ELECTRON MICROSCOPY OF PHAGOCYTIC CELLS

III. MORPHOLOGICAL FINDINGS RELATED TO ADHESIVE PROPERTIES OF HUMAN AND RABBIT GRANULOCYTES*

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POLYMORPHONUCLEAR granulocytes (PMNG) that are engaged in phagocytosis *in vitro* have a pronounced tendency to stick together and to form dense clumps (Allison, Lancaster and Crosthwaite, 1963; Allison and Lancaster, 1964). Furthermore, the processes of phagocytosis and of clumping are closely related and, in addition, extremely non-physiological conditions are required to prevent phagocytic PMNG from clumping (Allison *et al.*, 1963, Allison and Lancaster, 1964). This type of aggregation by PMNG in phagocytic systems resembles in some respects the adhesion between white blood cells and injured vascular endothelium *in vivo* and has been used as a model for study of leucocytic sticking.

Detailed electron microscopy of the clumping phenomenon has not been reported, although various structural changes within cells during phagocytosis have been described in several works (Lockwood and Allison, 1963, 1964; Zucker-Franklin and Hirsch, 1964; Horn, Spicer and Wetzel, 1964). This report is an extension of previous studies; it describes the electron micrographic appearance of clumped PMNG obtained from *in vitro* and *in vivo* preparations. Certain relationships between morphology and adhesive properties of the leucocytes are discussed.

MATERIALS AND METHODS

Leucocytes for the studies *in vitro* were collected from human venous blood and from sterile peritoneal exudates in rabbits. The cells were mixed with rough strain pneumococcus by rotation at 9 rpm in siliconized glass tubes at 25° or 37°. At intervals from 5 min. to 3 hr. the cell mixtures were fixed either in buffered osmium tetroxide or in glutaraldehyde followed by osmium tetroxide and subsequently prepared for electron microscopy. Details of the procedures have been previously described (Lockwood and Allison, 1963, 1964; Lockwood, 1964). Some preparations were treated with a solution containing 25 units of trypsin (Armour) per ml. for 15 min. prior to fixation, a procedure thought to improve the quality of preservation. In a few experiments the fixatives were buffered with cacodylate.

For study *in vivo*, rough strain pneumococcus, estimated to give a 10 : 1 bacterium-cell ratio, was introduced into sterile peritoneal exudates. After 30 min. the animals were killed with intravenous pentobarbital and the exudate-bacterium mixture was removed aseptically. Thereafter, the cells were prepared for electron microscopy by the same techniques used for *in vitro* experiments.

The magnifying power of the microscope was determined by photographing a grating replica containing 28,800 lines per inch. The photographic enlargement was determined for each print by comparison of the dimensions of easily recognized features on the original plate with the size of the same features on the print itself. The calibration of the microscope

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did not change significantly while these experiments were being performed. Errors in determination of the magnification were introduced by unavoidable variation in focusing the image, but it was estimated that such errors did not exceed 15 per cent.

RESULTS

Controls.—In all control preparations, the cells were found either singly or in small loose clumps containing only a few leucocytes. There were some differences between the electron microscopic appearances of the cells fixed only in OsO_4 and those fixed in glutaraldehyde plus OsO_4. The PMNG contained in control preparations fixed with osmium have been illustrated in earlier works (Lockwood and Allison, 1963, 1964). Their outlines were round or oval with relatively few cytoplasmic projections. The nuclei were multilobulated and were surrounded by an electron transparent space. The chromatin was clumped along the edges of the nuclear membrane and was finely granular. Mitochondria, ergastoplasm, Golgi complexes and the granules of light microscopy were identified within the cytoplasm. When osmium fixation was used, the cell membranes frequently were broken and the cytoplasmic organelles were disrupted.

In contrast, cells contained in preparations fixed with glutaraldehyde, (Figs. 1, 2 and 5) although generally round or oval, usually possessed numerous small cytoplasmic projections. Nuclear chromatin clumping was more pronounced than in osmium fixed material. Furthermore, in the glutaraldehyde treated material cell membranes were usually intact. In addition, small electron dense granules thought to be glycogen were often present in the cytoplasm, particularly in those cells that were exposed to trypsin prior to fixation.

