ASPECTS OF THE PATHOGENESIS OF THE RAUSCHER MURINE LEUKEMIA VIRUS INFECTION

PROEFSCHRIFT

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To all my teachers from whom I learned to write, to deduce, and to appreciate humanity.

CONTENTS

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CHAPTER 1

INTRODUCTION

> When, in 1928, I began the study of the etiology and pathogenesis of leukemia, it was an almost virgin field, ready for exploration.
> Jacob Furth, 1968, in *Experimental Leukemia* (ed. M.A. Rich)

Many physical, chemical, and biological factors have been thought to cause neoplasia. Soon after it had been suggested that some viruses could act as biological oncogenic agents in animals (SANARELLI 1898; ELLERMAN and BANG 1908; ROUS 1908; SHOPE 1932; FURTH 1933; BITTNER 1936), extensive studies on the etiology of neoplasms were undertaken with several kinds of oncogenic virus (GIRALDO et al. 1972; DMOCHOWSKI 1973). Several new oncogenic viruses have recently been added to the list of possible etiologic biological agents (DUTCHER 1973; HENLE and HENLE 1973; BORDEN 1974; CAWLEY and KARPAS 1975). The biological, immunological, and biochemical properties of these agents and their relationship to neoplasms continue to be matters of great importance. However, a cause-effect relationship in human neoplasms remains questionable, and the available information about the etiologic factors in these diseases in man is fragmentary (GROSS 1965).

Animal models of human diseases are widely used in experimental pathology (SNYDER et al. 1973; BLACK 1974; MILLER 1974; LONDON et al. 1974). Oncogenic viruses were discovered during the study of neoplastic diseases occurring naturally in animals (ELLER-

MANN and BANG 1908; ROUS 1911; GROSS 1951). Various forms of leukemia and lymphomas have been found in several kinds of domestic and laboratory animals such as chickens, rats, cats, and mice (DUNN 1954; FENNER 1968; GROSS and FELTMAN 1970). We are now convinced that in animals viruses can play a role in oncogenesis as etiological factors (GROSS 1970; BENTVELZEN 1972; KOBAYASHI and MUKAI 1974). The viral hypothesis might also be valid for human neoplasms, at least for some kinds of neoplastic diseases (e.g. leukemia), although this has not been proved convincingly (MANN et al. 1973; BAXT 1974; GALLO et al. 1974; de-THE and GESER 1974).

Among the oncogenic viruses, Rauscher murine leukemia virus (R-MuLV) (RAUSCHER 1962) occupies a relatively prominent place in experimental neoplasia. Therefore, we performed a series of experiments to acquire more information about the morphological changes occurring during R-MuLV-induced infection, which is also called Rauscher disease of mice (FINK et al. 1964). During these studies, we were able to determine the morphological expression of the cytopathic effects of R-MuLV and the neoplastic nature of Rauscher disease. Afterwards, the cytopathic effect of R-MuLV on adult and embryonic tissue was studied both light- and electron-microscopically.

Finally, we made an attempt to develop a convenient method for the transplantation of hemopoietic cells infected with R-MuLV. This investigation led to the identification of mesenteric hemopoietic colonies. The occurrence of mesenteric hemopoietic colonies was studied in several strains of mice, and to complete the investigation also in *Macaca fascicularis* monkeys.

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CHAPTER 2

VIRUSES AS BIOLOGICAL ONCOGENIC AGENTS

Viruses: The smallest infectious agents, the "infinitesimally small microorganisms" postulated by PASTEUR in 1881, were first demonstrated in the mosaic disease of tobacco plants by BEIJERINCK in 1898 (GROSS 1970). At the beginning of the twentieth century the term virus was applied to invisible, filtrable, pathogenic microorganisms, and so much knowledge was accumulated about the biological nature of viruses in this period that virology became a separate discipline.

Viruses cannot be seen with the optical microscope and have different biological properties than bacteria. Unlike bacteria, viruses pass through porcelain filters. The central core of viruses normally contains one kind of nucleic acid (BELLETT et al. 1973), i.e., either deoxyribose nucleic acid (DNA) or ribose nucleic acid (RNA), in other words genetic material. Viruses are obligate intracellular parasites on living cells, because they do not possess all of the enzymes and metabolic systems necessary for nucleic acid replication and protein synthesis (BELLETT et al. 1973).

