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THE ULTRASTRUCTURE OF PLATELET PSEUDOPODIA AND THE ADHESION OF HOMOLOGOUS PLATELETS TO TUMOUR CELLS

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SUMMARY.—When homologous platelets in plasma were mixed in a Chandler tube apparatus with Walker 256 carcinoma cells and mouse mammary adenocarcinoma cells a tumour-platelet body was formed in each case. Walker 256 carcinoma cells and rat platelets formed a loosely knit corpus of tumour cells, tumour cell debris, and activated platelets which showed a number of pseudopodia in contact with both intact and damaged tumour cells. Mouse mammary adenocarcinoma cells and mouse platelets formed a denser corpus consisting of platelets with numerous pseudopodia, fibrin and tumour cells. Mixed in this mass were " balloon " platelets. These were pear-shaped profiles of single platelets in which the larger spheroid was granule-free and the smaller granule-rich. No granules were seen external to the platelet plasma membrane. Platelet pseudopodia took various forms. Single filiform, bifid and staghorn types were seen. Some originated from the microtubular system and were supported by it. A peripheral bubble containing membranes arranged in a foamy fashion was present at the tip of some pseudopodia. If the leading portion of a pseudopodium had reached an attractive adhesive surface then it followed this surface for a little distance so as to result in a bend away from the main axis of the pseudopodium.

THE formation of platelet spiky processes or pseudopodia is the first gross alteration in the shape of the platelets in the processes of adhesion and aggregation. It probably represents an exploratory response by the cell to a stimulus detected by some sensor device. The sensor device may be alteration in the stability of the platelet plasma membrane in the immediate vicinity of the stimulus, resulting in the formation of a process in that direction. The phase of the formation of pseudopodia can be a transient phenomenon which rapidly progresses to either the adhesion of platelets to the stimulating object or the formation of platelet aggregates which may develop under certain circumstances into fully formed thrombi. Some types of tumours have long been known to be associated with thrombus formation, though the pathogenesis of this association probably is diverse in nature. Tumour cell emboli in the blood stream may not be solely composed of the neoplastic cells themselves but may be mixed or encrusted with platelets and fibrin. There is evidence to suggest that this occurs in some instances (Winterbauer, Elfenbein and Ball, 1968). The thromboplastic properties of certain tumour cells have been described repeatedly in various circumstances (Lawrence, Bowman, Moore and Bernstein, 1952; O'Meara, 1958; Koike, 1964; Holyoke and Ichihashi, 1966; and Frank and Holyoke, 1968).

A study was therefore conducted using the Chandler apparatus (Chandler, 1958; Poole, 1959) to mix homologous platelets in citrated plasma with rat Walker

256 carcinoma cells and mouse mammary adenocarcinoma. The tumour-platelet aggregates so formed were examined by electron microscopy and were found to contain numerous platelets exhibiting spiky pseudopodia, some of which were adherent to tumour cells.

MATERIALS AND METHODS

Walker 256 carcinoma.—This tumour has been carried in rats in this laboratory for some years. A résumé of the nature and history of the tumour is given by Stewart, Snell, Dunham and Schlyen (1959). The original tumour developed in the area of the mammary gland of a rat, and there is often a sarcomatous component to the tumour.

Mouse mammary adenocarcinoma.—C_3H mice which had spontaneously developed mammary tumours were donated by Dr. Hoshino of the Department of Anatomy, The University of Western Ontario.

Preparation of tumour suspension.—Small portions of the tumours were removed from the edge of the neoplasms in anaesthetized animals by clean surgical technique. Obvious fat and areolar tissues were stripped from these pieces of tumour and they were cut into 1 mm. cubes and placed in a small tissue grinder (Pyrex), together with 2 ml. of Hanks' balanced salt solution (Difco Laboratories, Detroit, U.S.A.). The tumours were gently ground several times and the supernatant containing tumour cells poured off. Most of the connective tissue of the stroma of the tumours remained in the grinder. This tumour cell suspension was used within 3 hr of its preparation.

Chandler's apparatus.—Chandler (1958) demonstrated that artificial thrombi could be formed *in vitro* if whole blood is rotated in a plastic tube on a turntable. Poole (1959) confirmed this and made certain modifications which were incorporated in the apparatus used here.

Platelet-rich plasma.—Blood was collected from rats and mice by cardiac puncture into plastic syringes containing 0·5 ml. of 10 per cent sodium citrate. The citrated blood was centrifuged for 5 min. at 1500 rpm and the platelet-rich plasma drawn off (Biggs and Macfarlane, 1962). This platelet-rich plasma was used within a short time of its preparation.

Preparation of tumour-platelet mixtures.—Platelet-rich plasma (0·5 ml.) was drawn into a syringe already containing 0·5 ml. of tumour suspension. Rat platelet-rich plasma was used with Walker 256 carcinoma and mouse platelet-rich plasma with mouse mammary adenocarcinoma. The tumour-platelet mixture was expelled into a length of clear plastic tubing of 4 mm. internal diameter which was curled to form a circle of 6 cm. radius. The interior of the clear plastic tubing had been previously carefully cleaned and finally syringed out with 10 per cent sodium citrate. The plastic tubing which now made a complete circle was fixed to a disc of perspex arranged at an angle of 60° with the horizontal, and rotated at 13 rpm. Following several trials it was found that, after a period of 10 min. tumour-platelet bodies were formed with both types of mixtures and this time was taken as the standard interval for mixing up in the tubing on the rotating disc.

