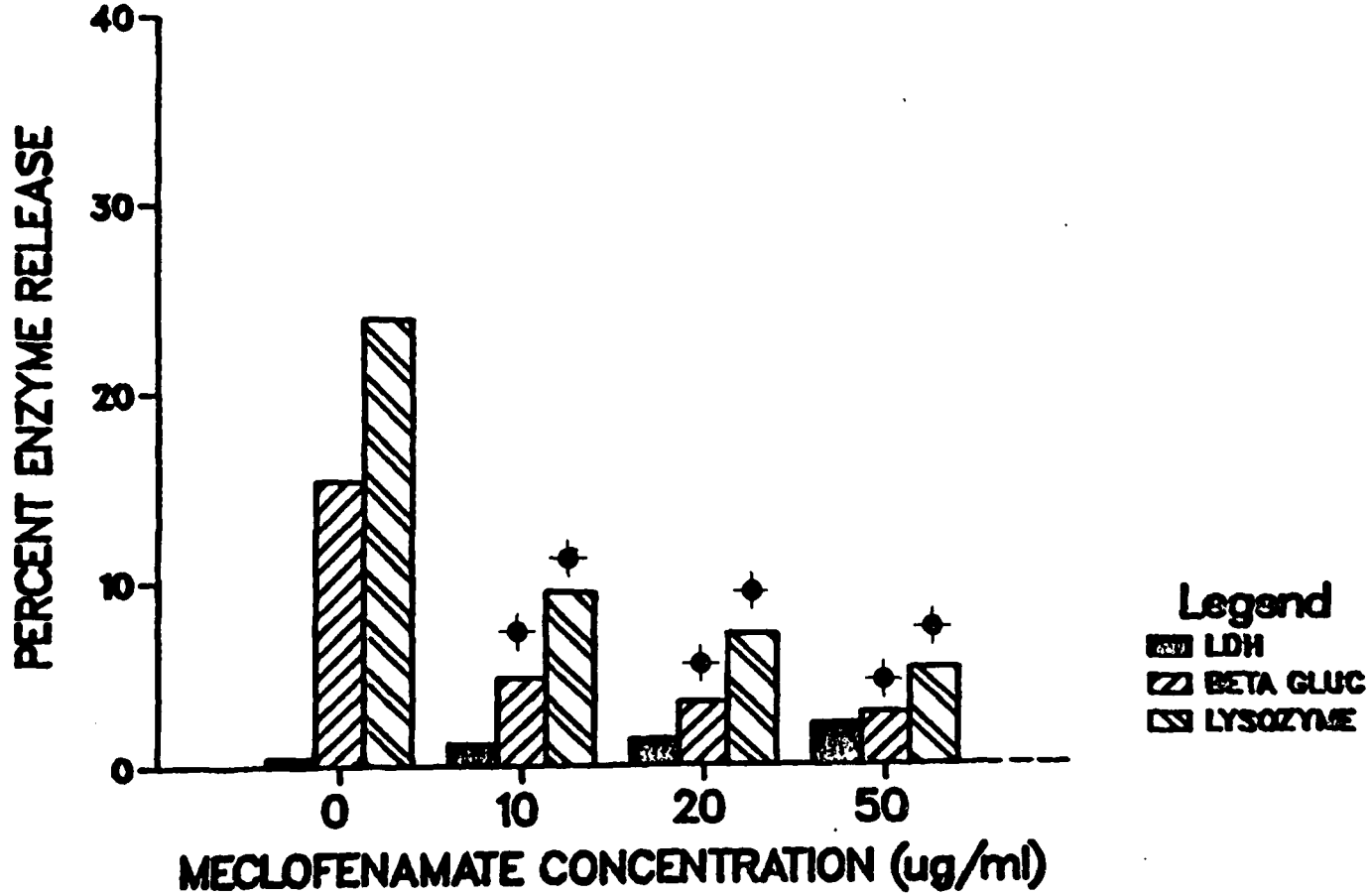


FIGURE 5: Meclofenamate-Degranulation

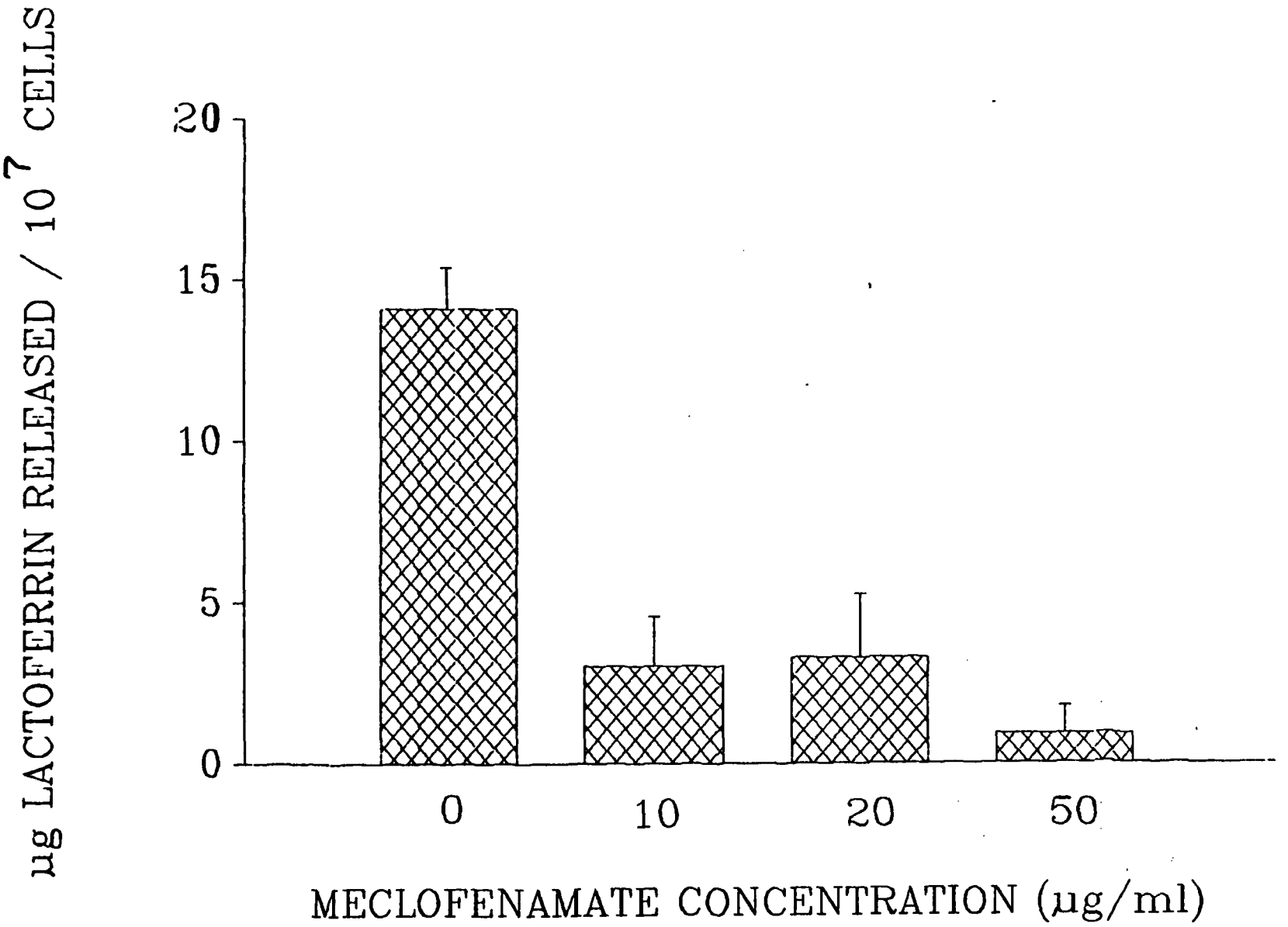


FIGURE 6: Meclofenamate-Lactoferrin

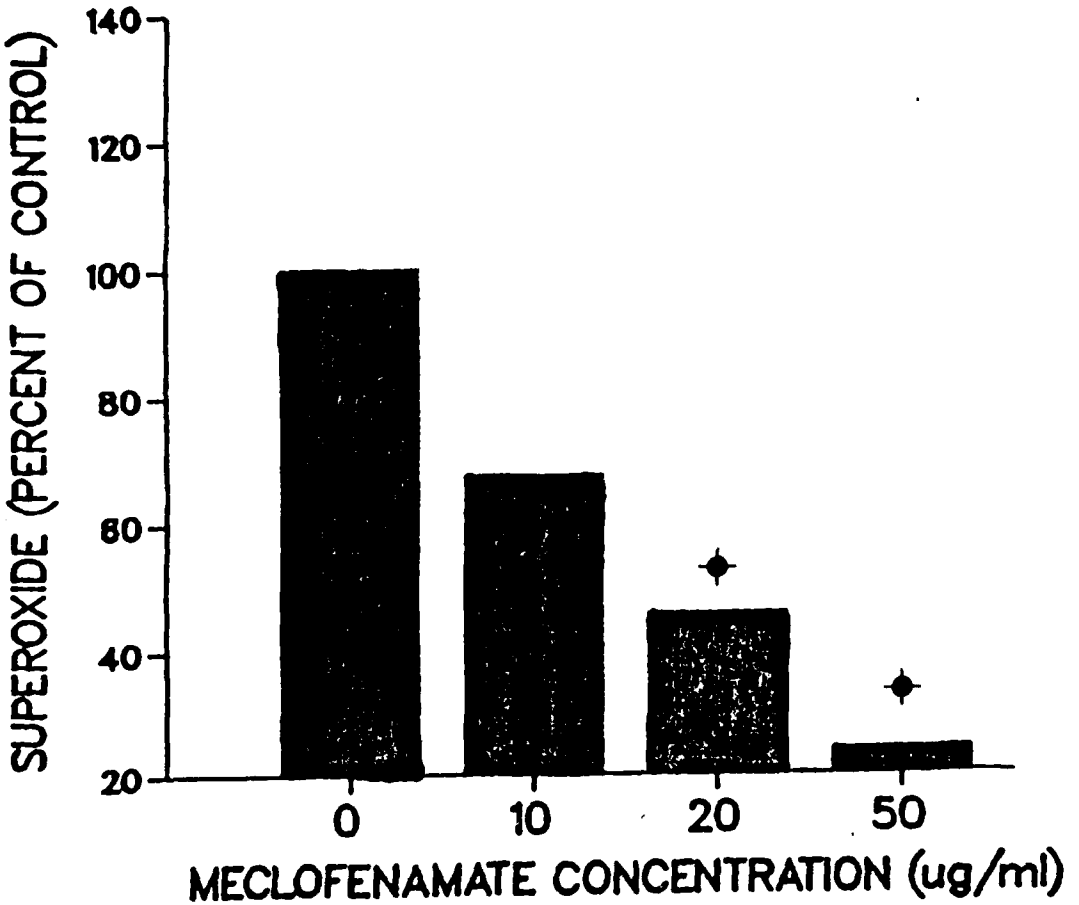


FIGURE 7: Meclofenamate-Superoxide

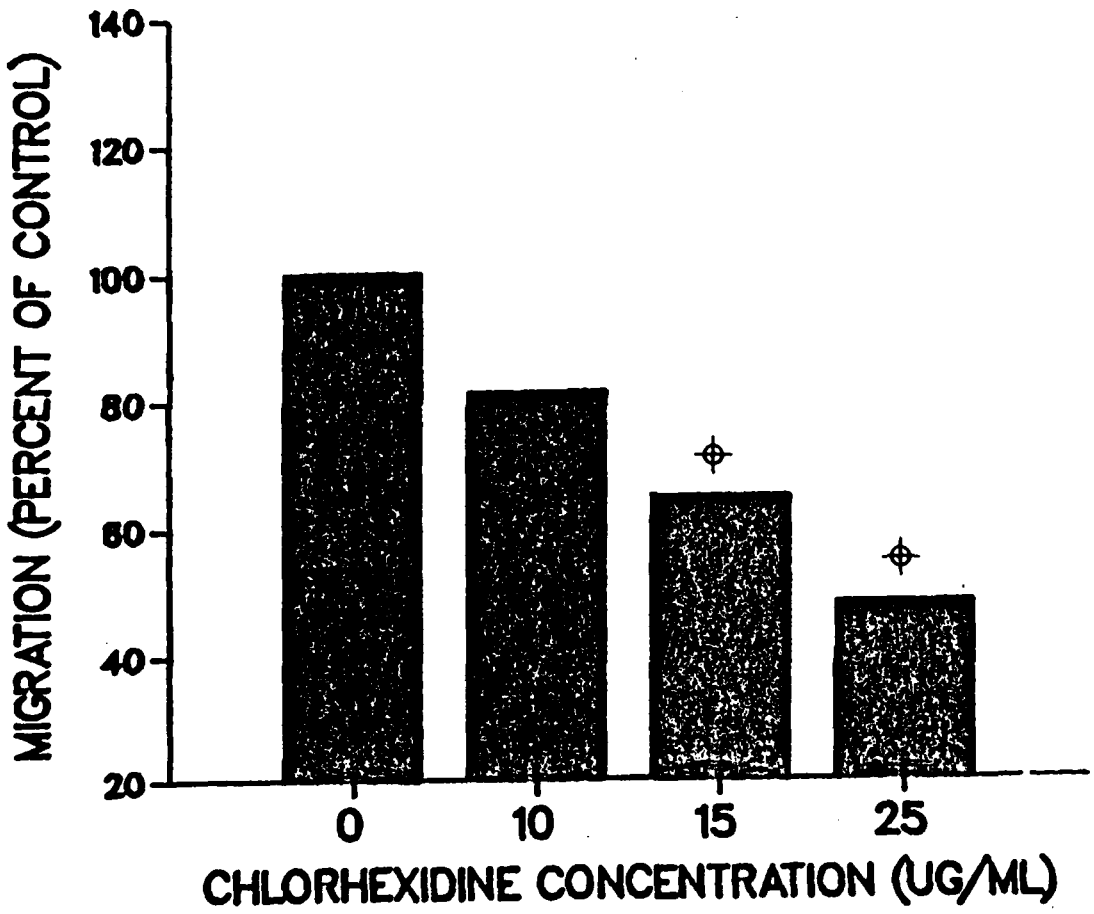


FIGURE 8: Chlorhexidine-Chemotaxis

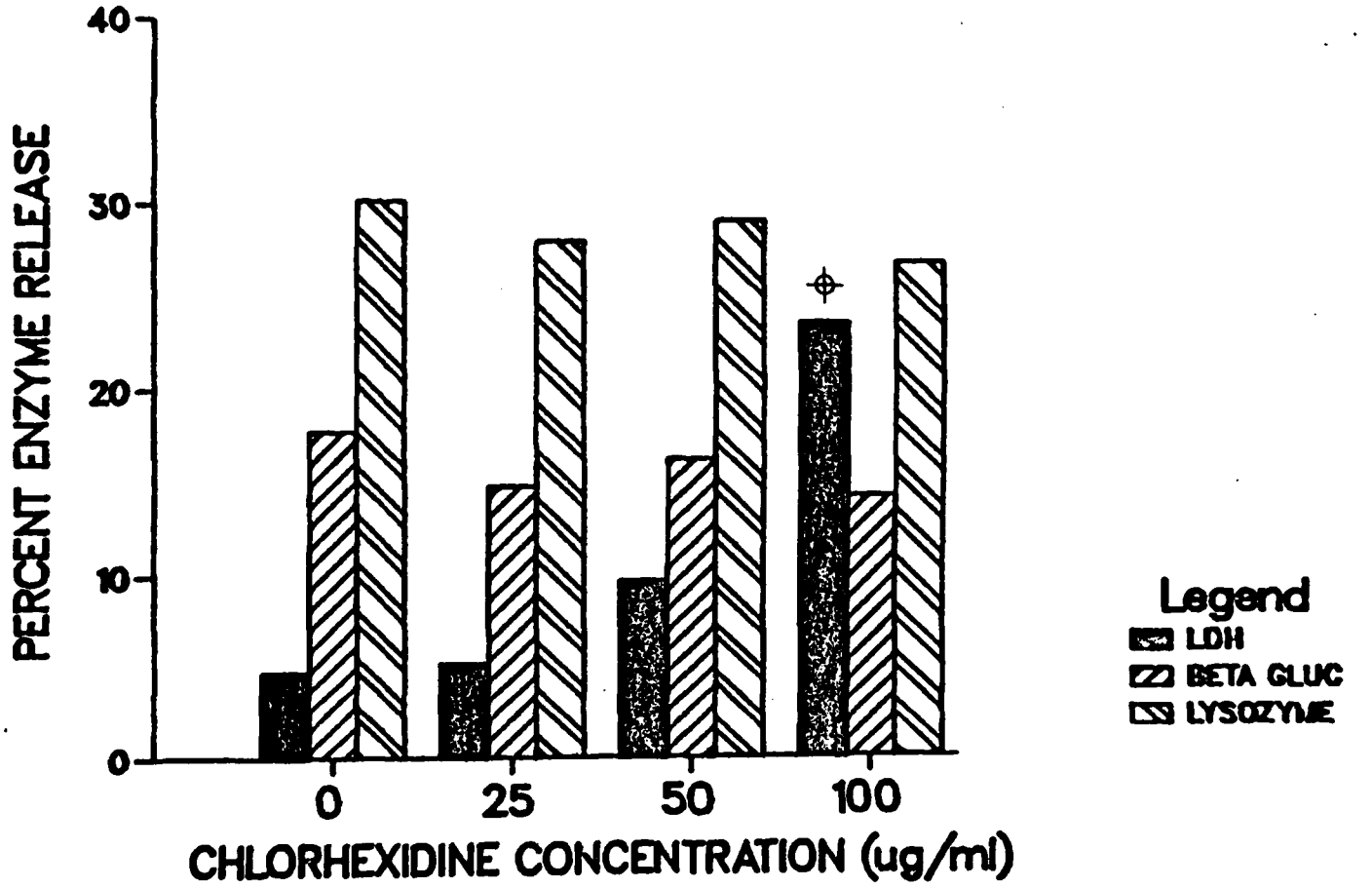


FIGURE 9: Chlorhexidine-Degranulation

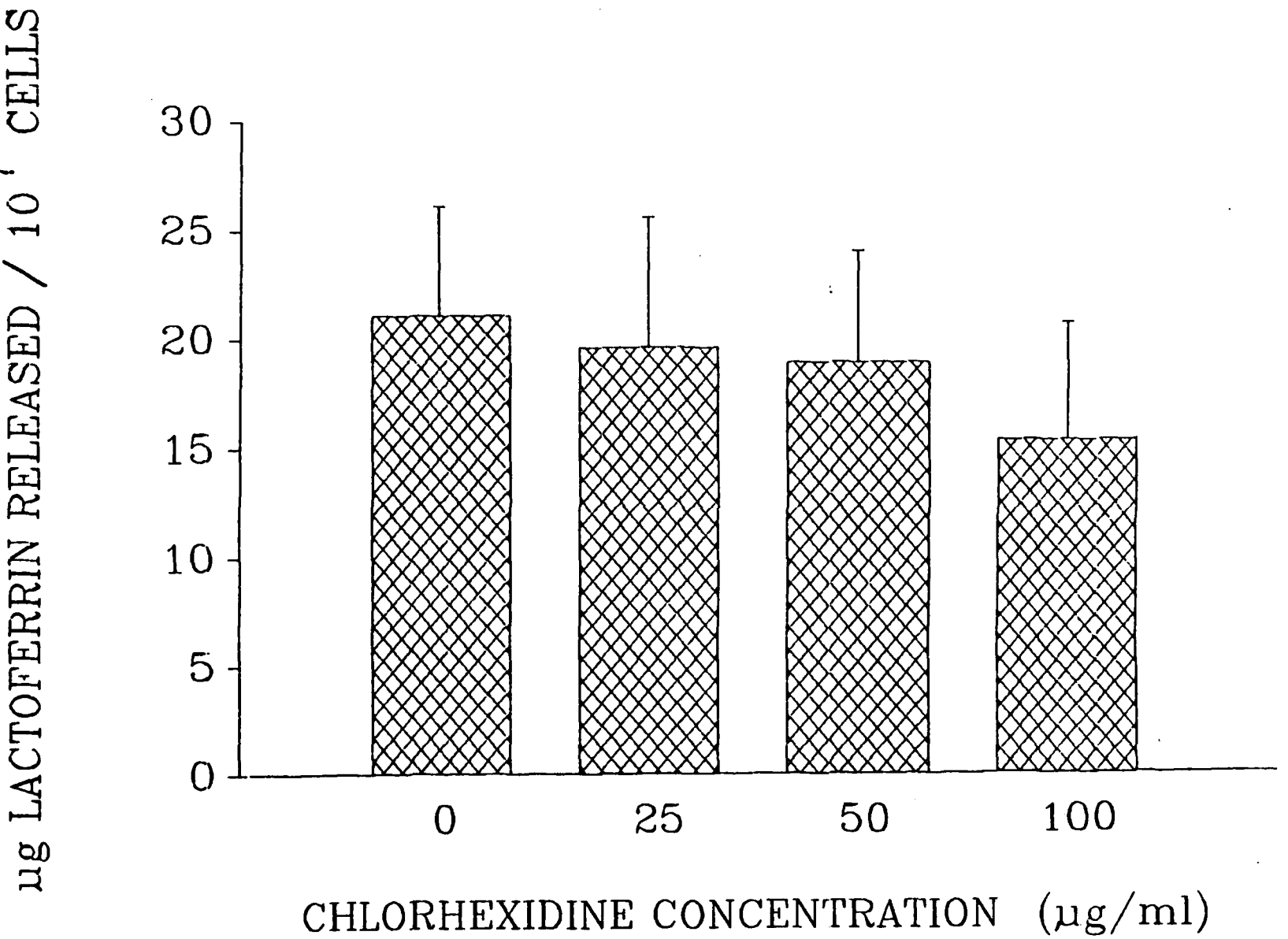


FIGURE 10: Chlorhexidine-Lactoferrin

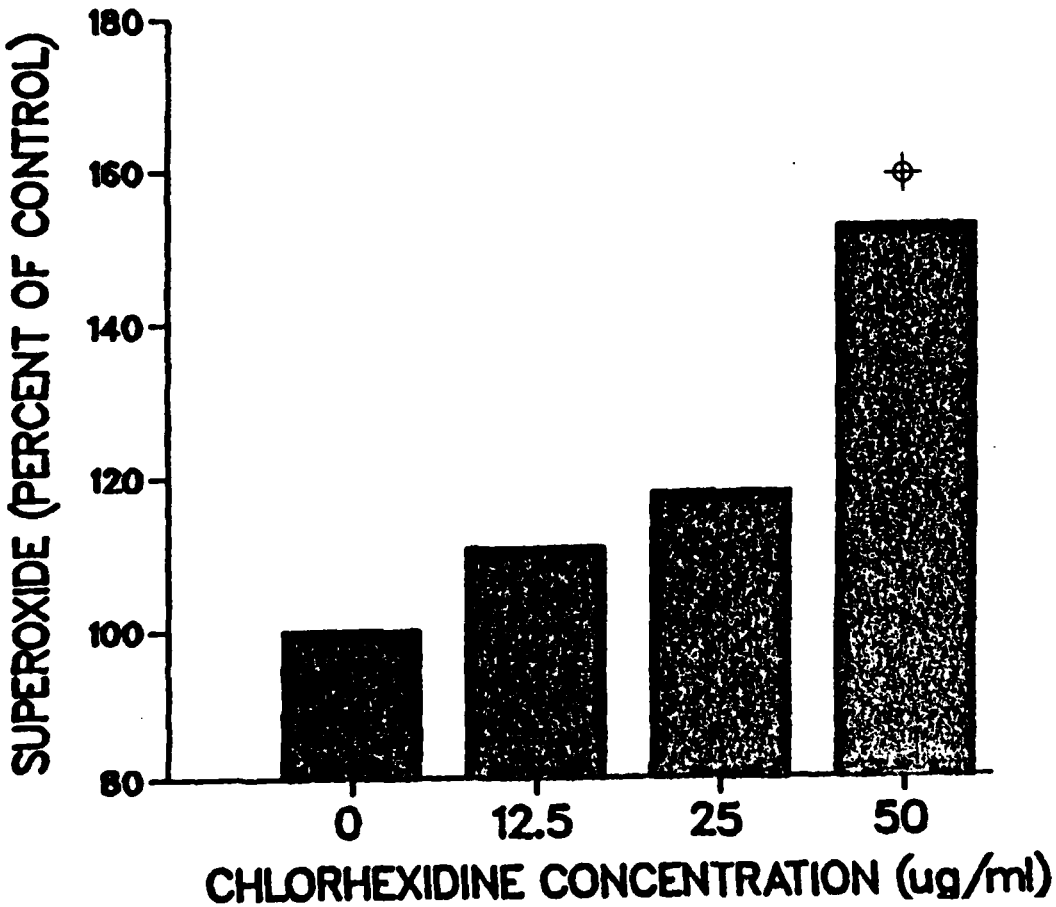
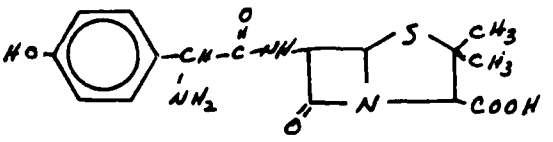


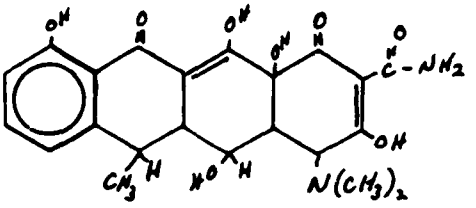
FIGURE 11: Chlorhexidine-Superoxide

AMOXICILLIN



Amoxicillin is a broad spectrum antibiotic from the penicillin family. It is beta-lactamase sensitive, so that its use in many staphylococcal infections is limited. However, when combined with a newly marketed compound, Clavulanic acid, which inhibits beta-lactamases, the spectrum of Amoxicillin is greatly extended. Amoxicillin had no significant effects on chemotaxis (ANOVA F=0.214, df5, p>.9) (Figure 12), or degranulation of beta-glucuronidase (ANOVA F=0.063, df4, p>.9) (Figure 13), lysozyme (ANOVA F=0.335, df4, p>.8) (Figure 13), and lactoferrin (ANOVA F=0.3687, df4, p>.8) (Figure 14). Also, there was no effect on the amount of cell death as measured by lactate dehydrogenase (ANOVA F=0.018, df4, p>.9) (Figure 13), or on superoxide production (ANOVA F=2.293, df3, p>.1) (Figure 15).

