# Letters to the Editor

### Changes in neutrophil alkaline phosphatase following splenectomy

Spiers et al (*J. clin. Path.*, |28, 517-523, 1975), in a recent lead article, postulated that more mature neutrophils exhibit weaker alkaline phosphatase activity than younger forms. They, furthermore, theorized that young neutrophils tend to be sequestered in such storage sites as the spleen and bone marrow, where they age and decrease in alkaline phosphatase activity. They hypothesize that neutrophil alkaline phosphatase levels are controlled by release of such sequestered neutrophils into the peripheral circulation. This concept is based in part on their observations that splenectomized patients exibit a transient rapid rise in neutrophil alkaline phosphatase activity (NAP) followed by a more gradual decrease to levels below normal.

Unfortunately, the authors did not control their experimental design and were apparently unaware of studies (Kaplow and Beck, 1964; Valentine et al, 1954; Wyllie, 1962) clearly demonstrating that surgery alone will cause an initial rise in NAP activity with peak elevations occurring two to three days after the start of surgery with a return to normal levels in five to nine days. These findings are almost identical with the observations reported after splenectomy by Spiers et al. Thus, the changes in NAP reported by these authors are very probably the result of surgical trauma and are not due to removal of a major neutrophil storage site. Changes in NAP are remarkably non-specific in nature, and responses are extremely sensitive to inflammatory reactions regardless of aetiology. It is likely that elevated NAP occurring after surgery is a non-specific response to tissue necrosis and/or accompanying changes in adrenal-pituitary hormonal activity.

The authors also emphasize the alleged increased sensitivity of the method for assessing NAP used in their study (Rutenberg et al, 1965), when compared to 'those which it now supersedes'. No references are provided, but presumably they refer to the technique originated by Kaplow (1955) or modifications thereof (Hayhoe and Quaglino, 1958; Kaplow, 1963). Their assumption of increased sensitivity is not supported. Indeed, the validity of comparing scores by the two methods is questionable since different

scoring criteria are used. The method recommended by the authors of the article in question is based on granule counts of individual azo dye particles, whereas, with conventional techniques, granules are not counted and instead each neutrophil is subjectively rated according to the distribution and intensity of the precipitated dye. The former method yields very discrete granular staining and is probably the method of choice in delineating enzyme localization sites. The latter yields a more diffuse reaction product. It is not recommended for determining loci of enzyme activity but has found general acceptance as being easy to score. Even if the objections to comparing scores by the two methods are ignored, the data presented by Spiers et al do not justify their conclusion. Their staining technique requires a 15-minute incubation period and they report a range of normal scores from 37 to 98, whereas conventional methods require only a 10-minute incubation period yielding scores ranging from 14 to 100 with a mean of 46 (Hayhoe and Quaglino, 1958) and 13 to 130 with a mean of 61 (Kaplow, 1963). An increase in the incubation period to 15 minutes would considerably raise these values. By prolonging the incubation time, stronger staining can be obtained with either method. However, increased sensitivity is not necessarily a desirable feature. For clinical studies, ideal staining conditions are those which will demonstrate the widest spectrum of scores as found in physiological and pathological states. Our own experience has indicated that a 10-minute incubation period is optimal for this purpose. To the best of my knowledge, no data have ever been presented which indicate a superiority of one method over the other in identifying untreated patients with CGL by cytochemically staining for NAP. It is regrettable that the authors so imply without offering evidence to support such a claim.

The relationship of NAP to neutrophil maturity is unclear. Available evidence would also suggest the opposite of what Spiers et al propose. Although a very rare band neutrophil may show weak staining for NAP activity, greater than 98% of such cells do not stain, and myelocytes and metamyelocytes are almost uniformly devoid of activity. Trubowitz et al (1959) have demonstrated that bone

marrow neutrophils exhibit considerably lower alkaline phosphatase activity than neutrophils of the peripheral circulation. Yet, if younger myeloid cells contained more alkaline phosphatase than older forms, one would expect strong activity in marrow neutrophils. Unpublished observations from this laboratory indicate that tissue neutrophils other than in bone marrow stain more strongly for NAP than peripheral blood neutrophils. The authors did not stain splenic imprints for NAP which would have confirmed or refuted their assumption that these cells are low in activity. The above considerations lend strong support to the concept contrary to that proposed by Spiers et al and suggest that physiologically or chronologically older neutrophils contain more not less NAP than younger cells. This is in accord with the views of Trubowitz et al (1959) and Pedersen and Hayhoe (1971).

