The Effect of Leukotriene B_4, Leukotriene C_4, Zymosan Activated Serum, Histamine, Tabanid Extract and N-formyl-methionyl-leucyl-phenylalanine on the *in vitro* Migration of Equine Eosinophils

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ABSTRACT

The migration of equine eosinophils under agarose in response to inflammatory mediators, an arthropod extract and a synthetic peptide was examined. A chemotactic index (CI) was calculated by determining the ratio of the distance of eosinophil migration towards the chemoattractant to the distance migrated towards a buffer. Differences between the CI of those eosinophils exposed to chemoattractants and those exposed only to buffer were assessed by an analysis of variance. All agents except leukotriene C_4 and the buffer induced statistically significant directional migration of eosinophils. Leukotriene B_4 (LTB_4) was the most effective chemotaxin for equine eosinophils. Migration of eosinophils stimulated by 10^{-9}M LTB_4 exceeded that induced by concentrations of histamine six orders of magnitude greater. The response of equine eosinophils to inflammatory mediators was similar to the reported behavior of human eosinophils. The ability of tabanid extract to attract equine oesinophils suggests that arthropod induced tissue eosinophilia many not depend entirely upon immunological mechanisms. The peptide, N-formyl-methionyl-leucyl-phenylalanine attracted equine eosinophils at 10^{-4}M and 10^{-3}M, concentrations that exceed those reported to be stimulatory for eosinophils of other species. The results of this study indicate that equine eosinophils are capable of migrating towards diverse stimuli, of which LTB_4 was the most effective. It is plausible that LTB_4 figures prominently in equine inflammation, particularly in lesions dominated by eosinophils.

RÉSUMÉ

Nous avons étudié la migration sur agarose d'éosinophiles équins en réponse à des médiateurs de l'inflammation, d'un extrait d'arthropode et d'un peptide synthétique. Un indice chémotactique (IC) a été calculé en établissant le rapport entre la distance migrée par les éosinophiles vers le chémo-attractant et la distance migrée vers un tampon. Les différences entre les IC des éosinophiles exposés aux chémo-attractants et ceux exposés uniquement au tampon ont été évaluées au moyen d'une analyse de variance. Tous les agents sauf le leucotriène C_4 et le tampon ont induit une migration directionnelle statistiquement significative des éosinophiles. Le leucotriène B_4 (LTB_4) était le chémo-facteur le plus efficace pour attirer les éosinophiles équins. La migration des éosinophiles stimulée par le LTB_4 (10^{-9} M) dépaissait celle induite par des concentrations d'histamine six fois plus élevées. La réponse aux médiateurs de l'inflammation était similaire à celle rapportée pour les éosinophiles humains. La capacité d'extraits de tabamid à attirer les éosinophiles équins suggère que l'éosinophilie tissulaire induite par les arthropodes ne dépendrait pas entièrement de mécanismes immunologiques. Le peptide, N-formyl-méthionyle-leucyle-phénylalanine attire des éosinophiles équins à des concentrations 10^{-4} M et 10^{-3} M, concentrations dépassant celles rapportées être stimulatoires pour les éosinophiles d'autres espèces. Les résultats de cette étude indiquent que les éosinophiles équins sont capables de répondre à divers stimuli en particulier le LTB_4. Ce dernier pourrait être un facteur important dans les processus inflammatoire chez le cheval et en particulier dans les lésions dominées par les éosinophiles. (*Traduit par Dr. Bernard Delorme*).

INTRODUCTION

The presence of eosinophils in inflammatory lesions, especially those of the skin, is frequently ascribed to type I hypersensitivity reactions or a response to parasites (1). Although these assumptions are valid in many instances, they may not be applicable to all eosinophil infiltrates (2). Eosinophils are recruited to and retained in inflammatory foci by a host of endogenous mediators which are generated by both immunological and nonimmunological mechanisms

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(2). Examination of eosinophil migration *in vitro* has established that autacoids such as the eosinophil chemotactic factor of anaphylaxis, and certain lymphokines attract eosinophils exclusively (2) whereas 12-mono-hydroxy-eicosatetranoic acid (12-HETE) (3) supposedly attracts eosinophils preferentially over other cells. The complement fragment, C5adesArg attracts eosinophils and neutrophils equally (4).