DOXYCYCLINE



Doxycycline is one of the broad spectrum tetracycline antibiotics. Doxycycline's effect on chemotaxis has been reported in the literature several times, and it causes a significant decrease in PMN chemotaxis (see Table I). It has no significant effect on degranulation, as the release of beta-glucuronidase (ANOVA F=1.683, df4, p>.2), lysozyme (ANOVA F=1.819, df4, p>.1) (Figure 16) and lactoferrin (ANOVA F=0.3952, df4, p>.8) (Figure 17) are not affected at any of the concentrations tested . Also, cell death is not significantly affected (ANOVA F=0.423, df4, p>.7) (Figure 16). It is interesting that Doxycycline causes a signficant reduction in superoxide production (ANOVA F=11.915, df3, p<.005) at the 5 and 10 ug/ml concentrations (Tukey-HSD, p<.05) (Figure 18).

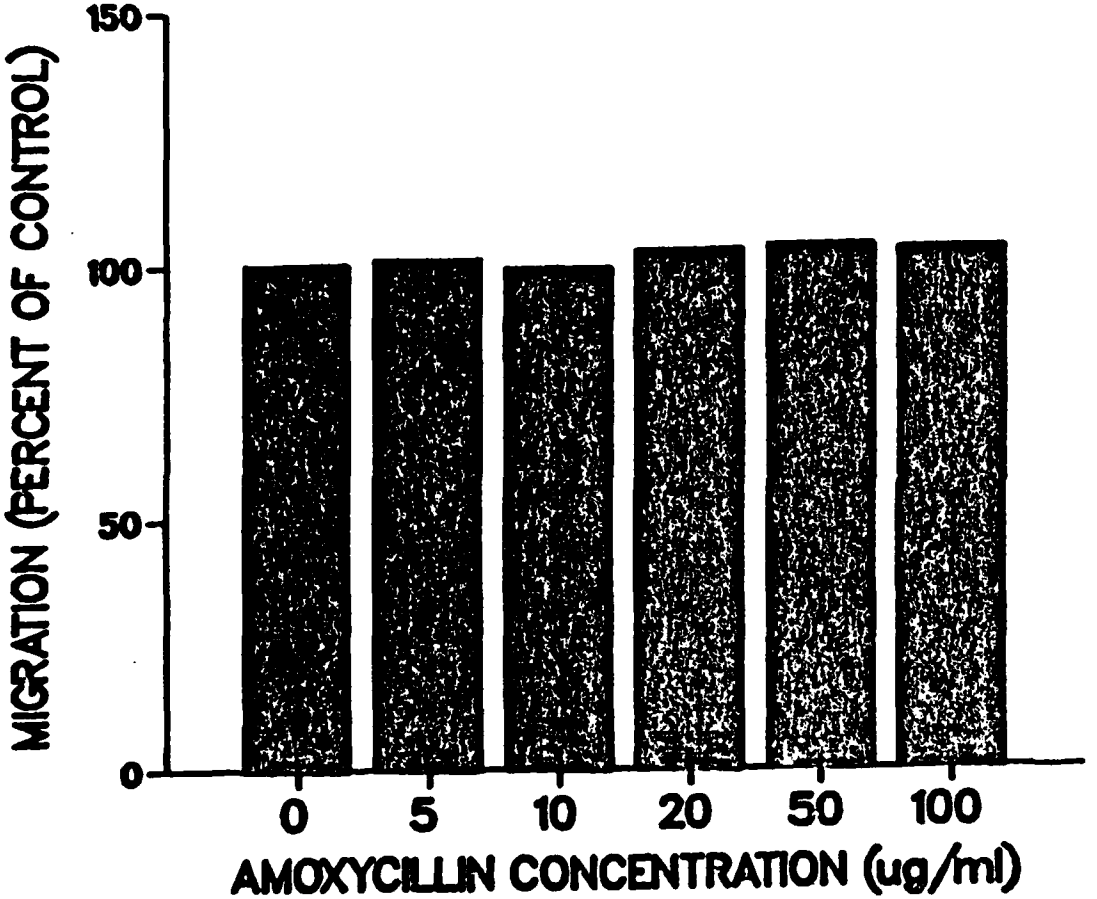


