Alkaline phosphatase as a marker of maturity in human neutrophils

Studies in normals dosed with aetiocholanolone and prednisolone

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SUMMARY Changes in the level of neutrophil alkaline phosphatase (NAP) in a population of peripheral blood neutrophils were determined in healthy subjects dosed with either aetiocholanolone (Aetio, 4 mg/m² im) or prednisolone sodium succinate (Pred, 30 mg/m² iv). Before dosing the mean NAP score, as measured by a modified Gomori azo-dye method, was 109 and 118 in the Aetio and Pred groups, respectively. Fifteen hours after injection the NAP score in the Aetio group had risen to 187 concomitant with the appearance of large numbers of juvenile (band) and mature neutrophils. Twenty-four hours after dosing, the NAP score increased to 213 with still further concentrations of juvenile cells, while the numbers of mature neutrophils returned to approximately baseline values. Five hours after injection, in subjects given Pred, the NAP score had fallen to 108 concomitant with a marked increase in the numbers of mature neutrophils. These data in normals dosed with either Aetio or Pred appear to substantiate a 'first-in, first-out' cellular progression with initial release of the oldest of the mature bone marrow reserve neutrophils containing less (relative to juvenile forms) NAP activity. These data also indicate that NAP activity is inversely related to cellular age and may support previous findings in the rat that enzyme levels are higher in bone marrow relative to peripheral blood.

In the rat, it has been shown that the levels of alkaline phosphatase in circulating neutrophil polymorphonuclear leucocytes are inversely related to the age of those cells, as determined by [³H]-thymidine labelling and changes taking place in nuclear morphology.¹ Further evidence for the inverse relationship between neutrophil age and alkaline phosphatase content is provided by a study of comparative neutrophil alkaline phosphatase (NAP) scores performed on rat bone marrow and blood.² This study demonstrated that alkaline phosphatase levels were higher in bone marrow neutrophils than in the older cells circulating in peripheral blood. In man, however, levels of NAP, as determined by both biochemical and cytochemical methods, are reported to be higher in peripheral blood than in bone marrow.³ This relationship implies that large numbers of juvenile (band) and mature neutrophils released from the bone marrow

reserve under stimulus would reduce the overall peripheral blood NAP score.

We have examined this hypothesis by dosing normal subjects either intramuscularly with aetiocholanolone (Aetio) or intravenously with prednisolone (Pred). Both these agents are known to increase the total blood pool (TBGP) by releasing neutrophils contained in the bone marrow reserve.^{4 5} We compared peripheral blood levels of NAP before and after the challenge (Aetio and Pred) using a quantitative enzyme cytochemical technique.

Material and methods

GENERAL PLAN OF INVESTIGATION

Eighteen normal healthy subjects (1 F, 17 M) were invited to participate in the study, and the possible risks were fully explained to them. In most instances, men rather than women were recruited because of the known influence of the hormonal cycle on leucocyte counts in the latter. In all subjects, the intake of food and the amount of physical activity were not controlled, but the subjects were asked not to participate in duties calling for strenuous exercise.

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Subjects asked to volunteer for control studies had similar intakes of food and degrees of physical activity to those experienced in the experimental portion of the study. In all instances subjects rested for (not less than) 15 minutes before the taking of blood samples to minimise variations due to extraneous factors.

AETIOCHOLANOLONE: EXPERIMENTAL AND CONTROL SERIES

A standardised dose (4 mg/m² im) of Aetio (Aetiocholan-3 alpha, 11β-diol-17-one, Sigma Chemie GmbH, FRG) suspended in propylene glycol (final concentration of 30 mg/ml) was given in the buttock of nine healthy men aged 19 to 28 between 0800 and 0900 on the day of the study. Three of the above test subjects were dosed on a separate occasion (approximately four weeks after the injection of Aetio) with an intramuscular injection of an equal volume of fluid as was used experimentally but consisting only of propylene glycol.

PREDNISOLONE: EXPERIMENTAL AND CONTROL SERIES

A standardised dose (30 mg/m² iv) of Pred ([11β, 17,21-Trihydroxypregna-1,4-diene-3,20-dione 21-(sodium succinate)], E Merck, FRG) was given in the forearm vein of nine healthy subjects (1 F, 8 M) aged 18 to 24 between 0800 and 1130 on the day of the study. Four of the men were dosed on a separate occasion (approximately three weeks after the infusion of Pred) with an intravenous injection of an equal volume of fluid used experimentally but containing only 0.9% isotonic saline.