Multiplication of virus takes place in the nuclei or cytoplasm of the host cell. After multiplication, hundreds of intact virus particles can together form intranuclear or cytoplasmic inclusion bodies (WATKINS et al. 1970; ZACKS 1971). The cell membrane and cytoplasm of the infected cells can determine the special shape and provide protein for the coat of the enveloped virus. Almost all viruses show antigenic properties (THORBECKE and BENACERRAF 1962; LANGENHUYSEN et al. 1974; GILDEN 1975). Certain viruses can

selectively infect a single species, and many others can show tropism to special cells or tissues (HOLLAND 1964; BODIAN and HORSTMAN 1965).

The effect of viral infection depends on the properties of both the host cells and the virus in question (ROBBINS 1974). A variety of possible effects of the cell-virus interaction can be cited, such as:

1. A cytocidal-lytic effect, e.g. infection by ECHO viruses (DALLDORF et al. 1955).
2. Polykaryocytosis by cell fusion, e.g. during herpes or rubeola infections, producing multinucleated giant cells (ROBBINS 1974).

These two possibilities are defined in many instances as cytopathogenic effects of viruses (PAUL 1975).

3. Non-cytocidal virus-cell interaction
   a) Unmanifested steady-state infection, e.g. avian leukosis virus infection in chickens (VOGT and FRIIS 1917).
   b) Transformation of cells by viral infection (PONTEN 1971).

The term transformation indicates not only morphological changes, but also alterations in genetic and biological properties of the cells accompanied by disturbances in the regularity of cell growth related to neoplasia. The "indefinite growth transformation", which is accompanied by loss of aging properties and the acquisition of unlimited division and survival potential, can be caused by an oncogenic virus infection (PONTEN 1971; TAMM 1975). The spontaneous occurrence of transformation has also been postulated (PONTEN 1971).

Oncogenic viruses: Oncogenic viruses are etiologically associated with a large number of neoplastic diseases in animals, including mammals. The central core of the oncogenic viruses contains either RNA or DNA (BIGGS et al. 1972). Since 1970, the group of oncogenic RNA viruses has also been designated as oncornaviruses (NOWINSKI et al. 1970). In mice leukemogenic biological agents – which are known to belong to the oncornaviruses – were conclusively determined by GROSS in 1951 after his discovery of an infectious virus preparation from a mouse with leukemia (GROSS 1951). Some years later, in 1957, a highly potent mouse leukemia virus strain called Gross passage A was established (GROSS 1957). This was followed by the isolation of various artificial and laboratory strains of murine leukemia viruses (SCHMIDT 1955; FRIEND 1956; FRIEND 1957; GRAFFI and GIMMY 1957; SCHOOLMAN et al. 1957; MOLONEY

helper-virus (PLUZNIK and SACHS 1964).

R-MuLV, like the other C-type virus particles, matures at the cell surface by an elaborate process of budding. The core of R-MuLV shows a hexagonal inner shell which houses an electron-dense nucleoid is composed of protein and single-stranded RNA (BLAIR and DUESBERG 1968). Electron-microscopical studies support the view that both core and nucleoid are assembled at the bud site (de-THE 1964; BOLOGNESI 1974).

R-MuLV has an antigenic potential on the immune system, especially in susceptible animals (TOTH et al. 1971). Interaction between R-MuLV and specific antiserum results in agglutination, as shown electron-microscopically by MAYYASI et al. (1966). Mice can be protected against R-MuLV infection by passive immunization with homologous specific antiserum (FINK and RAUSCHER 1964) or by active immunization (BARSKI and YOUN 1966).

Murine leukemia virus-associated cell-surface antigens are classified into two major groups, viz. G (Gross) and FMR (Friend-Moloney-Rauscher) (OLD et al. 1964; ROWE et al. 1965; AOKI 1974). Type-specific virus envelope antigens are a characteristic feature of individual strains in the FMR group (ECKNER and STEEVES 1972; AOKI 1974).

R-MuLV can infect mouse embryo fibroblasts in tissue cultures and can also propagate in human embryonic kidney cells (ABLASHI et al. 1972), in which they induce mouse group-specific antigen synthesis.

Finally, the occurrence of a humoral immune response (HANNA et al. 1973) and cell-mediated immune functions (MORTENSON et al. 1973) has been proven in mice by means of purified R-MuLV antigens and in human patients with formalin-killed R-MuLV (HERSH et al. 1974).