Acquisition of sufficiently large numbers of eosinophils for study has necessitated the experimental induction of eosinophilia in laboratory animals or obtaining cells from people with preexistent eosinophilia. The responsiveness and capabilities of such cells may not be representative of eosinophils in an unstimulated state (5). The equine eosinophil has gained attention because of the ease with which a large, relatively pure population of cells can be isolated from the peripheral blood of normal animals (6). Cells from such collections possess phagocytic (7), bactericidal (8) and cytotoxic capabilities (9). Leukotrienes which are produced (6) and metabolized (10) by equine eosinophils also induce the chemotaxis of these cells (11). Species differences exist among the relative chemotactic activities of the mono-HETEs and leukotrienes (11).

Similarly, there is an apparent disparity between the chemotactic behaviour of eosinophils from other species *in vitro* and our current understanding of the biology of equine inflammation. In an *in vivo* study we found that leukotriene B_4 induced a substantial eosinophilic and neutrophilic infiltrate (12) which contrasts with the predominant neutrophilic accumulation reported to occur following intradermal injection of leukotriene B_4 in laboratory animals and people (2, 13-16). Additionally, the crude form of $C5a_{desArg}$ (zymosan activated serum) does not recruit eosinophils when injected into the skin of horses (12), unlike the purported eosinophil chemotactic ability of C5adesArg *in vitro* (4).

The purpose of this study was to further characterize the migration of equine eosinophils to a variety of inflammatory mediators and phlogistins in order to determine if species differences could account for the observed idiosyncrasies of the equine inflammatory response.

MATERIALS AND METHODS

ISOLATION OF EQUINE EOSINOPHILS

Eosinophils were isolated from five clinically healthy horses according to the method of Bertram and Coignoul (17), with modifications. A total of 600 mL of whole blood obtained by jugular venipuncture, was drawn into heparinized syringes and transferred to large sterile test tubes. The following isolation procedures were performed at 4°C. After sedimentation for 45 min, the plasma, buffy coat and top 4 mL of red blood cells (RBC) was aspirated, then centrifuged at 1500 *g* for 5 min. The plasma was decanted and the RBCs were lysed by the addition of cold sterile double distilled water. Osmolality was restored by the addition of 5.4% sterile saline. The remaining leukocytes were centrifuged at 1500 *g* for 5 min and were washed twice with Hanks' balanced salt solution free of calcium and magnesium (HBSS-free, Grand Island Biological Company, Grand Island, New York). The cells were layered on to a mixture of Ficoll:Hypaque (Ficoll 400, Pharmacia Fine Chemicals, Piscataway, New Jersey; Hypaque sodium, Winthrop Laboratories, New York, New York) of specific gravity 1.085 and centrifuged at 470 *g* for 30 min. The granulocyte pellet was washed twice in HBSS-free, layered on to Ficoll: Hypaque (specific gravity 1.135) and centrifuged at 300 *g* for 20 min. Neutrophils formed a band in the middle of the test tube and the eosinophils formed a pellet at the bottom. The eosinophils were washed twice in H199 (Grand Island Biological Co., Grand Island, New York) supplemented with 1% equine serum albumin (ESA, Sigma Chemical Company, St. Louis, Missouri). A purity of at least 80-90% eosinophils of 95% viability as measured by trypan blue exclusion was obtained. An average of 8.3% of peripheral eosinophils were recovered using this method. The eosinophils were adjusted to a final concentration of 5×10^7/mL in H199-1%ESA.

CHEMOATTRACTANTS

To ensure sterility, all chemoattractants except the leukotrienes were passed through a 0.2 μm sterile filter (Gelman Science, Montreal, Quebec). Leukotrienes B_4 and C_4 (LTB_4, LTC_4) were kindly provided by Dr. J.D. Rokach, Merck-Frosst Canada, Inc., Montreal. Zymosan activated serum (ZAS) was made by adding 25 mg of washed zymosan (Zymosan A, Sigma Chemical Company, St. Louis, Missouri) to each mL of fresh equine serum. This mixture was placed in a shaking water bath (37°C, 120 oscillation/min) for 60 min. The mixtures were centrifuged at 1500 *g* for 20 min and the ZAS was aspirated by sterile Pasteur pipette. N-formyl-methionyl-leucyl-phenylalanine (N-FMLP, Sigma Chemical Company, St. Louis, Missouri), histamine diphosphate (Sigma Chemical Company, St. Louis, Missouri) and tabanid extract (Horsefly extract, Hollister-Steir, Rexdale, Ontario) were diluted with H199-1%ESA to achieve the appropriate concentrations. Histamine diphosphate and N-FMLP were adjusted to physiological pH.