Appearance of clumped cells in vitro.—Phagocytosis began within 5 min. after the rough pneumococcus was added to the suspension of leucocytes, and small dense aggregations of PMNG began to form shortly thereafter (Fig. 2). Nearly all cells eventually became clumped into 2 or 3 large masses that could be dispersed only by disrupting the cells themselves. The plasma membranes were thrown into complex, interdigitating folds throughout all layers of the clumps (Fig. 3). The closest approximation of cellular membranes was of the order of 200–250 Å (Fig. 3 and 4). Within the largest clumps the PMNG were found to be extensively vacuolated and pneumococci were numerous within the vacuoles as has been previously illustrated (Lockwood and Allison, 1963, 1964). Material thought to be derived from leucocytic granules was found associated with the intracellular bacteria, and, as noted previously (Lockwood and Allison, 1963, 1964; Zucker-Franklin and Hirsch, 1964; Horn, Spicer and Wetzel, 1964), communications between vacuolar and granule membranes were identified.

Leucocytes obtained from the peritoneal exudate following introduction of the pneumococci were found singly or in small loose clumps. Large dense clumps such as those found *in vitro* were not recovered *in vivo*. From the electron micrographs it was seen that the clumped leucocytes were closely approximated and as was recorded from the *in vitro* studies, complex interlocking projections were common. Pneumococci were found but they were rare, and most were within cytoplasmic vacuoles of the clumped cells although a few were in an extra-cellular position enmeshed within tiny projections from the cell. The cytoplasmic granules had the same morphology as those found in cells recovered from the experiments performed *in vitro*.

The appearance of extracellular material.—In essentially all preparations examined, small round deposits or short strands of amorphous, electron dense material, were found extracellularly (Figs. 4 and 5). This matter was present in control preparations as well as in mixtures of cells and bacteria. A small amount was found deep within the clumps in close proximity to neighbouring cells (Fig. 4). As shown in the illustrations, the spacing between cell membranes *per se* was never less than 200–250 Å, whereas, the amorphous material probably lay much closer to the cell membranes. The exact spacing could not be measured with certainty due to the ill-defined border of the electron dense material. The identity of this material was not established.

DISCUSSION

The preceding paragraphs have described the complex projections found on the surface of polymorphonuclear granulocytes (PMNG). It was thought that cells actively engaged in phagocytosis either *in vivo* or *in vitro* developed more extensive and complicated projections (Figs. 1, 2, 3 and 5) than resting PMNG. In addition, the projections from actively phagocytic cells were found to interlock (Fig. 3). The means by which phagocytosis of bacteria triggered the clumping together by leucocytes was not recognized in these experiments.

In those regions where the cells were closest together, the space between them most often was in the 500–1000 Å range (Figs. 3 and 4). The closest approach of one cell to another was about 200–250 Å, but such narrow spacing was rarely found. This distance between cell surfaces was somewhat greater than the 150 Å postulated by Curtis (1960), but it was of the same magnitude. There was no evidence that membranes of interlocked leucocytes approached the 5–10 Å range of separation that Bangham and Pethica (1960) and Pethica (1961) postulated for adhesion of cells by the bonding of chemical groups on the surfaces. It was pos-