TAKING OF SAMPLES OF WHOLE BLOOD

In all instances above (both experimental and control), samples of whole blood were taken from a 19-gauge indwelling cannula inserted in a forearm vein and collected into sterile plastic syringes (without anticoagulant) before (baseline) the injection of the active or control substance and at various intervals up to 24 hours after dosing. The samples of collected whole blood were either dispensed into EDTA, Na₂ tubes for determination of the full blood count (measured in triplicate with the Coulter Counter Model S), or small aliquots were placed on grease-free glass slides and duplicate smears were prepared. Dried smears were stored in a desiccator maintained at between 4 and 6°C until reacted chemically.

MEASUREMENT OF NAP AND DIFFERENTIAL COUNTS

Levels of NAP were determined using the cytochemical method (modified Gomori azo-dye tech-

Fig. 1 Changes in leucocyte count and NAP score in nine normal healthy men given Aetio intramuscularly (→) in a standardised dose of 4 mg/m² body surface area between 0800 and 0900 on the day of study. Each point is the mean ± SEM of the determinations on the nine subjects.

nique) previously described by Williams.¹ Smears made in duplicate were fixed in 60% acetone, buffered with citrate to pH 4.2 for 10 seconds at 20°C before incubation with Na alpha-naphthyl phosphate; the diazonium salt was Fast Blue RR. Finally, smears were counterstained with Giemsa buffered to pH 5.6 with M/15 Sørensen's buffer. Alkaline phosphate levels in individual cells were scored on a 0-4 scale, as described by Williams *et al.*,² who have shown that the results obtained in this manner correlated well with the results of the more lengthy microphotometric method.¹ One hundred cells, 50 being close to the edge of the smear and 50 from the centre, were scored on each of the two duplicate smears. The aggregate of these 200 individual scores was the NAP score for that sample. The differential

Table 1 Relationship between neutrophil count and NAP score, expressed as the ratio of post-values/pre-values, after dosing nine normal men with Aetio

	Hours after injection					
	3	6	9	12	15	24
Neutrophils-post/ Neutrophils-pre						
$\bar{x}$	0.99	2.14	2.28	2.32	2.07	1.29
SEM	0.05	0.21	0.15	0.18	0.21	0.08
NAP-post/NAP-pre						
$\bar{x}$	0.98	1.00	1.13	1.29	1.73	1.98
SEM	0.02	0.02	0.04	0.06	0.05	0.07

leucocyte count was determined by averaging the counts of 200 cells on each of the duplicate smears.

Results

SUBJECTS DOSED WITH AETIOCHOLANOLONE

Changes in peripheral blood leucocyte count

In nine normal men given an intramuscular injection of Aetio, the highest neutrophil count was reached 9 to 12 hours (ratio of neutrophils-post/neutrophils-pre ranged between 2.28 and 2.32) after dosing (Fig. 1, Table 1). The numbers of mature neutrophils then declined, and by 24 hours after injection the count was approaching baseline levels. The numbers of juvenile neutrophils in the peripheral blood did not rise until approximately 9 to 15 hours after injection (Table 2), and by 24 hours still greater numbers were observed. The numbers of lymphocytes remained relatively unchanged after dosing, even though a slight rise in the numbers of monocytes was observed.

Table 2 Relationship between appearance of juvenile neutrophils in peripheral blood of nine normal men after receiving an intramuscular injection of Aetio and the changes in the NAP score

	Control	Hours after injection	
		15	24
Juvenile neutrophils ($\times 10^9/l$)			
$\bar{x}$	0.10	0.31	0.38
SEM	0.05	0.03	0.09
Juvenile neutrophils (% of total leucocyte differential)			
$\bar{x}$	0.9	3.3	5.8
SEM	0.1	0.7	0.9
NAP score			
$\bar{x}$	109	187	213
SEM	6	5	4

In three of the above test subjects dosed on a separate occasion with only propylene glycol, this agent was observed not to influence the neutrophil

