CELL-MEDIATED CYTOTOXICITY: FINE STRUCTURAL STUDIES OF EFFECTOR CELLS AND THEIR INTERACTION WITH TARGET CELLS

by

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Abstract

In vitro cytotoxic reactions may reflect the role of lymphoid cells in tissue-damaging immune responses *in vivo*. When the present work commenced, the effector cells in antibody-dependent cell-mediated cytotoxicity, K cells, were generally considered to be a type of lymphocyte. The aim of this project was to identify K cells using electron microscopy. Particular emphasis was placed on the fine structure of effector-target cell interactions both in antibody- and phytohaemagglutinin (PHA)-dependent cell-mediated cytotoxicity.

Three antibody-dependent systems were examined. Effector cells were obtained from mouse peritoneal cavity, mouse spleen and human blood, while chicken red blood cells (CRBC) or *Cryptococcus neoformans* were used as target cells. Cytotoxicity was induced by small amounts of anti-target cell antiserum, normal serum having little effect.

Antiserum-treated CRBC were only associated with the macrophages among mouse adherent peritoneal cells, attachment usually being followed by ingestion. There was no morphological evidence of extracellular lysis and target cell death apparently resulted from phagocytosis.

Mouse splenic lymphocytes were attached to antibody- and less frequently to normal serum-treated CRBC; the contacts appeared the same whichever serum was used. Effector and target cell plasma membranes were usually parallel with 7.5-10 nm separating the cells; no intercellular junctions were observed. Despite low numbers of macrophages and neutrophils in the effector preparation, antibody-dependent

phagocytosis probably accounted for much of the observed cytotoxicity.

Purified human lymphocytes attached to antiserum- and normal serum-treated cryptococci with similar frequency. Lymphocyte projections never penetrated the cryptococcal capsule and extracellular lysis was considered unlikely. Although very few phagocytes remained after purification, target cell death apparently resulted from antibody-dependent phagocytosis.

This study has demonstrated that, in addition to lymphocytes, macrophages and neutrophils must be considered potential K effector cells. Furthermore, it is clear that morphological studies can make an important contribution to the assessment of the role of phagocytic cells in antibody-dependent cell-mediated cytotoxicity.

T lymphoblasts kill mastocytoma cells by an extracellular mechanism in the presence of PHA, and fluorescein transfer between labelled target and effector cells occurs. Axinella, another plant lectin, agglutinates lymphoblasts and mastocytoma cells but with less fluorescein transfer and cytotoxicity. Occasional gap junctions were observed between axinella-agglutinated cells, however in most of the PHA- and axinella-induced contacts 7.5-10 nm separated the cells. No intercellular junctions were seen between PHA-agglutinated cells, and cytotoxicity and dye passage probably occurred via the intercellular space.

SUMMARY

A number of *in vitro* systems have been designed to investigate the role of lymphoid cells in tissue-damaging immune responses *in vivo*. At the time the present work commenced, the effector cells in antibody-dependent cell-mediated cytotoxicity, K cells, were generally considered to be a type of lymphocyte, functionally distinct from conventional T and B lymphocytes. The aims of this project were to identify by electron microscopy the cell (or cells) involved and to study their role in cytotoxic reactions. Particular attention was paid to the areas of contact between effector and target cells, and the attachments formed during antibody-dependent and non-specific cytotoxic interactions were compared in an effort to elucidate the mechanism involved. Parallel functional studies were carried out by other members of the Division of Cell Pathology.

Three different *in vitro* antibody-dependent systems were examined. Effector cells were obtained from mouse peritoneal fluid, mouse spleen and human peripheral blood, while chicken erythrocytes or *Cryptococcus neoformans* were used as target cells. In all three systems cytotoxicity was induced by the addition of small amounts of anti-target cell antiserum, normal serum having little effect.

One of the properties, unusual for lymphocytes, attributed to K cells is "stickiness". When mouse adherent peritoneal cells were incubated with chicken erythrocytes and immune serum 2 types of rosette had previously been observed by phase contrast microscopy; "loose" rosettes formed around macrophages, and "tight" rosettes about round cells with cleft nuclei which were considered candidates for the cytotoxic lymphocyte. Investigation by electron microscopy established that most of the lymphocytes present were not independently

adherent to the glass, but were attached to macrophages which in turn were in contact with the coverslip. Antibody-treated chicken red blood cells were only associated with macrophages and attachment was usually followed by phagocytosis; up to 6 erythrocytes were found within a macrophage in a single section. After examination of serial thick (1 μm) and adjacent ultrathin sections it was concluded that tight rosettes were closely packed erythrocytes which had been engulfed by macrophages. There was no morphological evidence of extracellular lysis and target cell death appeared to result from phagocytosis.

Chromium release studies indicated that mouse spleen cell suspensions were a more suitable source of K effector cells, and the ultrastructure of spleen cells incubated with chicken erythrocytes and normal serum or specific antiserum was investigated. Antibody-treated chicken red cells were attached to macrophages, neutrophils and lymphocytes, but mostly the latter. Contact was not entirely antibody-dependent, occurring to some extent with normal serum, and attachments appeared the same whichever serum was present. Effector and target cell membranes were closely applied and parallel over considerable distances with 7.5-10 nm separating the cells; intercellular junctions were not observed. Although there was some evidence of extracellular lysis by effector cells, antibody-dependent phagocytosis of target cells accounted for much of the observed cytotoxicity, despite the relatively low numbers of phagocytic cells in the initial spleen cell preparations.

When purified human peripheral blood lymphocytes were used as effectors with C. neoformans as target cells attachment occurred with similar frequency whether antiserum or normal serum was present. Lymphocyte surface projections indented but never penetrated the cryptococcal capsule and extracellular lysis was considered unlikely.

Although very few phagocytic cells remained after the purification procedure, target cell death appeared to result from antibody-dependent phagocytosis by macrophages and neutrophils.

This study has demonstrated that, if K cell activity is defined as cell-mediated cytotoxicity dependent on antibody concentrations that do not induce complement lysis, then monocytes or macrophages and neutrophils, in addition to lymphocytes, must be considered potential K cells. Furthermore, since a small number of phagocytic cells can be responsible for considerable target cell killing, it is extremely important that the effector cell population should be examined thoroughly, by morphological as well as functional methods, so that the role of phagocytic cells can be accurately assessed.

Mastocytoma cells are killed by T lymphoblasts in the presence of phytohaemagglutinin (PHA) and transfer of fluorescein between target and effector cells can also be demonstrated. It was presumed that intercellular junctions, probably gap junctions, were formed which would allow the passage of dye from one cell to another, and the fine structure of contacts in this non-specific cytotoxic interaction was examined. Since there were no phagocytic cells present, cell-mediated lysis in this system could only have been effected through an extracellular mechanism. Numerous attachments formed between lymphoblasts and mastocytoma cells in the presence of PHA but, like the contacts between lymphocytes and antibody-coated chicken erythrocytes, 7.5-10 nm separated the plasma membranes and no intercellular junctions were seen. Axinella is another plant lectin which induces agglutination of T lymphoblasts and mastocytoma cells but with less fluorescein transfer and cytotoxicity. Although occasional gap junctions were found between axinella-agglutinated cells, the majority of the attachments was

morphologically indistinguishable from those induced by PHA and it was concluded that the cytotoxicity and dye transfer observed probably occurred via the 7.5-10 nm intercellular space.

DECLARATION

This work was initiated by Dr A.C. Allison, who suggested that morphological studies might permit the identification of cytotoxic effector cells and help to elucidate the mechanism involved in both antibody-dependent and phytohaemagglutinin-induced cell-mediated cytotoxicity.

I, therefore, examined the fine structural aspects of effector-target cell interactions in a number of experimental systems in collaboration with colleagues who carried out functional investigations.

I hereby declare that I was responsible for the preparation of the specimens, their examination and the production of the electron micrographs. The conclusions, though discussed with Dr Allison, are my own, as are the observations on the ultrastructure of the effector cells and their interaction with target cells. The thesis is my own composition and none of this work has previously been submitted in application for a higher degree to this or any other university.

ACADEMIC RECORD

I entered the University of St Andrews in 1965 and in 1969 graduated, having received the degree of Bachelor of Science. The following year I matriculated as a research student, and now submit the following thesis for the degree of Master of Science.

CERTIFICATE

The work included in this thesis was performed unaided by Mrs Joan Elizabeth Richmond in this Division.

A.C. Allison
Head of Division of Cell Pathology
Clinical Research Centre

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I am most grateful to Dr E.J. Wills for his encouragement throughout the course of this work, and for his help together with that of Dr J.J. Harvey in critically reading the manuscript. I am also grateful to Mrs Jean Ashley who kindly typed the manuscript and to Mr J. Gibson who photographed the diagrams.

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TABLE OF CONTENTS

1. GENERAL INTRODUCTION

1.1. OBJECTIVES

Lymphoid effector cells, or K cells, can destroy target cells *in vitro* in the presence of small amounts of specific anti-target cell antibody. This phenomenon, usually called antibody-dependent cell-mediated cytotoxicity, may reflect the role of lymphoid cells in tissue-damaging immune responses which occur in various autoimmune diseases and in allograft or tumour rejection (Perlmann & Holm, 1969).

Target cell damage induced by K cells has been assessed almost exclusively by functional means, but such methods provide limited information on the cellular events involved. By 1970 there had been only one ultrastructural study on K effector cells in human peripheral blood (Biberfeld & Perlmann, 1970). These authors concluded that lymphocytes were responsible for killing antibody-coated chicken erythrocytes by an extracellular mechanism of contact and osmotic lysis. Phagocytosis by lymphocytic cells was involved to a much lesser extent (Biberfeld & Perlmann, 1970).

Since morphological information was so limited when this project was undertaken, it was considered that examination of several other *in vitro* systems using electron microscopy would contribute to the understanding of antibody-dependent cytotoxic mechanisms. The aims of this project were twofold. In the first place, because the nature of K cells was by no means clear, an attempt was to be made to identify and describe the effector cell or cells involved. Secondly, to gain some insight into the cellular interactions, particular emphasis was to be placed on the fine structure of the areas of contact between effector and target cells. Both antibody-dependent and phytohaemagglutinin (PHA)--induced or non-specific cytotoxic interactions were considered worth investigation.

1.2. GENERAL MORPHOLOGY OF EFFECTOR CELL TYPES

Although many workers have tended to consider only lymphocytes, in the experiments to be described it will be shown that macrophages and polymorphonuclear leucocytes (neutrophils) also participate in killing antibody-treated target cells. It is convenient to consider here the fine structure of all three types of cells, but because there have been innumerable studies on their ultrastructure the descriptions will be deliberately brief.

1.2.1. Lymphocytes (Diag. 1)

Lymphocytes, which represent 20-25% of circulating human leucocytes, have traditionally been divided into small (4-5 μm), medium-sized (6-8 μm) and large (8-12 μm). Small lymphocytes usually have a higher nuclear: cytoplasmic ratio than the others but although such a division is useful it is somewhat arbitrary since cell diameter and organisation vary in a continuous fashion.

The nucleus may be rounded or reniform and frequently shows a deep invagination. Chromatin forms dense clumps along the inner aspect of the nuclear envelope, except in the region of nuclear pores, and surrounds the nucleolus. A pair of centrioles (the diplosome) and a few Golgi saccules, together with occasional mitochondria, lie in the cytoplasm adjacent to the nuclear cleft. Moderate numbers of free ribosomes are scattered throughout the cytoplasm and a few cisternae of rough endoplasmic reticulum can usually be seen. Pinocytic vesicles, multivesicular bodies, microtubules, bundles of 10 nm filaments and occasional lipid droplets are also present.

During locomotion lymphocytes display a characteristic "hand mirror" shape. The rounded anterior part of the cell contains the nucleus covered by a thin rim of cytoplasm whilst the rest of the cytoplasm

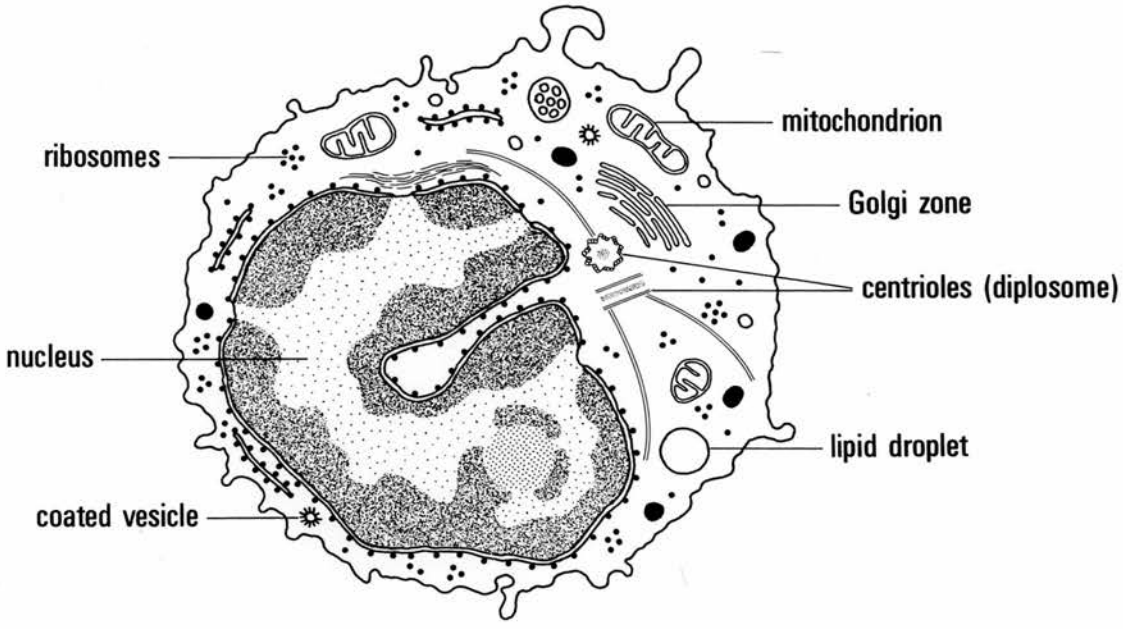


Diagram 1. Section through a lymphocyte.

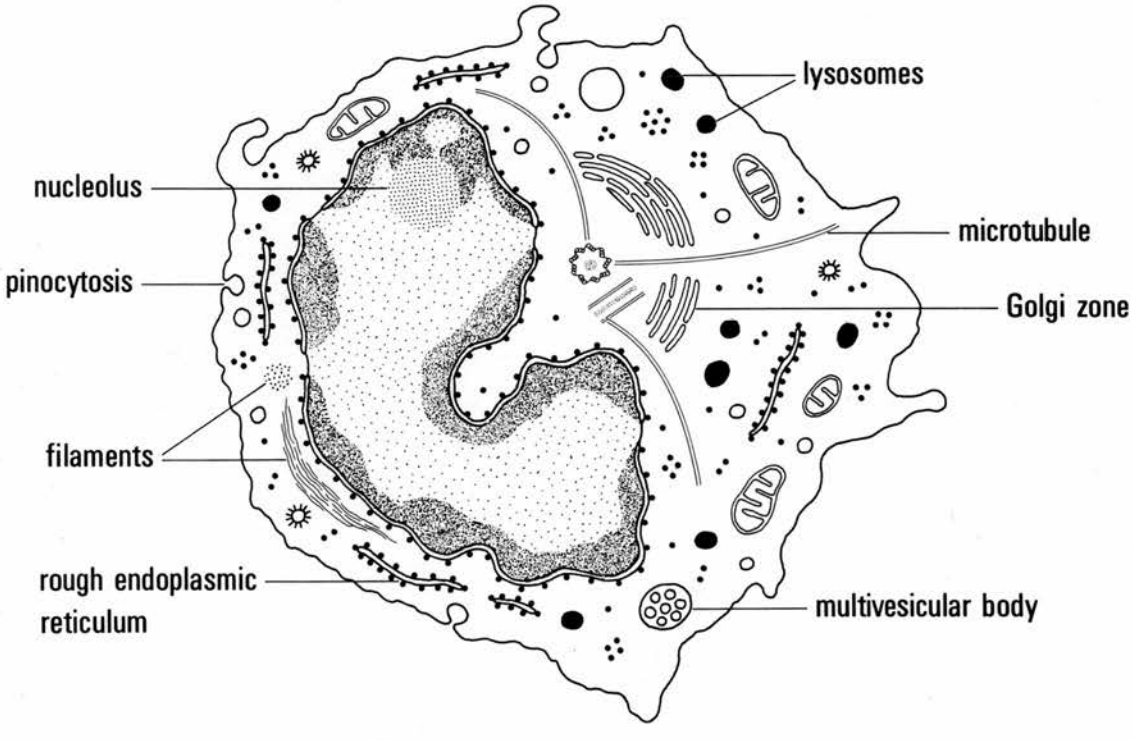


Diagram 2. Section through a monocyte.

with the Golgi zone, centrioles and most of the other organelles becomes extended behind in a uropod (the "handle" of the mirror) (Hirsch & Fedorko, 1968; Zucker-Franklin, 1969; Bessis, 1973; Oláh, Röhlich & Törö, 1975; Bloom & Fawcett, 1975).

1.2.2. Monocytes and macrophages (Diags. 2 & 3)

Mononuclear phagocytes can be divided into tissue macrophages (usually greater than 12 μm) and the circulating monocytes (9-12 μm) from which they are derived. Monocytes comprise 3-8% of the leucocytes in human peripheral blood and have many features in common with macrophages.

The nucleus varies from oval or kidney-shaped to vermiform and may occupy from more than $\frac{1}{2}$ to less than 1/10 of the overall cell area in section. One or 2 nucleoli, surrounded by nucleolar-associated chromatin, are found in the nucleus which consists largely of dispersed chromatin with varying amounts of marginated dense chromatin. A pair of centrioles and numerous Golgi cisternae with their associated vesicles are contained in the cytoplasm close to the nuclear indentation. The most characteristic cytoplasmic components are the many dense bodies comprised of small homogeneous primary lysosomes, phagocytic vacuoles, large heterogeneous secondary lysosomes (formed by the fusion of primary lysosomes with phagosomes or autophagic vacuoles), and residual bodies containing incompletely digested material. Smooth and coated vesicles, slim oval or elongated mitochondria and cisternae of rough endoplasmic reticulum are abundant. Scattered throughout the cytoplasm and in perinuclear bundles are numerous 10 nm filaments. Free ribosomes, multivesicular bodies, microtubules, lipid droplets and occasional dispersed glycogen particles are also present. Surface projections and invaginations produce irregularly shaped "vacuoles" in the region of 5-7 nm microfilaments beneath the plasma membrane. Pinocytosis and

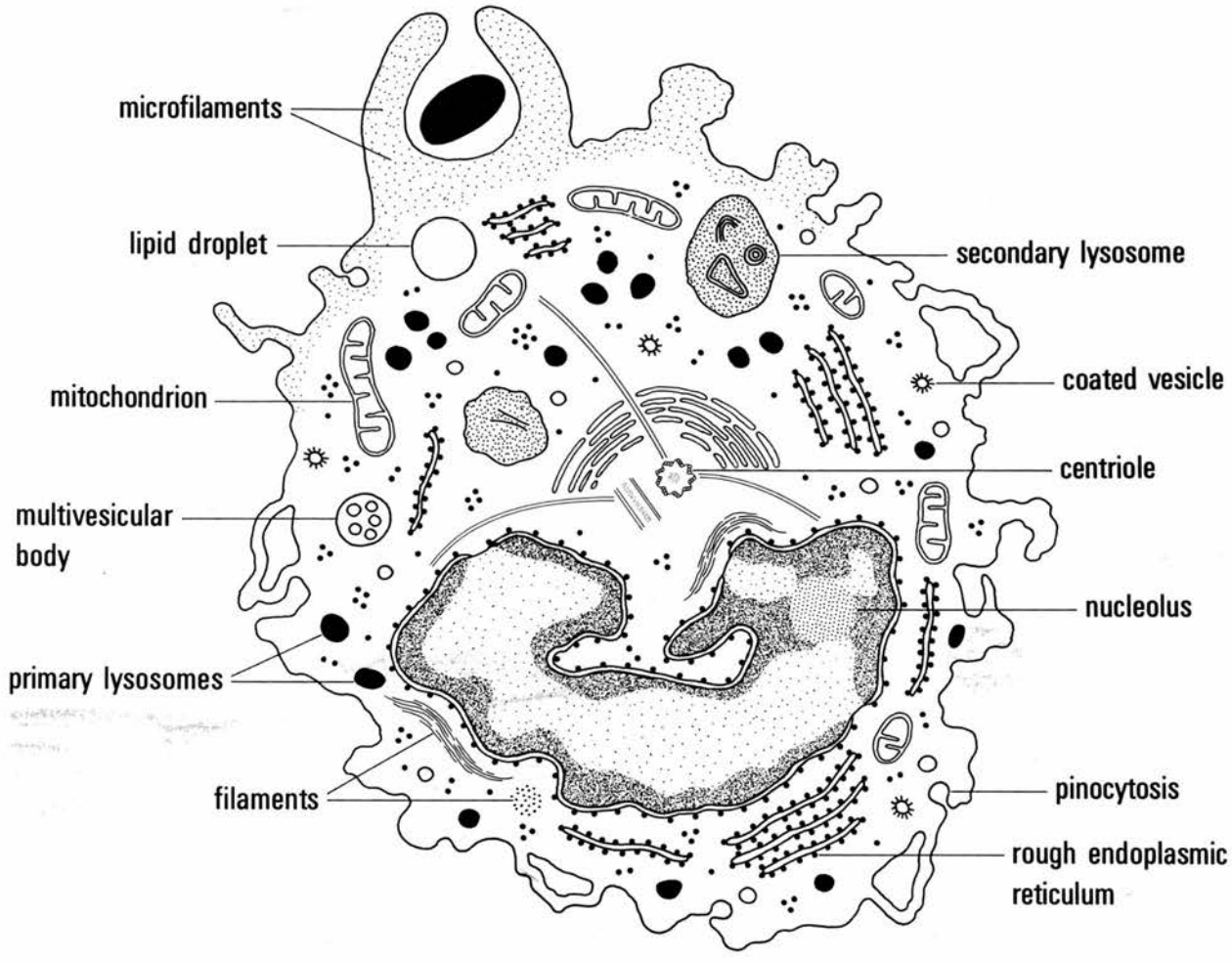


Diagram 3. Section through a macrophage.

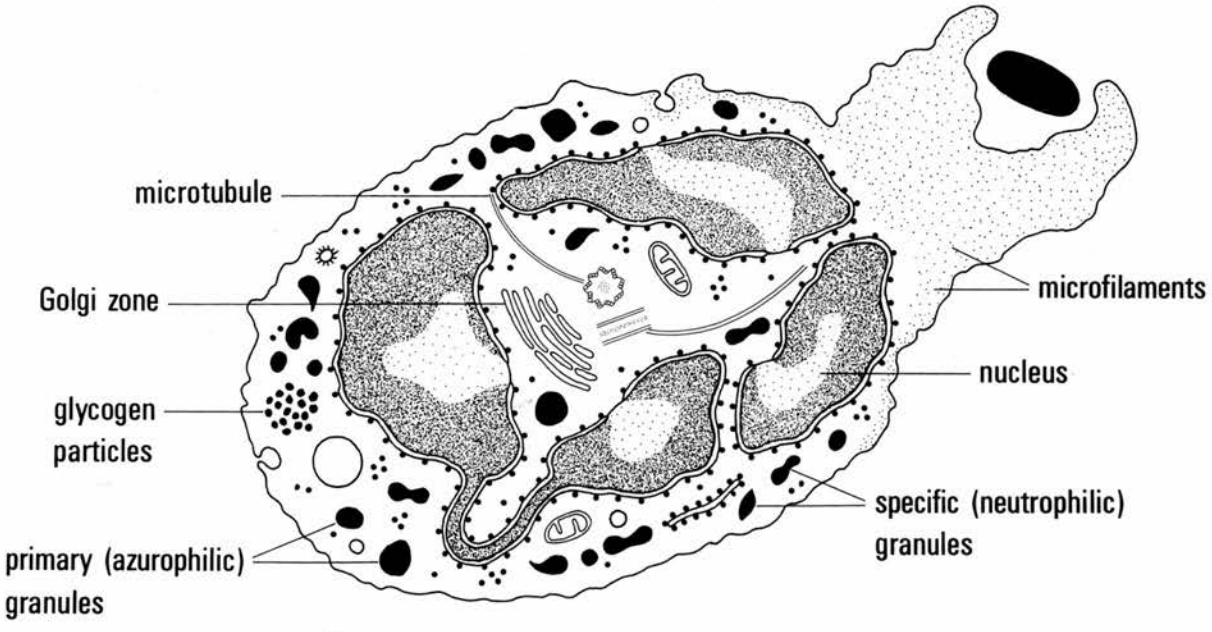


Diagram 4. Section through a neutrophil.

phagocytosis are often observed (de Petris, Karlsbad & Pernis, 1962; Carr, 1967; Cohn, 1968; Hirsch & Fedorko, 1968, 1970; Fedorko & Hirsch, 1970; Nichols, Bainton & Farquhar, 1971; Nichols & Bainton, 1973; Daems & Brederoo, 1973; Bessis, 1973; Reaven & Axline, 1973; Oláh et al., 1975; Bloom & Fawcett, 1975).

1.2.3. Neutrophilic polymorphonuclear leucocytes (neutrophils, Diag. 4)

Neutrophils (7-9 μm) are more abundant in blood than either lymphocytes or monocytes and account for 55-70% of circulating human leucocytes.

The highly characteristic nucleus which, in mature cells, is composed of up to 4 lobes connected by narrow strands surrounds the diplosome and small Golgi zone. The nucleus consists predominantly of dense chromatin with no trace of a nucleolus, thereby indicating that protein synthesis has ceased. At least 2 types of cytoplasmic granules are present - large, peroxidase-containing primary or azurophilic granules, and elongated or dumbbell-shaped specific neutrophilic granules which lack peroxidase activity. Neutrophils avidly ingest bacteria and other particulate matter and the granules coalesce with and discharge their contents into the phagocytic vacuoles thus formed. Abundant 5-7 nm microfilaments can be found in pseudopodia during phagocytosis or locomotion. The cytoplasm frequently contains aggregates of glycogen particles; small mitochondria, microtubules, cisternae of rough endoplasmic reticulum, pinosomes and lipid droplets are seen, while multivesicular bodies are rare (Hirsch & Fedorko, 1968; Farquhar et al., 1972; Bessis, 1973; Scott & Horn, 1970; West et al., 1974; Spitznagel et al., 1974; Bloom & Fawcett, 1975).

1.3. *IN VITRO* CONTACTS

There have been many ultrastructural studies of the contacts which occur between cells in "immunologically significant" *in vitro* systems. Such systems include rosette formation, whether spontaneous or the result of prior treatment of erythrocytes with antibody or antibody and complement, and cell-mediated cytotoxicity induced either by specifically sensitised cells, or by non-sensitised cells rendered cytotoxic by mitogens such as phytohaemagglutinin (PHA) or by specific antibody on the target cell surface.

1.3.1. Spontaneous rosettes

Most of the contacts between sheep erythrocytes and T lymphocytes in spontaneous rosettes are point attachments at the tips of microvilli (Lin, Cooper & Wortis, 1973; Polliack *et al.*, 1974; Levy, Knieser & Briggs, 1975; Tonietti *et al.*, 1975a) but large areas of apposition have also been seen (Wilson, Pang & Gavin, 1974; Polliack *et al.*, 1974; de Vries *et al.*, 1976). There have been conflicting reports on the shape of the rosetting cells; Levy and colleagues (1975) showed deformation of erythrocytes but Tonietti and co-workers (1975a) observed no alteration. In thin sections of T cell rosettes a constant distance of 7.5-10 nm between the plasma membranes in the region of contact was described (Levy *et al.*, 1975), but tight adherence with no visible interspace was reported when T cell rosettes were studied after freeze-etching (de Vries *et al.*, 1976).

1.3.2. Antibody rosettes

When rosette formation is induced by antibody on the erythrocyte surface it is generally agreed that broad areas of attachment predominate and that deformation of the rosetting cells occurs (Lo Buglio, Cotran & Jandl, 1967; Abramson *et al.*, 1970; Douglas & Huber, 1972;

Tonietti et al., 1975a; de Vries et al., 1976). Lymphocytes frequently develop a uropod to which the rosetting red cells are attached (Tonietti et al., 1975a; de Vries et al., 1976). In most studies the distance between the apposed cells has not been measured, but Douglas and Huber (1972) observed a space of 40 nm in thin sections and after freeze-etching de Vries and his colleagues (1976) reported tight adherence with no interspace; the discrepancy between these two observations is probably due to the different techniques used. Rare instances of fusion of the outer leaflets of the membranes of the apposed cells giving the appearance of tight junctions have even been described (Abramson et al., 1970). However, if such junctions were important in the destruction of the numerous red cells which swell and become increasingly fragile whilst in contact with the central cell (Abramson et al., 1970), greater numbers would be anticipated. Furthermore it is difficult to envisage a mechanism of cytotoxicity operating through tight junctions whose function is firm attachment and obliteration of the extracellular space (McNutt & Weinstein, 1973).

1.3.3. Complement rosettes

Erythrocytes treated with antibody and complement form multiple contacts of both point and broad types with human (Douglas & Huber, 1972; Lin et al., 1973; Polliack et al., 1974; Wilson et al., 1974; de Vries et al., 1976) or mouse (Suter, Probst & Dukor, 1972; Chen et al., 1972) lymphocytes or monocytes. Deformation of the red cells has frequently been observed (Lin et al., 1973; Polliack et al., 1974; Wilson et al., 1974; de Vries et al., 1976) and although the formation of polar "rosettes" has been described (de Vries et al., 1976), most investigators have shown that erythrocytes may attach to any part of the lymphocyte surface. Using lanthanum to penetrate the 10-20 nm space

between the central lymphoid cell and sheep erythrocytes, Douglas and Huber (1972) demonstrated 10 nm diameter subunits which they suggested were similar to those of gap junctions (Revel & Karnovsky, 1967). However in the contacts described by Douglas and Huber (1972) the distance between the apposed cells was considerably greater (at least 4 times) and the subunits were larger than those of true gap junctions. Furthermore, de Vries and his colleagues (1976) observed, after freeze-etching, particles on the surface of both rosetting and non-rosetting red cells which they therefore concluded were due to the coating of antibody and complement and not associated with rosette formation. Such particles could well account for the subunit-like appearance in attachments impregnated with lanthanum.

1.3.4. Sensitised cell systems - rosettes and cytotoxicity

A variety of cells injected into non-immune animals, usually mice, will induce the production of specifically sensitised lymphocytes and macrophages capable of rosette formation and cytotoxic interaction with the injected cell type. In rosettes both point and broad attachments develop between erythrocytes and sensitised cells (Storb et al., 1969; Elson, Bradley & Howells, 1972; Gudat & Villiger, 1973; Wilson et al., 1974) and, regardless of the area of contact, a gap of approximately 8 nm is present between the membranes (Gudat & Villiger, 1973). The attachments formed between sensitised effectors and target cells during cytotoxicity are similar (Weiss, 1968; Chambers & Weiser, 1969; Able, Lee & Rosenau, 1970; Koren, Ax & Freund-Moelbert, 1973) and frequently occur in the region of the effector cell uropod (Ax et al., 1968; Koren et al., 1973). Specialised junctional complexes have not been observed (Weiss, 1968) and a gap of 10-20 nm separates the cells (Firket & Degiovanni, 1975). Occasional regions of apparent membrane fusion (Weiss, 1968) or areas

where the cells were so closely apposed that it was impossible to distinguish between their plasma membranes have been reported (Koren et al., 1973). Discontinuities in the target cell membranes were also described (Koren et al., 1973) but the illustrations are unconvincing and these, together with instances of "fusion", almost certainly represent tangentially sectioned contacts.

1.3.5. PHA-induced cytotoxicity

Broad areas of attachment, either straight or undulating, are the predominant type of contact found in PHA-induced cytotoxicity (Biberfeld, Holm & Perlmann, 1968; Biberfeld, 1971a; Biberfeld & Johansson, 1975), but complex interdigitation has also been described (Tonietti et al., 1975b). Lymphocytes were usually attached by the pole of the cell containing most of the cytoplasmic organelles or by a uropod (Biberfeld, 1971a). Although a relatively constant space of approximately 20 nm separated the cells, lymphocytes were also observed in contact with Chang cells over "membrane structures reminiscent of intercellular junctions" (Biberfeld, 1971a). However, the presence of similar structures between adjacent Chang cells and on their free surfaces would indicate that they are unlikely to be associated with the cytotoxic interaction. Bridge-like contacts and regions in which blurred apposed membranes could not be separated were interpreted as the result of oblique sectioning (Biberfeld, 1971a). In this cytotoxic system no evidence of fusion or membrane discontinuity was found (Biberfeld & Johansson, 1975).

1.3.6. Antibody-dependent cell-mediated cytotoxicity

Although attachments through leucocyte filopodia to sheep erythrocytes were described (Inglis et al., 1975), contacts between

effector cells and other target cells such as Chang cells or chicken erythrocytes have in general been over large areas (Biberfeld et al., 1973; Biberfeld & Johansson, 1975; Fontana et al., 1975; Penfold, Greenberg & Roitt, 1976) with a gap of 15-25 nm separating the apposed cells (Biberfeld & Johansson, 1975). Frequently the contacts illustrated have been of the type described here as flat (Biberfeld et al., 1973; Biberfeld & Johansson, 1975) but varying degrees of interdigitation have been observed (Fontana et al., 1975; Penfold et al., 1976). Biberfeld and co-workers (1973) reported that many lymphocytes developed a uropod through which they established contact with monolayer cells. Areas of apparent membrane dissolution at the sites of contact were often seen which, it was suggested, might represent specialised junctions or membrane fusion (Biberfeld et al., 1973); however, it was considered that this appearance might be due to tangential sectioning, and Biberfeld and Johansson (1975) have recently found no evidence of fusion or membrane discontinuities.

1.3.7. Significance of contacts

In summary, point, flat and distorted contacts have all been observed in rosettes and cytotoxic interactions. No particular form of attachment has been consistently shown in any one system and it is probable that more than one type of contact is always present. There has been a considerable variation in the distances reported between the apposed cells in each system and this may in part reflect different experimental conditions or methods of preparation for electron microscopy. However, the intercellular gap is always somewhat variable, particularly if the effector cell is a monocyte or the contacts are greatly distorted, and can range from approximately 10 nm to 40 nm. Such genuine variations probably account for most of the apparent discrepancies.

Some form of contact is probably a prerequisite for target cell lysis (Perlmann & Holm, 1969). This applies both to sensitised effector cells (Ax et al., 1968; Weiss, 1968; Koren et al., 1973; Firket & Degiovanni, 1975) and to non-sensitised cells whether cytotoxicity is induced by PHA (Holm & Perlmann, 1967a; Biberfeld et al., 1968; Ax et al., 1968; Biberfeld, 1971a; Tonietti et al., 1975b) or by low concentrations of specific antibody (Biberfeld & Perlmann, 1970; Biberfeld et al., 1973, 1975; Inglis et al., 1975; Fontana et al., 1975; Penfold et al., 1976). From the widely varying observations already discussed it is hardly surprising that different types of attachment have been considered important. Firket and Degiovanni (1975) regarded parallel contacts or interdigitations between sensitised lymphocytes and target cells significant and point contacts non-specific; the attachments considered important by Biberfeld and his colleagues in both PHA- and antibody-dependent systems have usually been flat or slightly undulating (Biberfeld et al., 1968, 1973; Biberfeld, 1971a).

On the other hand the importance of interdigitating contacts between lymphocytes and target cells stressed by some investigators (Tonietti et al., 1975b; Fontana et al., 1975) must be viewed with some caution. It is probable that monocytes were present in both groups of experiments since erythrophagocytosis was observed by the former authors and Fontana and co-workers prepared effector cells by the same method. Lymphocytes are not always easy to distinguish morphologically from monocytes (Zucker-Franklin, 1974) and in a number of the published illustrations (Tonietti et al., 1975b; Fontana et al., 1975) where only a small portion, if any, of the nucleus is visible it is extremely difficult to classify the effector cell at all. At the 25:1 effector:target cell ratio used the presence of only 4% unrecognised

monocytes would be enough for such cells to be in a 1:1 ratio with target cells. Thus it is likely that some of the interdigitating contacts observed by these workers (Tonietti et al., 1975b; Fontana et al., 1975) were between monocytes and chicken erythrocytes and that both the number of distorted attachments with lymphocytes and their significance were overestimated.

The few functional studies that have been carried out suggest that there is more than mere contact between effector and target cells. Intercellular transfer of fluorescein from mastocytoma target cells to sensitised lymphocytes was described by Sellin, Wallach and Fischer (1971), and the demonstration of electrical coupling between PHA-agglutinated lymphocytes indicated ionic communication through low resistance junctions (Hülser & Peters, 1972). However, the occasional descriptions of junctional specialisation (Abramson et al., 1970; Biberfeld, 1971a; Douglas & Huber, 1972) and fusion or membrane discontinuities (Weiss, 1968; Koren et al., 1973; Biberfeld et al., 1973) already discussed, are rare and have not been confirmed by a substantial number of other investigations.

1.3.8. Significance of uropods

Motile lymphocytes present a characteristic appearance. They become elongated with the nucleus situated at the anterior end while the cytoplasmic pole containing the Golgi zone and most of the other organelles becomes extended behind to form a uropod or tail. Attachment at the uropod region has been considered an important feature of target cell lysis (Ax et al., 1968; Biberfeld, 1971a; Biberfeld et al., 1973) and it has even been suggested that the uropod plays a decisive role in the destruction of target cells by sensitised lymphocytes (Koren et al., 1973). In most of these studies the use of target cell monolayers

has predisposed towards both the formation and observation of uropods. For example, Ax and co-workers (1968) observed by microcinematography the typical "pear" or "hand mirror" shape of in vitro sensitised or PHA-treated lymphocytes actively crawling over monolayers of mouse embryo target cells. Biberfeld and his colleagues (1971a, 1973) examined the effect of lymphocytes on PHA- or antibody-treated Chang cell monolayers by fixing the cells in situ and cutting sections for electron microscopy perpendicular to the plane of the monolayer. With this technique all free cells would have been removed with the medium during fixation and the formation of uropods by a large proportion of the attached lymphocytes is only to be expected; perpendicular sectioning would then reveal the maximum number of uropods.

McFarland (1969) proposed that the uropod is not only a region of attachment, but also the main receptor organ of the cell and that its development illustrates the high degree of specialisation essential for lymphocyte function in immunological reactions. However, de Petris and Raff have convincingly demonstrated by ferritin labelling that lymphocyte antigen receptors occur diffusely over the entire cell surface; the rearrangement of such receptors into a cap over the cytoplasmic pole or uropod, which can also be shown by immunofluorescence or autoradiography, is artificial and the result of their backward flow (de Petris & Raff, 1972, 1973). Backward flow of membrane is also thought to occur during cell locomotion and would explain why lymphocytes appear to interact with target cells through their uropods (de Petris & Raff, 1972). Thus uropods are a general feature of lymphocyte mobility and although contact through these regions undoubtedly occurs, they are not specialised receptor or attachment sites.

1.4. IN VIVO CONTACTS

The parallel membranes of adjacent cells *in vivo* are separated by an extremely uniform intercellular space of 15-20 nm (Fawcett, 1966). In addition to this general attraction, intercellular junctions occur at regions of local differentiation. Various types of junctions were described in thin sections of mammalian epithelium and classified according to their ultrastructural characteristics by Farquhar and Palade (1963). For junctions with the membranes in contact and the extracellular space obliterated the term occludens was proposed, whereas the term adhaerens (more usually spelt adherens) referred to junctions in which the cell membranes were adherent but separated by an intercellular space. The extent of the cell surface involved in junction formation was described by the additional terms macula (spot), fascia (sheet) and zonula (belt or zone) (Farquhar & Palade, 1963). Thus, macula adherens describes the desmosome since the cell surfaces are adherent over a round to oval area, and zonula occludens the tight junction found at the apical surface of epithelial cells. It is generally agreed that desmosomes play an important role in cell-to-cell adhesion and that the adjacent cells cooperate by means of tight junctions to form a physical barrier between different compartments of the extracellular space (McNutt & Weinstein, 1973). Zonulae and fasciae adherentes (intermediate junctions) appear in thin sections as 2 parallel membranes separated by approximately 20 nm. The intercellular space frequently contains a faint condensation of amorphous material and fine filaments are found in the cytoplasm adjacent to the junctional membrane (Farquhar & Palade, 1963). Intermediate junctions provide sites for the mechanical attachment of cells.

In 1967, Revel and Karnovsky demonstrated that 2 types of junction

had inadvertently been included in the macula occludens (tight junction) category. These workers identified a junction in which 2 nm separated the plasma membranes of the apposing cells; such structures are usually called gap junctions to distinguish them from true tight junctions. The membrane substructure at gap junctions is composed of a hexagonal array of subunits which is visible only after lanthanum impregnation or freeze-fracture (Revel & Karnovsky, 1967; Goodenough & Revel, 1970; McNutt & Weinstein, 1970). Since these subunits bridge the intercellular space (McNutt & Weinstein, 1970) the term gap junction is somewhat misleading (Bennett, 1973) and nexus (McNutt & Weinstein, 1970) might be more appropriate.

Gap junctions have been demonstrated by electron microscopy in several situations both in vivo in mammalian tissue (for example Revel & Karnovsky, 1967; Goodenough & Revel, 1970; McNutt & Weinstein, 1970) and in invertebrates (Payton, Bennett & Pappas, 1969) as well as in vitro between mammalian cells in culture (Johnson & Sheridan, 1971; Pinto da Silva & Gilula, 1972; Leibovitz et al., 1973; Hülser & Demsey, 1973; Azarnia, Larsen & Lowenstein, 1974). The ultrastructural appearance of gap junctions has been correlated with ionic coupling (Bennett, 1973; McNutt & Weinstein, 1973; Hülser & Demsey, 1973; Azarnia et al., 1974) and with the transfer of dyes such as Procion yellow and fluorescein (Payton et al., 1969; Johnson & Sheridan, 1971; McNutt & Weinstein, 1973; Bennett, 1973). McNutt and Weinstein (1970) postulated that a hydrophilic channel through the centre of each subunit of the nexus would account for both the low resistance coupling and the direct transfer of dye between adjacent cells. In 1975, Simionescu, Simionescu and Palade proposed the term "communicating junction" (macula communicans) which would not only indicate the

function of gap junctions but also bring the nomenclature into line with that already in use for other junctional elements.

Antibody-dependent and PHA-induced cytotoxicity of non-immune lymphocytes resemble lysis mediated by sensitised lymphocytes. As already discussed (page 13), close contact is necessary for target cell destruction and there is evidence of communication between effector and target cells (page 14). Although no cytotoxic substances have been isolated, the local release of such factors cannot be entirely excluded. Nevertheless it was considered that some form of junctional specialisation may develop between effector and target cells, with gap junctions as the most likely structure through which cell-mediated cytotoxicity might operate. Consequently the fine structure of attachments formed during antibody-dependent and non-specific (PHA-induced) cytotoxicity was examined.

2. TECHNIQUES FOR ELECTRON MICROSCOPY

2.1. MATERIALS AND METHODS

2.1.1. Fixation

Sequential fixation with glutaraldehyde then osmium tetroxide

In preliminary fixation studies, suspensions of mouse peritoneal cells, mouse and human lymphocyte preparations were fixed in 2.5 or 3% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4 (Sabatini, Bensch & Barrnett, 1963) for 1-2 hours at 0°C. Samples were centrifuged at 1,500 g for 10 minutes, the resulting pellets washed 3 times in 0.25 M sucrose - 0.1 M sodium cacodylate solution (Sabatini et al., 1963), cut into fragments and maintained overnight at 4°C. The blocks were postfixed in phosphate buffered (Millonig, 1961) or cacodylate buffered (Glauert, 1975) 1% osmium tetroxide for 1 hour on ice and then briefly washed with deionised water before dehydration.

Monolayers of mouse peritoneal macrophages were fixed in cacodylate buffered glutaraldehyde and then either scraped off the petri dishes and pelleted, or processed in situ on coverslips for flat embedding. Both methods resulted in identical fixation but, since cell orientation was lost with the former, in situ processing of monolayers was preferred. A layer of carbon previously evaporated onto the coverslips to facilitate separation after polymerisation of the embedding medium (Robbins & Gonatas, 1964) did not affect cell adherence or spreading.

Since uranyl acetate is insoluble in both phosphate and cacodylate buffers, human lymphocytes were washed in Michaelis' veronal-acetate buffer, pH 7.4, after sequential fixation, treated with 0.25% uranyl acetate in the same buffer (Farquhar & Palade, 1965) for 45 minutes at 4°C and washed again before dehydration.

Simultaneous fixation with glutaraldehyde and osmium tetroxide

Mouse and human lymphocyte preparations, mouse peripheral blood and spleen cells were fixed in suspension in the combined glutaraldehyde/osmium tetroxide fixative of Hirsch and Fedorko (1968) for 45 minutes at 0°C. After fixation the cells were washed in veronal-acetate buffer as above (rather than the saline employed by Hirsch and Fedorko, 1968), postfixed for 45 minutes in 0.25% uranyl acetate (Hirsch & Fedorko, 1968) and then washed again in buffer solution.

Monolayers of mouse peritoneal macrophages on carbon-coated coverslips were treated in situ in the same manner prior to flat embedding.

Pellets obtained by centrifugation of cells fixed in suspension tended to break up, particularly during dehydration. Initially, attempts were made to avoid the use of agar, but since subsequent processing was distinctly easier and cellular fine structure was unaffected, the method of Hirsch and Fedorko (1968) was adopted. After postfixation, cells were centrifuged through warm 2% agar and the pellets cut into 0.5 mm cubes when the agar had solidified.

2.1.2. Dehydration and embedding

Fragments of pellets, cut agar blocks and monolayers on coverslips were all dehydrated in a graded series of aqueous ethanol solutions (50, 75, 90%), then in 3 changes of absolute ethanol followed by 2 changes of propylene oxide. Epikote 812 epoxy resin was mixed with accelerator in the proportion of A:B::3:7 according to Luft (1961) and either used immediately or after storage at -70°C (Minick, 1963). Specimens were rotated during infiltration in a 1:1 mixture of propylene oxide and epoxy resin for 1 hour at room temperature and 2 hours at 37°C, followed by 1 hour at 37°C in resin alone.