FIGURE 12: Amoxicillin-Chemotaxis

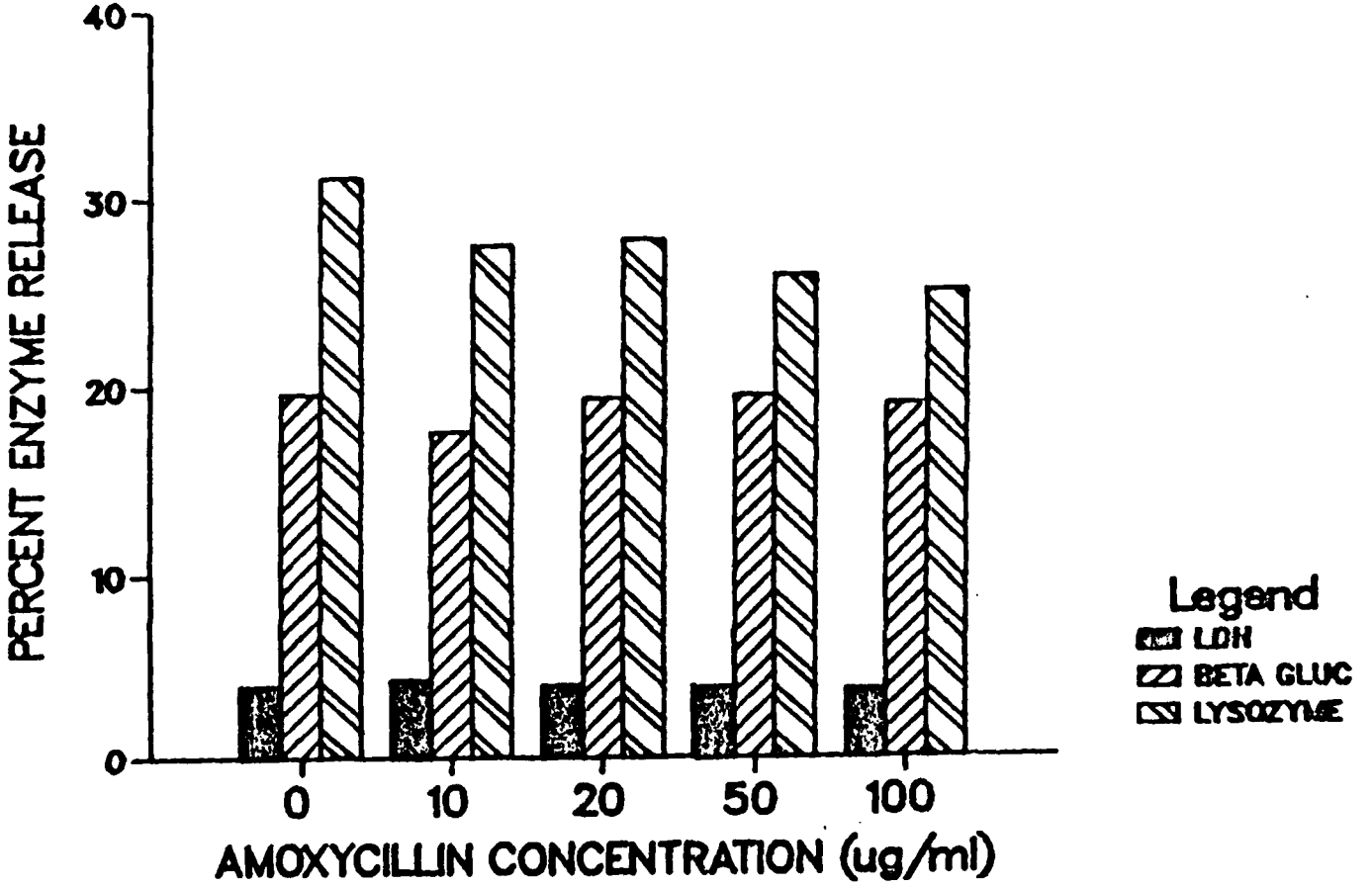


FIGURE 13: Amoxicillin-Degranulation

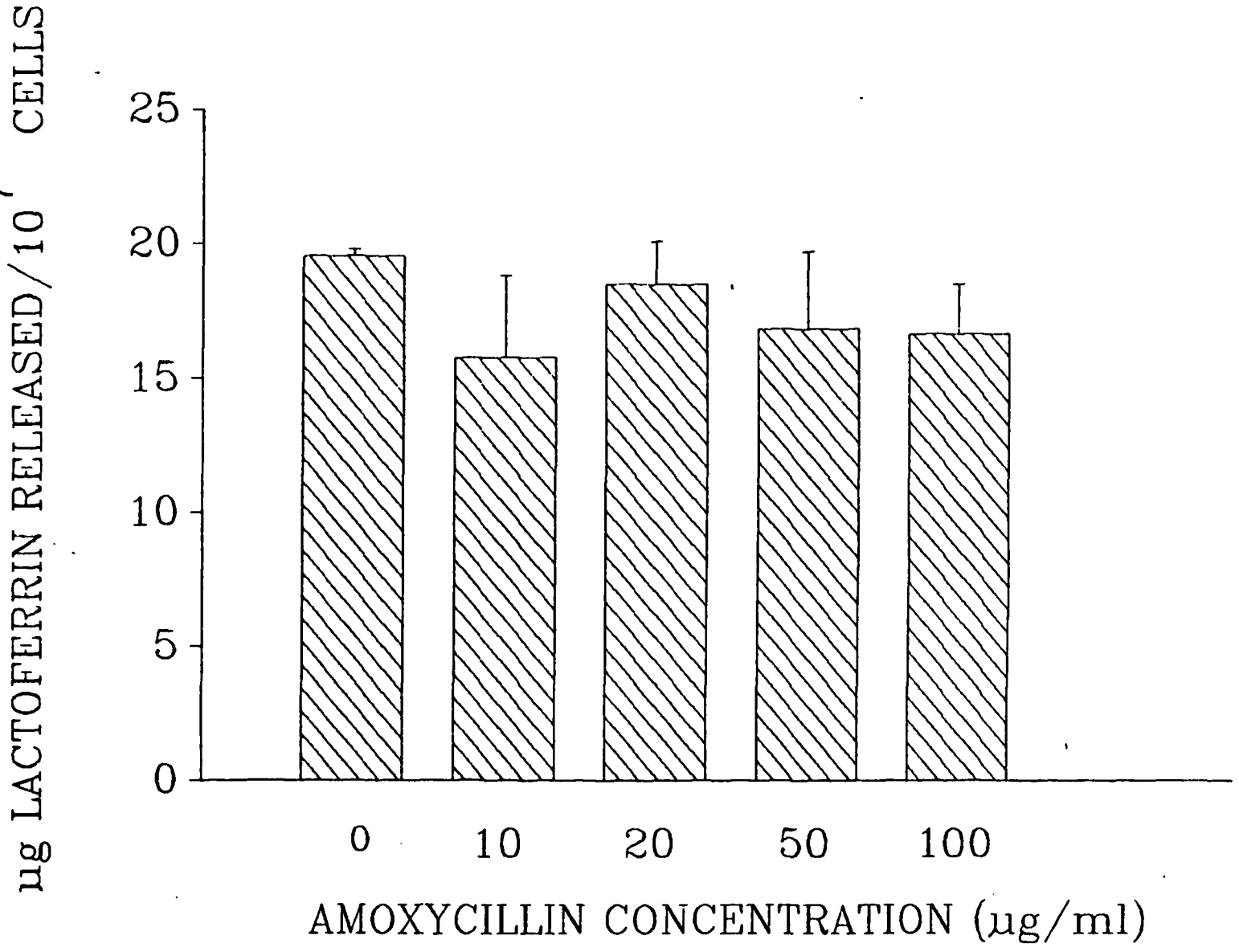


FIGURE 14: Amoxicillin-Lactoferrin

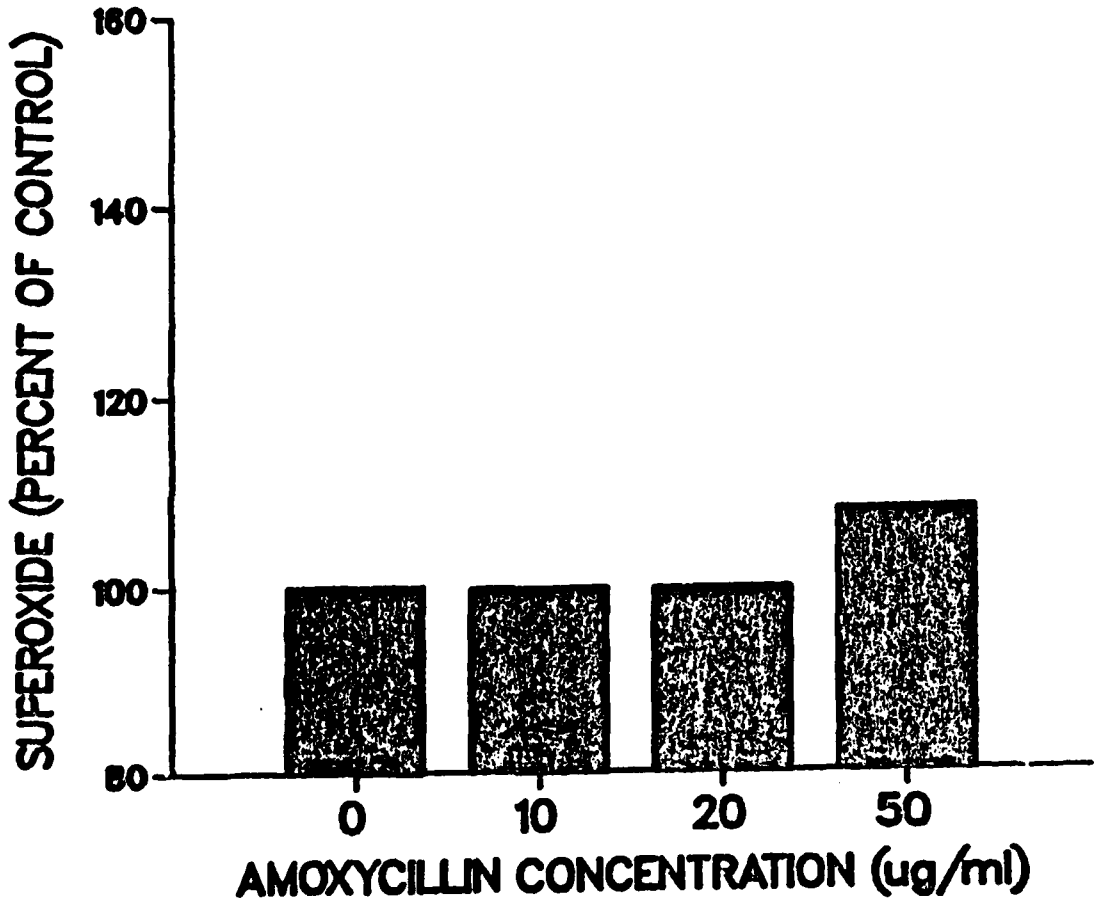


FIGURE 15: Amoxicillin-Superoxide

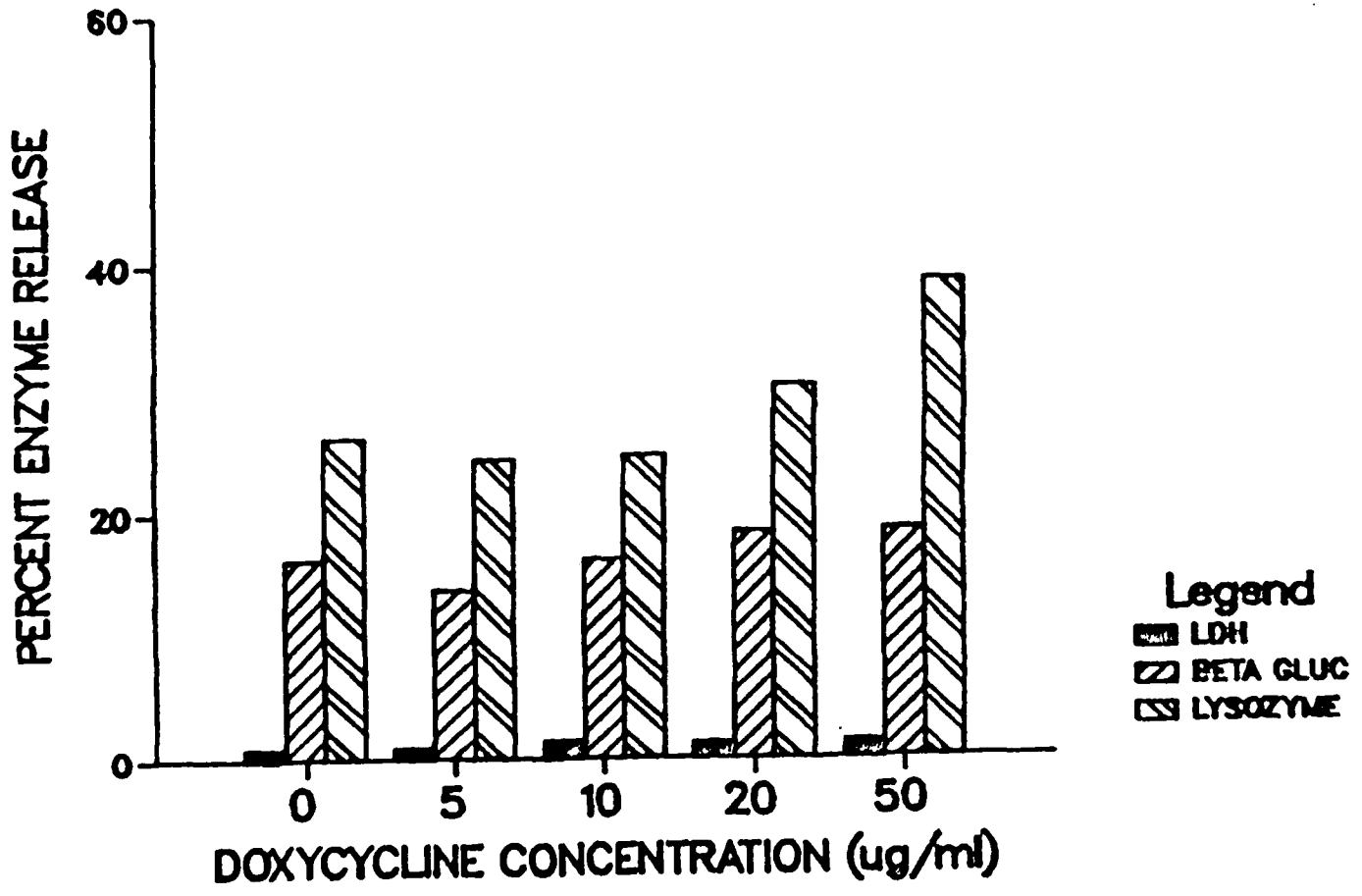


FIGURE 16: Doxycycline-Degranulation

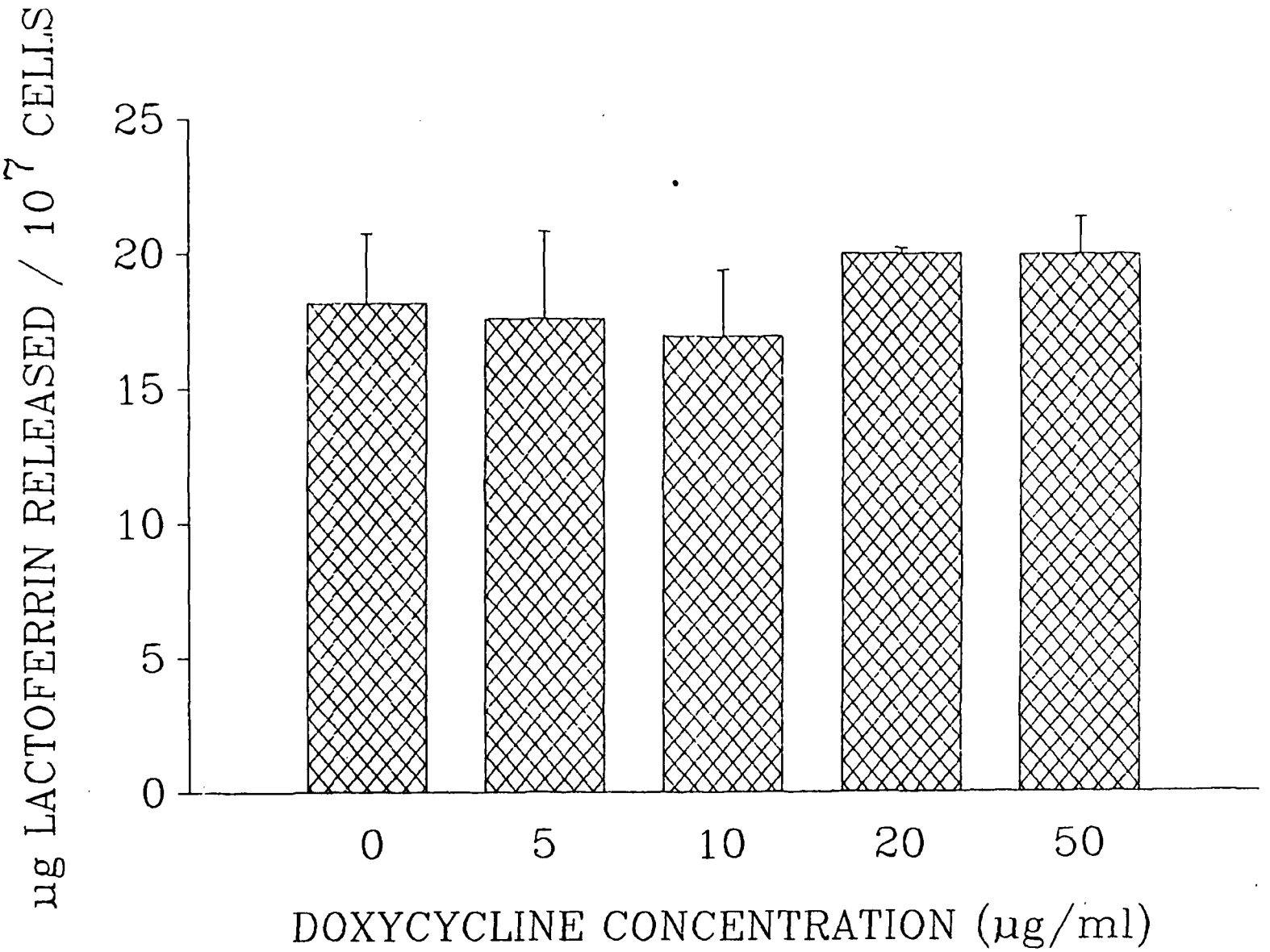


FIGURE 17: Doxycycline-Lactoferrin

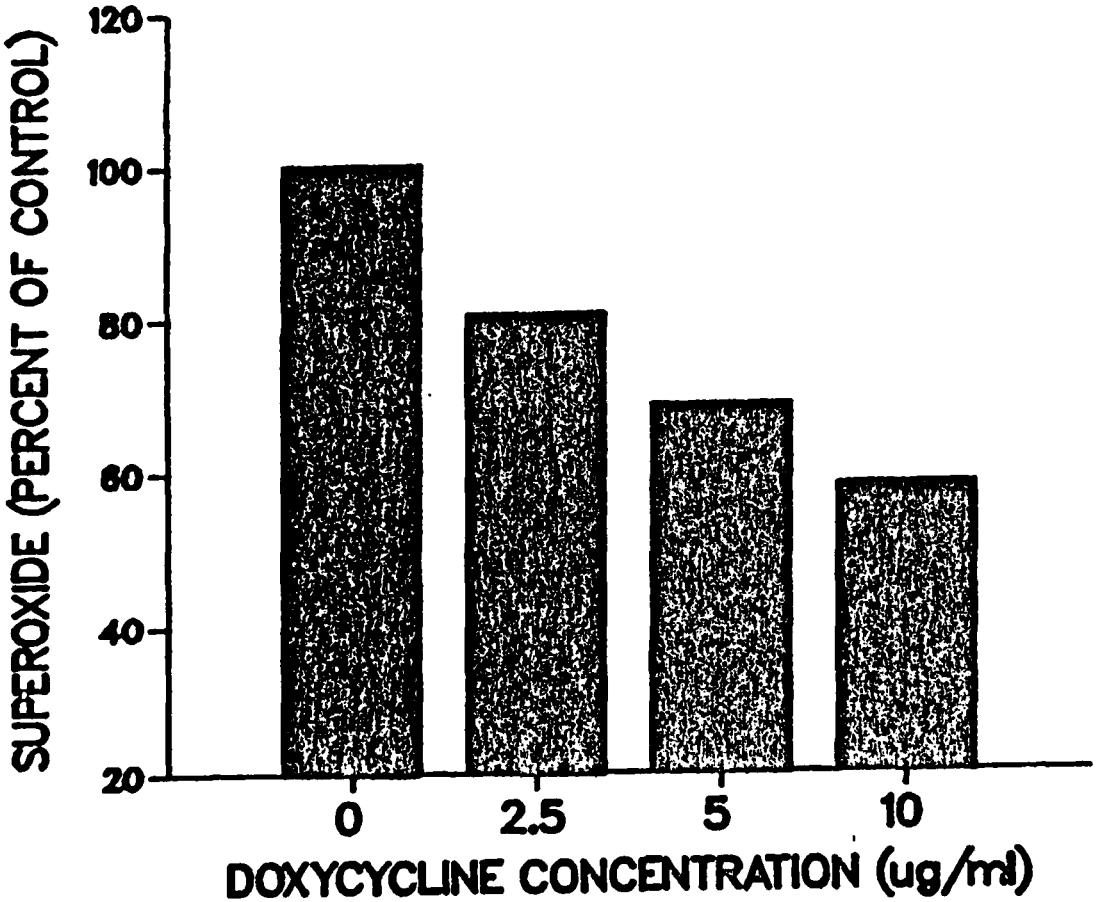


FIGURE 18: Doxycycline-Superoxide

DRUG COMBINATIONS:

Meclofenamate was chosen to be used with chlorhexidine or doxycycline in a study of the combined effects of a NSAID and an antimicrobial. Meclofenamate appeared to be the most potent of the NSAIDs in our model system and both antimicrobials had significant effects on PMN chemotaxis and superoxide production.

