A TRANSPLANTABLE PLASMA CELL TUMOUR OF MICE IN LATER GENERATIONS

Light and Electron Microscopic Studies, Behavioural Characteristics and Effects of Nitrogen Mustard

A Thesis

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By

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CONTENTS

I. GENERAL INTRODUCTION

Transplantable plasma cell neoplasias of mice resemble human plasma cell dyscrasias in their production of immunologically inert gammaglobulins (paraproteins). Morphologically the cellular component responsible for the elaboration of these proteins in both human and murine plasma cell disorders is a substantial network of rough endoplasmic reticulum.

With successive transplantation generations, several changes are known to occur in the behaviour and morphology of experimental tumours, until an 'ultimate' stage in the neoplastic process is reached. The behavioral changes are manifested by increased "takes" of inocula, a markedly accelerated growth of the neoplasm, and a pattern of metastasis differing from that seen in the original tumour. As regards to morphology, the changes are epitomized in the word "dedifferentiation". There is diminishing resemblance of tumour cells to their normal counterparts, while they appear more like primitive, embryonic cells, with increased nucleocytoplasmic ratio and a relatively simplified manner of organization in subcellular organelles.

The work described in this thesis was undertaken to study the cellular changes occurring in the later transplant generations of Adj-PC-5 type of murine plasma cell neoplasm. The morphological changes were correlated with the functional ability of the tumour to produce paraprotein.

The pattern of tumour cell dissemination in the later

transplant generations was found to be significantly different from that described in the original tumour or in earlier generations. The various forms of tumour spread were studied and a peculiar intracellular localization of tumour cells within hepatic parenchymal cells was investigated by electron microscopy.

The deposition of amyloid in the tumour tissue and various internal organs of tumour-bearing animals was sought for because this has been reported by previous investigators. Nitrogen mustard was administered in an attempt to enhance the deposition of amyloid in animals carrying the tumour, and its direct effect on the neoplastic cells at the ultrastructural level was studied.

II. Plasma Cells, Immunoglobulins, and Paraproteinaemias: Review of the Literature.

The following review of the literature dealing with plasma cells, immunoglobulins and paraproteinaemias apply mostly to human conditions.

1. PLASMA CELLS

(i) Normal distribution in human tissues:

Plasma cells are the major sites for synthesis of immunoglobulins, and thus constitute an essential component of the immunologic apparatus (1). They are normally distributed in many parts of the body; the lymph nodes and spleen are the principal sites of occurrence. They are frequently seen in the lamina propria of the digestive and urogenital systems. On occasion, they may also be seen in the interstitial connective tissue of normal exocrine organs such as mammary and submaxillary glands (2,3). In human bone marrow the plasma cell population varies from 0.1% to 3.5% (4).

(ii) Morphology:

Light microscopy:

The histologic appearance of plasma cells was described in detail by Marschalkó (5) at the end of the last century. The average plasma cell measures 10-20 microns in diameter, the smallest may have the size of a medium lymphocyte. The cell is ovoid or slightly polygonal with an eccentrically situated, round or somewhat oval nucleus, which occupies about one-third of the cellular mass. Coarse chromatin particles

are usually arranged at the periphery in interrupted aggregates, imparting a wheel-spoke ("Radkern") appearance. The nucleus contains a well-defined nucleolus. The cytoplasm stains blue except at a clear zone close to the nucleus (nuclear hof). Centrioles are generally seen in the paranuclear region.

Electron microscopy:

Ultrastructurally (6,7,8), the characteristic feature of plasma cells is the extensive lamellae of rough endoplasmic reticulum, occupying most of the cytoplasmic space. This accounts for the marked basophilia of the cytoplasm. The wide area occupied by the Golgi apparatus is represented by the appearance of the nuclear hof under the light microscope. It is mainly composed of flattened sacs accompanied by vacuoles and vesicles. Mitochondria are present in moderate numbers.

(iii) Histogenesis:

The theory of histogenesis of plasma cells is based on the concept (9,10) that the fixed reticular stem cell frees itself by breaking its syncytial links and gives rise to a haemohistioblast which then turns into a haemocytoblast. A parent haemocytoblast matures either into a plasma cell or a lymphocyte. In the process of maturation towards plasma cells the haemocytoblast transforms into a plasmablast, in which the nucleus takes up an eccentric position but the chromatin pattern remains the same. The enlarging cytoplasmic space exhibits progressive increase of rough endoplasmic reticulum.

In the proplasmocytic stage the nucleus becomes smaller with coarsening of chromatin, the Golgi apparatus becomes prominent and mitochondria becomes larger.

Whether plasma cells also develop from lymphocytes is a moot point. The morphological features of circulating small lymphocytes differ in many respects from those of plasma cells (11, 12). The round or oval nucleus, which is centrally placed and occupies almost the entire cell mass, often shows a deep indentation. The chromatin is either homogenously distributed throughout or clumped at the periphery in an irregular fashion. The nucleolus is small and frequently missed in a section. The sparse cytoplasmic rim is filled with dispersed ribosomal granules which are evenly distributed. The Golgi apparatus is inconspicuous and sometimes represented only by a few agranular vesicles. Mitochondria are few. (Comparison between plasma cells and small lymphocyte - Table 1). Under appropriate stimuli the small lymphocyte turns into a 'pyroninophilic cell' which, is particularly noticeable in the cortex of lymph nodes and in splenic white pulp (13,14). As the name implies, the cytoplasm of these cells demonstrates intense pyroninophilia due to increased number of free ribosomes. The cell is larger than an average lymphocyte. The nucleus contains a distinct nucleolus. The cytoplasm, apart from increased ribosomes, shows a variable amount of rough endoplasmic reticulum as well. Mitochondria are more numerous and larger compared to those of small lymphocytes, and a moderately developed Golgi apparatus

is evident (15). It has been claimed that a pyroninophilic cell is ultimately converted to a plasma cell (16, 14) but this is not universally accepted (17).

(iv) Alterations in population and morphology:

Chronic inflammation and antigenic stimulation:

It is well-known that the histologic manifestation of chronic inflammation is characterised by the presence of plasma cells and lymphocytes (18). The influx and proliferation of mononuclear cells, which are responsible for the production of immunoglobulins, are caused by the presence of foreign protein acting as antigenic stimuli, A series of changes has been observed in the morphology of plasma cells under antigenic stimulation (19). The lamellar endoplasmic reticulum undergoes cisternal dilatation and gradually accumulates material which later condenses into large spheres giving rise to Russell bodies. The Russell bodies were originally described (20) as fuchsionophilic bodies in the marginal cells of a malignant tumour nodule. They have been seen in normal, stimulated and neoplastic plasma cells. They are generally PAS-positive and are composed of mucoprotein (21), the protein component being gamma globulin (22). Russell bodies are not unique to plasma cells: they have been found in cells of malignant lymphomas (23) and in giant form in a case of leukaemic lymphosarcomatosis (24). Sometimes the cytoplasm of the stimulated plasma cell may be filled with numerous small hyaline vesicular bodies, and the

cell is then known as a Mott cell or a morular cell (25,26). These cells have also been seen in non-stimulated and neoplastic plasma cells. Occasionally the accumulated material in cisternae crystallizes, These crystals have a periodic structure and are birefringent in polarized light (27). These crystalline inclusions may be present in neoplastic plasma cells, and have once been reported in "lymphoplasmocytoid cells" of a lymphomatous disorder (28). The Golgi apparatus is well-developed in the paranuclear area of antigenically stimulated plasma cells, usually enveloping the centrosome. The lateral ends of the flattened sacs of the Golgi apparatus are often dilated and contain electron-dense material. Many small electron-dense vesicles found at the peripheral region of the Golgi apparatus are probably the results of sequestration of these lateral ends.

The so-called Türk's "irritation forms" are probably not plasma cells but a variant of myeloblasts (3).

Neoplastic plasma cells:

In humans the following types of plasma cell neoplasia are encountered:

1) Extraosseous plasmacytoma (29)
2) Plasma cell leukaemia (30)
3) Multiple myeloma
4) Waldenstrom's macroglobulinaemia
5) Heavy chain (Franklin's) disease

A long history of confusion in identifying the tumour cell type in multiple myeloma has been resolved with the general acceptance of the plasma cell as the neoplastic cell (31). The following light and electron microscopic descriptions of neoplastic plasma cells are of cells in multiple myeloma, however, they apply equally well to the cells of other forms of plasma cell neoplasia.

Light microscopy:

The myeloma cells - not including the multinucleated ones - may show a remarkable variation in size measuring from 12 to 60 microns, although the majority of them measure from 15 to 30 microns. Uniformity in the appearance of cells in some cases and marked polymorphism in others have no predictable difference in clinical prognosis. The deeply basophilic cytoplasm is usually abundant, often three to four times as voluminous as the nuclear mass. The nuclear hof is inconstant. As mentioned before, Russell bodies and Mott cells are frequently seen in myeloma. The "grape cells" (32), which are not seen in non-neoplastic plasma cells, have a cytoplasm containing multiple deeply basophilic, PAS-negative globular bodies, which sometimes may distort the size and shape of the nucleus.

The nuclei are eccentrically situated most of the time, with the chromatin showing finely reticulated to diffusely stranded patterns, but hardly ever the normal wheel-spoke type. Multinucleation is frequent. Nucleoli - usually one but

occasionally more - are almost always present. Non-specific hyalin spherules may be seen in any part of the tumour cell, including the Golgi apparatus and the nuclei (33).

It is well recognized that not infrequently there is a mixed cell proliferation in multiple myeloma. Many cells closely resemble lymphocytes and reticulum cells and many are of the "intermediate cell" type (34), in which the partial features of various lymphoid cell elements are exhibited.

Two morphologic variants of neoplastic plasma cells have been described in relation to IgA myeloma: the flaming cell and the thesaurocyte. The flaming cell (35) is large, possessing abundant eosinophilic cytoplasm which is compartmentalized by thin basophilic strands. Cytoplasmic extensions are frequent. The eosinophilic cytoplasm is PAS-positive which is resistant to diastase digestion and shows decreased pyroninophilia, in contrast to the basophilic strands which are PAS-negative and show marked pyroninophilia. The thesaurocyte (36) closely resembles the flaming cell in appearance and cytochemistry. In its fully-formed stage, there are no basophilic strands left in the eosinophilic cytoplasm which appears homogeneous. The nucleus is pressed against the periphery, becomes darker and smaller, and appears pyknotic. The nucleolus is no longer discernible in the nucleus.

There is a mixed cell proliferation in Waldenström's macroglobulinaemia, consisting of mature and immature

lymphocytes, plasma cells and intermediate cells. A good proportion of cells contain intranuclear and intracytoplasmic PAS-positive inclusions (37).

The proliferating cells in heavy chain disease predominantly are the atypical and immature plasma cells, mixed with reticulum cells, atypical lymphocytes and intermediate cells (38).

Electron microscopy (39, 40, 41):

The majority of tumour cells have the ultrastructural features of a mature plasma cell. Greater nucleocytoplasmic ratio with nuclear pleomorphism denote immature forms, while the cells exhibiting the combined features of both lymphocytes and plasma cells are indicative of intermediate cells. Surface modifications such as microvilli and pseudopods are frequent in myeloma cells. The endoplasmic reticulum of tumour cells may demonstrate the lamellar form which characterizes the normal plasma cell, but may also show a variety of shapes due to cisternal dilatation. Structural analogues of Russell bodies and Mott cells appear as dense osmiophilic intracisternal inclusions. Similar inclusions bound by a smooth membrane may be seen in the cytoplasm and also in the nucleus. The Golgi apparatus is usually prominent in the paranuclear area. More than two centrioles are not uncommon. Mitochondria are increased in number in some cases and may show alterations in size and shape such as elongation, and drum-stick or doughnut configurat-

ions. Intracytoplasmic inclusions resembling virus-like particles have been described in only one case of multiple myeloma (42). The particles were found, measuring 30-50 millimicrons in diameter. Most of them were bound by a single membrane but occasional particles demonstrated double membranes.

The nuclei of tumour cells are usually round with a large well-defined nucleolus. The nuclear shape and size undergo irregular alterations with the degree of anaplasia of the tumour cell.

Ultrastructural studies on Waldenström's macroglobulinaemia indicate predominance of plasma cell type in one series (43) and lymphocytoid type in another (44).

(v) Plasma cells and "host resistance" to cancer:

Significant infiltration of mononuclear cells within the substance and vicinity of a malignant tumour has been favourably correlated with the survival capacity of the host. Although plasma cells contribute to the population of these mononuclear cells to some extent, the predominant cell type in host resistance, both morphologically and functionally, is considered to be the lymphocyte (45,46).

2. IMMUNOGLOBULINS (47, 48, 49)

(i) Definition:

"Immunoglobulins are proteins of animal origin endowed with known antibody activity, and certain proteins related to them by chemical structure and hence antigenic specificity. Related proteins for which antibody activity has not been demonstrated are included - for example, myeloma proteins, Bence-Jones proteins and naturally occurring subunits of the immunoglobulins" (50). Apart from being in plasma, immunoglobulins are also present in physiological secretions such as urine, spinal fluid etc. and in tissues such as lymph nodes, spleen and others.

The major normal immunoglobulins are classified into five groups on the basis of their physiochemical characteristics and antigen specificity: 1) IgG, 2) IgM, 3) IgA, 4) IgD and 5) IgE.

(ii) Sites of synthesis:

Cells belonging to the lymphoid tissue synthesize immunoglobulins. Although plasma cells are the major producers, there is considerable evidence that under proper conditions large lymphocytes are capable of synthesizing all of the three common kinds of immunoglobulins, viz: IgG, IgA and IgM (51). Small lymphocytes are presumably restricted to producing IgM only (52). It has been shown that a clone of cells would synthesize only a

given subgroup of a given class of immunoglobulins, that is, one group of cells would synthesize, say, gamma heavy chain of IgG, another alpha heavy chain of IgA and so on (53). On occasion, however, the same cell has been found to produce more than one type of heavy chain (54).

(iii) Structure:

Each of the immunoglobulins consists of four polypeptide chains: two similar heavy chains and two similar light chains. There are two types of light chains - kappa and lambda - and both types are found in all classes of immunoglobulins, though molecules with kappa chains are more common than those with lambda chains. The individual heavy chain patterns are known as gamma in IgG, alpha in IgA, mu in IgM, delta in IgD and epsilon in IgE. The heavy chains and the light chains are interconnected with each other by disulfide bonds. Polymorphisms in the immunoglobulins of the same species expressed as antigenic differences are due to genetically determined Gm group of the heavy chains and Inv group of the light chains (55). Recently, an additional Oz group has been described in light chains (56).

(iv) IgG:

Normally IgG is the predominant type of immunoglobulin in the serum representing about 75% of the total gamma globulins with a concentration of 600-1200 mg/ 100 ml. The molecular

weight of each light chain is 20-25,000 and of each heavy chain is about 50,000. IgG contains 2% carbohydrate. Enzymatic dissociation of IgG protein with papain in the presence of cysteine results in two Fab (antigen binding) and one Fc fragment. The two Fab sites have the same specificity for combining with antigen, hence the IgG antibody molecule is bivalent. Each Fab fragment consists of a light chain and the N-terminal half of the heavy chain. The Fc fragment contains most of the carbohydrate moiety and the C-terminal half of the heavy chain. Most of the acquired antibodies, such as for neutralization of viruses and bacterial toxins are of the IgG type.

Ultrastructurally, the IgG molecule appears Y-shaped with Fab fragments as the arms and Fc as the base (57).

(v) IgM:

IgM represents about 8-15% of the total immunoglobulins with a plasma concentration of around 100 mg/100 ml. The carbohydrate content is 10%. IgM usually exists in the pentameric form consisting of 5 subunits, each of which has a molecular weight of about 170,000; therefore, the total weight of the IgM molecule is 850,000 to 900,000. The light chains have a similar molecular weight to those of the IgG, but the mu heavy chain is somewhat larger, weighing 70,000. According to the antigen binding sites the IgM appears to be pentavalent (58). Bactericidal antibodies against Gram-negative organisms, saline

isohaemagglutinins, saline Rh antibodies, cold haemagglutinins and "rheumatoid factor" belong to the IgM group.

Ultrastructurally the IgM molecule appears as a flexible spider-like particle with five appendages joining a central ring. The appendages measure 125 ± 30Å while the central ring measures about 100Å in diameter (59).

(vi) IgA:

IgA also represents about 8-15% of the total immunoglobulins with a plasma concentration of about 90 mg/ 100 ml. It has a carbohydrate content of 10%. Apart from its presence in the serum, IgA antibodies also possess the unique property of being secreted in physiological fluids such as colostrum, saliva, gastric juice, tears, urine etc. The secretory IgA molecule, however, is larger with a higher molecular weight of 400,000 in contrast to 160,000 of serum IgA (except higher molecular weight polymers) and also has antigenic determinants which are not present in serum IgA. Some isohaemagglutinins and antibodies against insulin belong to this class of immunoglobulin.

(vii) IgD:

The serum content of IgD type of immunoglobulin is in highly variable quantity; the total amount is small with a median level of 0.03 mg/ml (60). Its antibody function is not known.

(viii) IgE:

IgE consists of reaginic antibodies (61) and is shown

to have a molecular weight of about 200,000 (62).

3. PARAPROTEINAEMIAS

(i) Definition:

The paraproteinaemias are a group of lymphoid tissue proliferative disorders which give rise to qualitative and/or quantitative changes in the immunoglobulins. The paraproteins usually are structural analogues of normal immunoglobulins which are not capable of antibody activity. The term paraprotein (63) is not universally accepted because of its broad, nonspecific connotation and also because the term implies that these are aberrant proteins which is only rarely the case. Hence, alternative nomenclatures to paraproteinaemias such as gammopathies (64) or dysproteinaemias have been used.

(ii) Classification:

The gammopathies are classified into two broad groups according to their appearance in the classical electrophoresis: a sharp spike (M-component) in the gamma zone indicated a monoclonal type and a broad, elevated band indicates a polyclonal type (65). These terms assume that the monoclonal gammopathy is the result of proliferation of one clone of cells, while proliferation of multiple clones of cells is involved in the polyclonal type.

(iii) Myeloma proteins (66, 67, 68, 69):

Myeloma proteins are produced by the neoplastic cells of multiple myeloma, Waldenström's macroglobulinaemia, and Franklin's disease. Occasional cases of various forms of malignant lymphomas and leukaemias may produce myeloma proteins. These conditions are referred as "primary", because the cells producing myeloma proteins are neoplastic counterparts of cells which normally produce immunoglobulins under suitable conditions. Myeloma proteins generally are monoclonal. The increase is commonly manifested in a single immunoglobulin, or in the light or heavy chain components. The majority of the gammopathies are of the IgG type with preponderance of the K type over the L type in the ration of 70 to 30, which corresponds closely to the distribution of these two types in the normal serum. The next in frequency among myeloma patients is the IgA type of gammopathy in which the incidence of K type appears to be only slightly higher than the L type. IgM gammopathy, usually seen in Waldenstrom's macroglobulinaemia, has almost the same ratio of K and L types as the IgG gammopathy. Several cases of IgD gammopathy have also been reported (70).

About 40-60% of myeloma patients produce Bence Jones protein which is readily detectable in the urine because of its property of precipitating at 60°C, dissolving at a higher temperature and reprecipitating when the urine is cooled back to 60°C. Special techniques such as starch gel electrophoresis

are required to demonstrate this protein in the serum. It usually occurs concurrently with IgG or IgA gammopathies but rare cases with Bence Jones proteinopathy alone have been seen. Bence Jones protein which is composed of light chain only (71), is of either kappa or lambda chains in the ratio of 50:50.

In Franklin's disease the gammopathy consists of a heavy chain only, which resembles the heavy chain of IgG.

Rarely, mixed gammopathy, that is the presence of two myeloma globulins, occurs in the serum of the same patient (72).

(iv) Malignant lymphoma and monoclonal gammopathy:

The various forms of malignant lymphomas and leukaemias thus far reported to give rise to monoclonal gammopathy are: lymphosarcoma (73), chronic lymphatic leukaemia (74), Hodgkin's disease (75), giant follicular lymphoma (76), reticulum cell sarcoma (77), chronic myeloid leukaemia (78), and acute leukaemia (79).

(v) Secondary gammopathies (80, 81)

These gammopathies are associated with various neoplastic and non-neoplastic conditions in the absence of primary malignancies of the lymphoid tissue. Epithelial neoplasms originating from various organs have been reported to be associated with hyperimmunoglobulinaemia (82, 83). The gastrointestinal carcinomas constitute one-third of these neoplasms. The gammopathy is usually monoclonal. Monoclonal

gammopathy occasionally occurs in association with autoimmune diseases such as rheumatoid arthritis (84), periarteritis nodosa (85), Sjögren's syndrome (86), autoimmune haemolytic anaemia (87), etc. and not infrequently in hepatic diseases, both in cirrhosis (88) and in primary malignancy of the liver (89). Gammopathy associated with chronic bacterial or parasitic infections is usually polyclonal; but in a wide variety of conditions, isolated incidence of monoclonal rise has been reported. Gammopathy in non-specific granulomatous diseases such as sarcoidosis is more commonly polyclonal. Sporadic cases of unrelated diseases may manifest polyclonal or sometimes monoclonal gammopathy: e.g. Paget's disease of the bone (90), myasthenia gravis (91), etc.

"Secondary" gammopathy differs in the following respects from "primary" gammopathy: 1) it is more often polyclonal; 2) the population of lymphoplasmoid cells in the bone marrow is less than 25% - usually varying between 5-25%, whereas in primary conditions it is more than 25%; 3) the lymphoplasmoid cell morphology is usually normal in contrast to frequent abnormal cellular characteristics exhibited by the primary diseases (92); and 4) the removal or cure of the underlying causal disease often results in the disappearance of the gammopathy. Another differentiating characteristic which is thought to be significant is that in secondary conditions the other immunoglobulins are either increased or normal, whereas in primary conditions, the other immunoglobulins are

almost invariably decreased.

(vi) Essential benign gammopathy:

It is now generally accepted that in a certain number of individuals, there exists an essential benign monoclonal gammopathy without an apparent underlying primary or secondary cause (93). The plasma cell population in the bone marrow of these individuals is less than 5%. Clinical and relevant laboratory investigations are entirely negative. However, it has been pointed out that myelomatous conditions may manifest initially as monoclonal gammopathy for many years before any clinical or laboratory signs are evident (94).

TABLE I

Comparison of Morphologic Characteristics of the Mature Plasma Cell and the Small Lymphocyte

	Mature Plasma Cell	Small lymphocyte
Size	10 - 20 microns	Less than 8 microns
Shape	Ovoid / polygonal	Globoid / ovoid
Nuclear/cytoplasmic ratio	1 : 3	Very little cytoplasm
Nucleus	Eccentric	Central with thin rim of cytoplasm
Chromatin	Clock-face	Denser and diffuse
Nucleolus	Present in most cells	Absent in most cells
Cytoplasm	Basophilic	Basophilic
Mitochondria	3+	1+
Rough endoplasmic reticulum	3+ lamellar	Very little, small cisternae
Free ribosomes	1+	3+
Golgi apparatus	Prominent	Usually absent

III. Plasma Cell Neoplasms in Mice: Review of the Literature

1. SPONTANEOUS TUMOURS

Spontaneous plasma cell neoplasms in mice, both in generalized leukaemic form and in localized form, have been described in several strains and in some of their hybrids.

(i) Leukaemic form:

Plasma cell leukaemia was first described in the Street (ST) strain of mice (95), and later in AK strain (96), in DBA, DBA/2, C3H, C57BL and CBA strains as well as in the F_1 hybrids DBA x C57BL, C3H x C57BL, and DBA/2 x CBA (97). The incidence of leukaemia was 0.5% in all the inbred strains, while in the hybrids it reached as high as 4%. In contrast to other forms of spontaneous murine leukaemia, the plasma cell leukaemia did not occur before 14 months of age. The most conspicuous gross finding at autopsy was tumour infiltration of the perirenal tissue. Involvement of the reticuloendothelial tissue manifested by splenomegaly and hepatomegaly was frequent. The mesenteric, lumbar, inguinal, axillary, and sometimes bronchial lymph nodes showed varying degrees of macroscopic changes. The thymus was not involved in any of the cases.

Microscopically, diffuse infiltration by tumour cells to the spleen and perirenal tissue and in somewhat less frequency to the liver, kidneys and lymph nodes was noted. Neither the bone marrow nor the peripheral blood was examined. However, the usage of the term leukaemia was thought to be justified because there were significant numbers of tumour cells

in the vascular and sinusoidal spaces. The histologic appearance of neoplastic plasma cells was polymorphous; accordingly, a grading of differentiation was attempted. In Grade I, the majority of cells resembled normal, mature type of plasma cells, whereas in Grade IV, many cells resembled reticulum cells. Numerous transitional forms between plasmacytic and histiocytic types were found in association with the 'reticulum' cells. Special stains for reticulin fibres were faintly positive. In general, binucleated cells and irregular giant cells were common in all grades. Mitoses were rarely seen.

(ii) Localized form:

Spontaneous localized plasmacytomas have been observed in mice of ST strain (95), and in C3H and its hybrids (98). In C3H mice this form tended to occur in an advanced age and mostly in the ileocaecal region. The exact incidence was not ascertained as routine post-mortem examination of the intestinal tract was not done in all animals. The primary neoplasms were sometimes as small as 0.5 cm in diameter. In the region of the tumour the intestinal wall was thickened and hyperaemic. The mucosa overlying the lesion was ulcerated. Since mucosal ulceration always accompanied ileocaecal plasmacytomas, it was thought that ulceration preceded neoplasia. However, in routine animals without tumour, minute ulcerations were frequently seen which did not evoke plasma cell reactions. It was postulated therefore that ulceration was followed by chronic inflammation

which in some cases transformed into a neoplastic process (99). Histologically, well-differentiated plasma cells infiltrated the muscular layer. Lymphatic channels in adjacent areas of the mesentery and occasional sinuses of the mesenteric lymph nodes also contained neoplastic plasma cells. This type of small primary neoplasm could not be transplanted successfully.