Blocks were drained on absorbent tissue paper before being transferred

to dried, size 00 gelatin capsules containing fresh epoxy resin which was polymerised at 45°C overnight, then at 60°C for 4-6 days.

Coverslips were laid, cells uppermost, in disposable plastic petri dishes and size 2 gelatin capsules containing partially polymerised resin (3 hours at 60°C) were quickly inverted onto each coverslip. Excess resin was removed from the petri dishes and samples were polymerised as above. After polymerisation was complete, capsules with the embedded cells were snapped off the coverslips with pliers. Although it was considerably easier to remove capsules and their embedded monolayers from carbon-coated (Robbins & Gonatas, 1964) than from non-coated coverslips, it was necessary to cool the bottom of the petri dishes on solid carbon dioxide for 1 or 2 minutes as described by Howatson and Almeida (1958).

2.1.3. Sectioning and staining

A Sorvall MT-2 ultramicrotome was used to cut both thick and thin sections for light and electron microscopy.

Thick (1 μm) sections of flat embedded cells were cut using glass knives made with an LKB knifemaker, transferred on a drop of water in a wire loop to a clean glass slide and the water evaporated over a low bunsen flame. The sections were then stained with 1% toluidine blue in 1% borax (Meek, 1970) for 10 minutes at 60°C, rinsed with deionised water, dried and mounted in clearmount.

Ultrathin sections with gray to silver interference colours, 60-90 nm (Peachey, 1958), were cut with a diamond knife and mounted, after spreading with chloroform vapour (Sotelo, 1957), on cleaned uncoated 200 mesh copper grids or on 400 mesh grids which had been soaked briefly in a very dilute solution of Sellotape adhesive in chloroform (Pease, 1964). Thin sections were contrast stained for

10 minutes in a saturated solution of uranyl acetate (Watson, 1958) in 50% ethanol followed by 2 minutes in lead citrate (Reynolds, 1963) and washed thoroughly in several changes of deionised water.

2.1.4. Electron microscopy

All specimens were examined in an AEI EM 6B electron microscope. Electron micrographs were taken on Ilford EM4 plates using an accelerating voltage of 60 kV and an exposure time of 1 second with an appropriate level of illumination determined by matching with a spot photometer. Plates were developed in Ilford PQ Universal developer for 3-5 minutes at 20°C and photographic enlargements were made using an Ilfoprint autoprocessor and paper or, where necessary, Ilfobrom paper developed in PQ Universal developer.

2.2. OBSERVATIONS AND DISCUSSION

Sequentially fixed mouse (Fig. 1) and human (Fig. 2) lymphocytes show the general characteristics of lymphocytes already described (page 3). Variation in nuclear shape and size relative to that of the whole cell are clearly demonstrated.

The slightly denser appearance of mouse (Figs. 1 & 3) compared with human (Figs. 2 & 4) lymphocytes is probably due to postfixation of the former in phosphate buffered osmium tetroxide, and to the combined effects of cacodylate buffered osmium tetroxide followed by uranyl acetate in the latter, rather than to a species related difference. Millonig (1961) considered that phosphate buffer was superior to veronal-acetate because there was less extraction of the cytoplasmic matrix, but whether the variation observed here was due to the buffer used or the uranyl acetate treatment was not determined.

Both the nucleus and cytoplasm in mouse (Fig. 5) and human (Fig. 6) lymphocytes generally appeared less dense after simultaneous fixation (Hirsch & Fedorko, 1968) than with sequential fixation (Figs. 3 & 4); the difference between the two methods is more clearly demonstrated in mouse macrophages (Figs. 7 & 8). Simultaneous fixation of mouse (Fig. 9) and human (Fig. 10) neutrophils also produced good results; in particular the granules, which frequently appear mottled after sequential fixation (Hirsch & Fedorko, 1968), were well preserved, as were the microfilaments in the cytoplasm of pseudopodia (Fig. 10).

Hirsch and Fedorko (1968) considered the term "postfixation" in uranyl acetate might have been incorrect because uranyl ions were probably acting primarily as a stain rather than a fixative. In the present study, however, it was found that when the period of washing before uranyl acetate treatment was prolonged the plasma membrane was often discontinuous, although the overall contrast of the cells was high, indicating that uranyl acetate does indeed act as a fixative as well as a stain. Other workers have also suggested that treatment with uranyl acetate, buffered or aqueous, before dehydration enhances preservation (Ryter et al., 1958; Silva, Guerra & Magalhães, 1968; Terzakis, 1968) but a deleterious effect on glycogen has been observed (Hirsch & Fedorko, 1968; Vye & Fischman, 1970).

Although there is probably some extraction of material after simultaneous fixation (Hirsch & Fedorko, 1968) despite, or perhaps because of postfixation in uranyl acetate, most of the organelles including centrioles (Figs. 4, 6 & 9), Golgi vesicles and cisternae (Figs. 8 & 9), smooth and rough endoplasmic reticulum (Fig. 8) are well preserved. Simple fibrillar nuclear bodies, which are considered normal nuclear organelles (reviewed by Ghadially, 1975) are clearly

evident in lymphocytes (Fig. 5) and macrophages (Fig. 8). The appearance of 10 nm filaments in lymphocytes (Figs. 5 & 6) has probably been accentuated by postfixation in uranyl acetate in a manner similar to that noted by Daems and Brederoo (1973).

"Bubbles" and myelin figures at the cell surface (Figs. 1, 2 & 3) are artefacts of primary glutaraldehyde fixation (Trump & Ericsson, 1965 ; Oláh & Röhlich, 1966; Curgy, 1968) and were rarely seen after simultaneous fixation. Since the investigation was to involve the examination of plasma membranes and intercellular contacts at high magnification, simultaneous fixation with postfixation in uranyl acetate (Hirsch & Fedorko, 1968) was obviously preferred for the cytotoxicity experiments.

3. ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

3.1. INTRODUCTION

3.1.1. Functional and morphological aspects of antibody-dependent cell-mediated cytotoxicity

Humoral antibodies, besides destroying target cells in the presence of complement, may induce cell-mediated cytotoxic reactions in vitro. Specific antibody can both increase phagocytosis of target cells by macrophages (Bennett, Old & Boyse, 1963) and promote target cell damage by lymphoid cells from non-sensitised donors (Möller, 1965). In the first clear example of antibody-dependent cell-mediated cytotoxicity, Perlmann and Holm (1968) used fowl erythrocytes coated with purified tuberculin antigen as targets. Blood lymphocytes from normal guinea pigs were found to be cytotoxic for the target cells in the presence of antiserum from guinea pigs immunised with Bacillus Calmette-Guérin (BCG) (Perlmann & Holm, 1968). Subsequent systems have mostly utilised natural target cell antigens. Human peripheral blood lymphocyte activity against Chang cells coated with rabbit or rat anti-Chang cell antibody (MacLennan & Loewi, 1968), and against chicken erythrocytes exposed to rabbit anti-chicken erythrocyte serum (Perlmann & Perlmann, 1970) has been demonstrated.

In early experiments the concentrations of antibody chosen could have produced lysis of target cells in the presence of complenent. However, it is clear that antisera induce cell-mediated cytotoxicity at dilutions too high to permit conventional complement-dependent lysis (Perlmann & Holm, 1969; MacLennan, Loewi & Harding, 1970; Perlmann & Perlmann, 1970).

Radioactive chromium (^{51}Cr) has proved an excellent label for measuring target cell death (Holm & Perlmann, 1967b; MacLennan & Loewi, 1968; Perlmann, Perlmann & Holm, 1968), and because of the

obvious advantages associated with non-dividing cells, antibody-coated erythrocytes have generally been preferred. Furthermore, Perlmann, Perlmann and Holm (1968) considerably reduced spontaneous lysis of labelled erythrocytes by incorporating an appropriate excess of unlabelled red cells (or other suitable cells) as "fillers". Under these conditions spontaneous release of isotope during 24 hours incubation was usually less than 10% (Perlmann et al., 1968).

Effector cells have been obtained from various sources. Human peripheral blood lymphocytes (Perlmann & Perlmann, 1970), rabbit lymph node, spleen and peritoneal cells (Gelfand, Resch & Prester, 1972), mouse spleen (Dennert & Lennox, 1972, 1973; Greenberg et al., 1973 a & b) and mouse peritoneal cells (Dennert & Lennox, 1973) have all proved satisfactory against antibody-coated chicken erythrocytes. In addition, cells shown to be cytotoxic to antibody-treated human red cells have included human peripheral blood leucocytes (Holm, 1972; Holm & Hammarström, 1973) and human peritoneal cells (Holm, 1972).

There has been considerable interest in the nature of the effector cell responsible for antibody-dependent cell-mediated cytotoxicity. Various model systems involving cell separation techniques, kinetic analysis and demonstration of cell surface receptors have implicated lymphocytes (Perlmann & Holm, 1968; MacLennan & Loewi, 1968; Perlmann & Perlmann, 1970; Gelfand et al., 1972; Resch, Gelfand & Prester, 1974), "null" lymphoid cells (Greenberg et al., 1973a), granulocytes, monocytes or macrophages, either alone or in varying combination (Dennert & Lennox, 1972, 1973; Holm, 1972; Gelfand et al., 1972; Holm & Hammarström, 1973; Resch et al., 1974) as well as non-phagocytic monocytes (Greenberg et al., 1973b). This confusion has in large part resulted from failure to recognise the limitations of light microscopy.

In the few investigations combining light microscopic examination with functional analysis (Perlmann & Perlmann, 1970; Holm, 1972), close contact between target and effector cells has been considered important but the details could not be resolved.

Despite the wealth of investigations into the functional characteristics of the cells involved, there have been very few ultrastructural studies of antibody-dependent cell-mediated lysis. Biberfeld and Perlmann (1970) examined the interaction between purified human peripheral blood lymphocytes and antibody-coated chicken erythrocytes by electron microscopy. More than 98% of the effector cells were small and medium-sized lymphocytes, many showing uropod formation. Correlation of ^{51}Cr release with morphological findings indicated osmotic damage to the target cells but although erythrophagocytosis was occasionally observed it was not regarded as significant. Contact between target and effector cells occurred and it was suggested that only temporary contact was necessary for target cell killing (Biberfeld & Perlmann, 1970).

Similar morphological observations with human blood lymphocytes and Chang cell monolayers in the presence of rabbit anti-Chang cell serum were reported by Biberfeld, Biberfeld, Perlmann and Holm (1973). During clearance of the monolayer (plaque formation), many lymphocytes made close contact with large areas of target cells, often by their uropods. The shortest distance between the apposing membranes was approximately 20 nm and although there were many areas of apparent membrane dissolution the authors could not determine whether these represented specialised junctions, areas of membrane fusion or merely tangential sectioning. In the absence of antiserum only occasional lymphocytes were attached to the monolayers and plaques did not develop

(Biberfeld et al., 1973). In neither of these investigations, however, was the contact between target and effector cells in the presence of normal serum without specific antibody, examined by electron microscopy.

3.1.2. Selection of the experimental systems

The morphological investigations were planned in collaboration with members of the Division of Cell Pathology as a result of their preliminary findings in functional studies on antibody-dependent cell-mediated cytotoxicity. Initially, mouse adherent peritoneal cells were selected as effectors with chicken erythrocytes as targets. A.C. Allison and P. Davies, using phase contrast microscopy, observed 2 different types of rosette in this system when low concentrations of immune serum were added. "Loose" rosettes of red cells surrounded macrophages, whereas "tight" rosettes, which were less frequent, formed about round cells with cleft nuclei. The round cells were considered likely candidates for the effector cell responsible for antibody-dependent cytotoxicity.

Cells from mouse peritoneal fluid were separated in velocity-gradient columns by A.C. Allison, P. Davies and M. Denman (Allison, 1972). Although the yield was low, a population of round, adherent cells, distinct from macrophages and from smaller T or B lymphocytes was obtained. Such cells were thought to be a type of lymphocyte, and because they were involved in antibody-dependent reactions, adhered to glass, and were thought to kill target cells by attachment rather than by phagocytosis, it was proposed that they be called A cells (Allison, 1972). Allison, Davies and Page (1973) reported that A cells were also present in mouse adherent peritoneal cultures. At a symposium on Immunopathology at Cavtat, Yugoslavia, it was suggested that the lymphoid cells which kill antibody-coated target cells should

be called K cells (Nature new Biol., 1973) and this terminology has now been generally adopted.

Examination by electron microscopy of the antibody-mediated cytotoxic effect of mouse peritoneal cells on chicken erythrocytes was undertaken to investigate the specific effector cell (or cells) involved. In view of the paucity of K cells obtained by separation techniques, mouse adherent peritoneal cells were used as effectors in preliminary experiments. After work commenced ^{51}Cr release studies by I.A. Clark indicated that mouse spleen cell suspensions were more suitable and in subsequent ultrastructural studies, therefore, the antibody-dependent cytotoxic action of these cells on chicken red blood cells was investigated. In both systems particular emphasis was placed on the fine structure of the areas of contact between effector and target cells. The frequency and appearance of the attachments formed in the presence of both normal serum and antiserum were compared in an effort to determine both their significance and the mechanism of lysis.

3.2. THE CYTOTOXIC ACTION OF MOUSE ADHERENT PERITONEAL CELLS ON CHICKEN ERYTHROCYTES

3.2.1. Materials and methods

a. Effector cell preparation

Mice were injected intraperitoneally with Parker's medium 199 containing 5% heat-inactivated foetal calf serum (FCS) and cells obtained by peritoneal lavage (Cohn & Benson, 1965). Monolayers of adherent cells were then prepared by P. Davies as described by Davies, Page and Allison (1974). In summary, cell suspensions were incubated at 37^{o}C in culture dishes or on coverslips for 10 - 180 minutes to permit cell attachment. Non-adherent cells were removed by washing with warm phosphate buffered saline (PBS) and the adherent cells then

maintained in fresh medium at 37°C for 18-24 hours to allow spreading.

b. Target cells and estimation of target cell death

Cytotoxicity studies by I.A. Clark followed the general method of Perlmann and Perlmann (1970). Heparinised chicken blood was diluted with medium 199 + 5% FCS and centrifuged at 1,000 g for 10 minutes. Chicken red blood cells (CRBC) were removed from the bottom of the pellet and diluted fivefold with the medium. One hundred microlitres of the erythrocyte suspension was incubated with 100 μCi ^{51}Cr for 60 minutes at 37°C. The cells were washed 5 times to remove unabsorbed chromium and finally resuspended in medium.

The release of radioactivity from ^{51}Cr labelled chicken erythrocytes was measured, and the degree of target cell lysis was expressed as the percentage cytotoxicity.

$$\% \text{ cytotoxicity} = \frac{\% \ ^{51}Cr \text{ release}}{\text{maximum } \% \ ^{51}Cr \text{ release}}$$

where $\% \ ^{51}Cr$ release $= \frac{\text{counts per minute in the supernatant}}{\text{total counts per minute in the sample}} \times 100$

and maximum ^{51}Cr release = $\% \ ^{51}Cr$ release after distilled water lysis of all CRBC.

c. Serum

The source of antibody did not affect the amount of chromium released (I.A. Clark), so that anti-chicken erythrocyte serum from guinea pigs or rats was used. Because the cytotoxic potency of the immune serum was not related to its haemagglutination titre (Perlmann & Perlmann, 1970) the antiserum concentration producing optimal cell-mediated lysis was chosen. Control serum was obtained from normal guinea pigs or rats and all sera were inactivated at 56°C for 30 minutes.

d. Experimental procedure

Cytotoxicity experiments (I.A. Clark)

Mouse peritoneal cells were incubated with ^{51}Cr labelled chicken erythrocytes, usually in a ratio of 25:1, for up to 10 hours at 37°C. Medium contained either antiserum or normal serum at a final concentration of 1:1,000 and mouse erythrocytes were added as fillers to prevent spontaneous lysis of chicken red blood cells (page 28). Chromium release from the target cells was measured in triplicate incubations after various time intervals and the percentage cytotoxicity calculated.

Phase contrast microscopy. Rosette formation

The formation of rosettes of chicken red blood cells around adherent peritoneal cells was monitored by A.C. Allison and P. Davies. In brief, monolayers of adherent cells on coverslips were incubated at 37°C for 6 hours with chicken erythrocytes in the presence of normal serum or antiserum. The coverslips were then washed in PBS, fixed in 2.5% glutaraldehyde/cacodylate, and examined by phase contrast microscopy for tight and loose rosettes.

Samples for electron microscopy

In different experiments peritoneal cells were allowed to attach to 22mm^2 glass coverslips for 10, 60, 90 or 180 minutes before removal of non-adherent cells. After 18-24 hours incubation at 37°C adherent cells were fixed <u>in situ</u> as described previously (page 20). Duplicate samples of monolayers formed after the 90 minute attachment period were incubated as follows:

i. adherent peritoneal cells alone.

ii. adherent peritoneal cells with chicken erythrocytes.

iii. adherent peritoneal cells with chicken erythrocytes and guinea pig normal serum 1:1,000.

iv. adherent peritoneal cells with chicken erythrocytes and guinea pig antiserum 1:1,000.

To permit formation of the maximum number of cell contacts excess chicken erythrocytes were used and therefore mouse filler cells were omitted from samples intended for electron microscopy. Coverslips were washed with warm PBS to remove unattached chicken red blood cells and then flooded with fixative.

The cells were processed in situ and flat embedded for both thick (1 μm) and thin sectioning parallel to the coverslip. Blocks were trimmed to give a face of approximately 1 mm^2. A number of specimens were serially thick sectioned for light microscopy (page 22) through the entire monolayer. In general, however, only 1 or 2 thick sections were cut followed by serial thin sections. Ribbons of 3 or 4 thin sections were mounted on grids and contrast stained (page 22). Representative electron micrographs were taken of all samples but since cell counts were obtained directly from thin sections a standard procedure was adopted. To avoid counting the same cells at different levels, a single suitable section on each grid was selected at a scanning magnification of approximately x 400. Effector cells were then examined at a minimum magnification of x 5,000 - x 10,000 and identified by the morphological criteria already described (pages 3 - 7). Only cells with a nucleus in the plane of section were included. Because the total number of effector and target cells varied from one block to another, counts obtained from different blocks of the same sample were pooled.

3.2.2. Results and observations

a. Target cell death

Release of ^{51}Cr from chicken erythrocytes treated with normal serum or antiserum was followed over a 10 hour period. Almost all the lysis

of the antibody-coated cells occurred during the first 6 hours when a plateau was reached, and there was little additional release of chromium. After 6 hours incubation with antiserum an average of 45% of the target cells had been lysed compared with 15% in the presence of normal serum.

b. Phase contrast microscopy. Rosette formation

Rosettes were formed around 75% of the adherent cells after 6 hours incubation with chicken erythrocytes and antiserum. Seventy five per cent of these rosettes were described as loose, the typical ellipsoid shape of the red cells still being apparent. The remaining 25% were tight rosettes in which the erythrocytes were fewer in number but rounder and more closely packed over the central adherent cell. When incubated with normal serum only occasional chicken erythrocytes were observed attached to the adherent cells.

c. Morphology

Effector cells

Adherent peritoneal cells consisted mainly of macrophages with occasional lymphocytes. Most of the macrophages were well spread and, where sectioned near their site of attachment to the coverslip, were usually elongated or triangular in outline with a low nuclear:cytoplasmic ratio (Fig. 11). Where the plane of section passed higher through the cell the shape was typically round or oval and the nucleus occupied a relatively larger area (Fig. 12).

The number of lymphocytes among the adherent cells varied between 2 and 14% (6/270 to 100/609 cells counted in thin sections) in different experiments and was not related to the time allowed for initial attachment. Small, medium-sized and large lymphocytes, some of which contained bundles of 10 nm filaments (Figs. 13 & 14) were seen. Seventy per cent of the lymphocytes observed (63/90 in one sample) were in contact with macrophages,

occasionally where the macrophages were well spread (Fig. 13), but much more frequently in sections further away from the area of attachment to the glass (Fig. 14).

Target cells

The ultrastructure of the chicken erythrocytes was similar to that of other nucleated red cells (Davies, 1961; Fawcett & Witebsky, 1964; Fawcett, 1966). Preservation was good and the use of the Hirsch and Fedorko (1968) method of fixation gave favourable results compared with those obtained by Brown (1975) in a recent study on the fixation of avian erythrocytes. The flattened elliptical cells contained ovoid nuclei in which chromatin was condensed in irregularly shaped clumps (Figs. 15, 16 & 17). Electron-opaque cytoplasm was homogeneous, and occasional mitochondria or remnants of endoplasmic reticulum and Golgi lamellae were observed. When sectioned in a favourable plane, the equatorial marginal band of microtubules was apparent immediately beneath the plasma membrane of the cell (Fig. 16).

Contacts and phagocytosis

Light microscopic examination of toluidine blue stained, serial 1 µm epoxy sections completely through the monolayer showed that all the chicken erythrocytes were either attached to or had been phagocytosed by mononuclear cells. Comparison of adjacent thick (1 µm) and ultrathin sections always revealed the characteristic fine structure of macrophages (page 5) in the mononuclear cells involved. Chicken red blood cells were found only in association with macrophages; those that appeared to be merely lying close to a macrophage in one section (Fig. 15), were invariably seen to be attached when the same cells were examined at a different level.

The percentage of macrophages associated with chicken erythrocytes in the absence of serum was low, in the presence of normal serum slightly higher and markedly higher after the addition of antiserum (Table 1). In the controls (no serum or with normal serum) chicken red cells and macrophages were almost always associated in a 1:1 ratio. However, in the presence of immune serum, up to 7 erythrocytes were seen attached to and/or ingested by 1 macrophage.

Table 1. Attachment and phagocytosis of chicken erythrocytes (CRBC).

Mouse adherent peritoneal macrophages + excess CRBC

	Total no. macrophages	Macrophages with phago-cytosed CRBC	Macrophages with attached CRBC	Macrophages with phago-cytosed & attached CRBC	Macrophages associated with CRBC
No serum	517	16 (3%)	8 (2%)	0	24 (5%)*
Normal serum	258	13 (5%)	3 (1.5%)	1 (0.5%)	17 (7%)*
Antiserum	208	60 (29%)	35 (17%)	29 (14%)	124 (60%)**

Incubation at 37°C for 6 hours. Guinea pig normal serum or antiserum 1:1,000. Cells counted in thin sections (page 34).

* Usually 1 erythrocyte: 1 macrophage.

** Up to 7 erythrocytes: 1 macrophage.

Erythrocytes were usually attached at their equator, appearing rounded or elliptical depending on the plane of section. The area of contact varied greatly and frequently the region of the red cell near the macrophage showed considerable distortion (Figs. 16 & 17). Apart from the lower frequency of contacts in the controls (Table 1) the types of attachment in all samples were identical.

Phagocytosis was occasionally observed and the area of macrophage cytoplasm involved was usually devoid of organelles other than numerous

5-7 nm microfilaments (Figs. 15 & 16). Up to 6 erythrocytes were found within a macrophage in a single section, and ingested chicken cells could be seen in varying stages of degradation (Figs. 15 & 17).

3.2.3. Discussion

a. Target cell death

Rabbit (Gelfand et al., 1972) and mouse (Dennert & Lennox, 1973) peritoneal cells produce rapid, specific lysis of antibody-coated chicken erythrocytes which reaches a peak between 4 and 8 hours; after longer intervals the specific cytotoxicity decreases due to increasing lysis of non-antibody coated target cells (Gelfand et al., 1972). Similar results were obtained in the present experiments, and 6 hours was selected as a suitable incubation period for samples to be examined by phase contrast and electron microscopy.

b. Phase contrast microscopy. Rosette formation

Phase contrast microscopy confirmed that tight and loose rosettes of red cells were formed in the expected proportions after 6 hours incubation. Since 25% of these rosettes were classified as tight, it was assumed that the central cell, which accounted for 19% of all adherent cells, would be readily identifiable in parallel samples prepared for electron microscopy.

When attempting to identify the cells with cleft nuclei regarded by Allison (1972) as candidate effectors, adherent peritoneal cells were incubated with excess chicken erythrocytes to obtain the maximum number of contacts. Since it is technically difficult to measure ^{51}Cr release induced by adherent cells, whole peritoneal cell populations had to be used. For this reason the cytotoxicity assay served here only as a guide to the suitability of peritoneal cells as effectors.

c. Morphology

Effector cells

Weiss (1968) suggested that thick (1 µm) epoxy sections stained with toluidine blue were preferable to whole mount preparations for "cytologic analysis" and with this method Catanzaro, Graham and Hogrefe (1974) estimated that normal mouse peritoneal fluid contained 34% macrophages and 60% lymphocytes. However, by combining the property of adherence with uptake of neutral red (Lay & Nussensweig, 1968), phagocytosis (Krahenbuhl & Lambert, 1975) or cellular fine structure (Fishel et al., 1976) a more usual estimate of the number of macrophages is 60-70%. In the present study toluidine blue stained 1 µm sections alone were inadequate for the identification of many rosette-forming cells and so were examined in conjunction with the adjacent thin sections used for differential counts.

Ninety to 99% of mouse peritoneal cells which adhere to glass or plastic are macrophages (Fishel et al., 1976; Krahenbuhl & Lambert, 1975; Fedorko, Cross & Hirsch, 1973). Five to 8% of the adherent cells were lymphocytes (Fishel et al., 1976) and 2-14% were found in the present study. However, it was probable that 70% of these lymphocytes were "adherent" to the glass via the macrophages to which they were attached (Figs. 13 & 14). Similarly Fishel and co-workers (1976) illustrate a chain of 4 lymphocytes in contact through one another with a single peritoneal macrophage.

Some of the lymphocytes amongst the adherent cells possessed perinuclear bundles of 10 nm filaments (Figs. 13 & 14). Although such filaments are characteristic of monocytes and macrophages (de Petris et al., 1962) they can also be seen in lymphocytes (Bessis, 1973).

Contacts and phagocytosis

It was evident from light and electron microscopy of adjacent thick and thin sections that antibody-coated chicken red cells adhered only to macrophages, as has been reported in a human system (Douglas & Huber, 1972). Erythrocytes near macrophages in one section were invariably found in close contact at a different level and, because non-adherent red cells had been removed prior to fixation, all erythrocytes adjacent to macrophages in thin sections were counted as attached.

Seljelid, Melsom and Munthe-Kaas (1972) reported spontaneous attachment of untreated mouse erythrocytes to mouse macrophages but the cytotoxicity described by these authors may have been due to the combination of homologous target cells with high serum concentrations. In the present investigation there was little adherence or phagocytosis of chicken erythrocytes with normal serum or when serum was omitted (Table 1) and extracellular lysis was not observed. In a system in which the vast majority of effector cells was phagocytic it is hardly surprising that occasional red cells should have been ingested in the absence of antiserum and such non-specific phagocytosis could account for the relatively high cytotoxicity in control samples. In contrast, the addition of specific antiserum caused the attachment or phagocytosis of up to 7 erythrocytes by any 1 macrophage; similar results were obtained by Griffin and Silverstein (1974) and Tizard, Holmes and Parappally (1974).

Attachments between red cells and macrophages varied greatly in area and showed considerable distortion, resembling those illustrated by other investigators (Lo Buglio et al., 1967; Abramson et al., 1970; Douglas & Huber, 1972) between human monocytes or macrophages and antibody-coated erythrocytes. Abramson and his colleagues (1970)

described rare instances of tight junction formation but although the fine structure of contacts was examined very carefully no junctional specialisation was ever observed in the present study.

In both thick and thin sections macrophages with red cells attached appeared identical to the loose rosettes observed in whole mount preparations. Because thin sections were cut parallel rather than perpendicular to the plane of the coverslip, any erythrocytes attached to the "tops" of macrophages would not have been seen. Thus the number of macrophages with which antiserum-treated red cells were associated (60%, Table 1) has undoubtedly been underestimated and this would account for the 15% difference between the counts of rosette-forming cells obtained by phase contrast and electron microscopy. Similarly, a proportion of the 29% of macrophages which had apparently only phagocytosed red cells (Table 1) would also have had erythrocytes attached and been counted as loose rosettes in whole mount preparations.

Although it had been estimated by phase contrast microscopy that 19% of adherent peritoneal cells formed tight rosettes, none were found in either thick or thin sections. However, when macrophages with several ingested erythrocytes were sectioned at an appropriate level their appearance closely resembled that of tight rosettes. It was considered therefore that tight rosettes represented phagocytosed red cells, their intracellular localisation being obscured in whole mount preparations.

Numerous randomly arranged 5-7 nm microfilaments were observed in the region of macrophage cytoplasm involved in phagocytosis (Fig. 16). Fine filaments are a common, possibly universal, cytoplasmic component (Fawcett, 1966) and have been described, not only in muscle, but in fibroblasts, chondrogenic and nerve cells (Ishikawa, Bischoff & Holtzer, 1969), platelets (Behnke, Kristensen & Nielsen, 1971), reticuloendothelial

cells (Carr, 1972) and mast cells (Röhlich, 1975). Allison, Davies and de Petris (1971) demonstrated the actin-like nature of macrophage microfilaments, by heavy meromyosin binding, and proposed that a contractile system in the peripheral cytoplasm was involved in both cell movement and phagocytosis. Large numbers of microfilaments have been found in macrophage cytoplasm around forming phagosomes (Dumont & Robert, 1970) and their role in phagocytosis has been clearly demonstrated by Reaven and Axline (1973).

There have been several attempts to determine the viability of antibody-treated target cells once they become attached to macrophages or monocytes. Tumour cells could still exclude trypan blue (Bennett et al., 1963) and mycoplasmas incorporate radiolabelled thymidine into DNA (Jones & Hirsch, 1971), whereas the swelling and fragmentation of erythrocytes still attached to monocytes or macrophages suggested cell death (Lo Buglio et al., 1967; Abramson et al., 1970). In the present investigation, antiserum-treated red cells attached to macrophages appeared normal, apart from considerable deformation in the region of contact, and there was no evidence of extracellular lysis.

When heat-killed Candida albicans are used, 75-95% of adherent peritoneal macrophages are actively phagocytic (Fishel et al., 1976; Krahenbuhl & Lambert, 1975). The lower figure of 43% in the present study (Table 1) may be due partly to the different target cells and partly to an underestimate produced by counting only on thin sections.

The rate at which macrophages digest engulfed cells also varies. Mycoplasmas are partially degraded in 2 hours, and are completely digested 24 hours after ingestion (Jones & Hirsch, 1971). Intact intracellular group A streptococci are rare after 5-6 hours (Glick, Getnick & Cole, 1971), and there is total loss of viability of group E streptococci at 4 hours (Cargill & Olsen, 1973). Little remains of

erythrocytes 24 hours after they are phagocytosed by macrophages (Fedorko et al., 1973) and Holm and Hammarström (1973) calculated that each monocyte is capable of destroying 2-3 antibody-treated red cells in 18 hours. In the present study ingested erythrocytes were seen in varying stages of degradation after 6 hours incubation (Figs. 15 & 17) and although the possibility of extracellular lysis cannot be totally excluded it was considered that target cell death resulted from phagocytosis by macrophages.

3.2.4. Conclusions

Mouse adherent peritoneal cells consisted of macrophages to which a few lymphocytes were adherent. There was no evidence of a class of lymphocytes capable of independent attachment to glass.

Antiserum-treated chicken erythrocytes were never found associated with lymphocytes but formed loose rosettes around or were phagocytosed by macrophages. It was considered that the tight rosettes seen by phase contrast microscopy were in fact closely packed erythrocytes which had been ingested by macrophages.

The region of attachment between macrophages and antibody-coated chicken erythrocytes showed considerable distortion but no junctional specialisation. Extracellular lysis was not observed and target cell death appeared to result from phagocytosis.

Since the proportion of lymphocytes among mouse adherent peritoneal cells is lower than in mouse spleen suspensions, it was decided that the latter should be used as a source of effector cells in further studies of lymphocytes in antibody-dependent cell-mediated cytotoxicity.

3.3. THE CYTOTOXIC ACTION OF MOUSE SPLEEN CELLS ON CHICKEN ERYTHROCYTES

3.3.1. Materials and methods

Effector cells, target cells and sera were prepared by I.A. Clark who also measured the cytotoxicity.

a. Effector cell preparation

Mouse spleens were removed aseptically and placed in petri dishes with a small amount of HEPES buffered Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 5% heat-inactivated FCS. The tissue was squashed against the base of the dish with a plunger from a syringe and most of the stroma was removed by teasing with forceps. Cells were further dissociated by gentle squirting through the syringe. The suspension was left at room temperature for 15 minutes to allow large clumps of cells to settle and the supernatant, containing dispersed cells, then used.

b. Target cells and estimation of target cell death

Chicken erythrocytes were labelled and cytotoxicity measured as described previously (page 32) except that all dilutions were made in RPMI 1640 (as above) instead of Parker's medium 199.

c. Serum

Heat-inactivated mouse, guinea pig or rat anti-chicken erythrocyte serum was diluted to 1:1,000. Control serum was obtained from non-immunised mice, guinea pigs or rats.

d. Experimental procedure

Cytotoxicity experiments

Spleen cells were mixed with labelled chicken erythrocytes in a ratio of 25:1 and incubated at 37^{o}C for up to 20 hours in the presence of normal serum or antiserum and mouse filler cells. The release of

^{51}Cr from the target cells was measured at various times and the percentage cytotoxicity calculated. The effect of varying the ratio of effector to target cells was studied by measurement of ^{51}Cr release up to 20 hours.

Samples for electron microscopy

Mouse spleen cell suspensions were incubated at 37°C for 15 and 30 minutes, 1, 3 and 6 hours as follows:

i. mouse spleen cells alone

ii. mouse spleen cells + CRBC + rat or mouse normal serum 1:1,000

iii. mouse spleen cells + CRBC + rat or mouse antiserum 1:1,000

Effector to target cell ratios of 50:1, 25:1, 5:1 and 1:1 were examined. Cell mixtures were usually agitated during incubations of 1 hour or more.

Spleen cells with chicken erythrocytes (in ratios of 5:1 and 1:1) were also incubated for 1 hour at 4°C in the presence of either rat normal serum or antiserum 1:1,000.

Mouse filler cells were omitted from samples for electron microscopy and from duplicate samples in which ^{51}Cr release from the target cells was measured.

Cells were fixed in suspension (page 21), and differential counts were obtained directly from thin sections as previously described (page 34). Because the total number of effector and target cells varied from one block to another, the counts from different blocks of the same sample were pooled, and representative electron micrographs were taken.

To determine the proportion of lymphocytes present in the effector suspension approximately 230 control spleen cells from a number of different blocks were identified and counted (Table 4).

A minimum of 200 control lymphocytes and 100 lymphocytes attached

to antibody-coated target cells were classified according to whether their nuclei were rounded or irregularly shaped.

Approximately 200 lymphocytes from the 25:1 ratio and 100 lymphocytes from each sample at ratios of 5:1 and 1:1, were counted to determine the proportion which were attached to chicken erythrocytes (Table 5).

All of the contacts between effector and target cells (amounting to several hundred) were examined at high magnification for evidence of fusion or junction formation. In addition, 70-100 attachments per sample at a ratio of 5:1 were classified as point, flat or distorted (Table 6).

To assess the numbers of target cells which were phagocytosed, free, or attached to other cells (Table 7), 100 chicken erythrocytes from each sample at the 5:1 ratio were counted. However, because there were considerably fewer target cells in the 50:1 samples, the percentage of CRBC phagocytosed after 1 hour was based on the observation of 70 chicken erythrocytes.

3.3.2. Results and observations

a. Target cell death

Cytotoxicity figures for two representative experiments which were examined by electron microscopy are given in Tables 2 and 3.

Table 2. Cytotoxicity of chicken erythrocytes with and without mouse red cells as fillers. Ratio of spleen cells to CRBC 50:1

		1 hour	3 hours	6 hours
Antiserum	- fillers	16%	34%	72%
	+ fillers	n.t.	36%	62%
Normal serum	- fillers	9%	9%	10%
	+ fillers	n.t.	7%	8%

Spontaneous lysis of CRBC at 6 hours in the absence of effector cells = 9%

Incubation at 37°C. Rat normal serum or antiserum 1:1,000.

n.t. = not tested.

Table 3. Cytotoxicity of chicken erythrocytes without mouse red cells as fillers. Ratio of spleen cells to CRBC 5:1

	15 mins	30 mins	1 hour	3 hours
Antiserum	0.9%	1.9%	2.6%	5.4%
Normal serum	0.2%	0.3%	0.2%	0.2%

Spontaneous lysis of CRBC at 3 hours = 0.1%

Incubation at 37°C. Rat normal serum or antiserum 1:1,000.

As with the peritoneal effector cells (page 34), there was an initial period of rapid killing up to 6 hours (Table 2), after which the rate decreased. Approximately 50% of the total lysis occurred within the first 4 hours and maximum ^{51}Cr release was obtained after 18-20 hours incubation at 37°C. The percentage cytotoxicity after 20 hours incubation varied from one experiment to another but on average, when the ratio of spleen to red cells was 25:1, 75-95% of the antibody-treated target cells were lysed. Cytotoxicity in the presence of normal serum rarely exceeded 10% and was frequently as low as 5%.

Chromium was not released from cell mixtures maintained at 4°C but the normal pattern followed when they were subsequently incubated at 37°C.

It was established that the presence of filler cells did not significantly alter the release of ^{51}Cr from the target cells during short (up to 6 hours) incubation periods (Table 2).

After similar incubation times, the percentage lysis increased as the concentration of the effector cells was raised, such that there was a five to sixfold increase in the number of target cells killed at a ratio of 50:1 compared with 5:1 (compare 1 and 3 hour incubations, Tables 2 and 3).

b. Morphology

Effector cells

Ninety-five per cent of the cells in mouse spleen suspension were lymphocytes (Fig. 18, Table 4). The relatively small number of macrophages and neutrophils (Fig. 19) varied only slightly from one experiment to another. Occasional plasma cells (Fig. 19), platelets, megakaryocytes and maturing mouse erythrocytes were seen, and a number of damaged cells, of which all that remained was a nucleus and sometimes a plasma membrane, were also present.

Cells occurred singly or in clumps composed of lymphocytes (Fig. 18) and less frequently of mixed cell types (Fig. 19). Spleen suspensions fixed immediately after preparation contained many clumps (Fig. 19), but after incubation at 37°C for 1 hour or more most of the cells had separated. Cells in clumps, whether of the same (Fig. 18) or different types (Fig. 19), were separated by a gap of 15-20 nm which usually contained slightly electron-opaque, amorphous material.

The nucleus appeared round in approximately 60% of the lymphocyte population and cleft or irregularly shaped in the remaining 40% (Table 4).

Table 4. Proportion of lymphocytes with round and cleft or irregularly shaped nuclei in mouse control spleen cell suspensions

Lymphocytes with round nuclei	131
Lymphocytes with cleft or irregularly shaped nuclei	94
Total lymphocytes	225
Total spleen cells	234

Cells counted in thin sections (page 45).

Target cells

The morphology of chicken erythrocytes has already been described (page 36). Although filler cells were not added to samples for electron microscopy a number of mouse red blood cells were present in spleen cell suspensions. The only target cells which were counted, therefore, were those in which the nucleus was visible or which displayed the unmistakable ellipsoid shape of chicken erythrocytes.

Contacts

Contact between effector and target cells was considered established when the gap separating their plasma membranes was 25 nm or less. In the presence of normal serum or specific antiserum attachments were observed between chicken erythrocytes and macrophages, neutrophils or lymphocytes but the latter were by far the most frequent (Figs. 20-26).

The number of lymphocytes attached to target cells was not related to the length of incubation between 15 minutes and 6 hours. At each time sampled, at any given ratio, more lymphocytes were in contact with antibody-coated chicken erythrocytes than with normal serum-treated target cells (Table 5).

Variations of the effector to target cell ratio, however, considerably affected the proportion of lymphocytes attached to chicken erythrocytes. A relative increase in the number of target cells resulted in a marked increase in the number of contacts (Table 5).

Table 5. Effect of variation of cell ratio on the percentage of lymphocytes attached to chicken erythrocytes

Mouse spleen cells : CRBC	25:1	5:1	1:1
Lymphocytes attached to CRBC, antiserum	12%*	27%	53%
Lymphocytes attached to CRBC, normal serum	n.t.	7%	30%

Incubation at 37°C. Guinea pig or rat normal serum or antiserum 1:1,000.

n.t. = not tested.

* 29/241 lymphocytes counted in thin sections (page 45). At ratios of 5:1 and 1:1, 100 lymphocytes were counted in each sample.

The morphology of the lymphocytes was the same regardless of whether they were free, or in contact with normal serum- (Figs. 20 & 21) or specific antiserum- (Figs. 22 & 23) treated chicken erythrocytes. Neither size, nucleo-cytoplasmic ratio, amount of rough endoplasmic reticulum, development of the Golgi zone, nor number of lysosomes correlated with adherence of lymphocytes to chicken red cells. Furthermore, the nucleus of attached lymphocytes showed no distinctive shape; as with the control effector cells (page 48, Table 4), the nucleus was round in 60% (68/122) and cleft or irregular in 40% (54/122). Chicken erythrocytes were attached to any part of the lymphocyte surface and not just to the area of plasma membrane overlying the Golgi region (Fig. 21).

All areas of contact between effector and target cells were carefully examined for evidence of junctional specialisation. No gap or tight junctions were ever observed but three other types of attachment, best described as point, flat and distorted, were found. In each type of contact the plasma membranes of the effector and target cells were always separated by a gap of at least 7.5-10 nm. Point contacts usually consisted of attachment between the tips of small projections from the effector cell surface and the chicken erythrocyte. The length of membrane apposition was 0.25 µm or less.

A second type of contact, formed when the surfaces of adjacent cells in the region of attachment became slightly flattened (Figs. 21 & 22), was described as flat. In flat contacts (Figs. 24 & 25) the membranes of apposing cells ran almost parallel for distances up to 3.5 µm and were separated by a gap of 10 nm which here and there was reduced to 7.5 nm. Electron-opaque material was seen both in the narrow intercellular space and associated with the outer surface of the plasma membranes away from areas of contact (Fig. 25).

Attachments were classified as distorted when the apposing cells became deformed and projections from one cell fitted into depressions in the surface of the other (Figs. 20 & 23). The length of such contacts varied considerably, and was occasionally up to 8 µm.

Results from a differential count of lymphocyte attachments are given in Table 6. Contacts between macrophages or neutrophils and chicken erythrocytes were almost always distorted and sometimes so extensive that it was difficult to decide whether the two cells were merely attached or whether phagocytosis was taking place.

Table 6. Types of contact observed between lymphocytes and chicken erythrocytes. Ratio of mouse spleen cells to CRBC 5:1

	Point contacts	Flat contacts	Distorted contacts
30 mins at 37°C, antiserum	36%	60%	4%
normal serum	39%	60%	1%
60 mins at 37°C, antiserum	21%	76%	3%
normal serum	25%	75%	0
60 mins at 4°C, antiserum	9%	80%	11%
normal serum	57%	42%	1%

Rat normal serum or antiserum 1:1,000.

Seventy to 100 contacts between effector and target cells were counted in thin sections of each sample (page 45).

In general, chicken red blood cells in contact with lymphocytes appeared normal (Figs. 20, 22 & 23). "Leakage" of chromatin from the nucleus was occasionally evident both with normal serum (Fig. 21) and antiserum (Fig. 23). In 2 instances, in antibody-treated samples, attached chicken erythrocytes were swollen with rarefied cytoplasm (Figs. 26 & 27). However erythrocyte ghosts were never seen in any sample, either free or attached to effector cells.

Phagocytosis

Ingestion of chicken red cells by neutrophils (Fig. 28) and macrophages (Fig. 29) occurred in the presence of antiserum but not with normal serum. A slight increase in phagocytosis was observed during the first hour of incubation (Table 7), but after this the number of engulfed chicken erythrocytes was unrelated to time.

Table 7. Attachment and phagocytosis of chicken erythrocytes.

Ratio of mouse spleen cells to CRBC 5:1

	CRBC attached to lymphocytes	CREC attached to other cells*	Phagocytosed CREC	Free CREC
15 mins, antiserum	48%	18%	2%	32%
normal serum	23%	1%	0	76%
30 mins, antiserum	48%	17%	4%	31%
normal serum	33%	11%	0	56%
60 mins, antiserum	46%	15%	5%	34%
normal serum	36%	5%	0	59%

Incubation at 37°C. Rat normal serum or antiserum 1:1,000.
One hundred chicken erythrocytes were counted in thin sections of each sample (page 45).

* Including phagocytic cells (neutrophils and macrophages) and platelets.

Changes in the cell ratio, however, had a marked effect on ingestion. For example, after 1 hour at an effector:target cell ratio of 5:1, 5% (5/100) of chicken red blood cells had been phagocytosed, whereas at a 50:1 ratio the proportion was 24% (17/71). Numerous 5-7 nm microfilaments were seen in those regions of the cytoplasm of neutrophils and macrophages involved in phagocytosis (Figs. 28 & 29). On one occasion a neutrophil containing a swollen chicken red blood cell was observed (Fig. 30).

3.3.3. Discussion

a. Target cell death

The pattern of ^{51}Cr release from chicken erythrocytes during antibody-dependent cell-mediated cytotoxicity is well established (for example,

Biberfeld & Perlmann, 1970; Calder et al., 1974). After an initial period of rapid lysis the rate of killing decreases and maximum target cell death usually occurs after 12-24 hours incubation. In the present investigation cytotoxicity assays were always performed in parallel with fine structural studies; only if ^{51}Cr release followed the normal pattern, and the percentage cytotoxicity after 20 hours was within the range expected for the cell ratio used, were the samples examined by electron microscopy. The release of ^{51}Cr from antibody- and normal serum-treated chicken erythrocytes was comparable to that obtained in other ultrastructural studies (Biberfeld & Perlmann, 1970; Fontana et al., 1975; Penfold et al., 1976).