MECHLOFENAMATE + CHLORHEXIDINE

In this combination the drug concentrations chosen were those shown to be most effective when the drugs were used singly. When used together, there was a significant reduction in PMN chemotaxis (ANOVA $F=26.007$, df3, $p<.o5$), though the effect was not different from meclofenamate alone (Tukey-HSD, $p>.05$) (Figure 19). A similar effect was observed in the superoxide assay, where the combination produced a significant reduction in superoxide production (ANOVA $F=11.3513$, df3, $p<.05$), but not different from meclofenamate alone (Tukey-HSD, $p>.05$) (Figure 20).

MECHLOFENAMATE + DOXYCYCLINE

The drug concentrations for this combination were chosen as above. Chemotaxis was signficantly inhibited by this combination of drugs (ANOVA $F=194.3553$, df3, $p<.05$) (Figure 21), and some degree of synergy was apparent as the combination was significantly more potent than either of the drugs used alone (Tukey-HSD, $p<.05$). The combination also significantly reduced superoxide production (ANOVA 61.7368, df3, $p<.05$), but not different from meclofenamate alone (Tukey-HSD, $p>.05$) (Figure 22).

CONCLUSIONS:

We have identified at least two combinations of NSAIDs and antimicrobials which may have excellent potential for field use in the prevention of progressive periodontal disease. The combination of Meclofenamate with Doxycycline may be best as there is some synergy observed in the inhibition of PMN chemotaxis and no antagonism of the other drug effects. Meclofenamate and chlorhexidine may also be useful, as no antagonism is observed and chlorhexidine is applied topically, which may reduce the risk of drug side effects. From our literature search, we can also suggest that Clindamycin may be an appropriate antimicrobial to be combined with a NSAID. Also, Ibuprofen may be substituted for Meclofenamate. These combinations deserve further clinical study for their potential use under field conditions.

In addition we have established a set of assays, which can be used effectively in the future for the evaluation of new drugs,

which may be potentially useful agents in the treatment and prevention of periodontal disease. A complete list of the chemicals and solutions, as well as the complete assay protocols are provided in the following section of this report.