CHEMOTAXIS ASSAY

A modified version of the agarose plate method (18) was used to assess eosinophil migration. A mixture of 2% washed agarose (Type I agarose, Sigma Chemical Company, St. Louis, Missouri) in sterile distilled water was boiled for 15 min and allowed to cool to 48°C. An equal volume of pre-warmed 2xHBSS and 6 mL of pooled, heat inactivated equine serum (56°C for 30 min) were added to the agar. Seven mL of this suspension were dispensed into round 60 x 15 mm sterile tissue culture plates. After the agarose solidified, the plates were inverted and refrigerated overnight to allow the agar to harden. Four rows of three wells, 2.5 mm diameter, spaced 2 mm apart were cut in the agar using a plexiglass template and stainless steel punch. The agarose plugs were removed by suction.

Ten μL of the chemoattractant, H199-1%ESA and eosinophils were placed in the outer, inner and middle agar wells respectively. Each plate contained four replicates of one chemoattractant; up to three plates

were used for some chemoattractants. At least one plate containing buffer in both inner and outer wells was used to measure random eosinophil migration, which functioned as a negative control. Experiments were repeated on separate days.

Plates were incubated at 37° C in an atmosphere of 5% CO_2 in air at 100% humidity for 3 h for all chemoattractants. The inflammatory mediators ZAS, histamine and LTB_4 were also incubated for 1.5 h. The agar was fixed by flooding with a 1:1 formaldehyde:methanol mixture for 30 min. The agar was then gently removed from the plate. The plates were flooded with Giemsa stain for 20 min and then rinsed with distilled water.

The distance of cell migration towards the chemoattractant or buffer was measured with an ocular micrometer and a 1 mm^2 grid, partitioned into 100 equal squares. The leading front of the migrating cells (19), defined as two cells lying in a equal plane within 40 μm of each other was used to determine the distance the cell population had migrated. A chemotactic index (CI) was determined by the ratio of the distance migrated towards the chemoattractant to the distance migrated towards the buffer.

STATISTICS

A residual analysis (20) was first performed to ensure that the data obtained for each chemoattractant conformed to a normal distribution. A two-way analysis of variance, randomized complete block design (20) was used to assess differences between the CI of those eosinophils exposed to chemoattractants and those exposed only to buffer. Migration, in this study, was defined as a significant difference ($p < 0.05$ or $p < 0.01$) between the CI of the chemoattractant and the negative control. Tabulated values represent the mean CI ± the standard error of the mean.

RESULTS

Most of the agents tested except for the negative control buffer and LTC_4 stimulated the migration of equine eosinophils, but their efficacy was time and concentration dependent (Tables I and II). The chemotactic indicies of the negative controls for both incubation periods approximated 1.00 (Tables I and II).

Leukotriene B_4 was the most potent chemotaxin for equine eosinophils at both incubation times (Tables I and II). At 3 h incubation, even the lowest

TABLE I. Migration of equine eosinophils under agarose at three hours incubation in response to the negative control buffer H199-1%ESA, ZAS, N-FMLP, histamine, tabanid extract and leukotrienes B_4 and C_4

