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The role of neutrophils in acute and chronic inflammation in rats

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Summary

The effect of neutropenia on acute and chronic inflammatory oedema in rats was assessed using histamine, carrageenan and Freund's complete adjuvant as inducers. Neutropenia (about 85% reduction in peripheral blood neutrophil count) was induced with intraperitoneal administration of 2.5 mg/kg methotrexate for three consecutive days. Acute paw oedemas induced with carrageenan and Freund's complete adjuvant, but not that induced by histamine, were significantly decreased in neutropenic animals compared with controls. In adjuvant-induced chronic knee swelling (Adjuvant arthritis), neutropenia produced small, statistically non-significant, suppressive effect. In contrast, it significantly suppressed adjuvant-induced chronic paw swelling, although suppression was observed only in the late phase component of the swelling. The results suggest that neutrophils are involved in certain acute and chronic inflammatory responses but not in others.

Résumé

L'effet de la neutropenie dans l'edema inflammatoire aiguë et chronique chez les rats est éstimé avec l'histamine, le carrageenan et l'adjuvant complet du Freund comme des facteurs qui provoque ces edemas. La neutropenie (represent environ 85% de reduction dans le nombre des nuetrophiles présent dans le sang peripheral) est provoquée par la methotraxate administrée à 2.5mg/kg par la voie intraperitonéale pendent 3 jours de la suite. Les edemas aigue de la patte provoquees par le carrageenan et l'adjuvant complet du Freund mais pas celle provoquee par l'histamine sont significativement reduites chez les animaux neutropenique par rapport aux controles. Dans l'enflament chronique du genou provoquée peu de l'adjuvant (arthrite adjuvant), la nuetropenie a provoquée pou d'effect répressif (pas statistiquement significatif). Par contre, elle a supprimée de facon significatif l'enflement chronique de la patte provoquee par l'adjuvant, bien que la répression soit visible seulement dans un dernier stade constituent l'enflement. Les resultats laisse supposer que les neutrophiles sont impliquées dans quelques inflammations aigue et chronic mais pas dans d'autres.

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Introduction

A possible role for neutrophils in inflammation was clearly highlighted by Page and Good [1], who demonstrated that the acute inflammation that is usually observed following subcutaneous injection of egg white into rabbits failed to occur in neutropenic animals. Other reports employing experimentally-induced neutropenia have confirmed the observation, [2, 3, 4, 5, 6].

In chronic inflammation such as rheumatoid arthritis, there is also some evidence for a role for neutrophils. For example, Goldlust, and his co-workers [7], demonstrated large infiltration of polymorphonuclear leucocytes in synovial fluid of joints of patients with chronic joint inflammation which correlated with the severity of joint destruction. Similar results have also been observed in experimental chronic inflammation in animal models [8].

The mechanism whereby neutrophils may contribute to inflammatory oedema was believed to be the release of lysosomal enzymes in the process of phagocytosis [9]. The enzymes would then damage the vascular endothelium to allow leakage of plasma proteins. A more recent work by Wedmore and Williams [10] has however suggested a more direct involvement of these cells in inflammation. They showed that increased vascular permeability induced by many non-particulate factors (non-phagocytosable) such as C5a, formyl methionyl leucyl phenylalanine (FMLP) and leukotriene B4 are neutrophil-dependent, while those caused by other equally non-phagocytosable factors such as his-

tamine and bradykinin are neutrophil-independent. They thus concluded that for some agents, but not others, neutrophils interacted with endothelial cells in some way to cause increased vascular permeability without the involvement of phagocytosis. It has therefore become necessary that any role for neutrophils in inflammation would now have to be determined for individual defined experimental models.

In the present work, therefore, we have induced neutropenia in rats with methotrexate in order to examine the role played by neutophils in various acute and chronic inflammatory oedemas using established inducers — histamine, carrageenan and Freund's complete adjuvant.

Materials and methods

Animals

Male albino rats of Wistar strain weighing 100 – 200g were used in these studies. Animals were freely supplied with food and water.