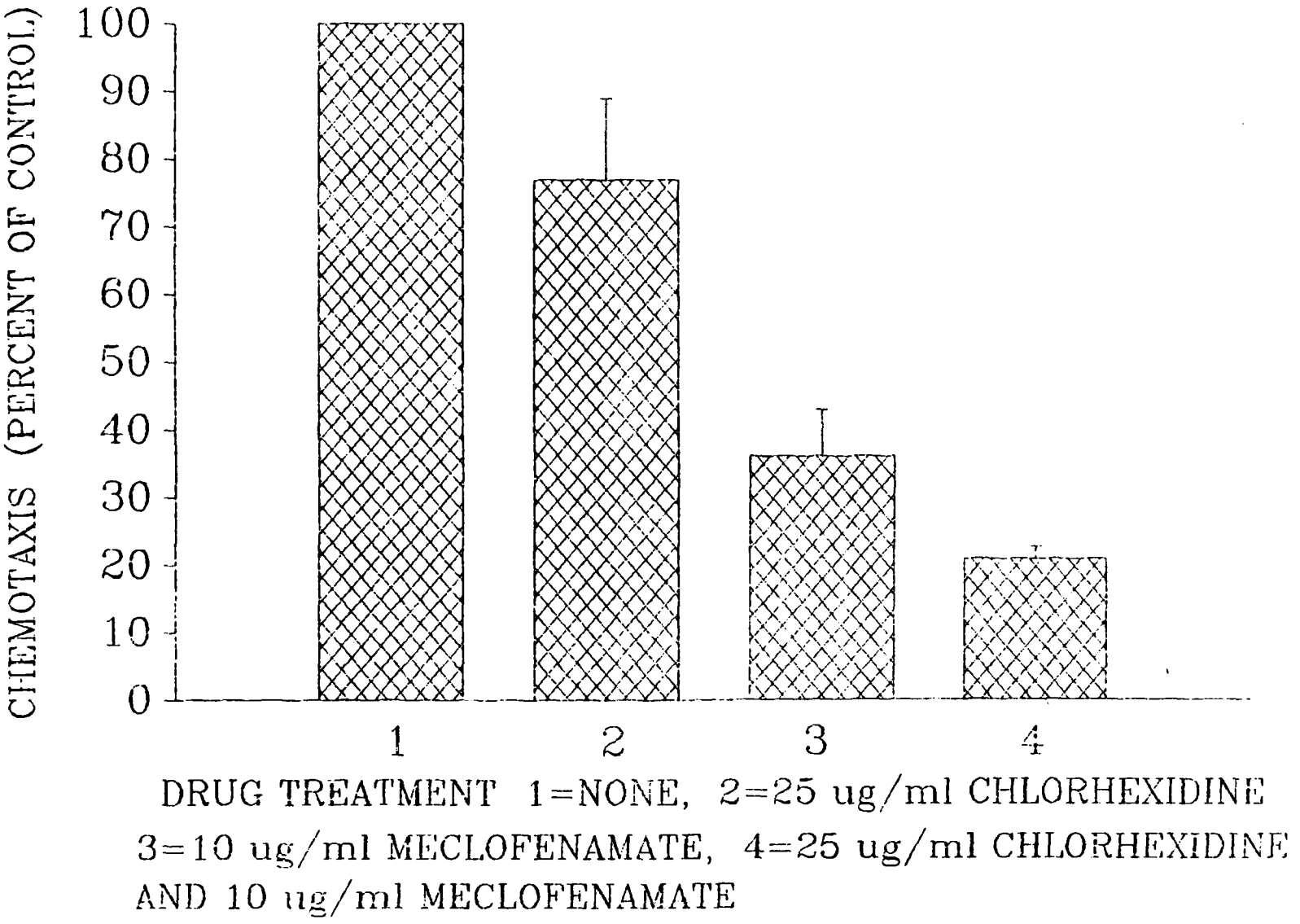


FIGURE 19: Meclofenamate + Chlorhexidine-Chemotaxis

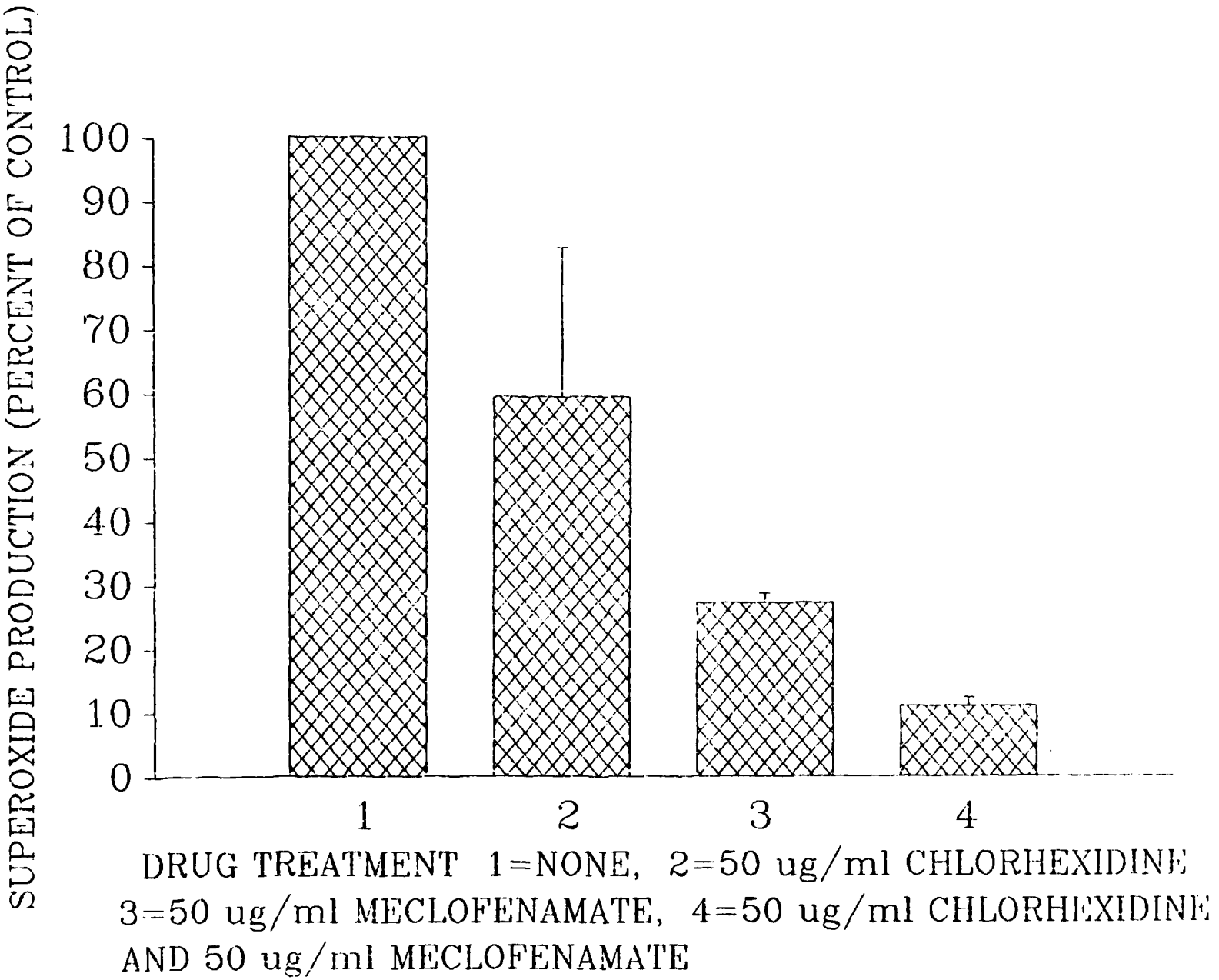


FIGURE 20: Meclofenamate + Chlorhexidine - Superoxide

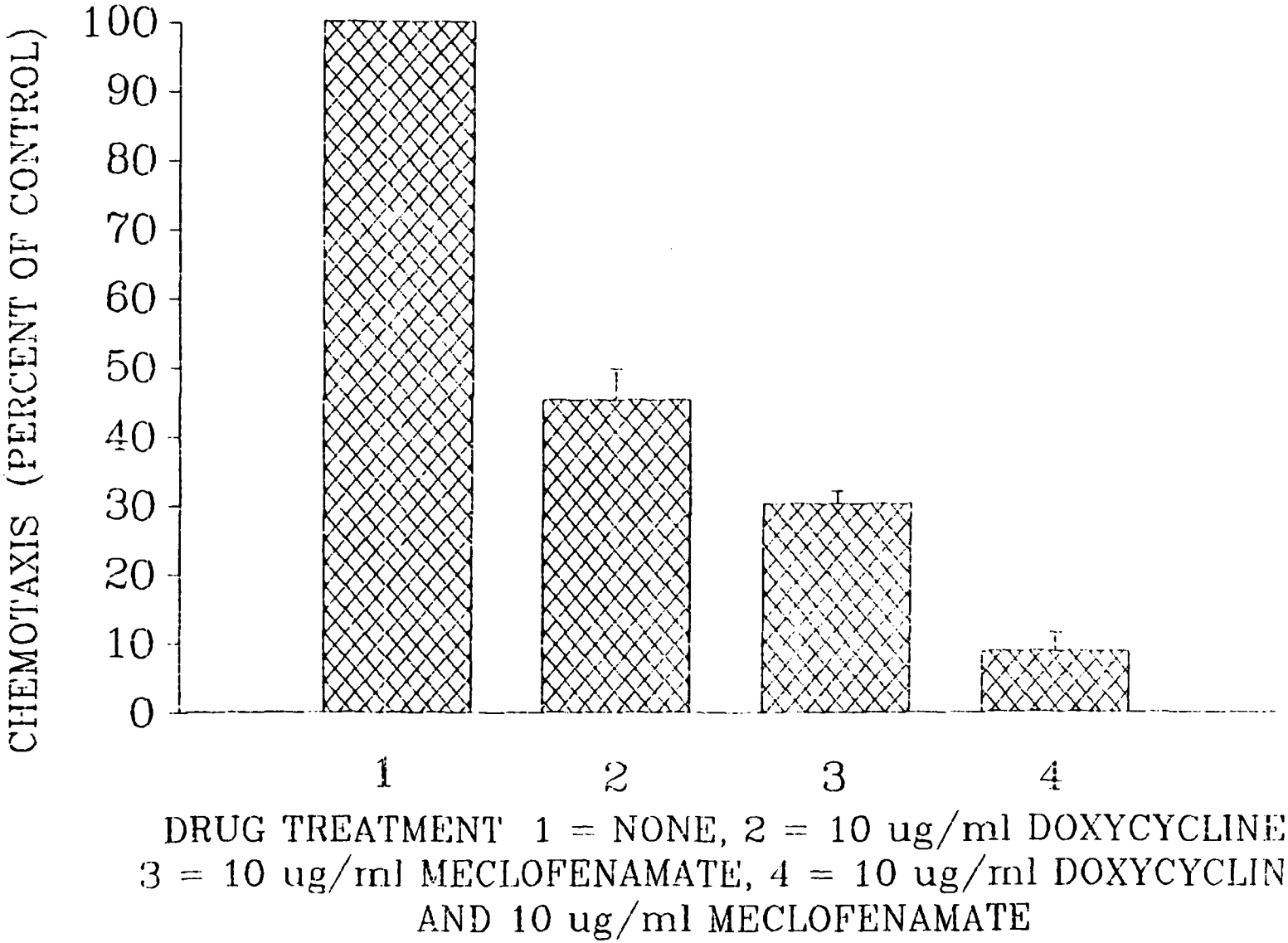


FIGURE 21: Meclofenamate + Doxycycline-Chemotaxis

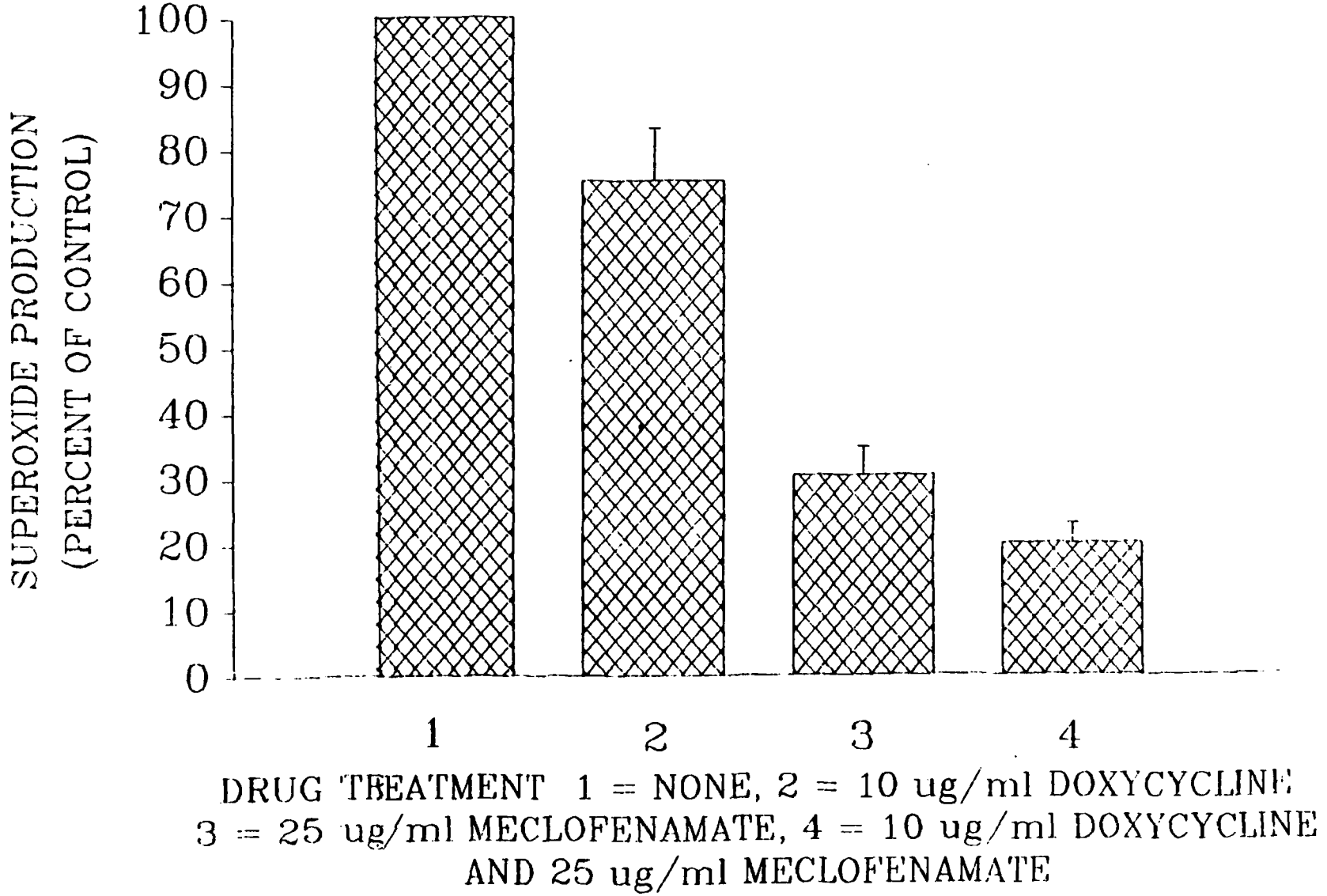


FIGURE 22: Meclofenamate + Doxycycline-Superoxide

MATERIALS AND METHODS:

STOCK SOLUTIONS

These may be prepared any time and stored (refrigerated at 4 degrees C unless otherwise noted). Sufficient quantities should be assured the day prior to an assay. Formulations for the stock solutions and solutions that are prepared the day of the assay follow, in alphabetical order, the list below.

Lactate Dehydrogenase
- Phosphate/Pyruvate Substrate Solution (50 mM/0.63 mM, pH 7.5)

Lysozyme
- Phosphate Buffer 66mM, pH 6.24

Beta-glucuronidase
- Acetate Buffer 0.2 M, pH 4.5
- Phenolphthalein glucuronide substrate solution
- Phenolphthalein standard solution
- AMP Buffer pH 11

Lactoferrin
- Lactoferrin Stock Buffer 1 - Barbital
- Lactoferrin Stock Buffer 2 - Tris-glycine
- Phosphate Buffered Saline pH 7.0
- Destaining Solution
- Staining Solution
- Lactoferrin Stock Standard

Chemotaxis
- Giemsa's Stain (Stock Solution)
- Tissue Culture Medium 199

Superoxide
- Superoxide Dismutase Solution

Incubation
- Triton X-100, 2%
- Serum Treated Zymosan (STZ)
- Cetyltrimethylammonium Bromide (CTAB) 3%
- Cytochalasin B - Stock Solution
- Incubation Buffer - 10x Stock (HBSS with Ca^{++} and Mg^{++})

Blood Separation
- Ficoll-Paque - use as purchased from Pharmacia
- Hanks Balanced Salt Solution (HBSS without Ca^{++} and Mg^{++})
- HEPES
- Dextran 6%

Formulations For Stock Solutions

Acetate Buffer 0.2 M, pH 4.5
3.72 ml glacial acetic acid
4.76 g sodium acetate trihydrate
Dissolve in 500 ml H_2O.

Ammonium Chloride Solution
8.3 g NH_4Cl
1 g potassium carbonate
37.2 mg EDTA (free acid)
Dissolve in 1 liter H_2O
Adjust pH to 7.4 with concentrated HCl
Filter sterilize, store frozen at -20 degrees C in 10 ml aliquots.

AMP Buffer pH 11
Place 100 ml graduated cylinder on balance and tare to zero
Add 8.91 g concentrated liquid Amino-2-Methyl-1-Propanol
Add distilled H_2O and mix thoroughly.
Dilute to 1 liter.
Add 2 g SDS
Stir gently to dissolve
Check pH, adjust if necessary with NaOH or HCl
If precipitate forms, warm to 37 degrees C and shake to dissolve.

Cetyltrimethylammonium Bromide (CTAB) 3%
1.5 g CTAB (Hexadecylmethylammonium Bromide)
Dissolve in 50 ml HBSS with Ca^{++} and Mg^{++}.

Cytochalasin B - Stock Solution
Add 0.23 ml dimethylsulfoxide (DMSO) to 5 mg vial of cytochalasin B.

Destaining Solution
450 ml 95% ethanol
100 ml glacial acetic acid
450 ml H_2O
Mix and store at room temperature.

Dextran 6%
Dissolve 9 g NaCl in 1 liter H_2O.
Add 60 g Dextran 70 (Sigma D-1390)
Stir until completely dissolved.
Filter sterilize in 15 ml aliquots into sterile 50 ml polypropylene tubes.

Dextrose
Dissolve 4.5 g dextrose in 45ml H_2O.
Filter sterilize and store 5ml aliquots in sterile tubes at room temperature.

FMLP Frozen Stock
Dissolve 44 mg fMLP in 10 ml DMSO. Store 0.5 ml aliquots in polypropylene tubes frozen at -70 degrees C.

Ficoll-Paque - use as purchased from Pharmacia

Giemsa's Stain (Stock Solution)

Disperse 1 g Giemsa powder in 66 ml glycerol and place in an oven at 60 degrees C for 2 hrs.

Add 66 ml methyl alcohol, absolute (acetone-free). Store tightly stoppered at room temperature. Dilute 1 part with 9 parts buffered water.

Buffered water: First make Clark and Lubs' Phosphate Buffer, pH 6.75 by mixing 6.8 g KH_2PO_4 and 0.9 g NaOH with 1 liter distilled H_2O. Adjust pH, if needed. Then dilute 1 part of the phosphate buffer with 5 parts distilled H_2O.

Hanks Balanced Salt Solution -- HBSS with Ca^{++} and Mg^{++}
(Incubation Buffer 10x Stock)

80 g NaCl
4 g KCl
2 g $MgSO_4.7H_2O$
0.9 g $Na_2HPO_4.7H_2O$
0.6 g KH_2PO_4
Dissolve first five ingredients in 700 ml distilled H_2O.
In 200 ml distilled H_2O, dissolve 2.76 g $CaCl_2$, anhydrous
While stirring, slowly add the dissolved $CaCl_2$ to the first beaker. Dilute to 1 liter. Add 1 ml chloroform as a preservative.

HBSS with Ca^{++} and Mg^{++} (Incubation Buffer) - <u>Working solution</u>

50 ml *HBSS with Ca^{++} and Mg^{++}, 10x stock*
Dilute with 440 ml H_2O.
Autoclave at 15 lbs for 20 min.
Sterile solution may be stored at room temperature.
Cool to room temperature and add:
5 ml sterile HEPES
5 ml dextrose (0.1 g/ml H_2O)

Hanks Balanced Salt Solution (HBSS <u>without</u> Ca^{++} and Mg^{++})
10x Stock

950 ml distilled H_2O
80 g NaCl
4 g KCl
0.9 g $Na_2HPO_4.7H_2O$
0.6 g KH_2PO_4
Make up to 1 liter.
Add 1 ml chloroform as a preservative.

HBSS without Ca^{++} and Mg^{++} Working solution

Dilute 50 ml of 10x stock with 440 ml H_2O.
Autoclave at 15 lbs. for 20 min.
(Sterile solution may be stored at room temperature).
Cool to room temperature and add 5 ml dextrose(0.1 g/ml)
Add 5 ml HEPES.

HEPES
Dissolve 23.8 g HEPES in 100 ml H_{20}.
Adjust pH to 7.5-7.6 with 10 M NaOH.
Filter sterilize and store 5 ml aliquots in sterile tubes at room temperature.

Lactoferrin Stock Standard
Human lactoferrin (Sigma L8010)
1 mg/ml in HBSS with Ca^{++} and Mg^{++}
Store 0.035 ml aliquots in 1.5 ml tubes at -70 degrees C.

Lactoferrin Stock Buffer 1 - Barbital
26 g sodium barbital
4.14 g barbital
Dissolve in 2 liters H_2O.

Lactoferrin Stock Buffer 2 - Tris-glycine
112.4 g glycine
90.4 g tris
Dissolve in 2 liters H_2O.

Lactoferrin Working Buffer
200 ml barbital stock buffer 1
200 ml tris-glycine stock buffer 2
Mix with 1600 ml H_2O and store at 4 degrees C.

Phenolphthalein glucuronide substrate solution - Use as purchased from Sigma.

Phenolphthalein standard solution - Use as purchased from Sigma.

Phosphate Buffer 66 mM, pH 6.24
4.49 g KH_2PO_4
Dissolve in 400 ml H_2O and adjust pH with 1 M KOH.
Dilute to 500 ml.

Phosphate Buffered Saline (PBS) pH 7.0
31.8 g NaCl
805.6 mg KCl
4.56 g Na_2HPO_4
816 mg KH_2PO_4
Dissolve in 4 liters H_2O.