Because approximately 50% of the target cells were lysed in the first 4 hours of incubation it was considered that, if contact was associated with cytotoxicity, the maximum number of attachments would be observed during this period. Samples for morphological examination were therefore incubated from 15 minutes to 6 hours.

Mouse erythrocytes were used as filler cells (page 28) in the cytotoxicity assays. However, since excess mouse red cells could have obscured effector-target cell interactions and their absence did not substantially alter the release of ^{51}Cr during short incubation periods (Table 2), fillers were omitted from samples for electron microscopy.

In agreement with other investigators (for example Greenberg et al., 1975) changes in effector:target cell ratios had a marked effect on the proportion of chicken erythrocytes lysed and ratios of 50:1, 25:1, 5:1 and 1:1 were examined in morphological studies. However at a ratio of 50:1, when killing was most efficient, there were very few contacts whilst at 1:1 the percentage cytotoxicity was low despite the formation of numerous contacts. Ratios of 25:1 and 5:1 were therefore considered most satisfactory for observing effector-target cell interactions.

b. Morphology

Effector cells

There is general agreement that most of the cells in mouse spleen suspensions are small and medium-sized lymphocytes (Waldo & Zucker-Franklin, 1972; Greenberg et al., 1973b; Catanzaro et al., 1974; Resch et al., 1974). However, reports of the number of macrophages and neutrophils present vary from 10% (Catanzaro et al., 1974) to 15-35% (Greenberg et al., 1973b).

The proportion of phagocytic cells found here was approximately 5% but the number of macrophages may have been underestimated because a purely morphological basis of identification was adopted. Difficulty was occasionally experienced in distinguishing between macrophages and large lymphocytes when only a small portion of the cell was visible, and differentiation often rested on the recognition of ingested material. Similar problems were encountered by Zucker-Franklin (1974) who used a functional approach, namely phagocytosis of latex particles to demonstrate that the percentage of monocytes in normal human peripheral blood was at least twice as high as is commonly recognised. Furthermore, some of the macrophages may have become attached to the plastic container during the initial 15 minute period allowed for the settling of large cell clumps (page 44). Greenberg and co-workers (1973b) reported a marked reduction in the proportion of phagocytes in spleen cell suspensions after as little as 5 minutes incubation with glass or plastic beads. To reduce further loss of adherent cells, therefore, cell mixtures for electron microscopy were agitated during incubations of 1 hour or more.

The clumps of lymphocytes (Fig. 18) and mixed cells (Fig. 19) in spleen suspensions presumably represented undissociated groups as they had existed in the Malpighian corpuscles and red pulp. The intercellular space was of the order of 15-20 nm and the extremely uniform spacing of

the apposed membranes suggested the operation of cohesive forces, either related to the properties of the membranes themselves or perhaps to the cell surface mucopolysaccharide. Thus these contacts resembled intermediate junctions or zonulae adherentes of epithelia (Farquhar & Palade, 1963) except that there was no condensed fibrillar material in the subjacent cytoplasm. Intermediate junctions, which provide a means of mechanical attachment between cells (McNutt & Weinstein, 1973), have been reported in vitro both between like cells (Armstrong, 1970; Johnson & Sheridan, 1971) and in heterotypic cell aggregates (Armstrong, 1970).

Lymphocytes have conventionally been divided according to size into small, medium and large (page 3). Substantial interest in their ultrastructure has been generated by the recognition of two major functionally distinct populations: thymus-derived (T) and bursa or bone marrow-derived (B) lymphocytes. Although there is a morphological spectrum, small lymphocytes by their dense nuclei, predominantly free ribosomes and few profiles of rough endoplasmic reticulum appear metabolically inactive or resting, whereas large lymphocytes appear more active having more abundant cytoplasm, paler nuclei, more rough endoplasmic reticulum and polyribosomes (Zucker-Franklin, 1969). Since Waldo and Zucker-Franklin (1972) always observed a few small cells with an "active" appearance, they suggested that a more appropriate classification was by the amount of rough endoplasmic reticulum. Even then, however, it was found that morphologically similar cells were not functionally the same (Waldo & Zucker-Franklin, 1972). Other attempts to define morphological differences between functional classes of lymphocytes on the basis of their ribosomes (Matter et al., 1972), nuclear bodies (Suter et al., 1972) and nucleoli (Stobo, Rosenthal & Paul, 1973; Catanzaro et al., 1974) have been equally unconvincing.

It is generally agreed that T and B lymphocytes have the same ultrastructural characteristics (Bessis, 1973; Janossy et al., 1973; Catovsky, Frisch & Van Noorden, 1975; Bloom & Fawcett, 1975); complement receptor and non-complement receptor lymphocytes are morphologically indistinguishable (Douglas & Huber, 1972; Chen et al., 1972); and the ultrastructure of cells which possess surface immunoglobulin is as variable as those which do not (Zucker-Franklin & Berney, 1972; Perkins, Karnovsky & Unanue, 1972; Waldo & Zucker-Franklin, 1972).

Because by light microscopy a nuclear cleft was considered a distinctive feature of the cells responsible for antibody-dependent cell-mediated cytotoxicity (Allison, 1972) the ultrastructure of lymphocytes in spleen cell suspensions was examined from this aspect. When the cells were classified according to whether they had a round or an irregularly shaped nucleus it was found that each category contained a range of typical small and medium-sized lymphocytes with no distinguishing features other than their nuclear shape. Whether or not nuclear shape was significant from a functional standpoint is considered below.

Contacts

Types of effector cells attached to target cells

The criterion of contact between cells adopted here was that of Biberfeld and Johansson (1975) namely, 25 nm or less separation of the apposing cells. Lymphocytes, macrophages and neutrophils from mouse spleen all formed attachments with both normal serum- and antiserum-treated chicken erythrocytes.

Lymphocytes

It has been suggested that phagocytic cells may be responsible for the initial period of target cell lysis (Perlmann & Perlmann, 1970;

Gelfand et al., 1972) but lymphocytes are usually considered the most important, if not the only, effector cell (Biberfeld & Perlmann, 1970; Biberfeld et al., 1973). Many more antibody-coated chicken erythrocytes were attached to lymphocytes than to any other cell type (Table 7) and since these lymphocytes may have possessed K activity, attention was directed to their morphology, particularly their nuclei. However, the ratio of round to cleft or irregular nuclei was the same in attached as in control cells and no other distinctive features were found. Thus in this system, nuclear shape, or indeed any other criteria considered, did not provide a means of identifying possible K lymphocytes. Even the single lymphocyte found in contact with an obviously damaged target cell (Fig. 26) was in no way remarkable.

The cytotoxic lymphoid cell with "distinct morphological features" described by Penfold, Greenberg and Roitt (1976) is indistinguishable from a typical medium-sized lymphocyte in which the nucleolus is not visible; such cells were abundant in mouse spleen suspensions here but, at effector:target cell ratios of 50:1 or 25:1 when cytotoxicity was greatest, few were associated with target cells. Furthermore, although small and large lymphocytes were illustrated in contact with somewhat deformed chicken erythrocytes, they were regarded by the authors as non-cytotoxic despite the fact that target cell distortion was one of their criteria of a cytotoxic reaction (Penfold et al., 1976).

The importance of uropods

The present study does not support the contention (Biberfeld et al., 1973) that uropods are associated with antibody-dependent cell-mediated lysis. When cytotoxicity was high, uropod formation was rare and antiserum-treated chicken erythrocytes were found in contact with all parts of the lymphocyte surface, not just the area overlying the Golgi

zone (Fig. 21). The lack of uropods is almost certainly due to the fact that the cells were usually agitated during the relatively short incubation period, fixed in suspension and randomly sectioned. Using suspensions of fibroblasts as target cells Ax and his colleagues (1968) demonstrated by microcinematography that uropod formation was infrequent and perambulation of effector lymphocytes restricted to a single fibroblast unless a confluent sheet had formed. Since chicken red cells do not form sheets the development of uropods would not be expected and close contact probably developed after chance collisions between lymphocytes and target cells.

Macrophages and neutrophils

Chicken erythrocytes were also found attached to macrophages and neutrophils. The relatively low number of phagocytic cells in the spleen suspensions used is probably the sole reason for the scarcity of contacts with these cells compared with lymphocytes. Other recent ultrastructural investigations have also demonstrated that macrophages, polymorphs and lymphocytes from human peripheral blood (Inglis et al., 1975) and mouse spleen (Penfold et al., 1976) can all kill antibody-coated target cells, and that granulocytes and macrophages, when present in sufficiently large numbers, can account for 90% of the attachments (Penfold et al., 1976). Although Biberfeld and Perlmann (1970) regarded only lymphocytes as important, they observed erythrophagocytosis by occasional "lymphocytic cells" from suspensions of human lymphocytes estimated to be 98-99.9% pure. Phagocytosis is not a function of lymphocytes and because it is not always easy to distinguish morphologically between lymphocytes and monocytes (Zucker-Franklin, 1974), the total number of the latter and their cytotoxic role may well have been underestimated by Biberfeld and Perlmann (1970). Furthermore, Biberfeld and

co-workers (1975) subsequently observed that lymphocytes, granulocytes and macrophages were all involved in plaque formation even when purified lymphocyte preparations were used, and that plaques also occurred with monocyte-rich suspensions.

In conclusion, if K cell activity is defined as cell-mediated cytotoxicity dependent on antibody at concentrations that do not induce complement lysis, then monocytes and neutrophils, in addition to lymphocytes, must be considered potential K cells.

Types of contact and their significance

Morphological evidence that antibody-coated target cell death is triggered by contact with effector cells has been presented (Biberfeld & Perlmann, 1970; Biberfeld et al., 1973) and is further extended in the present study. The observation that many more contacts were formed in the presence of antiserum than normal serum (Tables 5 & 7) accords with the findings of other investigators (Inglis et al., 1975; Yust et al., 1975). However, the decrease seen in target cell lysis despite the increased numbers of contacts when the proportion of target cells was raised (Table 5), indicates that many attachments were non-specific. Thus it is obvious that contact with an effector cell does not necessarily provoke lysis. There was no morphological distinction between functionally different types of contacts, which appeared identical in cytotoxic (Figs. 22 & 23) and non-cytotoxic systems (Figs. 20 & 21).

Despite diligent searching, no desmosomes, tight or gap junctions between effector and target cells were found in this investigation. In all instances when the plane of section was perpendicular to the contact and the trilaminar structure of both plasma membranes apparent, a relatively constant intercellular gap was observed (Figs. 24 & 25). Such attachments, like those between control spleen cells (Figs. 18 & 19),

could only be considered a form of intermediate or adhesive juction. Although it could be argued that contacts seen in tangential section may have been regions of fusion or junction formation, all such areas that were examined after tilting the specimen stage in the electron microscope showed complete and separate apposing plasma membranes (Biberfeld & Johansson, 1975).

The contacts observed in this study between lymphocytes and chicken erythrocytes are the same as those found in all "immunologically significant" systems, namely point, flat and distorted. Although at 37°C flat attachments were most frequent, no one type of contact was definitely related to cytotoxicity since all occurred in similar proportions whether antiserum or normal serum was used (Table 6). Biberfeld and Johansson (1975) also observed more broad than point contacts between lymphocytes and antibody-coated target cells, but this work and previous studies from the same group (Biberfeld & Perlmann, 1970; Biberfeld et al., 1973) can all be criticised for failing to examine normal serum-treated controls by electron microscopy.

The predominance of flat or point contacts between chicken erythrocytes and mouse lymphocytes (Table 6) is at variance with the observations of Fontana and his colleagues (1975). These authors found that interdigitating contacts in a human lymphocyte-chicken erythrocyte system formed 30% of the attachments present after 2 hours incubation, when ^{51}Cr release was 17%. Possibly there is a species-related difference in the way lymphocytes react with antiserum-treated target cells but it is notable that Biberfeld and co-workers (1970; 1973) failed to report distorted contacts with human effector lymphocytes. A more likely explanation for the apparent differences lies in the problems of obtaining pure effector cells and of distinguishing

morphologically between small portions of lymphocytes and monocytes as already discussed (page 13). It is probable that at least some of the interdigitating contacts observed by Fontana and his colleagues (1975) were between monocytes and target cells.

Although samples were examined during the period when 50% cytotoxicity occurred, distorted attachments between lymphocytes and target cells were rare and poorly developed (Figs. 20 & 23). Furthermore, in the only 2 contacts in which chicken erythrocytes were swollen (Figs. 26 & 27) the damage had apparently occurred with little surface distortion. Thus, although deformation was typical of interaction between phagocytes and target cells, it is clear that distorted contact with lymphocytes was not associated with red cell lysis.

Biberfeld and Perlmann (1970) suggested that only temporary contact is needed for a cytotoxic reaction and Koren, Ax and Freund-Moelbert (1973) observed that target cells were lysed after detachment from sensitised lymphocytes. It was possible that specific contacts, even junctional specialisation or cell fusion, might have developed but were no longer apparent once the cells had separated. An attempt was made to overcome this problem by adopting the technique devised by Morgan and his colleagues when investigating virus infection of cells in vitro. These workers (for example Morgan & Rose, 1968) found that attachment of the organism occurred at $4^{\circ}C$, but that cell penetration followed only when the temperature was raised to $37^{\circ}C$. By maintaining the specimens for 1 hour at $4^{\circ}C$ it was hoped that any specific attachments, that might be transient at $37^{\circ}C$, would be stable enough to be fixed and examined by electron microscopy. No target cell lysis occurred at $4^{\circ}C$, but the expected ^{51}Cr release followed when suspensions were subsequently incubated at $37^{\circ}C$. There was no

evidence of junction formation or membrane fusion at the lower temperature but a marked change in the distribution of contacts was apparent; considerably more flat contacts were found with antiserum than normal serum (Table 6). Thus flat attachments may well be associated with cytotoxicity.

Target cell damage

Leakage of chromatin from the nuclei of antibody-coated chicken erythrocytes attached to lymphocytes (Fig. 23) was initially considered a cytotoxic effect. However on further investigation "leaky" nuclei were found with equal frequency in both free and attached chicken red cells treated with normal serum (Fig. 21) so clearly breakdown of the nuclear envelope was not related to ^{51}Cr release. The appearance was not a fixation artefact since cells with damaged nuclei were otherwise well preserved and were usually surrounded by erythrocytes with intact nuclei. The most likely explanation is that such cells represent the older erythrocytes found in any normal circulating population and which might be more susceptible to damage during the ^{51}Cr labelling or some other part of the experimental procedure.

Chicken erythrocyte ghosts have been observed both free and attached to effector cells from human peripheral blood (Biberfeld & Perlmann, 1970) or mouse spleen (Penfold et al., 1976). However, in the present experiments control spleen suspensions always contained a variable number of damaged cells, making it impossible to identify genuine chicken erythrocyte ghosts with any degree of certainty in the "complete" cytotoxic mixture. Although damaged spleen cells might account for some of the "ghosts" observed by Penfold, Greenberg and Roitt (1976) after 1½ hours, the procedure adopted by Biberfeld and Perlmann (1970) for purifying human lymphocytes is unlikely to have

caused much damage to the effector cells and the demonstration of ghosts by these authors after 24 hours incubation is quite convincing.

Biberfeld and Perlmann (1970) also observed swollen chicken red cells both free and attached to effector cells but in the entire series of present experiments only 3 swollen, rarefied chicken erythrocytes were found. All 3 were in antiserum-treated samples and 2 were in contact with effector cells, 1 with a lymphocyte (Fig. 26), the other with a macrophage and a neutrophil (Fig. 27). Assuming that the target cell swelling was induced by the leucocytes to which they were attached, the appearance is consistent with the mechanism of extracellular osmotic lysis proposed by Biberfeld and Perlmann (1970); their rarity could optimistically be interpreted as the result either of extremely rapid lysis or of examining the samples after comparatively short incubation.

Phagocytosis

Phagocytosis by mouse neutrophils (Fig. 28) and macrophages (Fig. 29) occurred only in the presence of antiserum. Other fine structural investigations have demonstrated phagocytosis of antiserum-treated erythrocytes by human monocytes and "lymphocytic cells" (also probably monocytes, page 59) (Biberfeld & Perlmann, 1970), human polymorphs (Inglis et al., 1975) and mouse granulocytes and monocytes (Penfold et al., 1976).

In the present study the marked increase in the proportion of chicken red cells ingested when the ratio of effector to target cells was raised from 5:1 to 50:1 clearly reflects the number of macrophages and neutrophils present. Lysis also increased at the higher ratio and phagocytosis could easily have accounted for all the target cell death in the first hour of incubation. However, with longer periods there was no correlation between cytotoxicity and phagocytosis and ingestion

was probably not the only method of killing involved. From ultrastructural studies it has been proposed that polymorphs and macrophages can induce extracellular lysis of target cells (Inglis et al., 1975; Penfold et al., 1976) and such a mechanism might also operate here. In general, phagocytosed chicken erythrocytes became condensed and shrunken, then paler as their haemoglobin was extracted during digestion (Figs. 15 & 17). However, the ingested red cell illustrated in Figure 30 more closely resembles the swollen erythrocytes found attached to effector cells (Figs. 26 & 27), suggesting that this particular target cell had been damaged by contact prior to phagocytosis.

Functional studies have not established whether target cells are killed by phagocytosis or by an extracellular mechanism, and there have been a number of indications that more than one type of effector cell may be involved (Sanderson, Clark & Taylor, 1975; Perlmann et al., 1975; Papamichail & Temple, 1975; MacDonald et al., 1975; Greenberg et al., 1975). Resch, Gelfand and Prester (1974) demonstrated that phagocytes were responsible for much of the observed cytotoxicity, and the role of monocytes or macrophages and polymorphs has frequently been emphasised (Perlmann et al., 1975; Papamichail & Temple, 1975; MacDonald et al., 1975; Greenberg et al., 1975; Kovithavongs et al., 1975). Morphological investigations have so far failed to clarify the situation. Biberfeld and Perlmann (1970) regarded phagocytosis as unimportant. This assertion was unwise when their effector cell suspensions contained one to "a few per cent" of granulocytes and monocytes (Biberfeld & Perlmann, 1970; Biberfeld et al., 1975), and the same authors have calculated that as few as 1.5% of the effector cells actually possess K activity (Biberfeld et al., 1975). Indeed other morphological studies have indicated that both phagocytosis and extracellular killing by monocytes and polymorphs occurs (Inglis et al., 1975; Penfold et al., 1976).

3.3.4. Conclusions

In summary, lymphocytes, macrophages and neutrophils from mouse spleen were all found in contact with antiserum-treated chicken erythrocytes. Attachment was not, however, entirely antibody-dependent, occurring to a lesser extent in the presence of normal serum, and the contacts appeared the same whichever serum was used.

More significantly, phagocytosis of target cells by macrophages or neutrophils was only ever observed when antibody was present. The increase in cytotoxicity at high effector:target cell ratios resulted mainly from increased phagocytosis, but extracellular target cell lysis probably also occurred. However, the relative importance of these two mechanisms could not be determined in this or indeed any other reported fine structural study. Furthermore, it is still not clear whether extracellular lysis results from contact with lymphocytes, phagocytes or both.

It must be concluded that, although some lymphocytes may possess K activity, even a few phagocytic cells in the effector suspension can account for much of the cytotoxicity. To further investigate the role of lymphocytes in antibody-dependent cell-mediated cytotoxicity it was proposed that highly purified cell suspensions, from which cells with phagocytic potential had been eliminated, should be used as effectors.

3.4. THE CYTOTOXIC ACTION OF PURIFIED HUMAN PERIPHERAL BLOOD LYMPHOCYTES ON CRYPTOCOCCUS NEOFORMANS

3.4.1. Introduction

Cryptococcus neoformans, one of the Deuteromycetes or Fungi Imperfecti, is widespread in nature. The organism causes sporadic disease in man, inhalation being the most common mode of infection. The primary lesion is pulmonary but blood-borne dissemination may occur.

Meningeal involvement usually results in a slow, irregularly progressive meningoencephalitis, which is fatal when untreated (Cruickshank et al., 1973).

Although host defence mechanisms are incompletely understood, cellular reactions are considered more important than humoral responses (Abrahams, 1966; Abrahams et al., 1970; Gentry & Remington, 1971). C. neoformans is phagocytosed by human cells both in vitro (Bulmer & Sans, 1967; Cline & Lehrer, 1968) and in vivo (Emmons, Binford & Utz, 1970; Collins, Oppenheim & Edwards, 1971). Ingestion, and intracellular killing of the organism, by neutrophils or monocytes does not occur in the absence of serum or with 1% normal serum, but is maximal with either 10% normal serum or antiserum (Diamond, Root & Bennett, 1972).

There is no apparent cellular immune defect in patients with cryptococcosis (Diamond et al., 1972). The 40% of patients who produce antibody probably have a better chance of complete cure with chemotherapy than those who do not (Bindschadler & Bennett, 1968). Anti-cryptococcal antibody alone does not possess fungicidal activity (Diamond et al., 1972) but in the presence of extremely low concentrations of specific antiserum mononuclear cells can kill the organism by a non-phagocytic mechanism (Diamond, 1974). It was proposed that this mechanism is analogous to antibody-dependent cell-mediated lysis of erythrocytes or other target cells but, because of the heterogeneous nature of the mononuclear cell population, a specific effector cell could not be identified (Diamond, 1974).

Whilst visiting the Division of Cell Pathology, R.D. Diamond demonstrated that highly purified human peripheral blood lymphocytes possessed antibody-dependent anti-cryptococcal activity. It was considered that examination of this system by electron microscopy

might reveal the identity of the effector cell and demonstrate the mechanism of cytotoxicity.

3.4.2. Materials and methods

Effector cells, target cells and sera were prepared by R.D. Diamond who also measured the cytotoxicity.

a. Effector cell preparation

Human peripheral blood was defibrinated by shaking with glass beads. After incubation with carbonyl iron powder for 30 minutes at 37°C the phagocytic cells, which had ingested or adhered to iron particles, were sedimented by a strong magnet placed underneath the vessel (Lundgren, Zukoski & Möller, 1968). Lymphocytes were separated from the suspension by centrifugation through a mixture of Hypaque and Ficoll (Böyum, 1968). The proportion of monocytes contaminating the final preparation was monitored by light microscopy after neutral red treatment.

b. Target cells and estimation of target cell death

A small capsule isolate of C. neoformans was cultured for 72 hours on Sabouraud's agar and washed as described by Diamond (1974). The number of live organisms was estimated by plate counts (Diamond et al., 1972) and the cells killed during the incubation period expressed as a percentage of the original inoculum.

c. Serum

Control serum was obtained from a rabbit and anti-cryptococcal antibody, at an agglutination titre of 1:1024, was raised in the same animal. Both control serum and antiserum were inactivated at 56°C for 30 minutes.

d. Experimental procedure

Cytotoxicity experiments

Purified human lymphocytes and cryptococci were mixed in a ratio of 50:1. Medium consisted of rabbit normal serum or antiserum diluted 1:4,000 in Eagle's minimal essential medium (MEM) enriched as outlined by Simon and Sheagren (1971). Cells were incubated at 37°C for 4 hours, after which the percentage survival of the original inoculum was estimated.

Samples for electron microscopy

Identical samples were fixed in suspension for electron microscopy (page 20). Because of the relatively small size and low numbers of cryptococci, the procedure adopted previously for differential cell counts (page 34) was slightly modified. All the sections from each block were scanned and the number of cryptococci visible in each section then determined. Since some target cells were always obscured by grid bars, and their exclusion would have dramatically altered the counts, the section with the most cryptococci was selected. The average number of leucocytes per grid square was multiplied by the number of squares in that section to give the approximate total of leucocytes. The relative proportions of leucocytes and cryptococci per sample were obtained by adding the counts from several blocks. The entire experiment was repeated and the counts obtained from each were pooled.

3.4.3. Results and observations

a. Target cell death

After 4 hours incubation of C. neoformans with purified lymphocytes and antiserum approximately 60% of the cryptococci had been killed. This fungicidal activity occurred only in the presence of antibody;

after incubation with lymphocytes and normal serum there were more viable cryptococci than in the original inoculum.

b. Morphology

Effector cells

Ninety-nine per cent of the purified effector preparation were small and medium-sized lymphocytes (average diameter 6.6 μm). Only occasional monocytes, neutrophils and plasma cells were seen. Uropod formation by lymphocytes was observed, but very infrequently.

Target cells

The small capsule isolate of C. neoformans appeared round or oval in section, with an average diameter of 3.6 μm. The preservation was variable but in general the ultrastructure (Fig. 31) was similar to that described by other investigators (Edwards et al., 1967; Al-Doory, 1971; Dembitzer, Buza & Reiss, 1972). A plasma membrane surrounded the dense cytoplasm which contained numerous free ribosomes, mitochondria, lipid droplets, some smooth endoplasmic reticulum and glycogen particles. The nuclear envelope was formed by two well defined membranes with occasional pores; chromatin was dispersed and a nucleolus was often observed (Fig. 31).

The cell envelope, which varied in width from 0.25 μm to 0.45 μm consisted of a cell wall and capsule (Figs. 31 & 32). The cell wall, which was generally electron-opaque and either amorphous or composed of multiple fibrous layers, was closely applied to the plasma membrane. A clear zone frequently separated the cell wall from a spongy, fibrillar capsule (Figs. 31 & 32).

The appearance of the organism, in particular the capsule, was the same in control, normal serum- and antiserum-treated cryptococci.

Contacts

Similar numbers of cryptococci were found in contact with lymphocytes whether normal serum or antiserum was present (Table 8). Attachments were usually by lymphocyte projections which indented, but never penetrated, the capsule (Figs. 32, 33 & 34). Contacts were not restricted to the region of the uropod or opposite the Golgi zone, but occurred at any point of the lymphocyte surface. Antibody made no difference to the type of attachment.

Table 8. Attachment and phagocytosis of C. neoformans.
Ratio of purified human peripheral blood leucocytes to cryptococci 50:1

	Estimated number of leucocytes	Total number of cryptococci	Phagocytosed cryptococci	Cryptococci attached to lymphocytes	Free cryptococci
Normal serum	8600	109	1 (1%)	25 (23%)	83 (76%)
Anti-serum	6400	62	40 (65%)	12 (19%)	10 (16%)

Incubation for 4 hours at 37°C. Rabbit normal serum or antiserum 1:4,000. Cells counted in thin sections (page 69).

Assessment of damage to the target cells was hindered by the variable quality of their preservation, but there was no apparent morphological difference between normal serum- and antibody-treated cryptococci attached to lymphocytes.

In a single instance, in an immune serum-treated sample, a free misshapen cryptococcus was seen near a lymphocyte (Fig. 35).

Phagocytosis

Only one example of phagocytosis of a cryptococcus was seen when normal serum was present (Fig. 36), but with antiserum most of the target cells were engulfed (Table 8), by macrophages (Figs. 35 & 37) rather than neutrophils. Extracellular cryptococci usually remained round or oval even when preservation was poor (Fig. 36) whereas ingested organisms were generally distorted and crumpled (Figs. 35 & 37). Microfilaments were prominent in the region of phagocytic cell cytoplasm actively involved in engulfing cryptococci (Fig. 36) but not usually around formed phagocytic vacuoles (Figs. 35 & 37).

3.4.4. Discussion

a. Target cell death

Antibody-dependent killing of C. neoformans by purified human lymphocytes was rapid, only 40% of the organisms surviving after 4 hours. Because of the problems introduced by multiplication of the cryptococci, it was not possible to increase the amount of killing by extending the incubation period.

b. Morphology

Effector cells

Purification by a combination of Ficoll-Hypaque and iron-magnet separation yielded a 99% pure lymphocyte suspension. Although based solely on fine structure, this estimation correlates well with that of Zucker-Franklin (1974) who found that 0-1.5% of the cells remaining after similar treatment were capable of phagocytosing latex particles.

Target cells

Lymphocytes and cryptococci were incubated in a ratio of 50:1 but the effector to target cell ratio observed in thin sections was nearer 100:1 (Table 8). This was not unexpected since the average diameter of the effector lymphocytes was almost twice that of the cryptococci.

Contacts

It had been anticipated that, if non-phagocytic killing of cryptococci was analogous to antibody-dependent cell-mediated cytotoxicity in other systems (Diamond, 1974), close contact between lymphocyte and cryptococcal plasma membranes would be observed in the presence of antiserum. However contacts were non-specific and approximately equal numbers of lymphocytes were found attached to normal serum- and antibody-treated cryptococci (Table 8). Furthermore, the attachments were identical and lymphocyte projections were never seen penetrating the cryptococcal envelope even when antiserum was present (Figs. 32, 33 & 34). Similar results were obtained by Aronson and Kletter (1973) who observed pseudopodia from monocytes and polymorphs penetrating the capsule but not the cell wall during extracellular killing of C. neoformans.

Cryptococci, like chicken erythrocytes (page 50) were attached to any region of the lymphocyte surface. If lymphocytes were indeed responsible for extracellular lysis of cryptococci, the plasma membrane overlying the Golgi region or uropod was not in any way specialised (pages 15 & 58).

Even when inadequate fixation resulted in poor preservation of their subcellular structure, cryptococci remained round or oval (Fig. 36). Collapse of phagocytosed organisms was therefore the most

useful indication of damage (Figs. 35 & 37). By this criterion, the presence of only a single misshapen antibody-treated cryptococcus near a lymphocyte implied that extracellular lysis was extremely rare in this system.

Phagocytosis

Phagocytosis of cryptococci, unlike their attachment to lymphocytes, was specific and with one exception occurred only in the presence of immune serum (Table 8). Walters, Papadimitriou and Robertson (1976) have recently shown by scanning electron microscopy that even untreated cryptococci can attach to macrophages, so it is not unreasonable that normal serum-treated organisms should occasionally have been ingested.

Similar numbers of cryptococci are phagocytosed in 10% normal serum and antiserum (Diamond et al., 1972) but in the present study, with serum concentrations of 1:4,000, there was a striking difference between the two (Table 8). Since only 40% of antibody-treated organisms survived after 4 hours incubation with effector cells, it is obvious that phagocytosis can account for all the observed cytotoxicity.

Aronson and Kletter (1973) reported extracellular killing of a large capsule isolate of C. neoformans by rabbit monocytes which they considered equivalent to phagocytosis of smaller organisms. These authors suggested that naturally occurring, heat-labile opsonins in rabbit serum were necessary to overcome the slippery nature of the cryptococcal capsule and thus allow attachment of effector cells. In the present study, however, since sera were heat-inactivated to destroy the action of such opsonins (possibly complement components), phagocytosis was clearly induced by small amounts of specific antibody.

The significance of microfilaments in the region of effector cell cytoplasm actively involved in phagocytosis (Fig. 36) has already been

discussed (page 41). Such thin filaments were not usually observed around deeply situated phagocytic vacuoles (Figs. 35 & 37) and it is possible that they are responsible not only for formation of phagocytic vacuoles but also their transport from peripheral to central regions of the cytoplasm. Similar observations, during the phagocytosis of streptococci, were made by Glick, Getnick and Cole (1971).

On the assumption that cells which had not ingested iron particles during purification would remain incapable of phagocytosis, Greenberg, Shen and Roitt (1973b) suggested that a non-phagocytic monocyte might be the effector cell in antibody-dependent cell-mediated cytotoxicity. However the cells removed by the magnet probably have iron particles adsorbed to their surface rather than in phagocytic vacuoles (Zucker-Franklin, 1974) and it is clear that monocytes and neutrophils still present after purification are capable of ingesting antibody-coated target cells. A non-phagocytic monocyte, therefore, seems an unlikely candidate.

The relevance of these observations to <u>in vivo</u> conditions has not yet been established, but it would appear from this study that very small amounts of specific antibody are effective in stimulating phagocytosis which might be associated with eventual cure in patients who develop anti-cryptococcal antibody.

3.4.5. Conclusions

Contacts between human lymphocytes and cryptococci were not antibody-dependent and occurred with similar frequency in samples containing normal serum.

There was very little morphological evidence of extracellular lysis of antibody-coated cryptococci by lymphocytes, and if such a mechanism exists it is of minor importance. However, phagocytosis

of immune serum-treated cryptococci easily accounted for all the target cell killing observed after 4 hours incubation, even with a lymphocyte suspension containing only 1% monocytes and neutrophils.

Although it is theoretically possible that antiserum-treated cryptococci were altered in some morphologically indetectable way through contact with lymphocytes prior to ingestion, it is much more likely that phagocytosis was induced by specific antibody.

Since such a small number of phagocytic cells can be responsible for considerable target cell killing, it is extremely important that in all investigations of antibody-dependent cell-mediated cytotoxicity the effector cell population should be examined thoroughly, preferably by functional as well as morphological methods, to accurately assess the number of phagocytic cells and their role.

4. PHA-INDUCED CELL-MEDIATED CYTOTOXICITY

4.1. INTRODUCTION

4.1.1. Functional and morphological aspects of PHA-induced cell-mediated cytotoxicity

There are several *in vitro* systems in which lymphocytes are capable of specific cytotoxic killing without the addition of antibody, for example spleen cells from appropriately immunised mice will kill L cells (Able *et al.*, 1970), DBA/2 mastocytoma cells (Sellin *et al.*, 1971; Koren *et al.*, 1973) and YAC tumour cells (Firket & Degiovanni, 1975). Experiments with "educated" thymus cells (Golstein *et al.*, 1972) and cortisone-resistant thymus cells (Wagner, Harris & Feldmann, 1972) suggest that the effector cell is thymus derived.

T cells may also show non-specific cytotoxicity; non-sensitised lymphocytes, in the presence of phytohaemagglutinin (PHA), will kill Chang cells (Holm & Perlmann, 1967 a & b; Biberfeld *et al.*, 1968; Biberfeld, 1971a), mouse fibroblasts (Ax *et al.*, 1968) and chicken erythrocytes (Perlmann *et al.*, 1968; Tonietti *et al.*, 1975b). In addition Asherson, Ferluga and Janossy (1973) demonstrated PHA-induced killing of DBA/2 mastocytoma cells by concanavalin A (Con A)-stimulated T lymphoblasts. Target cells were efficiently killed only when PHA or other T cell mitogens were present and non-mitogenic leucoagglutinins such as axinella had a variable and usually small effect (Asherson *et al.*, 1973).

As with antibody-dependent cell-mediated lysis, both close contact between cells (Holm & Perlmann, 1967a; Biberfeld *et al.*, 1968; Ax *et al.*, 1968; Biberfeld, 1971a; Tonietti *et al.*, 1975b) and effector uropod formation (Biberfeld *et al.*, 1968; Biberfeld, 1971a) have been considered important in PHA cytotoxicity. Lymphocytes are electrically coupled within minutes of being agglutinated by PHA (Hülser & Peters, 1972) and, although these cells are usually separated by 10–25 nm

(Douglas, 1972; Biberfeld & Johansson, 1975), gap junctions or nexuses have been found (Gaziri et al., 1975). Johnson, Herman and Preus (1973) showed that gap junctions can develop between unlike cells, and such structures are associated with intercellular dye transfer (page 17). The passage of fluorescein between normal fibroblasts (Furshpan & Potter, 1968) and between hepatoma cells (Johnson & Sheridan, 1971) takes 1-3 minutes, and has been demonstrated in the course of target cell killing by sensitised lymphocytes (Sellin et al., 1971).

4.1.2. Selection of the experimental system

In addition to cytotoxicity measured by ^{51}Cr release (J. Ferluga), A.C. Allison observed PHA-induced passage of fluorescein from mastocytoma cells to Con A-treated T lymphoblasts. However, when the cells were agglutinated by axinella there was little target cell death or fluorescein transfer. The formation of gap junctions between PHA-treated effector and target cells could have accounted for both the high cytotoxicity and the number of dye permeable contacts, and it was considered that any junctions which might be formed would be detectable by electron microscopy.

Furthermore, although lysis was PHA- rather than antibody-dependent, T cells stimulated with Con A would provide an excellent source of phagocyte-free effectors for the further study of lymphocyte-mediated cytotoxicity.

4.2. MATERIALS AND METHODS

4.2.1. Effector cell preparation

Cortisone-resistant thymus cells were prepared by G. Janossy (Janossy et al., 1973). The cells were incubated with 3 µg/ml Con A (the optimal mitogenic dose) for 3 days at 37°C, at which time 70% were large lymphoblasts.

4.2.2. Target cells and estimation of target cell death

Cytotoxicity was assayed by J. Ferluga. Mastocytoma cells (P815), maintained in ascites form in DBA/2 mice, were labelled with $Na_2\ ^{51}Cr\ O_4$ (Asherson et al., 1973). Chromium release from the target cells was measured and, since the spontaneous release of ^{51}Cr from untreated mastocytoma cells in 16 hours was 25-30%, the amount of target cell lysis was calculated thus:

$$\frac{^{51}Cr\ (exp) - {}^{51}Cr\ (sp)}{^{51}Cr\ (max) - {}^{51}Cr\ (sp)} \times 100$$

where ^{51}Cr (exp) = chromium release in the experimental tube

^{51}Cr (sp) = spontaneous chromium release from mastocytoma cells without lymphoblasts

^{51}Cr (max) = the total amount of chromium released after freezing and thawing.

4.2.3. Agglutinins (PHA and axinella)

Dr M. Crumpton kindly provided the purified preparation from Axinella polypoides, the 50% agglutination dose of which was 13 µg/ml. The 50% agglutination dose of PHA (from Wellcome Research Laboratory, Beckenham, Kent) was 5 µg/ml (Asherson et al., 1973).

4.2.4. Experimental procedure

a. Cytotoxicity experiments (J. Ferluga)

Lymphoblasts and mastocytoma cells in ratios of 5:1, 2.5:1 or 1:1 were incubated in Eagle's MEM + 10% FCS, or in medium containing 2 µg/ml PHA or 15 µg/ml axinella for up to 16 hours at 37°C. Chromium release from the target cells was measured in duplicate incubations after various time intervals and the percentage target cell lysis calculated.

b. Fluorescein transfer

The passage of fluorescein from mastocytoma cells to lymphoblasts was studied by A.C. Allison. Target cells were labelled with fluorescein dibutyrate essentially as described by Rotman and Papermaster (1966), then incubated for 1 hour at 37°C with lymphoblasts (1:1) in medium, or in medium containing 3 µg/ml PHA or 20 µg/ml axinella. After lysis of tumour cells by the addition of anti-mastocytoma cell serum and complement, the relative number of dye-containing T lymphoblasts and hence the percentage of contacts permeable to fluorescein was determined.

c. Samples for electron microscopy

T lymphoblasts and mastocytoma cells were incubated, ratios 2:1 and 1:1, in medium with 3 µg/ml PHA or 20 µg/ml axinella for 1, 3, 5 and 6 hours at 37°C. The cells were then fixed in suspension in a mixture of glutaraldehyde and osmium tetroxide and processed for electron microscopy as already described (page 21).

4.3. RESULTS AND OBSERVATIONS

4.3.1. Target cell death

Chromium release from mastocytoma cells in the presence of PHA followed a similar pattern to that observed during antibody-dependent cell-mediated lysis of chicken erythrocytes (page 47) with an initial period of rapid killing. At an effector to target cell ratio of 1:1 5% of mastocytoma cells were lysed within 1½ hours and 50% by 6 hours; compared with 14% and 75% respectively when the ratio was increased to 2.5:1. At all ratios examined, 90-100% of the target cells were killed in 16 hours.

The axinella preparation used for fluorescence and electron microscopy induced comparatively little target cell death (Asherson et al., 1973) and 11% of the mastocytoma cells were killed in 16 hours

when the ratio was 5:1. At the same effector:target cell ratio, in the absence of PHA or axinella, there was no agglutination and 5% of the target cells were lysed.

4.3.2. Fluorescein transfer

The number of contacts between mastocytoma cells and lymphoblasts which were fluorescein permeable in the presence of PHA was 8%; with axinella 3%; and less than 2% when the cells were incubated in medium without agglutinins.

4.3.3. Morphology

a. Effector cells

Thymus cells stimulated for 3 days with Con A consisted mainly of large lymphoblasts (Fig. 38) with some medium-sized cells (average diameter 10 µm, range 5-15 µm). The preservation, apart from glycogen, was good and the cells displayed the fine structure described in detail by others (Biberfeld, 1971b; Janossy et al., 1973). Nuclear chromatin was predominantly dispersed and there were abundant polyribosomes and mitochondria in the cytoplasm. The Golgi region was well developed and aggregates of glycogen particles were often large. Variable numbers of lysosomes, cisternae of rough endoplasmic reticulum, vesicles and multivesicular bodies were present (Fig. 38).

b. Target cells

In general, mastocytoma cells (Fig. 39) were larger than lymphoblasts, having an average diameter of 14 µm (range 10-25 µm). Nuclei were pale with only small amounts of dense chromatin. The cytoplasm contained many large pleomorphic mitochondria, cisternae of rough endoplasmic reticulum, and free ribosomes which occurred singly or in clusters. A large Golgi area, lipid droplets and numerous vesicles and vacuoles

were also present (Fig. 39). A high proportion of the mastocytoma cells contained both intracytoplasmic and intracisternal A type virus particles (Fig. 39). Intracytoplasmic A particles could be seen budding into vacuoles (Fig. 40), and extracellular B type virus particles with eccentric nucleoids (Fig. 41) were also observed.

c. Contacts

In the presence of PHA, mixed agglutination of lymphoblasts and mastocytoma cells occurred (Fig. 42). Contact between the 2 cell types was usually over extensive flattened or slightly undulating areas (Fig. 42) but not by complex interdigitations. Some effector cells developed uropods, but mastocytoma cells were found attached to all parts of the lymphoblast surface. The plasma membranes of apposing cells ran parallel, sometimes for several micrometres, and a variable amount of amorphous material was present in the 7.5-10 nm intercellular space (Figs. 43 & 44); gap junctions were not observed but at their closest points the membranes were only approximately 5 nm apart.

Although agglutination was predominantly mixed when axinella replaced PHA, clumps consisting of only mastocytoma cells were also found (Fig. 45). Most of the axinella-induced contacts between effector and target cells were indistinguishable from those produced by PHA but 10-20 nm usually separated adjacent mastocytoma cells (Fig. 46). Regardless of the length of incubation, gap junctions were observed in approximately 1% of the axinella-induced contacts between lymphoblasts and mastocytoma cells (Figs. 47 & 48), and between mastocytoma cells. A space of 2.5-3 nm (Figs. 47b & 48b) separated the cells whose surfaces were frequently distorted; interdigitation varied from slight (Fig. 47) to complex (Fig. 48).

4.4. DISCUSSION

4.4.1. Target cell death

Con A-stimulated lymphoblasts were highly cytotoxic when incubated with PHA. The figure of 90-100% of mastocytoma cells killed in the present experiments is considerably greater than is usually obtained; for example 30-40% of Chang cells (Holm & Perlmann, 1967b) and up to 55% of chicken erythrocytes (Perlmann et al., 1968) are killed by normal human lymphocytes in the presence of PHA. The cytotoxicity induced by axinella, however, was similar to that obtained in the absence of PHA by these workers, namely 4% of Chang cells (Holm & Perlmann, 1967b) and approximately 14% of chicken erythrocytes (Perlmann et al., 1968).

Unlike antibody-dependent cell-mediated cytotoxicity (pages 47 & 54), lymphoblasts were effective in low proportions and so ratios of 2:1 and 1:1 were chosen to give the maximum number of contacts for fluorescence and electron microscopy. Since the pattern of ^{51}Cr release was similar to that observed during antibody-dependent killing of chicken erythrocytes incubation times of 1-6 hours were considered suitable for fine structural studies.

4.4.2. Fluorescein transfer

The number of fluorescein permeable contacts between lymphoblasts and mastocytoma cells after incubation with PHA for 1 hour was comparable to that observed by Sellin, Wallach and Fischer (1971) who reported dye movement between mastocytoma cells and sensitised spleen cells in 1%, 5% and 11% of the attachments formed by 1, 2 and 3 hours respectively. The percentage of contacts between target cells and normal spleen cells through which fluorescein passed varied from 0-2 (Sellin et al., 1971).

4.4.3. Morphology

a. Effector cells

Cortisone-resistant thymus cells are virtually pure preparations of T cells containing less than 1% B lymphocytes and macrophages (Janossy et al., 1973). Phagocytic cells were never observed in the effector suspension used and target cell lysis must have been due to the stimulated lymphoblasts.

The pale appearance of glycogen areas (Figs. 38 & 42) was attributed to extraction during treatment of the cells with uranyl acetate in veronal-acetate buffer prior to dehydration. Similar changes have been observed by other workers (Hirsch & Fedorko, 1968; Vye & Fischman, 1970). However, the clarity of the trilaminar configuration of plasma membranes at high magnification (Figs. 40, 41, 43, 44, 47b & 48b) obtained by the Hirsch and Fedorko (1968) fixation procedure more than compensated for such adverse effects.

b. Target cells

The classification of oncorna viruses into types A, B and C, devised by Bernhard and Guérin (1958), was reviewed by Dalton in 1972. The mastocytoma ascites tumour was chemically induced in DBA/2 mice (Dunn & Potter, 1957) and, since A type particles have been observed in tumours arising spontaneously in this strain (Brandes et al., 1966), their presence in mastocytoma cells was to be expected. A type particles proved particularly useful in identifying mastocytoma cells where only a small portion of the target cell was visible.

c. Contacts

Since cytotoxicity was low in the absence of agglutination, it is clear that contact between lymphoblasts and mastocytoma cells is essential for target cell lysis. All the PHA attachments observed were relatively

flat and resembled those described by Biberfeld and his colleagues (1968; 1971a; 1975). Although Tonietti and co-workers (1975b) suggested that close contact was non-specific and that complex interdigitation between effector and target cells was necessary, it is worth noting that the effector population used by these authors was heterogeneous (page 13). Even if important in PHA-dependent chicken red cell killing (Tonietti et al., 1975b), such complex contacts appear unnecessary when either Chang cells (Biberfeld et al., 1968; Biberfeld, 1971a) or mastocytoma cells are the targets.

Effector lymphocytes were usually attached to Chang cells by their cytoplasmic pole or uropod (Biberfeld, 1971a). However, although uropods were observed in the present study when cells were agglutinated with PHA, the mastocytoma cells were not preferentially attached to any particular area of the lymphoblast surface. This was not unexpected in view of the findings in antibody-dependent cell-mediated cytotoxicity (page 58) in which the cell mixtures were also incubated and fixed in suspension.