Induction of neutropenia

Acute neutropenia was induced by intraperitoneal injection of 2.5mg/kg methotrexate for three days. Eight days after the first injection, the animals were used for the experiments. Differential and total leucocyte count at this point confirmed neutropenia of about 85% in most of the animals. Animals having less than 80% neutropenia were excluded from the experiments.

In chronic experiments, the neutropenia induced as above was maintained for a further 14 days by injection of 0.8 mg/kg methotrexate every three days for the next 14 days, each injection being followed by intraperitoneal administration of folinic acid supplement (18mg/kg) to prevent toxicity, [11]. Cell counts (differential and total) were made in both control and methotrexate-treated animals just before the induction of inflammation. For chronic experiments, three additional counts were made in the course of 14 days, and for the analysis of results the mean of the four counts was used.

Neutrophil count

Blood for both total and differential counts was obtained from the tail vein. For the differential count, dried blood smear was fixed in absolute alcohol, stained in Giemsa and then counter-stained in Leishman's stain. A 100-cell differential count was done under high power (oil immersion) of a light microscope. Total count of appropriately diluted blood was performed under low power using improved Neubauer's haemocytometer. Absolute neutrophil count was therefore obtained by multiplying the total neutrophil count with the percentage neutrophil content.

Induction and evaluation of inflammation

Acute paw swelling was induced into the left hind leg by a subplantar injection containing 0.1ml of one of the following agents: 2mM histamine acid phosphate, Freund's complete adjuvant and 2% (w/v) carrageenan in normal saline. The same volume of saline was injected into the right hind paw to serve as control. Paw volume was measured using vernier calipers which measured up to 0.01mm, and measurement was made blind. Readings were taken at the point where free movement of the calippers just became restricted by the paw. Acute paw swelling in both legs was monitored at 10 minutes interval for 30 minutes in the case of histamine, and at 1 hour interval for 6 hours for the rest. Rat adjuvant arthritis (chronic inflammation) was assessed in those animals in which inflammation was induced with Freund's complete adjuvant. Injection of the latter into a foot pad of rat usually produced an acute swelling in the injected paw, followed by two types of chronic inflammation — a slowly evolving swelling of the contralateral paw and a similar swelling of the contralateral knee joint. In our chronic experiments, both paw and knee joint diameters were measured daily for 14 days using the method described above.

Drugs

Histamine acid phosphate and carrageenan were obtained from Sigma Chemicals (U.S.A), while Freund's complete adjuvant was a product of Difco Laboratories (U.S.A). Methotraxate was purchased from Lederle Laboratories (U.K).

Analysis of results

Quantitatively, inflammation was expressed as area under the inflammatory response versus time curve. Since the response was progressive in nature, this analysis was preferred to the use of the response at a particular time, as it takes better care of any skewed peaking of responses.

The percentage change in paw or knee which was used to plot the time-course graph was obtained from the following equations. For acute inflammation:

$$\text{Percentage increase in diameter} = \frac{(L_{id} - L_o) - (R_s - R_o)}{L_o} \times 100$$

where L_o = diameter of left paw before injection of inducer
L_{id} = diameter of left paw after injection of inducer
R_o = diameter of right paw before injection of saline
R_s = diameter of right paw after injection of saline

For chronic inflammation:

$$\text{Percentage increase in diameter} = \frac{L_2 - L_1}{L_1} \times 100$$

where L_1 = diameter of right paw or knee before induction
L_2 = diameter of right paw or knee after induction

Areas under the curve were determined planimetrically, and values expressed in arbitrary square units. Statistical comparison of results was carried out using student's *t*-test.