The larger lesions of the ileocaecal region which measured more than 1.0 cm in diameter, were usually associated with enlarged mesenteric lymph nodes and soft, greyish white nodular masses in the adjacent mesentery. The metastatic spread spared the lymph nodes in other parts of the body, but the spleen and thymus were grossly enlarged due to tumour involvement. Histologically, the neoplastic cells demonstrated a considerable variation in differentiation. They appeared more anaplastic in mesenteric nodes and thymus. The tumour cells replaced the centres of the splenic nodules and appeared in small aggregates in the liver. The larger lesions were readily transplantable, although for the first few generations the rate of growth at the site of inoculation and the pattern of dissemination varied in different hosts. Animals bearing transplanted tumours often had metastatic involvement of peripheral lymph nodes and gonads. With successive transplantations the pattern of growth became uniform in all animals. While the tumour grew to a considerable size at the site of inoculation in about 3 weeks, only microscopic accumulations of neoplastic cells were observed in the

internal organs. In later transplant generations tumour cells became larger with relatively large nuclei. Mitoses were frequent. The cell margin was less distinct, the nuclear hof was rarely seen and the chromatin pattern was no more distinctive of plasma cells. A moderately fuchsinophilic amorphous substance suggestive of Russell bodies was seen in many cells, particulary in tumour cells of early generations.

When the tumour type X5563 in the hybrid strain of C3H/He mice was allowed to grow for a period of 3 months, neoplastic cells sometimes disseminated into the bone marrow and caused osteolytic lesions.

(iii) Myeloma proteins in spontaneous tumours:

Serological investigations in (CBA x DBA/2)F_1 mice bearing transplantable spontaneous plasma cell leukaemia revealed that, although the total serum protein was within normal limits, there was a marked increase in the B-globulin fraction electrophoretically. The urine was free of paraprotein (100). It was shown in later investigations that in most instances the increased gamma globulin fraction was of IgG type and only infrequently of IgM or IgA types (101). The localized plasmacytoma designated as X5563 exhibited increased gamma peak in paper electrophoresis of the serum. The tumour-bearing animals did not have Bence Jones proteinuria (102). In C3H/He CRGL mice, carrying tumour type 5647 serum alterations occurred in the nature of an increase in gamma-1 globulin and beta-2

globulin. No Bence Jones protein was found in the urine (103). Myeloma proteins remained unchanged through several transfer generations over a period of years.

(iv) Chromosomal abnormalities:

Karyotypic studies of tumour cells from several lines of plasma cell leukaemia originating in (CBA x DBA/2)F_1 mice demonstrated that the number of chromosomes were variable, such as 39, 42, etc. Marker chromosomes were characterized by their excessive length, telocentricity or metacentricity (104).

2. INDUCED TUMOURS

(i) By carcinogenic hydrocarbons:

Induction of plasma cell leukaemia was first achieved in a mouse of ST strain (95) by injection of a large dose of a carcinogenic hydrocarbon (0.5 mg of benzpyrene subcutaneously into the left flank). The significant gross finding was a marked enlargement of the liver. With subcutaneous injection of 0.5 mg of 9, 10-dimethyl-1, 2-benzanthracene, localized plasmacytomas developed in two mice at the site of injection. Apart from the circumscribed, slightly ulcerated tumours, no other gross changes were demonstrable except in one, in which the lumbar lymph nodes were enlarged with tumour infiltration into lumbar muscles. In later investigations, plasma cell leukaemia was induced in several strains of mice and in their F_1 hybrids by injecting 0.02 mg of 9, 10-dimethyl-1,

2-benzanthracene into the thymus. The incidence of successful induction varied from 2.3% for C3H mice to 13% for C57BL mice (97). The mice developing the induced neoplasia ranged in age from 4 to 22 months (average age: 11.4 months); in contrast, the spontaneous form in respective strains of mice tended to occur between 7 and 29 months (average age: 19.3 months). In addition to thymic infiltration, the distribution and appearance of the tumour in the induced form were similar to that of the spontaneous form.

(ii) By intraperitoneal insertion of diffusion chambers:

Induction of plasma cell neoplasms by inserted diffusion chambers in the peritoneal space of BALB/c mice was first observed as an incidental finding, in the course of experiments designed to study passage of mammary tumour agents through pores of millipore membranes. The diffusion chambers contained pieces of mammary tumours that developed spontaneously in C3H mice carrying the milk agent (105). Later experiments (106) indicated: 1) that the BALB/c strain was more susceptible to this form of induction than other strains; 2) that the size of the chamber was an important factor as those with a diameter of 21 mm induced more tumours than 17.5 mm chambers, which in turn were more successful than those with a diameter of 14 mm; 3) that the presence of irritating plexiglas borings on the chamber increased the incidence of tumours possibly by causing more reactive fibrosis; and 4) that though empty chambers

induced the neoplasm, the presence of foreign living tissue inside the chamber enhanced the incidence.

A connective tissue capsule of varying thickness and vascularity formed around the chamber during the first few weeks. The capsule in most cases was adherent to the mesentery and serosal surfaces of abdominal organs causing thickening of the sites of attachment. The peritoneal surfaces exhibited small groups of hyperplastic plasma cells. Subsequently sanguineous ascites developed. At the time of autopsy the peritoneal surfaces were covered with tumour nodules of various sizes. The ascitic fluid contained neoplastic cells. The tumour cells resembled well-differentiated plasma cells. In several instances the plasma cell tumour was accompanied by a sarcoma.

During the process of transplantation these plasma cell neoplasms showed individual variations in the percentage of takes and the rate of growth for the first few generations. However, by the fifth generation the takes were uniformly successful and a detectable tumour in the transplanted host appeared in 2 to 3 weeks. Serologically the majority of these tumours produced B2A-type of myeloma protein. In a few instances Bence Jones proteinuria was present (107). The amount of protein excreted was directly proportional to the size of the tumour.

Only occasionally plasma cell neoplasms could be induced in C3H mice by intraperitoneal diffusion chambers. In this strain the size of the chamber or the presence of foreign

tissue did not have any bearing on the incidence of tumour induction.

(iii) By Freund's adjuvant:

Plasma cell neoplasms in BALB/c mice could be induced by intraperitoneal injections of a mixture of heat-killed staphylococcus aureus and incomplete Freund's adjuvant (108), or by incomplete Freund's adjuvant alone (109). Incomplete Freund's adjuvant was prepared by intimately mixing Bayol F and Arlacel A (mannide mono-oleate). Tumour with ascites developed 5 to 6 months after the injections.

(iv) By mineral oils:

Induction of plasma cell neoplasms in BALB/c mice by intraperitoneal injections of bland mineral oils such as Bayol F and Primol D (110) demonstrated that, given a favourable host, an irritating substance which is not readily removed by the reticuloendothelial system can eventually cause neoplasia. The first neoplasms appeared between 5 and 6 months after the initial injection of oil. Over 80% of the injected animals developed ascites within 14 months after the initial injection. The ascitic fluid contained neoplastic plasma cells.

The initial reaction was manifested by development of numerous oil granulomas on the peritoneal surfaces (111). They appeared as soon as 3 days after the first injection of the oil and were characterized by lipid droplets surrounded by macrophages. They had a rich vascular supply. A fully formed

granuloma was composed of undifferentiated mesenchymal cells, fibroblasts, scattered lymphoid cells and plasma cells. The plasma cells were considered immature because their nuclei did not have the clock-face appearance and the juxtanuclear hof was absent. One consistent site of extraperitoneal granulomas was the medullary cords of superior mediastinal lymph nodes which, as a consequence, became markedly enlarged. The lungs and liver occasionally contained these granulomas.

The proliferation of plasma cells in relation to oil granulomas preceded the progression of the granuloma to a plasma cell neoplasm. The granulomas which were not accompanied by plasma cell infiltration did not transform into a neoplastic process. Plasma cell neoplasms were always associated with ascites. Examination of the aspirated ascitic fluid in Wright-stained smears offered a ready clue to development of neoplasia. The neoplastic cells were large and had an intensely blue cytoplasm with a perinuclear clear zone. Nuclei were often multilobed. Macrophages could be distinguished from neoplastic cells by their vacuolated cytoplasm.

(v) Myeloma proteins:

It was noted in induced neoplasms that although all the peritoneal tumour nodules of a single host animal had a similar morphological appearance, each nodule had the ability to produce its own distinct immunoprotein. Thus, several plasma cell tumour lines differing according to the immunoprotein

type, could be produced in transplantation from one primary animal. Once the tumour line was established in transplantation, successive transfer generations produced the same type of paraprotein (112).

Plasma cell neoplasms in mice produced a variety of immunoglobulin alterations (113). Only one type of immunoglobulin was involved in a particular tumour. Any of the 3 immunoglobulins (IgG, IgM, IgA) showed increased serological levels. Bence Jones proteinuria was observed in several tumour-bearing animals.

(vi) Chromosomal abnormalities:

Karyotypes of 16 plasma cell neoplasms induced by mineral oil or mineral oil adjuvants in BALB/c mice (114) showed hypotetraploid chromosome numbers in 13 instances; while one line was hyperdiploid, one was hypotriploid and the remaining one with hypertetraploid. The chromosome number of a particular line varied more in earlier generations than in later ones. One to 4 marker chromosomes were present in many tumours with characteristics of being metacentric, submetacentric, minute and long telocentric. Marker chromosomes in tumour cells of various lines appeared alike in size and shape.

3. ULTRASTRUCTURE OF SPONTANEOUS AND INDUCED TUMOURS

Ultrastructural studies of plasma cell neoplasia, both spontaneous and induced, arising either in C3H or in BALB/c strains of mice, indicated that most of the morphological characteristics were similar in all tumour types (115, 116). There was a significant amount of rough endoplasmic reticulum in all tumour cells. The Golgi apparatus was well developed. There was an absolute increase in the amount of free ribosomal particles when compared to that of normal plasma cells from other sites of the host animal. Virus-like cytoplasmic particles were constantly present in every tumour cell. Particles of the A-1 type of Bernhard were seen lying free in the cisternae of endoplasmic reticulum. In some cisternae they appeared to be budding from the membrane. The rough endoplasmic reticulum was devoid of ribosomes at the site of budding and the area immediately adjacent to it. The particles were globoid or ovoid measuring 70 millimicrons in average diameter. They consisted of two concentric membranes surrounding an electron-lucent centre. The membranes were 5 millimicrons thick and were separated from each other by an electron-lucent space approximately 6 millimicrons wide. The central electron-lucent zone was slightly less than 30 millimicrons in diameter. The number of A-1 particles varied in various types of neoplasms. In occasional tumours they were seen in the space between two nuclear membranes and were

sometimes attached to them. Particles of A-2 type were seen in tumour types X5563 and DPC-1. These were aggregated in a circumscribed area near the nucleus in close association with but not actually within the Golgi apparatus. They were outside the cisternal space and occasionally appeared just beneath the plasma membrane. The outside diameter of these particles was 75 millimicrons. Each concentric lamina measured 6 millimicrons in thickness, and the central clear zone measured 50 millimicrons in diameter. The mitochondria in some tumours were swollen with formation of vacuoles and disruption of cristae. Granular bodies were present in a number of tumour cells. Nuclei were large and irregularly lobulated.

IV. The Adj-PC-5 Type of Plasma Cell Tumour in BALB/c Mice from 40th to 100th Transplant Generations: Present Study.

1. INTRODUCTION

(i) The induction and characterization of the primary Adj-PC-5 tumour:

The Adj-PC group of tumours was induced in the laboratory of Dr. Michael Potter, National Institute of Health, by a single injection of a mixture of incomplete Freund's adjuvant and heat-killed staphylococci (109). Incomplete Freund's adjuvant was prepared by intimately mixing 8.5 parts of Bayol F (Esso Standard Oil Co, New Jersey) and 1.5 parts of Arlacel A (Atlas Powder Co, Wilmington, Delaware), in a Waring blender. The mixture was sterilised in an autoclave for 15 minutes at 121°C and stored at 4°C. A trypticase soy-broth (Baltimore Biological Laboratories, Baltimore, Maryland) culture of Staphylococcus aureus, first incubated at 37°C under continuous aeration for 18 hours, was later heat-killed by placing it twice in a water bath at 60°C. The culture was then tested for sterility, adjusted to contain 16 x 10^9 bacterial cells per ml with turbidometric and centrifugal methods and stored at 4°C. Equal parts of incomplete Freund's adjuvant and heat-killed trypticase soy-broth culture of S. aureus were thoroughly mixed immediately before use. A single injection of 0.3 ml was given intraperitoneally to each mouse. This amount was chosen because it was not enough to produce ascites but sufficient to induce antibody formation (117). Seven out of a total of 64 mice used in the experiment developed plasma cell

neoplasms. The Adj-PC-5 type of tumour grew in a 10½ month old mouse (injection given at the age of 6 weeks). It was accompanied by ascites which contained histiocytes, lymphocytes and granulocytes as well as neoplastic plasma cells. These neoplastic cells were 6 to 8 times larger than lymphocytes or granulocytes. They had an irregular outline and a finely reticulated deep blue cytoplasm which contained a translucent juxtanuclear zone. Nuclei were either lobulated or duplicated. Mitotic figures were present.

Two pathologic processes were found in tissues of mice which developed plasma cell neoplasia: 1) a lipogranulomatous reaction, and 2) a proliferation of neoplastic plasma cells. The reactive lipogranulomatous nodules, which were found on both visceral and parietal surfaces of the peritoneum, were composed of cyst-like spaces and intervening connective tissue. The cysts had either thick or thin walls, showing endothelial-type cells, histiocytes and large cells with a foamy cytoplasm. The connective tissue between the cysts contained plasma cells. In mice with tumours, the lipogranulomas were largely replaced by neoplastic plasma cells. These cells had a deep-staining cytoplasm with a juxtanuclear hof. Nuclei were hyperchromatic, lobulated or duplicated. The lymph nodes which were involved had neoplastic cells in the marginal or hilar sinuses. The tumour did not spread to any abdominal organ. There were no osteolytic lesions and the kidneys did not show "myeloma"

changes. With paper electrophoresis a narrow gamma-peak was demonstrated, which later investigations confirmed to be elevated IgG levels in the serum (113). The tumour was successfully transplanted.

(ii) The "progression" of a neoplasm in the primary site and in transplantation:

A neoplastic process, in its primary growth and in serial transplantation, acquires novel characteristics over a period of time. This "progression" of a tumour has been defined as "development of a tumor by way of permanent, irreversible qualitative change in one or more of its characters" (118). The "progression" includes a continuous or intermittent series of alterations until the tumour reaches "a state of ultimate malignancy, so to speak, in which every cell acts for itself" (119). The unit characters of a particular tumour, such as the rate of growth, invasiveness, morphologic peculiarities, functional capacities, etc., may not alter simultaneously, and in fact, exhibit an "independent progression" (120). The end-point is not always reached in the original host and may be achieved during serial transplantation in normal isogenic hosts. Although the most common manifestations of progression in transplanted tumours are an increase in frequency of "takes" and a significant increase in the rate of growth, alterations in morphological appearance and even biochemical properties (121) are not unusual. It was shown in serial transplantation

of pulmonary adenomas of mice that a certain number retained their original adenomatous pattern, while others grew as spindle cell tumours (122) with the characteristics of a fibrosarcoma (123). A certain number of transplantable hepatomas in inbred mice were also shown to undergo drastic morphological change, the majority transforming into haemangioendotheliomas (124). Contrariwise, the pulmonary adenocarcinoma C4461 retained the original histology even at its 150th generation of transplanted growth (125).

Experimental plasma cell neoplasms in mice have been observed to undergo degrees of anaplastic change in subsequent transfer generations (98). A marked degree of anaplasia in tumour type 70429 with concomitant lack of endoplasmic reticulum was accompanied by loss of protein-producing capacity of the tumour cells (115).

(iii) The present study:

The following study of Adj-PC-5 type of plasma cell neoplasm in BALB/c mice, at the site of inoculation, from the 40th to 100th transplant generations, describes the morphological changes acquired in the later transplant generations. These changes, though considerable, were not accompanied by lack of protein synthesis by tumour cells.

2. MATERIALS AND METHODS

(i) Animals:

The BALB/cAn mice bearing the Adj-PC-5 type of experimental plasma cell neoplasm were obtained from the laboratory of Dr. Michael Potter, National Institute of Health, Bethesda, Maryland. The BALB/cAn strain is an inbred variant of BALB/c mice maintained at the National Institute of Health, which is not supplied to other institutions. Consequently in our laboratory the tumour was initially transplanted and subsequently maintained in BALB/cJ mice obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals were received at 6 weeks of age and kept under ordinary animal house conditions. The majority of animals at the time of transplantation were between 8 and 14 weeks of age; however, animals up to 26 weeks of age were used on occasion. In the beginning both males and females were used. The takes were uniformly successful in all animals of both sexes. However, after 2 or 3 generations the use of male mice was abandoned because of difficulty in keeping more than one animal in a cage as they tended to fight causing wounds which became infected.

(ii) Transplantation:

A group consisting of 3 mice of the same age was chosen for each serial transfer. The tumour-bearing donor animal was selected at random from the preceding group of 3

animals, between the 9th and 16th day of tumour growth, usually on 12th or 13th day. It was sacrificed by severance of the spinal cord. The skin surface overlying the tumour and the surrounding areas was liberally bathed with 70% ethyl alcohol as an antiseptic measure. On reflection of the skin the tumour tissue for transplantation was obtained from the peripheral region to avoid the central zone of necrosis. It was immediately put in ice-cold Medium 199(containing Hank's solution and l-glutamine, supplied by Grand Island Biological Company, Grand Island, New York) or sterile isotonic saline. The tissue was finely minced with scissors and the pieces were rinsed with the same fluid to remove the freely floating cells. Following this, several tiny pieces in 0.5 ml of the medium were injected with a 20-gauge needle subcutaneously into the right axillary region of the next group of mice.

(iii) Serology:

Serological examinations of tumour-bearing animals were performed to determine possible immunoglobulin alterations. Altogether 10 serological investigations were done, spaced irregularly between the 50th and 95th transfer generations. The animals were allowed to develop large tumours measuring at least 3.0 cm in diameter. Depending on the growth rate of the individual tumour this occurred between the 12th and 18th day. The blood was obtained by decapitating the animal. For each examination, sera of 3 tumour-bearing animals and that of

3 control mice of the same age and from the same shipment batch were pooled. Paper electrophoresis was done by the standard method with veronal buffer at pH 8.6. Immunoelectrophoretic analysis was done with a LKB immunophor instrument (LKB Producter, Stockholm), using agar gel as the supporting medium with a current of 20 milliamperes and 200 volts over a 1 hour period. Antiserum to mouse serum produced in rabbits (Hoechst Pharmaceutical Company, Kansas City) was used. The pooled samples of test and control sera were run simultaneously on the same slide and the slides were stained with amido black 10B.

(iv) Haematology:

Blood films were prepared and stained with Wrights' stain to look for tumour cells.

(v) Light microscopy:

Following a detailed post-mortem examination of each tumour-bearing animal, tissues were fixed in 10% formalin. Sections from the tumour, liver, spleen, kidneys, heart and lungs were routinely examined. Tissues from the thymus, right limb bone and intestine were frequantly examined. The brain and the internal genital organs were occasionally examined. Sections from paraffin-embedded tissues were stained with haematoxylin and eosin, Giemsa, Wilder's reticulin, Masson's trichrome, methyl green pyronin and periodic acid-Schiff stains. Lendrum's stain for intracellular inclusion bodies was done on

a few tumours.

(vi) Electron microscopy:

As soon as the skin overlying the tumour was reflected the surface was flooded with phosphate buffered 5% glutaraldehyde. The tumour tissue was selected from the peripheral region for securing the actively growing area of the tumour. Tiny pieces were immediately immersed in ice-cold phosphate buffered 5% glutaraldehyde for fixation. The glutaraldehyde solution was prepared according to the formula of Karlsson and Schulz (126). However, the strength of the solution was changed from 2.5% to 5%. Hence, though the tonicity of the phosphate buffer was maintained at 320-327 milliosmol, the final tonicity after addition of glutaraldehyde was higher. The tissue fragments in this fixative were kept at $4^{o}C$ for $1\frac{1}{2}$ to 2 hours. They were then washed with 0.2M phosphate buffer at pH of 7.3 (127), and post-fixed in phosphate buffered 1% osmium tetroxide (128) for $1\frac{1}{2}$ hours at $4^{o}C$. Following post-fixation the specimen was dehydrated in increasing concentrations of ethyl alcohol and finally in propylene oxide. The concentrations of ethyl alcohol in successive changes were 50%, 70%, 80%, 95% and 100% (absolute). The alcohols were changed once in the first 4 concentrations and 3 times in the absolute phase. The fragments were embedded in epoxy resin (129) after the final dehydration in propylene oxide. One-half micron sections were cut with a Servall Porter-Blum microtome and stained with toluidine blue

for preliminary light microscopic assessment. Sections for electron microscopy in the range of thickness 600-900Å were made using a Reichert "OmU2" ultramicrotome and received on either formvar-coated or uncoated grids. The grids were double stained with uranyl acetate (130) and lead citrate (131), and examined with the Philips EM 75B and EM 100B instruments.

3. OBSERVATIONS

Transplantation of the neoplasm through 60 transfers did not exhibit any observable variations from one generation to the other with respect to pattern of tumour growth and cellular morphology. Hence the following descriptions pertain to all the transplant generations studied.

(i) Gross findings:

In animals with subcutaneous minced tissue implantation the tumour became apparent on the 5th or 6th day as a diffuse swelling at the base of right forelimb. The swelling increased in size and soon became a well demarcated subcutaneous tumour nodule. By the time the animals were sacrificed (between the 12th and 16th day of tumour growth) the lesions measured slightly more than 3.0 cm in principal dimensions. At this time the host animals were lethargic and moved with difficulty. The right forelimb was almost immobilized from being partially incorporated in the tumour mass. The fur in general lost its sheen and became relatively sparse on the skin

overlying the tumour. Clinically this was the pre-mortem state as the animal usually died within a few hours if not sacrificed at this time. On palpation the tumour was firm in the early stage of growth but became progressively softer. Occasionally there was ulceration of the skin over the tumour.

At autopsy the skin over the subcutaneous tumour was focally adherent to the underlying mass in the central part. As a result of adhesion, a portion of the tumour usually became detached with reflection of skin. The tumour was monencapsulated but fairly well demarcated except at sites of adhesion. It was very soft and thus difficult to grasp with forceps. The lesion grossly appeared to involve the skeletal muscles surrounding the bone of the right forelimb and also the underlying intercostal muscles. With infiltration into the muscles the mass became adherent to the ribs and to the humerus. The cut surface of the tumour appeared chalky white and necrotic, with large areas of recent and old haemorrhage. However the peripheral regions were tan and glistening and appeared viable. Occasionally the tumour mass penetrated into the right pleural cavity causing haemorrhagic effusion. This incidence was so rare that it seemed probably that the thoracic cage might have been inadvertently punctured during inoculation.

(ii) Serology:

Quantitative estimations of protein fractions were not done. Compared with the control sera, the sera of tumour-

bearing animals showed a higher gamma peak in paper electrophoresis (Fig. 1), and a deeper staining IgG band in immunoelectrophoresis (Fig. 2), suggesting increased levels.

(iii) Haematology:

No abnormal cells resembling tumour cells were seen in the blood films.

(iv) Histology:

Neoplastic cells tended to disseminate from the tumour growing at the site of implantation through the vascular channels to various other organs. The pattern of dissemination and the morphological characteristics of tumour cells, both in contiguous structures and in distant sites, are described in a later section (section V). The following light and electron microscopic descriptions of tumour cells pertain to the cells growing at the site of subcutaneous implantation.

Light microscopy:

The tumour mass was composed of large roughly ovoid or polygonal cells arranged in lobules (Fig. 3). The lobules were formed by delicate strands of reticulum fibres surrounding a group of tumour cells (Fig. 4). In the actively growing areas the lobules were continuous giving a compact appearance. As the major part of the tumour mass was haemorrhagic and necrotic the lobules often appeared as isolated islands, separated from each other by non-staining areas or by

acidophilic amorphous material containing red blood cells.

The lobules were of various shapes and sizes, containing as few as 10 or as many as hundreds of tumour cells. Cells resembling fibroblasts with spindle-shaped nuclei and scant cytoplasm were attached to the reticulum fibres, but collagen could not be demonstrated within the substance of the tumour with Masson's trichrome stain. The peripheral areas of lobules contained moderate number of capillaries, the majority of which had tumour cells in their lumens. Fibrin thrombi were not infrequently found within the tumour substance (Fig. 5). Scattered thin wisps of reticulum fibres were occasionally seen within the lobules between tumour cells but not surrounding individual cells.

Tumour cells measured from 35 to 50 microns in diameter. Within individual lobules they were not attached to each other and showed no polarity in distribution. The cells had a distinct cytoplasmic outline. The cytoplasm was scanty, moderately basophilic and often vacuolated. With methyl green pyronin stain the cytoplasm appeared intensely pyroninophilic, indicating a considerable amount of ribonucleic acid in the cytoplasm. In general, the tumour cells stained negatively with PAS, but occasional cells near the necrotic zones contained a few tiny PAS-positive droplets. No cytoplasmic inclusions could be demonstrated with Lendrum's inclusion stain.

The nuclei occupied two-thirds or more of the cellular volume. They were irregularly lobulated and frequently demonstrated deep indentations which sometimes created the impression of binucleation. Only rarely multinucleated giant cells were seen. Nuclei were vesiculated and contained one or more nucleoli. The peripheral chromatin was of irregular thickness and did not reproduce the clock-face pattern of mature plasma cells. Mitotic figures were frequent and included various abnormal tripolar, star-shaped and other forms.