Agent	Concentraion	Chemotactic index[a]
H199-1%ESA	NA	0.97 ± 0.15
ZAS[b]	NA	2.56 ± 0.33*
Leukotriene B_4	3.0×10^{-5} M	1.32 ± 0.17NS
	1.5×10^{-5} M	2.36 ± 0.2**
	6.0×10^{-6} M	4.04 ± 0.93**
	3.0×10^{-6} M	3.82 ± 0.84**
	3.0×10^{-7} M	3.78 ± 0.73**
	3.0×10^{-9} M	3.64 ± 0.89**
Leukotriene C_4	4.0×10^{-5} M	1.29 ± 0.17NS
	2.0×10^{-5} M	1.42 ± 0.15NS
	4.0×10^{-6} M	1.21 ± 0.18NS
Histamine	10^{-3} M	3.01 ± 0.51**
	10^{-4} M	2.70 ± 0.36**
	10^{-5} M	2.70 ± 0.39**
	10^{-6} M	2.41 ± 0.37**
	10^{-7} M	1.10 ± 0.18NS
N-FMLP[c]	10^{-2} M	0.95 ± 0.11NS
	10^{-3} M	3.18 ± 0.75**
	10^{-4} M	2.00 ± 0.27**
	10^{-5} M	0.87 ± 0.30NS
	10^{-6} M	1.00 ± 0.04NS
Tabanid extract	1000 PNU mL	2.38 ± 0.31**
	100 PNU mL	2.10 ± 0.23*
	10 PNU mL	0.90 ± 0.33NS
	1 PNU mL	0.90 ± 0.33NS

[a]Values are expressed as mean chemotactic index ± SEM
[b]Zymosan activated serum
[c]N-formyl-methionyl-leucyl-phenylalanine
*Migration as compared to negative control at a significance of $p < 0.05$
**Migration as compared to negative control at a significance of $p < 0.01$
NS = No significant difference between negative control and chemoattractant
NA = Not applicable as agents were not diluted

TABLE II. Migration of equine eosinophils under agarose at 1.5 hours incubation in response to the negative control buffer H199-1%ESA, histamine and leukotrienes B_4 and C_4

Agent	Concentraion	Chemotactic index[a]
H199-1%ESA	NA	1.01 ± 0.18
ZAS[b]	NA	2.30 ± 0.27*
Leukotriene B_4	3.0×10^{-6} M	1.08 ± 0.20NS
	3.0×10^{-7} M	4.54 ± 1.15**
	3.0×10^{-9} M	4.30 ± 1.03**
Leukotriene C_4	4.0×10^{-5} M	1.29 ± 0.17NS
	2.0×10^{-5} M	1.42 ± 0.15NS
	4.0×10^{-6} M	1.21 ± 0.18NS
Histamine	10^{-3} M	1.25 ± 0.02NS
	10^{-5} M	1.42 ± 0.24NS
	10^{-6} M	2.30 ± 0.30**
	10^{-7} M	1.63 ± 0.44NS

[a]Values are expressed as mean chemotactic index ± SEM
[b]Zymosan activated serum
*Chemotaxis as compared to negative control at a significance of $p < 0.05$
**Chemotaxis as compared to negative control at a significance of $p < 0.01$
NS = No significant difference between negative control and chemoattractant
NA = Not applicable as agents were not diluted

concentration of LTB_4, 3 x 10^{-9} M exerted a greater effect on migration than 10^{-3} M histamine. At 1.5 h, the response of eosinophils to LTB_4 demonstrated maximal activity at 3 x 10^{-7} M with inhibition of migration at 3 x 10^{-6} M. The optimal activity of LTB_4 at 3 h was expressed at the higher concentration of 6 x 10^{-6} M with evidence of deactivation at 3 x 10^{-3} M.

Eosinophil migration to histamine was stimulated only at 10^{-6} M. At 3 h, the response of equine eosinophils from 10^{-6} M to 10^{-3} M histamine was linear, with maximal migration at the highest concentration.

The synthetic peptide N-FMLP and tabanid extract were assessed only at the 3 h incubation period (Table I). N-FMLP stimulated eosinophil migration over a narrow concentration range, from 10^{-4} M to peak activity at 10^{-3} M. Exposure of eosinophils to concentrations of N-FMLP higher and lower than these failed to induce migration. Tabanid extracts stimulated migration at concentrations of 100 and 1000 protein nitrogen units (PNU/mL) (Table I).

DISCUSSION

The result of this study indicate that equine eosinophils are capable of migrating towards diverse chemotactic stimuli and that for some agents, the response is dose and time dependent.

Leukotriene B_4 was the most effective chemoattractant examined. The chemotactic activity of equine eosinophils exposed to the lowest concentration of LTB_4 assayed, surpassed the maximal response induced by much higher concentrations of histamine, N-FMLP and ZAS. Optimal chemotactic activity of LTB_4 was obtained at 1.5 h incubation at concentrations between 10^{-7} and 10^{-9} M. These are equivalent or slightly lower than peak stimulatory concentrations of LTB_4 required for the migration of equine eosinophils in Boyden chambers (1) or for human eosinophils under agarose (21).