EXPLANATION OF PLATES

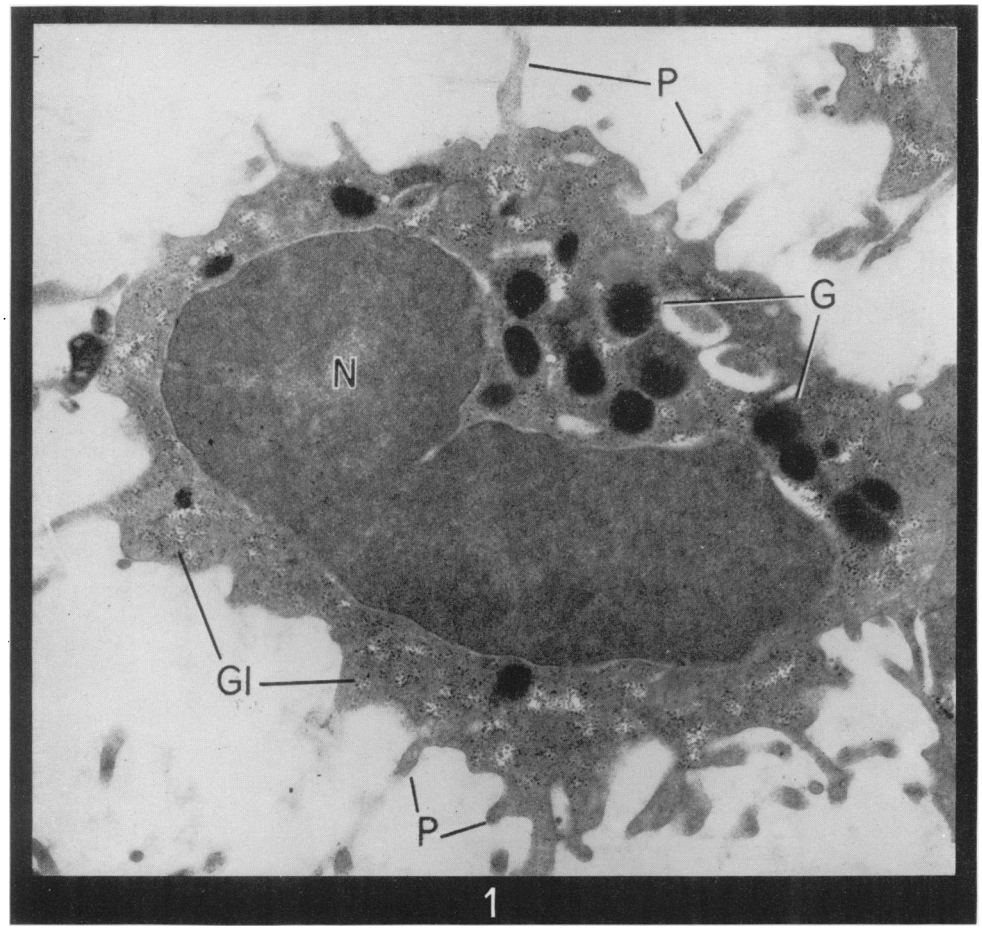
FIG. 1.—Granulocytes from rabbit peritoneal exudate incubated with pneumococci for 15 min. Note the numerous cytoplasmic projections (P). Small black granules (Gl), thought to be glycogen, are abundant in the cytoplasm. The nucleus is labelled " N ". Glutaraldehyde and osmium fixation. Dow epoxy embedding. Lead stain. ×14,300.

FIG. 2.—A small loose clump of granulocytes formed after 15 min. incubation. Pneumococci (Pn) are illustrated in various stages of the phagocytic process. Cytoplasmic projections (P) are numerous but do not interlock. From a preparation treated with trypsin, 25 units/cc. for 15 min. prior to fixation with glutaraldehyde and osmium. A vacuole is labelled " V ". Dow epoxy embedding. Lead stain. ×7,500.

FIG. 3.—Interlocking projections on rabbit cells after 1 hr. incubation with pneumococci. The average separation of the membranes is of the order of 500–1000 Å, and the closest approach of one to another is about 250 Å. The upper cell (C) contains numerous small black particles, probably ribonucleic acid, and it is thought to be a monocyte. The small white specks in the lower cell represent areas from which glycogen has been leached by the processes of fixation and embedding. Epon 812 embedding. Combined uranyl acetate and lead staining. ×16,200.