Fig. 2 Changes in leucocyte count and NAP score in three healthy men given Aetio (●) intramuscularly (→) in a standardised dose of 4 mg/m² body surface area. Approximately four weeks after the dose of Aetio these same test subjects were given an intramuscular injection of an equal volume of fluid to that used above, but consisting only of propylene glycol (▲). In both instances, subjects were injected between 0800 and 0900 on the day of study. Each point is the mean $\pm$ SEM of the determinations on the three subjects. Cell counts (neutrophils, lymphocytes, and monocytes) $\times 10^9/l$.

count up to 24 hours after an intramuscular injection (Fig. 2). A typical diurnal rise was observed in the lymphocyte count, while the numbers of monocytes varied throughout the period of sample taking.

Changes in the NAP score

The average NAP score of a population of neutrophils residing in the peripheral blood of nine men before dosing with Aetio was 109 ± 6 (SEM) (Fig. 1). This value rose slowly and by 12 hours after injection the ratio of NAP-post/NAP-pre was

Table 3 Summary of direct NAP scores in nine men dosed with Aetio

Time interval after dosing (h)	Mean NAP levels per band cell	Mean NAP levels per non-Band cell
Baseline	...	1.1*
3	3.0	1.2
6	2.5	1.2
9	3.0	1.4
12	2.3	1.4
15	2.3	1.9
24	2.6	2.2

*Score ranges from 0 to 4.
Each mean (N = 9) is derived from the individual scores on each cell in the sample for every smear at every time interval shown above.

Fig. 3 Changes in leucocyte count and NAP score in nine normal healthy test subjects administered Pred intravenously (→) in a standardised dose of 30 mg/m² body surface area between 0800 and 1130 on the day of study. Each point is the mean $\pm$ SEM of the determinations on the nine subjects.

1.29. By 24 hours after injection the ratio had risen to 1.98. The steep rise in the ratio of NAP:post/NAP:pre was correlated with the appearance of greater numbers of juvenile neutrophils in the peripheral blood (Table 2). As the number of mature neutrophils in blood fell after 15 to 24 hours (neutrophils:post/neutrophils:pre ratio declined from 2.07 to 1.29), the NAP ratio rose from 1.73 to 1.98 concomitant with further increases in the numbers of juvenile neutrophils. In comparing the mean NAP levels *per cell* in juveniles with the levels in the other non-juvenile cells at various times after dosing, it was shown that the juvenile cell NAP content did not fluctuate noticeably throughout the experiment, but gradually the content of the remainder of the cell population rises (Table 3). However, at no point did the non-juvenile NAP content rise above that of the juveniles.

In three of the test subjects dosed on a separate occasion with only propylene glycol, the NAP score remained essentially unchanged throughout the 24-hour post-injection period (Fig. 2).

SUBJECTS DOSED WITH PREDNISOLONE

Changes in peripheral blood leucocyte count
In nine healthy subjects administered an intravenous injection of Pred, the highest concentration of peripheral blood neutrophils was reached 3 to 7 hours (ratio of neutrophils:post/neutrophils:pre ranged between 2.23 and 2.63) after dosing (Fig. 3, Table 4); concomitantly there was both a lympho- and monocytopenia. The numbers of mature neutrophils then declined, and by 24 hours after injection the count was approaching baseline levels. The concentration of juvenile neutrophils remained essentially unchanged after the injection of Pred.
In four of the above subjects dosed on a separate occasion with only 0.9% isotonic saline, the concentration of mature and juvenile neutrophils, as well as the numbers of lymphocytes and monocytes,

Table 4 Relationship between numbers of mature neutrophils and subsequent NAP score, both expressed as ratio of post-values/pre-values after dosing nine normal test subjects with Pred

	Hours after injection					
	1	3	5	7	9	24
Neutrophils:post/Neutrophils:pre						
$\bar{x}$	1.25	2.23	2.63	2.24	1.80	1.38
SEM	0.25	0.40	0.34	0.21	0.15	0.15
NAP:post/NAP:pre						
$\bar{x}$	0.97	0.96	0.91	0.92	0.92	0.95
SEM	0.03	0.03	0.02	0.07	0.03	0.02

Fig. 4 Changes in leucocyte count and NAP score in four healthy men given Pred (●) intravenously (→) in a standardised dose of 30 mg/m² body surface area. Approximately three weeks after the injection of Pred these same test subjects were administered an intravenous infusion of an equal volume of fluid as was used above but containing only 0.9% isotonic saline (▲). In both instances, subjects were injected between 0900 and 1005 on the day of study. Each point is the mean $\pm$ SEM of the determinations on the four subjects. Cell counts (neutrophils, lymphocytes, and monocytes) $\times 10^9/l$.

remained essentially unaltered during the 24-hour observation period (Fig. 4).