Phosphate/Pyruvate Substrate Solution (50 mM/0.63 mM;pH 7.5) - Store refrigerated.
2.55 g $K_2HPO_4.3H_2O$
250 mg KH_2PO_4
17.2 mg Sodium Pyruvate
Dissolve in 250 ml H_2O.

Saline 0.9%
Dissolve 2.25 g NaCl in 250 ml H_2O.
Autoclave at 15 lbs for 15 min.

Saline 3.5%

Dissolve 1.58 g NaCl in 45 ml H_2O.

Serum Treated Zymosan (STZ)

Suspend 50 mg zymosan in 5 ml 0.9% NaCl.
Transfer 1 ml of the suspension to each of 5-12x75 mm glass culture tubes.
Place tubes in boiling water bath for 15 min.
Centrifuge for 10 min at 2000 rpm at 4 degrees C.
Decant the salt solution, resuspend the pellet in fresh saline, and centrifuge.
Repeat the above wash procedure twice more.
Discard the supernatant, add 1 ml human autologous serum to each tube and resuspend the pellet.
Incubate for 30 min at 37 degrees C in a shaking water bath.
Centrifuge and wash with saline twice as above.
Discard the supernatant and add 2 ml HBSS with Ca^{++} and Mg^{++} to each tube to obtain a 5 mg/ml suspension.
Transfer to a bio-vial and store at -70 degrees C.

Staining Solution

Dissolve 2.5 g Coomassie Brilliant Blue R in 500 ml destaining solution. Allow to stand overnight at room temperature and then filter. Store at room temperature.

Superoxide Dismutase Solution

Dissolve 4mg/ml superoxide dismutase in H_2O. Store aliquots of 200 µl at -70 degrees C.

Tissue Culture Medium (TCM)

Add the powdered Medium 199 (Sigma M-0393, unit size-1 liter) to 450 ml distilled H_2O while stirring. Water temperature should be 15-20 degrees C. Do not heat. Rinse all traces of the powdered medium from the bottle with a small amount of water and add to the stirring solution. Add 5 ml of stock Hepes solution. Adjust pH to 7.3 (with dilute NaOH or HCl). Dissolve 1.0 g BSA (Bovine Serum Albumin) in the medium. Bring the volume to 500 ml with distilled water. The conc. is now 2X. Add 250 ml of the 2X medium to 250 ml H_2O, bringing the conc to 1X. Filter sterilize the 1X medium into 2-250 ml sterile bottles. Filter sterilize the remaining 2X medium into a 250 ml sterile bottle. Store at 4 degrees C in darkness.

Triton X-100 (2%)

Dilute 2 ml Triton X-100 to 100 ml with HBSS with Ca^{++} and Mg^{++}.

BLOOD SEPARATION PROCEDURE

(Have the following on hand)

Heparin	3.5% Saline
Dextran (one 15 ml tube for each 30 ml blood drawn)	PBS
	Ficoll-Paque
Ice	HBSS without Ca^{++} and Mg^{++}
Sterile H_2O	HBSS with Ca^{++} and Mg^{++}

Dextran Separation

1. Draw blood slowly into 50 cc heparinized syringes (10 units heparin/ml blood). The amount of blood needed varies by assay; 60 ml for chemotaxis, 120 ml for superoxide, and 180 ml for the degranulation assays.

2. Add 30 ml blood to each tube containing dextran and mix gently by inversion. Allow to stand undisturbed at room temperature for 1 hour. Turn on centrifuge, set temperature at 4 degrees C.

3. Transfer the top layer to fresh 50 ml tubes on ice. Note volume transferred to each tube.

4. Centrifuge at 100 xg for 12 min at 4 degrees C.

RBC Lysis

1. Immediately after centrifugation, aspirate the supernatant.

2. Using a vortex mixer at low speed quickly and gently resuspend the pelleted cells in ice cold sterile distilled H_2O (0.1 ml/ml of volume in 3 above).

3. 30 sec after adding the water, add a volume of ice cold 3.5% NaCl equal to one third the volume of water added in previous step.

4. Bring the volume of each tube to approximately 15 ml with ice cold PBS.

5. Centrifuge at 160 xg for 4 min at 4 degrees C.

6. Immediately after centrifugation, decant supernant. A pasteur pipet may be used to carefully remove the ring of RBC's which may be found on the top of the pellet.

Ficoll Separation

1. Resuspend each pellet in about 5 ml ice cold PBS. [Combine two tubes to a single tube and add PBS to bring volume to 20 ml if less than 100 ml of blood is being processed or 40 ml if 100-200 ml of blood is used.]

2. Using a sterile pipet, carefully layer 20 ml of the cell suspension over 10 ml Ficoll-Paque which has previously been dispensed into 50 ml polypropylene tubes. Be careful to avoid mixing the layers.

3. Centrifuge at 250 xg for 20 min at 4 degrees C.

4. Carefully aspirate the mononuclear cell layer at the interface, then aspirate the rest of the supernatant.

5. Resuspend cells in 20 ml cold HBSS without Ca^{++} and Mg^{++}.

6. Centrifuge at 160 xg for 4 min at 4 degrees C.

7. Discard the supernatant, resuspend the pellets in 10 ml ice cold HBSS without Ca^{++} and Mg^{++}. Combine the suspensions into a single tube and centrifuge at 160 xg for 4 min at 4 degrees C.

8. Discard supernatant and resuspend the pellet in the appropriate buffer for the given assay (see table below) using about 0.5 ml buffer for each 10 ml whole blood.

ASSAY	HBSS FOR RESUSPENSION	CONC (cells/ml)	VOLUME NEEDED (ml)
CHEMOTAXIS	w/out Ca^{++}	$1x10^7$	3
SUPEROXIDE	with Ca^{++}	$1x10^7$	10
DEGRANULATION	with Ca^{++}	$2x10^7$	9

COUNTING CELLS

1. Add 50 µl cell suspension to 450 µl HBSS in 12x75 mm polypropolene tube. Vortex gently.
2. Add 450 µl of diluted cell suspension to 50 µl Trypan blue (0.4% solution) in a 12x75 mm tube. Vortex gently.
3. Carefully load both sides of a haemocytometer with a pasteur pipette.
4. Count cells in at least 2 large squares.
5. Adjust the cell concentration to what is appropriate for the assay by diluting with the appropriate buffer (See table above).

ASSAY PROCEDURES

Incubation for Degranulation Assays
(Lactate Dehydrogenase, Lysozyme, Beta-glucuronidase, Lactoferrin)

A. Preparation

Have the following ready for use
1. STZ(Serum Treated Zymosan)
2. HBSS with Ca^{++} and Mg^{++}
3. Set water bath at 37 degrees C
4. Set centrifuge at 4 degrees C
5. Ice

These solutions are prepared the day of the assay:
1. fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine)
   Remove frozen aliquot of fMLP (2.2 mg in 0.5 ml DMSO) and dilute 0.1 ml with 9.9 ml HBSS with Ca^{++} and Mg^{++}. Repeat hundredfold dilution.
2. Concanavalin A - 1 mg/ml
   Weigh out approximately 1 mg concanavalin A and dissolve in HBSS with Ca^{++} and Mg^{++} so that volume buffer (ml) equals weight (mg) of Con A.
3. Cytochalasin B - Working Solution
   0.01 ml cytochalasin B stock
   Dilute to 10 ml with HBSS with Ca^{++} and Mg^{++}.

B. Incubation Procedure
1. Pipet 0.1 ml STZ or Triton into the appropriate incubation tubes. Blanks may be prepared containing all stimulants to be used plus Triton.

2. Add drug solution and incubation buffer to bring total volume to 0.5 ml for tubes receiving cell suspension and 1 ml for blanks.

3. Add 0.5 ml cell suspension and place in shaking water bath set at 37 degrees C for 30 min.

4. Add cytochalasin B and incubate for 10 min at 37 degrees C.

5. Add fMLP, incubate 15 min longer at 37 degrees C; then remove tubes from water bath and place on ice.

6. Centrifuge at 750 xg for 10 min at 4 degrees C.

7. Transfer supernatant to a separate set of 10x75 mm glass tubes for enzyme assays.

Lactate Dehydrogenase

A. Preparation

1. Reduced nicotinamide adenine dinucleotide (NADH) solution
   30 mg $NaHCO_3$
   Dissolve in 3.0 ml H_2O, then add 28 mg disodium NADH
   ** Protect from light and keep at room temperature.
2. Remove phosphate/pyruvate solution from refrigerator and bring to room temperature.
3. HBSS with Ca^{++} and Mg^{++}.

B. Procedure

1. Pipet 3.15 ml H_2O into acrylic cuvette for reference standard.
2. Pipet 0.1 ml HBSS with Ca^{++} and Mg^{++} into blank cuvette and 0.09 ml into cuvettes to receive tritonized sample for 100% value.
3. Pipet 0.1 ml sample supernatant into approriate cuvettes in duplicate. Use 0.01 ml tritonized sample supernatants for 100% value.
4. Turn on spectrophotometer and set wavelength to 340 nm.
5. Place reference standard in spec and set to zero.
6. Pipet 0.05 ml NADH solution into the first cuvettes to be read.
7. Start stopwatch and after 10 seconds add 3.0 ml phosphate/pyruvate solution to first cuvette. Continue additions to the first set of cuvettes at 10 second intervals.
8. Record absorbance at 10 s intervals for each sample. Take 3-4 readings once a minute for each sample of the set.
9. Repeat steps 6-8 until all samples have been run.

C. Calculations

1. Subtract the final absorbance reading for each sample from the initial reading for that sample and divide by the number of minutes recorded to obtain the average change in absorbance per minute.
2. Multiply the average change value for the 100% sample by 10 to obtain the average change/minute/0.1 ml.
3. Divide the sample values by the 100% value obtained in the previous step to obtain the % LDH release and thus % cell death.

Lysozyme

A. Preparations

1. Micrococcus Suspension
   Dissolve 15 mg micrococcus lysodeikticus in 100 ml of 66 mM phosphate buffer. Bring to room temperature.
2. Lysozyme Standards (Keep Refrigerated)
   a. 600 units/ml - Dissolve 15 mg egg white lysozyme in 10 ml HBSS with Ca^{++} and Mg^{++}. Dilute 0.1 ml to 10ml HBSS with Ca^{++} and Mg^{++}.
   b. 400 units/ml - 0.6 ml a + 0.3 ml HBSS with Ca^{++} and Mg^{++}.
   c. 200 units/ml - 0.3 ml a + 0.6 ml HBSS with Ca^{++} and Mg^{++}.

B. Lysozyme Procedure

1. Pipet 1.3 ml H_2O into 1.5 ml polystyrene cuvette for reference standard.

2. Pipet 0.05 ml HBSS with Ca^{++} and Mg^{++} into blank cuvette, 0.04 ml into cuvettes to receive tritonized samples for 100% values, and an approriate intermediate volume into cuvettes to receive samples stimulated to degranulate. (e.g. If the stimulus is expected to produce 50% degranulation 0.025 ml buffer should be added here.)

3. Pipet 0.05 ml 200 U/ml - 600 U/ml standards into cuvettes. All standards and samples should be run in duplicate.

4. Pipet 0.05 ml sample supernatant into cuvettes except use 0.01 ml for tritonized samples and an approriate intermediate volume for samples stimulated to degranulate as discussed in step 2 above.

5. Turn on spectrophotometer and set wavelength to 450 nm.

6. Place reference standard in spec and set reference value.

7. Start stopwatch and after 10 s add 1.25 ml micrococcus suspension to first sample. Continue additions of micrococcus suspension to the first set of cuvettes at 10 s intervals.

8. Record absorbance at 10 s intervals for each sample. Take 3-4 readings once a minute for each sample in the set.

9. Repeat steps 7-8 until all samples have been run.

10. Calculations are performed essentially as for LDH. Assay linearity may be checked by plotting the standard curve.

Beta-glucuronidase

A. Preparations

1. Diluent Solution
   3.0 ml acetate buffer
   3.0 ml HBSS with Ca^{++} and Mg^{++}
   25 ml AMP buffer
2. Diluted Phenolphthalein Solution
   0.075 ml phenolphthalein standard
   4.925 ml diluent solution

Set water bath at 56 degrees C
Acetate buffer
Phenolphthalein-glucuronide substrate solution
AMP buffer
HBSS with Ca^{++} and Mg^{++}

B. Procedure

1. Pipet 0.15 ml acetate buffer into 10x75 mm glass culture tubes.

2. Add 0.05 ml phenolphthalein glucuronide substrate solution.

3. Add 0.1 ml HBSS with $Ca^{++}$and Mg^{++} to 2 blank tubes, 0.075 ml to tubes to receive tritonized samples, and an intermediate volume to tubes stimulated to degranulate as discussed in step 2 of the lysozyme procedure.

4. Add 0.1 ml sample supernatant except add 0.025 ml tritonized sample supernatant and intermediate volumes of samples stimulated to degranulate.