FIG. 4.—From the same experiment as Fig. 3. The large vacuole (V) has distended the cell and crowded the cytoplasm and nucleus to the periphery. As in Fig. 3 the separation of the cells is never less than 200–250 Å. In the space between the opposing membranes small collections of unidentified material (A) are present. ×14,500.

FIG. 5.—From the same experiment as Fig. 1. Note the extensive cytoplasmic projections from the cells and the amorphous extracellular material (E). Leucocytic granules and glycogen are abundant in the cytoplasm. This material consistently lacked the periodic structure characteristic of fibrinogen and was not identified. Lead stain. ×19,400.



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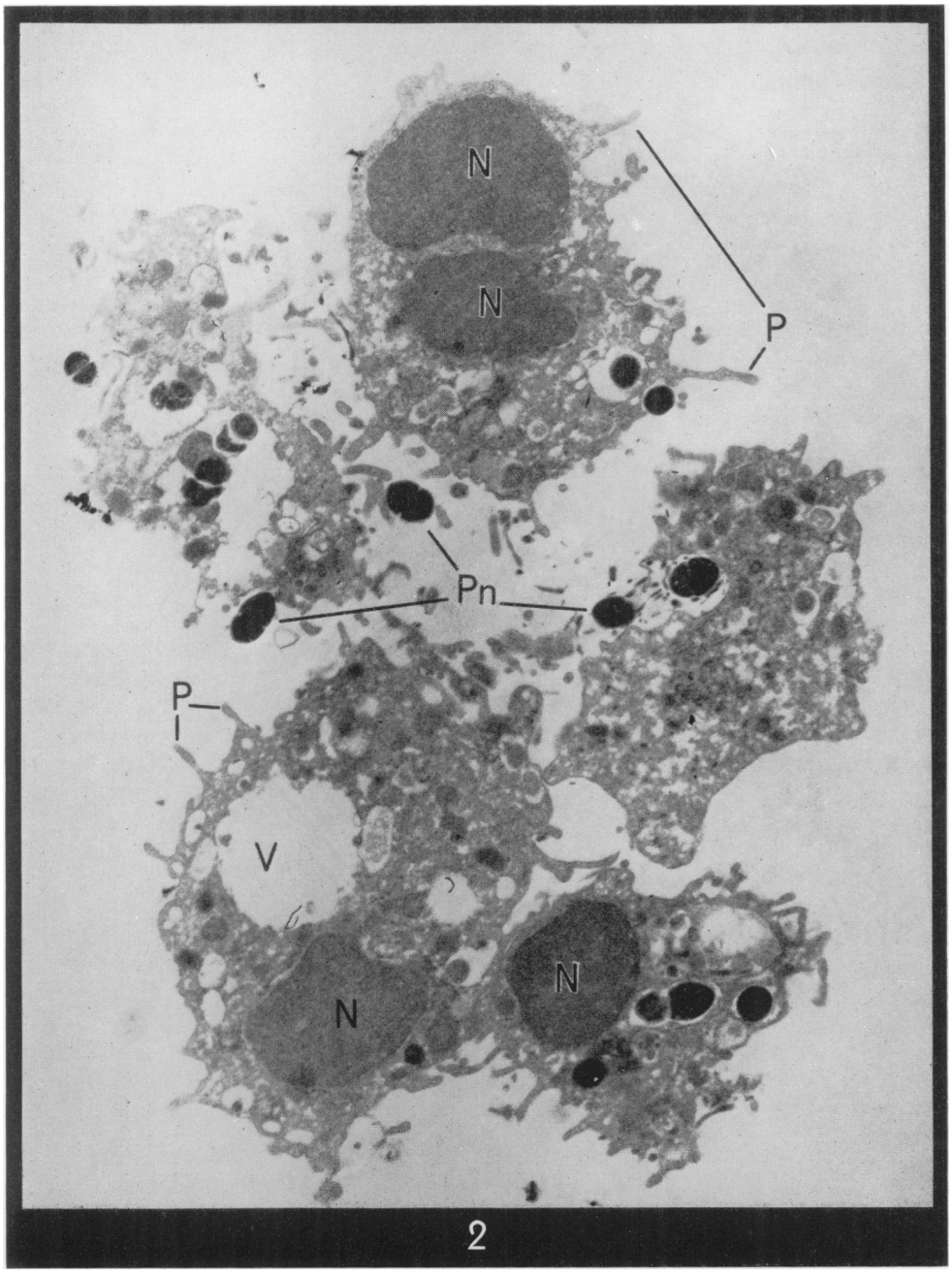

