The Effect of Incubation and Storage on Human Platelet Structure as Studied by Electron Microscopy

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Despite the well-known susceptibility of platelets to damage on handling or storage comparatively little has been written about the changes which platelets undergo on incubation or storage. Nevertheless, numerous studies have been undertaken employing platelets incubated at 37°C for various times to study certain aspects of their metabolism, or preincubating them with enzymes or radioisotopes to study their behavior in certain in vitro conditions. It is obvious that interpretations of such experiments could be fallacious unless allowance is made for the effect of incubation itself.

Platelet transfusions may be strikingly effective in certain clinical situations and radiation injury, but their routine usage is precluded by a number of practical problems. One of the most important of these is the lack of the practical method for storing platelets so that they can retain their viability and a platelet transfusion bank be established. Most attempts to prolong platelet viability during storage have followed technics successfully employed for the erythrocyte, such as nucleotide "additives," but while yielding useful information they have not completely answered this problem. This is not surprising when the in vivo life span, structural, and metabolic differences between the platelet and erythrocyte are considered.

The present study was undertaken in an attempt to define the morphologic and certain chemical changes occurring in platelets during incubation and storage, to enable a better understanding of, and perhaps approach, to the above problems. These studies suggest that autolytic or lysosomal activity in the platelet may be a major factor in the platelet's susceptibility to handling and storage, and show that marked morphologic and metabolic changes may occur within the platelet after short periods of in vitro incubation.

Methods

Five hundred-mi. samples of whole blood were collected from healthy volunteer donors into plastic bags containing 50 ml of 1.5 per cent Na EDTA in 0.85 per cent saline. This was then centrifuged at 320 g. for 20 minutes at 0°C in an International Centrifuge Model...
The resultant platelet-rich plasma was carefully expressed into a transfer pack. These transfer packs were then either incubated at 37 C. for 3, 6 and 24 hours with occasional shaking or stored in a cold room at 4 C. for a period of up to 2 weeks; a zero time sample was also obtained. At the end of their incubation or storage the contents of the plastic bags were centrifuged at 1000 g. for 30 minutes at 0 C. and the platelet button resuspended in a small portion of 0.85 per cent saline and the overall volume measured. A 1 ml. aliquot of this was then processed for electron microscopy as outlined below. A nucleotide extract was prepared from the remainder.

**Electron Microscopy**

The platelet sample was centrifuged at 3000 g. at 0 C. for 10 minutes and the platelet button covered with 2 per cent osmic acid for 1 to 1 1/2 hours. It was then processed through 50–100 per cent alcohol, "stained" 30 minutes in phosphotungstic acid in absolute alcohol, and then embedded in Araldite; sections with silver to gold interference colours were prepared with an L.K.B. ultramicrotome and examined in a Siemen's Elmiskop I.

**Nucleotide Extraction and Determination**

The platelet suspension was centrifuged at 3000 g. for 10 minutes and the platelet button extracted with 1.5 M perchloric acid at 0 C. in an all glass homogenizer, 1 volume of platelet suspension being added to 2 volumes of 1.5 M perchloric acid. The homogenate was then centrifuged and the precipitate re-extracted with 0.6 M perchloric acid and again centrifuged. The supernatants from both extractions were pooled and neutralized with 12.0 M KOH to pH 8.9 The potassium perchlorate was removed by centrifugation at 0 C. and the extracts applied to 15 cm. columns of Dowex — 1 Formate. The nucleotides were eluted with stepwise addition of appropriate concentration of formic acid and formic acid and ammonium formate commencing with 0.13 N formic acid.1 The nucleotides were identified by their optical density ratios at 260 and 280 m_, their position of elution from the column, and by paper chromatography. The concentration of ATP and ADP was calculated as described by Bishop et al.10

In one experiment platelet-rich plasma obtained from two 500 ml. samples of whole blood was pooled, a platelet count performed by the method of Brecher & Cronkite,11 and 50-ml. aliquots incubated or stored under sterile conditions as already outlined. At the end of incubation the platelet-rich plasma was centrifuged at 3000 g. for 15 minutes at 4 C. and the platelet button washed once with cold 0.85 per cent NaCl and a nucleotide extract prepared as above. This was lyophilized and taken up in 2.5 ml. of distilled water. Descending paper chromatography was performed on 100 _m_., samples using Whatman No. 1 Paper and a solvent system of isobutyric acid and 1 N ammonia (150:90) Krebs & Hems.12 The nucleotide spots corresponding to ADP and ATP were located with a U-V lamp, eluted with 0.01N HCl and the optical densities determined at 250, 260, 280 and 290 m_. Their identity was confirmed by comparison with Rf values of known standards and by the 250/260 260/280 ratios. The concentration of ATP and ADP was calculated from the molar extinction coefficient.