The necrotic areas did not elicit reactive infiltration of macrophages or neutrophils. The zone of necrosis initially appeared in the centre of the lobule and progressed centrifugally. Frequently the lobule lost its surrounding sheath of reticulum fibers and the cells became dispersed in the extralobular space. The necrotic cells were smaller and more or less rounded. The cytoplasm was acidophilic and completely devoid of pyroninophilia. Nuclei were either pyknotic or fragmented.

In cases where the skin overlying the subcutaneous tumour was ulcerated, large circumscribed areas of neutrophils were seen within the rumour mass, although purulent material was not evident grossly.

Electron microscopy:

The general appearance of the tumour cell under the electron microscope corresponded to that seen with the light

microscope. The cells were polygonal with large nuclei and relatively small amounts of cytoplasm. Under the light microscope the cells were often dissociated from each other within a lobule, but ultrastructurally they were in close apposition (Fig. 6). This difference in cellular disposition was considered to be the result of fixation. The plasma membrane of tumour cells was stretched out with slight undulations but without deep infolding. No surface microvilli were present. Small parallel areas of contiguous plasma membranes sometimes appeared relatively more electron-dense, but this was attributed to variabilities in image formation rather than to the presence of desmosomes. The intercellular space between plasma membranes of two neighbouring cells measured 35-40 millimicrons in width. As the cells were polygonal the space between 3 neighbouring cells often assumed a triangular shape which varied considerably in size. These spaces contained amorphous electron-dense granules (Fig. 7). Larger intercellular spaces contained either amorphous homogeneous electron-dense substance or distorted red blood cells.

The cytoplasmic volume was considerably less than the nuclear volume and contained the endoplasmic reticulum, ribosomes, the Golgi apparatus, virus-like particles, mitochondria and dense bodies (including multivesicular bodies).

The endoplasmic reticulum was neither abundant nor

arranged in typical lamellar fashion. Although present in every cell the amount was markedly decreased. Several flattened or dilated cisternae were scattered irregularly in the hyaloplasm all around the nucleus. Cisternae were of various sizes and shapes. The space within the dilated cisternae was usually electron-lucent and only rarely contained moderately electron-dense amorphous material. Dense spherical bodies - ultrastructural analogues of Russell bodies - were not seen in these tumour cells. The greater part of the surface of cisternae was granular, alternating with smaller agranular areas of variable length (Fig. 8). Free ribosomes were abundant in the hyaloplasm. Small ribosomal aggregates in the nature of polysomes were moderate in number. The Golgi apparatus, though not always appearing in the plane of section, was well developed and usually situated in close proximity to the indented region of the nucleus (Fig. 9). It was comprised of sacs or cisternae in parallel arrangement, electron-lucent vacuoles and also small vesicles with dense content. Not infrequently the cisternae or vacuoles were cystically dilated. There was no appraciable increase in the number of mitochondria. Although they were scattered around the nucleus there were small clusters in certain areas, particularly near the indented region of the nucleus. They presented a round, ovoid or elongated profiles and showed considerable variation in sizes. Many mitochondria showed swelling and vacuolation with loss

of intramitochondrial matrix and disruption of cristae. The cristae were moderate in number, sometimes showing a regular parallel arrangement but often disposed in a disorderly fashion (Fig. 10).

Characteristic cytoplasmic components of transplantable plasma cell neoplasms were virus-like particles (Fig. 11), resembling type A particles of Bernhard. Their number and distribution in individual cells varied considerably. They measured 80-130 millimicrons in diameter and had a round or somewhat ovoid contour. Almost all of them were found within the cisternal spaces of the endoplasmic reticulum. Only rarely an isolated particle was seen in the space between two nuclear membranes. The cisternal spaces contained from one to several of these particles. The surrounding endoplasmic reticulum showed alternate smooth and rough areas but infrequently it was entirely smooth. The majority of the particles were found free within the cisternae but occasional particles were attached to the inner surface of the membrane (Fig. 12). At the site of attachment the particle seemed incomplete, having a semicircular or a horseshoe appearance and the outer surface of the cisterna was devoid of ribosomes. The particles had a concentric double membrane with space between the two membranes. The inner membrane was slightly more electron-dense than the outer one. The double membranes contained an electron-lucent centre, the diameter of which was approximately

half of that of the particle itself.

Electron-dense bodies limited by a single membrane were seen in irregular numbers in numerous tumour cells. These bodies were globular or ovoid and differed from each other in density and size, measuring from 90 to 225 millimicrons in diameter. The majority of them were multivesiculated (Fig. 13). Although some were seen in the vicinity of the Golgi apparatus, they were scattered at random in the hyaloplasm even close to the plasma membrane. They bore no relation to the endoplasmic reticulum. Osmiophilic lamellated structures resembling myelin figures were seen in many cells. They were generally in relation to the endoplasmic reticulum but were infrequently found near the Golgi apparatus or the outer nuclear membrane.

The nuclei had lobulated configurations (Fig. 14). They usually showed indentations which sometimes were deep enough to simulate binucleation under the light microscope. The hyaloplasm extended into the indented region, and cytoplasmic inclusions within nuclear substance were not infrequent. The outer nuclear membrane was separated from the inner one by an electron-lucent space of variable width. The outer membrane was studded with ribosomes along the major part of its length, alternating with a smaller extent of smooth areas. The nuclei had an irregular chromatin pattern. Usually clumps of peripheral chromatin protruded into the depth of the nucleus,

giving the appearance of nucleoli under the light microscope. Nucleoli were rarely seen.

Cells undergoing mitosis contained the virus-like particles also (Fig. 15). Small cisternae of endoplasmic reticulum along with mitochondria and dense bodies were scattered in the peripheral region of mitotic cells.

4. SUMMARY OF FINDINGS

In later generations of Adj-PC-5 type of plasma cell neoplasms in BALB/c mice, the tumour grew rapidly at the site of subcutaneous implantation. At the 12th or 13th day of tumour growth, the mice were sacrificed and transplantation to the next group of animals was done. At that time the tumour had infiltrated into adjacent skeletal muscles and widely disseminated through vascular channels to various internal organs.

Serologically the tumour-bearing animals showed elevated levels of IgG immunoprotein. Cells resembling tumour cells were not recognized in blood films.

Under light microscopy tumour cells were large, measuring 35-50 microns in diameter and arranged in lobules which were surrounded by delicate strands of reticulum fibres. These tumour cells had very little resemblance to normal mature plasma cells, with reversion of nucleocytoplasmic ratio and frequent abnormal mitotic figures. The cytoplasm was intensely

pyroninophilic. Ultrastructurally, cells were polygonal with stretched out plasma membranes. The cytoplasm contained a markedly decreased amount of endoplasmic reticulum with alternating rough and smooth surfaces. Free ribosomes were abundant. The cisternae which were frequently dilated contained virus-like particles. The Golgi apparatus was well developed. Electron-dense bodies, particularly multivesiculated ones, were often present. Nuclei, devoid of distinct nucleoli, showed deep indentations, with clumps of chromatin protruding into the nuclear substance. Mitosing cells contained all cytoplasmic components, including virus-like particles and multivesicular bodies.

5. DISCUSSION

Light microscopic and ultrastructural characteristics of earlier generations of plasma cell neoplasms resembled well differentiated plasma cells (132, 115, 116). It appears that tumour cells in later generations develop considerable morphological changes towards anaplasia.

Several features characterize the nuclei and cytoplasm of cancer cells (133), although it is generally agreed that cancer cells may display a wide spectrum of morphological changes. Basically the cells acquire a resemblance to embryonic cells, thus becoming "dedifferentiated". They are larger than their normal counterparts. The increase in size is primarily due to enlarged nuclei without proportionate increase in

cytoplasm, resulting in a raised nucleocytoplasmic ratio. The nuclear enlargement is presumably due to the increased volume of chromosomes, frequently associated with their increased number (polyploidy). Some degree of aqueous imbibition also contributes to the enhanced nuclear size. Nuclei are irregular in contour with deep fissures which impart a lobulated configuration. Depending on the plane of section cytoplasmic invaginations into these nuclear indentations may create pseudoinclusions in the nuclei, containing cytoplasmic organelles. Chromatin clumps are usually coarser. The number of nucleoli is increased: individual tumour cells frequently exhibiting 2 or more nucleoli. Abnormal mitoses are common, and in many cells the number of chromosomes shows polyploidy or aneuploidy. A remarkable feature noted in a majority of tumour cells is the cytoplasmic basophilia. The basophilia in normal cells is mainly due to the presence of endoplasmic reticulum with attached ribosomal particles. In cancer cells, however, the loss of rough endoplasmic reticulum is directly proportional to the degree of differentiation. In spite of that, dedifferentiated cells may remain basophilic, largely due to the dispersed granules of ribosomes which are considerably increased in the hyaloplasm. These dispersed granules tend to be disposed singly rather than as polyribosomes. Swelling of cisternae of the endoplasmic reticulum is very common in cancer cells. The Golgi apparatus may either be

hypertrophied or atrophied, although there is a tendency to reduction in size with the degree of dedifferentiation. Mitochondria, though their number varies immensely from one cell to another even in the same tumour, are generally believed to be decreased. Their size and shape in cancer cells appear to be of no significance. The matrix often is swollen with consequent alterations in the internal structure.

It can be concluded then that the tumour cells in this study show most of the principal characteristics of dedifferentiated cells. Larger cell size, increased nucleo-cytoplasmic ratio, abnormal nuclear configuration, frequent mitoses, decrease in rough endoplasmic reticulum with increase in free ribosomal particles, denote their marked dissimilarity to normal mature plasma cells. Therefore they can be justifiably categorized as dedifferentiated cells.

It has been shown that cells which synthesize a large amount of secretory proteins, such as pancreatic acinar cells and plasma cells, have an elaborate system of rough endoplasmic reticulum which accounts for their marked basophilia under the light microscope. The pancreatic acinar cells which secrete digestive enzymes have well developed rough-surfaced cisternae of endoplasmic reticulum in the basal region. With the aid of ultrastructural radioautography and differential fractionation, it is known that the protein is synthesized on ribosomes attached to this membranous system (134), and

subsequently transported across the membrane to be segregated within the cisternae (135). Then they are condensed in the small, smooth-surfaced vesicles which form the peripheral component of the Golgi apparatus (136, 137) before appearing as zymogen granules at the apical region of the cell. Antibody-producing plasma cells under antigenic stimulation also display abundant rough endoplasmic reticulum (19, 14). Tumour cells of earlier generations of experimental plasma cell neoplasms contained similar amounts of rough endoplasmic reticulum (115) synthesizing high levels of immunoprotein. In comparison in this study tumour cells of later generations exhibited a considerable reduction of this organelle. This was evidenced not only by haphazard distribution of small cisternae around the nucleus,but also by alternate smooth areas of variable length on the membrane. There was no proliferation of non-neoplastic plasma cells in the bone marrow, in lymph nodes or in tissues around the tumour; therefore the continually elevated serum level of the immunoprotein in host animals could reasonably be attributed to the activity of tumour cells, despite the relative decrease in rough endoplasmic reticulum. Sustained IgG production due to increase in cell numbers, although with decreased output per cell, may be the explanation of this apparent paradox. However, certain primitive cells such as myoblasts can synthesize proteins through free ribosomes (138). Hence it is conceivable that these tumour

cells in the process of dedifferentiation have acquired the embryonic property of synthesizing proteins through free ribosomes.

All primary and transplantable plasma cell neoplasms in BALB/c and C3H mice described to date contain virus-like particles, which resemble Type A particles of Bernhard. Bernhard's description of these particles followed his observations on mammary tumours in mice which were known to carry Bittner's milk factor (139). As a matter of convenience he classified these particles into Type A and Type B. The Type A particles were predominantly intracytoplasmic, had a concentric double membrane with an electron-lucent centre, and measured 65-70 millimicrons in diameter. These particles were found in close topographical relation to the Golgi apparatus. Type B particles were predominantly extracellular, had an eccentric nucleoid body and measured an average 105 millimicrons in diameter. Occasionally both types could be found inside or outside the cell. It appeared that Type A could transform into Type B particles. In tumour cells of plasma cell neoplasms virus-like particles were almost exclusively found within cisternae of endoplasmic reticulum. Only rarely an isolated particle was seen between two nuclear membranes. In cisternae, incomplete particles could be seen "budding" from the inner surface of the membrane, which was devoid of ribosomes at the site of budding. This budding was presumably followed

by completion of the membranes and falling off within the cisternal space. This led to the view that the particles arose from the endoplasmic reticulum. However, how the particles acquire double membranes has not been fully explained. A morphological variant of these particles was described in X5563 type of plasma cell neoplasm where the central region of a bilaminated particle appeared dense or nucleoid (140, 116). The only difference between the particles of earlier and later generations of the tumours was in size; while in earlier generations they measured 75 millimicrons in average diameter, in the present study of later generations they measured over 80 millimicrons in diameter, and particles as large as 130 millimicrons were seen. The ability of these virus-like particles to induce neoplasia has not been proven. Ultracentrifugal fractions of cell-free extracts of MPC types, when injected subcutaneously in BALB/c mice, did not induce plasma cell neoplasms (106).

One characteristic of tumour cells in later generations observed in this study which was not described either in the primary or in earlier generations of plasma cell neoplasms, was the presence of several multivesicular bodies. There is controversy about the origin of multivesicular bodies; however, most observers believe that they arise from the Golgi apparatus. According to the GERL (Golgi endoplasmic reticulum lysosomes) theory of Novikoff, "Golgi vesicles move into a

pinocytotic or phagocytic vacuole forming from cell surface and into a multivesicular body" (141). There may be a wide range of qualitative and quantitative difference in size, shape and vesicles of mutivesicular bodies (142). They are believed to be closely related to the lysosomal system. The majority of them exhibit acid phosphatase activity. They can digest both exogenous and endogenous proteins; in the process of digesting endogenous proteins they become transformed into dense bodies (143). In some forms of tumour cells such as HeLa and Mac-21 (mucoid adenocarcinoma of the lung) they have been implicated in the formation of lipid-rich myelinoid structures and the possible formation of myelin figures (144). Although many myelin-like figures in tumour cells were seen in the present study of later generations of plasma cell neoplasm their mode of formation were often difficult to determine because of the propensity of these tumour cells to degenerate rapidly and the possibility of artefactual origin due to immersion fixation. Dense bodies were often seen in close proximity of multivesicular bodies, and the probability remains that these bodies were involved in digestion of endogenous proteins.

Significant morphological differences exist between tumour cells of the later generations of Adj-PC-5 plasma cell neoplasms and those of human multiple myeloma, the cellular features of which have been described earlier. In contrast to the larger cells with indented nuclei of these experimental

tumours, the typical myeloma cell is about one-half the size with usually one eccentric round nucleus occupying only one-third of the cellular volume. The cytoplasm of the myeloma cell is deeply basophilic and frequently contains Russell bodies and exhibits PAS-positivity. Large, round, dense intracisternal material which accounts for the light microscopic appearance of Russell bodies were absent in experimental tumour cells. PAS-positivity of the myeloma cell is due to the presence of dense amorphous intracisternal substance (145), whereas dilated cisternal spaces in experimental tumours are usually empty, correlating with the lack of PAS staining. Experimental tumour cells are devoid of surface microvilli which are characteristically seen in myeloma cells. Virus-like particles and a few multivesicular bodies are invariably present in these tumour cells, while they are generally absent in myeloma cells. However, both single and double-membraned particles, measuring 30-50 millimicrons, have been described in myeloma cells on rare occasions.

Table II

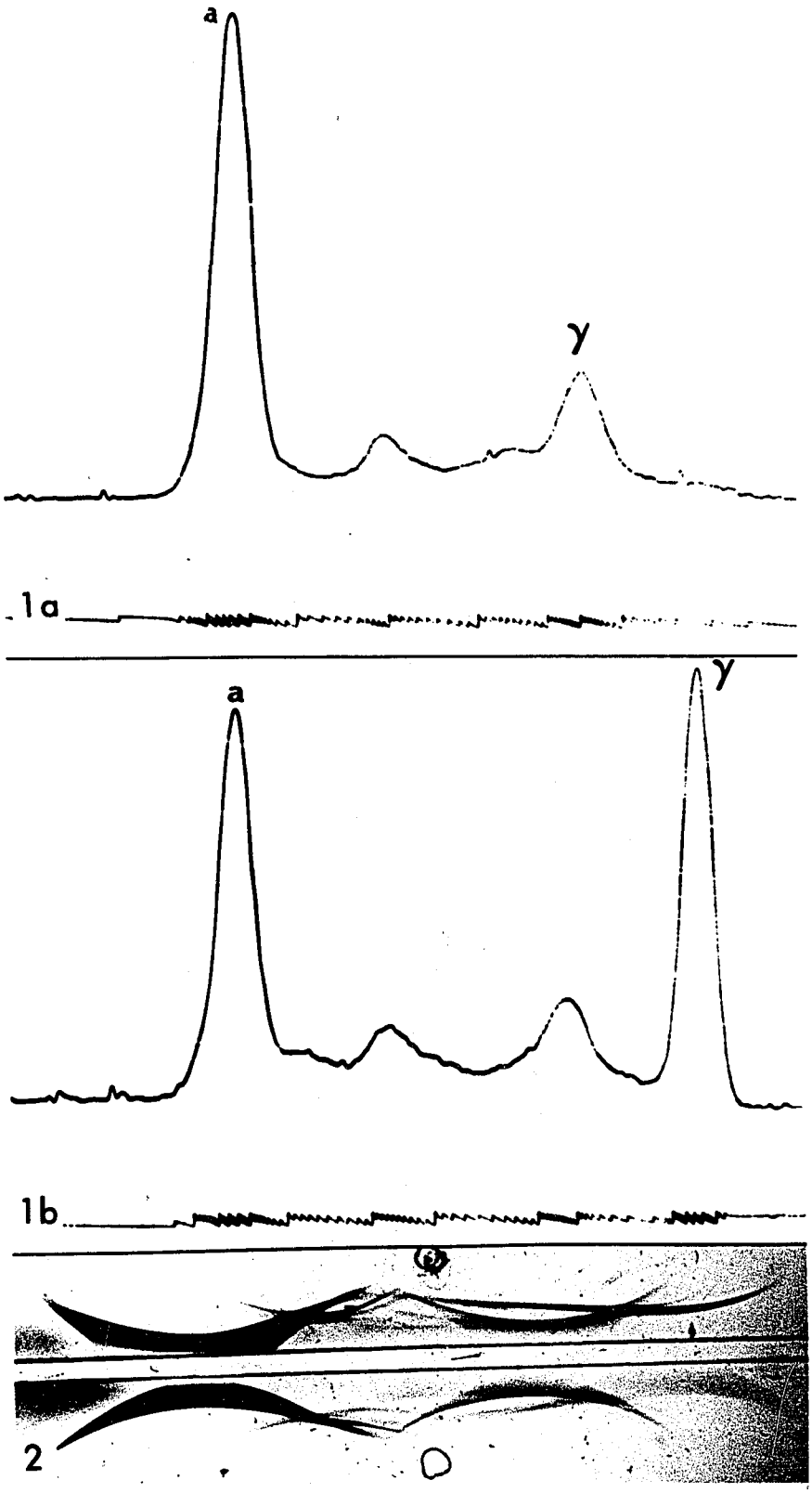
Comparison Between Neoplastic Cells of Human Multiple Myeloma and of Adj-PC-5 Tumour of Mice in Later Generations.

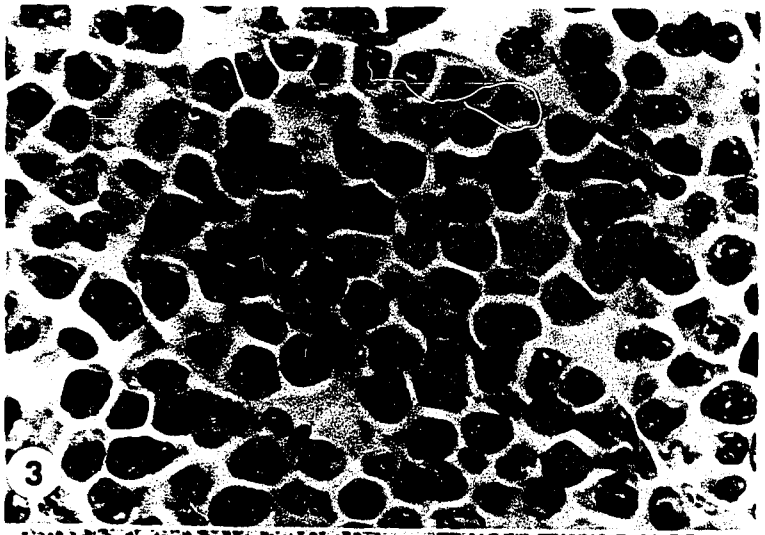
	Multiple myeloma	Experimental Tumor
Size	15-30 microns	35-50 microns
Plasma membrane	Microvilli	Stretched out
Rough endoplasmic reticulum	Abundant	Decreased
Russell bodies	Present	Absent
PAS-positivity	Often present	Very rare
Virus-like particles	None	Invariably present
Multivesicular bodies	None	More than one

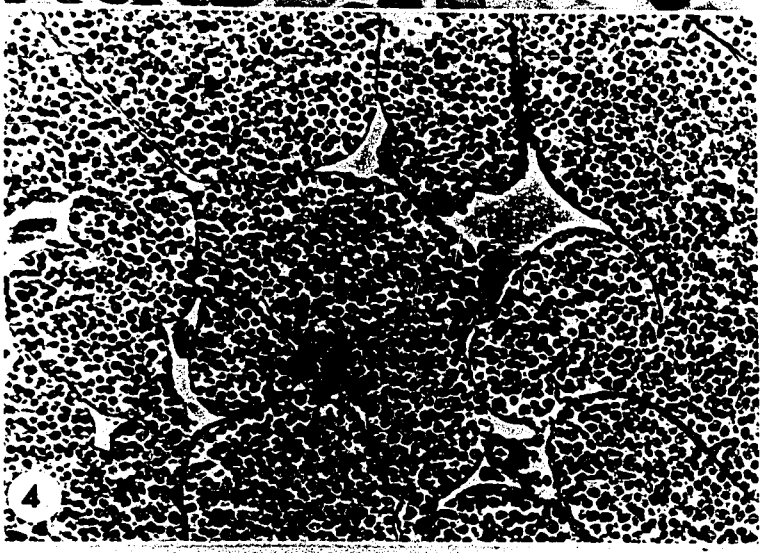
Figure 1a. Paper electrophoresis of pooled sera of 3 normal mice.

Figure 1b. Paper electrophoresis of pooled sera of 3 tumour-bearing mice shows elevated gamma peak.

Figure 2. Immunoelectrophoresis of pooled sera of tumour-bearing mice above, shows increase of IgG (arrow, right), as compared to the pooled sera of control animals below.


a
γ
1a
a
γ
1b
2


3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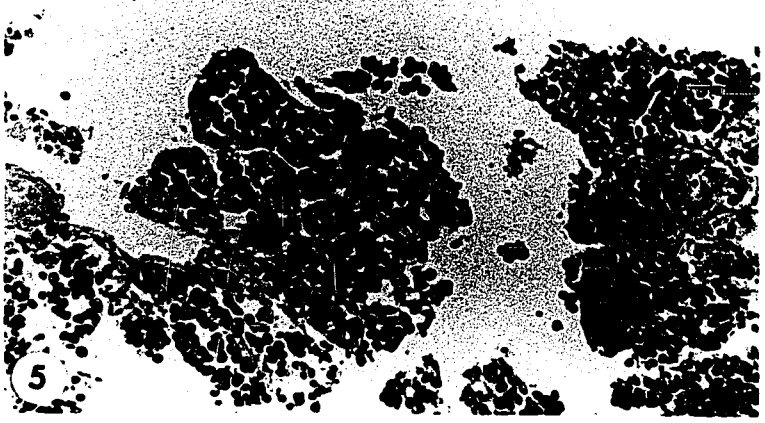

5

Figure 1. Low-power electron micrograph of the subcutaneous tumour. There are a group of polymorphonuclear cells with large, lobulated nuclei and a proportionately smaller amount of cytoplasm. Deformed red blood cells and electron-dense amorphous material occupy some intercellular spaces. Uranyl acetate and lead citrate. X3,000.


6

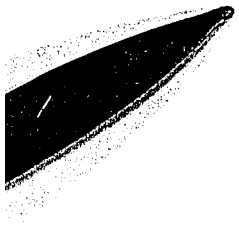
Figure 7. A triangular intercellular space (ISC) contains amorphous granular material. The plasma membranes of contiguous cells show slight undulations but are devoid of microvilli. Uranyl acetate and lead citrate. X55,000.


7

Figure 8. Smooth agranular areas, alternating with rough areas on the surface of the endoplasmic reticulum, are indicated by arrows. Numerous free ribosomes are present in the hyaloplasm. Two mitochondria show vacuolation of matrix. Uranyl acetate and lead citrate. X55,000.


8

Figure 9. A well-developed Golgi apparatus (GA) in the paranuclear area shows elongated sacs in parallel arrangement, and electron-dense and electron-lucent vesicles. A few electron-lucent vesicles are dilated. On the left is the nucleus (N). Uranyl acetate and lead citrate. X55,000.




9

Figure 10. Mitochondria (M) are present along the plasma membranes of two contigous cells which show vacuolation of the matrix. In some mitochondria the cristae are arranged in a parallel fashion, but in others they are irregularly disposed. Uranyl acetate and lead citrate. X30,000.


10

Figure 11. Virus-like particles in the paranuclear area have a concentric, bilaminated appearance. They are located within the cisternal spaces of the endoplasmic reticulum. Part of the nucleus (N) is seen on the right. Uranyl acetate and lead citrate. X40,000.


N
11

Figure 12. Virus-like particles with incomplete rings seem to be budding from the inner surface of the endoplasmic reticulum (arrows). At the areas of budding, the outer surface of the endoplasmic reticulum is devoid of attached ribosomes. Uranyl acetate and lead citrate. X40,000.