Long-term *in vitro* culture of R-MuLV results in attenuation of the leukemogenic capacity, but causes no morphological alteration of viral particles (BARBIERI and BARSKI 1973). Several low-leukemogenic variants of R-MuLV can be obtained, also in long-term cultures at supra-optimal temperature (40°C ± 0.5°C) (BARSKI et al. 1973). These temperature-sensitive mutants of R-MuLV are unable to reach target cells or to trigger the transformation process *in vivo* (STEPHENSON and AARONSON 1973).

R-MuLV is infectious for many strains of mice and also for

Osborne-Mendel rats (RAUSCHER 1962). Being a biological oncogenic agent, R-MuLV induces a neoplastic disease in mice, the infection being characterized by erythroleukemia, splenomegally, and a fatal outcome (PLUZNIK et al. 1965; CAMPBELL and LEVINE 1969; BROMMER 1972; LOZZIO et al. 1974; NOOTER and BENTVELZEN 1976).

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CHAPTER 3

MATERIALS AND METHODS

Of mice and men
John Steinbeck

Animals: The experiments were carried out in mice, mouse embryos, golden hamsters, and monkeys.

Mice: Various strains of mice, viz. BALB/c, CBA, C75BL, C3H, Swiss, CPB-Q, and F_1 (CBA x C75BL), were used (STAAT 1972). Food pellets and tap water were supplied *ad libitum*. The drinking water was acidified to pH 3.6 with HCl to avoid bacterial growth. Age, sex, and strains of mice were specified for each experimental design. The mice were usually killed by cervical dislocation to avoid the excessive capillary congestion associated with asphyxia in ether.

Mouse embryos: These were used to investigate the effect of R-MuLV on the embryos of infected mice. To obtain embryos, a male BALB/c mouse was caged with four female BALB/c mice. Vaginal plugs were controlled for determination of pregnancy. The pregnant mice were then caged separately. Uteri were dissected between the 6th and 12th day of the pregnancy. The embryos obtained were either immediately frozen in liquid nitrogen for the immunofluorescence studies or minced and treated with 0.25% trypsin in phosphate-buffered saline (PBS) for the XC test (see page 64). Fixation of embryos for both light and electron microscopy was performed *in utero* by total body perfusion via the left atrium or via the uterine artery according to REINIUS (1965). Perfusion fixation was followed by immersion fixation for at most 3 days in 1.5% glutaralde-

hyde (pH 7.4 and 320 mOsm); this fixative solution also contained 0.05 M $CaCl_2$ anhydride and 0.1 M sodium cacodylate as buffer (de BRUIJN 1973).

Preparation of mice for autopsy: Immediately after death, the animal was fixed to a cork table and the abdomen wiped with 70% alcohol. The abdominal wall was opened and bilaterally fixed to allow examination and manipulation of the internal organs and vessels. The thorax was opened through the diaphragm and costae; in this way, the sternum and thymus could be prepared *in toto*.

Isolation of bone marrow cells of mice: Femurs of the mice were dissected from the muscles. Bone marrow cells from the femurs were collected in Hanks' balanced salt solution (HBSS) by simple aspiration and washing from the long marrow cavity. Nucleated cells were counted in a Bürker chamber. The number of viable cells was determined by the trypan blue technique (TENNANT 1964).

Isolation of murine spleen cells: Spleen cells from non-infected or R-MuLV-infected mice were isolated immediately after the animals were killed. Spleens were collected into HBSS, in which pieces of spleen were minced. The nucleated-cell content of the suspension was determined in a Bürker chamber. The number of viable cells was determined with the trypan blue technique. The initial cell suspension was diluted with HBSS to obtain the concentration required for the inocula.

Golden hamsters (Mesocricetus auratus): Ten-week-old females were used to investigate the transplantability of the hemopoietic cells of mice infected with R-MuLV. Food pellets and acidified tap water were supplied *ad libitum*. The cheek pouches, which are highly distensible and evaginable diverticula of the buccal cavity, were used as transplantation sites for hemopoietic cells. The hamster cheek pouch technique described by LUTZ et al. (1950) was applied in our experiments because it is a simple method giving easily observable results. Cell suspensions prepared under sterile conditions were inoculated under the epithelium of the wall of both cheek pouches of hamsters anesthetized intraperitoneally with Nembutal® (Abbott) (0.16 ml/100 g), and 2.5 mg cortisone acetate (Adreson®, Organon) was given subcutaneously twice a week, starting on the day of transplantation. The pouches were everted and pinned to a suitable cork for daily observation. Sixty days after the transplantation, the cheek pouches were excised and studied histologically in serial pafaffin sections.