Preparation of material for electron microscopy.—The tumour-platelet bodies were fixed in 3 per cent glutaraldehyde in phosphate buffer for 2 hr, washed in buffered 5 per cent sucrose wash water and post-fixed in osmium tetroxide. The fixed specimens were dehydrated in graded alcohols, stained by 1 per cent phosphotungstic acid at the stage of absolute alcohol dehydration and embedded in araldite. The blocks were cut with glass knives using an LKB ultratome III and the sections floated on distilled water. The sections were mounted on either formvar coated or plain copper grids and most were then stained by 6 per cent uranyl acetate in 50 per cent alcohol. They were observed and photographed in a Philips EM200 or EM300 electron microscope using 60 and 80 kV.

RESULTS

The ultrastructure of the adhesion of homologous platelets to Walker 256 carcinoma cells and mouse mammary adenocarcinoma was studied. In general adhesion between platelets and tumour cells in a species-homologous system consisted, at least initially, of attachment of the thin pseudopodia of "activated" platelets to the microvilli of the tumour cells themselves. This contact progressed

by dint of enlargement of the pseudopodia of the platelets. The tumour cell-platelet contact did not trigger platelet-platelet adhesion and therefore the change in the platelet of first contact differs from that found in a similar platelet at the

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EXPLANATION OF PLATES

Fig. 1, 2, 3a and 3c are electron micrographs of mouse platelet mammary adenocarcinoma preparations, and Fig. 3b and 4 are electron micrographs of rat platelet–Walker 256 carcinoma cell preparations.

FIG. 1.—Electron micrographs of activated platelets in mouse platelet–mammary adenocarcinoma preparation.

(a) Section through a wedge of activated platelets at low power to illustrate the intermingling and adhesion of pseudopodia of the platelets without approximation of the platelet cell bodies. Fibrin strands (F) are interlaced between the platelet bodies (Pb) and pseudopodia (Ps). The pseudopodia are, for the most part, filiform processes. They do not contain granules. Sections of the platelet cell bodies are identified by their size and content of granules. × 10,600.

(b) Higher power magnification to show a section through several platelet cell bodies (Pb) and pseudopodia (Ps) in continuity. × 19,600.

(c) A fine fibrin network is present between the platelet pseudopodia and several platelet cell bodies. The section through a platelet cell body shows several granules, including mitochondria (M). × 19,600.

FIG. 2.—Balloon platelets in mouse platelet–mammary adenocarcinoma suspension.

(a) Lower power electron micrograph to show a mixture of balloon platelets (B), platelets with filiform pseudopodia (P), red cells (R) and fibrin strands (F). Three of the balloon platelets present show granules (B1, B2 and B3) and there are several spheroids (S) which do not show granules and which probably represent sections through the non-granular portion of the balloon platelets. × 5100.

(b) Higher power magnification of portion of Fig. 2a illustrating a single balloon platelet. This type of platelet is composed of a larger non-granular spheroid (NGS) and a smaller granulated spheroid (GS). The three types of platelet granulomere are present in the smaller spheroid. An isthmus joins the two spheroids and there is a mitochondrion (M) near the junction. Filiform platelet pseudopodia (FP) are present in the lower right hand corner of the micrograph. × 19,600.

(c) Balloon platelet in contact with fibrin (F) and red cells (E). There is a granulated smaller spheroid containing a mitochondrion (M) dense granules (DG) and γ-granules (G). The cytoplasm in the larger spheroid which does not contain granules (NGS) is sparse when compared with that in the smaller spheroid. × 31,000.

FIG. 3.—Varieties of platelet pseudopodia.

(a) This electron micrograph shows a mouse platelet–mammary adenocarcinoma with a long filiform pseudopodia (Ps) from a platelet cell body (P) in contact with a damaged cell (C). The pseudopodium contains a dilated tubule (Dt) and at one point has an ovoid swelling with microtubules (M) in cross-section. × 31,000.

(b) A region at the edge of a damaged Walker 256 carcinoma cell (C). There are sections of two platelet pseudopodia (Ps and S), one of which is of the staghorn type (S). The platelet cell bodies of these pseudopodia lie inside the plane of this section. One platelet (P) has been cut through its central portion. × 19,600.