### References

Hayhoe, F. G. J. and Quaglino, D. (1958). Cytochemical demonstration and measurement of leucocyte alkaline phosphatase activity in normal and pathological states by a modified azo-dye coupling technique. *Brit. J. Haemat.*, 4, 375-389.

Kaplow, L. S. (1955). A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow. *Blood*, 10, 1023-1029.

Kaplow, L. S. (1963). Cytochemistry of leukocyte alkaline phosphatase. Use of complex napthol AS-phosphates in azo dye coupling techniques. *Amer. J. clin. Path.*, 39, 439-449.

Kaplow, L. S. and Beck, R. E. (1964). Leukocyte alkaline phosphatase response to surgical stress. *Surg. Gynec. Obstet.*, 119, 97-102.

Pedersen, B. and Hayhoe, F. G. J. (1971). Relation between phagocytic activity and alkaline phosphatase content of neutrophils in chronic myeloid leukaemia. *Brit. J. Haemat.*, 21, 257-260.

Rutenberg, A. M., Rosales, C. L., and Bennett, J. M. (1965). An improved histochemical method for the demonstration of leukocyte alkaline phosphatase activity: clinical application. *J. Lab. clin. Med.*, 65, 698-705.

Trubowitz, S., Feldman, D., Benante, C. and Hunt, V. M. (1959). The alkaline phosphatase content of the human polymorphonuclear leukocyte in blood and marrow. *Amer. J. clin. Path.*, 31, 483-486.

Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S. (1954). Studies on leukocyte alkaline phosphatase activity: relation to "stress" and pituitary-adrenal activity. *J. Lab. clin. Med.*, 44, 219-228.

Wyllie, R. G. (1962). Neutrophil alkaline phosphatase. Response to acute inflammation. *Aust. Ann. Med.*, 11, 118-125.

LEONARD S. KAPLOW
*Laboratory Service,*
*Veterans Administration Hospital,*
*West Haven, Conn. 06516*
*and Yale University School of Medicine,*
*New Haven, Conn. 06510, USA*

The authors have commented as follows:

We appreciate Professor Kaplow's comments but he has not correctly understood some of our points. We believe that other work, which he interprets as contradicting our hypothesis, actually confirms it. We postulated that young neutrophils possess alkaline phosphatase activity whereas elderly or postmature forms do not. In chronic granulocytic leukaemia (CGL) the well-documented expansion of the neutrophil storage pools and prolonged intravascular life span of neutrophils tend to lower the proportion of young neutrophils in the blood because transit time through the expanded pools is prolonged and, in addition, young neutrophils in the circulation are diluted by the population of postmature cells without NAP activity. Drug treatment for CGL reduces the size of the storage pools and frequently produces a modest increase in the NAP score: removal by splenectomy of a major site of storage is consistently followed by a more marked elevation of NAP.

Direct controls for our experiments were not available, because in our patients with CGL surgical procedures other than splenectomy are rare. We are aware of the publications cited by Professor Kaplow (Kaplow and Beck, 1964; Valentine *et al*, 1954; Wyllie, 1962) which describe changes in NAP score in patients without CGL who underwent surgery. However, their findings are *not* 'almost identical with the observations reported after splenectomy'; there are important differences. (A) All three reports describe a peak in NAP score at 48 to 72 hours, whereas in our patients with CGL the peak occurred as early as 18 hours after operation and always before 48 hours. (B) In nine patients without CGL studied by Kaplow and Beck, the mean increase in NAP score after surgery was 180%, whereas in eight patients with CGL we found a median increase of 700% (ie, eight times the preoperative median). (C) In all the reports cited, the NAP score returned to normal levels within six to 10 days of operation if no infection occurred: in half of our CGL patients the score remained above its preoperative value for six or more months after operation. The stress effects of surgical trauma cannot explain this whereas removal of a storage site can. Even if surgical trauma influences the immediate postoperative NAP score to some extent, the observation that patients with CGL can produce large numbers of NAP-positive cells remains valid.

After surgery in patients without CGL (Kaplow and Beck, 1964) a neutrophil leucocytosis with a left shift occurred at 24 hours and declined at 48 to 96 hours, while the peak in NAP score was at 48 to 72 hours. This supports our view that NAP-positivity is a property of the young mature neutrophil: apparently NAP is absent from the band form just as it is from the ageing neutrophil, and, as Professor Kaplow also found, NAP activity is not maximal in the presence of a marked left shift. The neutrophilia and *simultaneous* peak NAP score after splenectomy in CGL is not accompanied by a left shift and is a different phenomenon—young mature neutrophils with NAP activity are reaching the circulation instead of being filtered off by the spleen.