Results

Effect of neutropenia on acute paw inflammation

Three consecutive administration of methotrexate (2.5mg/kg) into rats produced by the eight day a profound (80 – 90%) neutropenia. Other cell types were only slightly affected (less than 10% increase or decrease). Figure 1 shows the time course of the effect of neutropenia on acute paw oedema induced by three different agents – histamine, carrageenan and Freund's complete adjuvant. In control animals histamine produced a rapid but moderate paw swelling that reached a peak of about 40% increase within 30 min. In neutropenic animals, this response was essentially unaffected, (Fig 1a). When injected into normal animals both adjuvant and carrageenan produced profound paw swelling, but while carrageenan-induced swelling reached a peak of 94.7% increase within 4 hours and declined thereafter, that of adjuvant continued to increase after 6 hours. In neutropenic rats, the responses were substantially reduced (Figs 1b and 1c)

In Fig. 2, the results were expressed as areas under the time-course curves, and superimposed on the corresponding mean neutrophil count. In the histamine model, (Fig 2a), the mean inflammatory

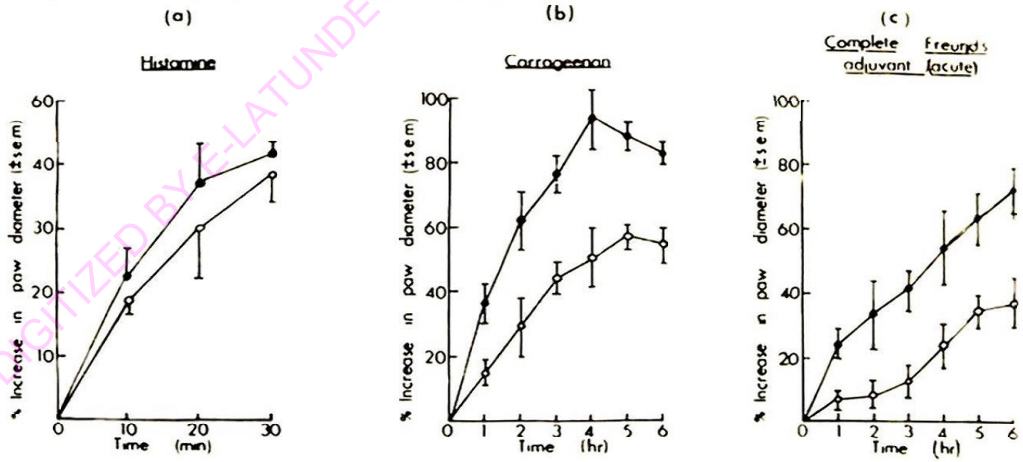

Fig 1: Time-course of acute inflammatory rat paw oedema induced with histamine, carrageenan or Freund's complete adjuvant in normal (●) and neutropenic (O) rats. Mean neutrophil counts are as in Fig 2. Values are means of 5 – 6 experiments.