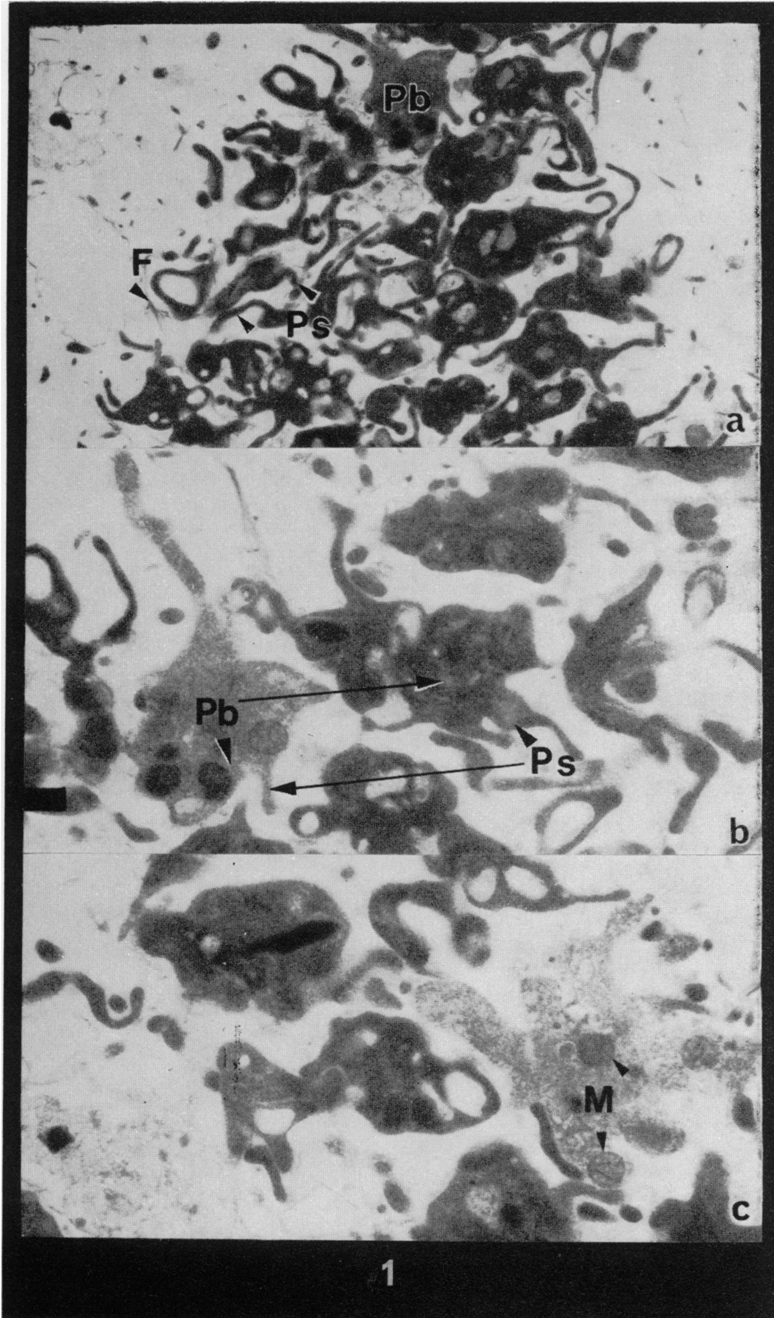
(c) Platelets showing complex forms of pseudopodia. As well as the intertwining of platelet filiform processes there are in this field a staghorn pseudopodium (SP) and a bifid pseudopodium (BP). The staghorn pseudopodium has four " points " and a stem which unites it with the platelet cell body in a different plane. Fibrin filaments (F) are scattered between the platelets. Mouse platelet-tumour preparation. × 19,600.

Fig. 4.—Saccular membranes at platelet tips. These electron micrographs show various alterations to the tips of the platelet pseudopodia when they have contacted other surfaces (a) or are in close propinquity (b) or have contacted another pseudopodium (c).

(a) This electron micrograph shows a platelet cell body (PB) from which extends a pseudopodium supported by microtubules and which has a saccule (S) at its tip. Within this dilated tip are numerous bubbles. The pseudopodium is in contact with a damaged Walker 256 tumour cell (T) over a major part of its length. × 19,600.

(b) Section of a portion of a platelet shows part of its cell body (B) and a saccular dilatation containing secondary membranes (SM). × 40,800.

(c) Two platelet pseudopodia (P1 and P2) are seen here in contact. In one (P1) the typical thin main portion of the pseudopodium continues to a bulbous end (B) which is in contact with the second pseudopodium (P2). The second pseudopodium possesses a saccular ending of similar structure (S) to those shown in Fig. 4a and b. × 40,800.



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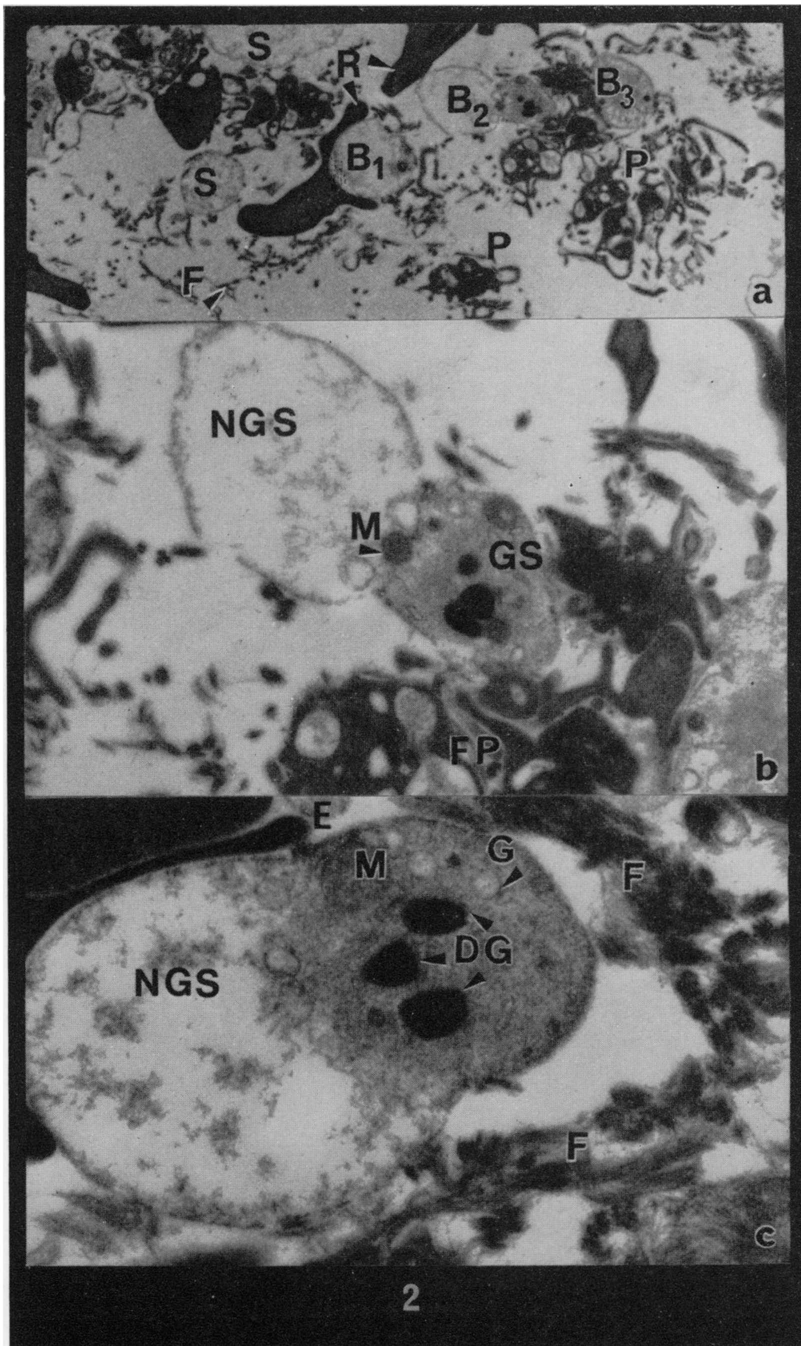