Hamsters were killed by intraperitoneal (ip) injection of 1 ml Nembutal.

Monkeys: *Macaca fascicularis* (syn. *Cynomologus*) monkeys were obtained from Sumatra, Indonesia, and kept in quarantine for at least 3 months. During this period they were tested twice for tuberculosis (Mantoux skin test and erythrocyte sedimentation rate), with negative results. They were also screened and treated for endo- and ectoparasites, and a fecal culture was made for the screening of bacterial pathogens.

Maintenance care: The diet consisted of commercial pellets (Hope Farms) and half an apple a day. This was supplemented by weekly injections of a multivitamin mixture (Duphaphal Multi). The housing consisted of two animal-rooms completely separated from other animals. The monkeys were maintained as a group, without introduction of new ones. Technicians and caretakers always wore boots and a surgical gown with cap and mask when they entered the rooms. The monkeys thrived and gained weight during and after the quarantine period. Animals weighing between 2.5 and 3 kg were used as donor and at the same time as recipient of the autogeneic bone marrow cells.

One week before the start of the experiment, the monkeys were again tested for tuberculosis and a fecal culture was made to determine the sensitivity of the enteric flora to antibiotics.

Preparatory procedures for the operation: The monkeys were fasted for 24 hours before the operation. They were anesthetized with ketamine hydrochloride (Kethalar®, Parke Davis), 2 mg/kg given intramuscularly, and 0.5 mg atropine sulfate was also injected. After intubation, anesthesia was maintained by inhalation of a mixture of fluothane, nitrous oxide, and oxygen.

The skin and the distal ligament of the patella were incised, and a hole was drilled in the articular surface between the condyles of the femur. A plastic catheter with a diameter corresponding to the drill hole was introduced into the femoral cavity and pushed toward the proximal end of the femur. The content of the femoral cavity was then aspirated into a heparinized syringe. On average, 40 ml was usually collected. This material was stored at room temperature for at most 2 hours. Counting of the nucleated bone marrow cells was performed in a Bürker chamber. At the end of the storage period 95% of the cells were viable, as determined by the eosin resistance test.

Irradiation: For irradiation, the monkey in a rotating transparent cylindrical cage (ϕ 20 cm) was placed in a Philips roentgen generator which was operated at 200 kV and 20 mA and was provided with a 1.5 mm Cu-filter. The tube-animal distance was 70 cm. The total body radiation dose was 800 rad. If irradiated monkeys refused to eat, they were given cooked rice, bananas, and apples. When non-specific diarrhea occurred, opium derivatives were injected to suppress intestinal motility.

Transplantation: Transplantation was performed by ip injection of the entire contents of a femur, comprising approximately 10^8 to 10^{10} living, nucleated, autogenic bone marrow cells.

Pathology: The indication for autopsy was heavy bleeding. After being anesthetized with Ketalar, the animal was killed by an intracardiac injection of 600 mg Triotal (Gist-Brocades N.V., Delft, The Netherland). Immediately after death, the monkey was fastened to the autopsy table and the abdomen wiped with 70% alcohol. The abdominal wall was opened and bilaterally fixed. The omentum and mesentery were carefully examined and then cut into several pieces, which were placed in fixative solution for 24 hours.

Several specimens were taken from the liver, spleen, kidney, adrenals, pancreas, lung, thymus, and sternum for histological examination. The tissue specimens were fixed in Bouin's solution or in a buffered neutral formalin solution. Sternal specimens were decalcified after fixation. Paraffin-embedded tissue were cut into 5 μm thick sections. All sections were routinely stained with hematoxylin-azophloxin (H & A) and in some instances with PAS, Turnbull blue, Toluidine blue, von Kossa's calcium, and Masson's trichrome.

Cytology: Imprints of spleen, liver, and bone marrow on glass slides were stained according to the May-Grünwald-Giemsa technique (LUNA 1968).

Histology: Tissue specimens were fixed in Bouin's solution or buffered neutral formalin solution. Sections were cut 5 μm thick from paraffin-embedded material. All sections were routinely stained with hematoxylin-azophloxin (H & A) and in some instances with PAS. Gömöri stain was used for reticular fibers, Turnbull blue for iron pigment, and toluidine blue for metachromasy.