12

Figure 13. Four multivesicular bodies (MV) in the centre of the electron micrograph show single limiting membranes. They present globoid profiles and are smaller than mitochondria (M). They are not in close relation to the Golgi apparatus or to the endoplasmic reticulum. Frequently they are accompanied by electron-dense bodies (DB). Uranyl acetate and lead citrate. X55,000.

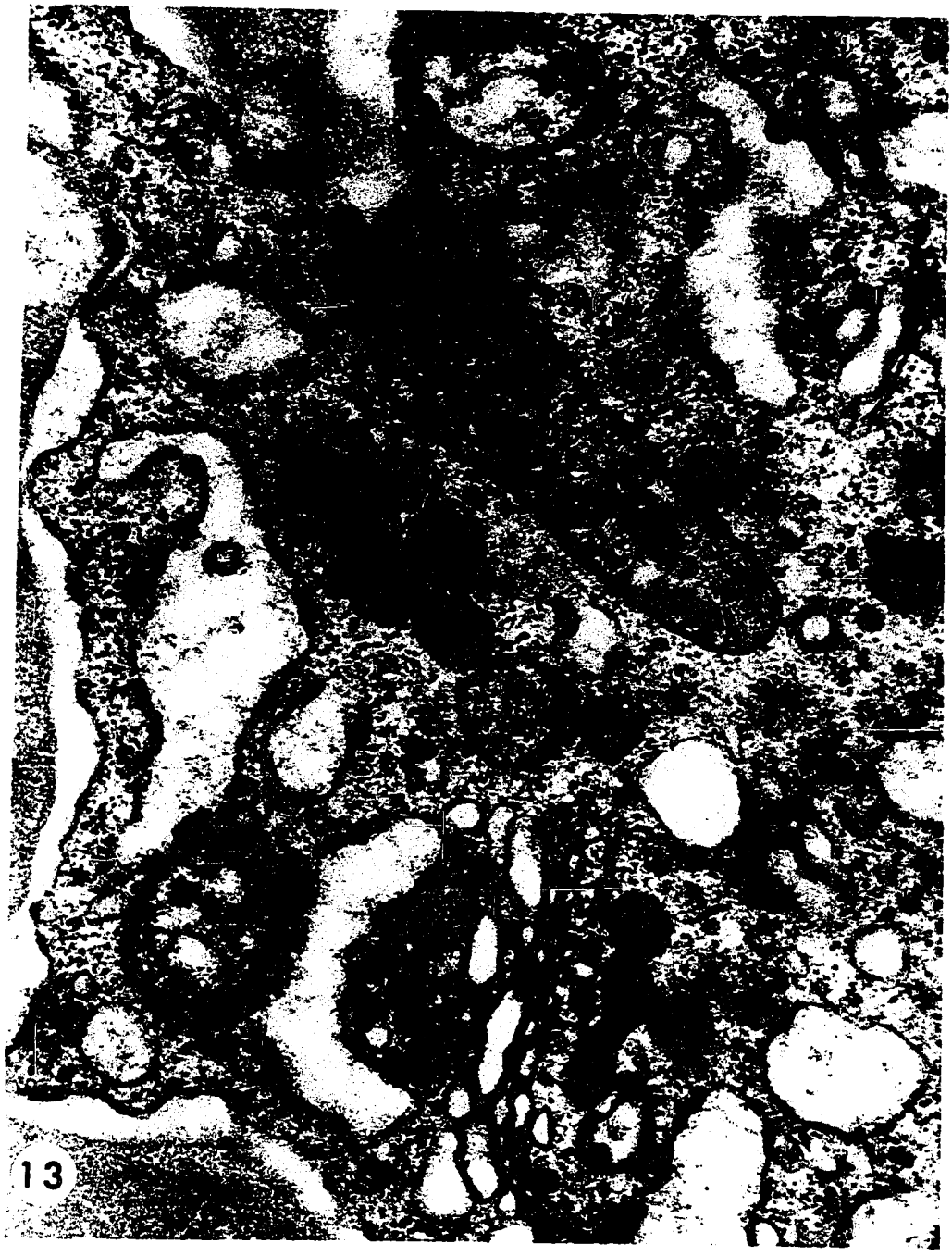
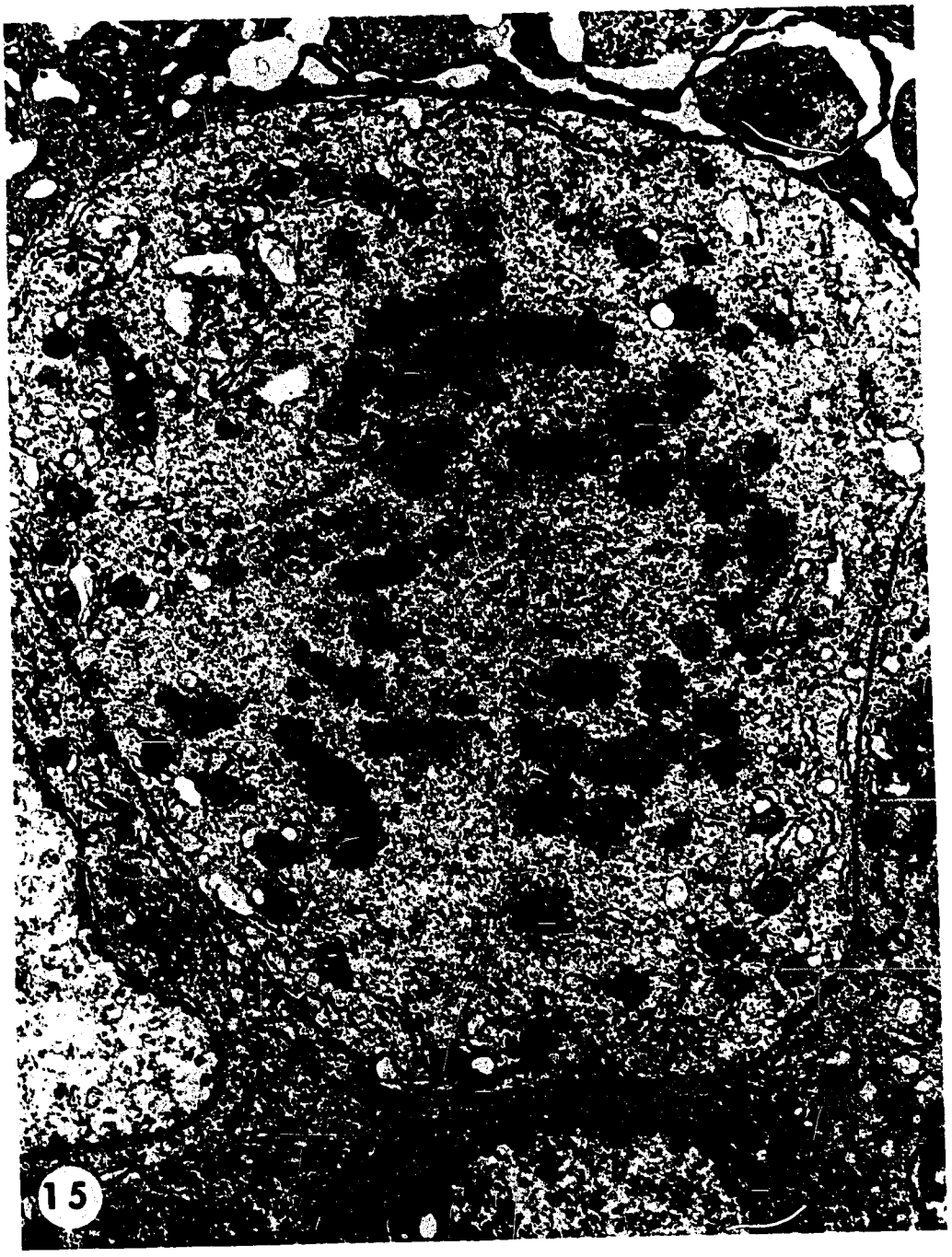

13

Figure 14. A large nucleus showing irregular indentations, and clumps of peripheral chromatin extending towards the central region of the nucleus. Virus-like particles are seen in the upper right corner of the electron micrograph. Uranyl acetate and lead citrate. X16,500.


14

Figure 15. A tumour cell in mitosis with a centriole (arrow). Mitochondria and cisternae of the endoplasmic reticulum are present at the periphery of the cell. Uranyl acetate and lead citrate. X9,000.


15

V. The Pattern of Spread of Tumour Cells in Later Transplant Generations of Adj-PC-5 Type of Plasma Cell Neoplasm: Present Study.

1. INTRODUCTION

(i) Local invasion, dissemination and metastasis:

After a variable period of growth at the site of origin (the primary site), the neoplastic cells of a malignant lesion tend to detach themselves from the parent mass and spread by aggressively infiltrating into the surrounding tissue and by disseminating into vascular and lymphatic channels. Local invasion is considered a prerequisite for dissemination. Dissemination often leads to metastatic lodgement in distant organs or in draining lymph nodes. Infrequently tumours metastasize by direct implantation, spreading over serosal surfaces.

An interplay of various factors - pertaining to the lesion itself, the cells and the associated matrix - has been postulated in the release of tumour cells from the parent mass. In a continuously growing lesion an internal tension develops which exerts a pressure on the budding marginal cells to invade the accessible crevices in the surrounding tissue. "The growth of the tumour thus resembles that of a root and its attached rootlets; the main root enlarges expansively, while the rootlets and roothairs thrust their way along lines of least resistance in the surrounding soil; and the growth and soil-disrupting powers of the whole structure are attributable to the progressive proliferation of its cells" (146). When tumour cell lines are inoculated in sponge-matrix tissue

culture it is seen that a nest of cells gives rise to other nests. Such spread of tumour, by progressively forming multicellular nests, is called "aggregate replication" (147). A rapidly expanding mass may be inadequately nourished by the available vascular supply, causing degenerative changes and subsequent cellular detachment (148). Presence of degeneration itself in the tumour may provide a stimulus for further rapid growth. Experimentally, irreversibly damaged tumour cells exert a proliferating effect on the admixed viable cell fraction (149) and thus enhance the occurrence of local invasion. By mechanical means, such as with microdissection needles or a shaking device, it has been shown that cancer cells are deficient in mutual adhesiveness; compared to normal cells they dissociate from each other more easily (150). Decreased cellular adhesiveness is attributed to deficiency of calcium, presumably at the surface of cancer cells. It is postulated that a molecular bond between apposed cells exists by linkage of the calcium to the carboxyl groups of proteins and to the phosphate groups of lipoids (151), and the lack of this bond renders cancer cells less adherent to each other. This property of cancer cells is complemented by active amoeboid movements. In tissue culture tumour cells exhibit motility as a single cell and also as small clusters; however, compared to polymorphonuclear leucocytes their movement is slower (152). Interestingly, different strains (developed in different

animals) of rat hepatomas demonstrate dissimilar rate of motility (153), indicating that the motility rate is a property peculiar to the individual tumour. Presence of a "spreading factor" or a hyaluronidase-like substance in human and animal tumour tissue has been disputed (154). A protein-like substance is thought to emanate from tumours stimulating the growth of undifferentiated young connective tissue in the adjacent region of the tumour. The presence of this tissue is considered essential for invasion (155). The quantity and morphology of this connective tissue may vary. Reticulum fibres and acid mucopolysaccharide may or may not be present. This young connective tissue is rich in ribonucleoprotein and PAS-positive polysaccharide.

Malignant cells do not exhibit "contact inhibition". The phenomenon of contact inhibition between normal cells, both fibroblastic and epithelial, can be demonstrated in tissue culture. When two explants of epithelial tissue are grown a short distance apart, the developing edges of both will move towards each other. The cells at the edge are the pacemakers. As soon as the two advancing edges meet the speed of the cells at the site of contact drops sharply, and the density of cellular population in the interexplant region becomes stable. In case of cancer cells this immobilization does not occur and the malignant cells continue to grow into the normal cell population (156). Certain types of malignant cells (Ehrlich

ascites tumour, Klein lymphosarcoma, sarcoma 37) show an increase in the negative charge on the cell surface (157); however, how the surface electrical property influences the invasiveness of malignant cells has not been determined.

Dissemination of cancer cells through the vascular system apparently occurs in several stages. The cancer cells first penetrate through the vascular wall into the lumen. Then they are transported to various sites, aided by the flow of blood to which they add their own motility. Primarily it is the venous system which transports the cells, but arterial dissemination has also been demonstrated. Finally the embolic cancer cells traverse the vascular wall to form the metastatic nidus in the parenchyma of the host organ under suitable conditions. It is generally accepted, however, that both in experimental animals and in man, vascular invasion and tumour cell embolization do not necessarily result in distant metastases (158). Nevertheless, since 1955 (159), numerous studies on circulating cancer cells have appeared. Although a few interesting facets of tumour cell dissemination have emerged from these studies, the search for circulating cancer cells in no way offers an aid to preliminary diagnosis, therapy or prognosis. Techniques in three principal categories - enzymatic, floatation and filtration - are applied to separate the normal cellular elements from cancer cells, and then the latter are studied on glass slides or membrane filters with

suitable staining. The success of identifying tumour cells increases with the degree of dedifferentiation of the primary tumour, and in tumours which have undergone considerable necrosis. Patients with widely disseminated cancer are more likely to have tumour cells in their blood (160). A higher yield in percentages of cases and also in harvesting of cells is obtained if blood samples can be procured from regional and local sites of the tumour - usually during surgical procedures - rather than from the peripheral blood (161). In one large series (162), based upon an analysis of 981 specimens obtained from 376 patients, it was observed that in resectable cases cancer cells could be demonstrated in 9% and 30% of the peripheral and local blood samples respectively, in contrast to 15% and 48% among non-resectable cases. Percentagewise, circulating tumour cells are more likely to be seen in patients suffering from sarcoma than adenocarcinoma, and least likely in epidermoid carcinoma (163).

In both human and experimental conditions the majority of circulating tumour cells perish in the course of time. Certain factors that are related to cancer cells, to the components of the vascular system, and to the host organs, exert modifying influences on the formation of metastases by the surviving cells. Greater number of viable circulating tumour cells are more likely to cause metastases. It has been shown with Ehrlich ascites tumour cells that the number of

pulmonary metastases could be raised when increased number of viable cells were injected intravenously (164). Metastasizability appears to be a property of late cancers rather than of early ones, and concurrently the cancer acquires heterotransplantability (165), thus indicating an enhanced virulence in the cells of a late cancer. Assuming that metastasis in the human is a type of autotransplantation, ploidy of tumour cells from the metastatic and primary sites of mammary adenocarcinoma was studied by the microspectrophotometric technique. It was found that metastatic cells demonstrated greater ploidy than the cells from the primary site; and hence the suggestion was made that hetero-aneuploidy favoured the survival of cells in the circulation that metastasize (166).

Several forms of coagulopathy appear to be the preeminent factor in the vascular component for the occurrence of metastases. In general, in the presence of blood-borne metastases, there is elevation of fibrinogen levels with concomitant increase in fibrinolysin inhibitors (167). Some cancer cells are rich in thromboplastin (168). With radioactive isotope labelling an intimate relationship between fibrin and tumour cells has been demonstrated (169).

Morphologic investigations at the site of metastases suggest a series of events beginning with the attachment of embolic cells to the wall of the vessel (170, 171). The histological studies have been corroborated by employing a

cinephotomicrographic technique (172), which visualized the following process. When V2 carcinoma cells are injected into the central artery of the rabbit ear chamber, proximal to the transparent membrane, the majority of cells die. The cells which survive tend to form small aggregates by sticking together. Apart from transient adherence to arteriolar and venular walls, the cells attach themselves predominantly to the capillary wall. Although this initial adherence is "independent of leucocyte sticking, capillary diameter, rate of blood flow or vasomotor activity", experimental alterations which induce leucocyte sticking and/or coagulopathy, increase the incidence of adhesion. A microcoagulum forms around the adhered tumour cells containing delicate meshworks of fibrin and platelets. Tumour cells grow in this clot and mitotic figures can be seen. Scattered leucocytes are frequently found in the vicinity of these dividing tumour cells. The endothelium is damaged at the site of adhesion by an undetermined mechanism. The tumour cells pass into perivascular connective tissue through this damaged endothelium. Active amoeboid movements of cancer cells during passage are seen. Following penetration by cancer cells the intactness of the endothelium is restored, but the sites of earlier penetration exhibit increased leucocyte sticking and thus stay vulnerable to further adhesion by tumour cell emboli. As the tumour grows into the perivascular connective tissue, lymphocytic

infiltration in the surrounding zone is conspicuous.

Whether the selectivity of organs for metastases is due purely to anatomical and mechanical factors (173) or to the metabolic properties of the host organ - "soil hypothesis" - (174), is a matter of contention. However, it has been shown that once the circulating tumour cells reach an organ such as liver, a prior alteration of its metabolic status by trauma, chemical agents (175), or cirrhosis (176), will provide a better environment for the lodgement and growth of metastases.

As has been described above, hypercoagulability, whether induced by physical means such as trauma or by chemical agents, increases the incidence of metastases. Conversely, the anticoagulating agents such as heparin and fibrinolysin decrease the incidence. Cortisone appears to have a double effect on tumours. It inhibits the local growth of implanted tumour cells, but augments blood-borne metastases.

Direct penetration is not the only way by which cancer cells gain entry into the lymphatic system. The lymphatic system has points of contact and even of intersection with the haematogenous system and it is not unusual for circulating tumour cells in the blood flow to disseminate into the lymph flow and vice versa (177). Free-floating tumour cells are found in the thoracic duct in about 20% of all cancer patients and their presence can be significant in relation to developement of pulmonary metastases (178). Circulating

tumour cells in the lymph fluid do not necessarily pass through lymph nodes: there are connections between afferent and efferent lymph vessels in the pericapsular tissue of the lymph node (179), and cancer cells can go to the venous system completely by-passing the intermediate lymph node.

(ii) The present study:

The following gives an account of the pattern of local invasion and dissemination of tumour cells observed in later generations of Adj-PC-5 type of transplantable plasma cell neoplasm. Even with a considerably short life-span the tumour-bearing animals showed widespread vascular dissemination by tumour cells. This is understandable, as it has been shown with suspension of radioactive chromium (Cr51) labelled Walker carcinoma cells that soon after subcutaneous inoculation, the cells disseminate through venous and lymphatic channels very rapidly. Only profound hypothermia can delay this dissemination by appreciably slowing the flow (180).

Ultrastructural aspects of tumour dissemination were studied in two organs; liver and spleen. In liver, tumour cells were contained in dilated sinusoidal spaces and the purpose of the study was to observe the effect of a restricted vascular space on tumour cells. In spleen - which is non-sinusoidal in mice - the purpose of the study was to observe the effect of direct contiguity between tumour cells and the splenic tissue cells.

2. MATERIAL AND METHODS

It was mentioned in the preceding section (Section IV, Materials and Methods) that all of the tumour-bearing animals in each group of transplant generations were routinely autopsied and specimens of tissues from various sites as well as samples of tumours at the sites of inoculation were obtained. While the previous section was concerned with the study of subcutaneous tumours, the present section will describe tissues collected from various sites in the animals. In order to study the pattern of local invasion, specimens from the skin overlying the lesion and from the skeletal muscles in the adjacent region of the growth were also obtained from the majority of the animals carrying the tumour. In an additional method of transplantation, minced pieces of tissue in ½ ml. of media were injected into the peritoneal cavity of animals, and, following tumour growth, tissues were collected and processed by the standard methods.

3. OBSERVATIONS

(i) Gross findings:

The growth of tumours after subcutaneous implantation has been described in the previous section. In cases of intraperitoneal injections of minced tissue, a discernible swelling of the abdomen appeared on the 8th or 9th day. With progression of the abdominal swelling the animals reached

premortem stage between the 17th and 20th day. Multiple nodules of irregular shapes and sizes grew on the peritoneal surfaces and on the mesentery. The nodules were pink tan and friable. A moderate amount of serosanguineous ascites was present.

Irresepective of the site used for transplantation the general pattern of tumour cell dissemination in all tumour-bearing animals showed only minor variations. Therefore the following descriptions pertain to all tumours. Among all organs the spleen consistently showed gross abnormalities. The normal spleen is soft and flat with sharp margins. The spleens in tumour-bearing animals were much larger and firm with rounded edges. While the normal spleen measures about 1.5 x 0.3 cm, the involved spleen measured 2.0 x 0.7 cm or more, with an average weight of 0.28 gm. In the thoracic cavity the heart, lungs and thymus were of normal appearance. The mediastinal nodes were not enlarged. In the abdominal cavity the liver, gastrointestinal tract, the mesentery (except with intraperitoneal tumour transplantation), and the internal genital organs were within normal limits. The kidneys were somewhat pale. No gross abnormality was observed in the brain. If the tumour extended into the neck the contiguous cervical lymph nodes were enlarged; the thyroids were of normal size in all tumour-bearing animals.

(ii) Light microscopy:

Skin:

In haired areas the normal mouse epidermis was very thin, usually consisting of two cell layers of stratum germinativum. Stratum spinosum and stratum granulosum were absent. As the subcutaneous tumour grew to a considerable size, the overlying skin began to lose hair, became dark and almost gangrenous in appearance and less elastic in consistency. These changes suggested impending ulceration and if the tumour-bearing animal survived long enough focal ulceration ensued. Microscopically the necrotic skin appeared as a thick cornified layer containing ghost nuclei or nuclear fragments. In the adjacent region the epidermis became multilayered with the appearance of stratum spinosum and stratum granulosum containing keratohyalin granules (Fig. 16). The stratum granulosum was usually one layer thick and frequently covered by a parakeratotic layer of variable thickness. In the dermis the tumour first infiltrated into thin sheets of striated muscle (the panniculus carnosus), gradually obliterating them. At this stage the dermal connective tissue between the epidermal layer and the zone of tumour became oedematous, manifested by loosening and widening of this space. Slight cellular infiltration of polymorphonuclear leucocytes and lymphocytes was present. The tumour relentlessly progressed by forming tongue-like projections which first surrounded the skin appendages and

and later obliterated them. However, even when the tumour was in close proximity to the epidermal layer it induced only minimal changes in the epidermis.

Skeletal Muscle

At the site of subcutaneous inoculation the tumour grew by invading adjacent skeletal muscles of the forelimb and of the intercostal region. Tumour cells appeared first to make their way into the interstices between muscle fibres (Fig. 17). During this process of infiltration the morphology of tumour cells was altered, resulting in elongated nuclei and cytoplasm with irregular projections at the advancing margin. With the continuation of growth, muscle fibres lost their reticulin sheath in focal regions, and tumour cells came to lie in direct contact with the muscle fibres. The muscle nuclei began shrinking and gradually the fibres lost their cross-striations, became hyaline and finally disappeared. When sufficient numbers of tumour cells had grown into an area the cells reformed into a lobulated pattern with each lobule being surrounded by reticulum fibres. The isolated atrophying muscle fibres could be seen both inside and outside these tumour lobules. Only rarely a mesenchymal reaction between the advancing tumour and muscle fibres was seen, consisting of young fibroblastic cells with capillaries and moderate polymorphonuclear and mononuclear infiltrate (Fig. 18).

Bone and bone marrow.

The tumours did not invade the periosteal connective tissue of the humerus or of the ribs (Fig. 19). Tumour cells dissected through muscle fibres right up to the periosteum but did not progress further. The bone marrow was hypercellular, and the hypercellularity appeared largely due to increase in granulocytes. Megakaryocytes were slightly increased. Tumour cells were generally absent either in marrow spaces or in vascular spaces. No plasma cell hyperplasia was seen in the bone marrow.

Lymph nodes:

A systematic dissection of lymph nodes during post-mortem examination was not done. However, sections of the heart and thymus, removed en bloc during autopsy, usually contained mediastinal lymph nodes. In the majority of these nodes there were free floating tumour cells in the subcapsular cortical sinuses (Fig. 20), which seemed to stream down towards the medulla. Infrequently metastatic foci were seen in these lymph nodes. In several cases, cervical, contralateral axillary and mesenteric nodes were examined. The contralateral axillary and mesenteric nodes were uninvolved; the cervical nodes had tumour cells in the subcapsular sinuses. Secondary effects possibly attributable to the tumour such as follicular hyperplasia or sinus histiocytosis were absent in these lymph nodes.

Small lymph nodes of microscopic size were sometimes seen in the vicinity of the pulmonary hilus and renal pelvis; these did not demonstrate morphologic change. Extranodal lymphoid aggregates such as Peyer's patches in the small intestine were unremarkable.

Liver:

Sections of liver from normal mice showed parenchymal cells arranged two deep in cords separated by collapsed sinusoids which were lined by cells with small elongated nuclei and indistinct cytoplasm. In all of the tumour-bearing animals, scattered tumour cells in the hepatic sinusoids appeared as early as 7th or 8th day after tumour inoculation. With the appearance of tumour cells the sinusoids became dilated. A moderate number of polymorphonuclear leucocytes with a sprinkling of lymphocytes were admixed with tumour cells. The number of tumour cells in the sinusoids grew with progression of the neoplasm. Although the sinusoids communicate not only with central veins but also directly with hepatic veins, tumour cells were restricted to sinusoidal spaces and not seen in other hepatic vascular spaces (Fig. 21). Bizarre-shaped nuclei, multinucleated giant cells and abnormal mitotic figures were frequently found among these tumour cells. The cytoplasm was usually scanty and slightly basophilic. The hepatic parenchymal cells exhibited a moderate degree of morphological changes. Normally these cells were large containing one or two round nuclei, fairly uniform in size with an occasional mitotic

figure. Simultaneously with the infiltration of tumour cells the parenchymal cell nuclei showed general enlargement and marked dissimilarity in size while retaining their round or globoid shape. The number of mitoses was moderately increased. A study of interrelationship between tumour cells and parenchymal cells not only revealed direct adhesion, but also an actual entry of tumour cells into parenchymal cells (Figs. 22, 23). The engulfed tumour cells had very little cytoplasm and there was a clear halo around them. Nuclei of the host parenchymal cells were displaced and deformed by the space-occupying tumour cells. In general, the parenchymal cells had a coarsely granular cytoplasm. No abnormality was noted in structures of the portal spaces.