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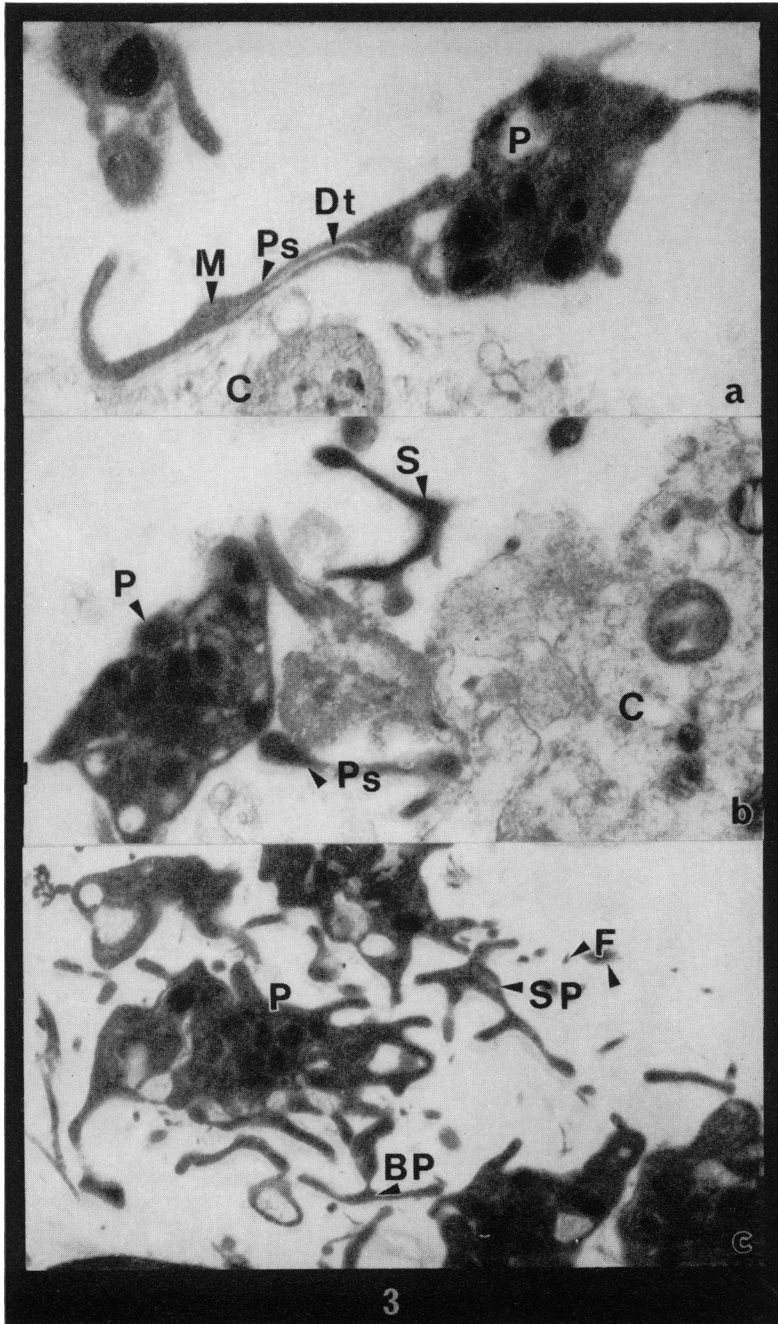