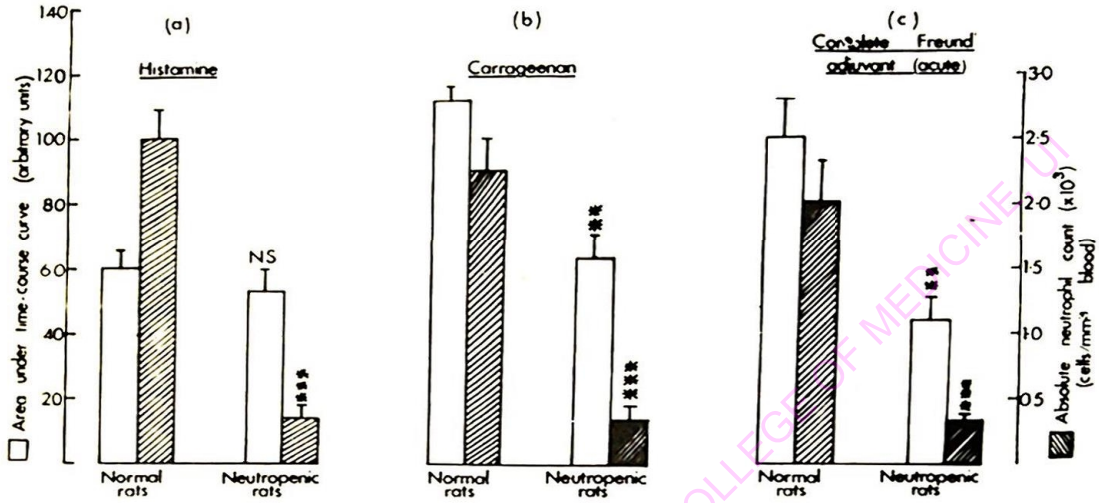

Fig. 2: Effect of neutropenia on acute paw oedema induced by histamine, carrageenan and Freund's complete adjuvant. Results are expressed as areas under the response-time curves (open histogram), juxtaposed to the corresponding neutrophil count (hatched histogram). In all cases, statistical comparison was made between neutropenic and normal animals. $^{***}P < 0.001$; $^{**}P < 0.01$; NS = non-significant.

response in normal animals (mean neutrophil count (MNC) 1005 ± 129 cell/mm^3 blood) was 60.3 ± 5.5 sq. units. This was reduced to a mean value of 53.1 ± 6.9 sq. units in neutropenic animals, (MNC = 145 ± 18 cells/mm^3). The difference was not statistically significant ($P > 0.05$), even though there was 85.5% mean reduction in neutrophil number.

In carrageenan-induced inflammation, (Fig. 2b), the mean response in control animals of 111.6 ± 4.0 sq. units (MNC 902 ± 115 cells/mm^3) was reduced to 63.6 ± 6.8 sq. units in neutropenic animals with MNC of 131 ± 13 cells/mm^3. The reduction was statistically significant at ($P < 0.01$). Similarly, the acute phase of adjuvant-induced paw oedema was reduced from 100.1 ± 12.7 sq. units in control rats (MNC 806 ± 103 cells/mm^3) to 43.9 ± 7.2 sq. units in neutropenic rats (MNC = 133 ± 10 cells/mm^3), (Fig 2c). The reduction was also statistically significant ($P < 0.01$).

Effect of neutropenia on chronic paw inflammation

Injection of Freund's complete adjuvant into the left hind paw of rats produced, in addition to the acute left paw swelling, a slowly evolving (chronic) swelling of the right paw and knee joint by the 14th day, which are correlates of rat arthritis.

Figures 3(a) and 3(b) show the time-courses of the right paw and knee respectively following injection of adjuvant into the left paw. Paw swelling was triphasic reaching the first peak at day 2, (12.8%), decreased slightly at day 4, and then increased steeply to a second peak (23.4%) on day 7. Finally there was a third increase which continued without peaking up to day 14 (last day). A similar but non-identical pattern of response was observed in knee swelling but with the first and second peaks occurring on days 4 and 9 respectively. The third increase continued up to day 14. In neutropenic animals the triphasic response was also observed and in these animals, only the third phase of swelling (day 10 – 14) was reduced. Expressed quantitatively, as areas under dose response curves, (Fig 4a and 4b), swelling in normal rats (MNC = 908 ± 103 cells/mm^3) was reduced from 68.3 ± 4.0 sq. units to 42.5 ± 5.9 sq. units in neutropenic animals, (MNC = 133 ± 10 cells/mm^3), Fig 4a.