That the staining technique used (Rutenburg *et al*, 1965) is more sensitive than many older techniques is substantiated by our finding that NAP scores of zero are extremely rare in CGL and never observed in normal subjects, whereas with some techniques the 'normal range' extends to zero. We concede Professor Kaplow's point that, to have optimal discriminant value, it is sometimes undesirable for a method to have maximum positivity. We do not consider the NAP score to be of great value in establishing the diagnosis of CGL, since it is often subnormal in other haematological conditions, and the diagnosis is better established by other means (Galton and Spiers, 1971). Our principal reasons for preferring Rutenburg's technique are its consistent success, even in inexperienced hands, and the lack of fading (coverslipped slides may be stored for weeks and checked or reviewed when desired).

Methods based on brentamine fast garnet staining possess neither of these advantages.

We confirm the finding of Trubowitz *et al* (1959) that the NAP score in bone marrow films is characteristically low: this was also the case in our CGL patients. But it is not reasonable to assume that the bone marrow must contain a high proportion of young mature neutrophils; these are diluted by band forms (newly-produced) and also by postmature neutrophils (because the marrow is a storage pool as well as a site of production). Both the diluting populations are NAP-negative. We have recently studied the NAP score in imprints of CGL spleens (unpublished) and have confirmed our assumption that these cells are low in activity, which we attribute to their postmaturity. The relationship of NAP score to neutrophil maturity has been further clarified by the recent findings of Williams (1975) that in animals given pulses of tritiated thymidine, the levels of NAP within peripheral blood neutrophils are inversely related to the age of those cells. Thus the NAP levels are highest in the youngest circulating neutrophils and lowest in the oldest circulating cells. The low levels of NAP in CGL are therefore probably due either to a decreased rate of entry of neutrophils into the circulation associated with an increased half life, or to the selective entrapment of young cells in the spleen. The marked effect of splenectomy suggests that the latter mechanism is important.

We feel that our clarification of the low NAP activity of the spleen and bone marrow cells in CGL, and of the time relations of the peak NAP score after splenectomy, which are different from those observed after surgery in patients without CGL, should answer Professor Kaplow's main objections. Furthermore, animal experiments support our view that NAP is present in the young mature neutrophil and absent from the ageing postmature neutrophil. The abnormalities of NAP score observed in CGL are most economically explained by the hypothesis that in this disease there is accumulation of postmature NAP-negative cells.

References

Galton, D. A. G. and Spiers, A. S. D. (1971). Progress in the leukemias. In *Progress in Hematology*, vol. VII, edited by G. B. Brown and C. V. Moore, pp. 343-405. Grune & Stratton, New York.

Kaplow, L. S. and Beck, R. E. (1964). Leukocyte alkaline phosphatase response to surgical stress. *Surg. Gynec. Obstet.*, **119**, 97-102.

Rutenburg, A. M., Rosales, C. L., and Bennett, J. M. (1965). An improved histochemical method for the demonstration of leukocyte alkaline phosphatase activity: clinical application. *J. Lab. clin. Med.*, **65**, 698-705.

Trubowitz, S., Feldman, D., Benante, C., and Hunt, V. M. (1959). The alkaline phosphatase content of the human polymorphonuclear leukocyte in blood and marrow. *Amer. J. clin. Path.*, **31**, 483-486.

Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S. (1954). Studies on leukocyte alkaline phosphatase activity: relation to "stress" and pituitary-adrenal activity. *J. Lab. clin. Med.*, **44**, 219-228.

Williams, D. M. (1975). Leucocyte alkaline phosphatase as a marker of cell maturity: a quantitative cytochemical and autoradiographic study. *Brit. J. Haemat.*, **31**, 371-379.

Wyllie R. G. (1962). Neutrophil alkaline phosphatase. Response to acute inflammation. *Aust. Ann. Med.*, **11**, 118-125

A. S. D. SPIERS and
D. M. WILLIAMS
*MRC Leukaemia Unit and*
*Department of Histopathology,*
*Royal Postgraduate Medical School,*
*Du Cane Road, London W12 0HS*

## Platelet Counts in Normal Pregnancy

We are pleased to be able to confirm the findings of Sejeny et al (*J. clin. Path.*, **28**, 812-813, 1975) that, using a Coulter Counter, the platelet count decreases progressively and significantly in normal pregnancy. Sejeny et al suggest, without adducing any evidence, that this decrease in count may be due to an increase in plasma volume. We, however, have evidence that does not prove, but strongly suggests, that this haemodilutional effect

is not nearly sufficient to account for the observed drop in platelet count.