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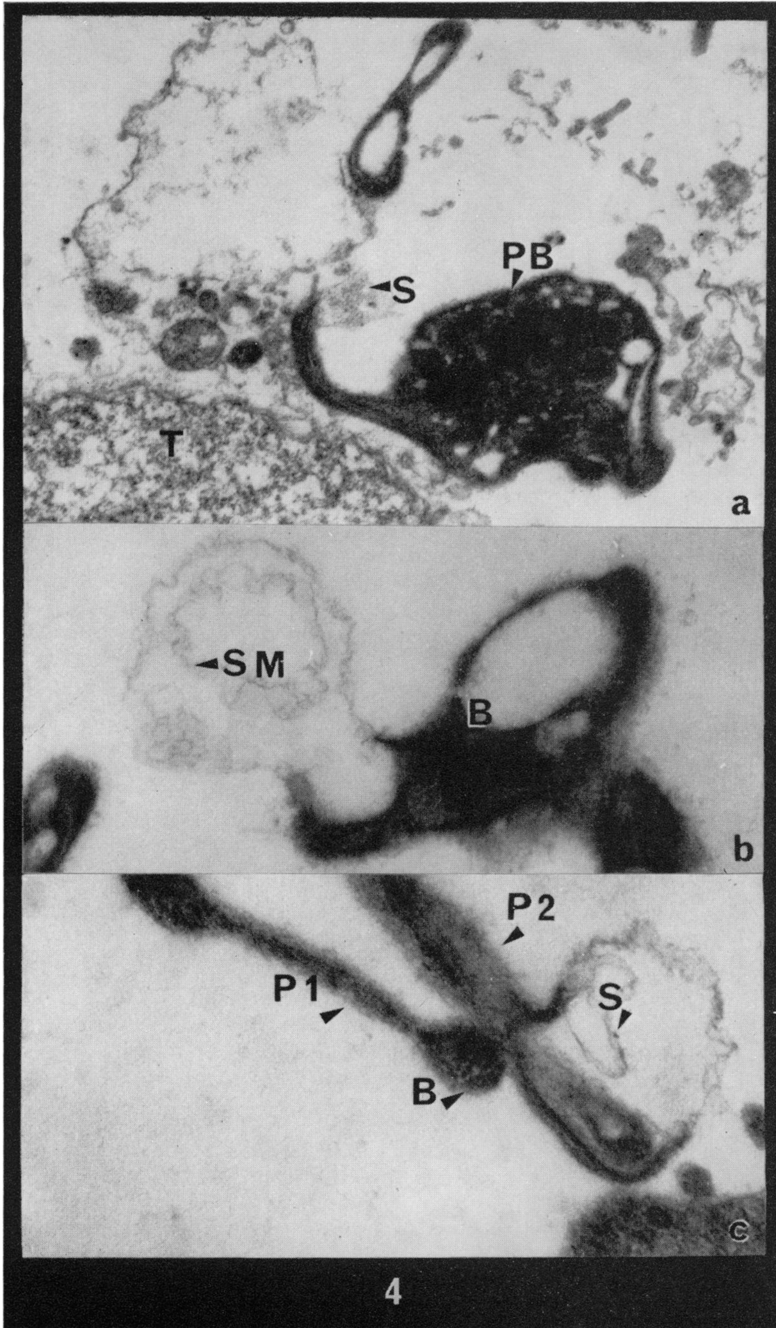
PB
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commencement of the formation of the haemostatic plug, though this may be quantitative in nature.

Ultrastructure of agitated suspension of rat platelet and Walker 256 carcinoma cells

Change in the shape of the platelets occurred and the platelets put out (in one plane) about 4–8 long spiky pseudopodia each, which contacted microvilli of the Walker 256 tumour cells. No fibrin was visible. Adhesion of thrombocytes to cells in mitosis was seen. The spiky pseudopodia of the platelets did not contain organelles and the α-, β- and γ-granules appeared to remain within the core cytoplasm of the thrombocytes. In some instances pseudopodia thicker than this variety were seen and in these cases the pseudopodium was adherent to the tumour cell over a wider area than the spiky variety.

Fine collagen fibre remnants of the stroma of the tumour were present occasionally and in these preparations platelet pseudopodia were found in contact with the fibres. A string of platelets was formed, each platelet being attached *via* pseudopodia to the central group of collagen fibres.

Ultrastructure of agitated suspension of mouse platelet and mammary adenocarcinoma cells

Unlike the rat platelet tumour suspension which was relatively loosely held together the mouse platelet tumour suspension formed a closely knit corpus with the formation of a small amount of fibrin in certain areas. The earliest stage was the formation of a phalanx of single platelets separate with regard to each main cell mass but having multiple pseudopodia, some of which were in contact and all of which were complexly intermingled (Fig. 1*a*). These pseudopodia were spiky in form (Fig. 1*b* and 3*a*), *i.e.* if they are compared with the thicker varieties which are seen following contact with an attractively adhesive surface. Some of the pseudopodia were supported by the microtubular system of the platelets while others were not supported by this framework. Granules were discernible within the cell bodies of the platelets (Fig. 1*b*, *c*) and no granules were observed external to the platelet plasma membranes.

In some preparations there were occasional red cells (Fig. 2*a*) and alterations in the form of local platelets to produce balloon-shaped structures. These " balloon " platelets consisted of pear-shaped masses containing granules in the smaller spheroids (Fig. 2(*b*), (*c*)). Mitochondria and dense granules could readily be identified grouped together in the granulated spheroid. The larger or non-granular spheroid appeared to possess few solids and seemed fragile. These balloon platelets occurred mixed with red cells (Fig. 2*a*), spiky pseudopodia (Fig. 2*b*) and fibrin (Fig. 2*c*). Sections of " balloon " platelets which passed through the larger spheroid alone did not contain any granules. This was interpreted as indicating that the granular complement of such platelets might be redistributed and was fully represented in the smaller spheroid. Not all pseudopodia were single elongated filiform structures whose length in some cases was 30 times its width (*e.g.* Fig. 3*a*). In some instances they were bifid (Fig. 3*c*) or staghorn in type (Fig. 3*b*, *c*). The forms of platelet pseudopodia seen are listed in the Table.

The adhesive process of platelets

Many pseudopodia showed peculiar formations at their tips. A sequence could be arranged from the observations under the electron microscope and electron

TABLE.—*Morphology of Platelet Pseudopodia*

Origin . . .	(a) May arise directly from circumferential microtubular system. (b) May occur apparently without involvement of microtubular system and be unsupported by that system.
Intermediate region .	Usually of even width and finger-like. Sometimes supported by microfilaments in longitudinal section. Occasionally ovoid enlargement of transverse section of portion of microtubular system.
Tip	Usually bulbous and frequently shows transverse striations.
Contact process .	Tip becomes deflected by object and follows attractive adhesive surface so that a bend is formed with the main axis of the pseudopodium.
Types . . .	Unsupported single filiform process. Supported bifid process. Unsupported bifid process. Supported staghorn process.