Like the contacts between spleen cells and antibody-coated erythrocytes, all of the PHA- (Figs. 42, 43 & 44) and most of the axinella-induced attachments between lymphoblasts and mastocytoma cells can only be regarded as a form of intermediate or adhesive junction (pages 55 & 60). Since the nexus is the most likely junction through which cell-mediated cytotoxicity might occur (page 18), it was anticipated that such structures, if they formed at all, would develop when PHA was present. The gap junctions between cells incubated with axinella (Figs. 47 & 48) could theoretically account for both the dye transfer and the lysis observed but if so they ought to have been present in greater numbers in the presence of PHA. Innumerable contacts between PHA-agglutinated cells were carefully examined but gap junctions were not found and some

other mechanism must be sought.

Although correlation between cell coupling and gap junctions is extensive and convincing (McNutt & Weinstein, 1973; Bennett, 1973), adjacent cells may be electrically coupled without obvious morphological specialisation if regions of low resistance membrane are 20 nm apart (Bennett & Auerbach, 1969). Fluorescein can cross membranes by virtue of its lipid solubility (Bennett, 1973) and may pass into adjacent cells after a high concentration is reached in the extracellular space without the agency of a specialised junction (Furshpan & Potter, 1968; Payton et al., 1969; Bennett, 1973). If it is assumed that the effector and target cell membranes in the region of close contact are functionally but not morphologically (at least in thin sections) different from the rest of the plasma membrane, it is possible that both dye transfer and cytotoxicity could be mediated via intermediate junctions. It is clear, however, that mere attachment is not sufficient since both cell-mediated lysis and fluorescein transfer occurred only to a limited extent in axinella-treated samples despite a large number of morphologically similar contacts. It must be concluded that PHA, in addition to inducing agglutination, activated the lymphoblasts in some morphologically indistinguishable manner.

4.5. CONCLUSIONS

Since there were no phagocytic cells present, cell-mediated lysis in this system could only have been effected through an extracellular mechanism by T lymphoblasts.

Lymphoblasts and mastocytoma cells were as efficiently agglutinated by axinella as they were by PHA and contact between target and effector cell, although essential, did not necessarily result in target cell death.

No morphological difference could be found in thin sections to account for the functionally different nature of PHA- and axinella-induced attachments between lymphoblasts and mastocytoma cells.

Although occasional gap junctions were present between axinella-agglutinated cells it was concluded that the cytotoxicity and dye transfer observed probably occurred via the 7.5-10 nm intercellular space.

5. GENERAL CONCLUSIONS

In the antibody-dependent cell-mediated cytotoxic systems studied three types of cell, lymphocytes, macrophages and neutrophils were associated with target cells. Contact between any of these cells and target cells was not entirely antibody-dependent, always occurring to some extent with normal serum. Attachment to either macrophages or neutrophils in the presence of antiserum, even in extremely low concentration, usually resulted in phagocytosis, but the possibility of prior interaction between lymphocytes and antibody-coated target cells could not be excluded. Phagocytosis was rarely seen when normal serum was used rather than antiserum.

Lymphocytes, macrophages and neutrophils were found attached to antibody-treated chicken erythrocytes with ultrastructural features indicating that all these effectors could produce extracellular osmotic lysis. In the non-specific system studied, in which cytotoxicity was induced by PHA and was not antibody-dependent, the effector cells could only have been T lymphoblasts. There was no evidence that any specific form of contact between effector and target cells developed in either the antibody- or PHA-dependent systems examined. Although the number of attachments depended on whether specific antiserum or normal serum was added, or whether PHA or axinella was present, the vast majority of contacts appeared identical. In all cases in which the plasma membranes of the cells were closely apposed, they were always separated by a gap of approximately 10 nm. However, there remains the theoretical possibility that specific attachments, morphologically indistinguishable in thin sections from non-specific contacts, did form in the presence of antibody or PHA.

On general grounds, gap junctions were considered the most likely structure through which cell-mediated cytotoxicity might operate. Damage could be effected either by formation of the junction followed

by separation of the cells without repair to the target cell, or by a cytotoxic factor passed directly from the K cell to the target cell. For the former mechanism K cell intramembranous particles would have to be capable of rearrangement to their normal state, whilst those of the target cell would have to remain in hexagonal array thus leaving the cell "leaky" and susceptible to lysis. As there is no reason for supposing that one type of plasma membrane has a greater potential for rearrangement than any other, the more likely alternative is the passage of a cytotoxic factor between the cells. However, nexuses were not seen in the PHA- or antibody-dependent systems examined, and were only observed after axinella treatment, where both cytotoxicity and fluorescein transfer were low. The possibility that gap junctions form and dissociate too rapidly to be seen cannot be excluded, but no evidence for such a transient nature of these junctions can be found in the literature. It seems unlikely that such junctions form during cell-mediated lysis.

In conclusion, therefore, this investigation has indicated that lymphocytes, macrophages and neutrophils are all involved in K (antibody-dependent cell-mediated cytotoxic) activity. There is some evidence to support a mechanism involving extracellular lysis. Nevertheless, ingestion by macrophages and neutrophils plays a considerable part which must not be overlooked, even when only a few phagocytic cells are present in the effector cell population.

SUPPLIERS OF MATERIALS AND EQUIPMENT

CHEMICALS AND REAGENTS

All chemicals were of 'AnalaR' grade or equivalent quality and were obtained from BDH Chemicals Ltd., Poole, Dorset, except where otherwise stated.

Agar (Ionagar)	Oxoid Ltd., London.
Barbitone sodium	
Chloroform	
Ethanol, absolute	
Epikote 812 (Epon) epoxy resin embedding components	Emscope Laboratories, London.
D-Glucose	
Glutaraldehyde EM (25% aqueous solution specially purified for use in electron microscopy)	Taab Laboratories, Reading, Berks.
Hydrochloric acid solution, N (M), concentrated volumetric solution	
Lead nitrate	
Michrome Clearmount (xylol miscible neutral mountant)	E. Gurr Ltd., London.
Osmium tetroxide	Johnson Matthey Chemicals Ltd., Royston, Herts.
Propylene oxide	
Sodium acetate trihydrate	
Sodium cacodylate trihydrate EM	Taab Laboratories, Reading, Berks.
tri-Sodium citrate	
Sodium dihydrogen orthophosphate	
Sodium hydroxide solution, N (M), free from carbonate, analytical volumetric solution	

di-Sodium tetraborate	
Sucrose	
Toluidine blue	G.T. Gurr (Searle Diagnostic), High Wycombe, Bucks.
Uranyl acetate	

PHOTOGRAPHIC MATERIALS

All photographic materials were supplied by Ilford Ltd., Basildon, Essex.

Developer, PQ Universal

Fixer, HYPAM

Paper, ILFOBROM

Paper, ILFOPRINT processor

Plates, Electron microscope plates type EM 4

Processor solutions, ILFOPRINT Activator and Stabilizer

EQUIPMENT

Autoprocessor, ILFOPRINT processor 1502	Ilford Ltd., Basildon, Essex.
Carbon or graphite rods	Johnson Matthey Chemicals Ltd., Royston, Herts.
Diamond knives (42 - 47° included angle)	Ge-Fe-Ri s.n.c., 03100 Frosinone, Italy.
Electron microscope, EM 6B	AEI Scientific Apparatus Ltd., Manchester.
Enlarger, Beseler model 45 MCX	C.A.V. Services Ltd., Maidenhead, Berks.
Enlarger lenses, El Nikkor 50 mm, f 2.8; 80 mm, f 5.6; 105 mm, f 5.6	Rank Pullin Commercial, Perivale, Middx.

Gelatin capsules	Polaron Equipment Ltd., Watford, Herts.
Glass Knifemaker and glass strips	LKB Instruments Ltd., South Croydon, Surrey.
Glazer, F.C. Glazing Machine, model S2	Pelling & Cross Ltd., London.
Grids, Maxtaform copper	Taab Laboratories, Reading, Berks.
Ultramicrotome, Sorval MT-2	V.A. Howe & Co. Ltd., London.
Vacuum coating unit, Metrovac type 12	AEI Scientific Apparatus Ltd., Manchester.

REFERENCES

ABLE, M.E., LEE, J.C. & ROSENAU, W. (1970)
Lymphocyte-target cell interaction in vitro: ultrastructural and cinematographic studies. Amer. J. Path., 60: 421-434.

ABRAHAMS, I. (1966)
Further studies on acquired resistance to murine cryptococcosis: enhancing effect of Bordetella pertussis. J. Immunol., 96: 525-529.

ABRAHAMS, I., STOFFER, H.R., PAYETTE, K.M. & SINGH, F.R. (1970)
Cellular immunity in experimental murine cryptococcosis. Fed. Proc., 29: 503 (Abs.).

ABRAMSON, N., LO BUGLIO, A.F., JANDL, J.H. & COTRAN, R.S. (1970)
The interaction between human monocytes and red cells: binding characteristics. J. exp. Med., 132: 1191-1206.

AL-DOORY, Y. (1971)
The ultrastructure of Cryptococcus neoformans. Sabouraudia, 9: 113-118.

ALLISON, A.C. (1972)
Immunity and immunopathology in virus infections. Ann. Inst. Pasteur, 123: 585-608.

ALLISON, A.C., DAVIES, P. & de PETRIS, S. (1971)
Role of contractile microfilaments in macrophage movement and endocytosis. Nature new Biol., 232: 153-155.

ALLISON, A.C., DAVIES, P. & PAGE, R.C. (1973)
Effects of endotoxin on macrophages and other lymphoreticular cells. J. infect. Dis., 128 Suppl. (July), S212-S219.

ARMSTRONG, P.B. (1970)
A fine structural study of adhesive cell junctions in heterotypic cell aggregates. J. Cell Biol., 47: 197-210.

ARONSON, M. & KLETTER, J. (1973)
Aspects of the defence against a large-sized parasite, the yeast, Cryptococcus neoformans. In: Dynamic aspects of host-parasite relationships, edited by A. ZUCKERMAN & D.W. WEISS, Vol. 1. London, Academic Press. pp.132-162.

ASHERSON, G.L., FERLUGA, J. & JANOSSY, G. (1973)
Non-specific cytotoxicity by T cells activated with plant mitogens in vitro and the requirement for plant agents during the killing reaction. Clin. exp. Immunol., 15: 573-589.

AX, W., MALCHOW, H., ZEISS I. & FISCHER, H. (1968)
The behaviour of lymphocytes in the process of target cell destruction in vitro. Exp. Cell Res., 53: 108-116.

AZARNIA, R., LARSEN, W.J. & LOEWENSTEIN, W.R. (1974)
The membrane junctions in communicating and non-communicating cells, their hybrids, and segregants. Proc. nat. Acad. Sci., Wash., 71: 880-884.

BEHNKE, O., KRISTENSEN, B.I. & NIELSEN, L.E. (1971)
Electron microscopical observations on actinoid and myosinoid filaments in blood platelets. J. Ultrastruct. Res., 37: 351-369.

BENNETT, B., OLD, L.J. & BOYSE, E.A. (1963)
Opsonization of cells by isoantibody in vitro. Nature, Lond., 198: 10-12.

BENNETT, M.V.L. (1973)
Function of electrotonic junctions in embryonic and adult tissues. Fed. Proc., 32: 65-75.

BENNETT, M.V.L. & AUERBACH, A.A. (1969)
Calculation of electrical coupling of cells separated by a gap. Anat. Rec., 163: 152 (Abs.).

BERNHARD, W. & GUÉRIN, M. (1958)
Présence de particules d'aspect virusal dans les tissus tumoraux de souris atteintes de leucémie spontanée. C. R. Acad. Sci., Paris, 247D: 1802-1805 + Figs. 1-6.

BESSIS, M. (1973)
Living blood cells and their ultrastructure, translated by R.I. WEED. Heidelberg, Springer-Verlag.

BIBERFELD, P. (1971a)
Cytotoxic interaction of phytohemagglutinin-stimulated blood lymphocytes with monolayer cells: a study by light and electron microscopy. Cell. Immunol., 2: 54-72.

BIBERFELD, P. (1971b)
Morphogenesis in blood lymphocytes stimulated with phytohaemagglutinin (PHA): a light and electron microscopic study. Acta path. microbiol. scand. Sect. A. Suppl. 223: 1-70.

BIBERFELD, P., BIBERFELD, G., PERLMANN, P. & HOLM, G. (1973)
Cytological observations on the cytotoxic interaction between lymphocytes and antibody-coated monolayer cells. Cell. Immunol., 7: 60-72.

BIBERFELD, P., HOLM, G. & PERLMANN, P. (1968)
Morphological observations on lymphocyte peripolesis and cytotoxic action in vitro. Exp. Cell Res., 52: 672-677.

BIBERFELD, P. & JOHANSSON, A. (1975)
Contact areas of cytotoxic lymphocytes and target cells: an electron microscopic study. Exp. Cell Res., 94: 79-87.

BIBERFELD, P. & PERLMANN, P. (1970)
Morphological observations on the cytotoxicity of human blood lymphocytes for antibody-coated chicken erythrocytes. Exp. Cell Res., 62: 433-440.

BIBERFELD, P., WÅHLIN, B., PERLMANN, P. & BIBERFELD, G. (1975)
A plaque technique for assay and characterization of antibody-dependent cytotoxic effector (K) cells. Scand. J. Immunol., 4: 859-864.

BINDSCHADLER, D.D. & BENNETT, J.E. (1968)
Serology of human cryptococcosis. Ann. intern. Med., 69: 45-52.

BLOOM, W. & FAWCETT, D.W. (1975)
A textbook of histology. 10th Ed. Philadelphia, Saunders.

BÖYUM, A. (1968)
Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. clin. Lab. Invest. 21 Suppl. 97: 77-89.

BRANDES, D., SCHOFIELD, B., SLUSSER, R. & ANTON, E. (1966)
Studies of L1210 leukemia. I. Ultrastructure of solid and ascites cells. J. nat. Cancer Inst., 37: 467-485.

BROWN, J.N. (1975)
The avian erythrocyte: a study of fixation for electron microscopy. J. Micr., Lond., 104: 293-305.

BULMER, G.S. & SANS, M.D. (1967)
Cryptococcus neoformans II. Phagocytosis by human leukocytes. J. Bact., 94: 1480-1483.

CALDER, E.A., URBANIAK, S.J., PENHALE, W.J. & IRVINE, W.J. (1974)
Characterization of human lymphoid cell-mediated antibody-dependent cytotoxicity (LDAC). Clin. exp. Immunol., 18: 579-593.

CARGILL, C.F. & OLSON, L.D. (1973)
Viability and ultrastructural change in group E streptococci after in vitro phagocytosis by swine macrophages. Canad. J. comp. Med., 37: 256-260.

CARR, I. (1967)
The fine structure of cells of the mouse peritoneum. Z. Zellforsch., 80: 534-555.

CARR, I. (1972)
The fine structure of microfibrils and microtubules in macrophages and other lymphoreticular cells in relation to cytoplasmic movement. J. Anat., Lond., 112: 383-389.

CATANZARO, P.J., GRAHAM, R.C. & HOGREFE, W.R. (1974)
Mouse peritoneal lymphocytes: a morphologic comparison of normal and exudate peritoneal lymphocytes. J. reticuloendothel. Soc., 16: 161-174.

CATOVSKY, D., FRISCH, B. & VAN NOORDEN, S. (1975)
B, T and "null" cell leukaemias: electron cytochemistry and surface morphology. Blood Cells, 1: 115-124.

CHAMBERS, V.C. & WEISER, R.S. (1969)
The ultrastructure of target cells and immune macrophages during their interaction in vitro. Cancer Res., 29: 301-317.

CHEN, L-T., EDEN, A., NUSSENZWEIG, V. & WEISS, L. (1972)
Electron microscopic study of the lymphocytes capable of binding antigen-antibody-complement complexes. Cell. Immunol., 4: 279-288.

CLINE, M.J. & LEHRER, R.I. (1968)
Phagocytosis by human monocytes. Blood, 32: 423-435.

COHN, Z.A. (1968)
The structure and function of monocytes and macrophages. Advanc. Immunol., 9: 163-214.

COHN, Z.A. & BENSON, B. (1965)
The differentiation of mononuclear phagocytes: morphology, cytochemistry and biochemistry. J. exp. Med., 121: 153-170 + plates 5-14.

COLLINS, D.N., OPPENHEIM, I.A. & EDWARDS, M.R. (1971)
Cryptococcosis associated with systemic lupus erythematosus: light and electron microscopic observations on a morphologic variant. Arch. Path., 91: 78-88.

CRUICKSHANK, R., DUGUID, J.P., MARMION, B.P. & SWAIN, R.H.A. (1973)
Medical microbiology: a guide to the laboratory diagnosis and control of infection. 12th Ed. Edinburgh, Churchill Livingstone. Vol. 1. Microbial infections. pp. 541-548.

CURGY, J-J. (1968)
Influence du mode de fixation sur la possibilité d'observer des structures myéliniques dans les hépatocytes d'embryons de poulet. J. Micr., Paris, 7: 63-80.

DAEMS, W.Th. & BREDEROO, P. (1973)
Electron microscopical studies on the structure, phagocytic properties, and peroxidatic activity of resident and exudate peritoneal macrophages in the guinea pig. Z. Zellforsch., 144: 247-297.

DALTON, A.J. (1972)
RNA tumour viruses - terminology and ultrastructural aspects of virion morphology and replication. J. nat. Cancer Inst., 49: 323-327.

DAVIES, H.G. (1961)
Structure in nucleated erythrocytes. J. biophys. biochem. Cytol., 9: 671-687.

DAVIES, P., PAGE, R.C. & ALLISON, A.C. (1974)
Changes in cellular enzyme levels and extracellular release of lysosomal acid hydrolases in macrophages exposed to group A streptococcal cell wall substance. J. exp. Med., 139: 1262-1282.

DEMBITZER, H.M., BUZA, I. & REISS, F. (1972)
Biological and electron microscopic changes in gamma radiated Cryptococcus neoformans. Mycopathologia et Mycologia applicata, 47: 307-315.

DENNERT, G. & LENNOX, E. (1972)
Cell interactions in humoral and cell-mediated immunity. Nature new Biol., 238: 114-116.

DENNERT, G. & LENNOX, E.S. (1973)
Phagocytic cells as effectors in a cell-mediated immunity system. J. Immunol., 111: 1844-1854.

de PETRIS, S., KARLSBAD, G. & PERNIS, B. (1962)
Filamentous structures in the cytoplasm of normal mononuclear phagocytes. J. Ultrastruct. Res., 7: 39-55.

de PETRIS, S. & RAFF, M.C. (1972)
Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy: antibody-induced, temperature-dependent redistribution and its implications for membrane structure. Europ. J. Immunol., 2: 523-535.

de PETRIS, S. & RAFF, M.C. (1973)
Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. Nature new Biol., 241: 257-259.

de VRIES, E., van de PUTTE, L.B.A., LAFEBER, G.J.M., van BUYSEN-NUTTERS, A.C., de VETTEN-van der HULST, H.L.M. & MEYER, C.J.L.M. (1976)
Morphological differences in the interactions between human mononuclear cells and coated or uncoated sheep red blood cells: a freeze-etch study of different types of rosettes. Cell Tiss. Res., 168: 79-88.

DIAMOND, R.D. (1974)
Antibody-dependent killing of Cryptococcus neoformans by human peripheral blood mononuclear cells. Nature, Lond., 247: 148-150.

DIAMOND, R.D., ROOT, R.K. & BENNETT, J.E. (1972)
Factors influencing killing of Cryptococcus neoformans by human leukocytes in vitro. J. infect. Dis., 125: 367-376.

DOUGLAS, S.D. (1972)
Electron microscopic and functional aspects of human lymphocyte response to mitogens. Transplant. Rev., 11: 39-59.

DOUGLAS, S.D. & HUBER, H. (1972)
Electron microscopic studies of human monocyte and lymphocyte interaction with immunoglobulin- and complement-coated erythrocytes. Exp. Cell Res., 70: 161-172.

DUMONT, A. & ROBERT, A. (1970)
Electron microscopic study of phagocytosis of Histoplasma capsulatum by hamster peritoneal macrophages. Lab. Invest., 23: 278-286.

DUNN, T.B. & POTTER, M. (1957)
A transplantable mast-cell neoplasm in the mouse. J. nat. Cancer Inst., 18: 587-601.

EDWARDS, M.R., GORDON, M.A., LAPA, E.W. & GHIORSE, W.C. (1967)
Micromorphology of Cryptococcus neoformans. J. Bact., 94: 766-777.

ELSON, C.J., BRADLEY, J. & HOWELLS, R.E. (1972)
The mechanism of rosette formation between Rh (D)-positive erythrocytes and peripheral blood lymphocytes from Rh isoimmunized individuals: the role of surface micro-projections. Immunology, 22: 1075-1086.

EMMONS, C.W., BINFORD, C.H. & UTZ, J.P. (1970)
Medical mycology. 2nd Ed. Philadelphia, Lea & Febiger. pp.186-206.

FARQUHAR, M.G., BAINTON, D.F., BAGGIOLINI, M. & DE DUVE, C. (1972)
Cytochemical localization of acid phosphatase activity in granule fractions from rabbit polymorphonuclear leukocytes. J. Cell Biol., 54: 141-156.

FARQUHAR, M.G. & PALADE, G.E. (1963)
Junctional complexes in various epithelia. J. Cell Biol., 17: 375-412.

FARQUHAR, M.G. & PALADE, G.E. (1965)
Cell junctions in amphibian skin. J. Cell Biol., 26: 263-291.

FAWCETT, D.W. (1966)
The cell, its organelles and inclusions: an atlas of fine structure. Philadelphia, Saunders.

FAWCETT, D.W. & WITEBSKY, F. (1964)
Observations on the ultrastructure of nucleated erythrocytes and thrombocytes with particular reference to the structural basis of their discoidal shape. Z. Zellforsch., 62: 785-806.

FEDORKO, M.E., CROSS, N.L. & HIRSCH, J.G. (1973)
Appearance and distribution of ferritin in mouse peritoneal macrophages in vitro after uptake of heterologous erythrocytes. J. Cell Biol., 57: 289-305.

FEDORKO, M.E. & HIRSCH, J.G. (1970)
Structure of monocytes and macrophages. Semin. Hemat., 7: 109-124.

FIRKET, H. & DEGIOVANNI, G. (1975)
Ultrastructural study of the relationship between immune cytotoxic lymphoid cells and target cells in vitro. Virchows Arch. Abt. B. 17: 229-238.

FISHEL, C.W., HALKIAS, D.G., KLEIN, T.W. & SZENTIVANYI, A. (1976)
Characteristics of cells present in peritoneal fluids of mice injected intraperitoneally with Bordetella pertussis. Infect. & Immun., 13: 263-272.

FONTANA, L., de SANCTIS, G., PECCI, G. & TONIETTI, G. (1975)
Ultrastructure of lymphocyte-mediated cytotoxicity. II. Interaction between human lymphocytes and antibody-coated chicken erythrocytes. Experientia, 31: 1231-1233.

FURSHPAN, E.J. & POTTER, D.D. (1968)
Low-resistance junctions between cells in embryos and tissue culture. In: Current topics in developmental biology, edited by A.A. MOSCONA & A. MONROY. New York, Academic Press. Vol. 3, pp.95-127.

GAZIRI, I.F., OLIVEIRA-CASTRO, G.M., MACHADO, R.D. & BARCINSKI, M.A. (1975)
Structure and permeability of junctions in phytohemagglutinin stimulated human lymphocytes. Experientia, 31: 172-174.

GELFAND, E.W., RESCH, K. & PRESTER, M. (1972)
Antibody-mediated target cell lysis by non-immune cells: characterization of the antibody and effector cell populations. Europ. J. Immunol., 2: 419-424.

GENTRY, L.O. & REMINGTON, J.S. (1971)
Resistance against Cryptococcus conferred by intracellular bacteria and protozoa. J. infect. Dis., 123: 22-31.

GHADIALLY, F.N. (1975)
Ultrastructural pathology of the cell: a text and atlas of physiological and pathological alterations in cell fine structure. London, Butterworth. pp.80-83.

GLAUERT, A.M. (1975)
Fixation, dehydration and embedding of biological specimens. Practical methods in electron microscopy. Vol. 3, pt. 1. Amsterdam, North-Holland Pub. Co. p.27.

GLICK, A.D., GETNICK, R.A. & COLE, R.M. (1971)
Electron microscopy of group A streptococci after phagocytosis by human monocytes. Infect. & Immun., 4: 772-779.

GOLSTEIN, P., WIGZELL, H., BLOMGREN, H. & SVEDMYR, E.A.J. (1972)
Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. J. exp. Med., 135: 890-906.

GOODENOUGH, D.A. & REVEL, J.P. (1970)
A fine structural analysis of intercellular junctions in the mouse liver. J. Cell Biol., 45: 272-290.

GREENBERG, A.H., HUDSON, L., SHEN, L. & ROITT, I.M. (1973a)
Antibody-dependent cell-mediated cytotoxicity due to a "null" lymphoid cell. Nature new Biol., 242: 111-113.

GREENBERG, A.H., SHEN, L. & ROITT, I.M. (1973b)
Characterization of the antibody-dependent cytotoxic cell: a non-phagocytic monocyte? Clin. exp. Immunol., 15: 251-259.

GREENBERG, A.H., SHEN, L., WALKER, L., ARNAIZ-VILLENA, A. & ROITT, I.M. (1975)
Characteristics of the effector cells mediating cytotoxicity against antibody-coated target cells. II. The mouse nonadherent K cell. Europ. J. Immunol., 5: 474-480.

GRIFFIN, F.M. & SILVERSTEIN, S.C. (1974)
Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. exp. Med., 139: 323-336.

GUDAT, F.G. & VILLIGER, W. (1973)
A scanning and transmission electron microscope study of antigen-binding sites on rosette-forming cells. J. exp. Med., 137: 483-493.

HIRSCH, J.G. & FEDORKO, M.E. (1968)
Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. J. Cell Biol., 38: 615-627.

HIRSCH, J.G. & FEDORKO, M.E. (1970)
Morphology of mouse mononuclear phagocytes. In: Mononuclear phagocytes, edited by R. VAN FURTH. Oxford, Blackwell. pp.7-28.

HOLM, G. (1972)
Lysis of antibody-treated human erythrocytes by human leukocytes and macrophages in tissue culture. Int. Arch. Allergy, Basel, 43: 671-682.

HOLM, G. & HAMMARSTRÖM, S. (1973)
Haemolytic activity of human blood monocytes: lysis of human erythrocytes treated with anti-A serum. Clin. exp. Immunol., 13: 29-43.

HOLM, G. & PERLMANN, P. (1967a)
Cytotoxic potential of stimulated human lymphocytes. J. exp. Med., 125: 721-736.

HOLM, G. & PERLMANN, P. (1967b)
Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. Immunology, 12: 525-536.

HOWATSON, A.F. & ALMEIDA, J.D. (1958)
A method for the study of cultured cells by thin sectioning and electron microscopy. J. biophys. biochem. Cytol., 4: 115-118 + plates 56, 57.

HÜLSER, D.F. & DEMSEY, A. (1973)
Gap and low-resistance junctions between cells in culture. Z. Naturf., 28C: 603-606.

HÜLSER, D.F. & PETERS, J.H. (1972)
Contact cooperation in stimulated lymphocytes. II. Electrophysiological investigations on intercellular communication. Exp. Cell Res., 74: 319-326.

INGLIS, J.R., PENHALE, W.J., FARMER, A., IRVINE, W.J. & WILLIAMS, A.E. (1975)
Observations on the antibody-dependent cytotoxic cell by scanning electron microscopy. Clin. exp. Immunol., 21: 216-225.

ISHIKAWA, H., BISCHOFF, R. & HOLTZER, H. (1969)
Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. J. Cell Biol., 43: 312-328.

JANOSSY, G., SHOHAT, M., GREAVES, M.F. & DOURMASHKIN, R.R. (1973)
Lymphocyte activation. IV. The ultrastructural pattern of the response of mouse T and B cells to mitogenic stimulation in vitro. Immunology, 24: 211-227.

JOHNSON, R.G., HERMAN, W.S. & PREUS, D.M. (1973)
Homocellular and heterocellular gap junctions in Limulus: a thin-section and freeze-fracture study. J. Ultrastruct. Res., 43: 298-312.

JOHNSON, R.G. & SHERIDAN, J.D. (1971)
Junctions between cancer cells in culture: ultrastructure and permeability. Science, 174: 717-719.

JONES, T.C. & HIRSCH, J.G. (1971)
The interaction in vitro of Mycoplasma pulmonis with mouse peritoneal macrophages and L-cells. J. exp. Med., 133: 231-259.

KOREN, H.S., AX, W. & FREUND-MOELBERT, E. (1973)
Morphological observations on the contact-induced lysis of target cells. Europ. J. Immunol., 3: 32-37.

KOVITHAVONGS, T., RICE, G., THONG, K.L. & DOSSETOR, J.B. (1975)
Effector cell activity in antibody mediated cell dependent immune lysis. II. Evidence for different populations of effector cells for different targets. Cell. Immunol., 18: 167-175.

KRAHENBUHL, J.L. & LAMBERT, L.H. (1975)
Cytokinetic studies of the effects of activated macrophages on tumour target cells. J. nat. Cancer Inst., 54: 1433-1437.

LAY, W.H. & NUSSENZWEIG, V. (1968)
Receptor for complement on leukocytes. J. exp. Med., 128: 991-1009.

LEIBOVITZ, A., McCOMBS, W.B., JOHNSTON, D., McCOY, C.E. & STINSON, J.C. (1973)
New human cancer cell culture lines. I. SW-13 small-cell carcinoma of the adrenal cortex. J. nat. Cancer Inst., 51: 691-697.

LEVY, J.D., KNIESER, M.R. & BRIGGS, W.A. (1975)
Ultrastructural characteristics of the non-immune rosette-forming cell. J. Cell Sci., 18: 79-96.

LIN, P.S., COOPER, A.G. & WORTIS, H.H. (1973)
Scanning electron microscopy of human T-cell and B-cell rosettes. New Engl. J. Med., 289: 548-551.

LO BUGLIO, A.F., COTRAN, R.S. & JANDL, J.H. (1967)
Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science, 158: 1582-1585.

LUFT, J.H. (1961)
Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol., 9: 409-414.

LUNDGREN, G., ZUKOSKI, CH.F. & MÖLLER, G. (1968)
Differential effects of human granulocytes and lymphocytes on human fibroblasts in vitro. Clin. exp. Immunol., 3: 817-836.

MacDONALD, H.R., BONNARD, G.D., SORDAT, B. & ZAWODNIK, S.A. (1975)
Antibody-dependent cell-mediated cytotoxicity: heterogeneity of effector cells in human peripheral blood. Scand. J. Immunol., 4: 487-497.

MacLENNAN, I.C.M. & LOEWI, G. (1968)
Effect of specific antibody to target cells on their specific and non-specific interactions with lymphocytes. Nature, Lond., 219: 1069-1070.

MacLENNAN, I.C.M., LOEWI, G. & HARDING, B. (1970)
The role of immunoglobulins in lymphocyte-mediated cell damage, in vitro. I. Comparison of the effects of target cell specific antibody and normal serum factors on cellular damage by immune and non-immune lymphocytes. Immunology, 18: 397-404.

MATTER, A., LISOWSKA-BERNSTEIN, B., RYSER, J.E., LAMELIN, J.-P. & VASSALLI, P. (1972)
Mouse thymus-independent and thymus-derived lymphoid cells. II. Ultrastructural studies. J. exp. Med., 136: 1008-1030.

McFARLAND, W. (1969)
Microspikes on the lymphocyte uropod. Science, 163: 818-820.

McNUTT, N.S. & WEINSTEIN, R.S. (1970)
The ultrastructure of the nexus: a correlated thin-section and freeze-cleave study. J. Cell Biol., 47: 666-688.

McNUTT, N.S. & WEINSTEIN, R.S. (1973)
Membrane ultrastructure at mammalian intercellular junctions. Progr. Biophys. mol. Biol., 26: 45-101.

MEEK, G.A. (1970)
Practical electron microscopy for biologists. London, Wiley-Interscience. p.456.

MILLONIG, G. (1961)
Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. appl. Phys., 32: 1637 (Abs.).

MINICK, O.T. (1963)
Low temperature storage of epoxy embedding resins. Stain. Tech., 38: 131-133.

MÖLLER, E. (1965)
Contact-induced cytotoxicity by lymphoid cells containing foreign isoantigens. Science, 147: 873-879.

MORGAN, C. & ROSE, H.M. (1968)
Structure and development of viruses as observed in the electron microscope. VIII. Entry of influenza virus. J. Virol., 2: 925-936.

Nature new Biology (1973)
Advances in immunopathology. 243: 225-226.

NICHOLS, B.A. & BAINTON, D.F. (1973)
Differentiation of human monocytes in bone marrow and blood: sequential formation of two granule populations. Lab. Invest., 29: 27-40.

NICHOLS, B.A., BAINTON, D.F. & FARQUHAR, M.G. (1971)
Differentiation of monocytes: origin, nature and fate of their azurophil granules. J. Cell Biol., 50: 498-515.

OLÁH, I. & RÖHLICH, P. (1966)
Peculiar membrane configurations after fixation in glutaraldehyde. Acta biol. Acad. Sci. hung., 17: 65-73.

OLÁH, I., RÖHLICH, P. & TÖRÖ, I. (1975)
Ultrastructure of lymphoid organs: an electron-microscopic atlas. Paris, Masson.

PAPAMICHAIL, M. & TEMPLE, A. (1975)
Characterization of the human effector cell causing antibody-mediated cytotoxicity. Clin. exp. Immunol., 20: 459-467.

PAYTON, B.W., BENNETT, M.V.L. & PAPPAS, G.D. (1969)
Permeability and structure of junctional membranes at an electrotonic synapse. Science, 166: 1641-1643.

PEACHEY, L.D. (1958)
Thin sections. I. A study of section thickness and physical distortion produced during microtomy. J. biophys. biochem. Cytol., 4: 233-242 + plates 125, 126.

PEASE, D.C. (1964)
Histological techniques for electron microscopy. 2nd Ed. New York, Academic Press. p.208.

PENFOLD, P.L., GREENBERG, A.H. & ROITT, I.M. (1976)
Characteristics of the effector cells mediating cytotoxicity against antibody-coated target cells. III. Ultrastructural studies. Clin. exp. Immunol., 23: 91-97.

PERKINS, W.D., KARNOVSKY, M.J. & UNANUE, E.R. (1972)
An ultrastructural study of lymphocytes with surface-bound immunoglobulin. J. exp. Med., 135: 267-276.

PERLMANN, P. & HOLM, G. (1968)
Studies on the mechanism of lymphocyte cytotoxicity. In: Mechanisms of inflammation induced by immune reactions (5th Internat. Symp. Immunopath.), edited by P. MIESCHER & P. GRABER. Basel, Schwabe & Co. pp. 325-341.

PERLMANN, P. & HOLM, G. (1969)
Cytotoxic effects of lymphoid cells in vitro. Advanc. Immunol., 11: 117-193.

PERLMANN, P. & PERLMANN, H. (1970)
Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. Cell. Immunol., 1: 300-315.

PERLMANN, P., PERLMANN, H. & HOLM, G. (1968)
Cytotoxic action of stimulated lymphocytes on allogenic and autologous erythrocytes. Science, 160: 306-309.

PERLMANN, P., PERLMANN, H., LARSSON, Å. & WÅHLIN, B. (1975)
Antibody dependent cytolytic effector lymphocytes (K cells) in human blood. J. reticuloendothel. Soc., 17: 241-250.

PINTO da SILVA, P. & GILULA, N.B. (1972)
Gap junctions in normal and transformed fibroblasts in culture. Exp. Cell Res., 71: 393-401.

POLLIACK, A., FU, S.M., DOUGLAS, S.D., BENTWICH, Z., LAMPEN, N. & de HARVEN, E. (1974)
Scanning electron microscopy of human lymphocyte-sheep erythrocyte rosettes. J. exp. Med., 140: 146-158.

REAVEN, E.P. & AXLINE, S.G. (1973)
Subplasmalemmal microfilaments and microtubules in resting and phagocytosing cultivated macrophages. J. Cell Biol., 59: 12-27.

RESCH, K., GELFAND, E.W. & PRESTER, M. (1974)
Antibody-mediated target cell lysis by nonimmune cells: the use of anti-immunoglobulin to distinguish effector cell populations. J. Immunol., 112: 792-803.

REVEL, J.P. & KARNOVSKY, M.J. (1967)
Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J. Cell Biol., 33: C7-C12.

REYNOLDS, E.S. (1963)
The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17: 208-212.

ROBBINS, E. & GONATAS, N.K. (1964)
In vitro selection of the mitotic cell for subsequent electron microscopy. J. Cell Biol., 20: 356-359.

RÖHLICH, P. (1975)
Membrane-associated actin filaments in the cortical cytoplasm of the rat mast cell. Exp. Cell Res., 93: 293-298.

ROTMAN, B. & PAPERMASTER, B.W. (1966)
Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. nat. Acad. Sci., Wash., 55: 134-141.

RYTER, A., KELLENBERGER, E., BIRCH-ANDERSEN, A. & MAALØE, O. (1958)
Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. I. Les nucléoides des bactéries en croissance active. Z. Naturf., 13b: 597-605.

SABATINI, D.D., BENSCH, K. & BARRNETT, R.J. (1963)
Cytochemistry and electron microscopy: the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol., 17: 19-58.

SANDERSON, C.J., CLARK, I.A. & TAYLOR, G.A. (1975)
Different effector cell types in antibody-dependent cell-mediated cytotoxicity. Nature, Lond., 253: 376-377.

SCOTT, R.E. & HORN R.G. (1970)
Ultrastructural aspects of neutrophil granulocyte development in humans. Lab. Invest., 23: 202-215.

SELJELID, R., MELSOM, H. & MUNTHE-KAAS, A. (1972)
Lytic effect of mouse macrophages on red cells. Exp. Cell Res., 75: 534-536.

SELLIN, D., WALLACH, D.F.H. & FISCHER, H. (1971)
Intercellular communication in cell-mediated cytotoxicity: fluorescein transfer between H-2d target cells and H-2b lymphocytes in vitro. Europ. J. Immunol., 1: 453-458.

SILVA, M.T., GUERRA, F.C. & MAGALHÃES, M.M. (1968)
The fixative action of uranyl acetate in electron microscopy. Experientia, 24: 1074.

SIMIONESCU, M., SIMIONESCU, N. & PALADE, G.E. (1975)
Segmental differentiations of cell junctions in the vascular endothelium: the microvasculature. J. Cell Biol., 67: 863-885.

SIMON, H.B. & SHEAGREN, J.N. (1971)
Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. exp. Med., 133: 1377-1389.

SOTELO, J.R. (1957)
Technical improvements in specimen preparation for electron microscopy. Exp. Cell Res., 13: 599-601.

SPITZNAGEL, J.K., DALLDORF, F.G., LEFFELL, M.S., FOLDS, J.D., WELSH, I.R.H., COONEY, M.H. & MARTIN, L.E. (1974)
Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest., 30: 774-785.

STOBO, J.D., ROSENTHAL, A.S. & PAUL, W.E. (1973)
Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface Θ or immunoglobulin determinants. J. exp. Med., 138: 71-88.

STORB, U., BAUER, W., STORB, R., FLIEDNER, T.M. & WEISER, R.S. (1969)
Ultrastructure of rosette-forming cells in the mouse during the antibody response. J. Immunol., 102: 1474-1485.

SUTER, E.R., PROBST, H. & DUKOR, P. (1972)
The fine structure of complement-receptor lymphocytes in mice. Europ. J. Immunol., 2: 189-190.

TERZAKIS, J.A. (1968)
Uranyl acetate, a stain and a fixative. J. Ultrastruct. Res., 22: 168-184.

TIZARD, I.R., HOLMES, W.L. & PARAPPALLY, N.P. (1974)
Phagocytosis of sheep erythrocytes by macrophages: a study of the attachment phase by scanning electron microscopy. J. reticuloendothel. Soc., 15: 225-231.

TONIETTI, G., PECCI, G., d'ACUNTO, G., LIOY, E., MERCALLI, M.E. PERRICONE, R. & FONTANA, L. (1975a)
Ultrastructure of cell-mediated immunity. I. Comparison between E and EA rosettes. Experientia, 31: 1464-1465.

TONIETTI, G., PECCI, G., LIBERATORE, C., SALSANO, F. & FONTANA, L. (1975b)
Ultrastructure of lymphocyte-mediated cytotoxicity. I. Interaction between phytohaemagglutinin-stimulated human lymphocytes and chicken erythrocytes. Brit. J. Haemat., 30: 71-74.

TRUMP, B.F. & ERICSSON, J.L.E. (1965)
The effect of the fixative solution on the ultrastructure of cells and tissues: a comparative analysis with particular attention to the proximal convoluted tubule of the rat kidney. Lab. Invest., 14: 1245-1323

VYE, M.V. & FISCHMAN, D.A. (1970)
The morphological alteration of particulate glycogen by en bloc staining with uranyl acetate. J. Ultrastruct. Res., 33: 278-291.

WAGNER, H., HARRIS, A.W. & FELDMANN, M. (1972)
Cell-mediated immune response in vitro. II. The role of thymus and thymus-derived lymphocytes. Cell. Immunol., 4: 39-50.

WALDO, E.D. & ZUCKER-FRANKLIN, D. (1972)
Ultrastructure and immunofluorescence of mouse spleen cells obtained by discontinuous albumin gradient centrifugation. J. Immunol., 108: 1665-1674.

WALTERS, M.N-I., PAPADIMITRIOU, J.M. & ROBERTSON, T.A. (1976)
The surface morphology of the phagocytosis of micro-organisms by peritoneal macrophages. J. Path., 118: 221-226.

WATSON, M.L. (1958)
Staining of tissue sections for electron microscopy with heavy metals. J. biophys. biochem. Cytol., 4: 475-478 + plates 226-229.

WEISS, L. (1968)
Interactions of sensitized lymphoid cells and homologous target cells in tissue culture and in grafts: an electron microscopic and immunofluorescence study. J. Immunol., 101: 1346-1362.

WEST, B.C., ROSENTHAL, A.S., GELB, N.A. & KIMBALL, H.R. (1974)
Separation and characterization of human neutrophil granules. Amer. J. Path., 77: 41-66.

WILSON, J.D., PANG, G.T.M. & GAVIN, J.B. (1974)
A scanning electron microscope study of lymphocyte-erythrocyte binding in three different types of rosette. Pathology, 6: 337-342.

YUST, I., SMITH, R.W., DICKLER, H.B., WUNDERLICH, J. & MANN, D.L. (1975)
Human lymphocyte dependent antibody mediated cytotoxicity: adherence of lymphocytes to antibody treated target cells. Cell. Immunol., 18: 176-186.

ZUCKER-FRANKLIN, D. (1969)
The ultrastructure of lymphocytes. Semin. Hemat., 6: 4-27.

ZUCKER-FRANKLIN, D. (1974)
The percentage of monocytes among "mononuclear" cell fractions obtained from normal human blood. J. Immunol., 112: 234-240.

ZUCKER-FRANKLIN, D. & BERNEY, S. (1972)
Electron microscope study of surface immunoglobulin-bearing human tonsil cells. J. exp. Med., 135: 533-548.

FIGURES

All the figures are electron micrographs of thin sections. Samples were either fixed with glutaraldehyde followed by osmium tetroxide (sequential fixation) or in a mixture of glutaraldehyde and osmium tetroxide (simultaneous fixation). All simultaneously fixed material was postfixed with uranyl acetate prior to dehydration and embedding in epoxy resin; unless otherwise stated, sequentially fixed specimens were not treated with uranyl acetate.

The sections were contrast stained with uranyl acetate and then lead citrate.

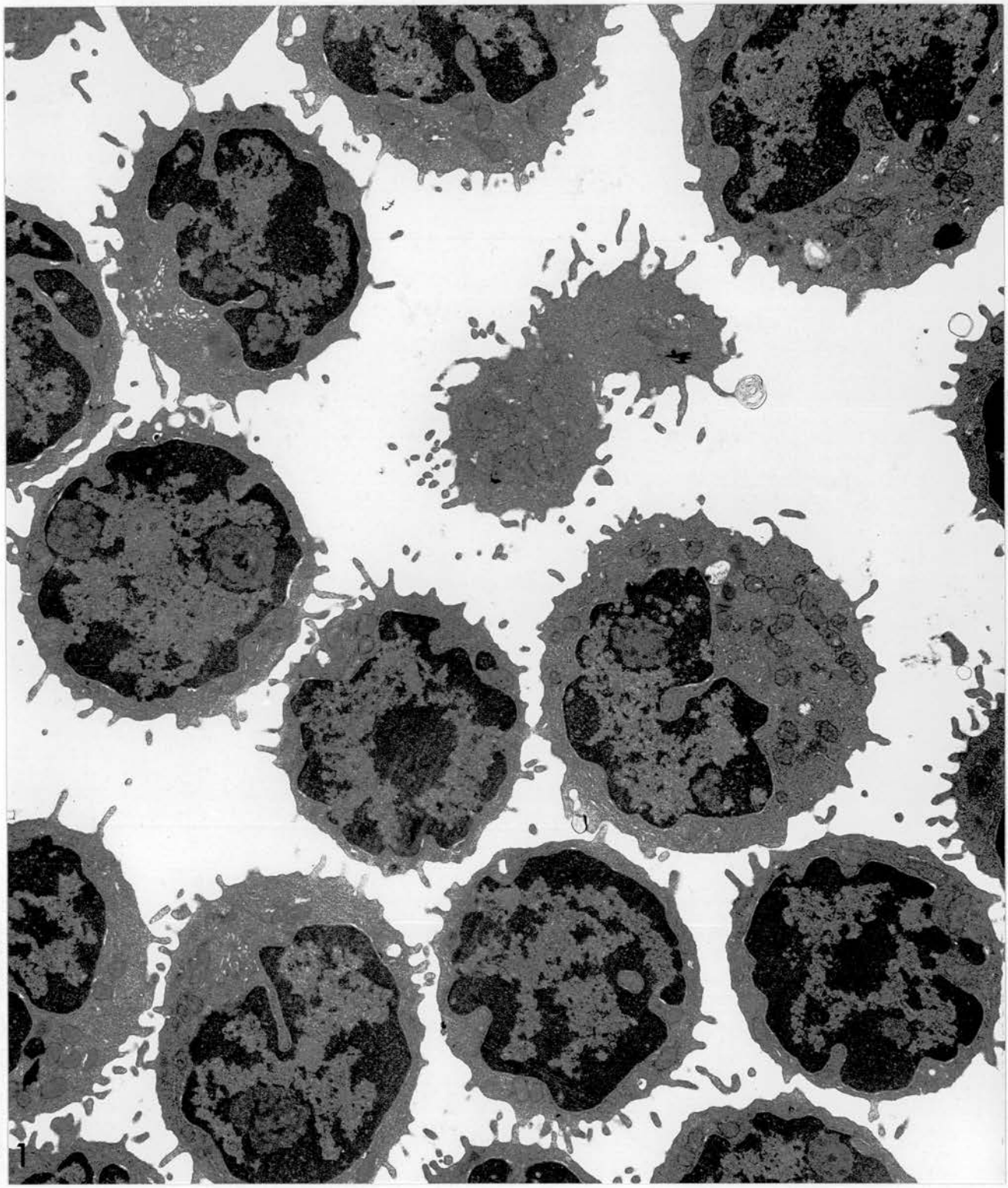


Fig. 1. Mouse peritoneal lymphocytes illustrating the range of variation in cell diameter and nuclear size. The predominantly dense nuclei are irregular in outline and frequently possess deep invaginations; the Golgi zone is situated in the cytoplasm opposite the nuclear cleft. Sequential fixation (phosphate buffered osmium tetroxide). "Bubbles" and myelin figures, evident at the surface of some cells, are artefacts of primary glutaraldehyde fixation. x 8,000.