Specimens embedded in Epon (McGEE-RISSEL and de BRUIJN 1964), with and without osmification, were cut 1 μm thick on a LKB Ultratome III (Sweden) with glass knives. The Epon matrix

was removed according to the method of LANE and EUROPA (1965) before staining of the semi-thin sections with H & A and the other stains mentioned above.

Hematoxylin-azophloxin staining technique for tissue embedded in Epon: For this purpose, the method routinely used for H & A staining was modified in our laboratory. In a series of experiments it was found that after removal of the Epon matrix, semi-thin sections of Epon-embedded specimens could be additionally stained with toluidine blue, methylene blue, van Gieson-elastica, PAS, and Jones' kidney stain. The staining methods applied to tissues embedded in paraffin are suitable for those embedded in Epon, with the exception of hematoxylin-azophloxin.

Hematoxylin-azophloxin staining technique for tissue embedded in Epon:

- Remove Epon matrix by immersion in saturated NaOH solution for 1 hour (LANE and EUROPA 1965)
- Immerse in hematoxylin (Mayer) for 25 minutes at room temperature
- Wash in running tap water for 5 minutes
- Short differentiation in 1N HCl in alcohol 70%
- Rinse in tap water
- Dip a few times in 1% NH_4OH in alcohol 70%
- Wash in running tap water for 5 minutes
- Immerse in azophloxin 1/4% for 25 seconds (pH 3.5, adjusted with glacial acetic acid according to de BRUIJN and McGEE-RUSSEL (1966)
- Rinse in tap water to remove excess dye
- Dehydrate in ascending alcohol series from 70% to 100%
- Immerse in xylene, two changes, 2 minutes each
- Mount

Perfusion fixation technique: In this study both immersion and perfusion fixation were performed. Livers were perfused through the portal vein with a fixative solution containing 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.05 M anhydrous $CaCl_2$ (pH 7.4). The osmolarity of this solution was 320 mOsmoles, as measured with an Advanced osmometer (Advanced Instruments Inc., Mass., USA). The rate of perfusion was 5 ml/100 g body weight per minute, the duration 3-5 minutes, the cooled bottle of fixative solution being

placed 75 cm above the operation table. The inferior caval vein was opened for the outflow of blood and fixative solution. Fixation of the spleen and of embryos *in utero* was performed by total body perfusion via the left atrium. Perfused tissues and embryos were then immersed in the same fixative for at most 72 hours at 4°C (de BRUIJN and den BREEJEN 1975). Specimens were stored in buffer solution for periods varying from a minimum of 24 hours to a maximum of 1 year, as described by de BRUIJN and den BREEJEN (1975).

Electron microscopy: The technique used for electron microscopy is described in detail by de BRUIJN (1973), and a brief outline will suffice here.

The tissues to be studied electron microscopically were fixed in a 1.5% glutaraldehyde solution containing 0.1 M sodium cacodylate buffer and 0.05 M anhydrous $CaCl_2$ (pH 7.4, 320 mOsm). Fixation took place either by perfusion or immersion, and in many cases both. Tissue blocks measuring approximately 1 mm^3 were then washed in 0.1 M sodium cacodylate buffer (pH 7.2) without $CaCl_2$ for 2 to 4 hours at room temperature. Preoxydation was carried out with 0.05 M $K_3Fe(CN)_6$ in 0.1 M sodium cacodylate for 2 hours at room temperature. In the following step OsO_4 was used as a second fixative, especially because of its contrast-augmenting property. This solution was composed of 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.05 M $K_3Fe(CN)_6$. The fixation time was 24 hours at 4°C. After this osmification, the blocks were washed twice in distilled water for 1 hour. Dehydration was then carried out with a graded acetone series from 30% to 100% at room temperature, each step lasting 30 minutes, followed by pure acetone for 1 hour. Next, a 1/1 mixture of acetone (100%) and catalyzed Epon was applied at room temperature for 2 hours, and lastly, catalyzed Epon for 1 hour at 37°C. After infiltration and impregnation of Epon, the specimens were placed in gelatin capsules and stored for polymerization at 37°C for 16 hours and then at 60°C for at least 24 hours. Tissues embedded in Epon were cut 1 μm thick for the light-microscopical studies. Ultrathin sections for electron microscopy were cut on a LKB Ultrotome III (Sweden) with glass knives and collected on carbon-coated Formvar films.