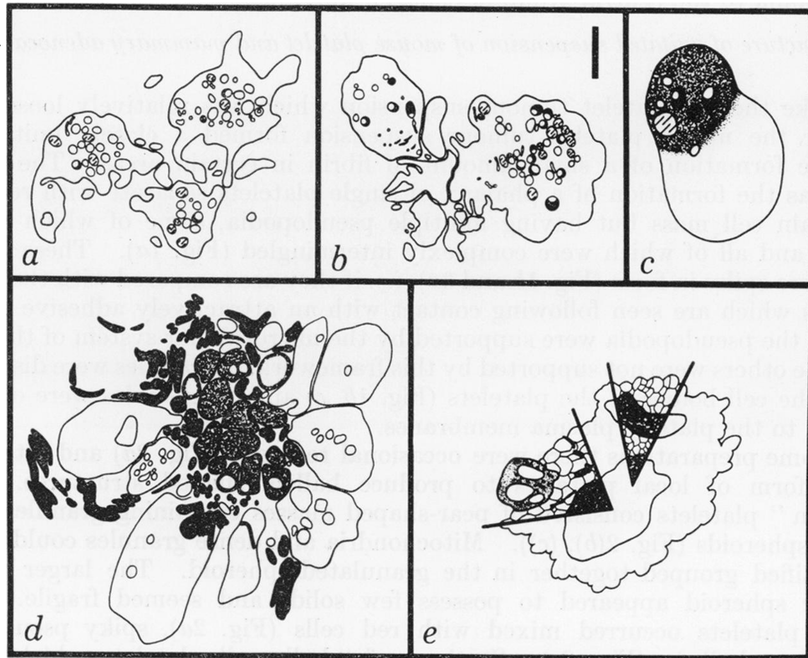

FIG. 5.—Stages in the aggregation of platelets.

(a) The production of spiky pseudopodia from the central cell mass of the platelet.

(b) The broadening of the initial points of adhesion and the closing of the wide spaces between the platelet cell bodies.

(c, d) The production of fibrin and the formation of balloon platelets, one of which is shown in (5c). These balloon platelets are orientated so that the platelet aggregate has a central granule-rich core and a peripheral rim of non-granular spheroids (5d). Fibrin strands are found between the peripheral non-granular bodies. In (5d) the granules of the central portion of the primary platelet aggregate have not been included in the drawing.

(e) Further growth by accretion and adhesion of the primary platelet aggregates. This gives rise to compound platelet aggregates as shown here. A leucocyte is stuck to the periphery of the compound platelet aggregate at one point.

The bar in Fig. 5b represents 1 μm. for Fig. 5a, 5b, and Fig. 5d; 0·6 μm. for Fig. 5c, and 9 μm. for Fig. 5e.

micrographs made which showed a series of changes in the ultrastructure of the platelet pseudopodia which would represent the adhesive process of the platelet. Usually in these instances at a site a little back from the tip of the platelet pseudopodium was formed a filamentous bubbly object consisting of little more than an empty lipoprotein membrane shell. These bubbles were of the order of 0·5 μm. in diameter and contained secondary bubbles of intermediate size (about 1/3 diameter of main bubble) with at their base a further number of much smaller bubbles (about 1/13 diameter of main bubble) applied one to the other. The formation of this object was possibly activated by a sensor device at the tip of the platelet pseudopodium. It was found when there was firm contact of the pseudopodium over some distance (Fig. 4*a*), just touching (Fig. 4*c*), or close propinquity (Fig. 4*b*) to an attractive adhesive surface. There was a suggestion in some fields that fibrin was formed at the periphery of these bubbles.

Fig. 5 illustrates the stages of platelet-platelet adhesion. Platelet-tumour adhesion was found to be identical to that shown in Fig. 5*a* and 5*b* with a tumour

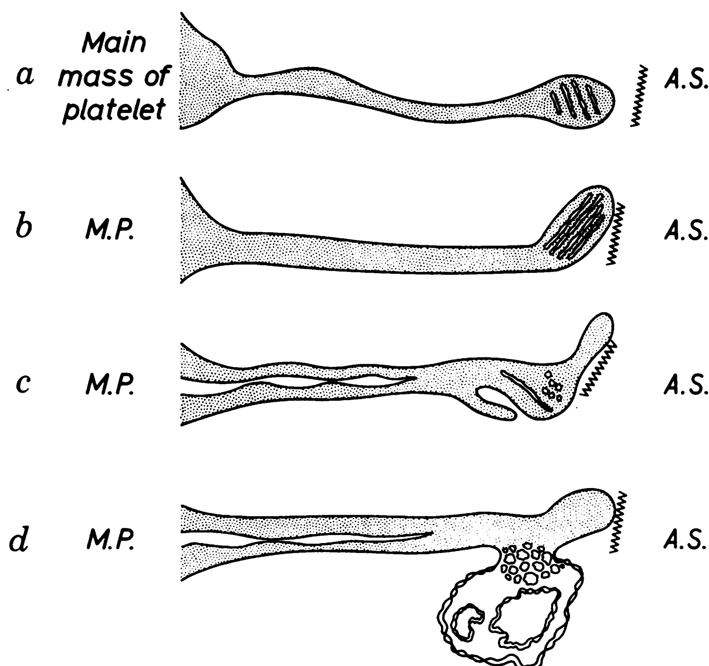

FIG. 6.—Adhesion of platelet pseudopodia. Schematic interpretation from an amalgam of many electron micrographs of platelet pseudopodia in tumour–platelet mixtures in which the platelet pseudopodia were adherent to intact and damaged tumour cells. The drawings are arranged in a sequence to illustrate:

(a) The attraction of a platelet pseudopodium from the main mass of the platelet (MP) towards an appropriate surface (AS).