**RESULTS**

**Morphologic Changes**

Figure 1 shows the electron microscopic appearance of a normal platelet. Its structural features have previously been described in detail.18 Particularly prominent are the vesicles, which usually have a lining membrane, the granules, which are approximately 0.4 _m_ in diameter, and the mitochondria. Small slit-like vesicles are also often noted (fig. 1) as well as background staining of the platelet cytoplasm with phosphotungstic acid.
Incubation at 37 C.

Figure 2 shows a typical appearance seen in platelets after 3 hours incubation at 37 C. The vesicles have become more prominent and enlarged with a resultant increase in the platelet’s diameter. In many platelets a system of canals appears to link some of the large vesicles and a number of granules are contained within a vesicle which occasionally appears to join the canal system. Some granules have become abnormal in appearance with central densities. After 6 hours, the changes are similar but more pronounced.

After 24 hours, incubation at 37 C, gross disorganization of the platelet’s internal structure occurs (fig. 3). Note the circumscribed central area containing a platelet granule, two swollen mitochondria identified by their double membrane and cristae, and a partly disintegrated granule and debris. This structure resembles an autophagic vacuole which de Duve has suggested is derived from lysosomes. Figure 4 and 5 show more advanced autolysis with complete loss of granules which have been replaced by smaller fragments. Again the area of autolysis is circumscribed. In other platelets vesicles are still present but are not nearly as evident as in the 3 or 6 hour samples. In only rare instances were normal platelet granules present and the overall picture was that of gross disorganization of the platelet’s internal structure.

Storage at 4 C.

After 48 hours, relatively little change was noted in the majority of platelets apart from some loss in density of the cytoplasm. The granules appeared
Fig. 2.—Platelets after 3 hours incubation at 37 C. Note the prominence of the canals and the inclusion of a granule in a vesicle-like structure (arrows). C, canals; V, vesicle, G, granule.

Fig. 3.—A platelet after 24 hours incubation at 37 C. There is a circumscribed area containing mitochondria and a granule, suggesting an autophagic vacuole.
normal but some mitochondria were more darkly staining than usual. In a few platelets however, grossly abnormal structures were evident and some granules now appeared to be contained in vesicles (fig. 6). At the end of 1 week the platelet was far less disorganized than after 24 hours at 37 C. Granules were present in normal numbers and many of them were normal in appearance. In some platelets, however, the granules had bizarre forms producing quite beautiful mosaic patterns and in some, fragments appeared to be forming within the granules (fig. 7). Mitochondria were readily identified but often were much more darkly stained than usual and appeared to vary more than usual in size. Other platelets revealed structures similar to autophagic vacuoles or the postulated end result of these structures, residual bodies14 (fig. 8). After 2 weeks, storage some precipitate formed in the plasma and the platelet count was halved. This precipitate was centrifuged off and consisted of grossly altered platelets on electron microscopy. Because of their appearance we have called these “seaweed” forms (fig. 9). The remaining platelets showed similar features to those described for 1 week storage except that autophagic vacuoles and residual bodies appeared more prominent.

Nucleotides

The ATP content of freshly isolated platelets was 5.9 \( \mu \text{M/10}^9 \) platelets whereas ADP was 2.3 \( \mu \text{M/10}^9 \) platelets. After 6 hours, incubation at 37 C. the ATP level dropped to 20 per cent of its initial value in one experiment and to
Fig. 5.—Platelets after 24 hours incubation at 37 C. Arrows point to vacuolated areas which appear to be preparing to discharge their contents to the exterior. Marked disorganization is apparent in the remaining platelets.

Fig. 6.—A platelet stored for 48 hours at 4 C. Near normal in appearance but note the three granules which appear to be contained by vesicles.
Fig. 7.—A platelet stored for 1 week at 4 C. Note the small bodies and appearance of fragments within granules giving a mosaic effect.

Fig. 8.—A platelet stored for 2 weeks at 4 C. There is still a full complement of granules, although some have eccentrically placed pale areas (arrows). The mitochondria are abnormal in appearance and one is markedly swollen. R, could represent a residual body; M, mitochondria.
Fig. 9.—Low power electron microscope view of platelets stored for 2 weeks at
4°C. S, “seaweed” form (see text).

40 per cent in another whilst GTP had completely disappeared. After 24
hours at 37°C, no ATP was detected. When platelets were stored at 4°C the
drop in ATP was far slower and after 48 hours it was approximately 70 per
cent of the initial level. At the end of 2 weeks this was further reduced to
20 per cent in one instance and could not be detected in the other.

DISCUSSION

The obvious morphologic changes occurring in platelets after 3 hours of
incubation at 37°C emphasize the importance of recognizing that platelets
handled in this way may differ significantly metabolically and otherwise from
normal platelets. This is also supported by the striking loss of platelet ATP
content which occurred under these conditions within 6 hours.