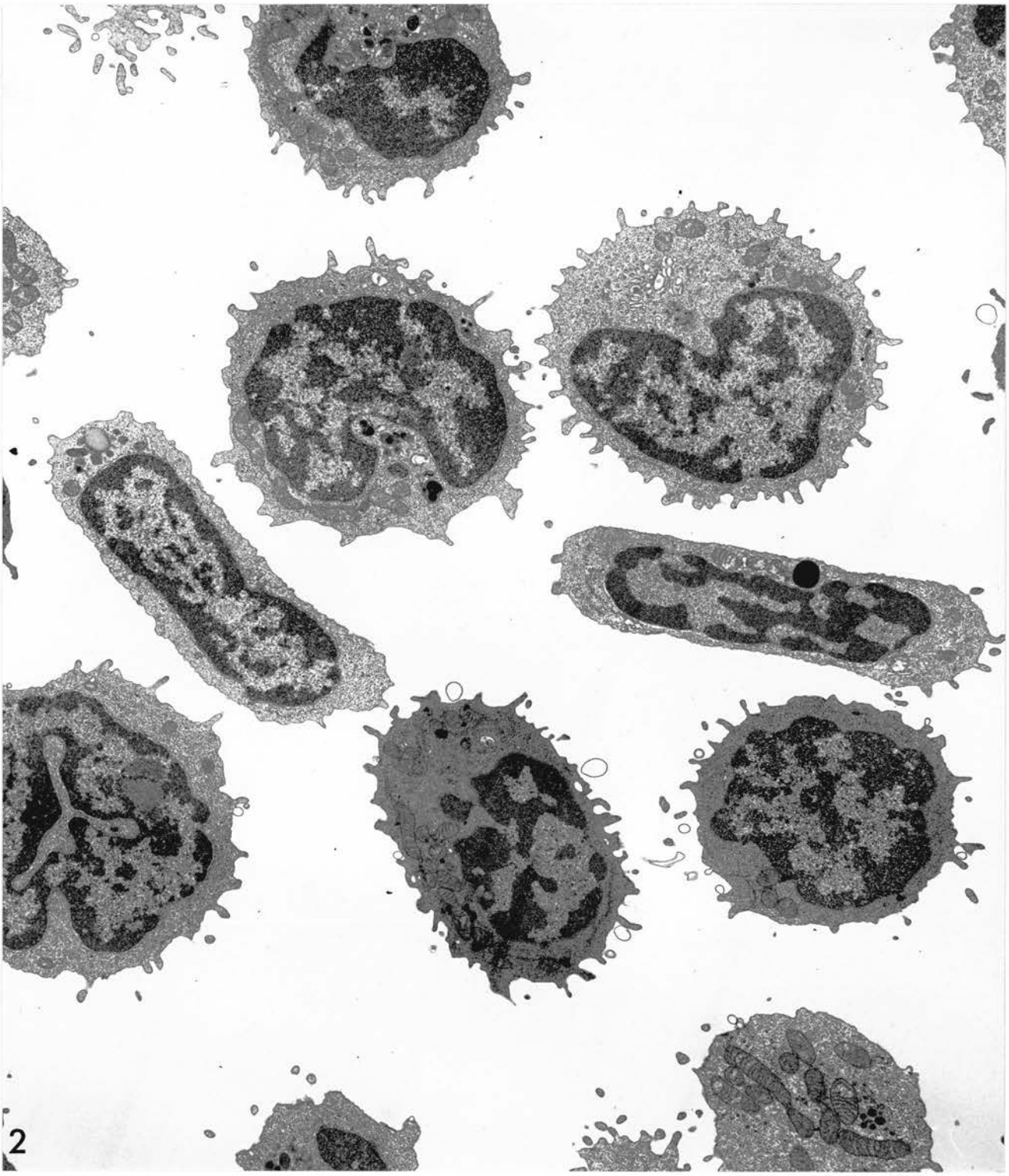
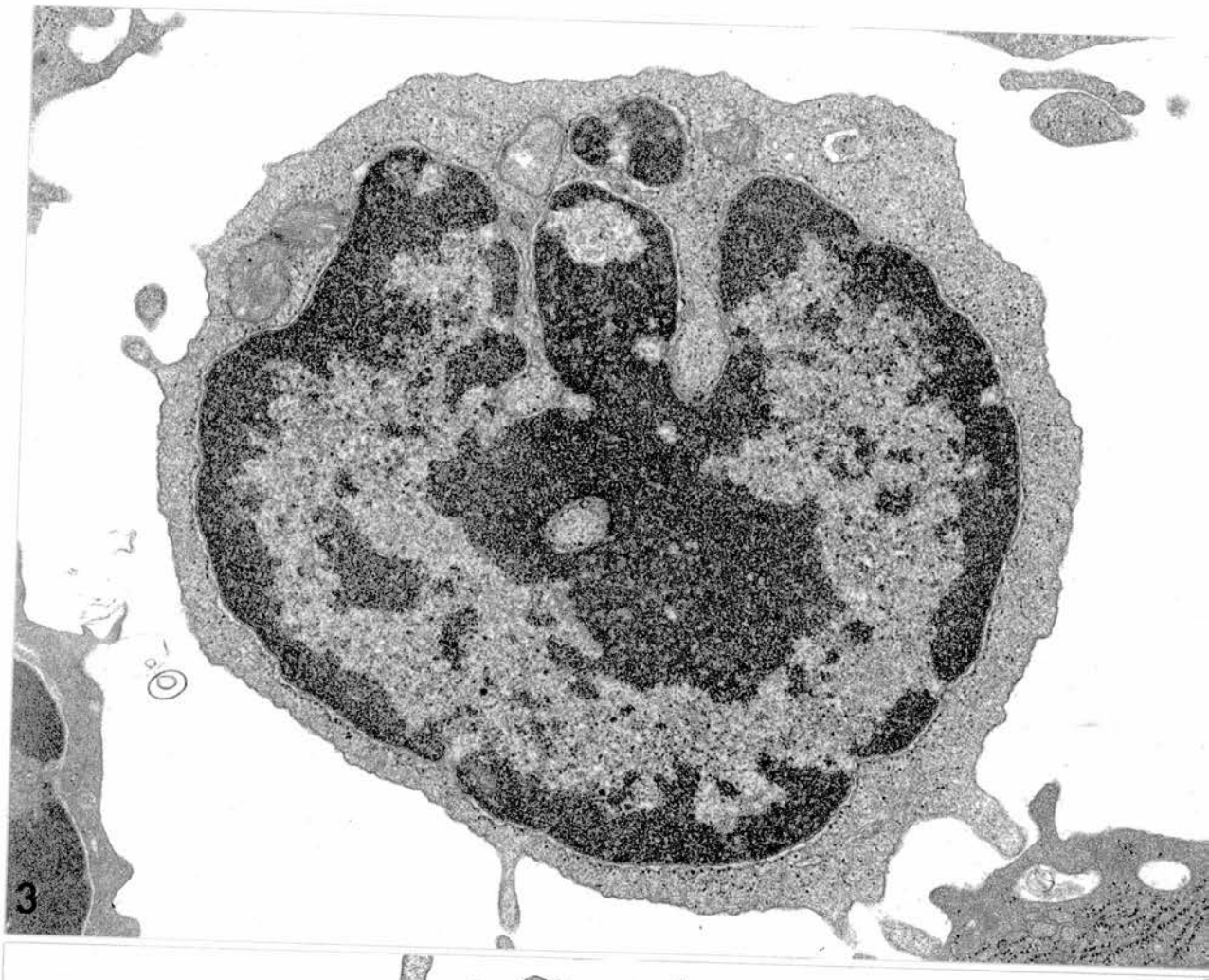


Fig. 2. Human peripheral blood lymphocytes with morphological diversity similar to the mouse lymphocytes in Fig. 1. Sequential fixation (cacodylate buffered osmium tetroxide). Postfixation in uranyl acetate has enhanced the detail of cell membranes. x 8,000.



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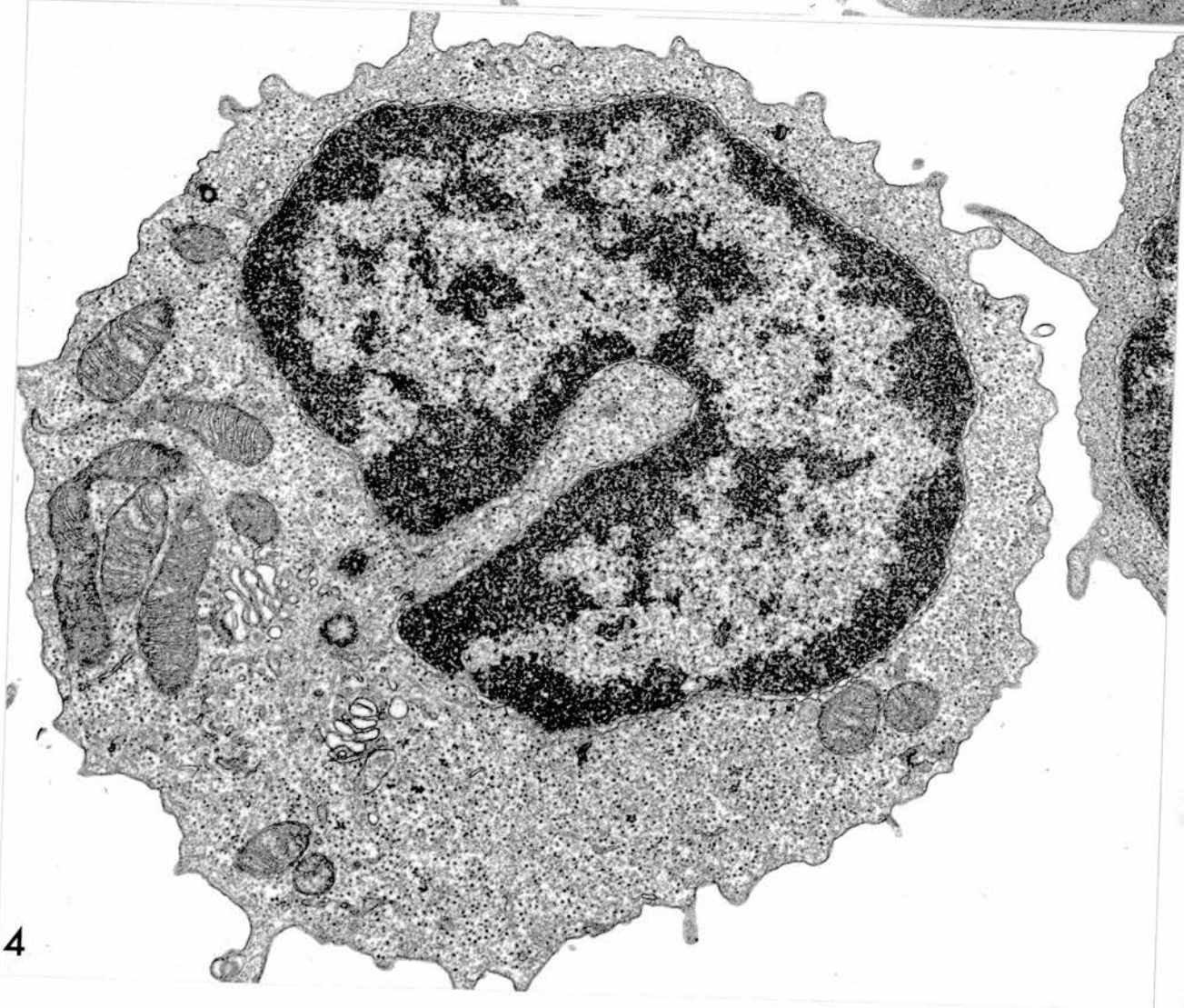

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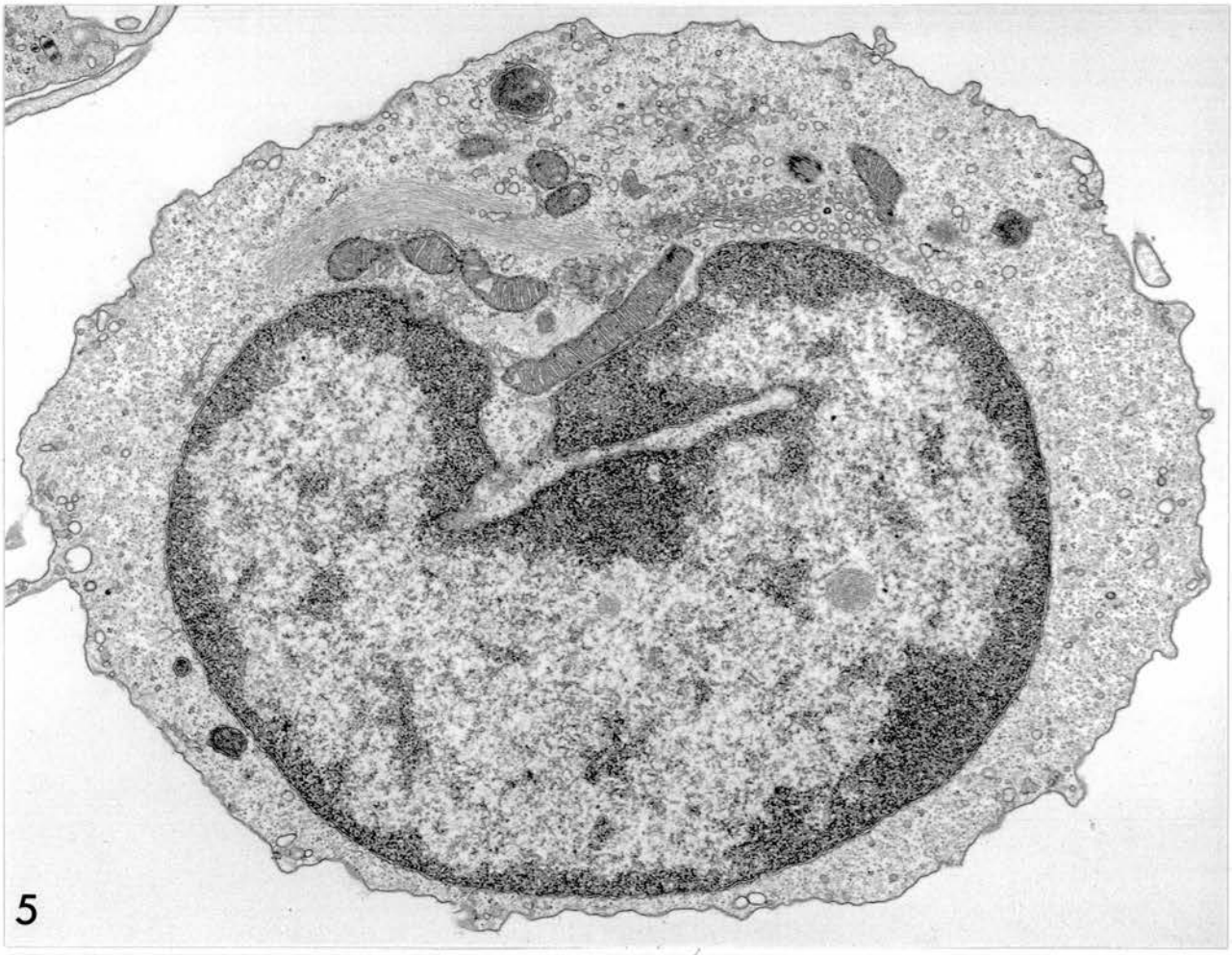
Figs. 3 & 4. Small lymphocytes. x 21,000.

Fig. 3. The irregularly shaped nucleus of a mouse peritoneal lymphocyte contains predominantly condensed chromatin. Few organelles other than ribosomes are visible in the thin rim of cytoplasm. Sequential fixation (phosphate buffered osmium tetroxide).

Fig. 4. A human peripheral blood lymphocyte. Golgi saccules and a centriole with its associated microtubules can be seen in the cytoplasm adjacent to the nuclear cleft. Free ribosomes are mainly single and mitochondria appear rounded or elongated. Sequential fixation (cacodylate buffered osmium tetroxide); postfixation in uranyl acetate.

Fig. 5. A large lymphocyte from mouse peritoneal cavity. Longitudinally sectioned 10 nm filaments interweave between mitochondria opposite the forked nuclear invagination. Two simple fibrillar nuclear bodies are present. Simultaneous fixation. x 13,000.

Fig. 6. A medium-sized, human peripheral blood lymphocyte. Situated below the pair of centrioles (which are more usually observed with the planes of their long axes perpendicular to each other) is a bundle of transversely sectioned 10 nm filaments. Occasional filaments which have been cut longitudinally can be seen in the perinuclear region of the cytoplasm and there is a multivesicular body close to the nuclear cleft. Simultaneous fixation. x 15,000.



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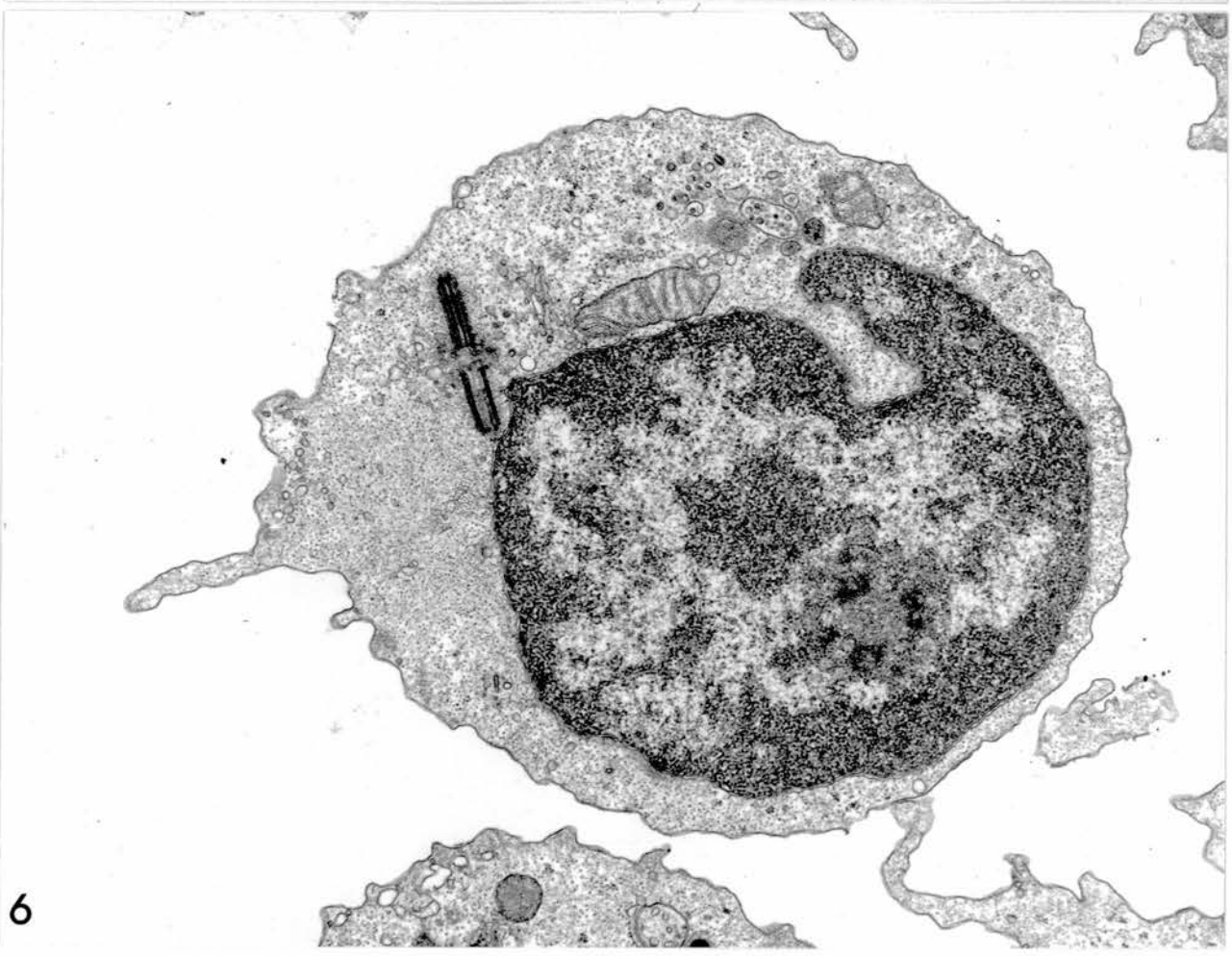

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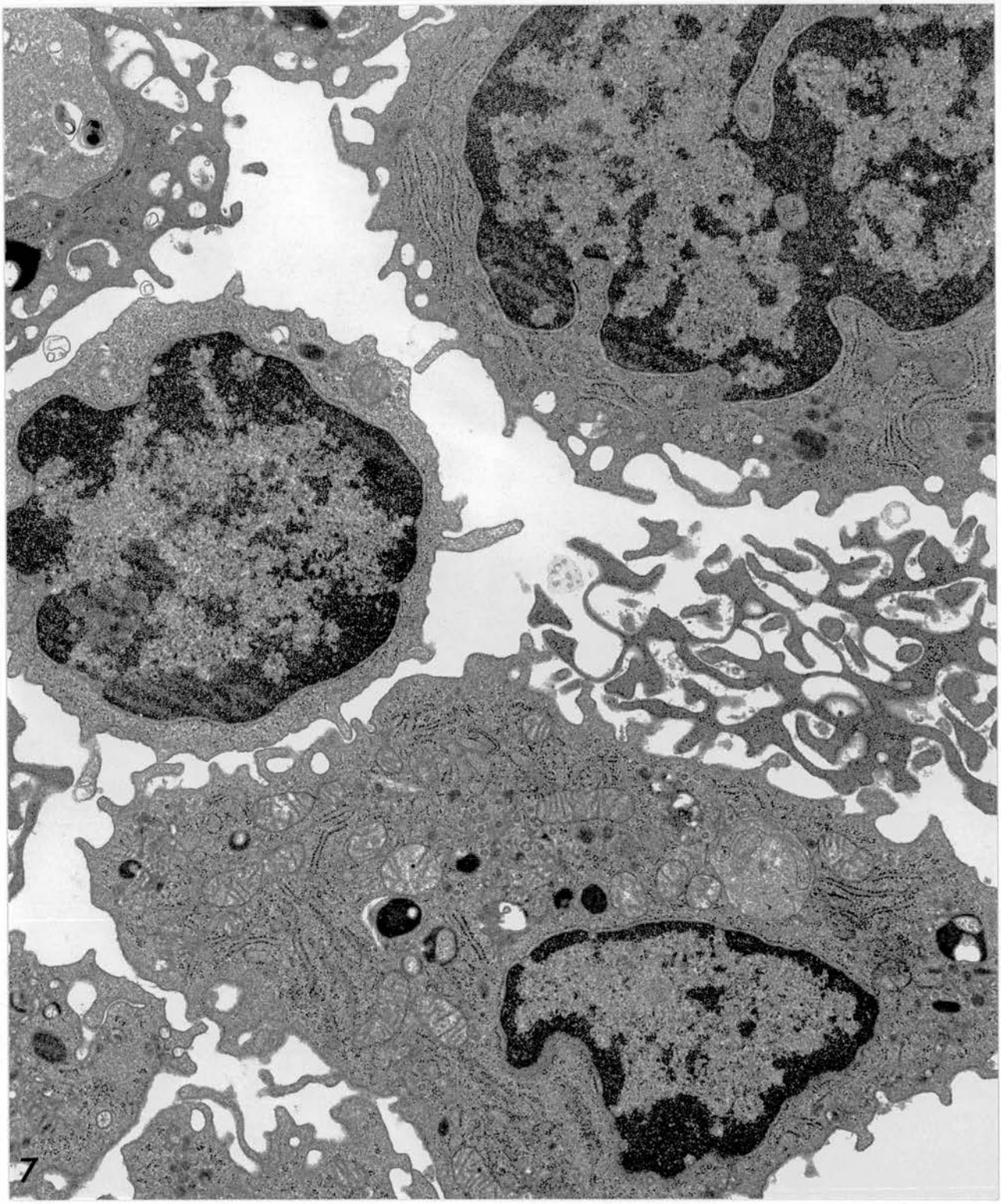


Fig. 7. Mouse peritoneal cells. A small lymphocyte lies to the left. On the right are 2 macrophages whose dense cytoplasm contains numerous free ribosomes, cisternae of rough endoplasmic reticulum, mitochondria and lysosomes. Sequential fixation (phosphate buffered osmium tetroxide). x 15,000.

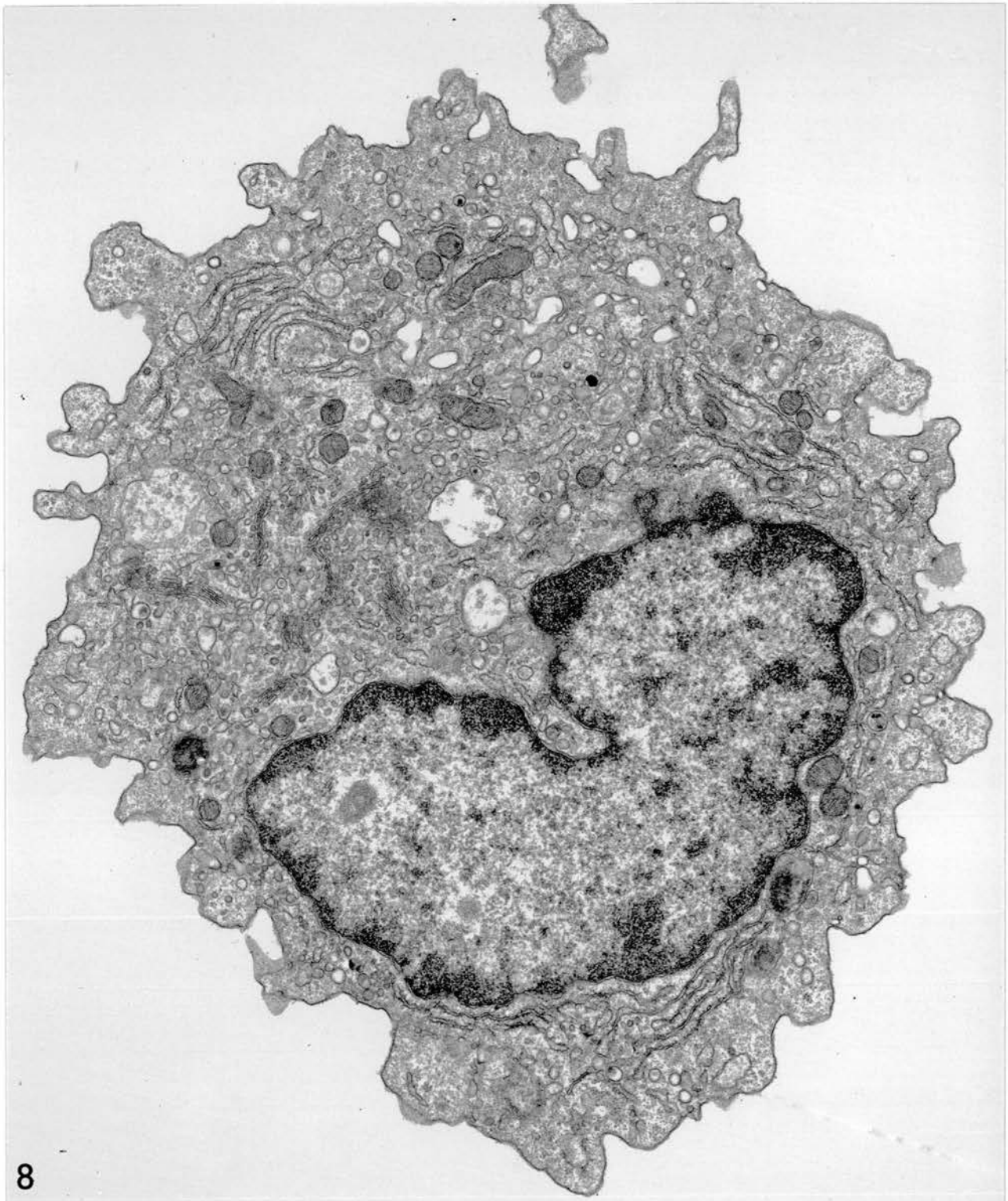
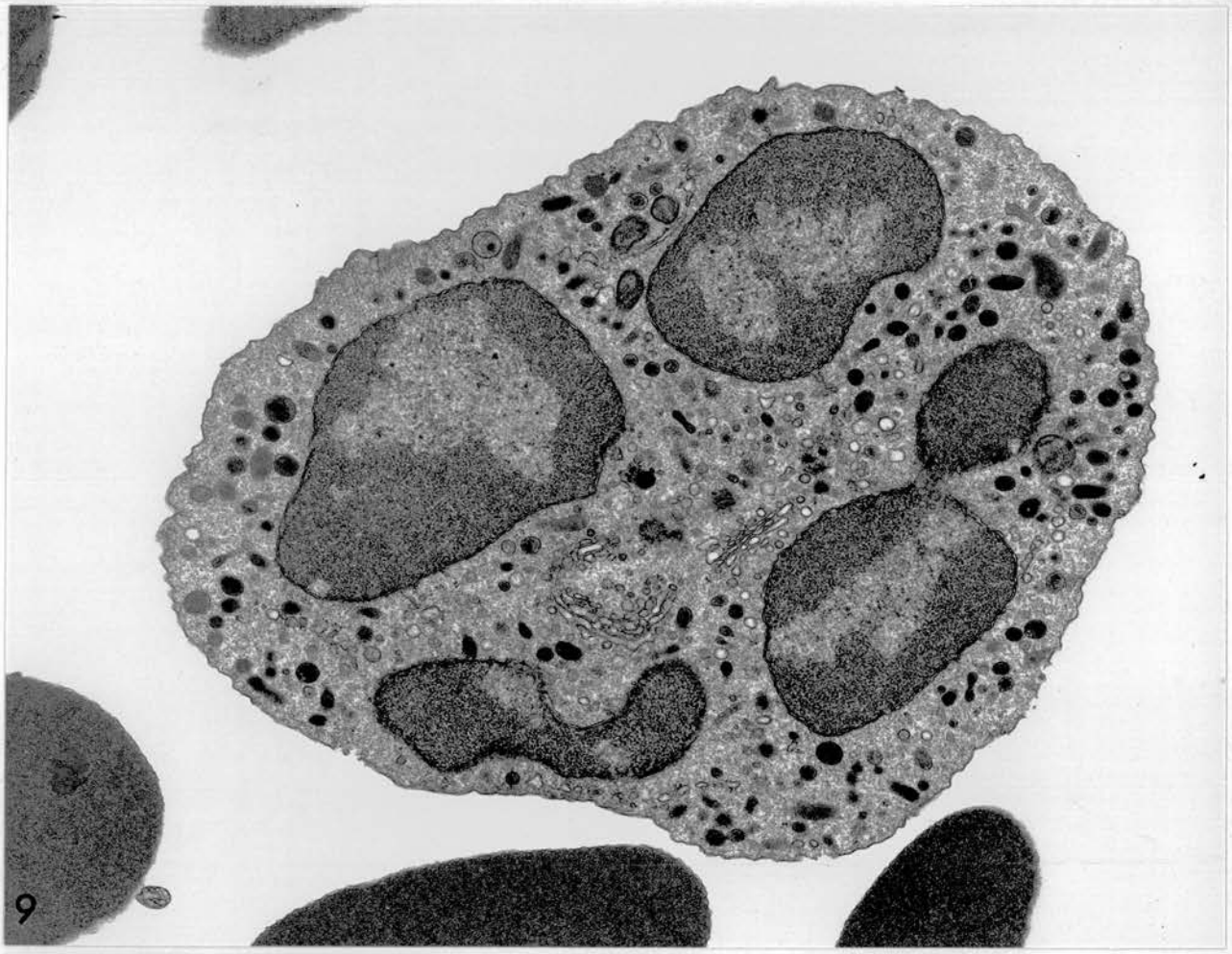


Fig. 8. A mouse peritoneal macrophage. Two simple fibrillar bodies are present in the typical reniform nucleus; the small amount of condensed chromatin is marginated. In addition to multiple stacks of Golgi saccules and cisternae of rough endoplasmic reticulum, numerous vesicles and vacuoles are apparent. Simultaneous fixation. Note the appearance after simultaneous fixation compared with the sequential method (Fig. 7). x 15,000.

Fig. 9. A neutrophil from mouse peripheral blood. Four lobes of the nucleus surround the Golgi region and diplosome. The cytoplasm is filled with numerous primary and neutrophilic or specific granules and occasional cisternae of rough endoplasmic reticulum. Simultaneous fixation. x 15,000.

Fig. 10. A human peripheral blood neutrophil. A thin strand containing condensed chromatin joins 2 of the nuclear lobes. Primary and specific granules, together with other cytoplasmic organelles, are absent from the filamentous region of cytoplasm forming the pseudopodium to the left of the cell. Simultaneous fixation. x 15,000.



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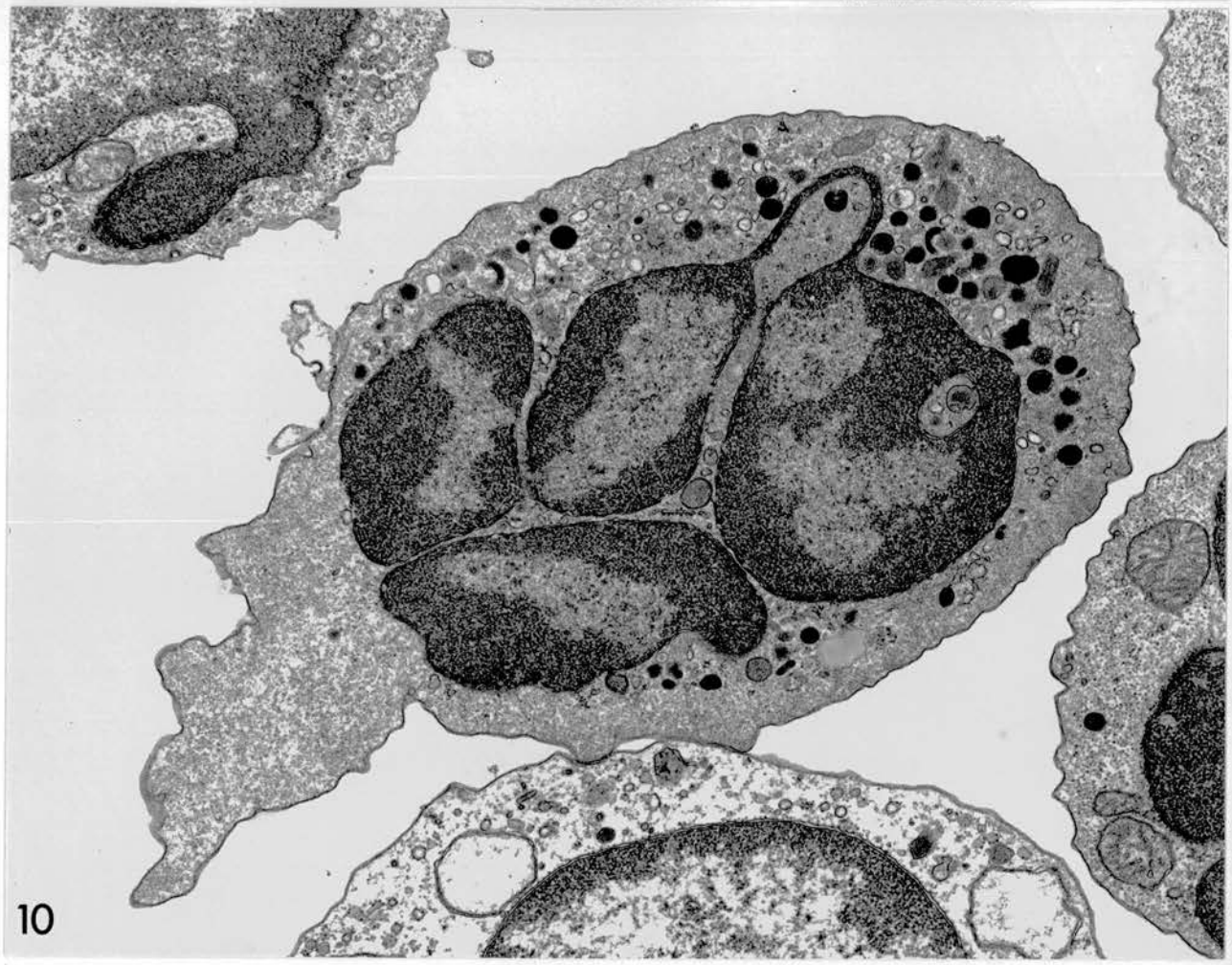

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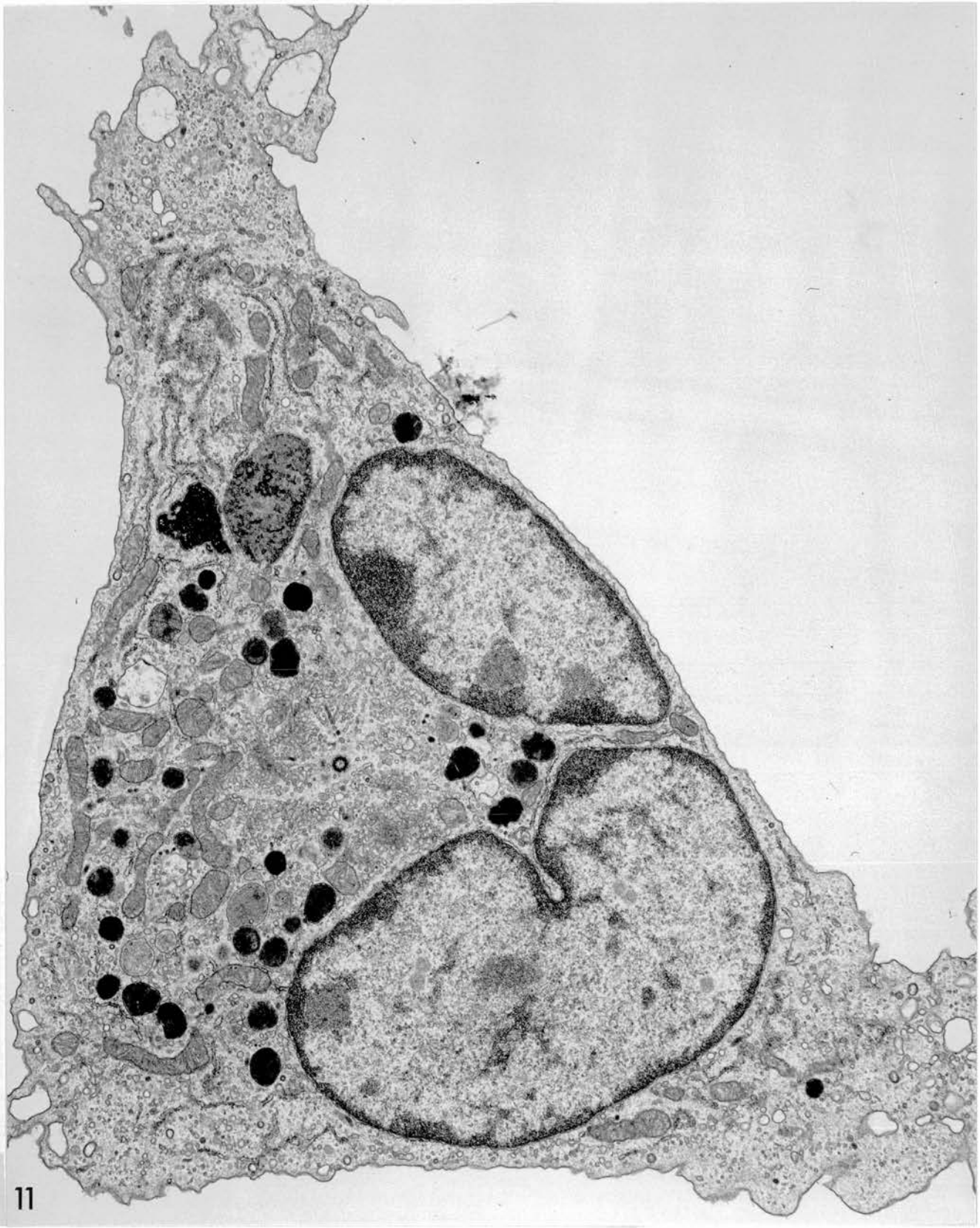

Fig. 11. A mouse adherent peritoneal macrophage. The triangular shape is characteristic of cells sectioned parallel to and near their attachment to the coverslip. To the left of the nucleus, which contains several fibrillar bodies, lies a centriole and the Golgi zone. Numerous primary and secondary lysosomes and mitochondria are also present. Simultaneous fixation. x 10,000.

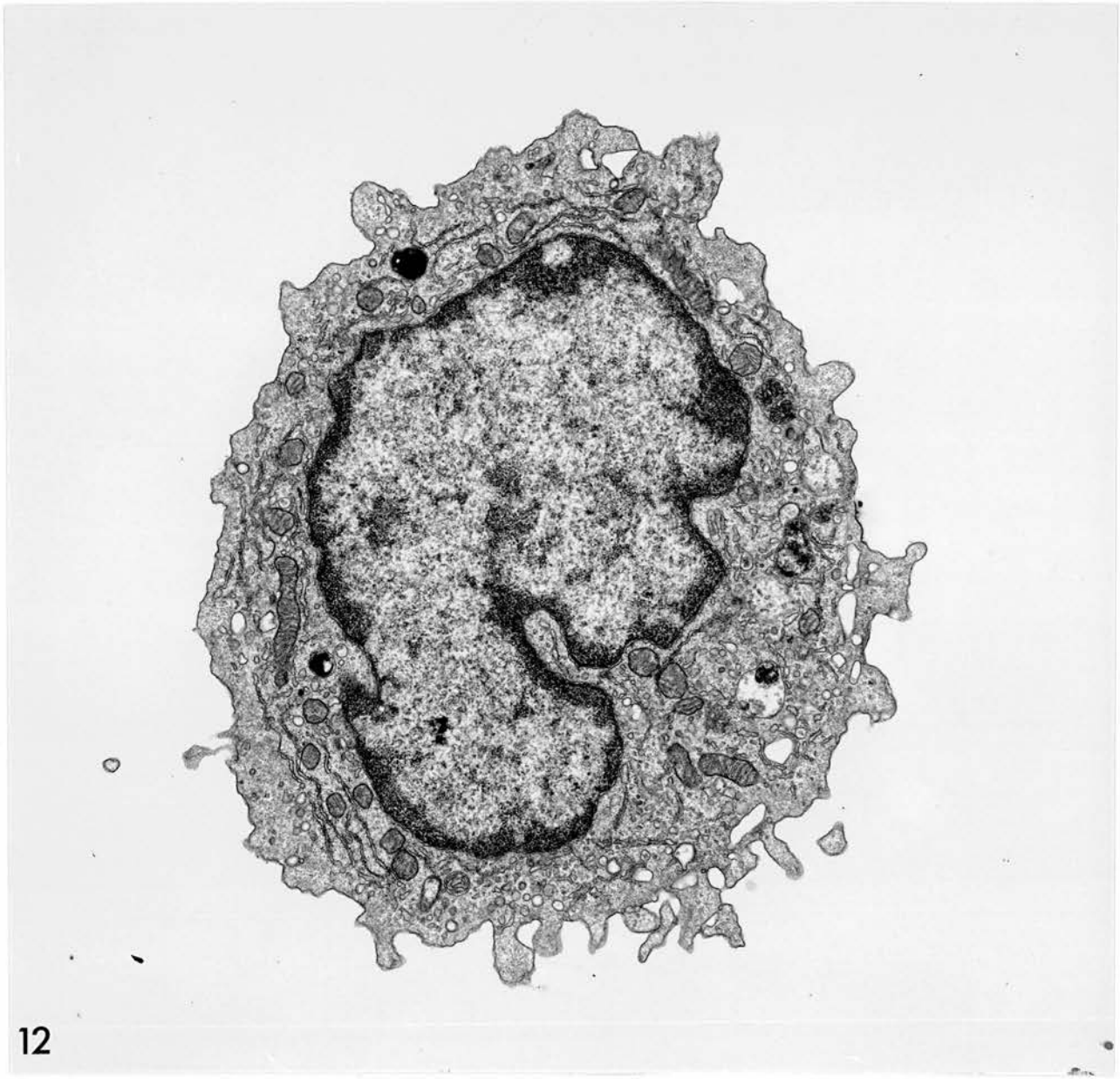

Fig. 12. A mouse adherent peritoneal macrophage. Macrophages sectioned parallel to and at some distance away from their attachment to the coverslip, as here, appear rounded or oval with the nucleus occupying a relatively large area. Numerous vesicles, cisternae of rough endoplasmic reticulum and mitochondria fill the cytoplasm. The "vacuoles" beneath the plasma membrane are probably invaginations of the cell surface. Simultaneous fixation. x 10,000.