(b) Bending of the terminal bulb of the pseudopodium once the adhesive surface has been reached.

(c) Alteration of the bulb and change in the stem of the pseudopodium and possibly dilatation of a supporting microtubule of the pseudopodium.

(d) The production of saccular membranes at the end of the pseudopodium. It is not suggested that this sequence is completed each time a platelet pseudopodium becomes attached to an attractive adhesive surface.

cell substituting for one platelet, *i.e.* microvilli of the tumour contacted pseudopodia of the platelet (Fig. 5*a*) and firmer contact was made by a thickening of the platelet pseudopodia on the adhesive surface of the tumour cell (Fig. 5*b*). Fig. 5*c* is a line drawing showing a balloon platelet. These have been described previously within platelet aggregates elicited by means of an extract of coagulant snake venom without fibrin production and in the platelet aggregates formed in blood during the production of an artificial thrombus in Chandler's apparatus (Warren and Davey, 1970). This is the first clear demonstration of their occurrence as completely separate structures. They appear to be the units from which the primary platelet aggregate is formed (Fig. 5*d*). Fibrin formation seems to follow their appearance under certain circumstances. Fig. 5*e* shows the formation of a complex platelet aggregate by the adhesion of the peripheral layers of two primary platelet aggregates. The line drawings comprising Fig. 5 are derived from tracings of actual electron micrographs. Fig. 6 consists of a diagram of the stages of adhesion of a platelet pseudopodium and is an interpretation of the appearances of a number of platelet pseudopodia under the various conditions represented, from the initial approach of a platelet pseudopodium extending from the main mass of the platelet (M.P.) to an attractive adhesive surface (A.S.) (Fig. 6*a*). The next drawing shows a bending of the bulbous ending, altering its axis to take up a position more parallel to the adhesive surface (Fig. 6*b*). This may be followed by alteration in the internal structure of the pseudopodium (Fig. 6*c*) and the eventual formation of a membranous bubble near its tip (Fig. 6*d*).

DISCUSSION

Thromboplastic properties of suspensions of tumour cells

There have been a number of biochemically orientated investigations concerning the coagulative properties of certain tumours. Lawrence *et al.* (1952) and O'Meara (1958) showed that tumours were able to increase the tendency of homologous blood to clot. Lawrence *et al.* (1952) described the thromboplastic activity of a suspension of V2 carcinoma. When a suspension of this tumour was injected i.v. into rabbits there was cardiac and pulmonary thrombosis resulting in the death of the animals.

O'Meara (1958) first put forward the idea of a " cancer coagulative factor " and more recently O'Meara and O'Halloran (1963) summarized the earlier publications of this group and the investigation of a labile coagulative factor which diffuses from tumour cells into the surrounding medium. The suggestion was made that this " cancer coagulative factor " is associated with invasive growth of neoplasms, and counteracting this factor might tend to diminish the rate of spread of the malignancy and perhaps cause a decrease in size of the established tumour. Differing thromboplastic activities were found in four different ascites tumours by Koike (1964) who examined the effects of intravenous administration of extracts of these tumours. Prior treatment with heparin protected mice against the lethal effects of i.v. administered supernatant from an homogenate of one of the tumours. Holyoke and Ichihashi (1966) examined the thromboplastin activities of extracts of C_3H spontaneous mammary tumour, mouse breast tissue and T241 Lewis sarcoma. Extracts of both the mammary tumour and the sarcoma contained both heat stable and heat labile thromboplastin activity.

The tumour homogenate of C_3H mouse mammary adenocarcinoma contained more thromboplastin activity than the mouse breast tissue.

Frank and Holyoke (1968) showed that the thromboplastin activity of fluid collected from the periphery of T241 Lewis sarcomas into Millipore filter chambers is higher than that from normal subcutaneous tissues, using a calcium reconstitution assay.

Rudenstam (1968) studied the thromboplastic activity of cell suspensions from DAB induced hepatoma, 20-methylcholanthrene induced sarcoma, Rous virus induced sarcoma, and spontaneous mammary carcinoma (C_3H mice). Cell suspensions of the DAB inducted hepatoma were most active. No definite differences were found in the thromboplastic activities of suspensions of the last three tumours. Mechanically produced cell suspensions were more thromboplastic than those produced by trypsin digestion. From the discussion of the literature above, it can be seen that a number of tumour cell suspensions have been shown to possess thromboplastic properties including mouse mammary adenocarcinoma (Holyoke and Ichihashi, 1966) and Walker cell carcinoma (Jones and Wallace, 1969). The present investigation utilized this property to study the early stages of platelet adhesion and aggregation which occur when platelets and tumour cells are mixed.

The progression of platelet-platelet adhesion towards thrombus formation was more advanced in the preparations of mouse platelets and mammary adenocarcinoma than in those formed from rat platelets and Walker 256 carcinoma. Using the staging of platelet adhesion illustrated in Fig. 5 the rat suspension had proceeded only to stages 1 and 2 while the mouse suspensions had proceeded to stage 3a as well, and there was the formation of fibrin. Not all tumours have thromboplastin activity (Roscher, 1969) and those that do, have this property in varying amounts. Roscher (1969) has suggested that a consumption coagulopathy may result from the release of tumour tissue factors into the blood stream. He suggested that thrombosis due to coagulation factors from tumour cells may eventually so deplete the circulating compounds involved in coagulation that a haemorrhagic diathesis may result.