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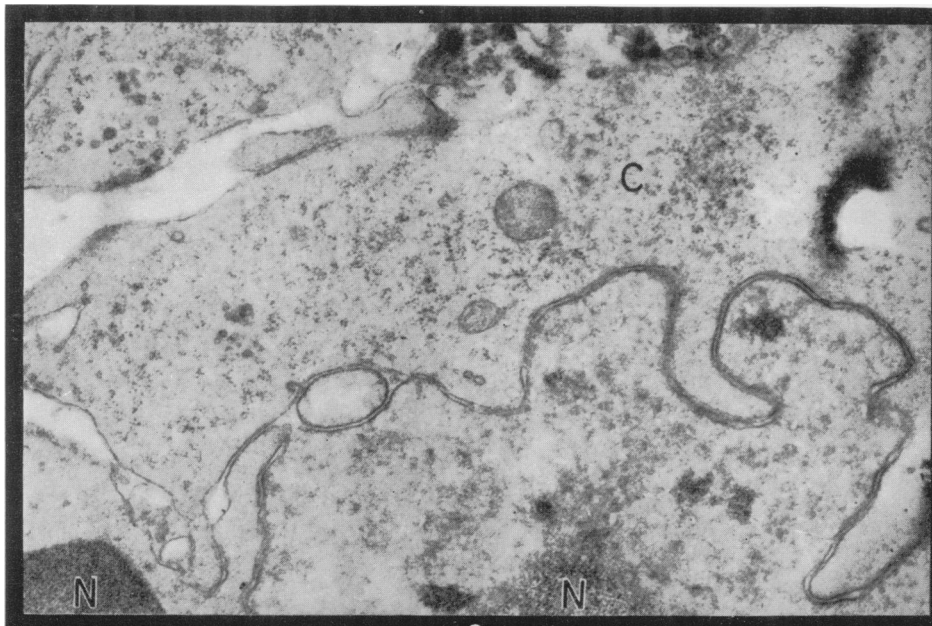

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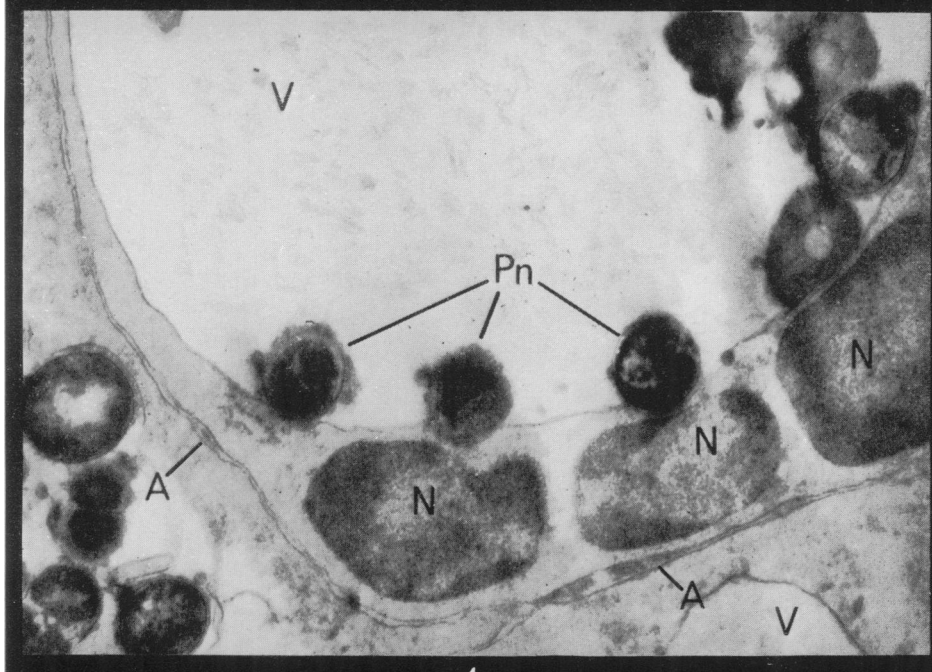