5. Incubate at 56 degrees C in shaking water bath for 1 hour.

6. While incubating prepare standard curve as follows.
   a. Pipet 0.05, 0.1, 0.2, 0.4, and 0.6 ml dilute phenolphthalein solution into 10x75 mm glass tubes for 0.5, 1, 2, 4, and 6 µg/ml standards respectively.
   b. Pipet 1.5 ml diluent solution into a tube for the standard blank and bring volumes of other tubes up to 1.5 ml by adding 1.45, 1.4, 1.3, 1.1, and 0.9 ml diluent solution respectively.

7. Remove assay tubes from water bath and place on ice.

8. Add 1.25 ml AMP buffer to each assay tube and pour contents into 1.5 ml polystyrene cuvette.

9. Record absorbance at 550 nm immediately or store refrigerated overnight and return to room temperature before reading.

10. Data are calculated as percent release essentially as for LDH and lysozyme.

Lactoferrin

A. Preparations

1. Lactoferrin working buffer (keep at 4 degrees C)

2. Agarose Gel
   a. Refrigerate cooling plate and cut gel bond film (Pharmacia) to 84x245 mm
   a. 350 mg agarose (Sigma Type I, Low EEO)
      35 ml working buffer
      dissolve agarose in buffer by heating in a boiling water bath
   b. Bring solution to 50 degrees C in a water bath
   c. Add 0.0525 ml lactoferrin antiserum (Calbiochem), vortex gently. AGAROSE MUST BE POURED WITHIN 5 MIN.
   d. Pour agarose quickly onto the gel bond film on a leveling plate.
   e. Allow to cool 10 min at room temperature followed by 20 min at 0-4 degrees C.
   f. Punch 3 mm wells, place gel on cooling plate, (which has been leveled on the gel box) set up wicks and pour lactoferrin working buffer into tanks.

3. Lactoferrin Standards
   a. 0.035 mg/ml - add 0.965 ml HBSS with Ca++ and Mg++ to 0.035 ml frozen lactoferrin stock standard
   b. 0.02 mg/ml - 0.2 ml a + 0.15 ml HBSS
   c. 0.01 mg/ml - 0.1 ml a + 0.25 ml HBSS
   d. 0.0035 mg/ml - 0.035 ml a + 0.315 ml HBSS

B. Lactoferrin Procedure

Gel Loading

1. Set up power supply and electrodes , have power supply ready to turn on. Set to constant voltage (100 V).

2. With agarose gel in place on electrophoresis apparatus note the position of any irregular or unusable wells. These should be filled with HBSS before beginning to load samples onto gel.

3. Load 0.01 ml standard or sample into wells noting what sample is pipetted into each well. Fill any unused wells with incubation buffer. THE ENTIRE LOADING PROCEDURE MUST BE COMPLETED WITHIN 10 MINUTES.

4. Cover with anti-condensation plate.

5. Hook up power supply and turn on.

6. Run electrophoresis overnight.

7. Turn off power supply, disconnect, remove cover and gel.

Gel Staining

1. Remove gel, cover with a sheet of filter paper and several layers of paper towels and sustain even pressure for 15 min using a smooth glass or metal plate weighted with one liter of bottled liquid.

2. Place gel in washing chamber, cover with approximately 125 ml PBS and agitate gently on shaker for 15 min.

3. Repeat wash in PBS, then wash in distilled H_2O.

4. Blot again as in step 1.

5. Dry gel in gel dryer at 60 degrees C for approximately 2 hours, making certain that the silicone gasket is sealed.

6. Place dried gel in staining chamber, cover with approximately 125 ml staining solution and agitate gently on shaker for 10 min.

7. Pour staining solution back into bottle and remove gel to destaining chamber.

8. Cover gel with 125 ml destaining solution and agitate gently for 10 min.

9. Repeat step 8 twice more.

10. Data are calculated by plotting lactoferrin concentration in the standards against the rocket height and determining the concentration corresponding to the sample rocket height.

Chemotaxis

A. Preparations

1. Drug Dilutions (the following is given as an example, drug concentrations can be varied). Prepare two sets of 6 sterile 15 ml tubes, A and B. Label and make the following additions of 2X tissue culture media(TCM) to each set. Set B will be mixed with agarose to yield the following drug conc.

TUBE #	2X TCM		Final Drug Conc (µg/ml)
1A	5.0 ml	1B	100
2A	2.5 ml	2B	50
3A	3.0 ml	3B	20
4A	2.5 ml	4B	10
5A	2.5 ml	5B	5
6A	2.5 ml	6B	0
1B-6B	2.0 ml each		

Add 5 mg drug in tube 1A. Allow plenty of time to dissolve.
Transfer 2.5 ml of 1A to 2A. Vortex.
Transfer 2 ml of 2A to 3A. Vortex.
Transfer 2.5 ml of 3A to 4A. Vortex.
Transfer 2.5 ml of 4A to 5A. Vortex.
Vortex 6A.
Transfer 0.5 ml of each A tube to its corresponding B tube through tube 6.

2. Cell Incubation Mixtures - Prepare 2 sets of 6 tubes, E and F. Set F should be 12 X 75 mm polypropylene tubes for incubation. Make the following additions to set E. Set F will be mixed with 0.5 ml of cell suspension to yield the following drug conc.

Tube #	1X TCM		Drug Conc (µg/ml)
1E	3 ml	1F	100
2E	2.5 ml	2F	50
3E	3 ml	3F	20
4E	2.5 ml	4F	10
5E	2.5 ml	5F	5
6E	2.5 ml	6F	0

Add 1 ml of 1A and 1 ml distilled H_2O to 1E. Vortex.
Transfer 2.5 ml of 1E to 2E. Vortex.
Transfer 2.0 ml of 2E to 3E. Vortex.
Transfer 2.5 ml of 3E to 4E. Vortex.
Transfer 2.5 ml of 4E to 5E. Vortex.
Vortex 6E.
Transfer 0.5 ml of each E tube into the corresponding F tube.

3. Prepare chemotactic solution-fMLP (10^{-7} M)
   1. Use frozen stock (2.2 mg/0.5 ml DMSO) and dilute 100 fold by adding 9.9 ml HBSS.
   2. Dilute 100 fold again by adding 9.9 ml HBSS to 0.1 ml from #1.
   3. Dilute 10 fold by adding 9.0 ml HBSS to 1.0 ml from #2.

B. Chemotactic plate preparation

1. Mix 250 mg agarose (Sigma type II) in 25 ml H_2O. Boil to dissolve. Dispense 2.5 ml aliquots into glass tubes.

2. Place still molten agarose tubes in 48 degree C water bath (or boiling water bath to redissolve, if needed).

3. Working in a clean area, lay out 1 60 X 15 mm sterile petri plate for each tube. Label plates with the drug dilution they will receive.

4. Place set B tubes containing TCM and drug in water bath with agarose tubes for 1-2 minutes only.

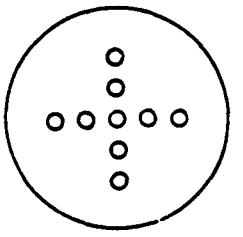
5. Remove 1 tube with tissue culture medium and carefully dry tube completely. Remove 1 agarose tube and using sterile technique, pour the TCM into the agarose tube. Flame top of tube, replace cap, dry, vortex, and pour the contents into a petri dish.(This step must be completed 1-2 min after mixing or the agarose will harden in the tube. Also avoid putting mixed tubes back into the water bath as excessive heat will break down the tissue culture medium.)

6. Allow plates to sit at room temperature for 20 min, then refrigerate until the wells are punched.

C. Assay Procedure

1. Add 0.5 ml cell suspension to each of the appropriate tubes in set F. Incubate at 37 degrees C for 30 min while agitating slowly.

2. During incubation the wells should be punched in the chemotaxis plates. The wells are cut using a 3 mm diameter punch, spaced about 6 mm apart, as shown in the diagram. The plugs are drawn out using a pasteur pipette attached to a vacuum. Fluid accumulating in the wells should be aspirated before the additions are made.



3. When incubation is completed, centrifuge tubes at 160 xg for 4 min. Pemove about 900 µl from each tube without disturbing the pellet. Gently resuspend cells in remaining supernatant.

4. Add 10 µl of cell suspension to each middle well of 3 in the appropriate plate. Add 10 µl fMLP to the well in the center of the plate and 10 µl TCM to each of the outer wells. Incubate 2 hours at 37 degrees C.

5. After incubation, cover the plates with 3 to 5 ml methanol for 30 min. Pour off the methanol and cover with 4.0 % formaldehyde. Let the plates stand overnight. Pour off formaldehye and carefully pop out agarose (cells will remain attached to the plate).

6. Prepare Giemsa's working solution by mixing 5 ml Giemsa Stock and 45 ml buffered H_2O. Stain for 30 min or longer, if necessary.

7. Measure the distance of cell migration and record as the percent of control (no drug) movement.

Superoxide

A. Preparation - On the day of assay, prepare these solutions.
   1. Cytochrome C (type VI or III)-- Dissolve 16 mg/ml in incubation buffer.
   2. fMLP-- Thaw frozen stock (2.2mg in 0.5 ml DMSO). Dilute 0.1 ml with 9.9 ml incubation buffer. Repeat 100-fold dilution.
   3. Beads-- 1µm carboxylated, latex coated styrene (Polysciences)
   4. Superoxide Dismutase-- Remove frozen aliquots from -70 degree C freezer just before needed.

B. Procedure
   1. Using 12 x 75 mm polypropylene tubes with conical bottoms, add cell suspension, cytochrome C, drug, and HBSS (volumes are given on the following page as an example). Incubate at 37 degrees C for 30 min.

   2. Add beads and fMLP and continue incubating for 20 min more. Place the tubes in ice.

   3. Centrifuge at 750 xg for 10 min at 4 degrees C. Tubes with beads must be transferred to eppendorf tubes and centrifuged again in microfuge for 4 min.

   4. Transfer 400 µl of each supernatant and 800 µl incubation buffer to 1.5 ml cuvettes and measure O.D. at 550 nm using the tubes with SOD as blanks.

C. Calculations
   Record percent release, using tube with fMLP as 100 % value.

APPENDIX A: PUBLICATIONS

1. Shelly, J., Jacoby, L., Walker, S., Hoff, S., Tolmetin modulates fomyl-peptide stimulated human neutrophil functions. Pharmacologist 29(3): 124, 1987

2. Hoff, S., Shelly, J., Walker, S., Alteration of human neutrophil functions in vitro by combinations of meclofenamate with doxycycline or chlorhexidine. IUPHAR, 1987.

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John A. Shelly
Department of Pharmacology
The Chicago Medical School
North Chicago, IL 60064

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TOLMETIN MODULATES FORMYL-PEPTIDE STIMULATED HUMAN NEUTROPHIL FUNCTIONS. J. Shelly, L. Jacoby, S. Walker, and S. Hoff (SPON: A. Vazquez). Department of Pharmacology, The Chicago Medical School, North Chicago, IL 60064.

Studies were conducted to determine how Tolmetin, a non-steroidal antiinflammatory drug (NSAID), would affect several neutrophil functions associated with the inflammatory response. Therapeutic concentrations of Tolmetin (50-100µg/ml) were found to increase significantly ($p<0.01$) FMP induced chemotaxis of human neutrophils under agarose in a dose dependent manner, and FMLP stimulated neutrophils demonstrated a significant ($p<0.01$) dose dependent decrease in lysosomal enzyme release (both specific and azurophilic granules) after Tolmetin treatment. Also, phagocytosis of latex beads and superoxide anion production were markedly reduced when compared to control stimulated neutrophils. Tolmetin differs in its actions from other NSAIDs, such as Indomethacin and Ibuprofen, in its ability to increase the chemotactic responsiveness of neutrophils. These data support the proposal that NSAIDs may differentially affect discreet neutrophil functions, and thus these drugs may have multiple but different sites of activity involved in their antiinflammatory actions.

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Author(s) BY COMBINATIONS OF MECLOFENAMATE WITH DOXYCYCLINE
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S.F. Hoff, J. Shelly, S. Walker, Department of Pharmacology, The Chicago Medical School, North Chicago, Illinois, USA.

Because non-steroidal anti-inflammatory drugs (NSAID) and some antibiotics may be used simultaneously to treat infected injuries, it was of interest to determine their combined pharmacological effects. Using relevant drug concentrations, we have found that meclofenamate (MF), doxycycline (DC) and chlorhexidine (CH) significantly decrease FMLP-stimulated chemotaxis. When combined, (MF + DC) or (MF + CH) appear to demonstrate a synergistic action in their reductions of neutrophil chemotaxis. Also, MF and DC significantly decrease superoxide production, while CH may cause an increased production. Again when combined (as above), there appears to be some synergism in the inhibition of superoxide production. With regard to degranulation of lysozyme or beta-glucuronidase, MF very effectively inhibits degranulation, while DC and CH have no effect. The combined drugs (as above) inhibit degranulation to the same extent as MF alone. Further study is required to determine if these effects are therapeutically advantageous.
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Author(s) Institution S.F. Hoff, J. Shelly, S. Walker, Department of Pharmacology, The Chicago Medical School, North Chicago, Illinois, USA.

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