Cliffton and Grossi (1955) examined over 200 tumours of several pathological types for fibrinolytic activity by the fibrin plate method. There was considerable variation in fibrinolytic activity of the tumours—ranging from squamous carcinomas with no significant activator or lytic activity to sarcomas with high fibrinolytic activity. Whole pieces of tumour were used and no attempt was made to partition the fibrinolytic activity into the effect of the tumour cells themselves or their stroma. Vascular endothelium under such circumstances will lyse fibrin and it is likely that there is a correlation between the vascularity of the tumour examined and its fibrinolytic activity. Blood containing spaces in tumours are not all lined by mature vascular endothelium, so that in tumours with rudimentary vascular channels such as malignant melanomas one would not expect fibrinolytic activity of the fragments of these neoplasms and this was the case here. The work of Cliffton and Grossi (1955) is therefore quite compatible with a cancer coagulative factor, if it is considered that they were measuring the total effect of the malignant cells and their stroma rather than determining the effect of the neoplastic cells alone.

The morphology of thrombosis associated with tumour emboli

Observations on the thromboplastic properties of tumour extracts were made in parallel with morphological studies of the mechanism of metastasis. Work by

Baserga and Saffiotti (1955), Wood (1964), Jones and Wallace (1969) has indicated that thrombosis or some manifestation of thrombosis is a frequent occurrence in the natural history of metastatic growth of several tumours.

Thrombus formation incorporating arrested tumour emboli is common (Baserga and Saffiotti, 1955; Wallace, 1956; Wood, 1964) though not invariable (Winterbauer *et al.*, 1968). These last mentioned workers found that if tumour emboli were associated with thrombosis then the tumour cells were in a peripheral position, though there was evidence suggesting that the thrombus and malignant cells embolized as a unit and that the thrombus was not secondary to the arrest of the tumour cells. Wallace (1956) suggested that while some tumours may be inhibited in their spread by inability or difficulty in invading blood vessels and once introduced therein, even if artificially, can readily produce metastases, others easily gain access to the vessel lumen only to die in the circulation and fail to propagate their line in distant sites. In a group of sarcomata arising from a transplantable rat fibroadenoma Wallace (1956) showed that artificial metastasis by intravenous inoculation was only successful in those sublines capable of spontaneous metastases and that this method of induction of metastases was not uniformly successful.

Normally functioning endothelial cells aid in the dissolution of thrombi because of their plasminogen activator activity (Warren, 1963), and it is probable that this property would tend to inhibit the firm adherence of tumour emboli to endothelium. Where adhesion occurs the balance between coagulating and fibrinolytic tendencies in the local circulation may have been tipped in favour of coagulation by the thromboplastic properties of tumour cells. The thromboplastic properties of tumour cells may thus play a central role in the initial process of adhesion to the vessel walls in the pathogenesis of the development of metastases.

Platelet pseudopodia and the adhesion of platelets to fibrin

Where there is thrombus formation platelets may appear stuck to fibrin and this situation may arise in two ways. Fibrin presents a surface which is attractive to circulating platelets and they stick to fibrin strands. However, no alteration in the shape of the platelets, no pseudopodia formation and no alteration in granule distribution occurs in such circumstances. Secondly, platelets occur in association with " nascent " fibrin that they have assisted in forming early in the course of a thrombotic process. In this instance pseudopodia are formed, granules remain, initially, in the main mass of the platelet (Fig. 1) which may retain some semblance to its original shape. Eventually the usual ovoid forms of the platelets become greatly altered and there results the development of balloon platelets (Fig. 2).

The morphology of individual platelets within platelet aggregates

When human platelets are aggregated by an extract of the venom of the snake *Trimeresurus okinavensis* no fibrin is formed and the form and content of the individual platelets within the aggregate are easily discernible (Warren and Davey, 1970). From a study of two such platelet aggregates, one containing 43 and the other 123 profiles it was found that most platelet profiles greater in sectional area than 1 $\mu m.^2$ were seen to contain granules and those smaller than this did not. No granules were seen external to the aggregates. Hence, within the platelet

aggregates certain sections of platelets, although apparently not containing granules, represented processes of granule-containing cell bodies, and did not indicate that there had been any discharge of the content of any granule external to the platelet plasma membranes (Warren and Davey, 1970).

Examination of platelet aggregates formed in the Chandler apparatus with the production of fibrin revealed within the aggregates similar balloon platelets to those shown here in Fig. 2. The granule-rich spheroid was orientated towards the central portion of the platelet aggregate.

The platelet pseudopodia stained by 1 per cent phosphotungstic acid formed under the influence of a snake venom extract and without the eventual formation of fibrin were apparently unsupported by microtubules and did not show any dilated saccular structures similar to those reported here.

A scheme has been suggested for the stages of platelet aggregation (Warren and de Bono, 1970) and these stages are shown in the line drawings of Fig. 5. The stages that the experiments reported here are particularly concerned with are stages 1, 2 and 3. Platelets in the Walker 256 cell-rat platelet suspension showed changes similar to those depicted in Fig. 5a and 5b. In the mouse mammary adenocarcinoma–mouse platelet suspension after agitation balloon platelets (Fig. 2), which are an early phase of stage 3, were seen.

In certain sections of platelet aggregates both with and without fibrin formation electron micrographs of platelet profiles have demonstrated that the granule-rich portions of the platelets (which are centrally situated within the aggregate) are connected to the granule-free blebs at the periphery (Warren and Davey, 1970).

The series of electron micrographs shown here (Fig. 2a, b, c) illustrating the balloon platelets confirm this earlier report (Warren and Davey, 1970) that there can be redistribution of granules within the main mass of the platelet under certain circumstances.

In the scheme of the stages of platelet aggregation (Warren and de Bono, 1970) this is the linking form between the platelets showing pseudopodia both spiky and thicker forms (stages 1 and 2), and the fully formed primary platelet aggregate associated with fibrin (stage 3). This goes on to form complex platelet aggregates (Fig. 5d).

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