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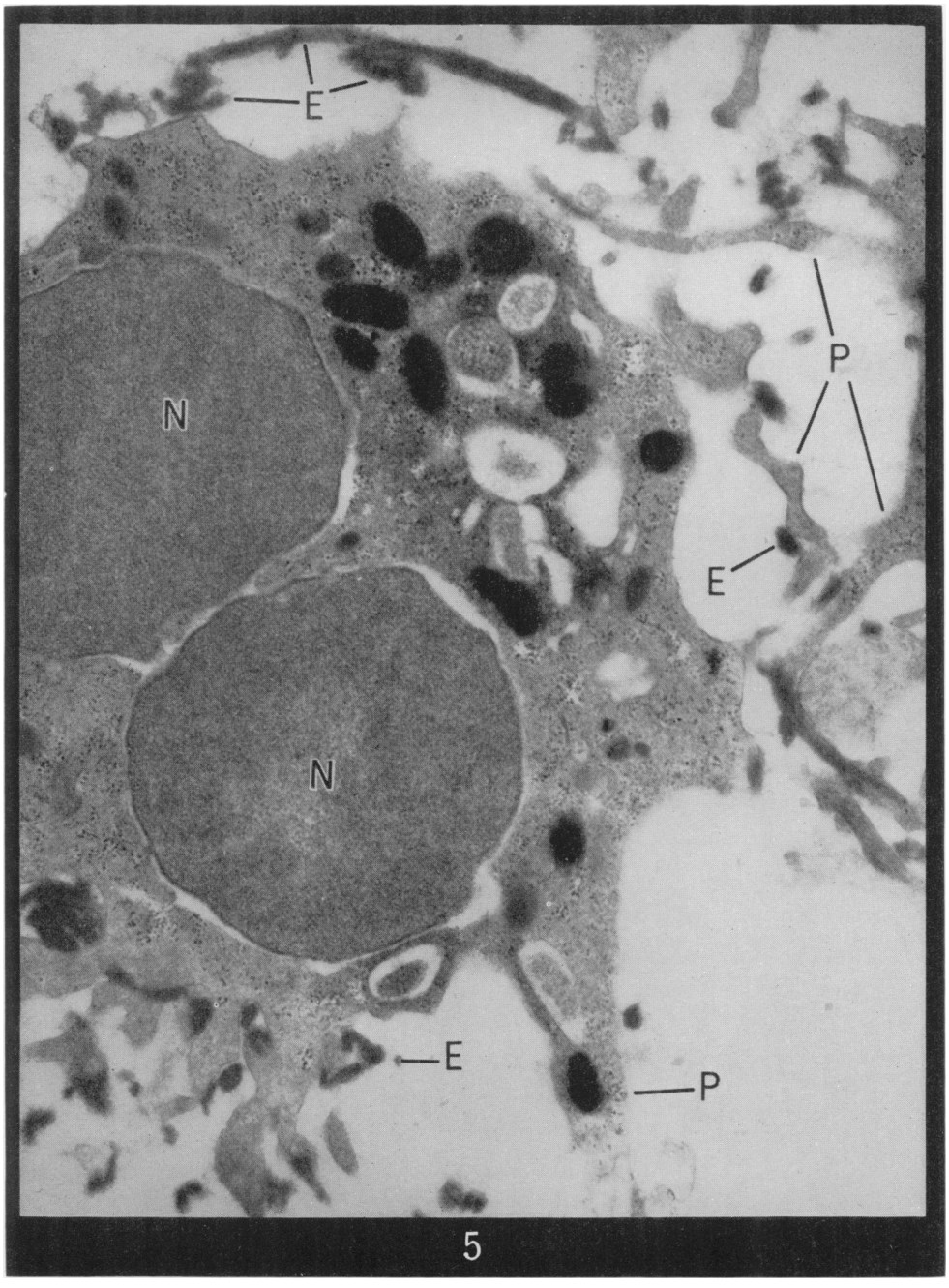