These observations also improve our concept of the normal platelets ultra-
structure. It would seem that an intricate canular system traverses the plate-
let (fig. 2) and that many of the granules may lie within this system (fig. 6)
or at least within a vesicle. These structures are not apparent on examination
of normal platelets although indefinite structures which probably represent
this canular system may be seen (small vesicle (fig. 1) and occasionally a
granule is seen within a vesicle. Fifteen to 30 seconds after calcium is added
to normal platelet-rich plasma platelet granules are observed free in the
plasma. At this stage, all the platelets appear intact and it is only at a later
interval—usually 3 minutes—that a few of the free lying platelets appear
to be disintegrating.13 French and Poole15 have suggested that a process re-
sembling pinocytosis in reverse may be an explanation of granule release
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while platelets remain intact. Certainly, although the opening up of the canals is a very striking effect of incubation, in no instance were canals observed to connect with the exterior of the cell to suggest the presence of pores through which granules could escape during the early phase of viscous metamorphosis.

Feissly et al.18 have previously studied the morphologic changes occurring in platelets after storage at 4 C. for periods up to 80 days. They comment on the disappearance of the dense granules, the development of vacuolization and the continued presence of mitochondria. In the present investigation the granules, although often unusual in appearance, were still present up to 14 days at 4 C. However, there was a striking loss of granules after only 24 hours incubation at 37 C. The granules were replaced by smaller fragments (figs. 4 and 5) contained within a well-demarcated area which in a number of instances appears to be preparing to discharge its contents to the exterior (fig. 5). It is possible that some of the inclusions observed in granules (fig. 7) may be the forerunners of these fragments.

Zucker et al.17 first drew attention to the presence of acid phosphatase in the platelet. This enzyme has been used by De Duve14 as a marker for lysosome activity and it has been suggested that some of the platelet granules may be lysosomes.18 This is supported by the morphologic studies of this investigation and the demonstration of acid phosphatase activity in washed isolated platelet granules.19 De Duve14 has postulated that these bodies play an important role in intracellular digestion following phagocytosis and in cellular breakdown by the release of the catalytic enzymes and he has picturesquely termed them “suicide sacs” in the process of cell autolysis.8 It has also been suggested that the stability of the lysosome membrane containing the catalytic enzyme may be influenced by certain agents: glucocorticoids appear to increase the lysosome’s resistance to breakdown, whereas Vitamin A has the opposite effect.20 Since many of the changes occurring in platelets on incubation and storage appear to be due to autolysis, further investigation of this process may be one promising approach to the problem of platelet storage. It is also interesting to speculate on the possible role of platelet lysosomes in the dissolution of in vivo thrombi.

Baldini et al.21 have shown that the loss of platelet viability as measured by platelet survival studies is very rapid even in platelets stored at 4 C. It is notable that the ATP level in platelets stored for 48 hours at 4 C. was still quite high being 70 per cent of the initial value. Such an ATP level would be compatible with a normal life span in the erythrocyte22 and yet Baldini et al.21 have shown that platelets stored for this period of time possess negligible viability. This re-emphasises the important metabolic differences between the platelet and erythrocyte which must be remembered when investigating the effects of storage on these cells. Cohen and Gardner23 have reported that stored platelets may retain a normal capacity for clot retraction after 54 hours’ storage at 0 C. although their viability was negligible. Since ATP is a requisite for clot retraction it is logical that the platelet ATP level should correlate with this function.24 Platelets stored for 2 weeks at 4 C. had markedly diminished levels of ATP despite the fact that mitochondria could
still be readily seen. These mitochondria were quite abnormal, however, varying greatly in size and frequently being very densely staining.

**Summary**

The electron microscopic appearance of platelets incubated at 37 C. and stored at 4 C. are described, and the relationship of these changes to normal platelet structure are discussed together with the hypothesis that the observation may be due to lysosome activity of some of the platelet granules.

The platelet's ATP level during incubation and storage was also followed and it was concluded that this relates more to the platelet's ability to sustain clot retraction than to its viability.

**Summario in Interlingua**

Es describite le apparentia electrono-microscopic de plachettas incubate a 37 C e immagasinate a 4 C. Le relation de iste alterationes al normal structura plachettal es discutite insimul con le hypothese que le observation es possibilemente le resultato del activitate lysosomatic de certes del granulos plachettal.

Le nivello plachettal de triphosphato de adenosina durante le incubation e le immagasinage eseva etiam observate, e il eseva conclusit que isto es relationate plus con le capacitate del plachettas de sustener le retraction del coagulo que con su viabilitate.

**REFERENCES**

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