We have studied about 30 patients in each trimester and 24 non-pregnant women. The results will be published elsewhere, but in brief we have found a significant progressive decrease in count and increase in the mean platelet volume so that the total platelet volume mass per ml (volume $\times$ numbers/ml) remained approximately constant. The concept of a constant total platelet mass has been reported before (O'Brien and Jamieson, 1974; Behrens, 1975). Additionally, we found marked shortening of the heparin thrombin clotting time of platelet-poor plasma. This may perhaps reflect the presence of platelet factor 4 liberated into the plasma as the result of thrombosis that occurs normally and extensively even in the healthy placenta.

From the table it will be seen that, as expected, the haemoglobin falls progressively through pregnancy. The packed cell volume also fell but there was no evidence of iron deficiency developing since the MCHC remained constant. Therefore it seems reasonable to assume that the fall in haemoglobin reflected a relative increase in plasma volume. Assuming that the original total platelet count remained constant, in this case the mean was $272 \times 10^9/1$, it is then possible to calculate what the count would have been if it had been diluted in the increased plasma volume as had the red cells. It will be seen that the observed count decreased far in excess of the calculated figure due to haemodilution. Thus very probably the total number of circulating platelets per unit volume decreases absolutely.

### References

Behrens, W. E. von (1975). Mediterranean macrothrombocytopenia. *Blood*, **46**, 199-207.

O'Brien, J. R. and Jamieson, S. (1974). A relationship between platelet volume

and platelet number. *Thrombos. Diathes. haemorrh.* (*Stuttg.*), **31**, 363-365.

Sejeny, S. A., Eastham, R. D., and Baker, S. R. (1975). Platelet counts during normal pregnancy. *J. clin. Path.*, **28**, 812-813.

J. R. O'BRIEN
*Central Laboratory,*
*St. Mary's General Hospital, Portsmouth*

## Amylase Assay by the Phadebas Method

In the past we have personally corrected a misconception among some colleagues concerning the assay of amylase by the Phadebas method but now that this erroneous notion has recently appeared in print it becomes necessary to respond likewise. Ojala and Harmoinen (1975), in the discussion on the methodology of the Phadebas amylase kit (Pharmacia AB), confirm the manufacturer's literature that the substrate does not remain suspended but sinks after the initial shaking at the beginning of incubation. They then go on to claim that 'a clearly higher and better reproduced amylase value was obtained by shaking the mixture by hand vigorously during the entire incubation'. We find no significant difference in activity or reproducibility of the assay whether the reaction tubes are (A) shaken or (B) remain unshaken during incubation.

A mean amylase value = $501 \pm 12$ U/l
$n = 10$
B mean amylase value = $500 \pm 12$ U/l
$n = 10$.

Higher absorbances were encountered with the shaken tubes (A), as were noted originally by Ceska et al (1969), but this was paralleled by a higher absorbance of the blank.

The implication of the statement of Ojala and Harmoinen is that while the substrate sediments the enzyme remains in solution in the supernatant. However, it can readily be shown by the experiments detailed below that the amylase is adsorbed on to the solid phase substrate during the initial mixing after addition of the tablet. A further series of reaction tubes containing the same serum was centrifuged immediately after addition of the tablet to the assay mixture. The supernatants were decanted into clean tubes, absorbances were noted, and incubation was carried out after the addition of a further tablet. The increase in absorbance in this series was minimal ($\Delta A < 0.01$). Simultaneously with this series, 4 ml of 0.9% saline was added to the residues in the original tubes and the assay procedure was continued. The

| | Controls | 1st Trimester | 2nd Trimester | 3rd Trimester |
|---|---|---|---|---|
| Haemoglobin (g/dl) | 13·4 | 12·7 | 12·5 | 12·2 |
| Packed cell volume | 0·41 | 0·39 | 0·38 | 0·37 |
| Haemoglobin per unit volume (MCHC) | 32·7 | 32·6 | 32·9 | 33·0 |
| Observed platelet count ($\times 10^9/1$) | 272 | 249 | 243 | 210 |
| Expected count due to haemodilution | — | 257 | 253 | 247 |

Table  *Possible effect of heamodilution on platelet count: means*