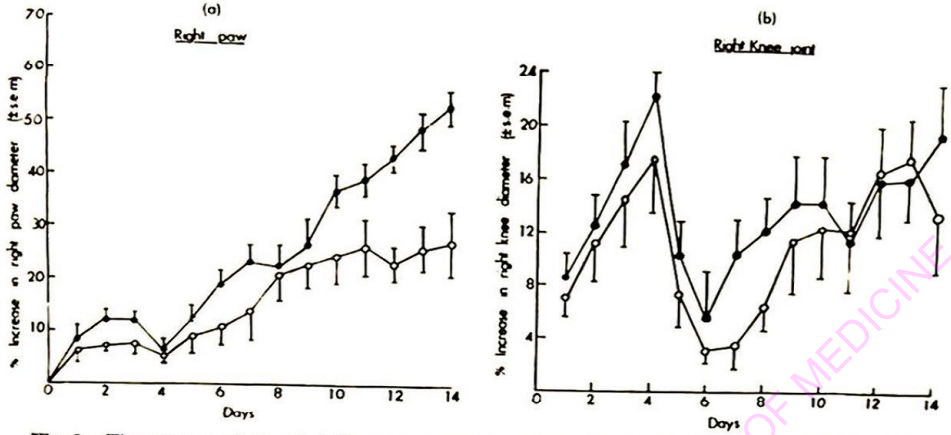

Fig. 3: Time-course of chronic inflammatory swelling of non- injected (right) paw and right knee joint induced by the injection of Freund's complete adjuvant into the left paw. (●) represent normal animals while (○) represent neutropenic animals. Neutrophil counts are as in Fig. 4. Values are means of 6 – 8 experiments.

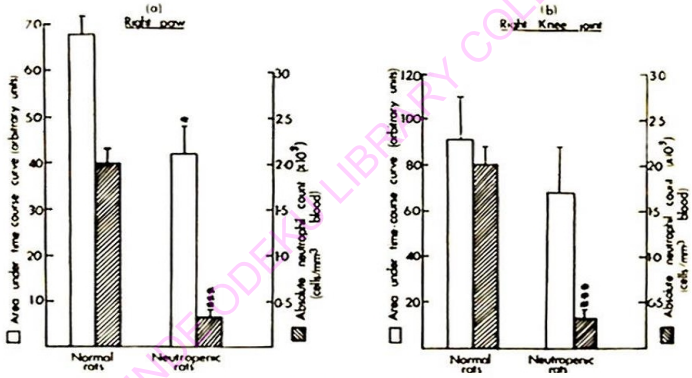

Fig. 4: Effect of neutropenia on chronic paw and knee (of non- injected right foot) inflammation induced by injection of Freund's complete adjuvant into the left hind paw. Results are expressed as area under the response-time curves (open histograms), juxtaposed to the corresponding mean neutrophil counts (hatched histogram). $^{***}P < 0.001$; $^{*}P < 0.05$.

The decrease was statistically significant at $P < 0.05$. Analysis of the right knee swelling showed that the reduction observed in neutropenic animals (from 91.1 ± 27.2 to 68.0 ± 24.6 sq. units) did not reach statistical significance.

Discussion

We have used methotrexate to induce a fairly selective neutropenia in order to study the involvement of neutrophils in various forms of acute and chronic inflammatory oedema in rats. Results show that circulating neutrophils are clearly involved in some but not other forms of rat oedema. For example, inspite of comparable neutropenia of 85.5%, histamine-induced paw oedema was completely unaffected, whereas those of carrageenan and adjuvant were highly suppressed. The result obtained with histamine is in agreement with recent findings in rabbit skin by Wedmore and Williams, [10], that oedema induced by certain mediators such as histamine and bradykinin are neutrophil-independent whereas those induced by others like C5a and leukotriene B4 are neutrophil-dependent.

In adjuvant-induced chronic inflammation, neutropenia significantly reduced swelling in the contralateral paw, (though this occurred only from the 10th

day after induction), but suprisingly failed to reduce knee joint swelling in the same limb. The reason for this differential effect is not readily apparent. The swelling of the knee of the uninjected leg is believed to resemble chronic arthritis in man. The lack of inhibition of this effect is therefore surprising since it is known that in rheumatoid arthritis in man and antigen-induced chronic joint disease in experimental animals, neutrophils often accumulate in the synovial tissues and that the extent of accumulation correlates with the severity of joint destruction [7, 12, 8].