Changes in the NAP score

The mean NAP score of peripheral blood neutrophils determined in nine subjects before dosing was 118 ± 2 (SEM) (Fig. 3). This baseline value fell slowly, and by 5 hours after injection the ratio of NAP-post/NAP-pre was 0.91, even though the ratio of neutrophils-post/neutrophils-pre was 2.63 (Table 4). These findings were in contrast to what we had observed in subjects dosed with Aetio. By 24 hours after injection the NAP ratio had risen to 0.95; concomitantly the neutrophil ratio had fallen to 1.38.

In four of the subjects dosed only with 0.9% isotonic saline on a separate occasion, the NAP score remained essentially unchanged throughout the 24-hour observation period (Fig. 4).

Discussion

While estimations of the alkaline phosphatase content of neutrophils are widely utilised in clinical medicine,⁶ there is disagreement as to the precise localisation of the enzyme. Bainton and her co-workers^{7,8} have claimed that the enzyme is associated with the specific (secondary) granules at the myelocyte stage of development, although it subsequently becomes latent to demonstration ultrastructurally until the cell has begun to phagocytose.⁷ However, Bainton's illustrations show the enzyme to be associated with the membrane of phagocytic vacuoles rather than with the granules, which remain unreactive. By contrast, Borgers *et al.*⁹ have shown the enzyme to be present on the plasma membrane in man, and we have demonstrated a similar location in the rat neutrophil.¹⁰ Biochemical studies on cell fractions from mature neutrophils also lend support to the view that the enzyme is membrane- rather than granule-associated.¹¹

There is, however, general agreement that maturation of the neutrophil before its release from the bone marrow reserve is accompanied by atrophy of the Golgi apparatus and an involution of the endoplasmic reticulum with concomitant diminution in the number of ribosomes.¹² Thus, as the cell matures, its ability to synthesise alkaline phosphatase ceases, so that levels of the enzyme might be expected to decrease as the cell ages. This view is supported by findings in the rat showing that the NAP score diminishes as nuclear segmentation increases¹ and that the score is higher in bone marrow neutrophils than in their older circulating counterparts.² In man, however, peripheral blood NAP activity is reported to be higher relative to bone marrow.³ This observation implies that neutrophils released from the bone marrow reserve under stimulus would 'dilute' the circulating pool with cells containing low alkaline phosphatase activity,

subsequently reducing the overall NAP score. Our results in normal subjects dosed with Aetio have, however, shown that a marked rise in the overall peripheral blood NAP score is paralleled by the release of juvenile and young non-band neutrophils from the bone marrow reserve (Fig. 1; Tables 2 and 3).

Aetio, a metabolite of dehydroepiandrosterone, produces fever and leucocytosis in man.¹³ The leucocytosis consists principally of an increase in mature neutrophils with small numbers of immature cells.¹⁴ The increment in circulating neutrophils is associated with an expanded TBGP, and cells are distributed into the circulating pool and marginated pool in equal proportions.¹⁵ After dosing normal subjects with Aetio, we observed increased numbers of both mature and immature neutrophils in the peripheral blood (Fig. 1; Table 2). However, of particular interest was the continual rise in the NAP score 15 to 24 hours after injection, concomitant with a steady diminution of the numbers of mature neutrophils (Table 1). The sustained elevated NAP score during this period was shown to correlate with increased numbers of juvenile and young non-band neutrophils (Tables 2 and 3). Godwin *et al.*,¹⁶ using [³H]-thymidine labelling in man, observed that when the bone marrow reserve is large (normal), Aetio mobilises neutrophils which have been in the reserve pool for a period of time before releasing more recently matured cells. On the other hand, a small bone marrow reserve will release newly formed cells as rapidly as they are produced. These data appear to substantiate an orderly 'first-in, first-out' progression, with preferential release of the oldest of the mature marrow neutrophils contained in a normal reserve pool.^{17, 18} Our test subjects given Aetio possessed a normal bone marrow reserve,* and thus we believe that the cells initially released from the reserve pool would be the most 'mature' and presumably contain relatively low NAP activity. This sequence would support the slow rise in the overall NAP score during the first 12 hours after injection, even though the neutrophil ratio was > 2 (Table 1). Juvenile cell numbers did not rise appreciably until 9 to 15 hours after dosing, indicating a delayed release from the bone marrow reserve. In this regard the overall NAP score rose in response to the release of juvenile and young non-band cells from the reserve pool (Tables 2 and 3). These data clearly support the bone marrow reserve cellular release mechanism induced by Aetio.¹⁶ We know that Aetio increases the circulating