Although the dose-response to LTB_4 was altered with time, the optimal chemotactic concentrations during both incubation periods were in the 10^{-6} M to 10^{-9} M range. These are tenable biological concentrations as they approximate the nanogram quantities of LTB_4 recovered from carrageenan-induced inflammatory exudate in rats and horses (22,23). Additionally, 10^{-7} M LTB_4 induced a significant eosinophil dermal infiltrate in an *in vivo* study of equine inflammation (12).

Histamine similarly recruits equine eosinophils *in vivo* (24) and induces their migration *in vitro*. In people, however, histamine is a controversial chemotaxin (25-28). It is selectively chemotactic for human eosinophils at 10^{-6} M to 10^{-7} M but inhibits chemotaxis at higher concentrations (27,28). However, by increasing the incubation period, a linear dose response curve is produced with maximal activity at 10^{-3} M (28). These changes in the dose-response relationship of human eosinophils to histamine are analogous to the response of equine eosinophils that we observed. Interpretation of results from chemotactic studies must therefore consider the effect of time on leukocyte responses.

In this study, equine eosinophil migration in response to LTB_4 and histamine was considerably influenced by time. Concentrations of LTB_4 and histamine that failed to induce migration at 1.5 h, attracted eosinophils at 3 h incubation, suggesting that eosinophils are a heterogenous population. The concept of functional aberrations due to population heterogeneity was invoked for the variable manner that human eosinophils respond to histamine (27). Two distinct eosinophil subpopulations have been identified in horses (29) and other species (5). Perhaps the affinity of receptors for specific chemotaxins also varies with cell density. If subpopulations of equine eosinophils differed with respect to chemotactic receptor density, those with a greater number of high affinity receptors may undergo receptor down-regulation first and subsequently exhibit deactivation of the chemotactic response at an earlier stage, as occurred in this study. Initial and selective down-regulation of a high affinity LTB_4-receptor on human neutrophils has been documented (30).

As with LTB_4 and histamine, tabanid extract attracted eosinophils both *in vivo* (12) and *in vitro*. The results of this study demonstrate that tabanid extract can also function as a primary chemotaxin for equine eosinophils. This activity is comparable to eosinophil chemotactic factors extracted from metazoan parasites (31,32). Although further investigation is needed, it is possible that arthropod induced eosinophilia may not entirely depend upon immunological mechanisms.

Unlike the preceeding agents, ZAS did not elicit a significant response *in vivo* (12) even though it induced the migration of equine eosinophils *in vitro*. Zymosan activated serum is chemotactically active for equine neutrophils both *in vivo* (15) and *in vitro* (33-35). Therefore, $C5a_{desArg}$, the putative, major constituent of equine ZAS (36) is a plausible inflammatory mediator in the horse. The ability for $C5a_{desArg}$ to effectively recruit human (4) and equine eosinophils *in vitro* is not reflected following its intradermal injection in either species (12,37). Reasons for this are obscure, but do illustrate the disparity that sometimes arises when attempting to extrapolate results of *in vitro* studies to complex physiological systems. The effects of zymosan activated serum, i.e. $C5a_{desArg}$ on equine eosinophils and its role in equine inflammation requires further investigation.

The biological significance of the formyl peptides on equine eosinophil migration also requires further clarification. The high concentrations of N-FMLP required to elicit the chemotaxis of equine eosinophils or neutrophils (33,38) contrasts with the nanomolar concentrations that induce granulocyte chemotaxis in other species (39,40). The effects of N-FMLP on equine leukocyte migration remain controversial and merit further examination as some groups do not recognize the chemotactic capabilities of the formyl peptides for equine granulocytes (34,35). The role of N-FMLP in equine inflammation *in vivo* is currently under investigation.

In summary, the results of this study indicate that equine eosinophils are attracted to a wide variety of chemoattractants. Overall, LTB_4 was the most potent chemoattractant tested. Leukotrienes are produced by equine neutrophils and eosinophils (6) and

have been recovered from equine inflammatory exudate (23). The results of this study and the findings of our investigation of equine inflammation *in vivo* (12) indicate that LTB_4 figures prominently in mediating equine inflammation. The relative biological importance of LTB_4 to other proposed mediators of equine inflammation, such as platelet activating factor (41,42), awaits further investigation.