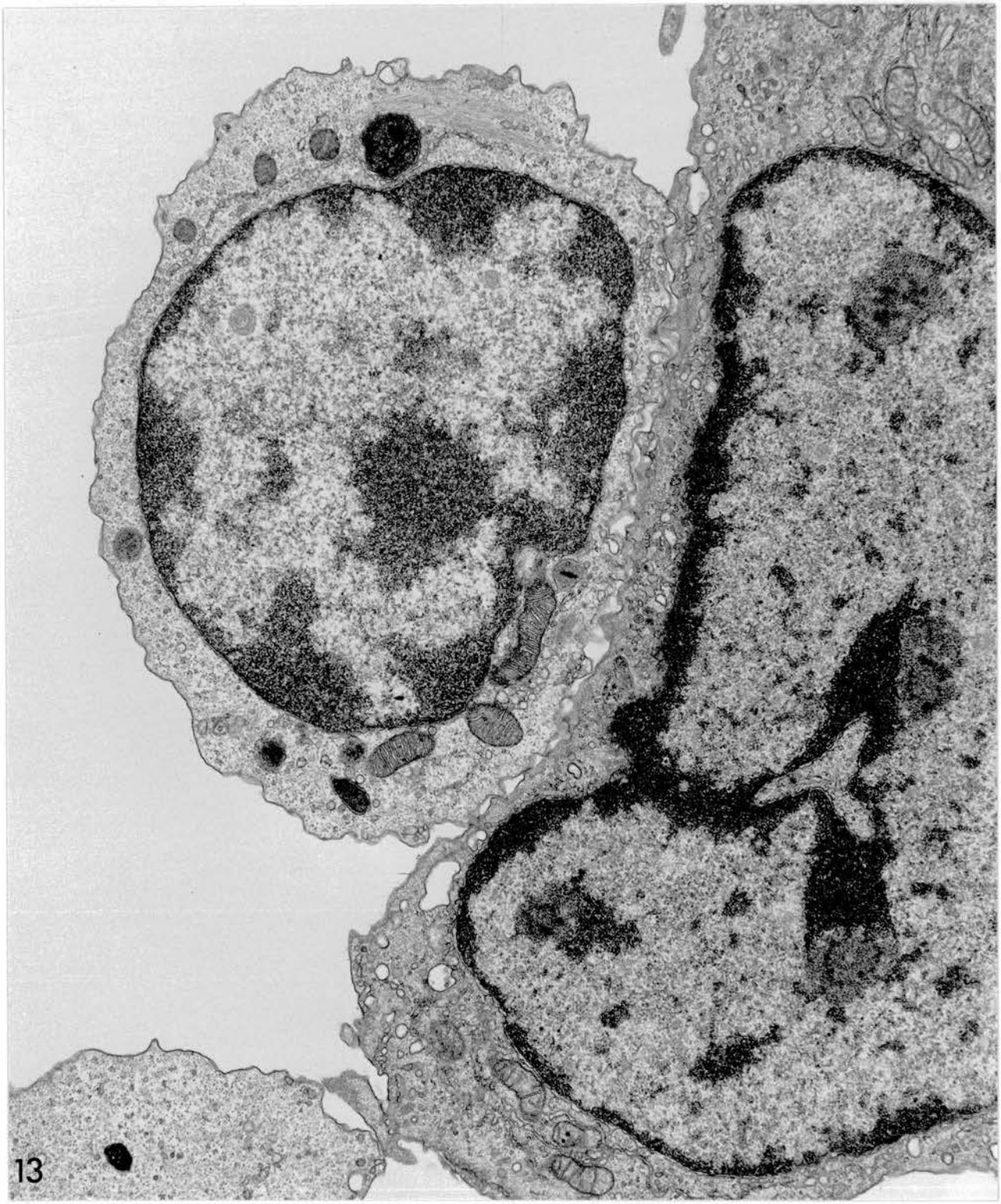

Fig. 13. Mouse adherent peritoneal cells. A lymphocyte can be seen attached to a macrophage sectioned near the coverslip. Simultaneous fixation. x 13,000.

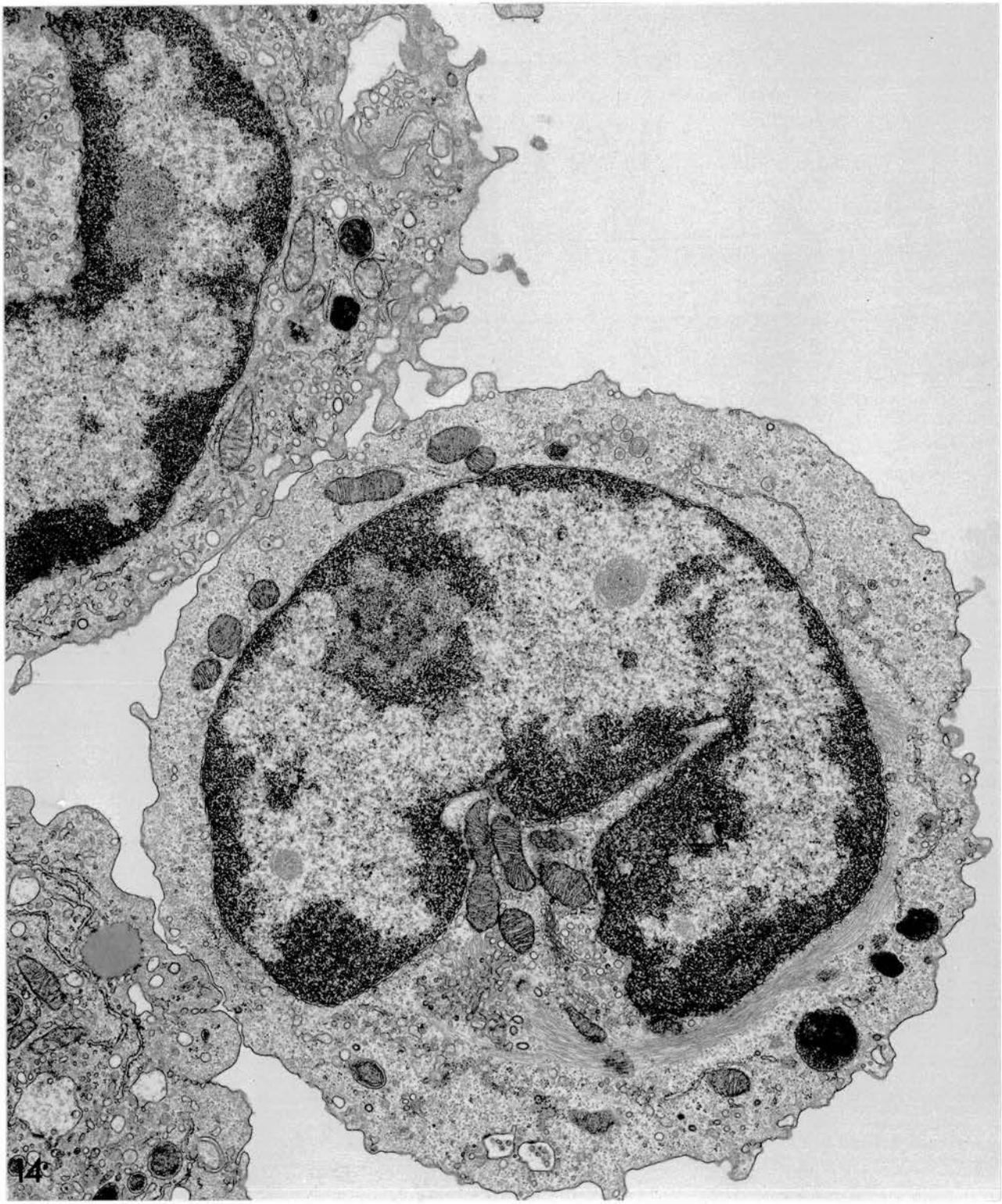

Fig. 14. Mouse adherent peritoneal cells. A lymphocyte, which has been sectioned away from the coverslip, is attached to 2 macrophages, only small portions of which are illustrated. The lymphocyte contains an unusually large bundle of perinuclear 10 nm filaments. Simultaneous fixation. x 13,000.

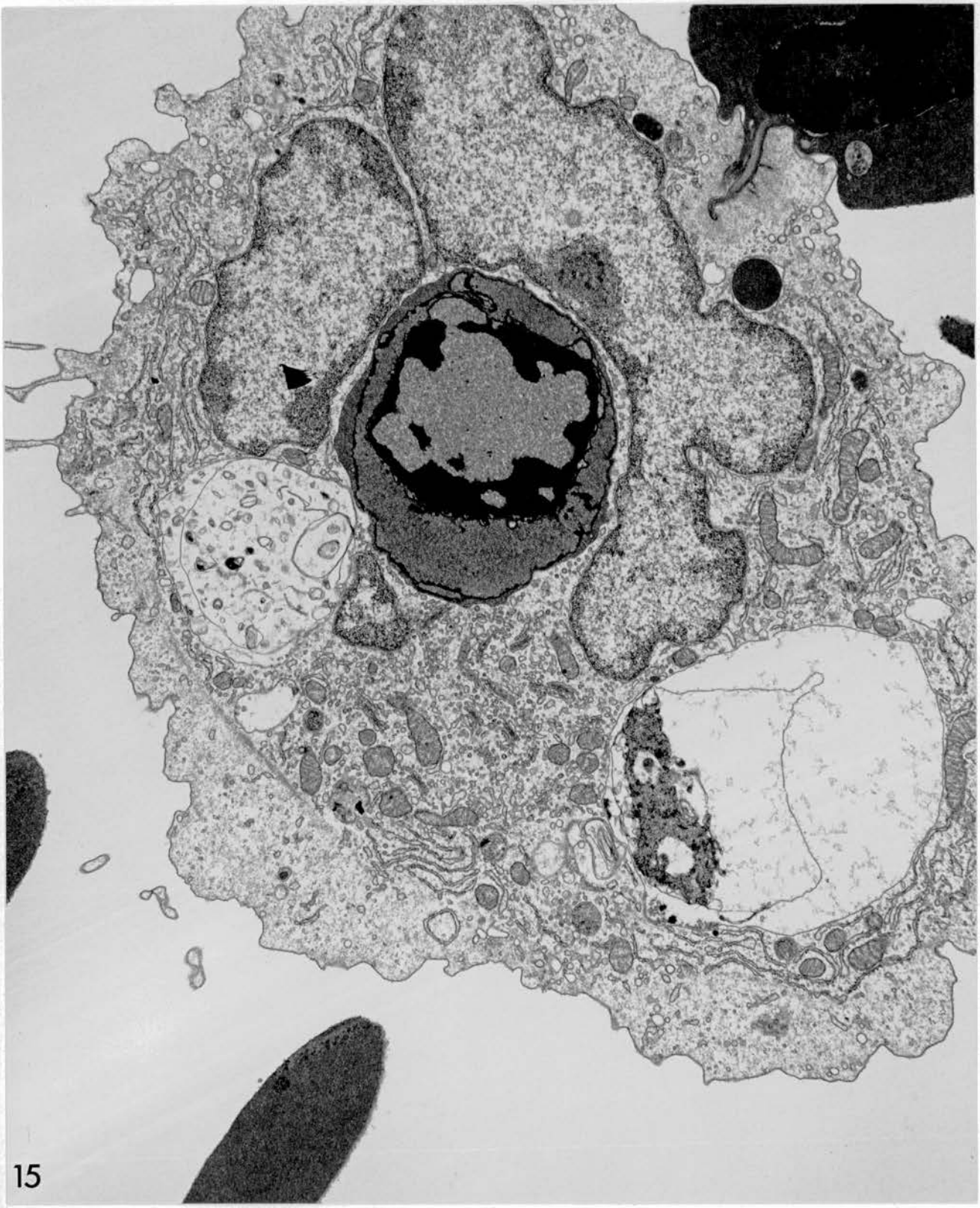

Fig. 15. Mouse adherent peritoneal cells + excess CRBC + guinea pig antiserum 1:1,000, 6 hours at 37°C. Part of a rosette showing 4 chicken erythrocytes around a macrophage; one of the erythrocytes is being ingested in the plane of section (top right) while the others are attached at different levels. The macrophage contains one recognisable, partially digested red cell and possibly the remnants of 2 others. Simultaneous fixation. x 10,000.

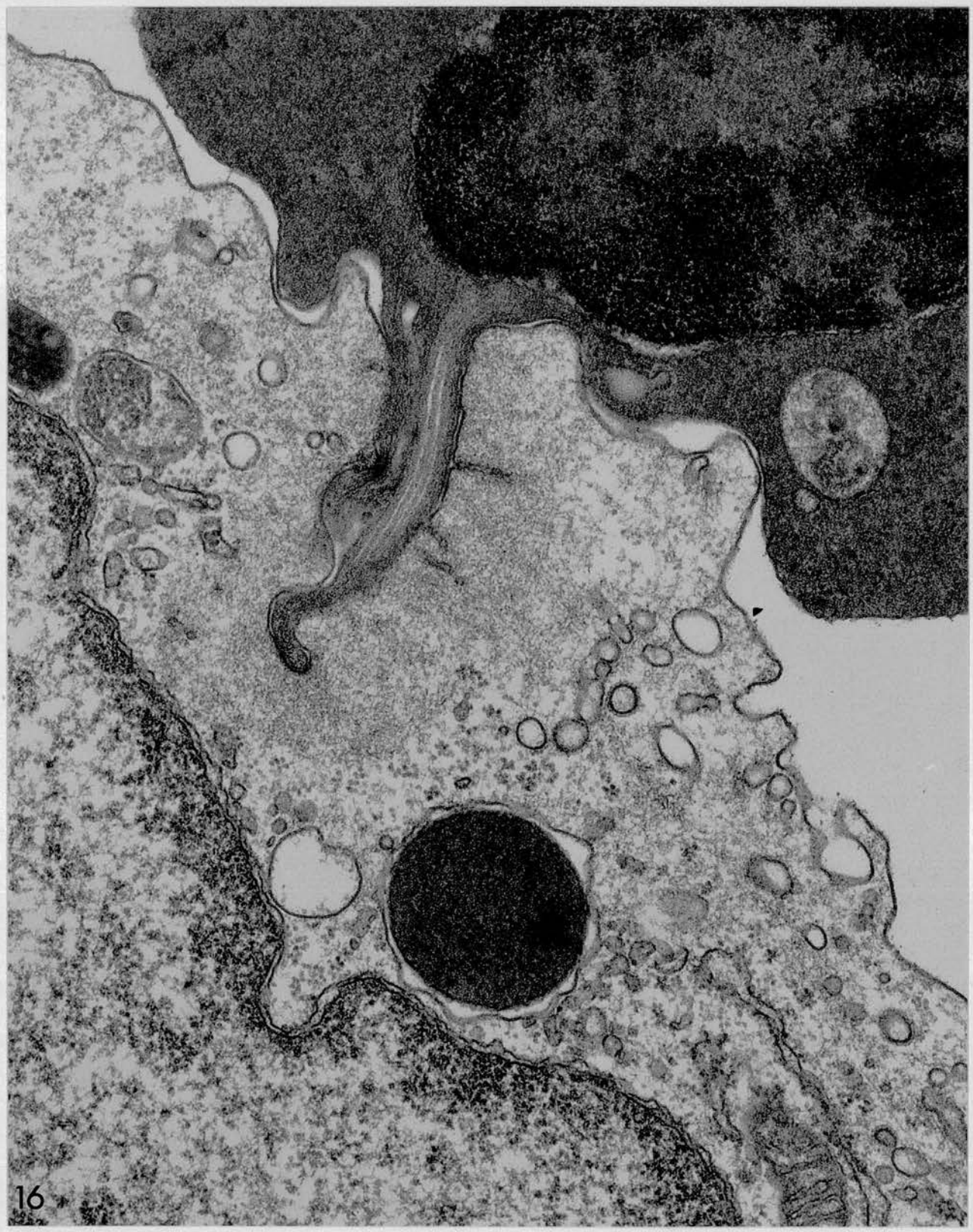

Fig. 16. Detail of erythrocyte ingestion shown in Fig. 15. Cytoplasmic organelles other than microfilaments are absent from that region of the mouse macrophage actively involved in phagocytosis. Microtubules of the equatorial marginal band can be seen in longitudinal section in the portion of chicken red cell being ingested. x 38,000.

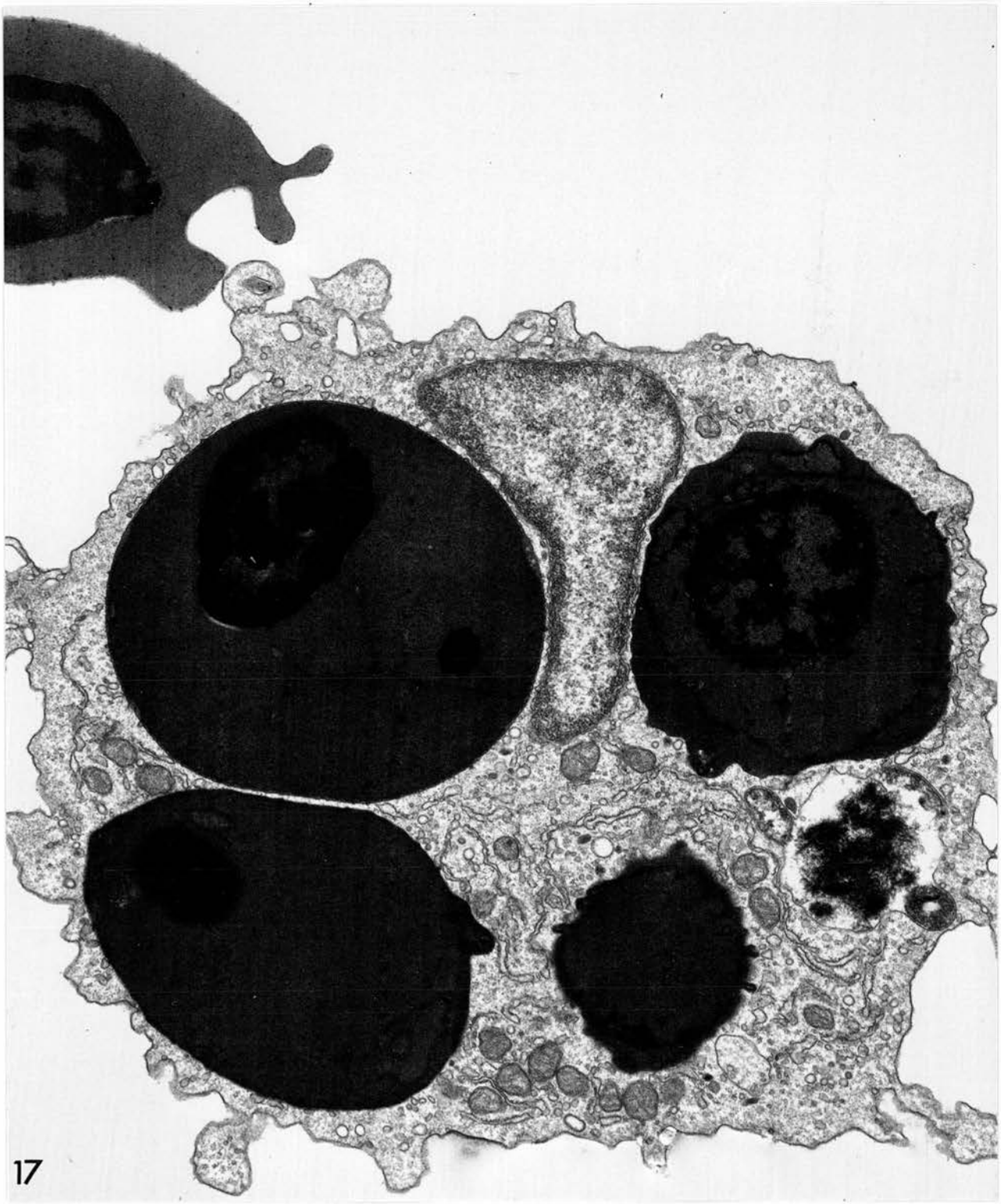

Fig. 17. Mouse adherent peritoneal cells + excess CRBC + guinea pig antiserum 1:1,000, 6 hours at 37°C. A single, distorted red cell is attached to a macrophage sectioned away from the coverslip and 5 phagocytosed erythrocytes are in various stages of degradation. Simultaneous fixation. x 13,000.

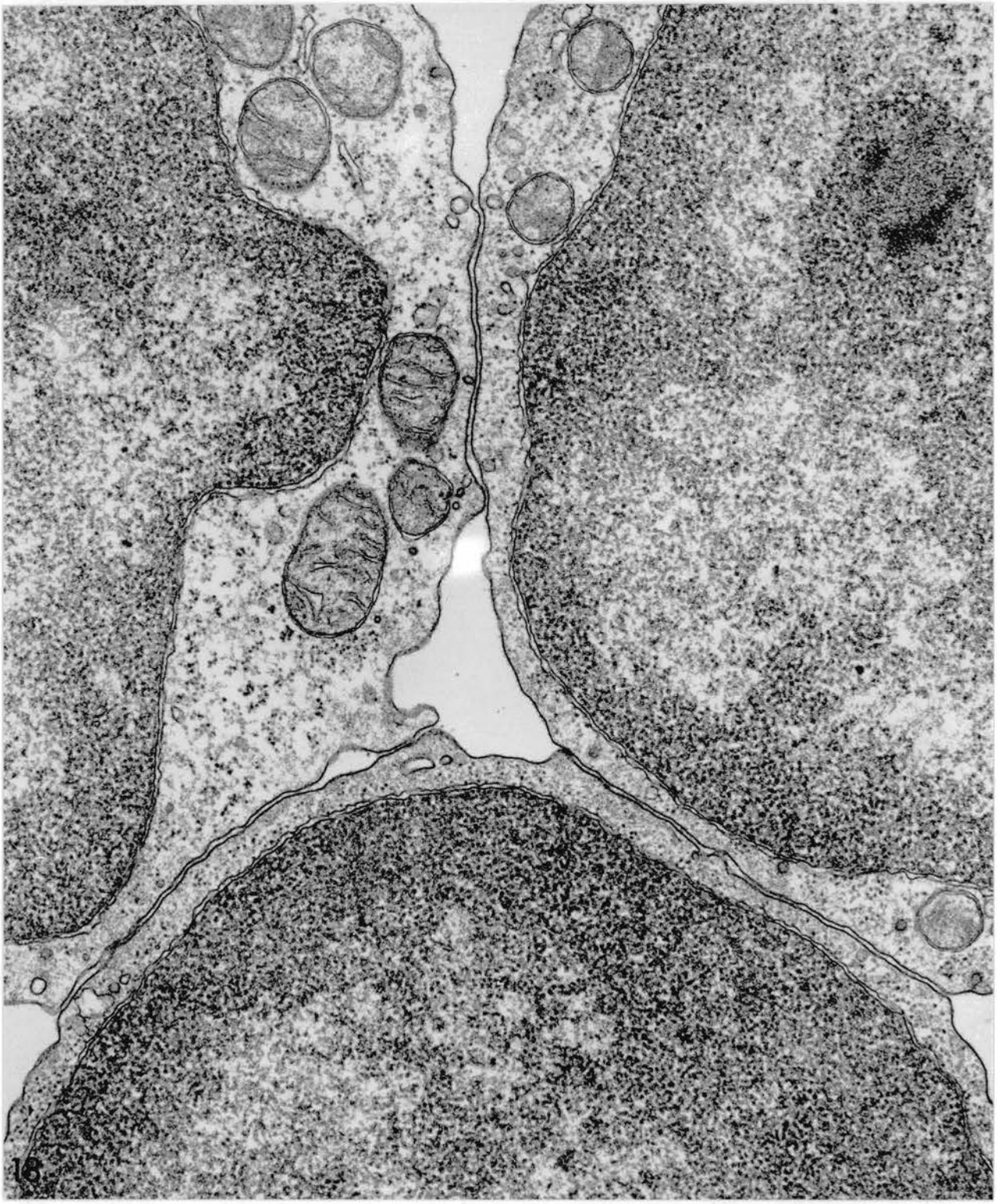

Fig. 18. Mouse spleen cell suspension, 3 hours at 37°C. Detail of the region of attachment between 3 lymphocytes in a clump. Amorphous material is present in the 15-20 nm intercellular space. Simultaneous fixation. x 32,000.

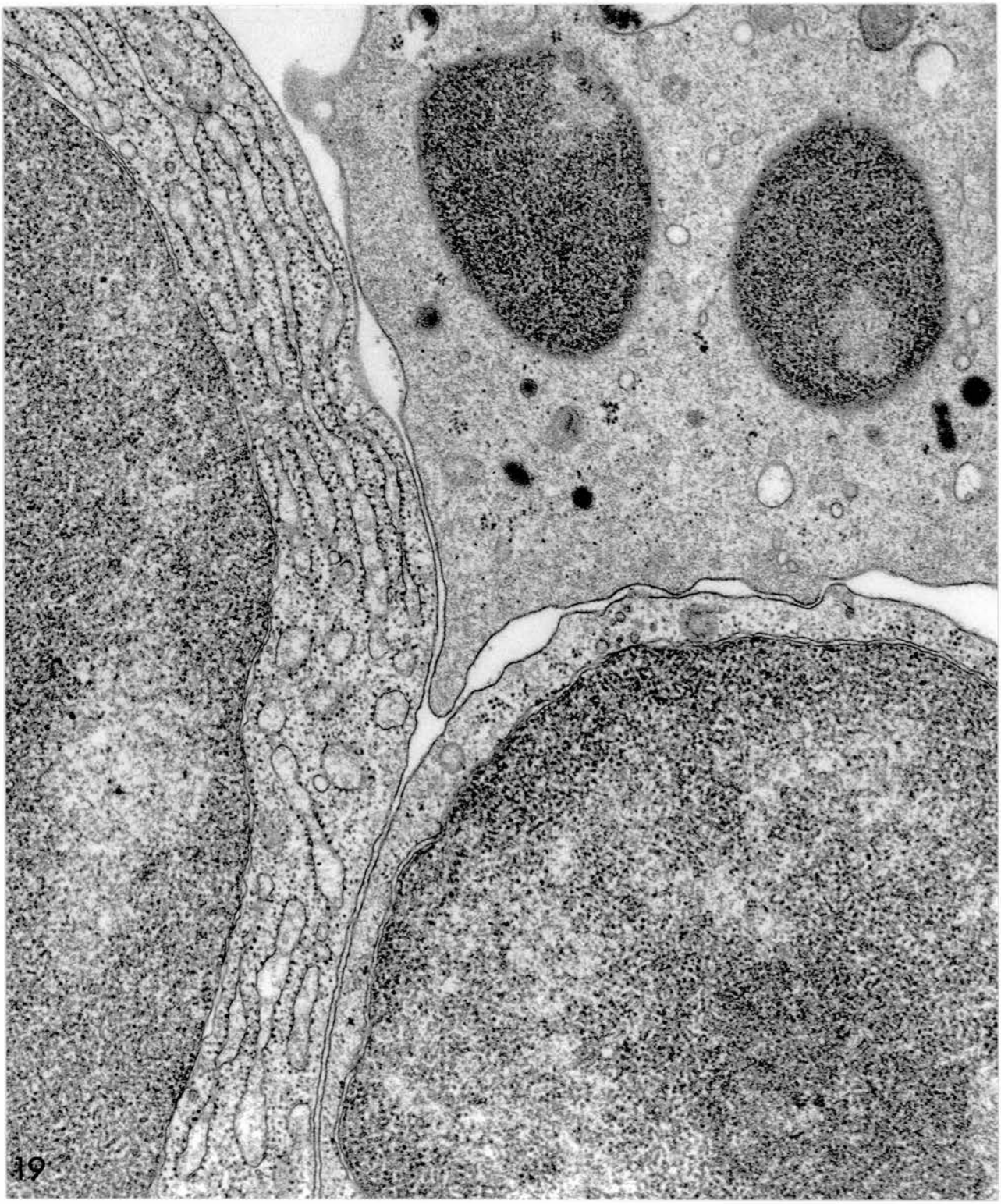

Fig. 19. Mixed cell types in a freshly prepared mouse spleen suspension showing details of contact between a plasma cell (left), a neutrophil (upper right) and a lymphocyte (lower right). A gap of 15-20 nm separates the cells. Simultaneous fixation. x 32,000.

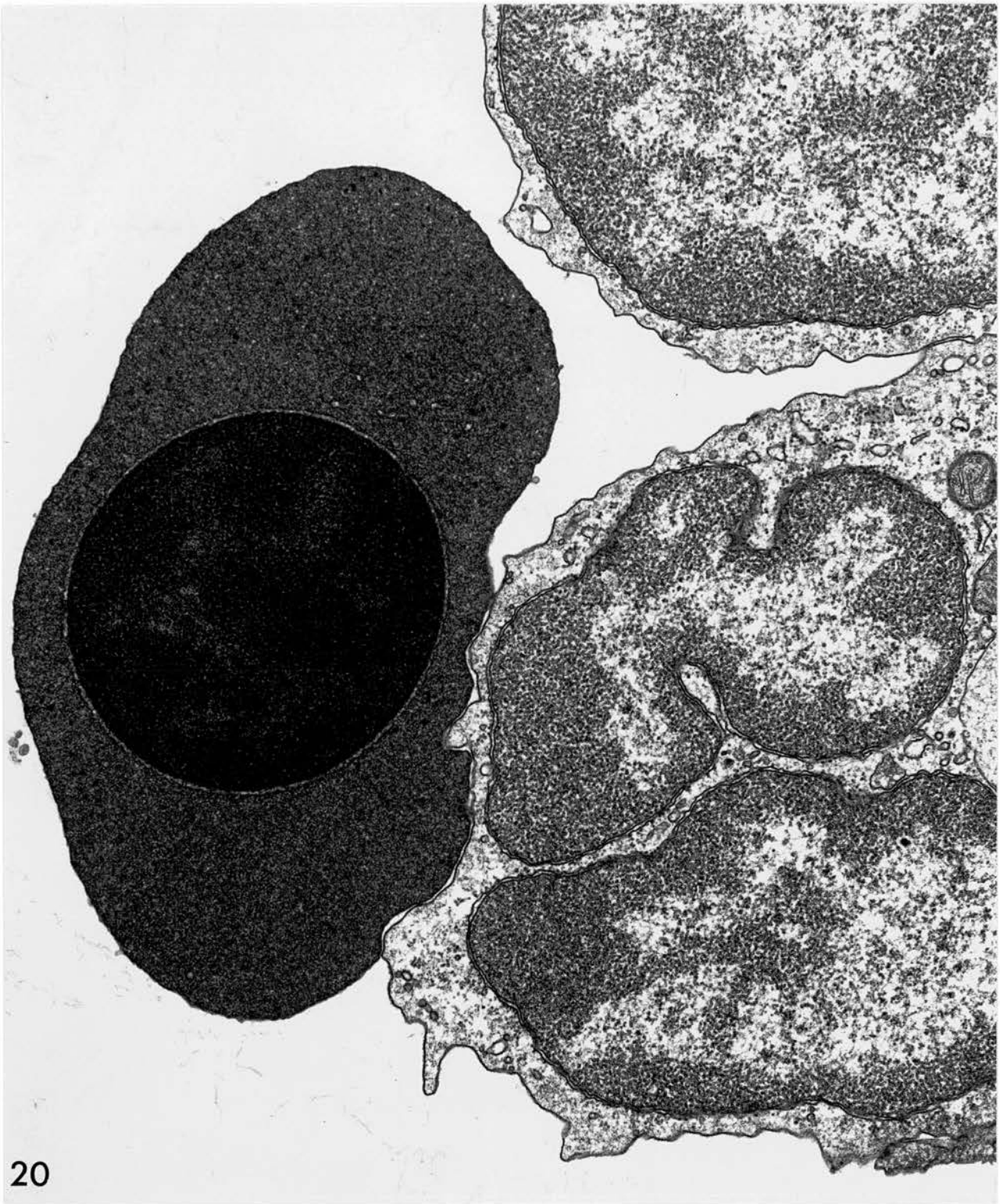

Fig. 20. Mouse spleen cells + CRBC (5:1) + rat normal serum 1:1,000, 15 minutes at 37°C. A slightly distorted attachment has formed between a chicken erythrocyte and a mouse lymphocyte. Simultaneous fixation. x 25,000.

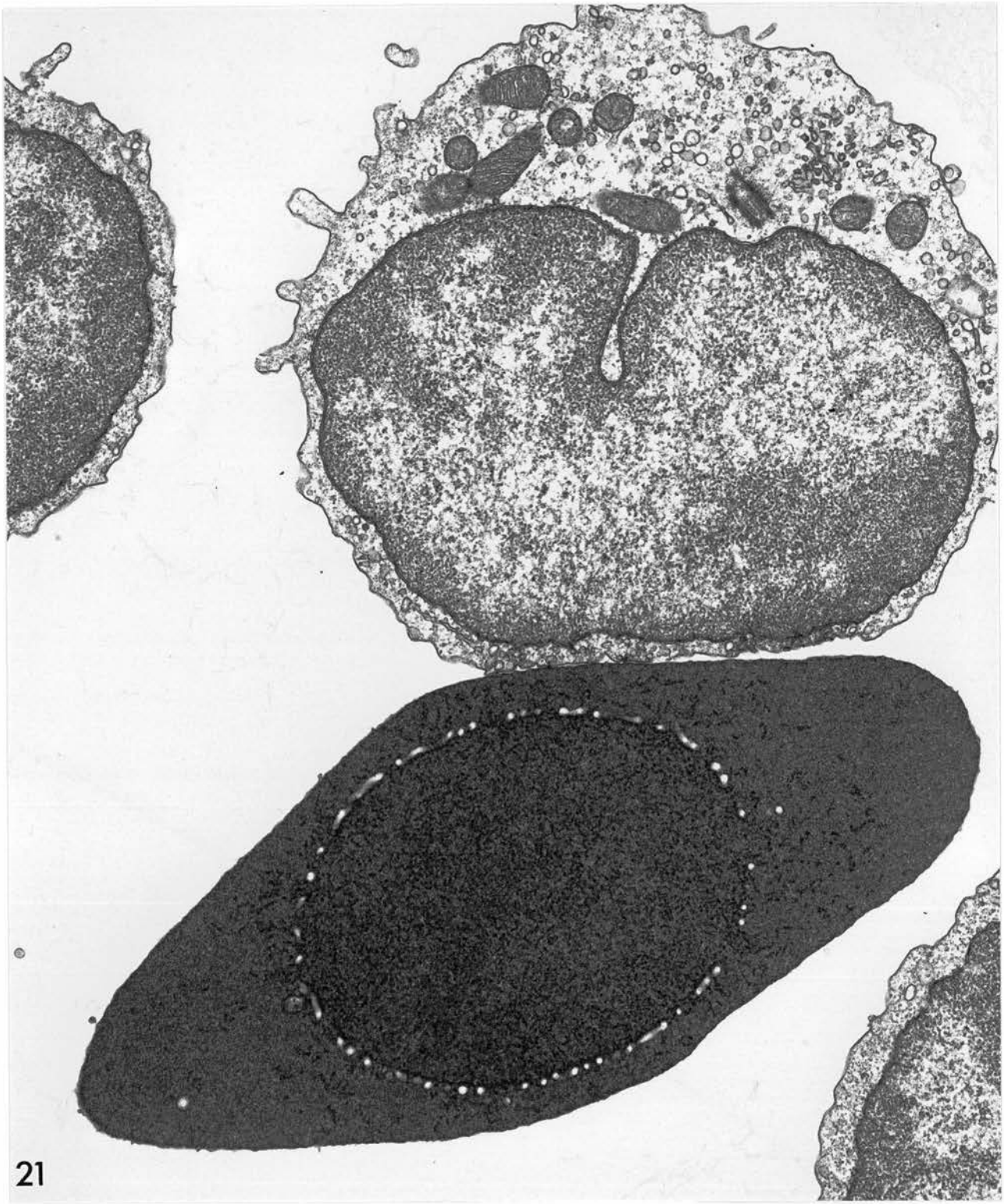

Fig. 21. Mouse spleen cells + CRBC (5:1) + rat normal serum 1:1,000, 30 minutes at 37°C. The surfaces of an erythrocyte and a lymphocyte are slightly flattened in the region of contact. Chromatin has leaked from the nucleus of the chicken red cell into the cytoplasm. Simultaneous fixation. x 18,000.

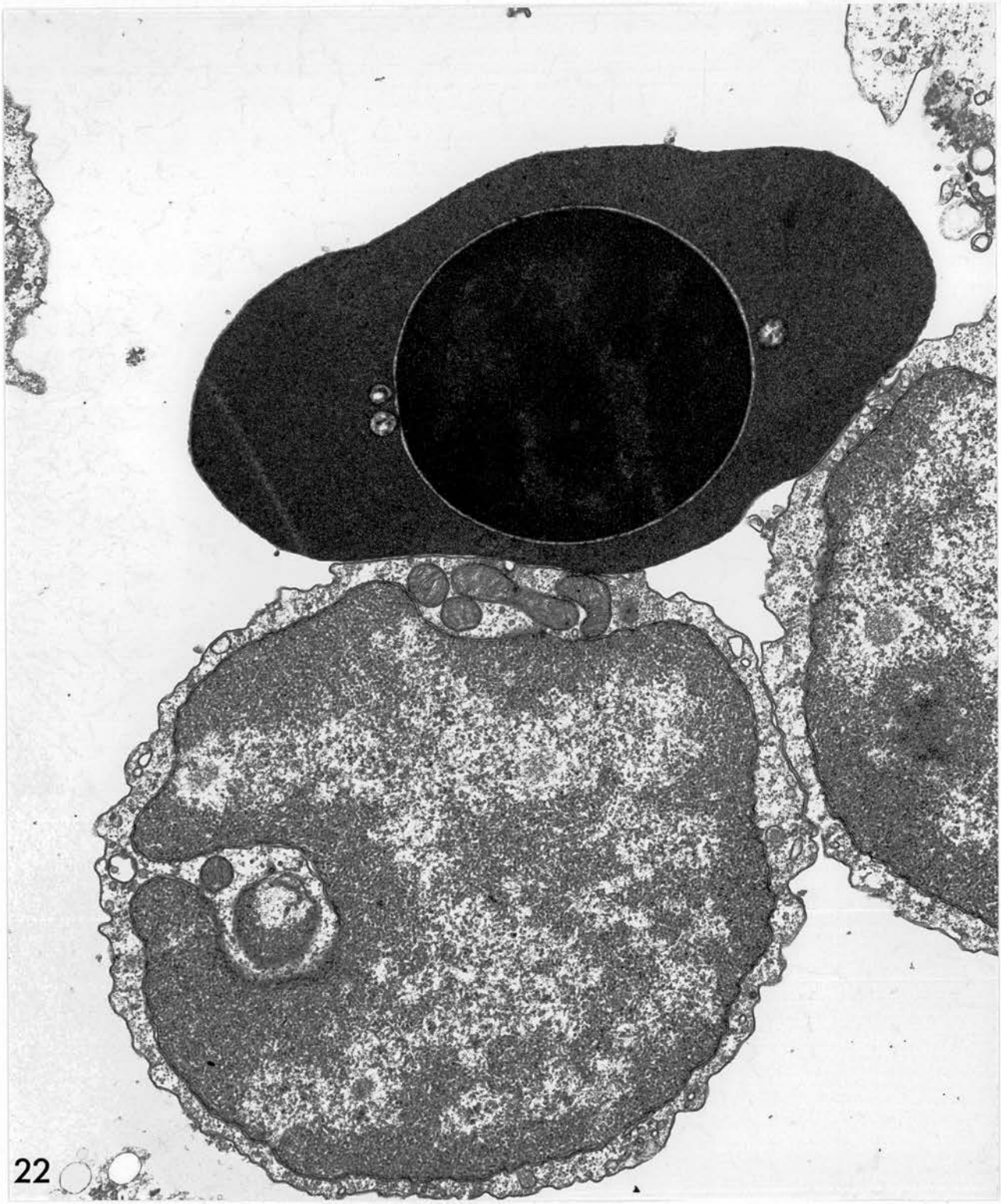

Fig. 22. Mouse spleen cells + CRBC (5:1) + rat antiserum 1:1,000, 30 minutes at 37°C. A target cell is attached to 2 mouse lymphocytes by flattened contacts. Simultaneous fixation. x 18,000.

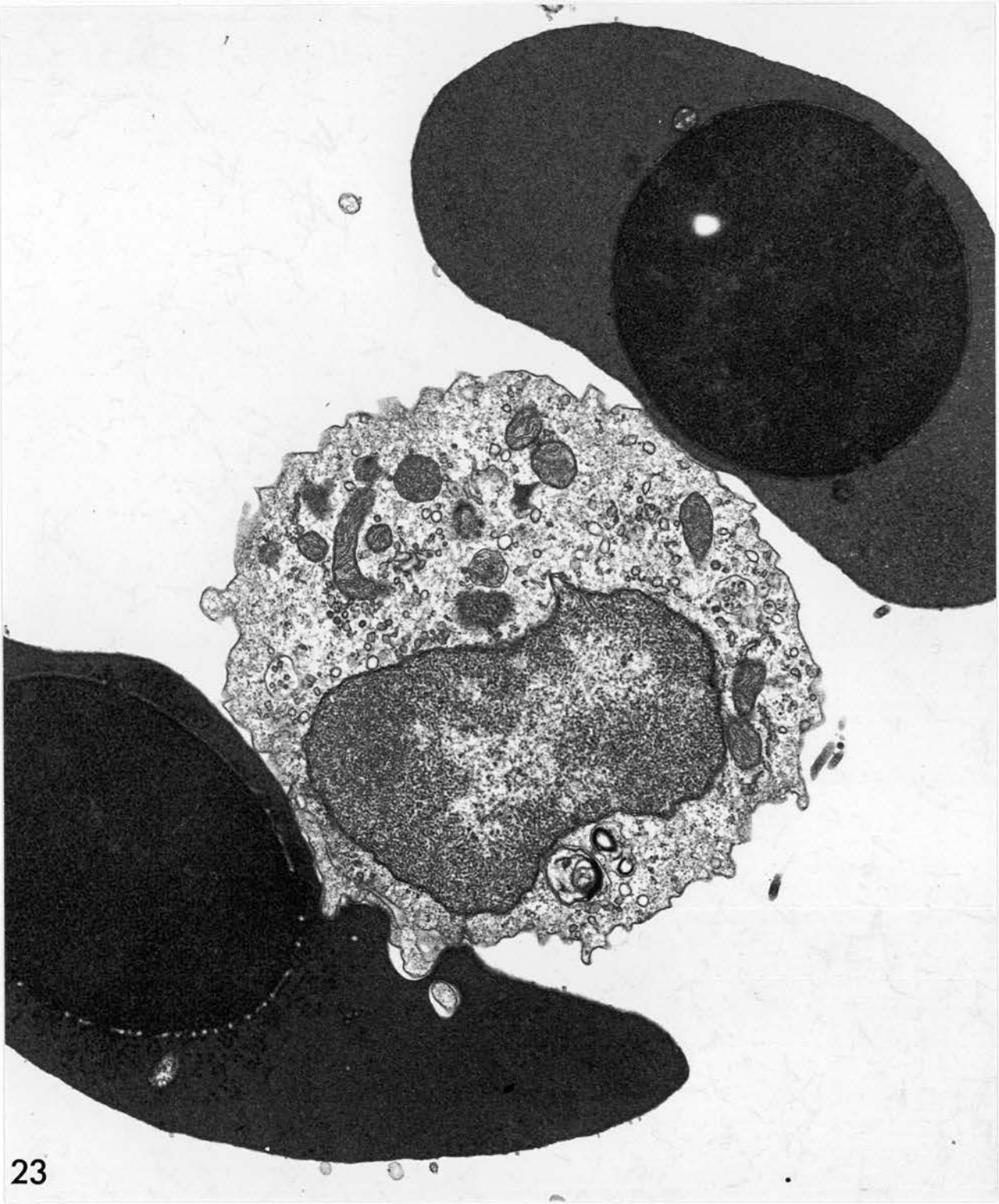

Fig. 23. Mouse spleen cells + CRBC (5:1) + rat antiserum 1:1,000, 30 minutes at 37°C. A flattened attachment (above) and a distorted contact (below) have formed between 2 chicken erythrocytes and a single mouse lymphocyte. Chromatin can be seen leaking from the nucleus of the lower chicken red cell. Simultaneous fixation. x 18,000.