Spleen:

In early stages of dissemination, neoplastic cells in all of the tumour-bearing animals were first seen in subcapsular spaces which became prominent compared to those of the spleen of the normal mouse. With the spread of tumour by infiltration around the malpighian corpuscles, the red pulp diminished considerably. As the vascular arrangement in the mouse spleen is non-sinusoidal (see discussion), tumour cells should be seen in direct contact with splenic lymphoreticular cells, mostly in the subcapsular and perifollicular regions (Fig. 24). Compared to the normal spleen it appeared that many splenic lymphoreticular cells in the perifollicular region

acquired an increased amount of somewhat basophilic cytoplasm following tumour dissemination. With methyl green pyronin stain this cytoplasm was deeply pyroninophilic. When there was a longer survival of the tumour-bearing animal there appeared to be hyperplasia of reticulum cells in the white pulp; only with prolonged survival did tumour cells invase the white pulp from the periphery and grow in it. In spite of the presence of numerous pyroninophilic cells, plasma cells were generally absent.

Pancreas:

Both exocrine and endocrine pancreas were unremarkable. In a small number of cases, tiny areas on the surface of the pancreas were attached to the splenic capsule by fibrous connective tissue which showed scattered mononuclear cell infiltrate but no tumour cells.

Kidney:

Glomerular capillaries contained isolated tumour cells (Fig. 25) in kidneys of all of the tumour-bearing animals. Tumour cells usually were represented as large, vesiculated, lobulated nuclei devoid of cytoplasm, the size and appearance of which were quite distinguishable from the neighbouring epithelial and endothelial cells. Not more than two or three tumour cells were seen in one glomerulus, and not all glomeruli were affected. Glomeruli harbouring tumour cells always

contained a few polymorphonuclear leucocytes. Thus the hypercellularity that these glomeruli demonstrated was thought to be due to the presence of tumour cells and leucocytes rather than to an increase in normal cellular components. Mitotic figures presumably of tumour cells, were occasionally seen in cells in the glomeruli. Hyaline, proteinaceous tubular casts were not constant, and when present were more often seen in the cortex than in the medulla. The interstitial tissue and the vascular system were unremarkable.

Adrenal:

Adrenals were free of tumour involvement.

Intestine:

When transplants were grown intraperitoneally tiny tumour nodules were seen on the serosal surface (Fig. 26). These nodules were separated from the intestinal wall by a dense layer of connective tissue.

Gonads:

Very rarely lumens of ovarian capillaries contained tumour cells. Otherwise sections of ovaries, uterus and fallopian tubes were unremarkable.

Lung:

Extensive dissemination of tumour cells in the alveolar septal capillaries were constantly seen (Fig. 27).

These cells, although lacking a discernible cytoplasm, could easily be recognized by their abnormal nuclear morphology in comparison to other cells in alveolar septa. Presence of tumour cells in capillaries was always accompanied by a moderate infiltration of polymorphonuclear leucocytes in the septa. Tumour cells were not seen in the vascular spaces around the bronchioles. In one mouse with longer than average survival sheets of tumour emboli in veins (Fig. 28) and numerous fibrin thrombi were seen.

Heart:

Isolated tumour cells were found in small myocardial capillaries. Typical lobulated nuclei with scant amount of pyroninophilic cytoplasm of tumour cells were readily distinguishable from other myocardial cells (Fig. 29).

Thymus:

The thymus of normal mice is composed of a dense cortex surrounding a pale medulla. In tumour-bearing animals density and thickness of the cortex showed a wide variation. Even when the adjacent adipose tissue was infiltrated with tumour, the thymus did not show direct invasion or vascular dissemination.

Brain:

No tumour cells were found in the cerebral vessels.

(iii) Electron microscopy:

Liver:

When tumour cells reached hepatic sinusoids they underwent morphological changes which were manifested principally in the cytoplasm. In contrast to tumour cells at the primary site, where the large nuclei occupied a central position surrounded by a rim of cytoplasm, in hepatic sinusoids the major portion of cytoplasm seemed to shift to one side of nuclei, apparently towards the direction of blood flow. The cytoplasm at this margin had irregular projections (Fig. 30). Many cells, probably due to crowding in sinusoids, had lost the greater part of their cytoplasm with consequent loss of organelles in varying degree, including virus-like particles (Fig. 31). The nuclei often retained their irregularly convoluted appearance with chromatin material mainly arranged at the periphery and lack of distinct nucleoli. The tumour cell surface was in close proximity to the absorptive surface of parenchymal cells which was represented by numerous microvilli.

It appeared, as noted with light microscopy, that some tumour cells gradually gained entrance into the cytoplasm of hepatic parenchymal cells. The following observations suggested that this migration represented an active invasion by tumour cells rather than a phagocytic engulfment of them by parenchymal cells. Tumour cells infiltrated through gaps

between Kupffer cells. If this occurred at the junction of two hepatocytes, the desmosomal attachments between two parenchymal cells stopped abruptly at or near the margin of the infiltrating tumour cell (Fig. 32). In the process of invasion, tumour cells in the majority of instances were seen with a depleted cytoplasm containing very few organelles but with a large nucleus; infrequently the nucleus was small and there was a moderate amount of cytoplasm containing a regular complement of organelles. Plasma membranes of both tumour cell and parenchymal cell were indistinct at the site of contact. When tumour cells were lodged within the cytoplasm of parenchymal cells a very thin rim of cytoplasm surrounded tumour nuclei, containing free ribosomal particles and several small cisternae of endoplasmic reticulum (Fig. 33). Occasional virus-like particles remained. There was a clear space between the lodged tumour cell and the cytoplasm of the parenchymal cell. Depending on the size and site of the lodged tumour cell, the parenchymal cell nuclei were distorted. The cells lining the nearby sinusoidal space did not show alteration, and it seemed that tumour cells preferentially invaded parenchymal cells

Spleen:

It has been mentioned before that the splenic circulation is non-sinusoidal. Therefore tumour cells were seen in direct contact with splenic lymphoreticular cells. It was observed that following tumour infiltration, many splenic

lymphoreticular cells turned into pyroninophilic cells. These pyroninophilic cells were larger than ordinary splenic lymphocytes with a considerable amount of free ribosomes and a moderate amount of rough endoplasmic reticulum dispersed throughout the hyaloplasm. These cells had a well developed Golgi apparatus. There appeared to be points of adhesion between tumour cells and splenic cells (Fig. 34, 35). At the site of adhesion the plasma membranes of both cells appeared deficient, and on occasion, virus-like particles were seen in the region of these contact zones. Many pyroninophilic cells contained virus-like particles, suggesting their migration from tumour cells (Fig. 36). The location of the particles in pyroninophilic cells was in the hyaloplasm and they tended to congregate in one area whereas in tumour cells they were almost exclusively seen within cisternal spaces of the endoplasmic reticulum.

4. SUMMARY OF FINDINGS

The pattern of dissemination of tumour cells in later transplant generations of Adj-PC-5 type of plasma cell neoplasm through 60 transfers remained the same. The sites of inoculation, subcutaneous or intraperitoneal, did not influence this pattern. Apart from tumour nodules developing at sites of implantation, the only other consistent gross abnormality was splenomegaly. Microscopically tumour cells were found in the subcapsular and perifollicular regions in spleens. Numerous

tumour cells were present in hepatic sinusoids, pulmonary alveolar septal vessels, glomerular capillaries and subcortical sinuses of mediastinal lymph nodes. When the survival time of tumour-bearing animals was restricted to between 12 and 20 days of tumour growth, metastatic nodular deposits were absent in organs which however demonstrated considerable number of tumour cells in lymphovascular spaces. Locally, the subcutaneous tumour infiltrated into adjacent skeletal muscles of the forelimb and of the intercostal regions. In mice receiving intraperitoneal transplants the peritoneal tumour nodules grew on serosal surfaces but failed to penetrate the intestinal wall.

In liver tumour cells sometimes actually gained entry into the cytoplasm of parenchymal cells. The engulfed cells were considered viable because their nuclei were intact and surrounded by scant cytoplasm containing a few organelles. This phenomenon was thought to be similar to emperipolesis, which is exhibited by lymphocytes.

Many splenic lymphoreticular cells became pyroninophilic following tumour dissemination. Under the electron microscope some of these altered cells were found in direct contact with tumour cells. These contacts were associated with the appearance of virus-like particles in the hyaloplasm of pyroninophilic cells.

5. DISCUSSION

The pattern of metastases described in this study of later transplant generations of Adj-PC-5 plasma cell neoplasm differed from that reported in the primary tumour and in the earlier transplant generations. Cells from the primary and early generations of tumours did not spread to abdominal organs and were inconstantly found in marginal or hilar sinuses of lymph nodes (109). Widespread dissemination of tumour cells however was found to be constant in later generations. On the average, tumour-bearing animals were sacrificed on the 12th day of tumour growth, and neoplastic cells were invariably found in considerable numbers in the hepatic sinusoids, in the spleen, and in pulmonary septal and glomerular capillaries. Isolated tumour cells were not unusual in myocardial capillaries and tumour cells in subcapsular sinusoids of lymph nodes were frequent.

That the pattern of metastases changes with the number of transplantations has already been described in relation to localized plasmacytomas spontaneously developing at the ileocaecal region of C3H mice (98). The primary tumour formed metastatic nodules in thymus, spleen and liver. It invariably metastasized to mesenteric nodes but spared lymph nodes in other parts of the body. In early transplant generations the tumour also spread to the gonads. With progress of transplant generations the tumour rapidly grew to an enormous size at the

site of subcutaneous inoculation, but only a few tumour cells were seen in the internal organs.

In spite of apparently increased malignant potential of later transplant generations in this study, no lesions was seen in bones. The inability of almost all types of experimental plasma cell neoplasms (except type X5563 with prolonged survival) to cause osteal lesions is in marked contrast to human multiple myeloma. The reported extraskeletal spread in human multiple myeloma was most frequently to the spleen, liver, lymph nodes and kidneys (181). Involvement of other organs such as lungs, gastrointestinal tract, pancreas, adrenal, bladder and testis has also been reported (182). In humans in the spleen, infiltration appeared first in sinusoidal spaces and later the malpighian corpuscles were invaded. In addition to parenchymal involvement in various organs myeloma cells were found in paravertebral venules and other vascular spaces including hepatic sinusoids. In kidneys both peritubular capillaries and the interstitial tissue contained myeloma cells.

Engorgement of hepatic sinusoids with neoplastic cells without lodgement in parenchyma is seldom seen in human neoplasms, except in such rare tumour-like conditions as histiocytic medullary reticulosis (183). In this study tumour cells in sinusoidal spaces seemed to burrow actively into the cytoplasm of hepatocytes. Transcellular migration is known to be a property of lymphocytes. The word "emperipolesis",

derived from the Greek and meaning "inside round about wandering", has been used to describe the "phenomenon whereby the living lymphocytes become adherent to other cells or apparently pass over and into them" (184). Examination of various normal tissues from different species including that of the human, has shown that healthy lymphocytes are found within the cytoplasm of a variety of cells, mostly epithelial. The main types of host cells include those of intestine, trachea, thyroid, liver, urinary tract and fibroblasts. The intestinal epithelium appears to be the only place where engulfed lymphocytes have been observed to undergo mitosis (185). Using phase-contrast time-lapse cinemicrography on serum-agar culture of cells it was shown that lymphocytes could enter malignant cells of both human and murine tissue origin (184). Conversely, leukaemic lymphoblastic cells, both of murine and human origin, could enter reticular and fibroblastic cells (186, 187). Following invasion the foreign cell lodges itself in a vacuole with a distinct membrane, surrounded by an unstained area appearing as a halo. Electron microscopy of mouse ascites lymphocytic tumour cells that had invaded macrophages (188) showed that two membranes separated the invading cell from the host cell, one belonging to the former and the other to the latter. However, the membrane of the macrophage was often deficient in focal areas, and thus the cytoplasm of host cell came in direct contact with the membrane of the invading cell.

With malignant lymphocytes, the consequence of emperipolesis was either the destruction and digestion of the invading cell (189), or the disintegration and death of the host cell (190). Until now, emperipolesis has been known to be exhibited only by lymphocytes, either normal or malignant (191).

Tumour cells in later generations of plasma cell neoplasms demonstrated the phenomenon of emperipolesis only in hepatocytes and not in other cells. The hepatic parenchymal cells in tumour-bearing mice were larger in size than tumour cells taking part in emperipolesis whereas at other sites such as spleen, lungs and kidneys, tumour cells were larger than the neibhbouring cells. In contrast to emperipolesis shown by lymphocytes, the membranes defining tumour cells and the cytoplasm of the host cell were not distinct, although an electron-lucent space was present between them. It did not appear that the host cell was exerting a digestive faculty on the invading cell, as lysosome-like structures were not seen in significant numbers in the vicinity of tumour cells. The engulfed tumour cells had scant cytoplasm, but the nuclei appeared intact. It was also noted that nuclei of tumour cells were larger after being lodged within the parenchymal cell than following penetration through the endothelial spaces. Therefore it seemed doubtful that emperipolesis of tumour cells resulted in their destruction; it appeared more likely that the host cell would eventually die and be replaced by the tumour

cell.

There were two interesting alterations in spleens containing tumour cells. Following tumour dissemination to the spleen many splenic cells became pyroninophilic and these altered cells contained virus-like particles. It has been shown with silver impregnation techniques that in mouse spleen (192), capillaries and penicilli arising from the central artery terminate by opening into the perifollicular space, after pursuing a radial course through the pulp and forming frequent anastomoses. The perifollicular space is open and not bound by sinusoidal walls, resulting in a "non-sinusal" type of splenic circulation. The narrow, branched venous twigs ("primordial veins") originate in the red pulp. Due to this "open" circulation tumour cells came in direct contact with splenic cells. Whether the transformation to pyroninophilic cells was due to humoural stimulation mediated by dysgamma-globulinaemia (see review of the literature on amyloidosis in next section) or through some influence of the tumour cells is a matter of conjecture. The virus-like particles which were present in pyroninophilic cells were morphologically similar to those in tumour cells. It has been shown that if isolated virus-like particles are put in the media with a culture of mouse embryo cells they fail to enter the cells (193). Therefore it appears that there might have been fusion between tumour cells and pyroninophilic cells for the transfer of virus-like particles.

Figure 16. A section of skin overlying the subcutaneous tumour shows multilayered epidermal cells, in contrast to the two-cell layer of normal mouse skin. The uppermost layer consists of stratum granulosum, usually absent in normal skin. The dermal connective tissue is loose and oedematous. Haematoxylin and eosin. X120.

Figure 17. A section of skeletal muscle shows separation of fibres by infiltrating tumour cells. Haematoxylin and eosin. X240.

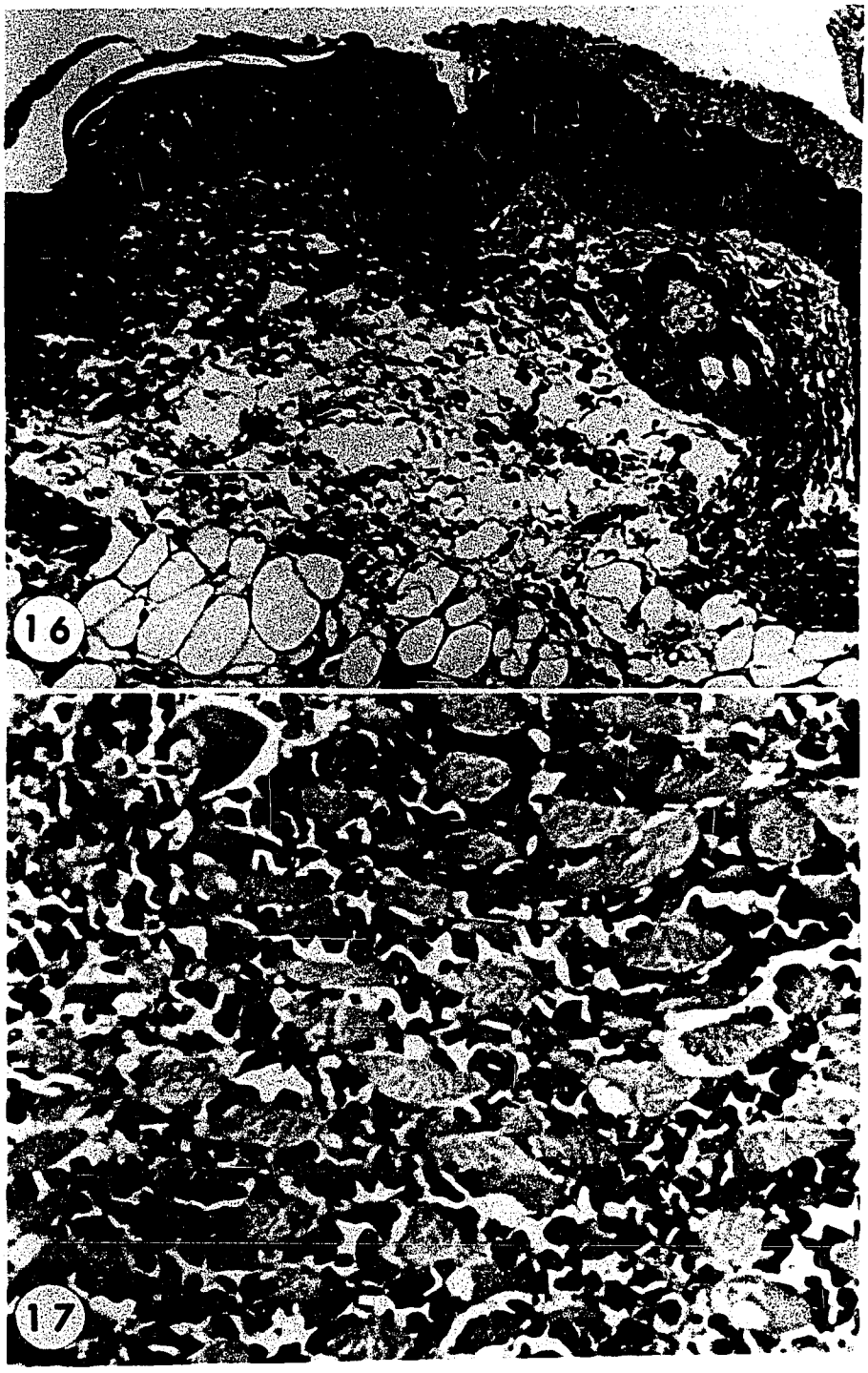

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Figure 18. The section shows a cellular reaction consisting of young fibroblasts and mononuclear cells between the advancing tumour and underlying skeletal muscle. Haematoxylin and eosin. X120.

Figure 19. The section shows the humerus with surrounding skeletal muscle massively infiltrated by tumour, which does not penetrate the periosteum. Haematoxylin and eosin. X120.

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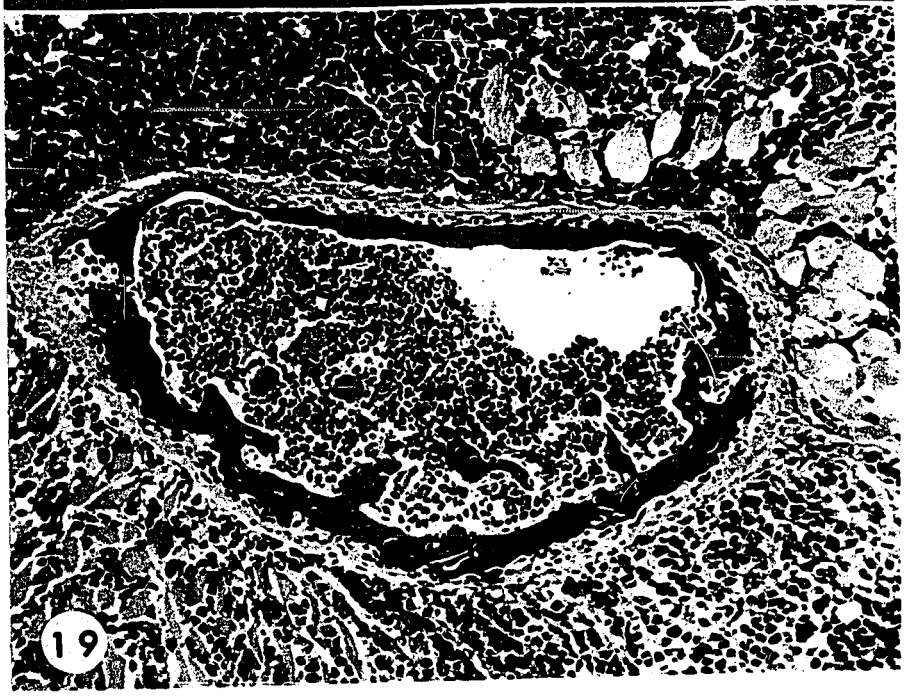

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Figure 20. A section of a mediastinal lymph node showing tumour cells in subcortical sinuses. Haematoxylin and eosin. X180.

Figure 21. A section of liver showing tumour cells in sinusoidal spaces in association with a sprinkling of polymorphonuclear leucocytes. Haematoxylin and eosin. X180.

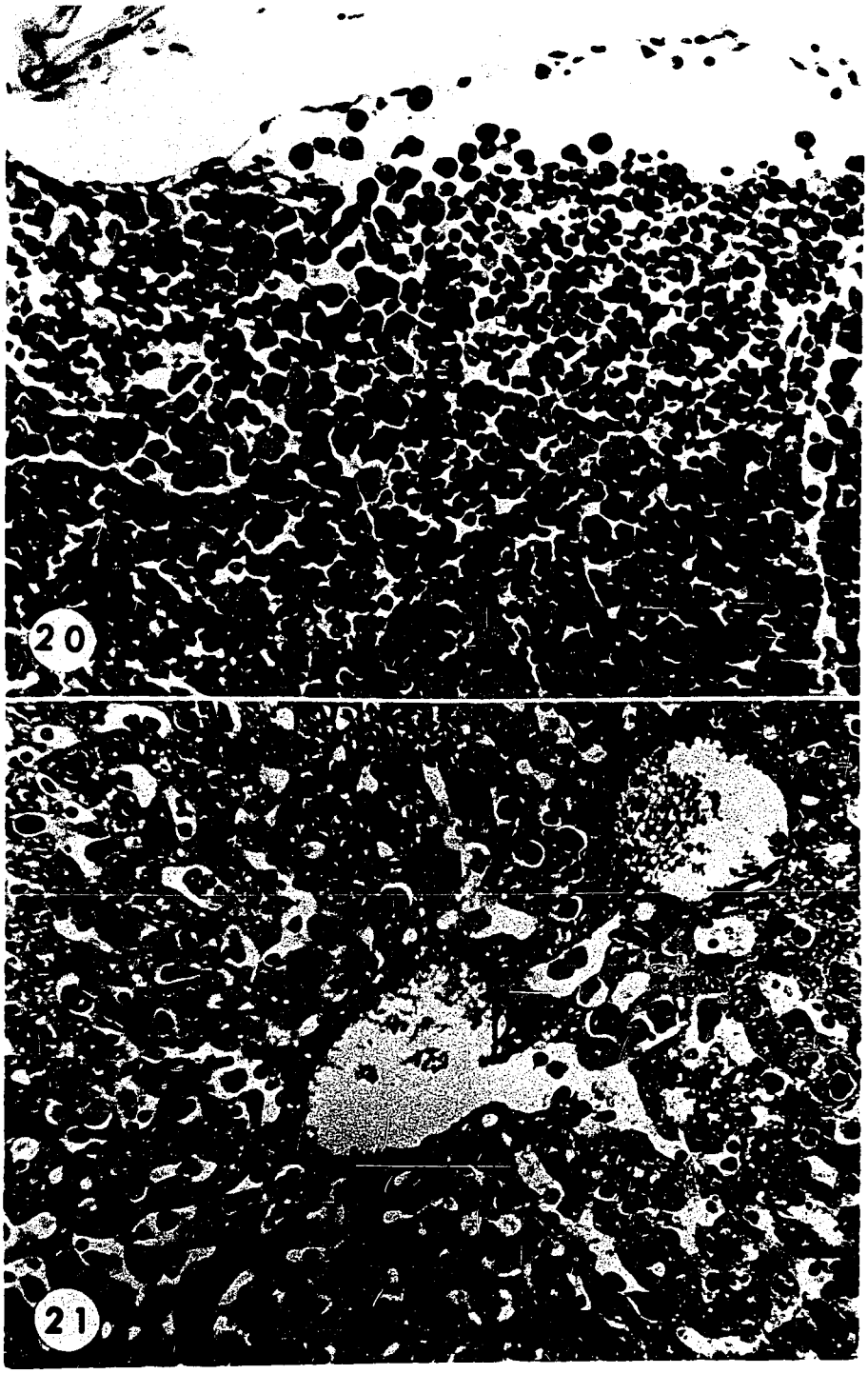
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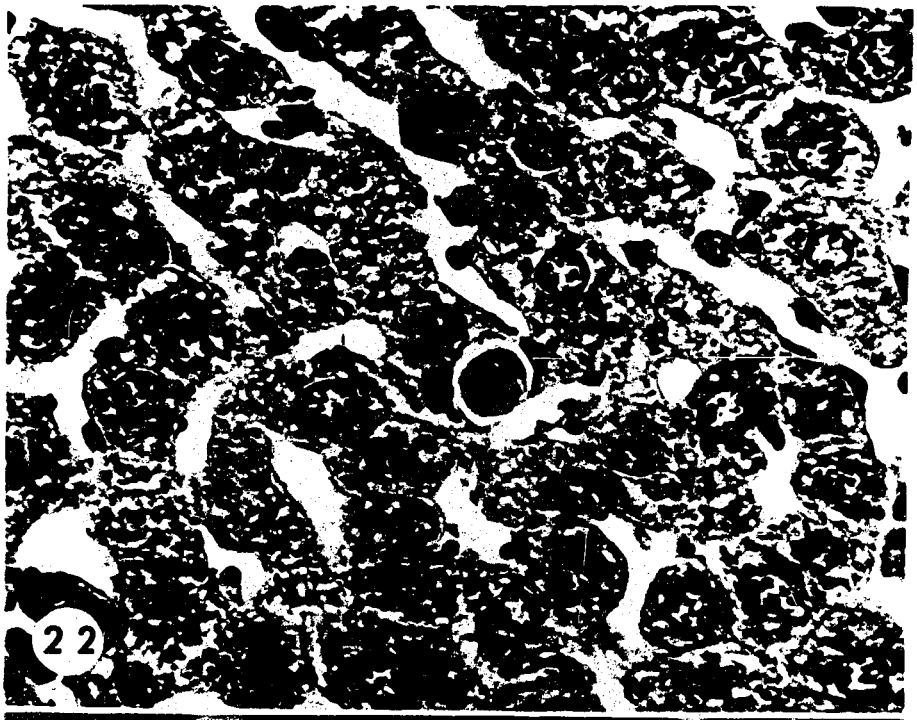
Figure 22. A section of liver shows a tumour cell lodged within a hepatocyte (arrow). A clear halo intervenes between the host and the foreign cell. A Kupffer cell is seen on the right of the tumour cell. Haematoxylin and eosin. X1250.