REFERENCES

1. **NUTMAN TB, COHEN EA, OTTENSEN SG.** Eosinophilia and eosinophil-related disorders. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, eds. Allergy. Principles and Practice. St. Louis: CV Mosby, 1988: 861-890.
2. **SPRY CJF.** Eosinophils: A Comprehensive Review and Guide to the Scientific and Medical Literature. Oxford: Oxford University Press, 1988.
3. **GOETZL EJ, WOODS JM, GORMAN RR.** Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetranoic acid. J Clin Invest 1977; 59: 179-183.
4. **FISHER N, CZARNETZKI BM.** Comparative studies on eosinophil chemotactic factors during leukocyte migration under agarose. J Invest Dermatol 1985; 79: 222-226.
5. **WHITE CJ, MAXWELL CJ, GALLIN JI.** Changes in the structural and functional properties of human eosinophils during experimental hookworm infection. J Infect Dis 1986; 154: 778-783.
6. **JORG A, HENDERSON WR, MURPHY RC, KLEBANOFF SJ.** Leukotriene generation by eosinophils. J Exp Med 1982; 155: 390-402.
7. **ARCHER GT, HIRSCH JG.** Motion picture studies of degranulation of horse eosinophils during phagocytosis. J Exp Med 1963; 118: 287-293.
8. **JORG A, PASQUIER J, KLEBANOFF SJ.** Purification of horse eosinophil peroxidase. Biochim Biophys Acta 1982; 701: 185-191.
9. **NATHAN CF, KLEBANOFF SJ.** Augmentation of spontaneous macrophage-mediated cytolysis of eosinophil peroxidase. J Exp Med 1982; 155: 1291-1308.
10. **HENDERSON WR, JORG A, KLEBANOFF SJ.** Leukotriene generation by eosinophils. J Exp Med 1982; 155: 1291-1308.
11. **POTTER KA, LEID RW, KOLATTUKUDY PE, ESPELIE KE.** Stimulation of equine eosinophil migration by hydroxyacid metabolites of arachidonic acid. Am J Pathol 1985; 121: 361-368.
12. **McEWEN BJ, WILCOCK BP, YAGER JA, EYRE P.** The response of the eosinophil in acute inflammation in the horse. Advances in Veterinary Dermatology, Vol 1. (in press).
13. **MOVAT HZ, RETTL C, BURROWES CE, JOHNSTON MG.** The in vivo effect of leukotriene B_4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement (C5adesArg) and enhancement by prostaglandin E_2. Am J Pathol 1984; 15: 233-244.
14. **CAMP RDR, COUTTS AA, GREAVES MW, KAY AB, WALPORT MJ.** Responses of human skin to intradermal injection of leukotrienes C_4, D_4, and B_4. Br J Pharmacol 1983; 80: 497-502.
15. **CAMP R, JONES RR, BRAIN S, WOOLLARD P, GREAVES M.** Production of intraepidermal microabscesses by topical application of leukotriene B_4. J Invest Derm 1984; 82: 202-204.
16. **CARR SC, HIGGS GA, SALMON JA, SPAYNE JA.** The effects of arachidonate lipoxygenase products on leukocyte migration in rabbit skin. Br J Pharm 1981; 73: 253P-254P.
17. **BERTRAM TA, COIGNOUL FL.** Morphometry of equine neutrophils isolated at different temperatures. Vet Pathol 1982; 19: 534-543.
18. **NELSON RD, QUIE PG. SIMMONS RL.** Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol 1975; 115: 1650-1656.
19. **SMITH GS, LUMSDEN JH, WILCOCK BP.** Chemotaxis of porcine neutrophils under agraose. Can J Comp Med 1985; 49: 43-49.
20. **SNEDECOR GW, COCHRAN WG.** Statistical Methods. Ames, Iowa: Iowa State University Press, 1980.
21. **PALMBALD J, UDEN AM, FIEBERG I, RINGERTZ B, RADMARK O, LINDGREN JA, HANSSON G, MALMSTEN CL.** Effects of novel lipoxygenase products on neutrophil and eosinophil function in vitro. In: Samuelsson B, Paoletti R, Ramwell R, eds. Advances in Prostaglandin, Thromboxane and Leukotriene Research. New York: Raven Press, 1983: 25-31.