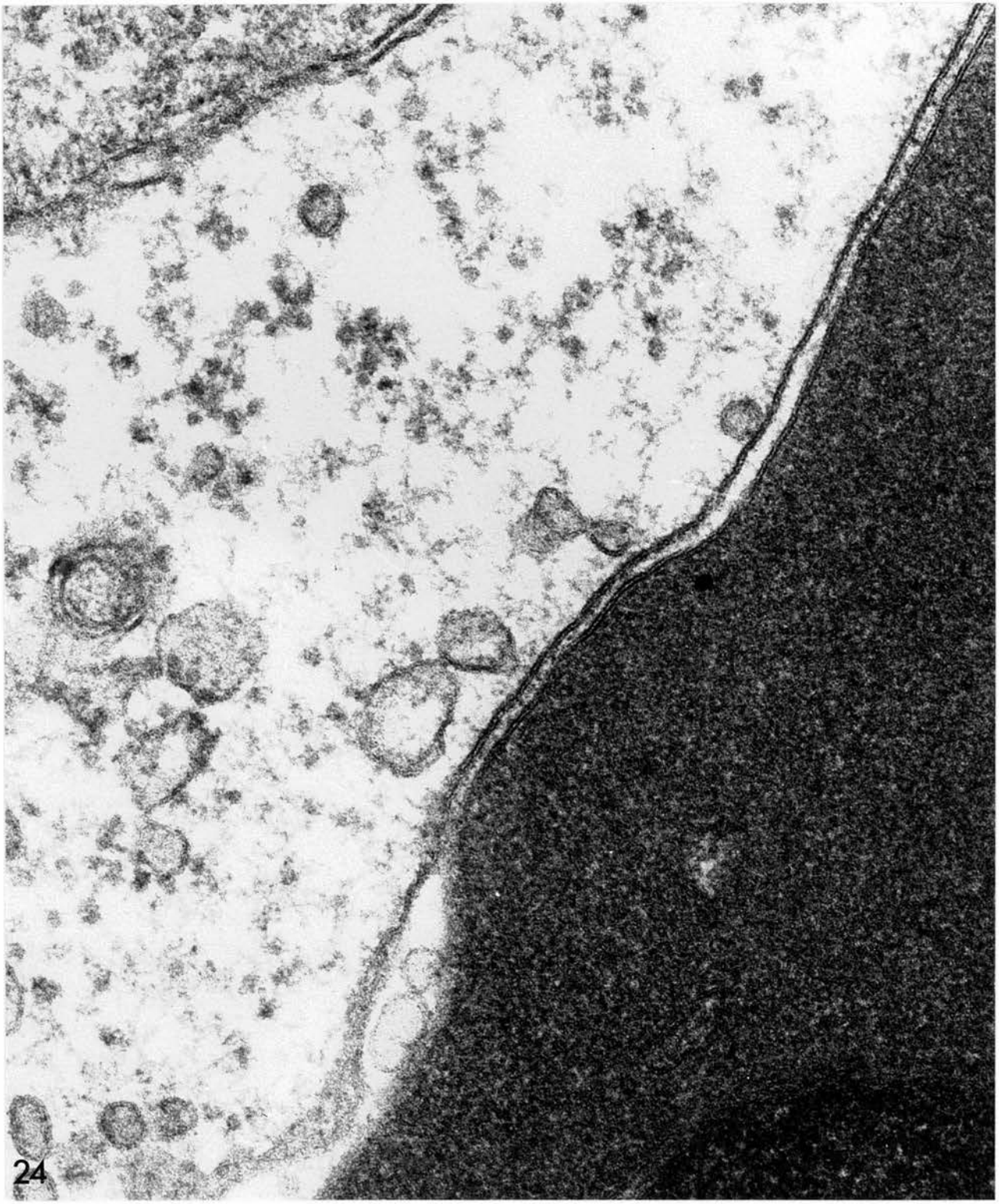

Fig. 24. Mouse spleen cells + CRBC (25:1) + guinea pig antiserum 1:1,000, 1 hour at 37°C. Detail of attachment of a chicken erythrocyte to a mouse lymphocyte. Where the apposing membranes are parallel, the cells are separated by a space of 7.5-10 nm containing electron-opaque material. Simultaneous fixation. x 150,000.

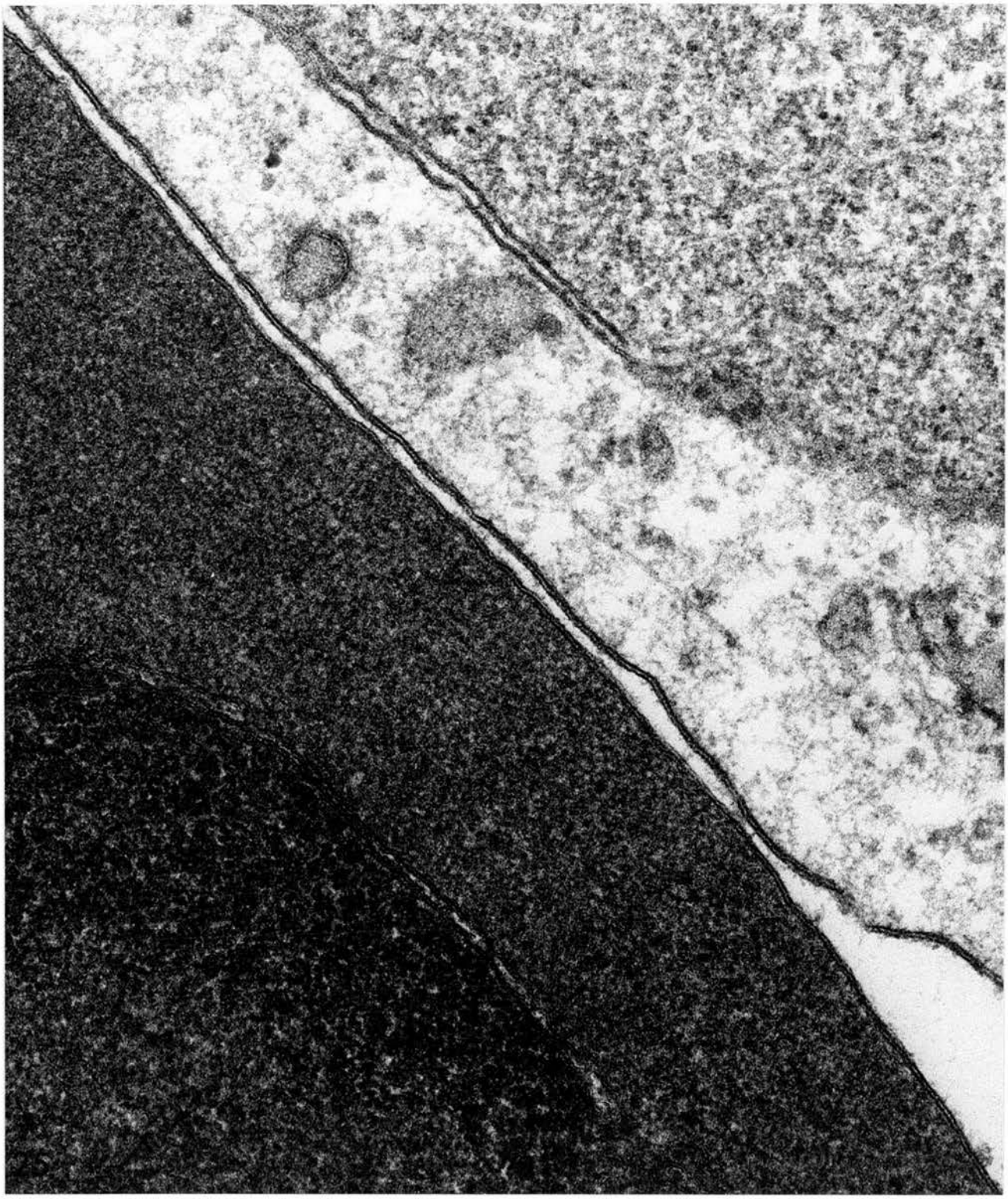

Fig. 25. Mouse spleen cells + CRBC (25:1) + guinea pig antiserum 1:1,000, 1 hour at 37°C. There is a 7.5-10 nm intercellular space between the parallel plasma membranes of a chicken erythrocyte and a mouse lymphocyte. Small amounts of slightly electron-opaque material are also present on the surface of both effector and target cell away from the region of contact. Simultaneous fixation. x 150,000.

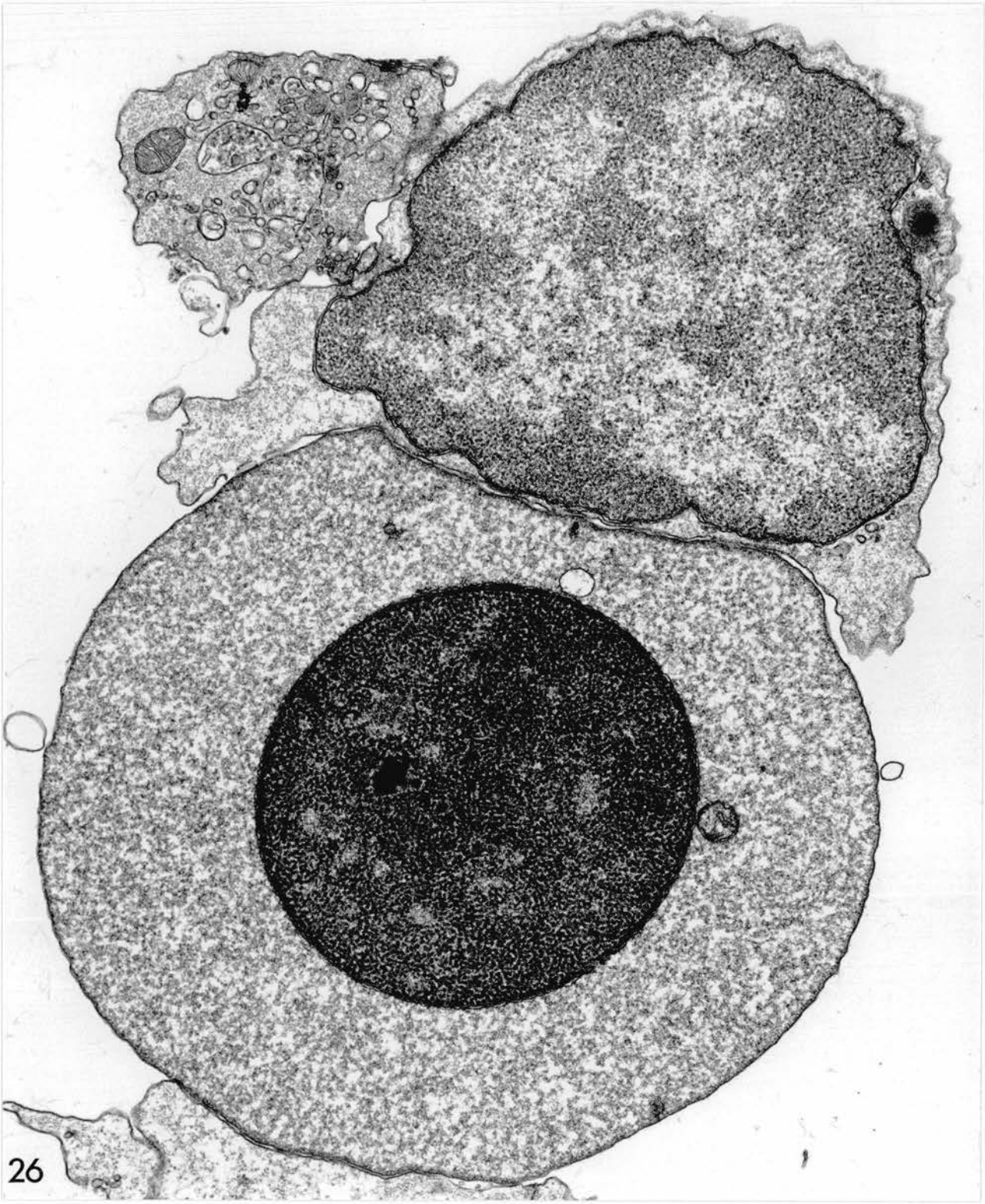

Fig. 26. Mouse spleen cells + CRBC (5:1) + rat antiserum 1:1,000, 15 minutes at 37°C. A swollen chicken erythrocyte with rarefied cytoplasm is attached to a mouse lymphocyte by a flattened contact. Simultaneous fixation. x 25,000.

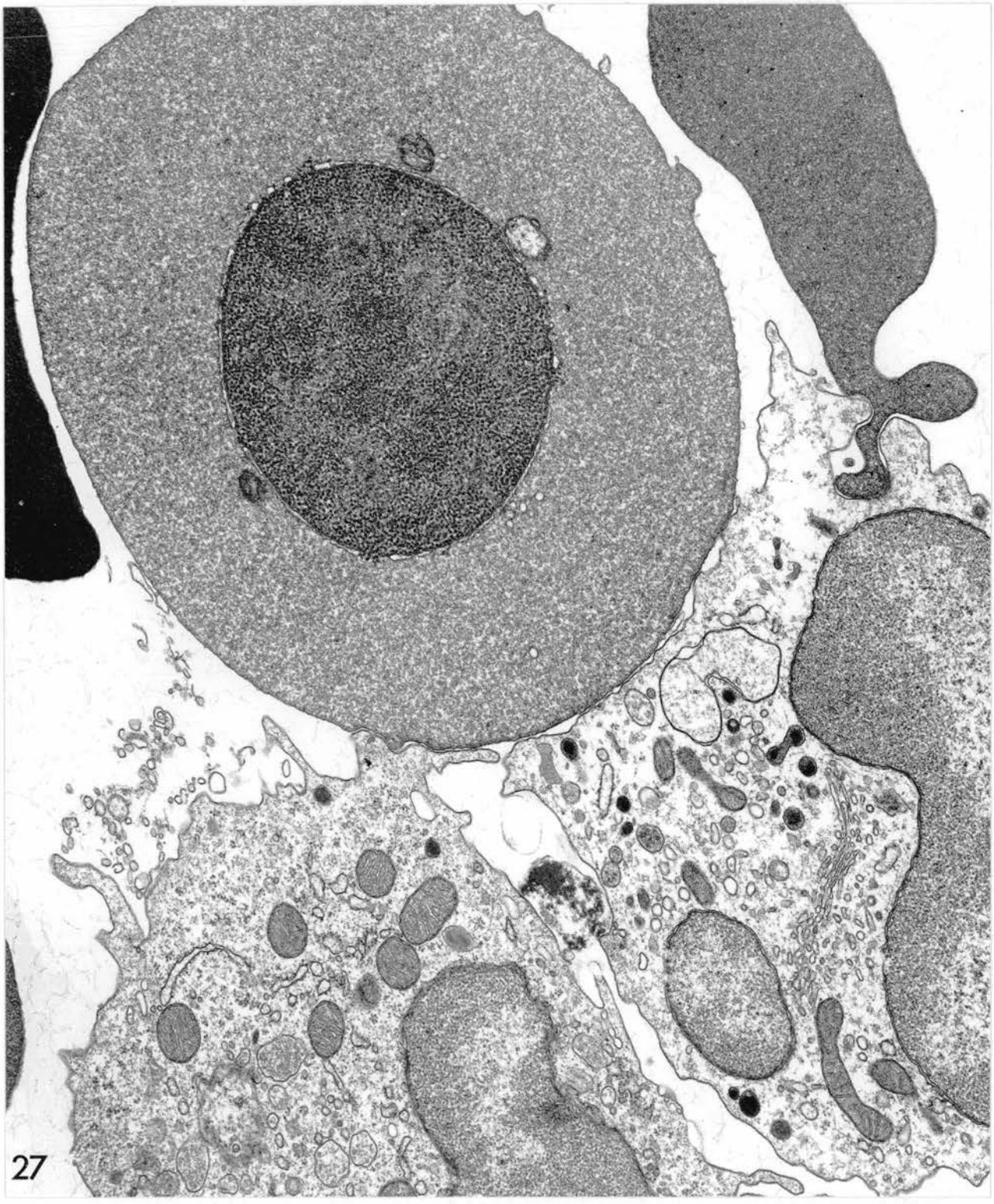

Fig. 27. Mouse spleen cells + CRBC (5:1) + rat antiserum 1:1,000, 1 hour at 37°C. A macrophage (left) and a neutrophil (right) have formed predominantly flattened contacts with a swollen chicken erythrocyte. Simultaneous fixation. x 21,000.

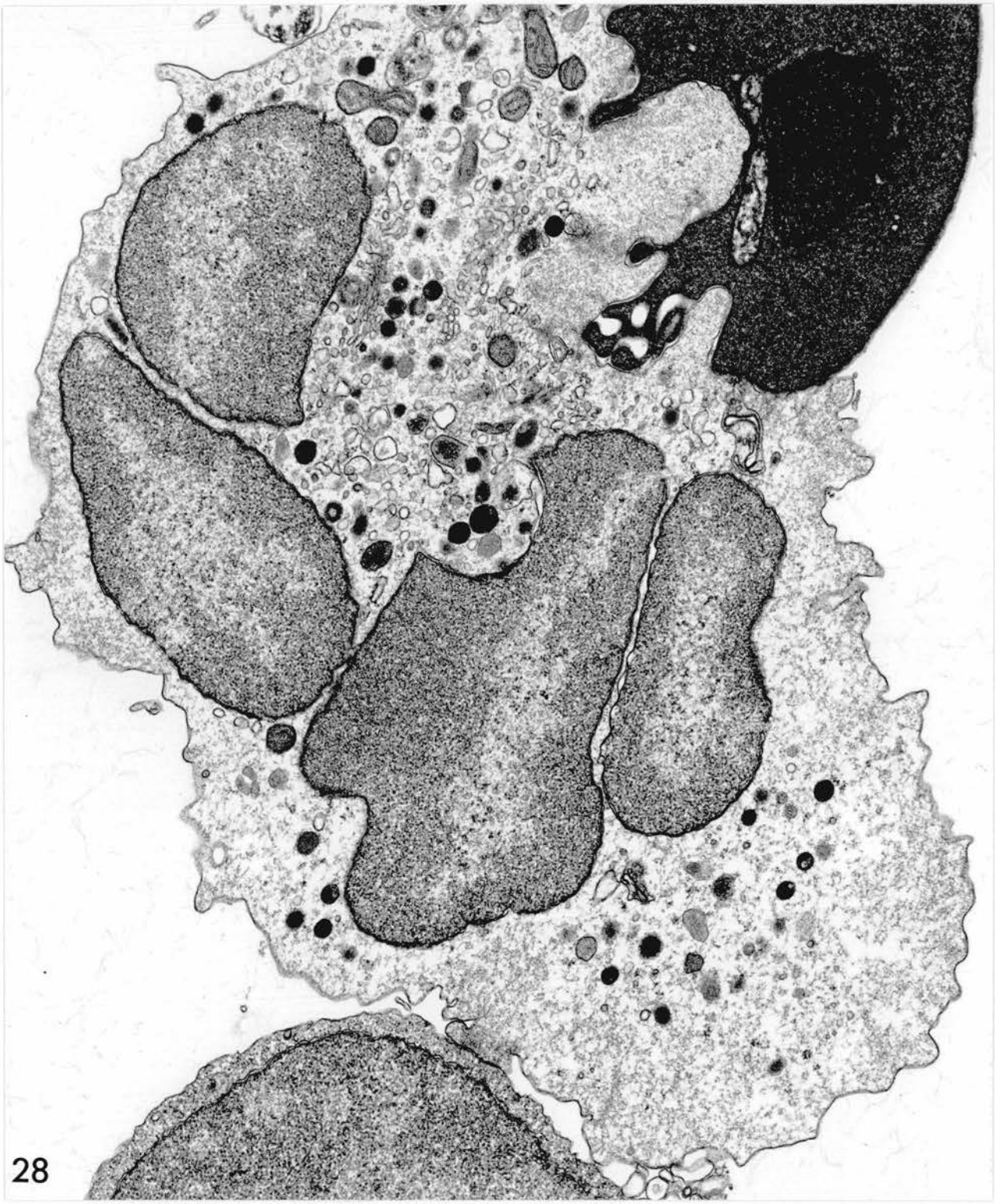

Fig. 28. Mouse spleen cells + CRBC (5:1) + rat antiserum 1:1,000, 30 minutes at 37°C. A chicken erythrocyte in the process of ingestion is severely deformed. The region of the neutrophil actively engaged in phagocytosis contains multiple microfilaments. Simultaneous fixation. x 18,000.

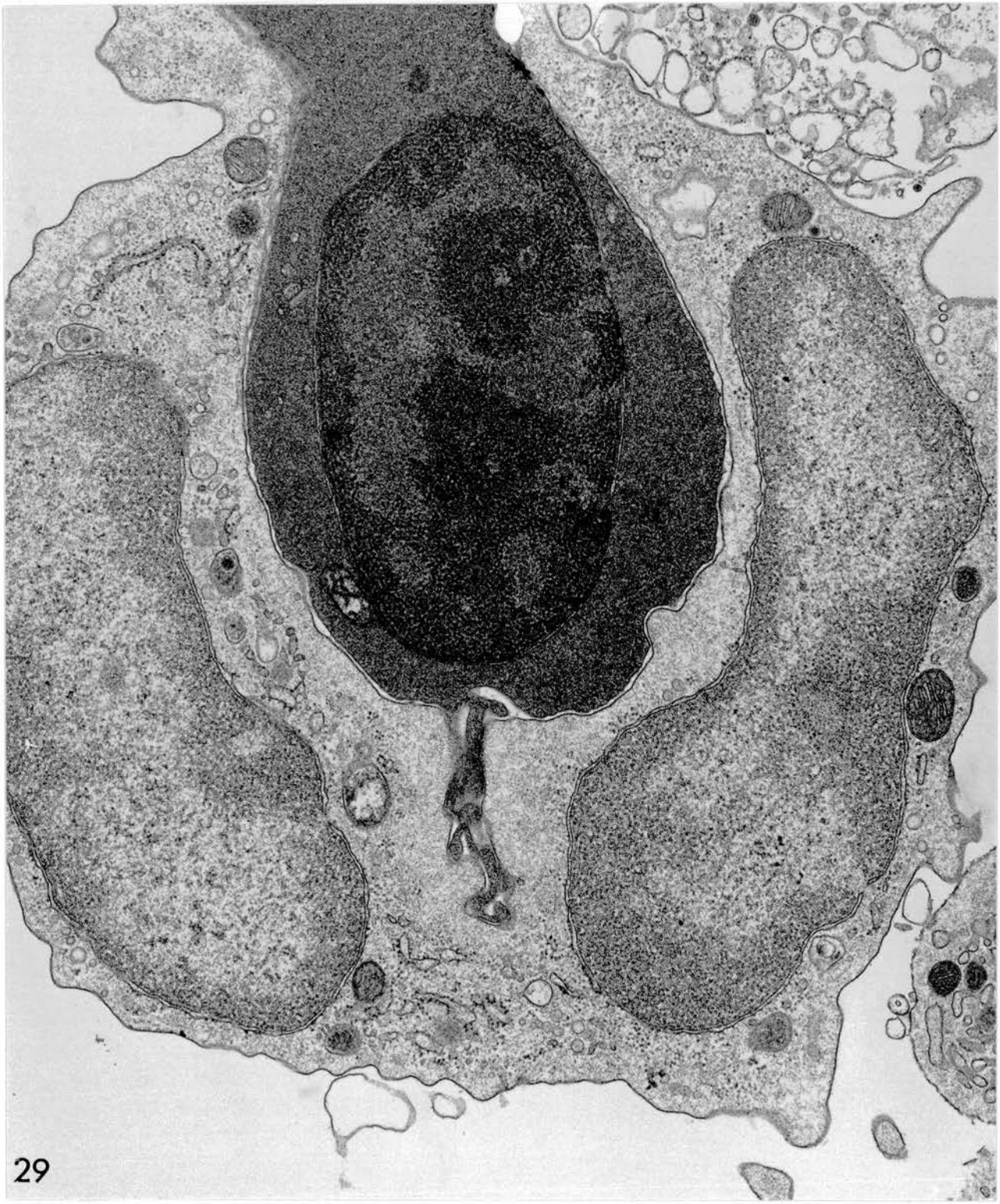

Fig. 29. Mouse spleen cells + CRBC (25:1) + guinea pig antiserum 1:1,000, 1 hour at 37°C. A mouse macrophage has almost completely engulfed a chicken erythrocyte. Distortion of the target cell surface and microfilaments in the effector cell cytoplasm are clearly demonstrated. Simultaneous fixation. x 25,000.

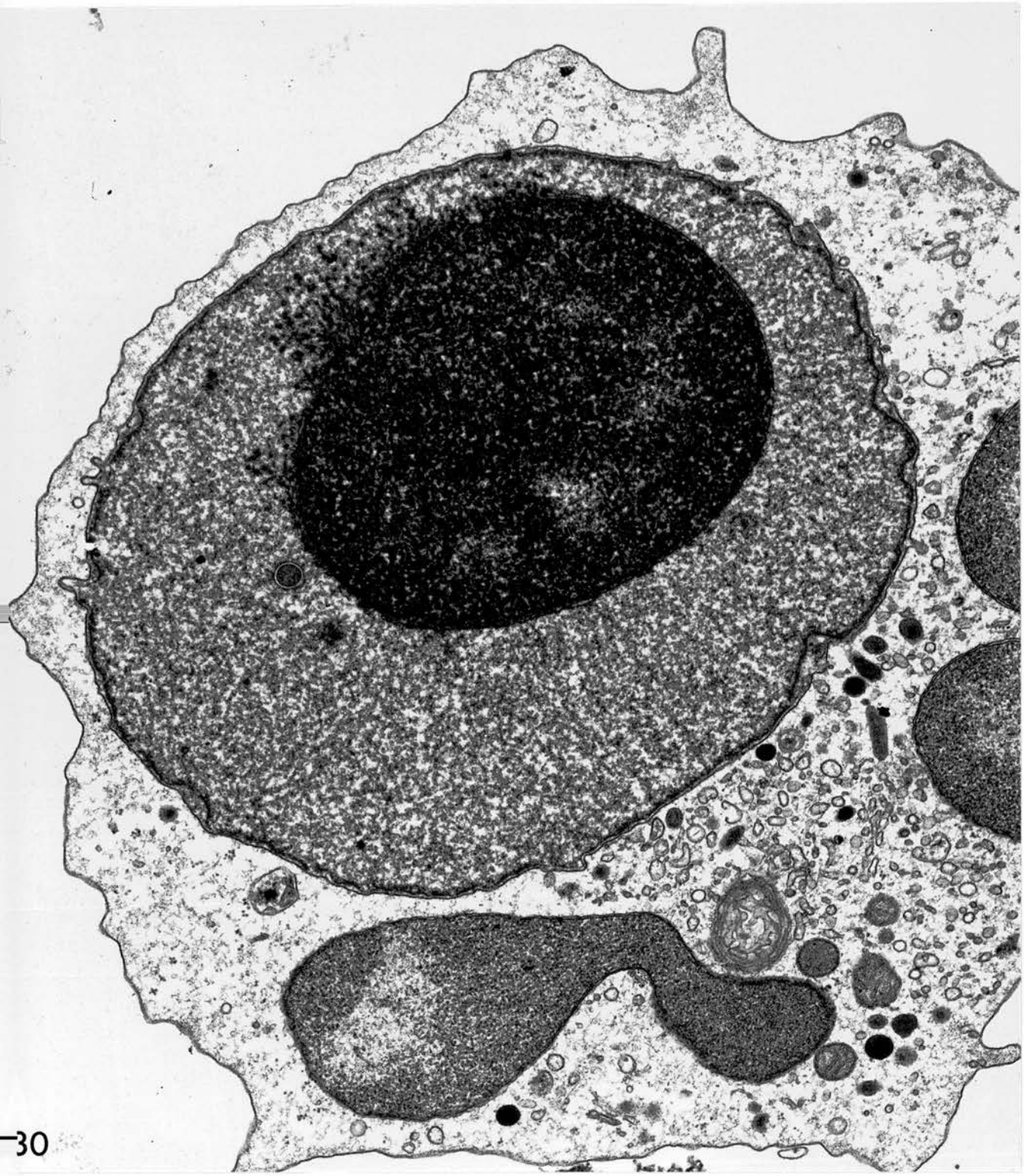

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Fig. 30. Mouse spleen cells + CRBC (50:1) + rat antiserum 1:1,000, 6 hours at 37°C. A pale, swollen chicken erythrocyte is enclosed within a neutrophil phagocytic vacuole. Simultaneous fixation. x 25,000.

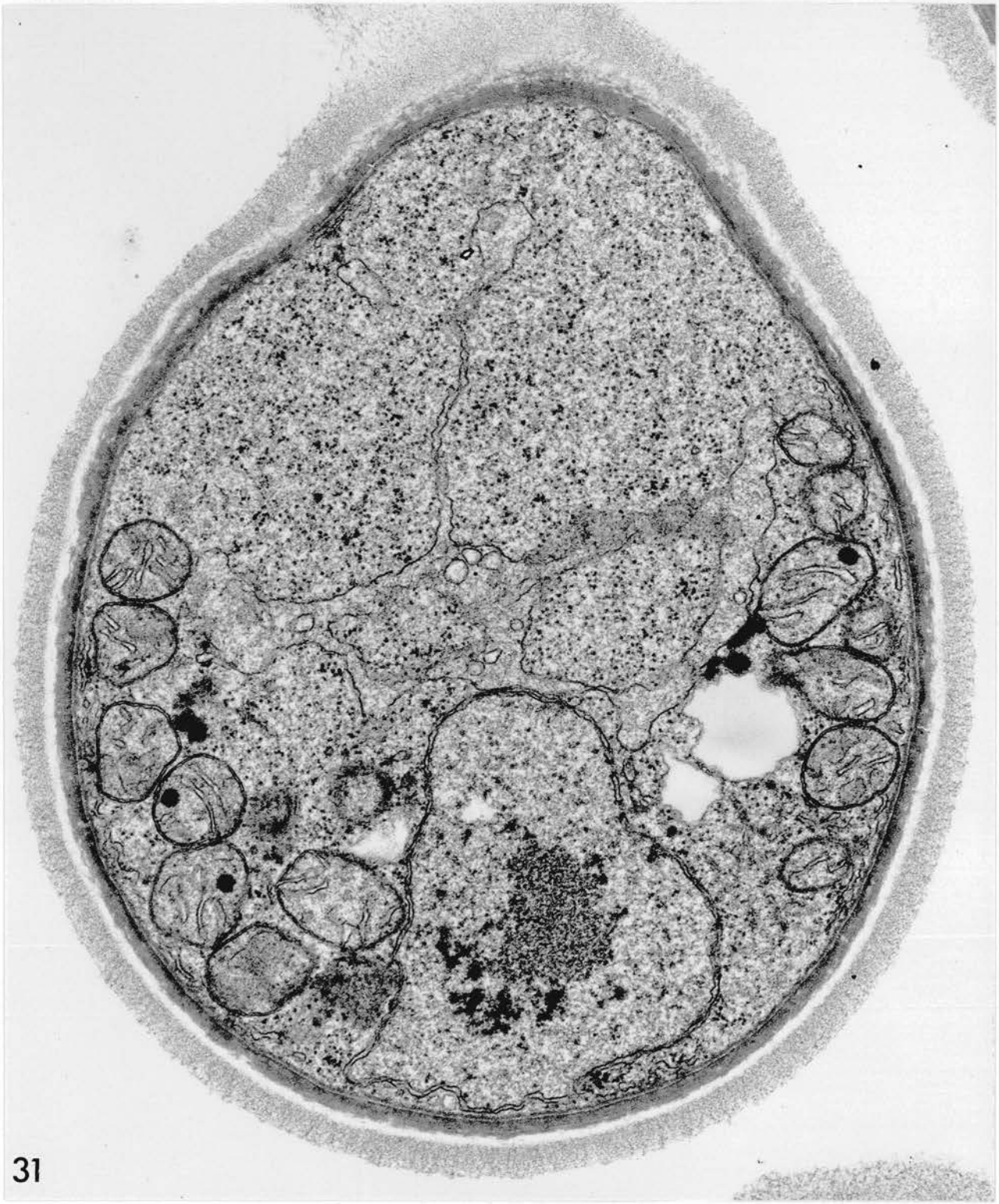


Fig. 31. Cryptococcus neoformans + rabbit antiserum 1:4,000. The single nucleus contains dispersed chromatin and a nucleolus. Within the cytoplasm are numerous free ribosomes and mitochondria, a few lipid droplets and smooth endoplasmic reticulum. There is a clear zone between the capsule and the electron-opaque wall which surround the plasma membrane. Simultaneous fixation. x 32,000.

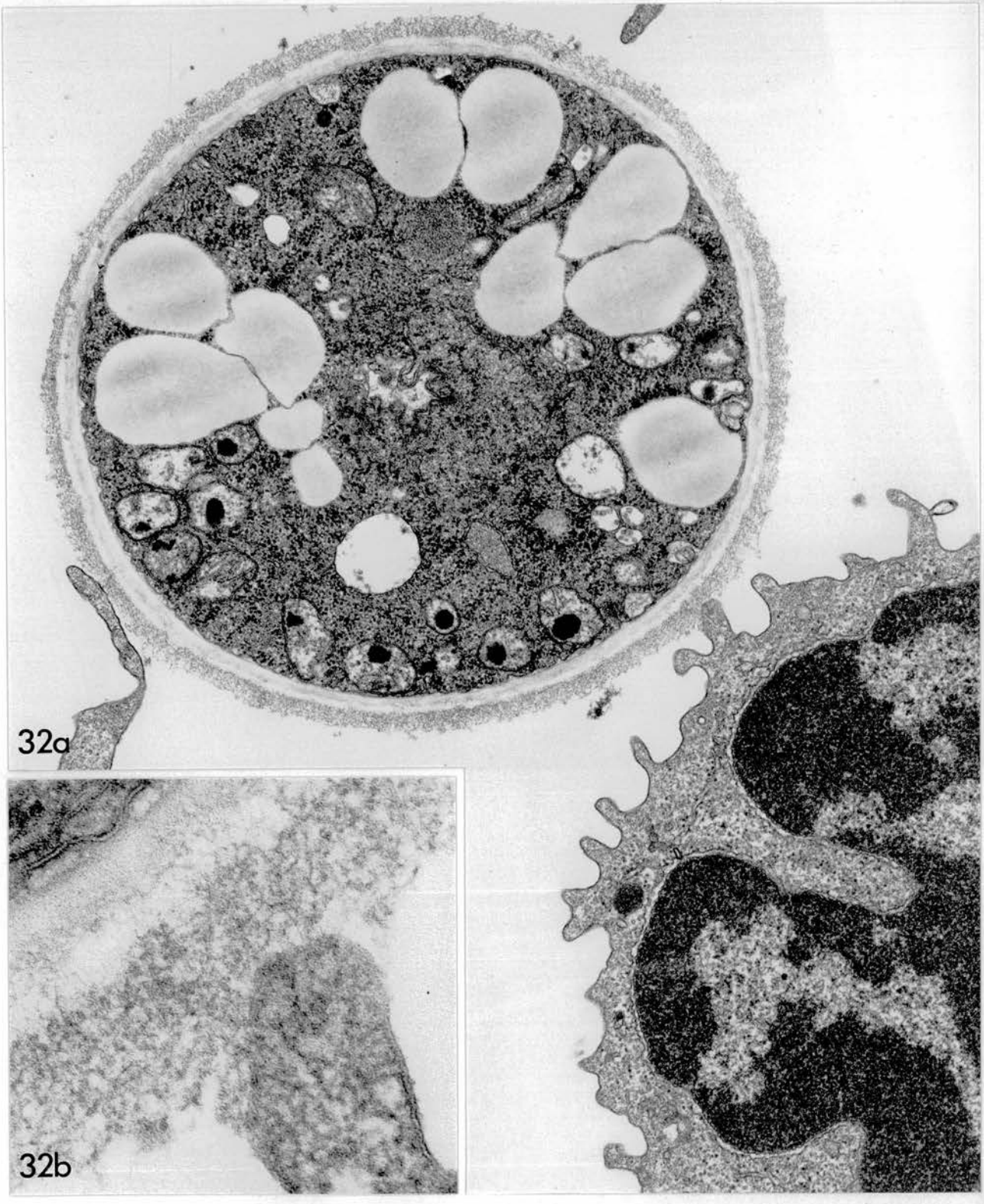


Fig. 32. Human peripheral blood lymphocytes + C. neoformans (50:1) + rabbit antiserum 1:4,000, 4 hours at 37°C. Sequential fixation; postfixation in uranyl acetate.

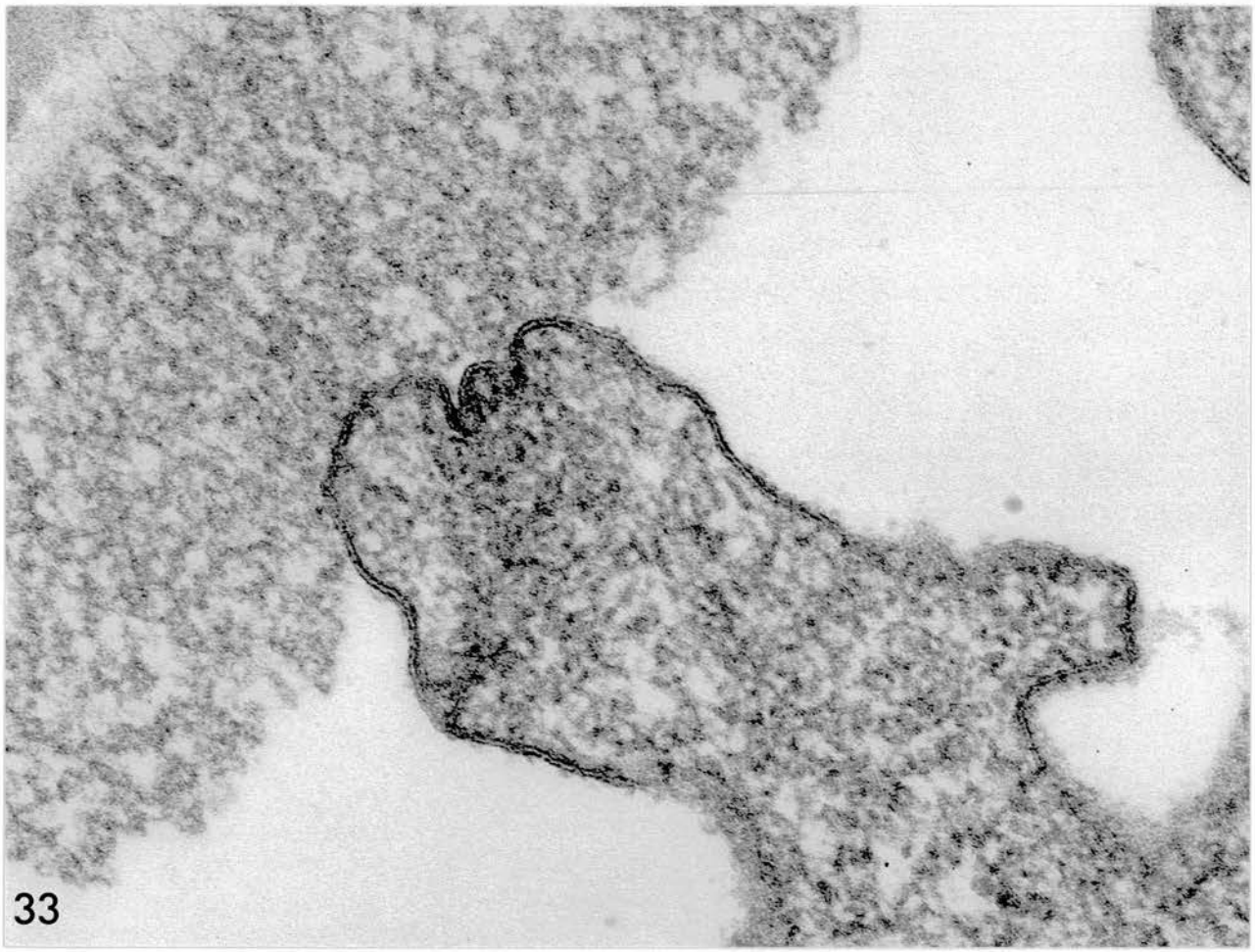
Fig. 32a. Part of a lymphocyte in contact with a cryptococcus. x 27,000.

Fig. 32b. Detail of the attachment showing that the wall and most of the capsule separate the tip of the lymphocyte projection from the cryptococcal plasma membrane. x 150,000.

Figs. 33 & 34. Human peripheral blood lymphocytes + C. neoformans (50:1) + rabbit antiserum 1:4,000, 4 hours at 37°C. Projections from lymphocytes indent but do not penetrate the spongy fibrillar cryptococcal capsule.

Fig. 33. Sequential fixation; postfixation in uranyl acetate. x 150,000.

Fig. 34. Simultaneous fixation. x 150,000.



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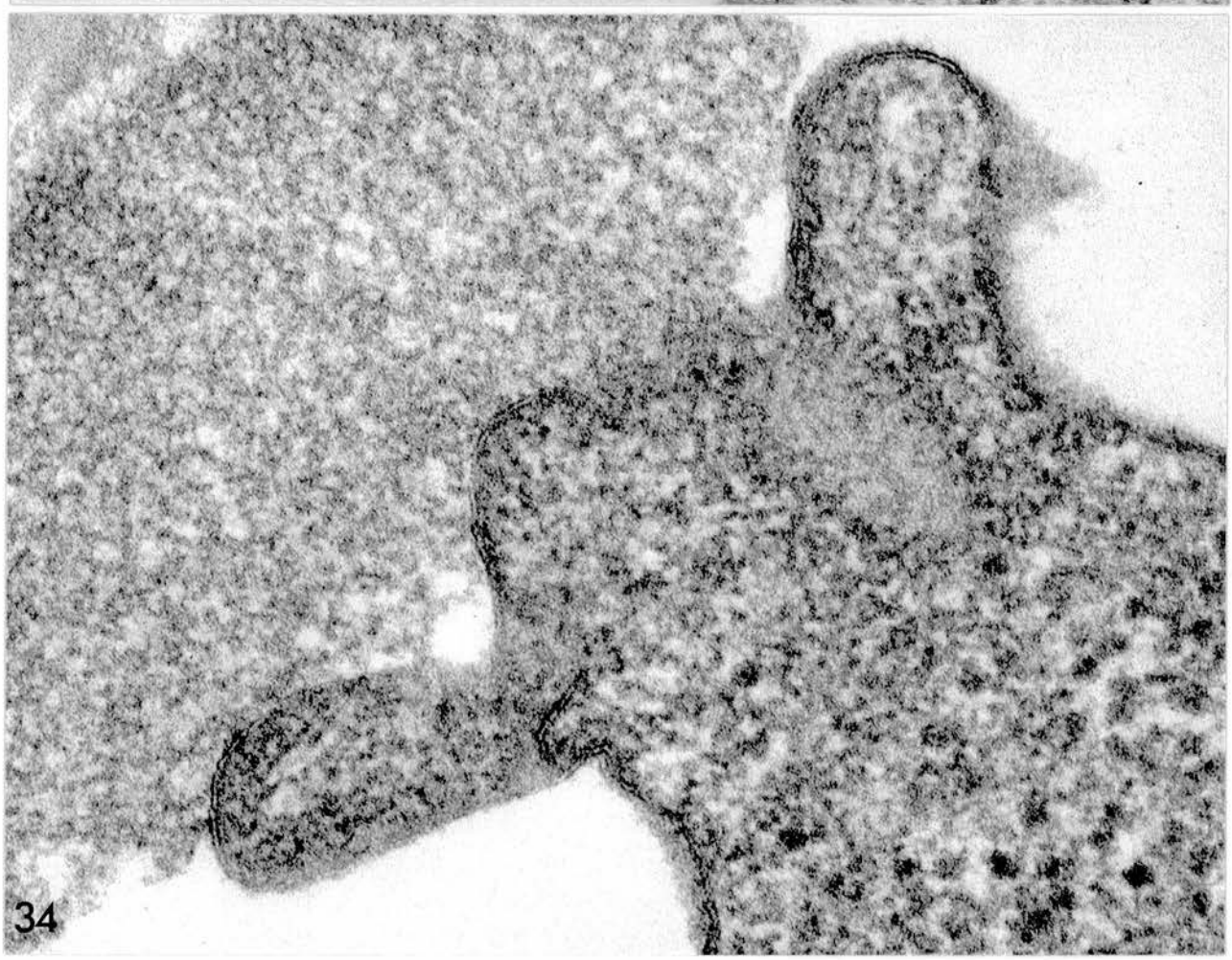

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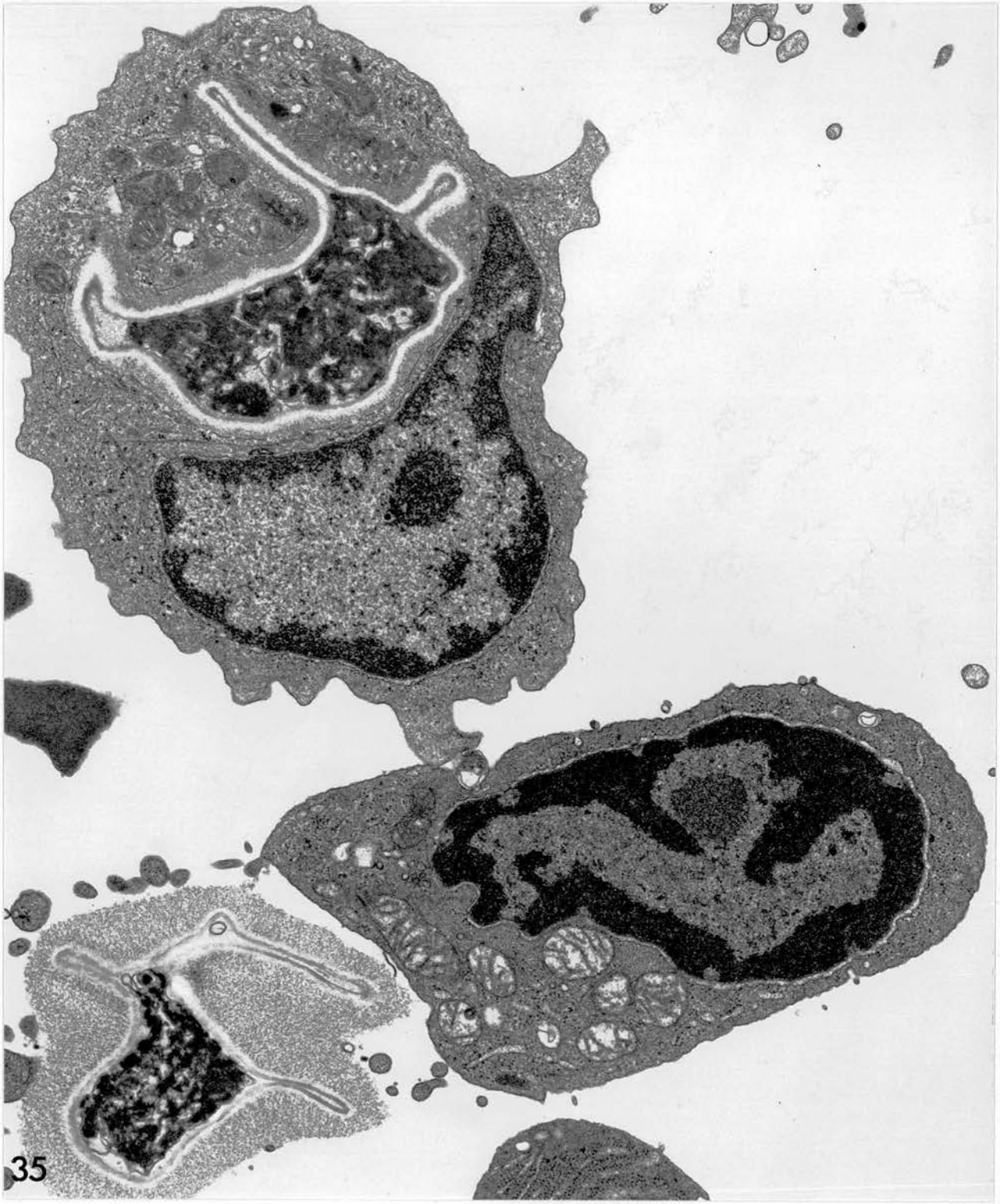


Fig. 35. Human peripheral blood lymphocytes + C. neoformans (50:1) + rabbit antiserum 1:4,000, 4 hours at 37°C. Two collapsed, misshapen cryptococci can be seen, one phagocytosed by a macrophage, the other near a lymphocyte. Sequential fixation; postfixation in uranyl acetate. x 20,000.