Figure 23. A section showing a tumour cell within a hepatocyte under higher power. The nucleus of the hepatocyte is deformed and pushed to the periphery. Haematoxylin and eosin. X1625.



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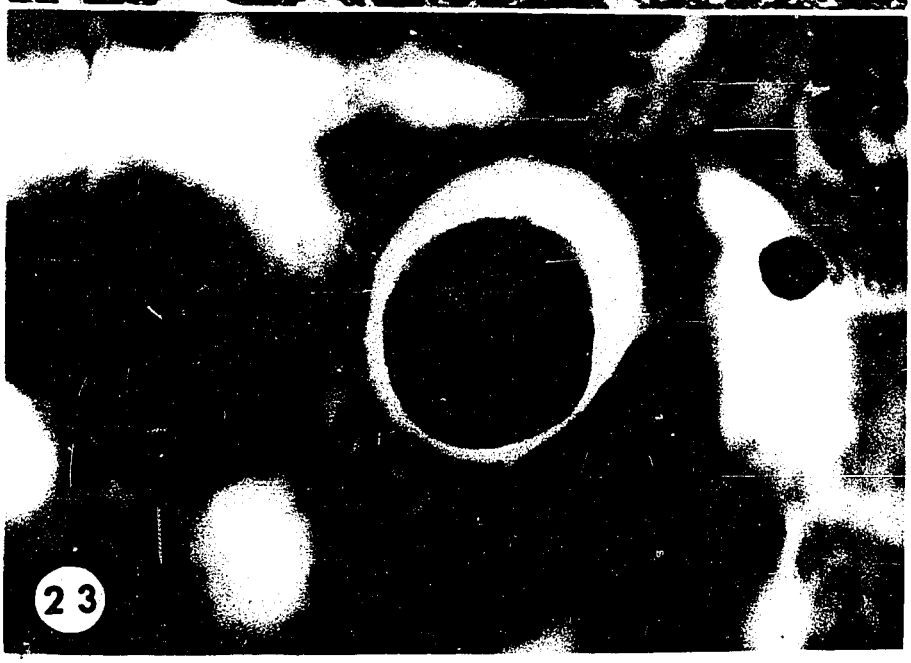

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Figure 24. A section of spleen showing tumour cell infiltration in the subcapsular region (arrow). Megakaryocytes (double arrows) are normally present in mouse spleen, and are distinguished from tumour cells by their larger size and abundant cytoplasm. Haematoxylin and eosin. X240.

Figure 25. A section of kidney showing tumour cells in glomerular capillaries (arrows). Haematoxylin and eosin. X210.

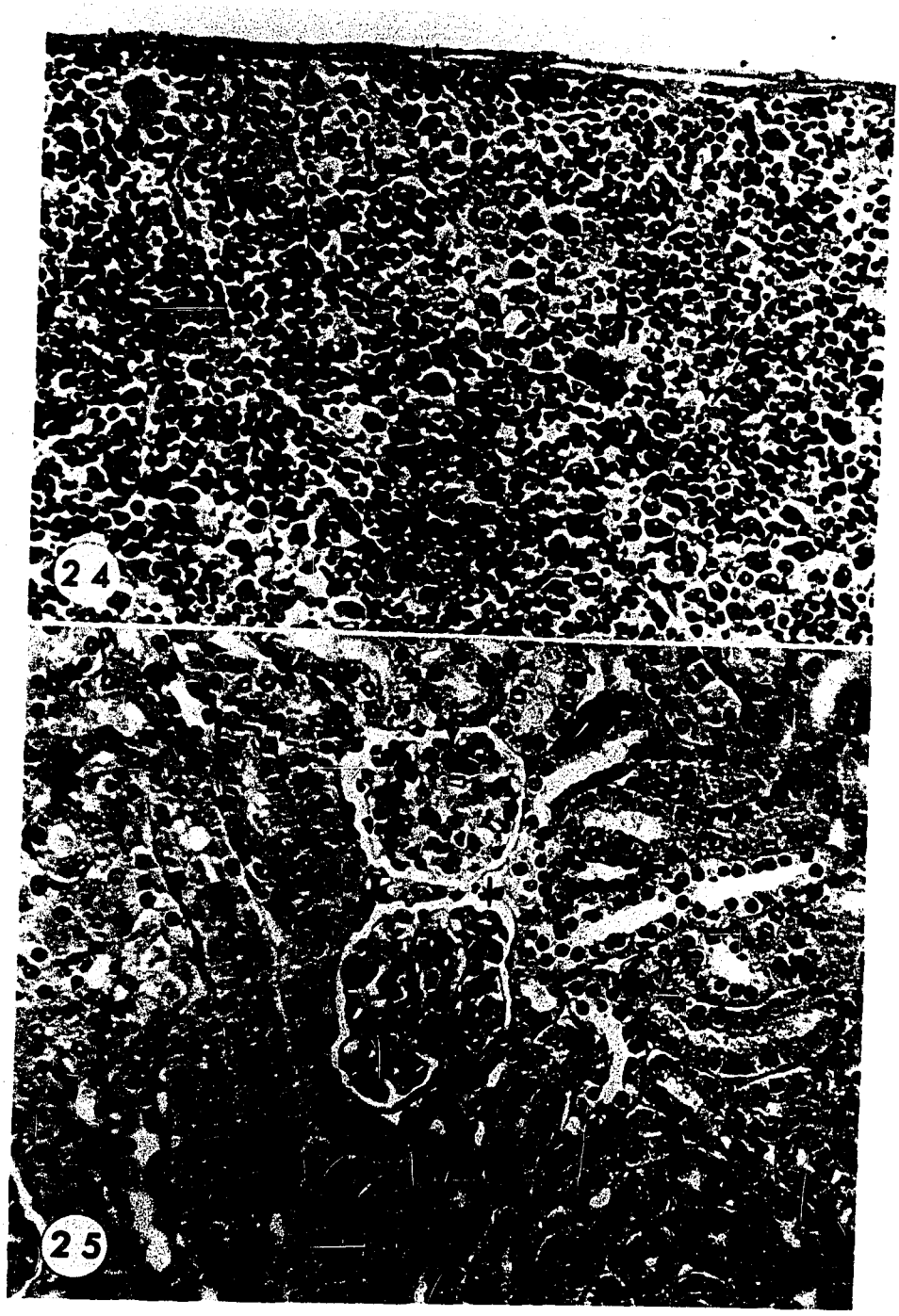
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Figure 26. A section of a tumour nodule on the serosal surface of the large intestine following intraperitoneal transplantation. The tumour does not infiltrate beneath the serosal surface. Haematoxylin and eosin. X210.

Figure 27. A section of lung showing large, hyperchromatic tumour cells in alveolar septae. Haematoxylin and eosin. X420.

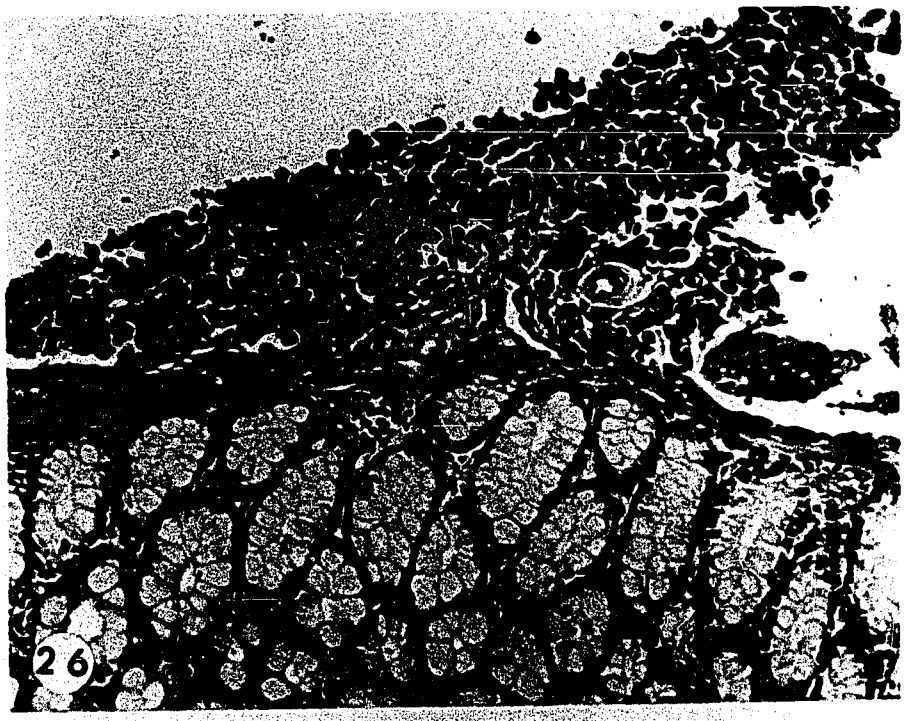

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Figure 28. A section of lung showing a tumour embolus in a vascular space. Haematoxylin and eosin. X210.

Figure 29. A section of myocardium showing a tumour cell in a capillary lumen (arrow). Methyl green pyronin stain. X420.

28

29

Figure 30. A tumour cell (TC) in a hepatic sinusoidal space showing irregular projections of the cytoplasm (arrows). The cytoplasm has a considerable number of free ribosomes but only a few small cisternae of endoplasmic reticulum. A portion of a hepatocyte (HC) is seen at the top of the electron micrograph. Uranyl acetate and lead citrate. X10,000.

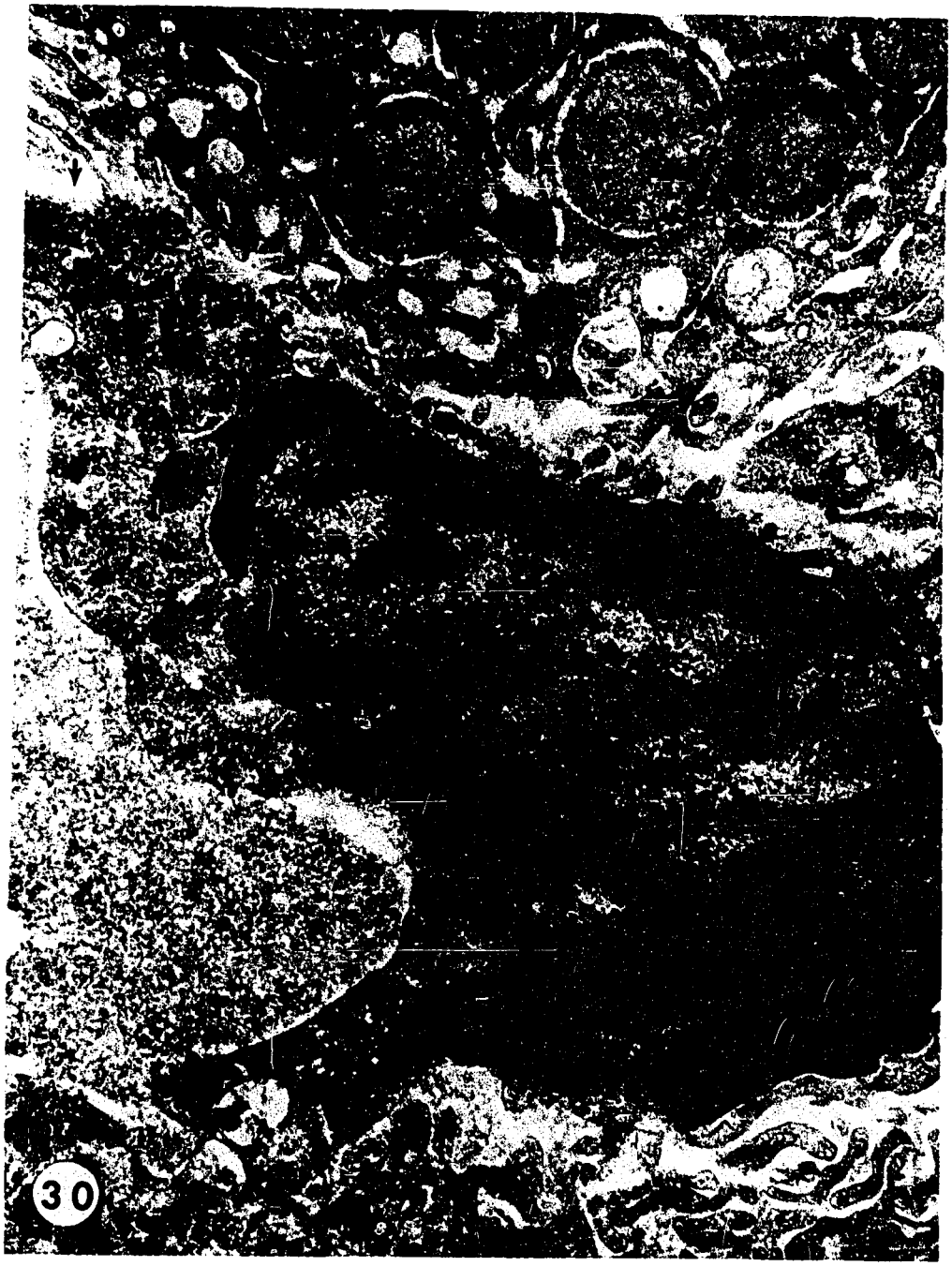
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Figure 31. A tumour cell (TC) in the sinusoidal space has a large nucleus and a cytoplasm with numerous free ribosomes but scant endoplasmic reticulum and a few mitochondria. A portion of a hepatocyte (HC) is seen at the top and on the left of the electron micrograph. Uranyl acetate and lead citrate. X9,000.

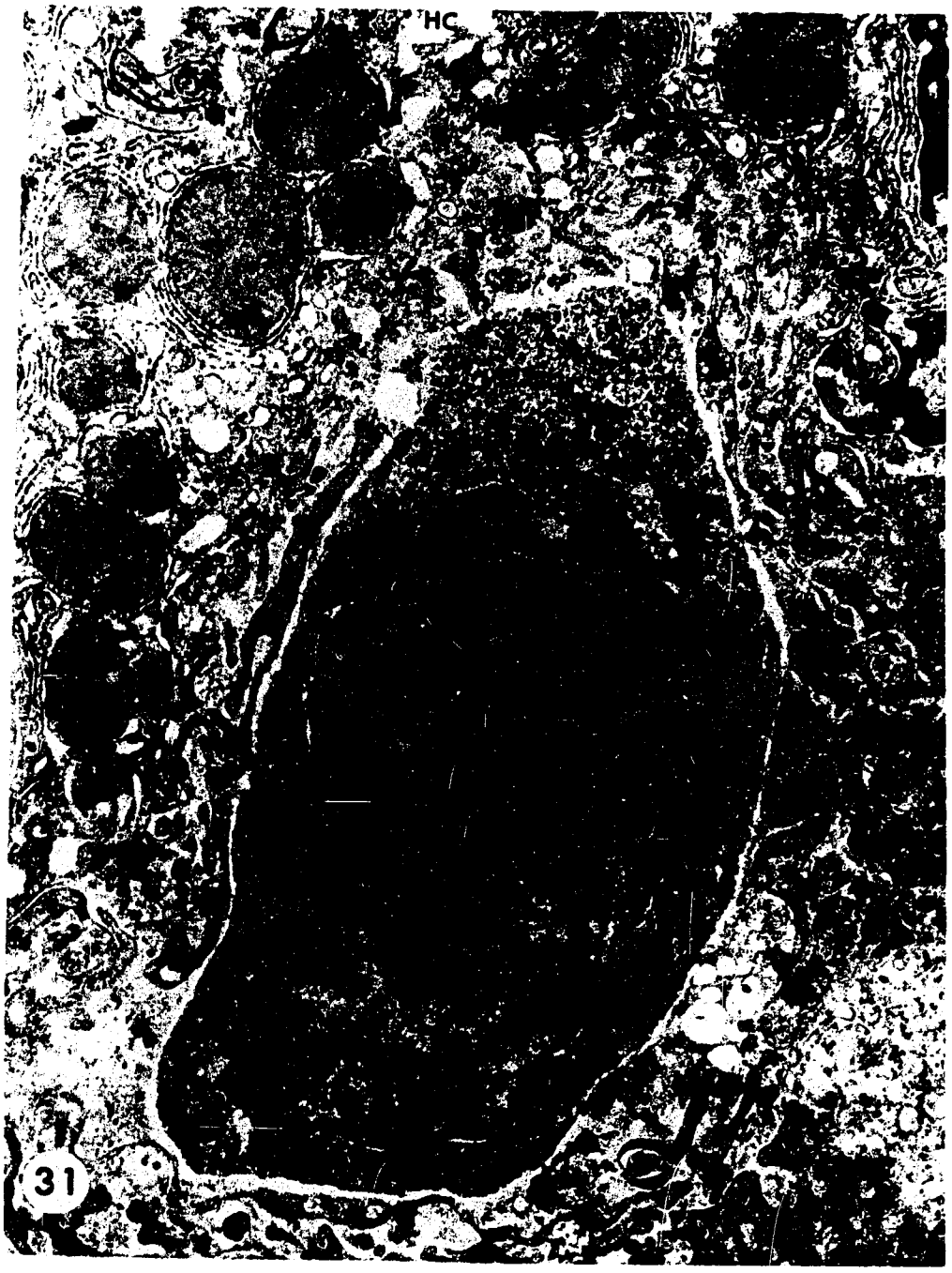
HC
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Figure 32. An electron micrograph showing a tumour cell between two hepatocytes (HC). The sinusoidal space is on the lower right, showing a portion of a red blood cell and a Kupffer cell. The desmosomal attachments (arrow) between the two hepatocytes are present above the tumour cell. Uranyl acetate and lead citrate. X8,000.

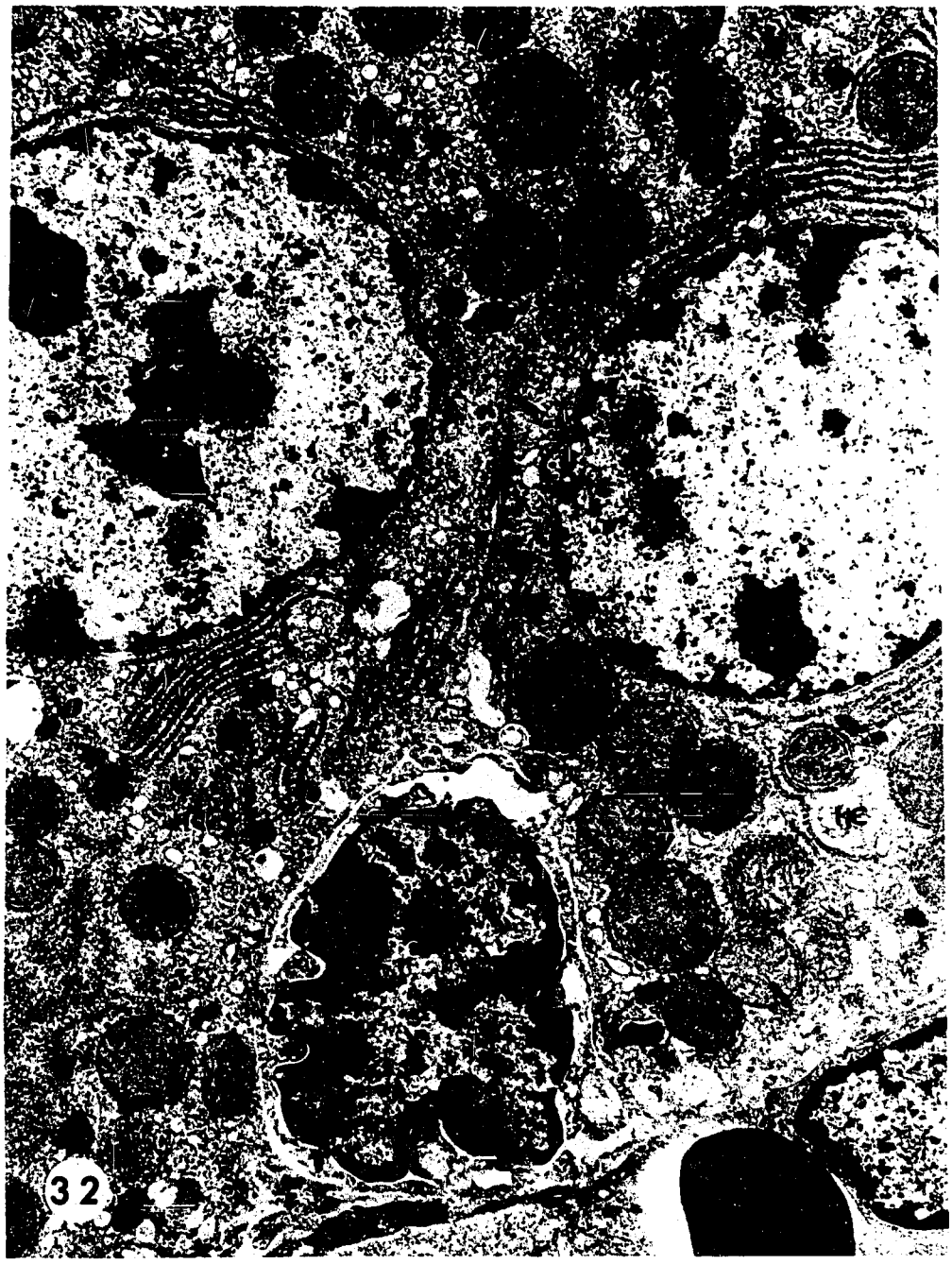
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Figure 33. An electron micrograph showing a tumour cell (TC) within a hepatocyte (HC). The tumour cell has scanty cytoplasm containing free ribosomes and a few tiny cisternae of endoplasmic reticulum. An electron-lucent space separates the tumour cell from the cytoplasm of the hepatocyte. Uranyl acetate and lead citrate. X8,000.

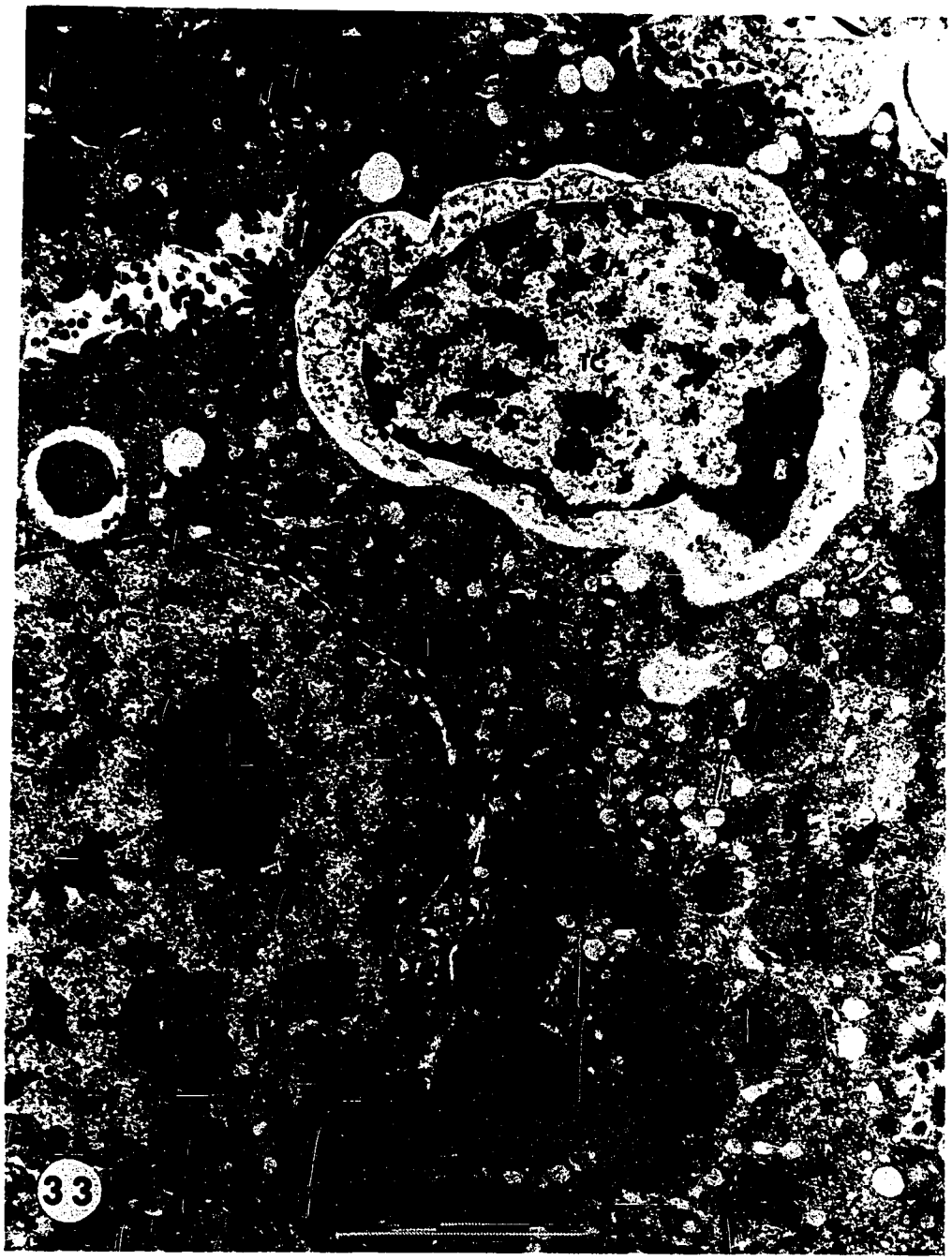
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Figure 34. An electron micrograph showing a large tumour cell (TC) in close proximity to two splenic cells (S). Uranyl acetate and lead citrate. X8,000.