22. **SIMMONS PM, SALMON JA, MONCADA S.** The release of leukotriene B_4 during experimental inflammation. Biochem Pharm 1983; 32: 1353-1359.
23. **HIGGINS AJ, LEES P.** Detection of leukotriene B_4 in equine inflammatory exudate. Vet Rec 1984; 115: 275.
24. **ARCHER RK.** Eosinophil leucocyte attracting effect of histamine in skin. Nature 1960; 187: 155-156.
25. **FELARCA AB, LOWELL FC.** Failure to elicit histamine eosinophilotaxis in the skin of atopic man. Description of an improved technique. J Allergy 1969; 41: 82-87.
26. **WADEE AA, ANDERSON, R, SHER R.** In vitro effects of histamine on eosinophil migration. Int Arch Allergy Appl Immunol 1980; 63: 322-329.
27. **CLARK RAF, GALLIN JI, KAPLAN AP.** The selective eosinophil chemotactic activity of histamine. J Exp Med 1975; 142: 1462-1476.
28. **TURNBULL LW, KAY AB.** Histamine and imidazole acetic acid as chemotactic agents for human eosinophil leukocytes. Immunology 1976; 31: 797-802.
29. **DENNIS VA, KLEI TR, CHAPMAN MR, JEFFERS GW.** *In vivo* activation of equine eosinophils and neutrophils by experimental *Strongylus vulgaris* infections. Vet Immunol Immunopathol 1988; 20: 61-74.
30. **GOLDMAN DW, GOETZL EJ.** Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B_4. J Exp Med 1984; 159: 1027-1041.
31. **OWHASHI M, HORII Y, ISHII A.** Eosinophil chemotactic factor in Schistosome eggs: A comparative study of eosinophil chemotactic factors in the eggs of *Schistosoma japonicim* and *S. mansoni* in vitro. Am J Trop Med Hyg 1983; 32: 359-366.
32. **SASAKI O, KATSUNO M.** Leukocyte chemotactic factors in soluble extracts of *Metastrongylus apri* at different stages. Jpn Vet Sci 1983; 45: 807-809.
33. **ZINKL JG, BROWN PD.** Chemotaxis of horse polymorphonuclear leukocytes to N-formyl-L-methionyl-L-leucyl-L-phenylalanine. Am J Vet Res 1982; 46: 613-616.
34. **CAMP CJ, LEID RW.** Chemotaxis of radiolabelled equine neutrophils. Am J Vet Res 1982; 43: 397-401.
35. **SNYDERMAN R, PIKE MC.** N-formylmethionlyl peptide receptors on equine leukocytes initiate secretion but not chemotaxis. Science 1980; 209: 493-495.
36. **SLAUSON DO, SKRABALAK DS, NEILSEN NR, ZWAHLEN RD.** Complement-induced equine neutrophil adhesiveness and aggregation. Vet Pathol 1987; 24: 239-249.
37. **YANCEY KB, HAMMER CH, HARVATH L, RENFER L, FRANK MM, LAWLEY TJ.** Studies of human C5a as a mediator of inflammation in normal human skin. J Clin Invest 1985; 75: 486-495.
38. **SEDGWICK AD, LEES P, DAWSON J, MAY SA.** Cellular aspects of inflammation. Vet Rec 1987; 120: 529-535.
39. **WILKINSON PC.** Synthetic peptide chemotactic factors for neutrophils: The range of active peptides, their efficacy and inhibitory activity, and susceptibility of the cellular response to bacterial toxins. Immunology 1979; 36: 569-588.
40. **SCHIFFMAN E, CORCORAN BA, WALSH M.** N-formylmethionyl peptides as chemoattractants for leukocytes. Proc Natl Acad Sci (USA) 1975; 72: 91-93.
41. **WIMBERLY HC, SLAUSON DO, NEILSEN NR.** Functional and biochemical characterization of immunologically derived equine platelet-activating factor. Vet Pathol 1985; 22: 375-386.
42. **DAWSON J, LEES P, SEDGWICK AD.** Platelet activating factor as a mediator of equine cell locomotion. Vet Res Comm 1988; 12: 101-107.