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sible that molecular structures undetected by the methods of fixation and staining employed in this study contributed to cellular adhesiveness. Unfortunately, techniques for exploration of this possibility were not available. Thus, the distance between the surfaces of adjacent leucocytes firmly adherent to one another did not correspond exactly with the figures proposed by either Curtis (1960) or Bangham and Pethica (1960). However, separation between PMNG was more nearly in keeping with that predicted by Curtis.

The intercellular matter illustrated in Figs. 4 and 5 was not identified. There was a possibility that this substance represented a partially polymerized form of fibrinogen that assisted in holding together the aggregates of PMNG. However, the evidence that the clotting mechanism played a role in leucocyte clumping was indirect (Knisely, Bloch and Warner, 1948), and, furthermore, no periodic structure such as that demonstrated in fibrin was found in any of the intercellular material. There was no suggestion from these observations to support either the concept that the blood coagulation mechanism was primarily involved in the clumping phenomenon or the theory that clumping was related to direct linkage of chemical groups upon the surfaces of adjacent cells.

On the other hand, the interlocking projections as illustrated in Fig. 3 may be significant. The projections were present before as well as after phagocytosis, and they have been seen on living PMNG using phase contrast optics. It was clear that the folds interlocked after phagocytosis (Fig. 3). It was also known that the aggregated, interlocked cells could not be dispersed. There was no convincing evidence of an extracellular cement substance, and as the separation of the cells was too great to invoke electrical bonding, the integrity of the clumps may have been maintained by mechanical factors.

Perhaps these observations bear on the problem of leucocyte margination during inflammation. At high magnifications, vascular endothelium has been seen to have fine processes that extend into the lumen (Kirsch, 1957) to which circulating leucocytes could attach mechanically if surface conditions were suitable. The observations of this experiment lend support to the concept that prior to diapedesis PMNG use their finger-like projections to adhere to the endothelial surface, but it is clear that some mechanism other than direct mechanical linkage is also involved. The intravascular projections from endothelial cells are present in normal as well as in inflamed tissue and they do not increase in size or number during inflammation (Lockwood, unpublished). Nevertheless, study of the clumping phenomenon *in vitro* suggests that mechanical linkage may play a role in leucocyte sticking, although other poorly defined factors are also involved.

SUMMARY

Leucocytes obtained from peritoneal exudate of rabbits and from peripheral venous blood of human donors were permitted to ingest rough strain pneumococcus *in vitro*. The aggregates of cells and phagocyted bacteria were then studied electronmicroscopically with particular attention directed to the surfaces of the clumped cells. It was found that delicate, finger-like, membranous projections extended into the medium from both phagocytic and non-phagocytic cells. After the engulfment of pneumococcus, these projections of clumped cells were found to be intimately intertwined and interlocked. A distance of approximately 500–1000 Å was noted between adherent cells and in no instance were they closer

than 200 Å. Scattered throughout the preparations were small amounts of matter that were more intimately associated with the surfaces of cellular aggregates. The structural configuration of this material was not characteristic of fibrin.

From these results it was concluded that the forces of electrical bonding *per se* were not solely responsible for the clumping together of phagocytes. It is not unlikely that the interdigitations of cell membranes from adjacent leucocytes assist in a mechanical sense to the sticking together by these cells. Finally, it is suggested that similar structural events may be operative in the adhesion of blood borne leucocytes to vascular endothelium during acute inflammation.

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