The mechanism whereby neutrophils may be involved in producing increased vascular permeability (oedema) is not clear. However, it is believed that neutrophils accumulate at sites of inflammation for the purpose of phagocytosis, and that during the process, lysosomal enzymes and oxygen-derived radicals are released which damage vascular endothelium and basement membrane to allow vascular permeability [9, 12]. While this may possibly explain the neutrophil-dependence of oedema induced by phagocytosable agents like zymosan, it may not explain that induced by soluble agents such as leukotriene B4 and C5a [10]. Thus, the question as to why oedema caused by some agents are neutrophil-dependent while that by others are not, still remains. It may be that, among other possibilities, the former agents act indirectly by releasing permeability-increasing factor from neutrophils while the latter act directly on the endothelial cells.

Methotrexate is frequently employed as a neutropenic agent in experimental animals [13, 4] and although the possibility that it exhibits a non-specific anti-inflammatory effect cannot be completely ruled out, this seems unlikely since only oedema induced by certain agents but not others are affected. It has also been shown that administration of the drug 3 hours before induction of inflammation, (by which time no neutropenia had developed) had no significant effect on the resulting inflammatory response [11]. Furthermore, since there is a 5 day gap between the last administration of the drug and the induction of inflammation, it is unlikely that there would be sufficient plasma level of the drug to exert such pharmacological effect.

In summary, it is concluded that acute rat paw oedema induced by carrageenan and Freund's complete adjuvant are neutrophil- dependent, while that due to histamine is not. Furthermore, the late phase component of adjuvant-induced chronic paw oedema appeared to be neutrophil-dependent whereas chronic knee joint oedema was not. Thus the involvement of neutrophils in inflammation may be dependent both on the type and site of the reaction. Further work is called for in order to understand why neutrophils are involved only in some forms of inflammatory conditions and the mechanism and conditions of their involvement.

References

1. Page AR, and Good RA. A clinical and experimental study of the function of neutrophils in inflammatory responses. Am. J. Pathol. 1958; 34: 645-656.
2. Vinegar R, Traux JF, and Selph JL. Pedal oedema formation in agranulocytic rats. Fed. Proc., 1971; 30: 385
3. Arrigoni-Martelli E, and Restelli A. Development of nystatin oedema and lysosomal enzyme release at the site of inflammation in normal and leukopenic rats. Abstract of the Fifth International Congress on Pharmacology, San Francisco, 1972; p. 10.
4. Higgs GA. Acute oedema formation in neutropenic rats. Abstract of IUPHAR 9th International Congress of Pharmacology, London, 1984; p. 445.
5. Hambleton P. and Miller P. Vascular changes associated with cellular accumulation in the rat. Abstract of IUPHAR 9th International Congress of Pharmacology, London, 1984; p. 446.
6. Williams TJ, Hellewell PG and Jose PJ. Inflammatory mechanisms in Arthus reaction. Agents and Actions, 1986; 19: 66 – 72.
7. Goldlust MB, Rich LC and Brown WR. Immune synovitis in rabbits: effects of differing schedules for intra-articular challenge with antigen. Am. J. Pathol. 1978; 91: 329-344.
8. Hunneyball IM, Ball M, Crossley M and Spow AGE. Pharmacological studies of antigen-induced arthritis in Balb/c mice. Agents and Actions 1986; 18: 384-393.
9. Burke JS, Uriuhara T, Macmorine DRL and Morat HZ. A permeability factor released from phagocytosing PMN leucocytes and its inhibition by protease inhibitors. Life Sci. 1964; 3: 1505.
10. Wedmore CV and Williams TJ. Control of vascular permeability by polymorphonuclear leucocytes in inflammation. Nature 1981; 289: 646-650.
11. Mansour A and Nelson DS. Effect of hydrocortisone, cyclophosphamide, azathioprine and methotrexate on cutaneous delayed and Arthus hypersensitivities in the rat. Int. Arch. Allergy. appl. Immunol. 1979; 60: 50-59.
12. Bainton DF. The cells of inflammation: a general review. In: Weisserman G. ed. The Cell Biology of Inflammation. North Holland: Elsevier, 1980: 1-25
13. Willoughby DA and Spector WG. Inflammation in agranulocytic rats. Nature 1968; 219: 1258.

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