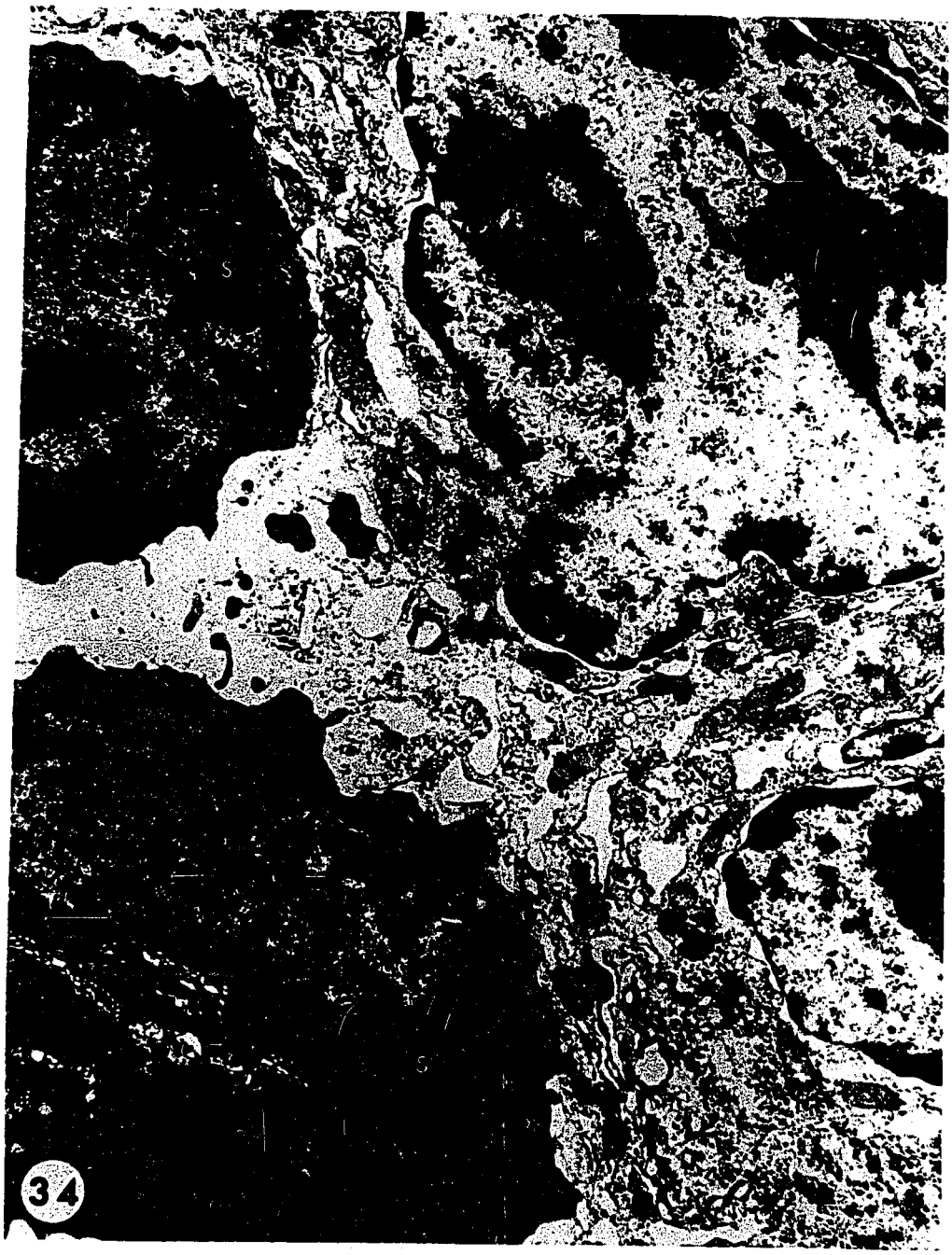
S
S
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Figure 35. An electron micrograph showing approximation of the cytoplasm of a tumour cell (TC) and a splenic cell (S). The plasma membranes in this area are indistinct (arrow). Uranyl acetate and lead citrate. X12,600.


35

Figure 36. An electron micrograph of a splenic cell having the characteristics of a pyroninophilic cell. The cell has an elaborate Golgi apparatus (GA), numerous free ribosomes and scattered cisternae of rough endoplasmic reticulum. Virus-like particles (V), similar to those of tumour cells, are present in the hyaloplasm. Uranyl acetate and lead citrate. X11,900.


GA
36

VI. Investigations Concerning Amyloidosis in Later Transplant Generations of Adj-PC-5 Plasma Cell Neoplasm: Present Study.

1. INTRODUCTION

(i) Amyloid and amyloidosis:

Virchow applied the name 'amyloid' to a substance which exhibited the property of a starchy element, turning blue or purple after iodine staining and gentle application of sulfuric acid. Analysis of chemically purified extractions has shown that amyloid is highly hydrophilic (water content about 35%), consisting of approximately 85% protein and 15% aminosugars and neutral sugars. About 18 different kinds of aminoacids have been found in the protein moiety with a relatively high amount of aspartic and glutamic acids. No hydroxyproline is present. In the sugar component glucosamine, galactosamine, glucose, mannose, rhamnose and fucose have been found. Sialic acid is present in small fractions. About 0.3% of uronic acid present in amyloid contains chondroitin sulfate, heparan sulfate and hyaluronic acid (194, 195). The histological staining characteristics of amyloid have been known for a long time; it stains with Congo red and the post-stained material shows a green birefringence and dichroism on polarization microscopy, and it demonstrates metachromasia with crystal violet. Newer staining methods such as Sirius red (196) and application of fluorescent dyes such as thioflavine T and S (197) are frequently used. Ultrastructurally human amyloid deposits consist of two types of fibres. Type I fibres (198), representing 90% of the total deposit, are composed of a number of filaments. Each

filament is 75-80Å in diameter and consists of 5 subunits (amyloid protofibrils) in parallel arrangement. These subunits are 25-30Å wide and show a helical arrangement with a 35-50Å repeat. Type II fibres (199), representing the remaining 10%, appear as 100Å rods, divided in to segments at 40Å intervals. On cross section, the segments consist of a pentagonal structure (the unit structure) composed of five globular sections surrounding a dense core.

Various cell types have been implicated in the formation of amyloid. Amyloid fibres were seen within the reticuloendothelial cells in the perifollicular zone of spleen in C3H mice treated with casein (200). Intracellularly these fibres were in close proximity to mitochondria and free ribosomes. In the spleen of casein-induced amyloidotic New Zealand rabbits (201), fibrils were found within reticuloendothelial cells of the red pulp. Radioautographic investigations suggested that the Golgi apparatus was involved in the preliminary elaboration of fibrils, which were at first localized at the cell surface and finally seen free in the extracellular space. Ultrastructural studies of bone marrow from a patient with multiple myeloma revealed the presence of amyloid fibrils in reticuloendothelial cells, in close association with free ribosomes (202). A scalp nodule in a patient with multiple myeloma contained amyloid fibrils and sparse cellular elements which were solely of fibroblastic type. From this observation

it was concluded that amyloid fibres were a variant of one of the normal scleroproteins and fibroblasts might be responsible for their formation in certain instances (203).

The following clinicopathological classification of human amyloidodsis (204) is widely used: a) primary: which is not associated with long-standing diseases and in which tissues such as muscles, blood vessels and skin are preeminently involved; b) secondary: which is usually associated with chronic infective conditions, rheumatoid arthritis, tumours etc. and in which the internal organs such as liver, spleen, and kidneys are principally involved; c) "tumour-forming": characterized by small, single or multiple masses of amyloid, especially in the respiratory tract; and d) amyloid associated with multiple myeloma.

It has become apparent in recent years that a number of heredofamilial forms of amyloidosis (205) cannot be conveniently fitted into the above classification. Hence a morphological classification has been proposed on the basis of localization of amyloid material in relation to the fibrous connective tissue. With the aid of polarization microscopy, it is seen that at the beginning amyloid is deposited either along reticulin fibres and reticulin containing basement membrane or along collagen fibres. Thus amyloidosis is classified in two broad groups: perireticulin and pericollagen (206). The perireticulin group includes: hereditary amyloidosis with

nephropathy, primary and secondary amyloidosis. The pericollagen group includes: hereditary amyloidosis with neuropathy or cardiomegaly, "tumour-forming" amyloidosis and amyloidosis associated with plasma cell dyscrasias. In systemic lupus erythematosus both forms can be present simultaneously.

The pathogenesis of amyloid formation is still a matter of speculation; the involvement of a variety of factors appears more likely than that of a single mechanism. In experimental animals numerous agents and methods have been used successfully to induce amyloidosis (207). It is believed that after injection of amyloidotic agents, an intermediate substance, amyloid-enhancing factor (AEF), develops which has been found to be antigenically related to mouse IgG (208). Observations of both human and animal conditions indicate that dysproteinaemia with associated disorders in immune mechanisms is generally the cardinal predisposing factor in the development of amyloidosis. The incidence of amyloidosis in human multiple myeloma is relatively high, ranging from 5 to 20% of cases (209), and this has led to considerable investigation of relations between plasma cell dyscrasia and amyloidosis. It has been suggested (210) that "in the majority, if not in all cases of 'amyloidosis', there is 1) an underlying proliferative disorder ('dyscrasia') of plasma cells, with an associated derangement in gamma-globulin synthesis; 2) that the gamma-globulin products of these abnormal plasma cells are directly responsible for the

production of 'amyloid infiltrates'". Particular stress is put on Bence Jones protein (L-polypeptide) which is thought to be directly responsible for the production and accumulation of amyloid. This concept is contrary to the demonstrated facts that plasmacytosis is not always present with amyloidosis (211), and that amyloidosis has been reported in cases of agammaglobulinaemia (212).

The "two-phase cellular theory of local secretion" of Teilum (213) is widely quoted as a possible mechanism for amyloid production. Initially, there is proliferation of reticuloendothelial cells in response to antigenic stimuli, with production of pyroninophilic cells and plasma cells and a concomitant rise in the level of serum gamma globulin. Under the stress of continued stimulation the altered cells (pyroninophilic cells and plasma cells) reach a state of exhaustion. At this stage a PAS-positive substance appears in the cytoplasm of the altered cells, the serum gamma globulin level decreases, and the dysfunctional cells begin to elaborate amyloid fibrils. An extension of this concept describes the process in three stages or phases (214). In the hyperglobulinaemic reticulum cell sarcoma of mice, pyroninophilic cells appear in the splenic perifollicular space. This is attributed to humoural stimulation and is similar to the splenic changes described in this thesis in Section V. With the appearance of pyroninophilic cells the first phase the proliferative stage has begun which later goes

into the inversion stage with change to PAS-positivity of the cytoplasm, and finally ends in the amyloid stage when fibrils are precipitated around cells.

Teilum first observed that amyloid production could be considerably increased in spleens of mice pretreated with casein by administration of nitrogen mustard (215). The basic mechanism was thought to be the suppression of actively proliferating mesenchymal (pyroninophilic) cells by nitrogen mustard, and thus acceleration of the second phase and amyloid production.

(ii) Amyloidosis in murine plasma cell neoplasms:

Out of 7 transplantable sublines of a spontaneous plasma cell leukaemia (216) occurring in (CBA x DBA/2)F_1 mice, amyloid was found exclusively in glomeruli in 2 sublines with electrophoretically normal serum protein pattern. There were neither Bence Jones proteinuria nor 'myeloma kidney'. In a third subline with hypergammaglobulinaemia the spleen was the only organ to exhibit amyloidosis. It was deposited in the peri-follicular region in close association with pyroninophilic cells and plasma cells. Among the induced forms of plasma cell neoplasms, several types of MOPC tumours demonstrated renal interstitial infiltration of amyloid (217).

It has been shown in Adj-PC-5 plasma cell neoplasm that amyloid can be detected in the tumour and the liver as early as 15 days after transplantation. After 20 days the

kidneys are involved and at 24 days, amyloid was also found in the heart, lungs and spleens of a few mice (218).

(iii) The present study:

The present study was undertaken to seek confirmation of the findings of the above investigators (218) who also worked with Adj-PC-5 tumours in later generations. Attempts were made to enhance the life-span of tumour-bearing animals by varying the methods of transplantation. Nitrogen mustard was used for two reasons: 1) its enhancing effect on the production of amyloid; and 2) its ability to increase the life-span of tumour-bearing animals without completely eradicating the tumour, thus prolonging the hypergammaglobulinaemic state which might induce amyloidosis.

2. MATERIALS AND METHODS

To increase the life-span of tumour-bearing animals, 50 mice were given decreased quanta of inocula and the minced fragments were repeatedly washed before injection. In an additional 50 animals, known numbers of tumour cells were inoculated instead of tissue fragments. For this the tumour tissue from the donour mouse, immersed in the medium, was minced on a fine wire mesh in an attempt to ensure that free cells would pass through and tissue fragments would be eliminated. The cells were counted using a haemocytometer; necrotic cells were identified by their affinity for the eosin stain and were

not counted. A known number of cells in 0.5 ml of the medium was injected subcutaneously in the right axillary region or intraperitoneally. At the onset a cell count of 2 millions per injection was given, and in the succeeding 10 transplants the cell count was reduced to 4000 per injection. Further reductions in numbers were avoided to maintain reasonable accuracy in counts.

According to the purpose of experiments with nitrogen mustard, the animals were divided into 3 groups. However, methods of initiating tumours and the scheduling of the 1st injection were similar in all of the animals. Minced fragments were injected subcutaneously in the right axillary regions of female BALB/cJ mice between 12 and 16 weeks of age. When the subcutaneous lesion achieved a moderate size around the 8th to 10th day, nitrogen mustard (Mustargen, Merck Sharp and Dohme of Canada Limited, Montreal; 10 mg dissolved in 10 ml of distilled water) was injected subcutaneously at the base of the left forelimb, on the contralateral side of the neoplasm. The dosage given was 2.5 mg per kg of body weight (the average weight of a mouse is 20 mg). The time of the 1st nitrogen mustard injection varied from the 8th to the 10th day due to the fact that the growth rate of the tumours was inconstant, presumably because of quantitative differences in the inocula. The size of the growth was visually assessed before the administration of nitrogen mustard.

After the 1st injection of nitrogen mustard, the

Group I animals received 2 further injections of the same dose at 2 days intervals. They were sacrificed on the 3rd day following the last injection. The dosage and the schedule of nitrogen mustard administration were according to Teilum's experiments on casein-treated mice (mentioned in the Introduction of thepresent Section; Ref. No. 215). Five such experiments, each consisting of 3 animals, were carried out.

The Group II experiments were carried out to prolong indefinitely the survival of animals carrying a grossly demonstrable subcutaneous tumour. Hence the schedule of injections followed in the Group I experiments was interrupted and the 2nd injection did not follow the 1st one after a 2 day interval. Instead, the 2nd injection was withheld until the subcutaneous tumour regained its original size. Altogether 15 animals were used in the Group II experiments; in 5 of these, a 3rd injection of nitrogen mustard was administered, depending on the clinical state of the animal.

The Group III experiments were designed for an electron microscopic study of the effect of nitrogen mustard on tumour cells at the site of inoculation. Altogether 5 tumour-bearing animals were used and the mice were sacrificed on the 3rd day following the 1st injection of nitrogen mustard.

Methods of sacrificing animals, post-mortem examinations, collections of tissues, and processing for light and electron microscopy, have been described under Materials and Methods of Section IV.

3. OBSERVATIONS

(i) Gross findings:

In spite of varying the methods of transplantation the longest survival achieved was 23 days. Nearly 90 out of 100 lived from 16 to 20 days, while the remaining 10 lived from 21 to 23 days. The tumour at the site of inoculation took longer to appear, but once formed the rate of growth was similar to that of tumours grown by the usual quanta of inocula. The difference in the number of dispersed cells injected did not have much appreciable effect on the growth.

In experiments with nitrogen mustard, the subcutaneous tumour rapidly abated in size after the 1st injection, but did not completely disappear. At the site of nitrogen mustard injection in the left axillary region, an oedematous swelling appeared within a short time following the 1st injection, that extended to the forelimb. The swelling progressed to focal ulceration, and the forelimb itself appeared shrivelled and atrophied.

In Group I experiments, the subcutaneous tumour at the site of inoculation disappeared soon after the 2nd injection of nitrogen mustard (Fig. 37); therefore at the time of sacrifice on the 3rd day following the 3rd injection, the animal was devoid of a grossly visible tumour. When the skin overlying the site of tumour inoculation was reflected no tumour mass was present. Among the internal organs the spleen appeared markedly decreased

in size, in contrast to the findings in untreated tumour-bearing animals, in which the spleen was the only organ to show gross enlargement.

In Group II experiments, the longevity of tumour-bearing animals ranged from 26 to 39 days. At the time of sacrifice the tumour mass showed relatively more necrosis than what was seen in untreated cases. Among the internal organs the spleen was markedly enlarged with a bosselated capsular surface. In some instances greyish-white pin-head sized deposits resembling metastatic nodules were seen on the capsular surface of liver.

In Group III experiments, the tumour mass was comparatively smaller in size, and the lesion showed extensive necrosis.

(ii) Light microscopy:

In animals with longer survival, patterns of local invasion and of dissemination were similar to that seen in animals with an average survival (described in Section V); although there were numerical increase in tumour cells both in areas of local spread and in lymphovascular spaces. Polymorphisms of neoplastic cells were more conspicuous. Amyloid was not detected in tumour tissue, liver, kidneys, spleen or lungs.

In Group I experiments, no tumour cells were found in the subcutaneous tissue and adjacent skeletal muscles at the site of tumour implantation. The wide dissemination of

tumour cells in untreated animals was absent in treated ones. Bizarre hyperchromatic cells resembling tumour cells but not definitely identifiable as such, were occasionally seen in the alveolar septal capillaries or in the splenic perifollicular space; but in general, the treated animals were found to be free of tumour. There was generalized depletion of lymphoid tissue, and lymphoid cells demonstrated a marked degree of pyknosis and karyorrhexis. As a result of lymphocytic depletion, the morphological demarcation between cortical and medullary zones in the thymus and lymph nodes was lost in most areas. In the spleen the white pulp was markedly diminished with increase of red pulp (Fig. 38). The connective tissue septae were unusually prominent. The perifollicular areas contained moderate amounts of haemosiderin granules. Pyroninophilic cells disappeared. Megakaryocytes were decreased in number. The bone marrow was remarkably hypocellular with large lakes of haemorrhage and a few small clusters of cells, mostly of granulocytic nature. No significant abnormality was noted in such organs as liver, lungs, kidneys and heart. No amyloid was detected in any of the internal organs.

In Group II experiments, sections of the subcutaneous tumour showed large areas of necrosis in the central region of lobules with only two or three layers of viable cells remaining at the periphery. In morphology and in its manner of local invasion the tumour closely resembled those of untreated animals.

However, the disseminating tumour cells were more numerous and more bizarre in shape in alveolar septal capillaries, hepatic sinusoids and glomerular capillaries. In the spleen the perifollicular areas were packed with tumour cells with frequent encroachment of white pulp (Fig. 39). Compared to spleens in Group I animals, the number of megakaryocytes was increased. Small metastatic foci were infrequently seen in liver (Fig. 40) with tumour emboli in venous spaces (Fig. 41). Frequent metastases were found in cervical and mediastinal lymph nodes. No amyloid was present.

(iii) Electron microscopy (Group III experiments):

Ultrastructurally, tumour cells tended to be dissociated from each other, sometimes separated by moderately clectron-dense amorphous material (Fig. 42). Numerous myelin-like figures were present both intra-and extracellularly, in a variety of shapes and sizes. In the cytoplasm the amount of free ribosomes was markedly decreased. Cisternae of endoplasmic reticulum were markedly dilated showing intricate convolutions. The majority of them were electron-lucent, many contained virus-like particles (Fig. 43). Electron-dense bodies were increased in number (Fig. 44) in many cells. The Golgi apparatus was conspicuous in several cells. In nuclei the peripheral chromatin was sparse and clumps of chromatin-like material were often present within the nuclear substance. The overall impression was that in most regions the tumour did not consist of a

uniform cell population but contained both degenerating and viable cells.

4. SUMMARY OF FINDINGS

In this study amyloid was not detected in tissues of tumour-bearing animals. By varying methods of transplantation the longest survival achieved was 23 days. Within this period amyloid did not appear in the tumour substance, liver, kidneys, spleen or lungs.

Nitrogen mustard was used because of its enhancing effect on amyloid formation, but the results were negative. With 3 consecutive injections at 2 days' interval, the tumour not only disappeared at the site of inoculation but also from the spleen and from the vascular spaces of other internal organs. When nitrogen mustard was administered at irregular intervals the animals survived between 26 and 39 days and their tumours persisted. Necrosis was prominent in the tumour at the site of inoculation. The spleen was massively infiltrated with tumour cells. There were metastatic foci in the liver in addition to tumour cells in vascular spaces. Again, no amyloid was detected in tissues including the subcutaneous tumours. Ultrastructurally, tumour cells demonstrated numerous myelin-like figures, decreased free ribosomes and markedly dilated cisternae of endoplasmic reticulum. Dense bodies were present in increased numbers. Nuclei had irregular chromatin distribution.

5. DISCUSSION

The findings of previous investigators in detecting amyloid in later generations of Adj-PC-5 plasma cell neoplasm (218) were not confirmed in this study. Although the longest survival achieved in these experiments fell short of that achieved by previous investigators, the life-span was considered to be of sufficient length to permit amyloidotic deposits to occur at least in the tumour substance and in liver.

In the present study, in which hypergammaglobulinaemia was associated with a significant number of perifollicular pyroninophilic cells, administration of nitrogen mustard was followed by disappearance of pyroninophilic cells without resultant amyloid.

Effectiveness of nitrogen mustard in causing complete regression of Adj-PC-5 tumour in BALB/c mice has been described before (219). The results of this study were similar. In previous studies the ultrastructural effects of nitrogen mustard on cells of plasma cell neoplasms has not been described. In mouse epithelial cells (220) administration of nitrogen mustard was followed by marked decrease of rough endoplasmic reticulum and free ribosomes. In this study the effect of nitrogen mustard on tumour cells seemed more widespread. The presence of numerous myelin-like figures indicated a toxic effect on membranous structures; ribosomes were decreased and the chromatin pattern of nuclei was altered.

Figure 37. Photograph of 2 mice inoculated with tumour at the same time. The tumour growth in the mouse on the right disappeared 2 days after the second of 2 injections of nitrogen mustard. The mouse on the left was untreated.

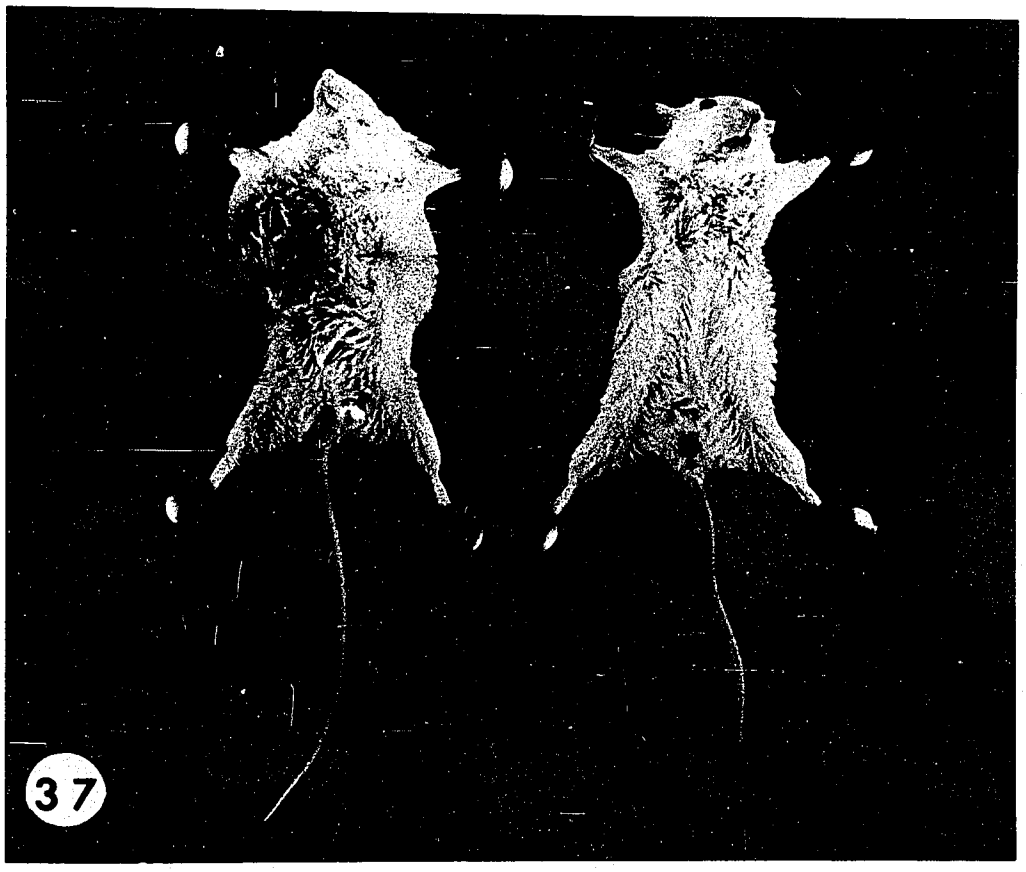

37

Figure 38. A section of spleen following 3 spaced consecutive injections of nitrogen mustard. The spleen is free of tumour cells. There is marked depletion of the white pulp and megakaryocytes have disappeared. The red pulp is increased. Haematoxylin and eosin. X210.