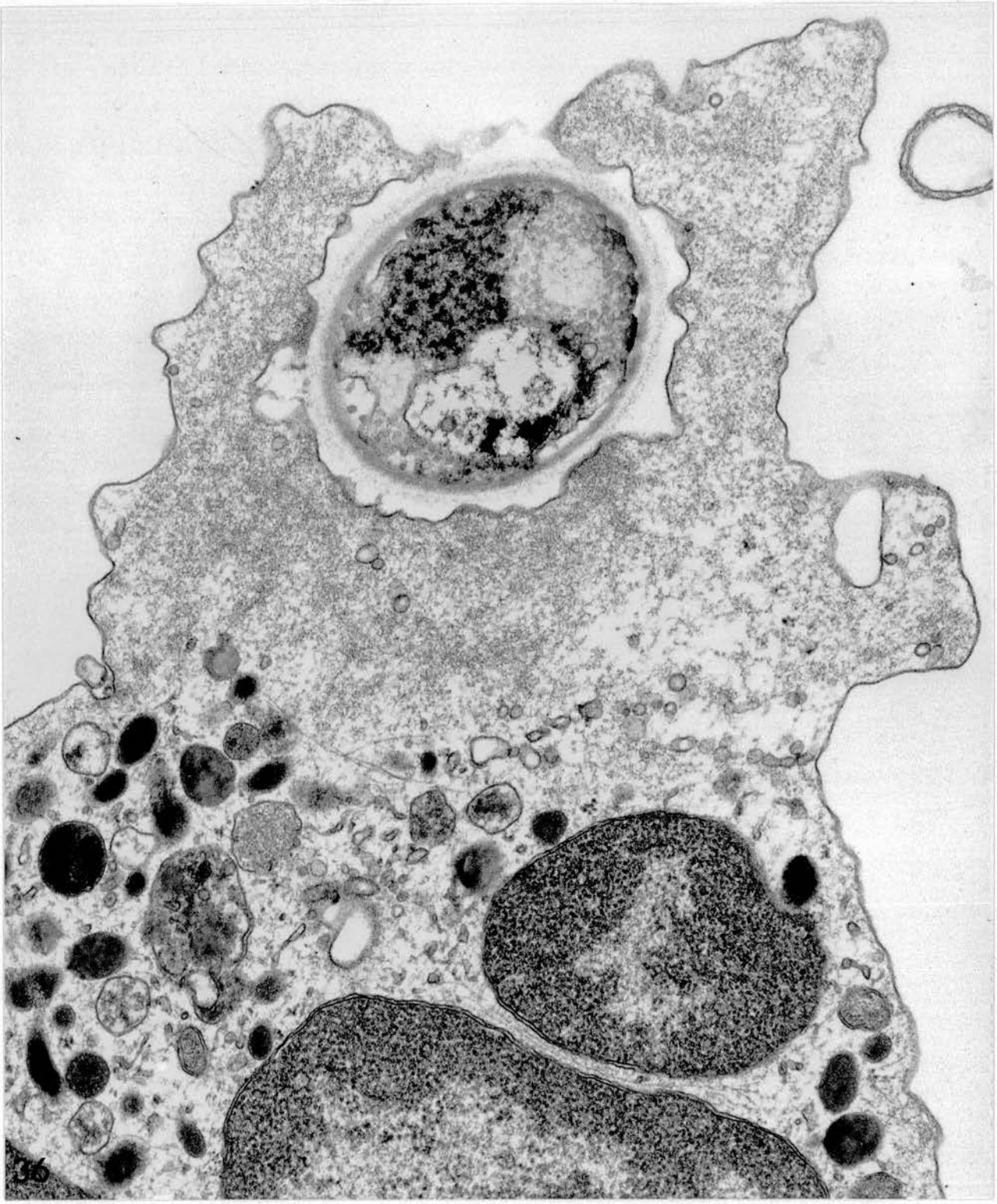


Fig. 36. Human peripheral blood lymphocytes + C. neoformans (50:1) + rabbit normal serum 1:4,000, 4 hours at 37°C. The neutrophil pseudopodium engulfing a cryptococcus contains numerous microfilaments. The characteristic shape of the target cell has been maintained although the subcellular structure is not well preserved. Simultaneous fixation. x 32,000.

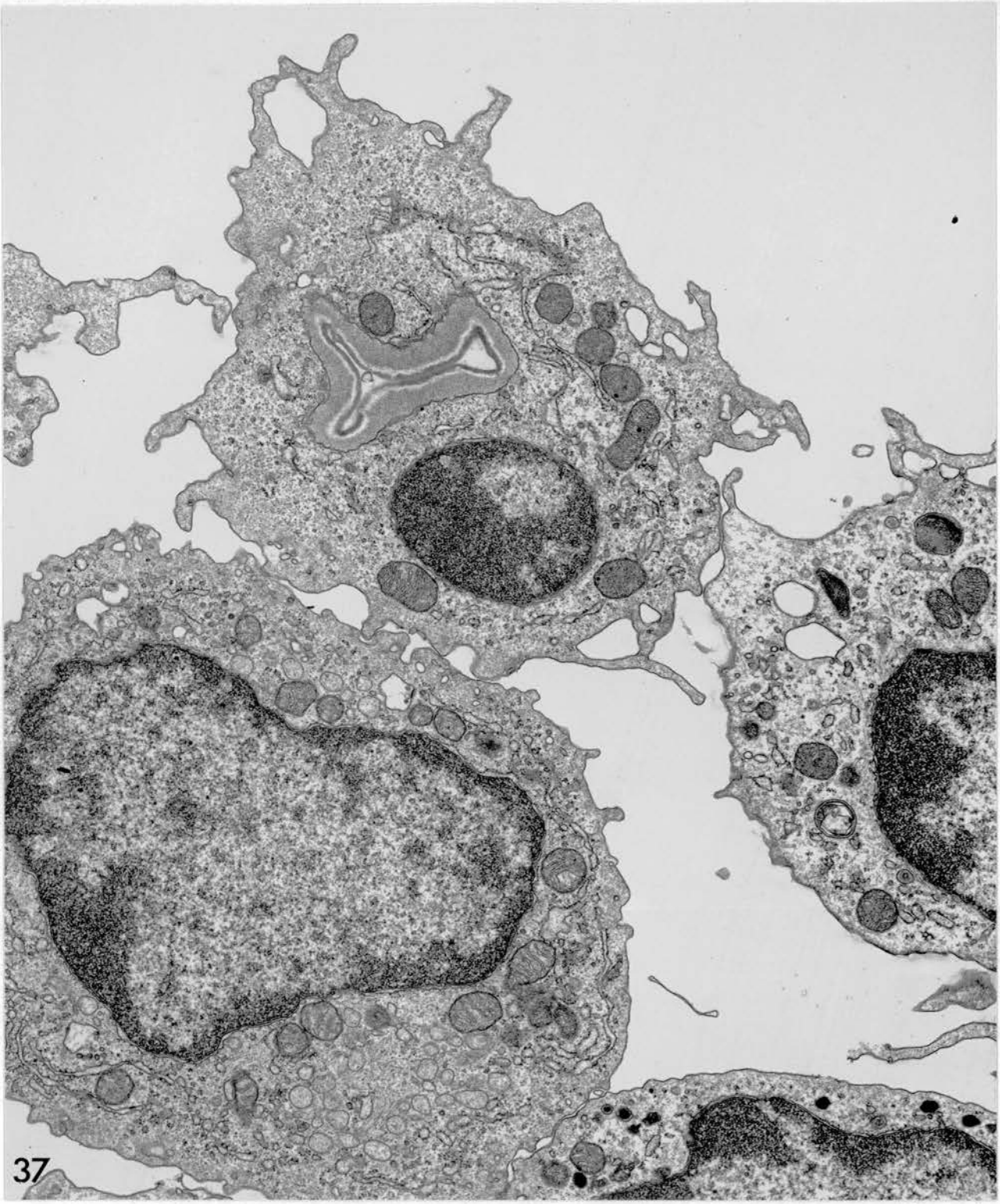


Fig. 37. Human peripheral blood lymphocytes + C. neoformans (50:1) + rabbit antiserum 1:4,000, 4 hours at 37°C. A distorted, crumpled cryptococcus is present within a macrophage phagocytic vacuole. Simultaneous fixation. x 13,000.

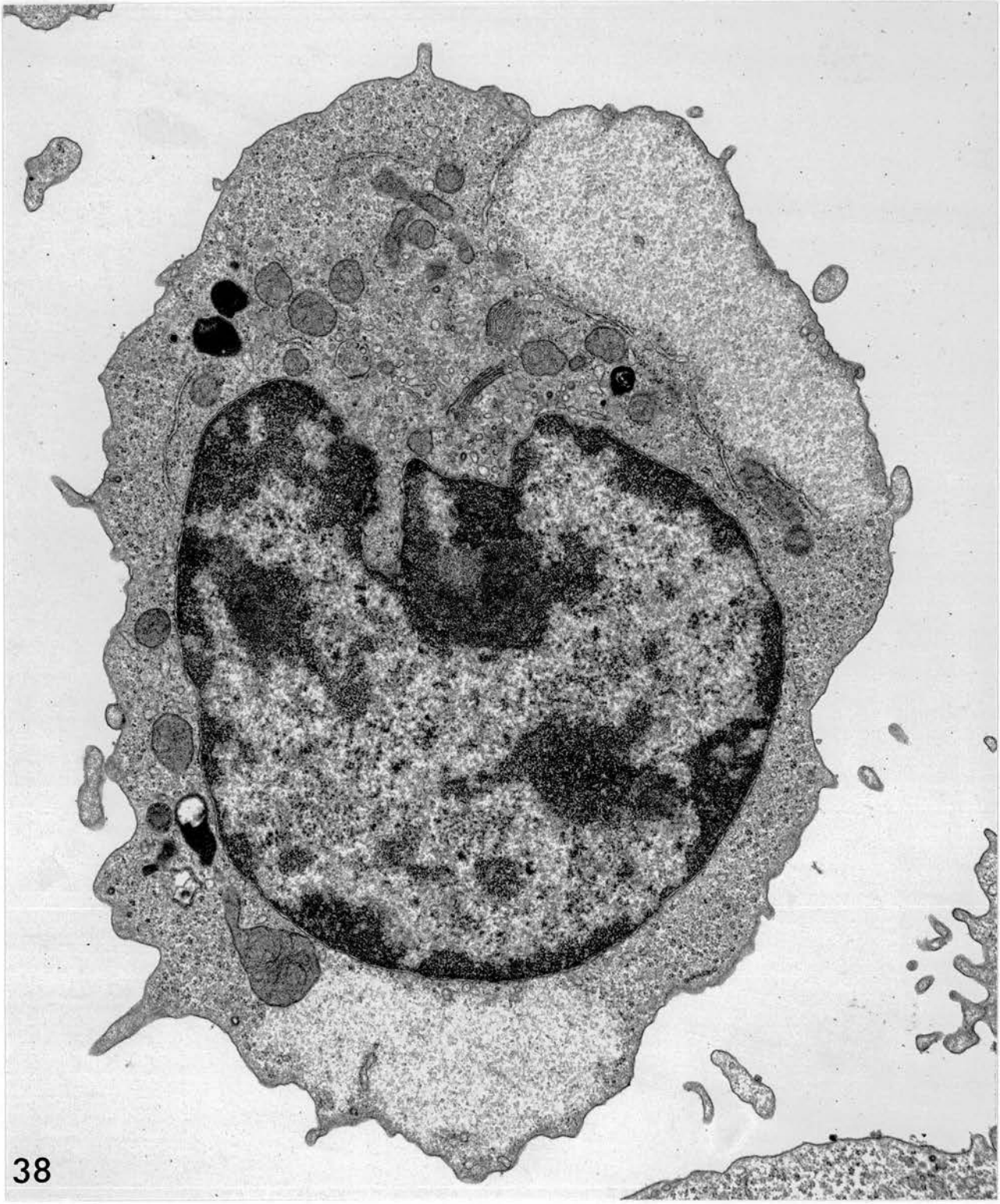


Fig. 38. A mouse T lymphoblast after 3 days stimulation with Con A. Abundant polyribosomes and 2 large accumulations of pale staining glycogen particles are prominent features of the cytoplasm. A number of mitochondria, lysosomes and cisternae of rough endoplasmic reticulum are also present and a multivesicular body lies near the nuclear invagination. Simultaneous fixation. x 13,000.

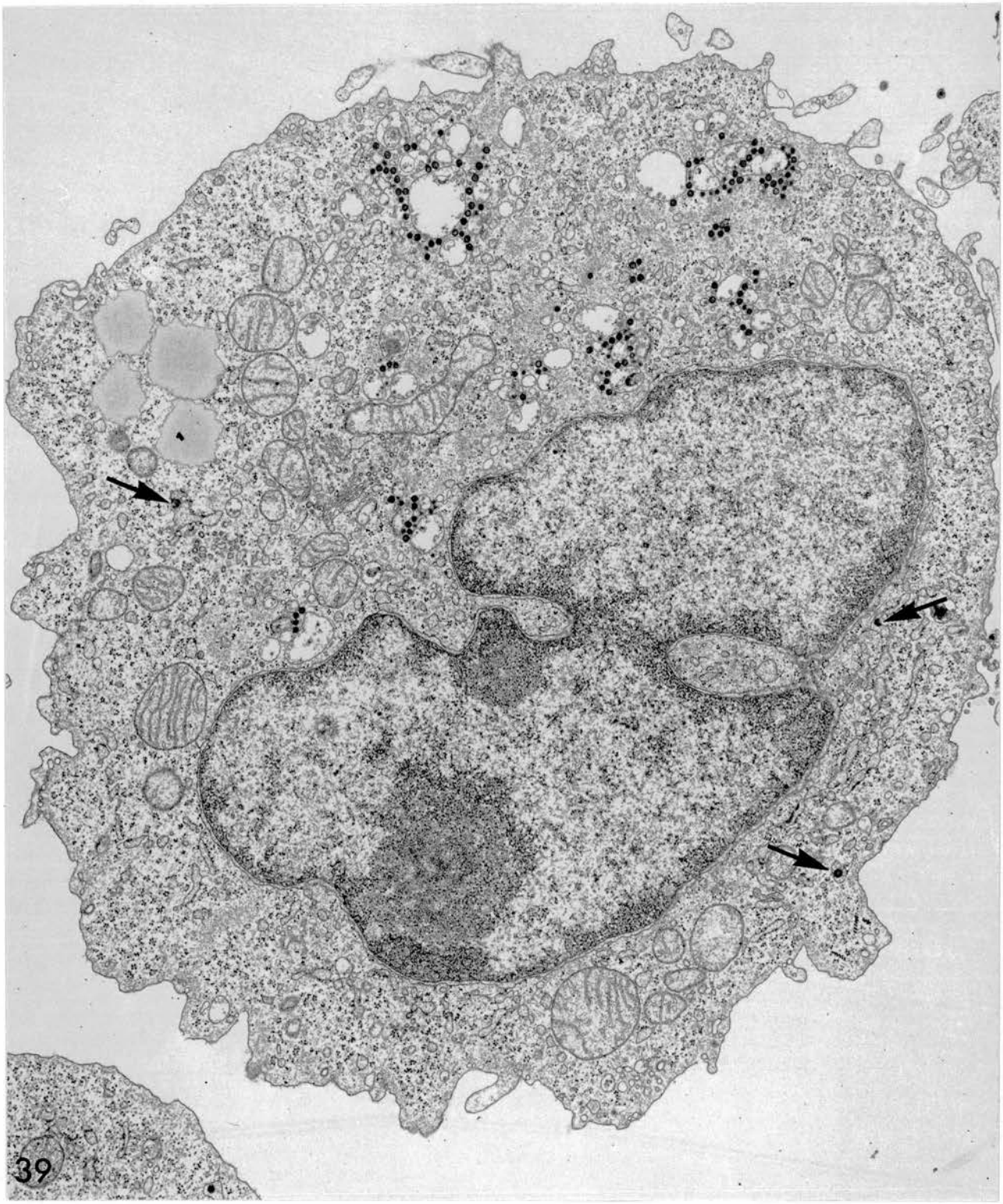
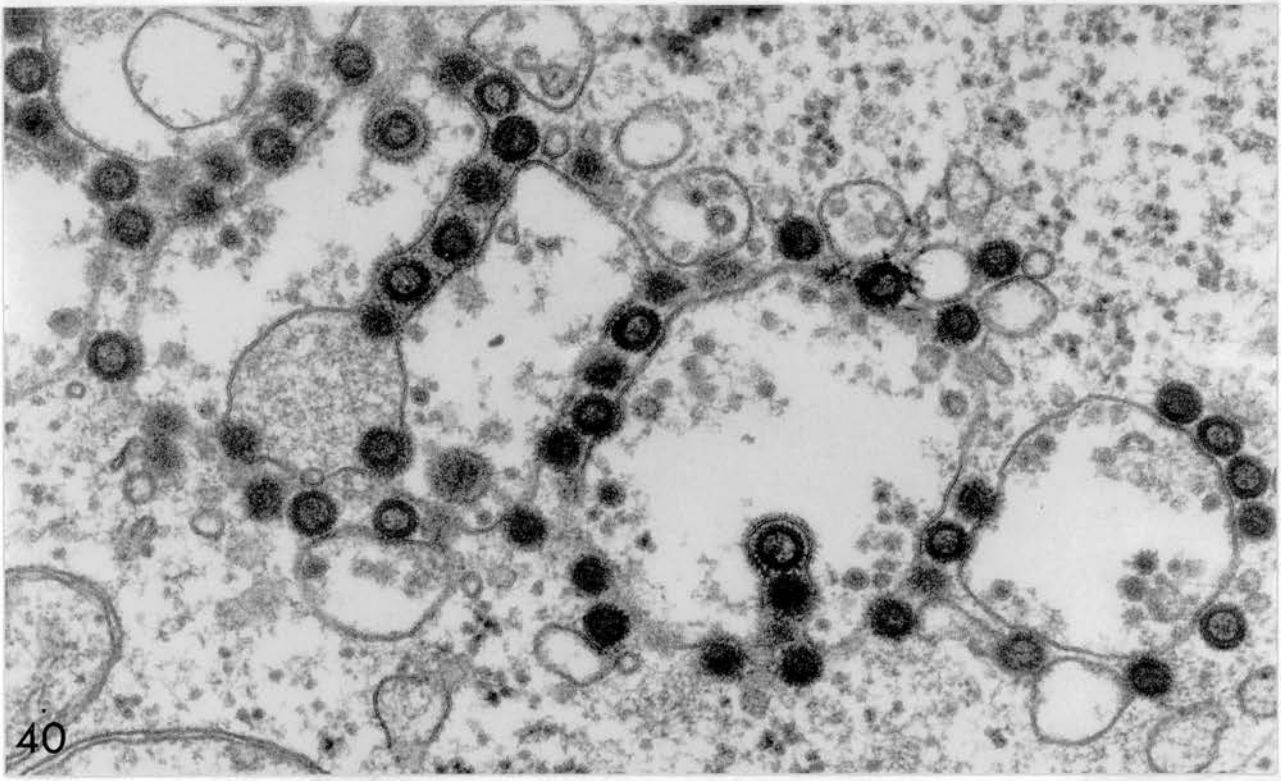
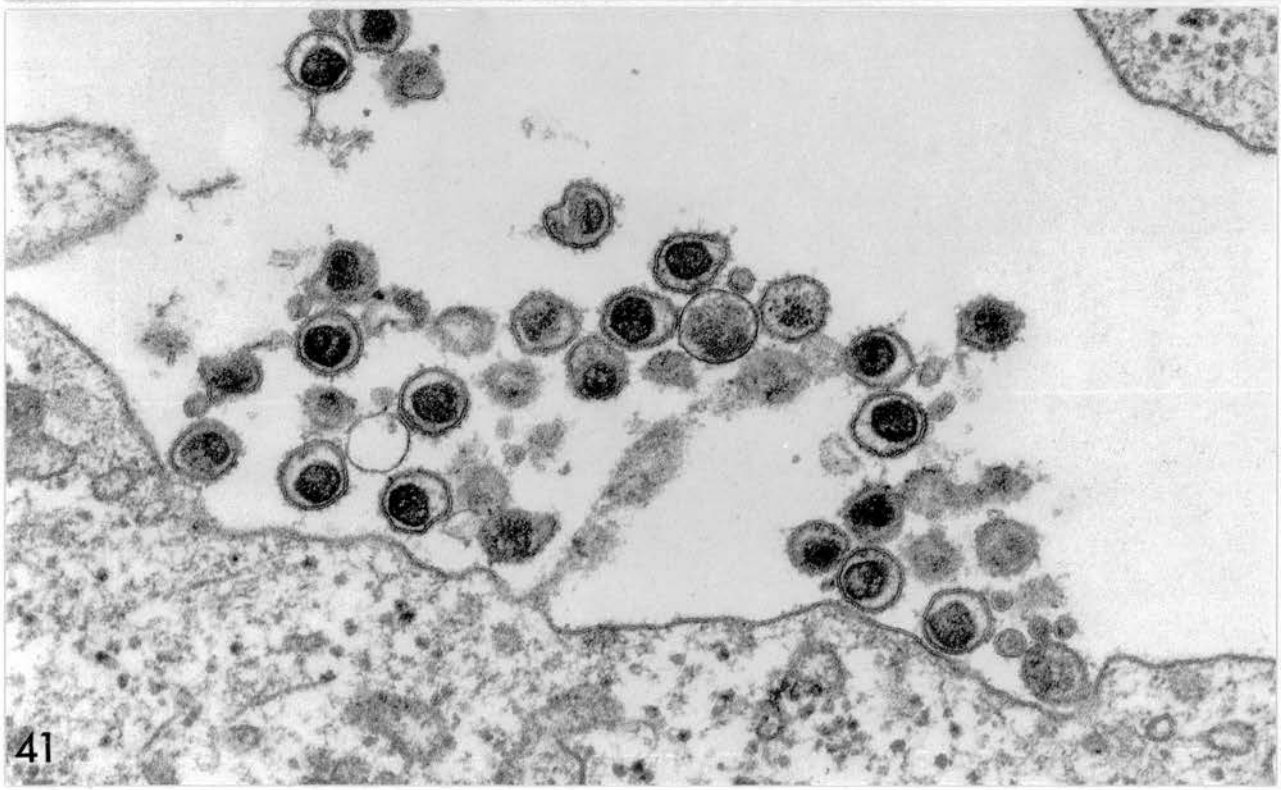


Fig. 39. A DBA/2 mouse mastocytoma ascites tumour cell. Nuclear chromatin is predominantly dispersed, and a nucleolus is evident. The cytoplasm is filled with large mitochondria, polyribosomes, vesicles, cisternae of rough endoplasmic reticulum and some lipid droplets. Numerous intracytoplasmic A type virus particles are associated with vacuoles and occasional intracisternal A type particles (arrows) are also visible. Simultaneous fixation. x 13,000.





Figs. 40 & 41. Details of mastocytoma cells. Simultaneous fixation.

Fig. 40. Intracytoplasmic A type virus particles are budding into vacuoles. x 86,000.

Fig. 41. A group of extracellular B type virus particles with characteristic eccentric nucleoids. x 86,000.

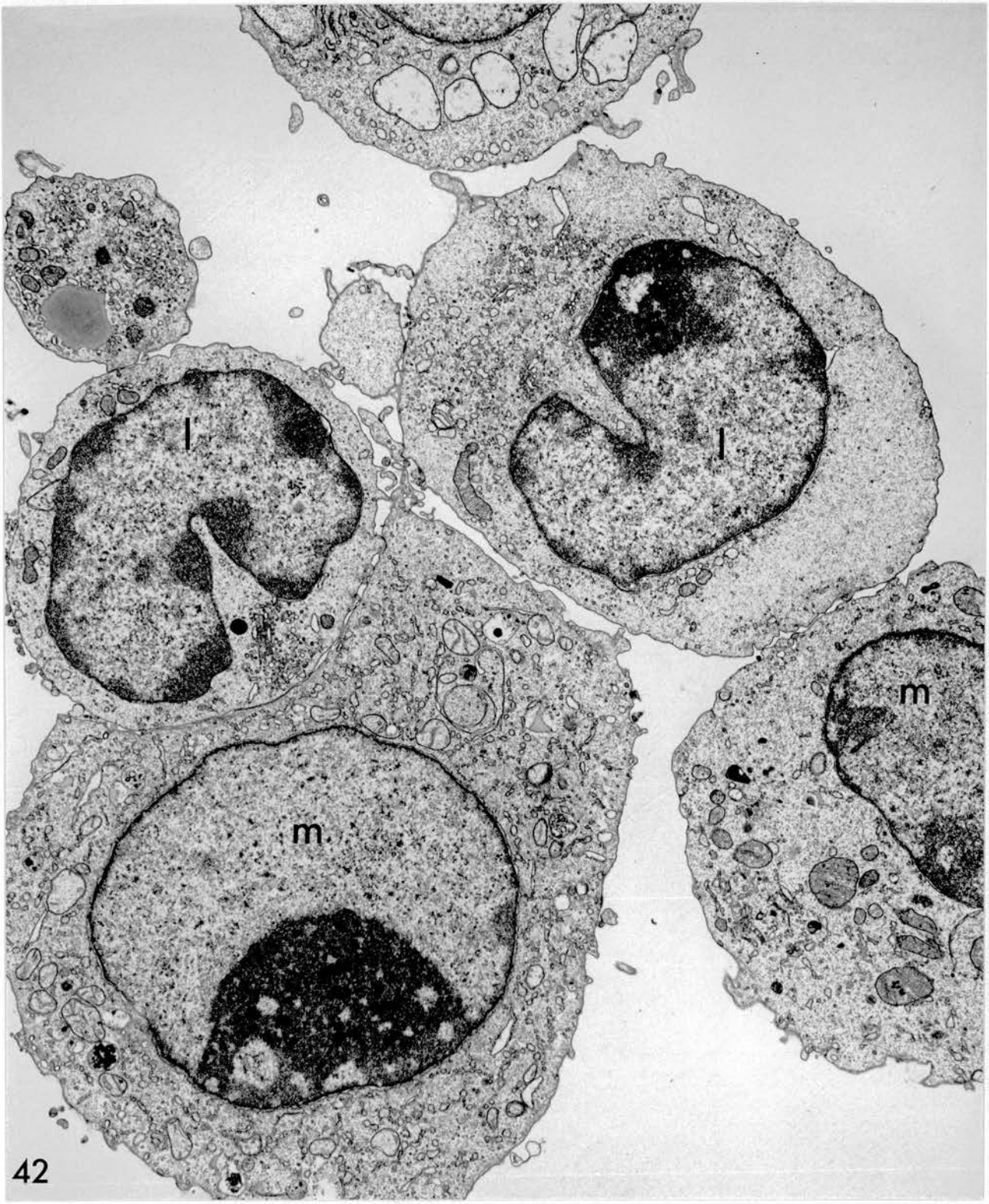
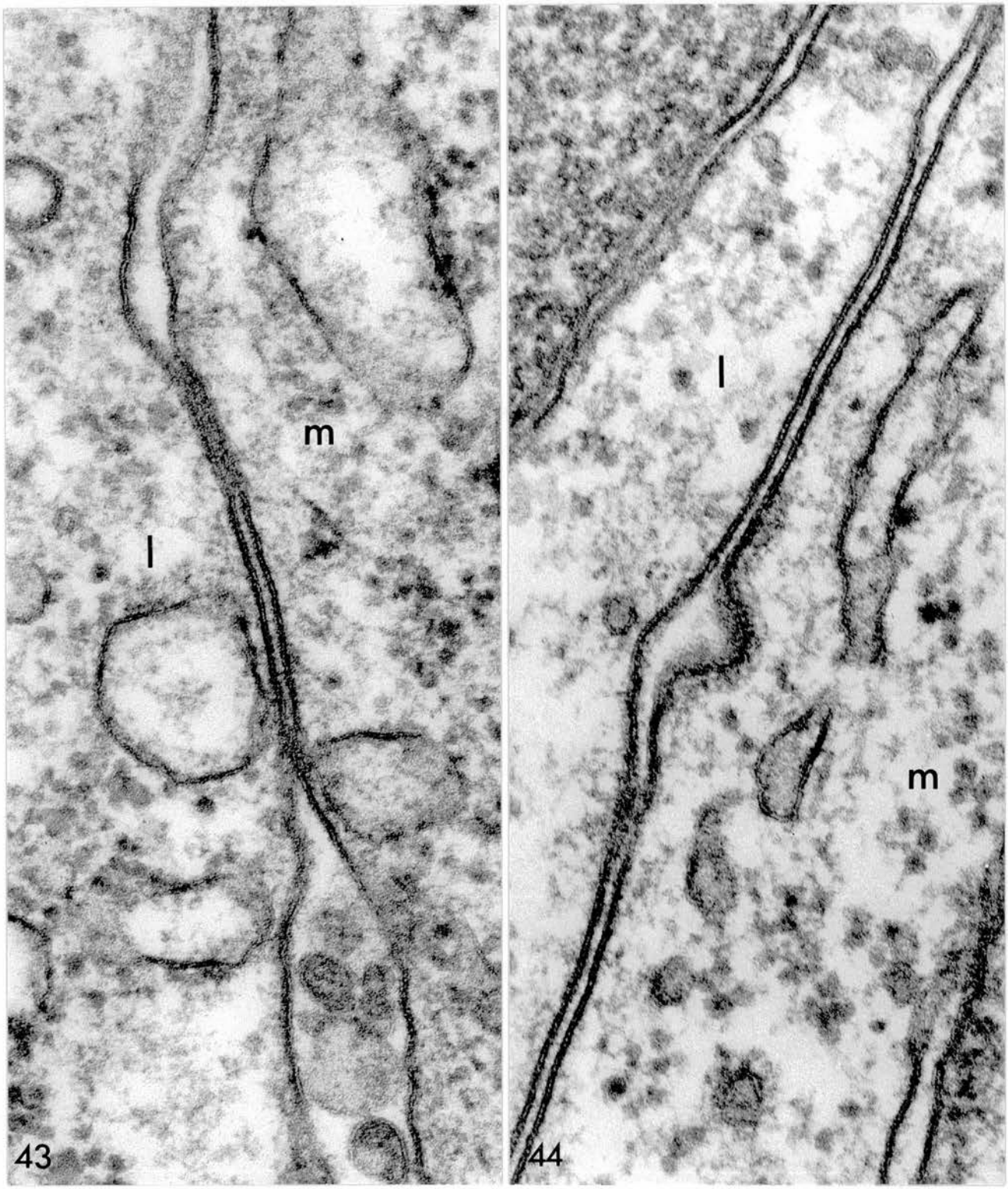
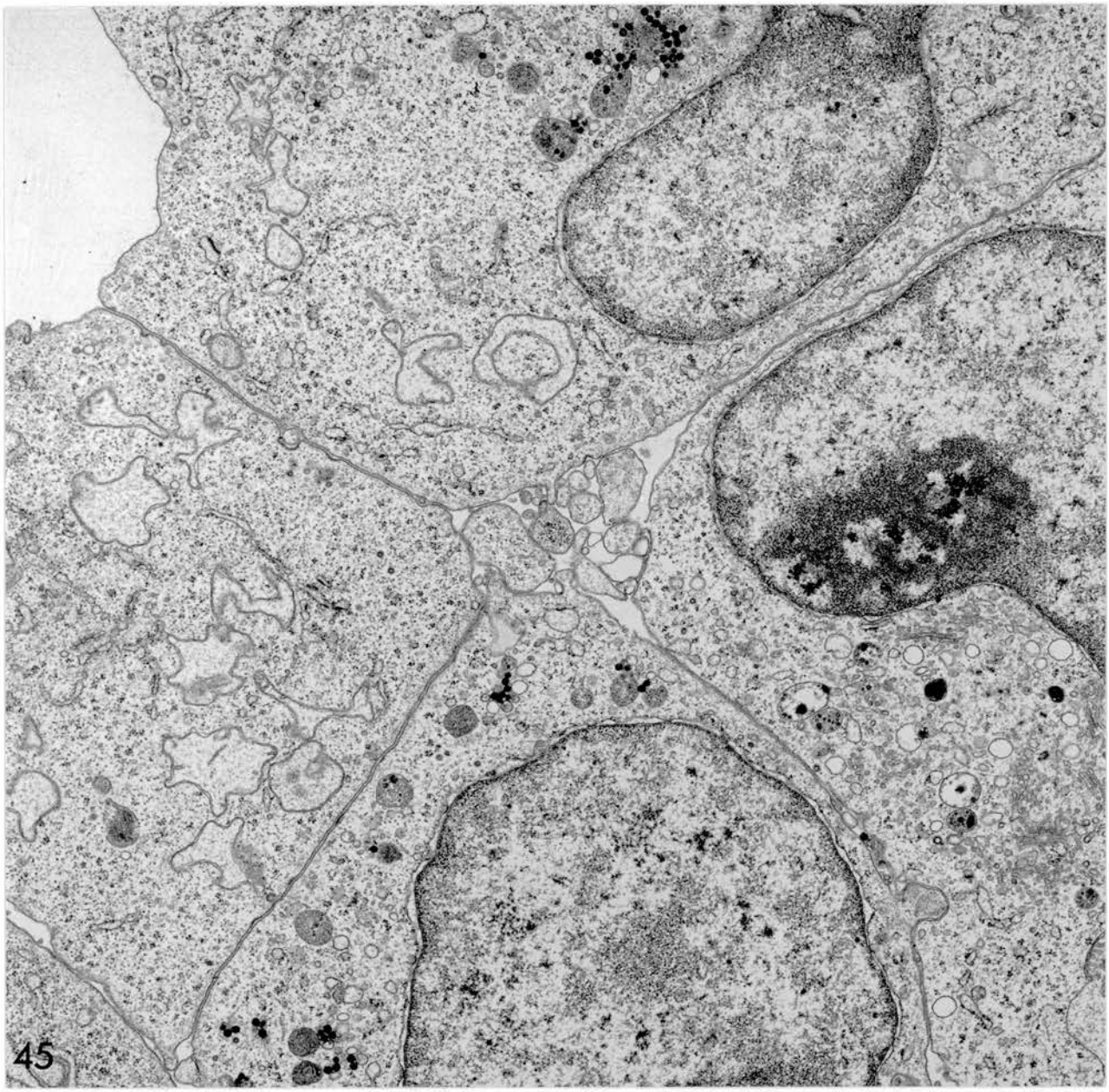
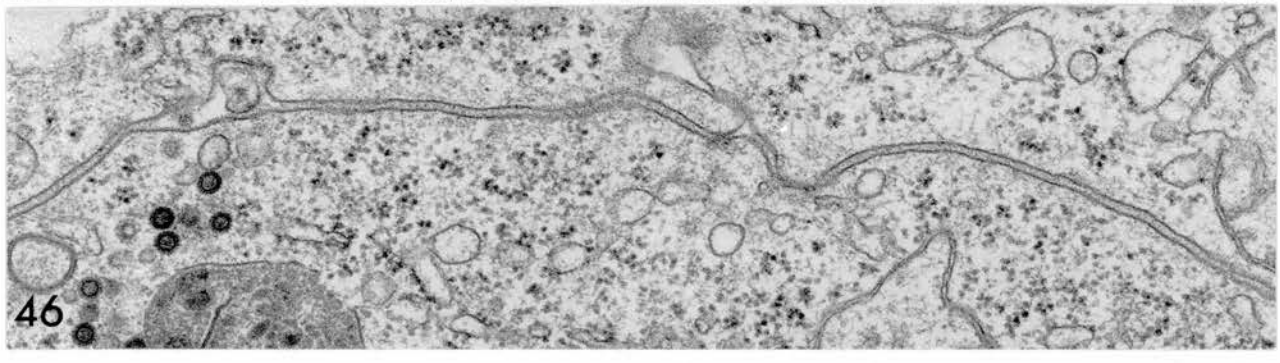


Fig. 42. Mouse T lymphoblasts + mastocytoma cells (2:1) + PHA, 3 hours at 37°C. Two lymphoblasts (l) lie in contact with 2 mastocytoma cells (m). Simultaneous fixation. x 8,000.



Figs. 43 & 44. Mouse T lymphoblasts + mastocytoma cells (Fig. 43 2:1, 6 hours, Fig. 44 1:1, 3 hours) + PHA at 37°C. The 7.5-10 nm intercellular space between lymphoblasts (l) and mastocytoma cells (m) contains amorphous slightly electron-opaque material, which is also associated with the cell surfaces away from the region of contact (Fig. 43). Simultaneous fixation. x 150,000.





Figs. 45 & 46. Mouse T lymphoblasts + mastocytoma cells (1:1) + axinella, 3 hours at 37°C. Simultaneous fixation.

Fig. 45. A clump of 4 mastocytoma cells with large areas of flattened contact. x 13,000.

Fig. 46. Detail of attachments between 3 mastocytoma cells. There is amorphous material within the 10-20 nm intercellular gap. x 75,000.

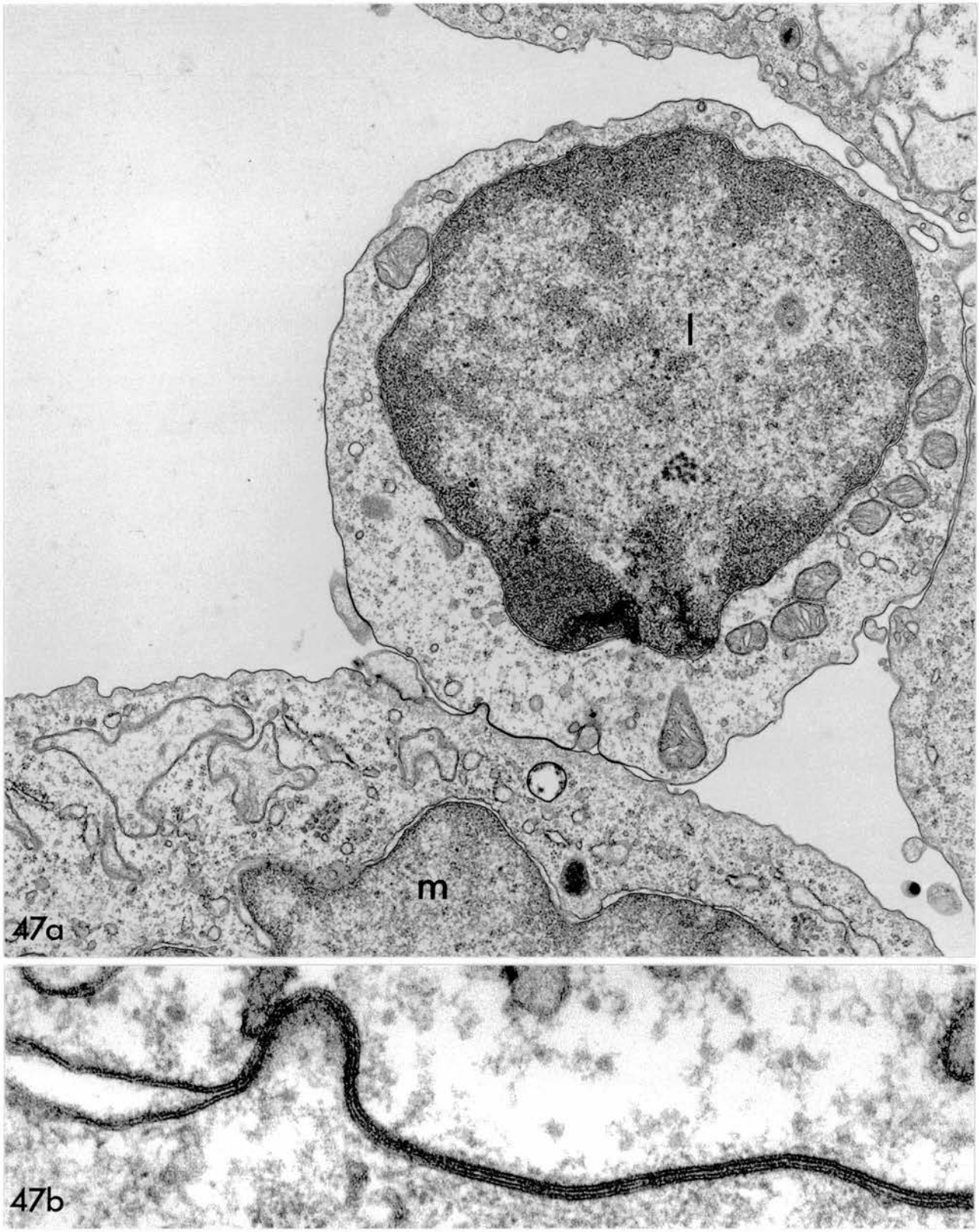


Fig. 47. Mouse T lymphoblasts + mastocytoma cells (2:1) + axinella, 3 hours at 37°C. Simultaneous fixation.

Fig. 47a. A lymphoblast (l) is attached to a mastocytoma cell (m) by means of a gap junction. x 18,000.

Fig. 47b. Detail of part of the gap junction in Fig. 47a showing the 2.5-3 nm space between the apposing cells. x 150,000.

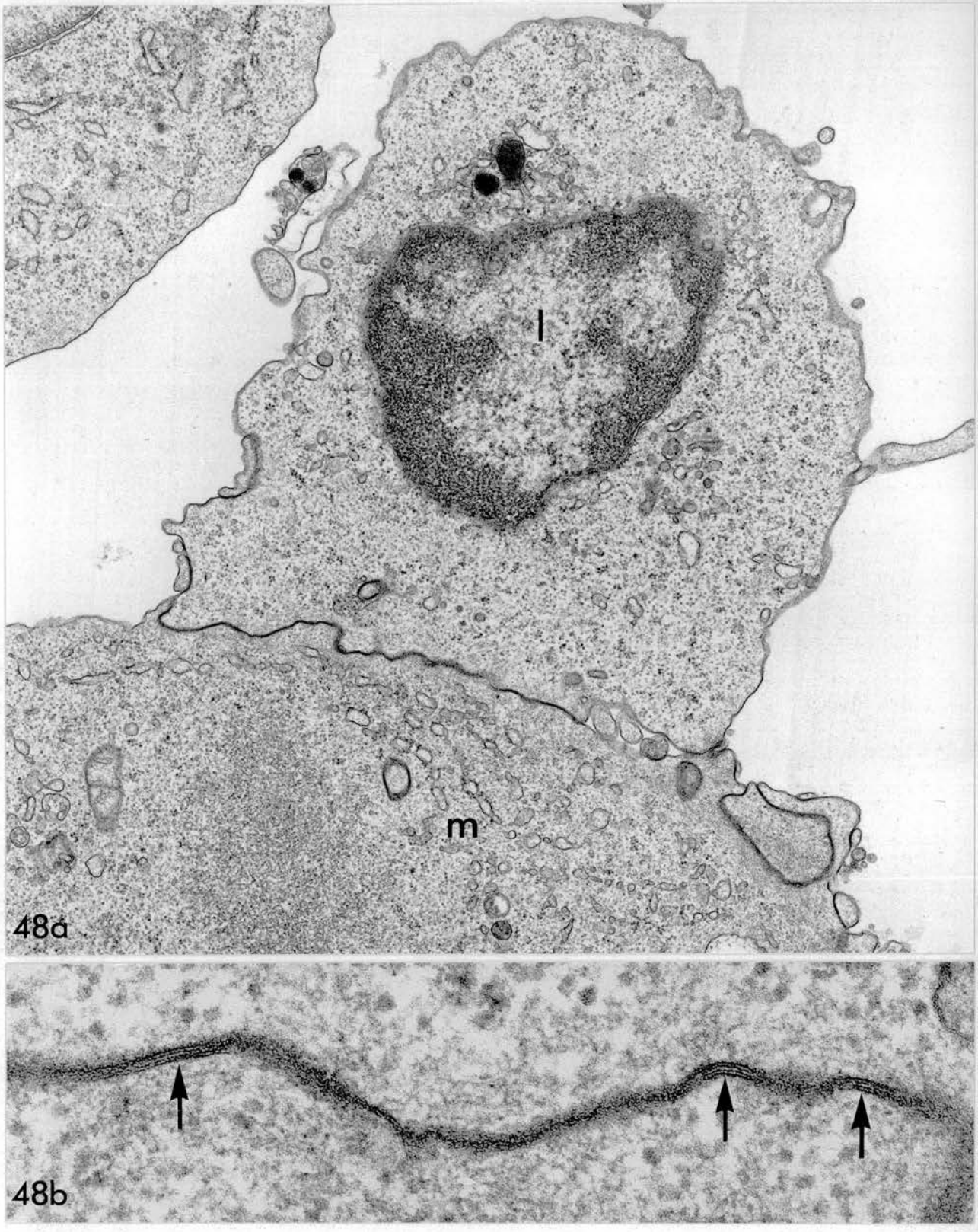


Fig. 48. Mouse T lymphoblasts + mastocytoma cells (2:1) + axinella, 1 hour at 37°C. Simultaneous fixation.

Fig. 48a. A gap junction is present between a lymphoblast (l) and a mastocytoma cell (m). x 21,000.

Fig. 48b. Although the plane of section is tangential to most of the junction, the 2.5-3 nm intercellular space is clearly visible where the membranes have been sectioned perpendicularly (arrows). x 150,000.