pool by depleting the bone marrow reserve,^{15, 16} causing a 'shift to the left',¹⁴ and the NAP score rises in response to the presence of large numbers of juvenile and non-band neutrophils (Table 2 and 3). We believe, therefore, that the measurement of the NAP score can be utilised to assess the age of neutrophils in peripheral blood, the levels of the enzyme being inversely proportional to the age of the cells.

Large numbers of mature neutrophils are seen in peripheral blood 4 to 6 hours after dosing man with Pred.¹⁹ The induced neutrophilia observed after administering glucocorticoids is due to an increase in the TBGP,²⁰ even though the increase in the circulating pool is slightly greater relative to the marginated pool.⁵ The expanded TBGP results from changes in both the accelerated release of cells from the bone marrow reserve and from a decrease of cellular egress from blood, but primarily the former.⁵ In the present study, the peak blood neutrophilia appeared 5 hours after Pred had been administered to normal subjects (neutrophil ratio 2.63); concomitantly the NAP ratio was 0.91 (Fig. 3, Table 4). Throughout the 24-hour period after dosing, the numbers of juvenile neutrophils remained essentially unaltered. These data, combined with a relatively unchanged NAP score, suggest that Pred 'selectively' released the most 'mature' of the reserve pool neutrophils possessing lower (relative to juvenile cells) NAP activity. These findings are similar to those previously reported in normal subjects dosed with either dexamethasone²¹ or Pred.²² With reference to our findings in subjects given Aetio, we believe that Pred 'preferentially' mobilises mature neutrophils stored previously for some time in the reserve pool, and this observation may explain the peripheral blood neutrophilia seen in the absence of a profound 'shift to the left'. The exact mechanism of cellular release from the reserve pool under the influence of either Pred or Aetio remains unclear, even though the mode of administration (intravenous or intramuscular) would differentiate between acute and prolonged exposure to the stimulus. It appears, however, that Aetio has a much more profound depleting effect on the reserve pool than Pred under the conditions of the present investigation.

The present study also seemed an indirect test of the hypothesis that, as in the rat, NAP activity is higher in bone marrow relative to peripheral blood.^{1, 2} Our results in normal subjects appear to substantiate this relationship, and this can be clearly seen in Table 3, in which juvenile cells (youngest cells circulating in normal blood) have higher alkaline phosphatase activity relative to non-band neutrophils. Our results also show that, under stimulus, the bone marrow initially mobilises the more 'mature'

*A rise in the neutrophil count of greater than 2600 μ l after a dose of 0.1 mg Aetio per kg is regarded as evidence of a normal bone marrow reserve.¹⁴

neutrophils that have been previously stored in the reserve pool for a longer period of time before releasing younger cells possessing higher alkaline phosphatase activity. This release sequence substantiates a 'first-in, first-out' cellular progression^{17 18} and accounts for the delayed rise in both the numbers of juvenile and non-band neutrophils possessing higher NAP activity (Table 3). It could be hypothesised that peak levels of alkaline phosphatase are probably attained somewhere around the juvenile stage (or even earlier) and decrease thereafter. This would account for the lower (relative to juvenile cells) alkaline phosphatase activity observed in mature neutrophils released initially from the reserve pool after Aetio stimulation (Table 3).

From a more practical standpoint, our results in normal subjects indicate that NAP may be utilised as an approximate marker of cellular maturity relative to normal baseline values in a population of neutrophils residing in the peripheral blood.

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