Figure 39. A section of spleen from a tumour-bearing mouse that survived for 35 days after 3 injections of nitrogen mustard were given at irregular intervals. Only small clusters of dark-staining splenic cells remain, while tumour cells occupy most of the parenchymal substance. Haematoxylin and eosin. X210.

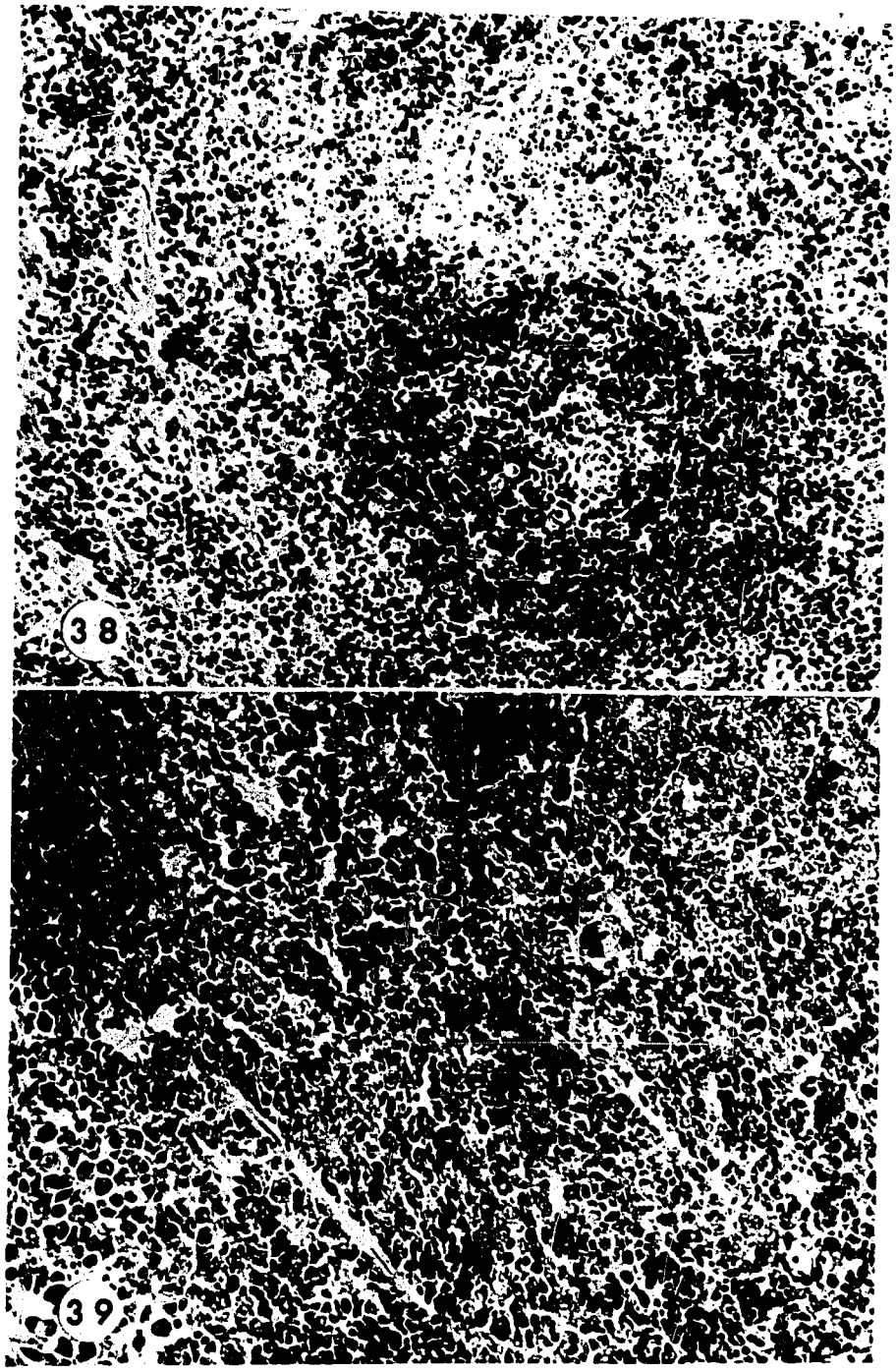

38
39

Figure 40. A section of liver showing a metastatic tumour nodule on the right. On the left, a few cords of liver cells still remain (arrow). The tumour cells are arranged in a lobular fashion, similar to that seen in the subcutaneous tumour at the site of implantation. Haematoxylin and eosin. X210.

Figure 41. A section of liver showing a tumour embolus in a vascular space (arrow). Haematoxylin and eosin. X210.

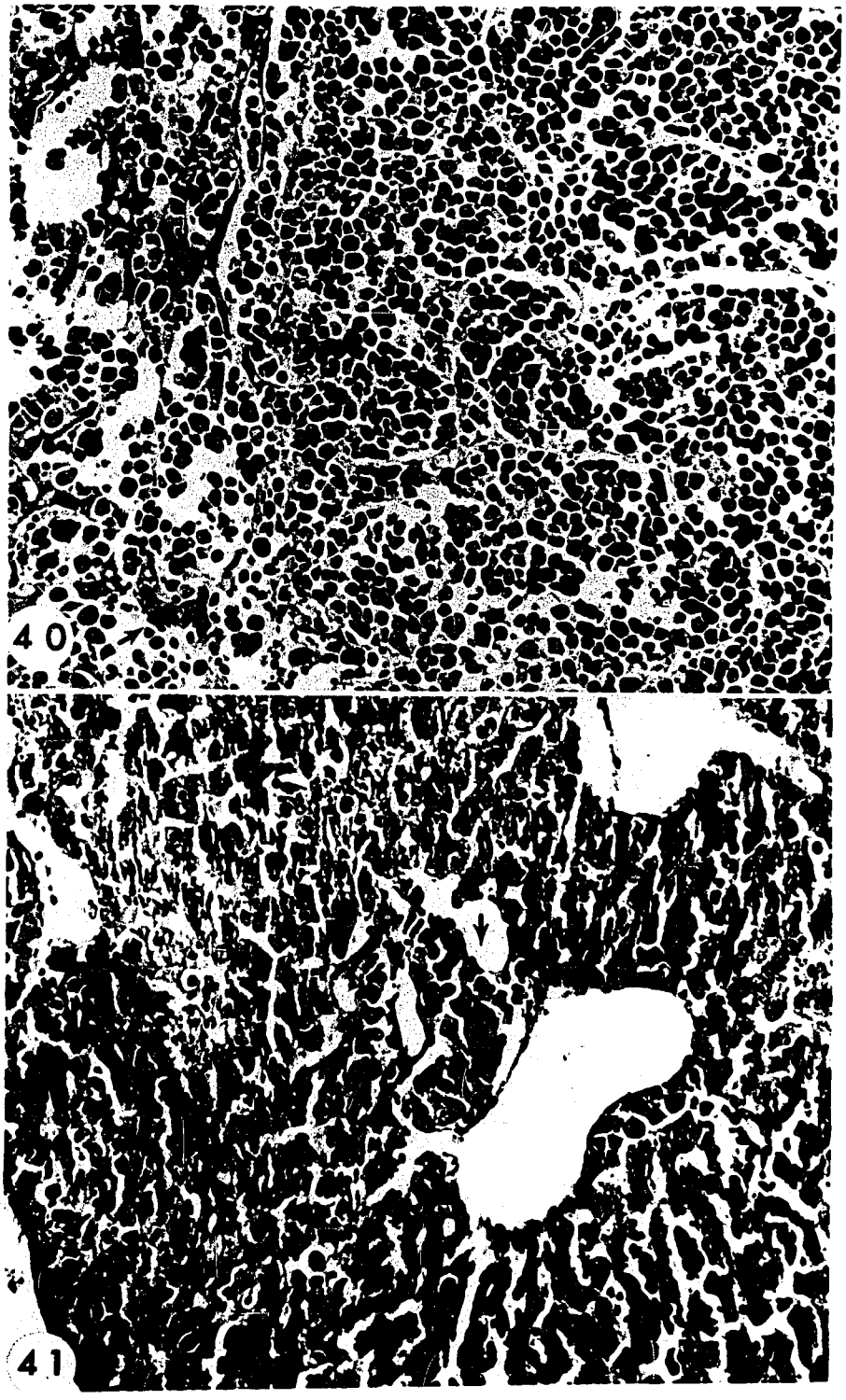

40
41

Figure 42. An electron micrograph of a tumour cell, 2 days following an injection of nitrogen mustard. Cisternal spaces are markedly dilated and free ribosomes are considerably reduced in number. The chromatin is in condensed clumps towards the centre of the nucleus. The intercellular space in the lower left corner (IS) is filled with amorphous material. Uranyl acetate and lead citrate. X25,000.

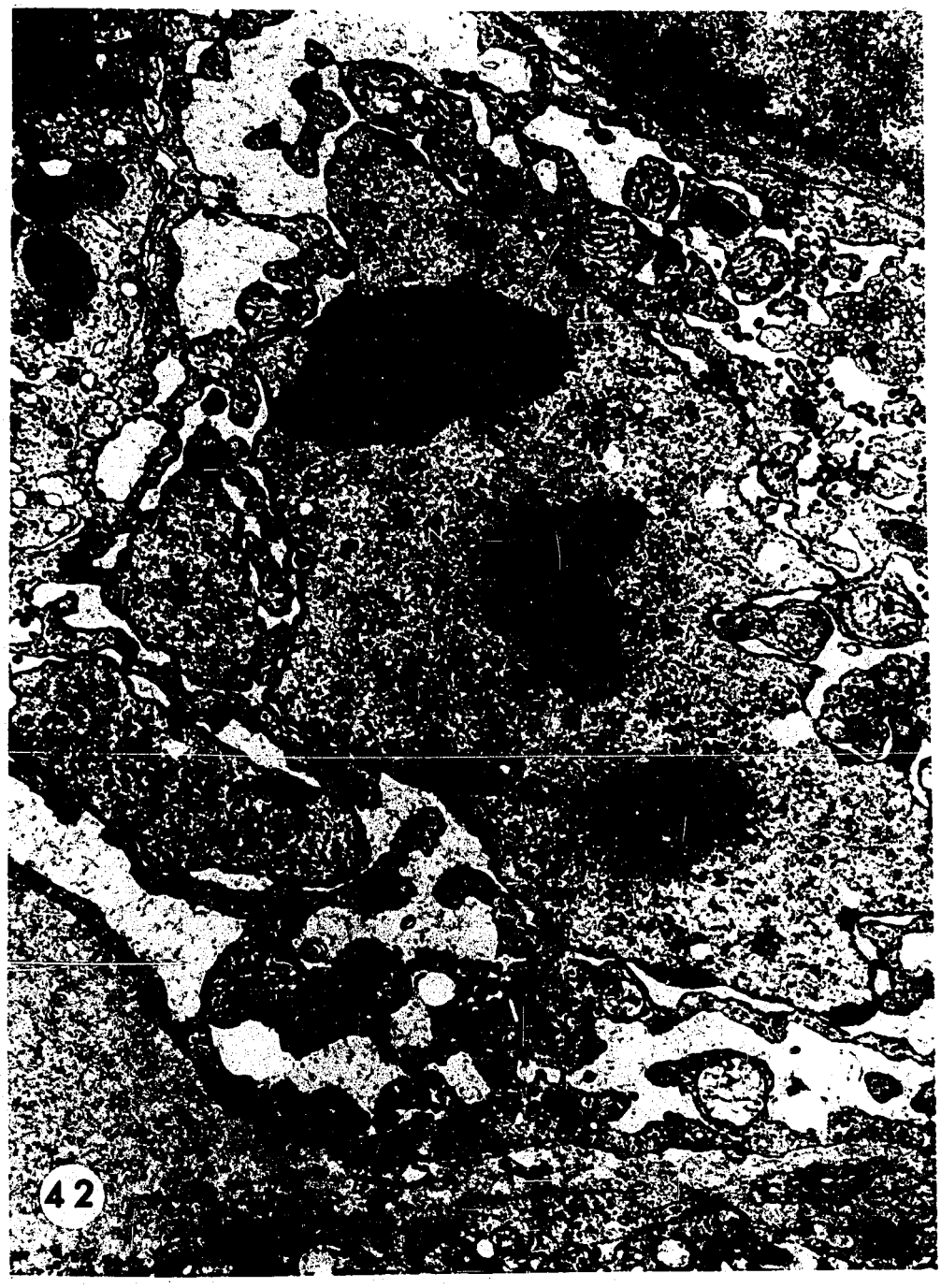

42

Figure 43. An electron micrograph of a tumour cell, 2 days following an injection of nitrogen mustard. The cisternae of the endoplasmic reticulum show marked dilatation. Virus-like particles are seen in a few cisternae. Scattered vesicles of the Golgi apparatus are present. Uranyl acetate and lead citrate. X54,000.

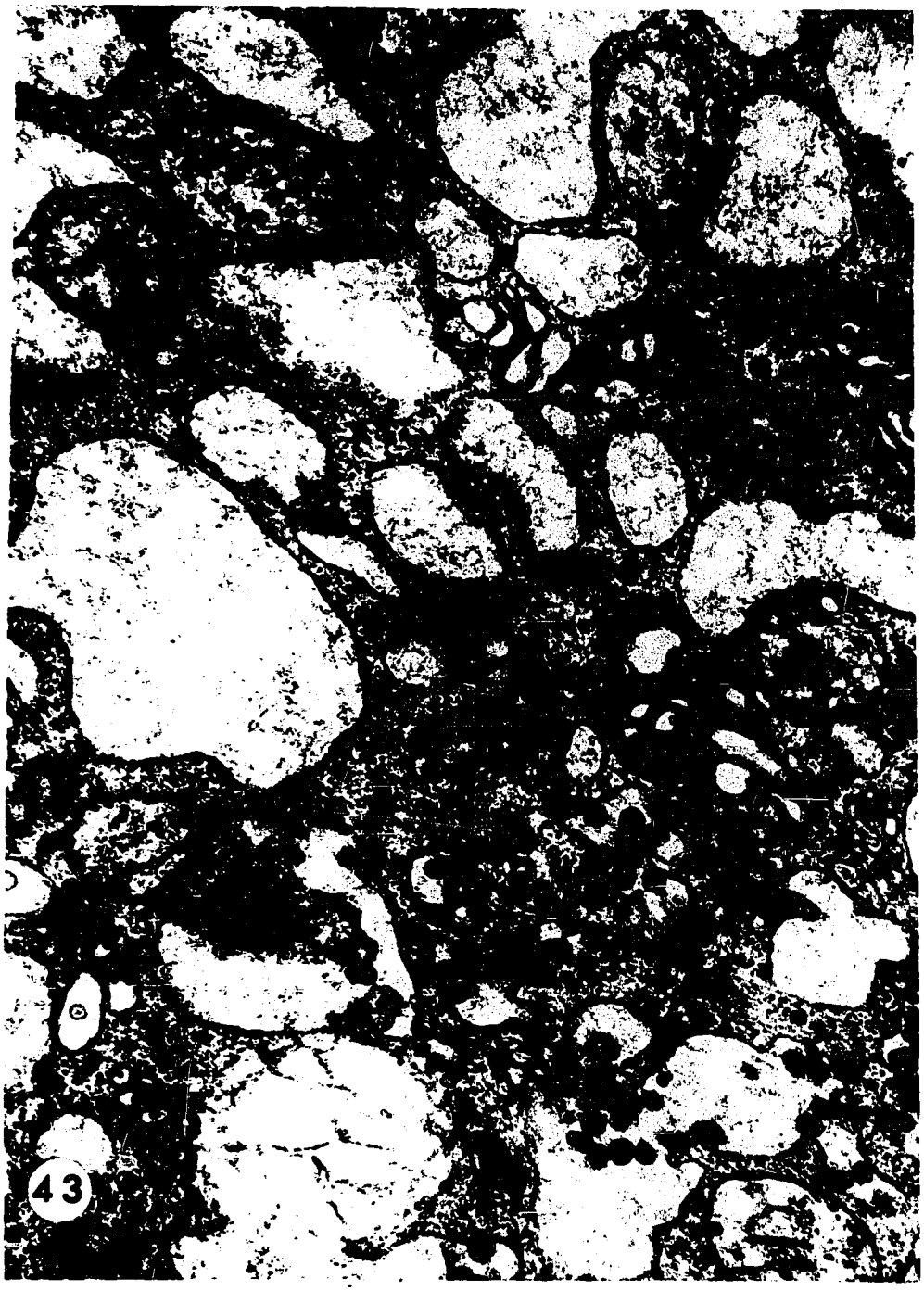
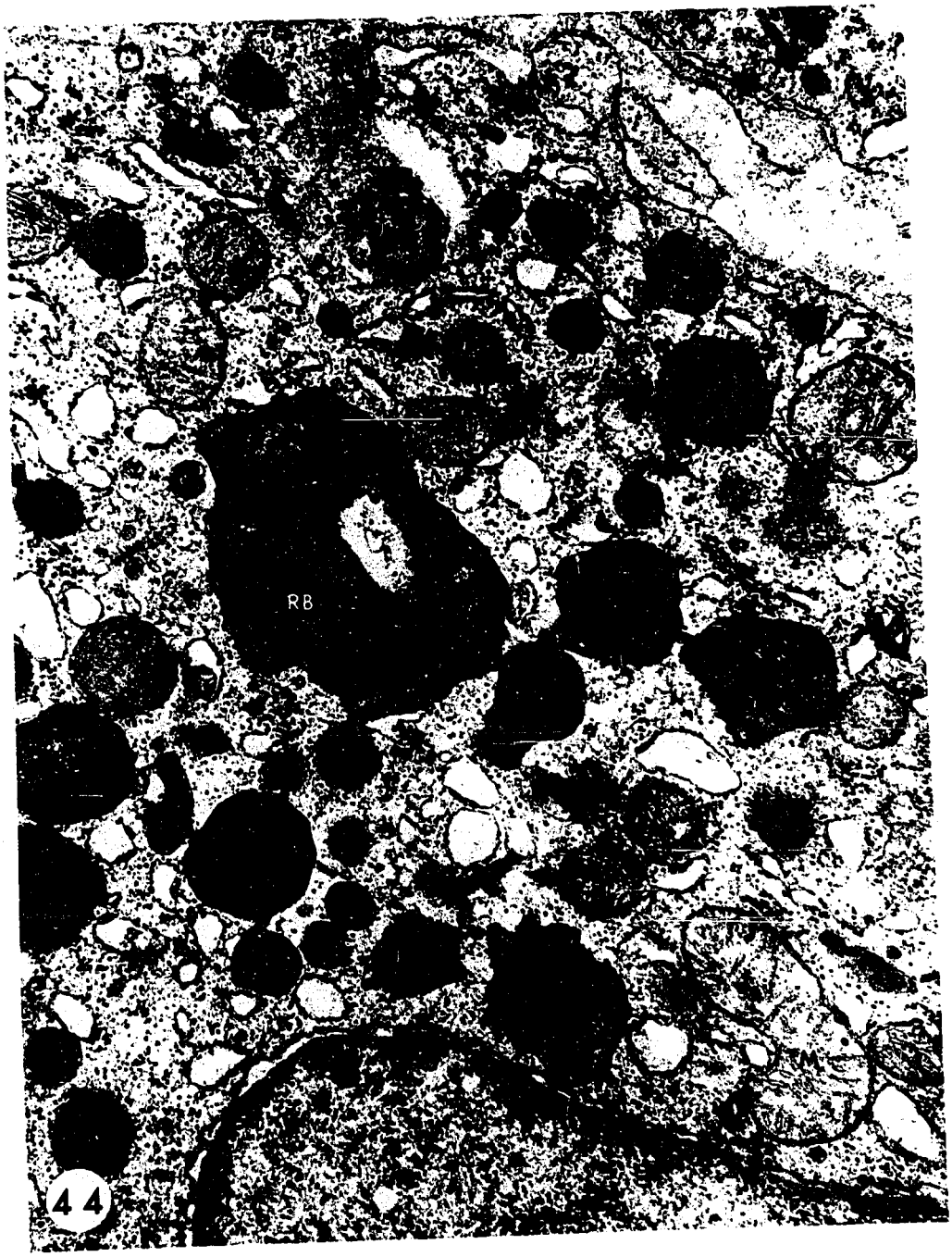

43

Figure 44. An electron micrograph of a tumour cell, 2 days following an injection of nitrogen mustard. The cytoplasm shows many electron-dense bodies of irregular sizes; some of which appear to contain membrane-like structures and may be residual bodies (RB). M = mitochondrion. Uranyl acetate and lead citrate. X42,000.


RB
44

VII. Summary and Conclusion

The Adj-PC-5 plasma cell tumour in BALB/c mice was induced in the laboratory of Dr. Michael Potter, National Institute of Health, Bethesda, Maryland, by a single injection of a mixture of Freund's adjuvant and heat-killed staphylococci. The neoplastic plasma cells of the primary tumour are described as having hyperchromatic, lobulated nuclei and deeply basophilic cytoplasm with a juxtanuclear hof, resembling mature plasma cells, although without the clock-face pattern of nuclei. Lymph nodes, when involved, had neoplastic cells in sinuses. The tumour did not spread to thoracic or abdominal organs, there were no osteolytic lesions, and so-called myeloma kidneys were not observed. With paper electrophoresis an elevated narrow gamma peak was demonstrated in the serum.

The present study was of later transplant generations of this plasma cell neoplasm, from the 41st to the 100th transfer. The tumour was transplanted to groups of 3 mice every 12th day, usually by subcutaneous injection of minced tumour tissue, and the local growth was studied by light and electron microscopy. The morphology of the neoplasm did not alter through the 60 generations studied. Tumour cells were arranged in lobules of various sizes, separated by delicate strands of reticulum fibres. The cells measured 35 to 50 microns in diameter, with large lobulated nuclei occupying more than two-thirds of the cell volume and scant, vacuolated, moderately basophilic and pyroninophilic cytoplasm. Mitotic figures were

frequent. The tumour cells no longer bore a close resemblance to mature plasma cells.

Ultrastructurally, the closely apposed tumour cells had slightly undulated plasma membranes devoid of desmosomes and without surface microvilli. The amount of rough endoplasmic reticulum was considerably diminished, as compared to that seen in a mature plasma cell. Small cisternae, frequently dilated, were scattered around the nuclei with alternate rough and smooth surfaces. Free ribosomes were abundant in the hyaloplasm. The Golgi apparatus was well developed. Electron-dense bodies, many of them multivesiculated, were usually present. Intracisternal virus-like particles measured 80-130 millimicrons in diameter. Mitochondrial cristae were sometimes in parallel arrangement but fregquently showed irregular alterations in disposition.

Ten serological examinations spaced irregularly between the 50th and 95th transfer generations demonstrated elevated levels of IgG immunoglobulin. This elevation occurred in spite of a scant amount of rough endoplasmic reticulum, which normally is the protein-producing apparatus in cells, particularly in plasma cells. It is possible that elevated levels of protein production were maintained by increased total numbers of neoplastic cells, or by increased numbers of free ribosomes.

At the site of inoculation the tumour infiltrated the adjacent skeletal muscles around the humerus and ribs, but was arrested at the periosteum. Gross splenomegaly was due to

tumour infiltration in subcapsular and perifollicular regions. Tumour cells were found in large numbers in hepatic sinusoids, in capillaries of pulmonary alveolar septa and glomeruli, and in sinuses of lymph nodes. In spite of engorgement of vascular spaces by tumour cells, the parenchyma of liver, lung, kidney and lymph nodes was free of metastatic deposits.

In the liver, tumour cells were seen within hepatocytes. From the appearance of the lodged tumour cells, with their intact nuclei surrounded by a rim of cytoplasm, it was considered that the tumour cells may have gained entry by the process of emperipolesis rather than by that of phagocytosis by hepatocytes.

In the spleen, which is non-sinusal in mice, many splenic lymphoreticular cells became pyroninophilic following tumour dissemination. These altered cells came into direct contact with tumour cells and may have formed cytoplasmic fusions, because virus-like particles, which are characteristic of tumour cells, were seen in pyroninophilic cells.

Amyloidosis has been reported to occur in mice bearing Adj-PC-5 plasma cell tumour. By variations in the method of transplantation and the size of the inoculum the survival of many of the tumour-bearing animals was increased up to 23 days. Although in the reported study amyloidosis developed after 15 days of tumour growth, in our mice amyloid was not detected either in the tumour substance or in any of

the internal organs. Nitrogen mustard is known to enhance amyloid formation in casein-treated mice. In the present study, when nitrogen mustard was administered to tumour-bearing animals in 3 consecutive injections at 2 days' interval, complete regression of tumours occurred with disappearance of splenic pyroninophilic cells, but without amyloid deposition. When nitrogen mustard was given at irregular intervals, to cause partial regression of tumours and thus enable the animals to maintain a hypergammaglobulinaemic state for a prolonged period, amyloid was still undetectable either in the tumour substance or in internal organs after a life-span ranging from 26 to 39 days. In these animals, in addition to vascular dissemination, metastatic foci were found in the liver and there was massive replacement of splenic parenchyma by tumour cells. Two days following nitrogen mustard administration, the ultrastructural changes in tumour cells were manifested in the appearance of numerous myelin-like structures and in marked decrease of free ribosomes. The chromatin pattern of nuclei was altered.

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