For contrast staining reactions, uranyl acetate or lead citrate (MERCER and BIRBECK 1972) were used or, in selected cases, both.

Sections were studied in a Philips EM 300 operated at 40 or 60 kV, with a 20 μm objective aperture. Gevaert Scientia 23 D 50 photographic plates, used throughout this study, were developed in Kodak HRP developer. The whole procedure can be symbolized by the formula $G^{Ca}/Os^{K_3Fe(CN)_6}/Ac/E/(-,UrAc,Pb)$ (de BRUIJN and den BREEJEN 1975).

Origin of our R-MuLV stock: The R-MuLV stock was kindly supplied to us by P. Bentvelzen, Radiobiological Institute TNO, Rijswijk, The Netherlands, and was serially propagated in inbred BALB/c mice in our laboratory. Over a period of five years, the symptoms of the disease caused by this virus have not changed. On the 26th day of infection, animals with huge spleens were killed. The spleens, and in some instances venous blood obtained from the portal vein, were used for virus isolation.

Virus preparations: Three methods were used for the purification of R-MuLV, yielding:

a. A crude virus preparation made from spleens of mice infected with R-MuLV 26 days earlier.
b. A purified virus preparation, extracted from spleens of infected mice by repeated ultracentrifugation and sucrose gradient centrifugation (CHENAILLE et al. 1968).
c. A purified virus preparation, extracted from the plasma of infected mice (O'CONNOR et al. 1964) by repeated ultracentrifugation and sucrose gradient centrifugation.

Virus extraction from spleens of infected mice: The procedure used for R-MuLV extraction can be briefly outlined as follows:

- Four weeks after ip injection of 4 mg R-MuLV, 100 enlarged spleens were collected at 0°C.
- Spleens were homogenized in Dulbecco's PBS in a pre-cooled Waring Blendor for approximately 2 minutes.
- The homogenate was centrifuged at 5,000 rpm for 10 minutes at 4°C, giving a crude viral preparation as supernatant.
- The supernatant was centrifuged for 45 minutes at 30,000 rpm in a Beckman rotor R 30 at 4°C.
- The pellet, which contained a large amount of virus, was dissolved in PBS, after which this suspension was centrifuged for 5 minutes at 10,000 rpm.

- The suspension was then carefully layered onto a discontinuous sucrose gradient (20% / 50% w/v sucrose in PBS).
- Centrifugation was carried out for 150 minutes at 27,000 rpm at 4°C in a Beckman rotor Sw 27, after which the interphase band was collected. With this procedure, ribosomes and membraneous remnants were spun to the bottom of the tubes. Semi-purified and highly concentrated virus was found in the interphase band between the two columns of sucrose solutions in different concentrations. The resulting virus solution was diluted to an extinction $E^{1\ cm}_{280\ nm}$ of 40.0. The stock of extracted R-MuLV, to which gentamycine sulphate, 5 µg/10 ml, (Garamycin®, Schering) and streptomycine sulphate, 5 µg/10 ml, (Streptomycine, Specia) were added, was then divided into 10 ml aliquotes and frozen at −70°C until use.

Virus extraction from plasma of infected mice: Immediately after ether asphyxiation, the venous blood of mice infected 26 days before with 4 mg R-MuLV, was collected from the portal vein at 4°C. Serum was separated from the blood clot by initial centrifugation at 5,000 rpm for 10 minutes at 4°C, after which the method described above for the isolation of R-MuLV from spleens by repeated ultracentrifugation and sucrose gradient centrifugation was applied. The presence of R-MuLV particles was confirmed electronmicroscopically under negative staining with phosphotungstic acid (MERCER and BIRBECK 1972).

The infectivity of the extracted R-MuLV was tested in 32 BALB/c mice divided into four equal groups. Fractionated doses of virus, i.e., 4 mg (10^1), 10^{-1}, 10^{-2}, 10^{-3}, were injected ip. Twenty-six days after this injection, the size and weight of the spleen were determined as one of the parameters of the infection (van GRIENSVEN et al. 1973). The spleens were also studied histologically.

Immunofluorescence studies: The presence of active R-MuLV antigens in the mouse embryos infected *in utero* was demonstrated by the fluorescent antibody technique with specific rabbit antisera to R-MuLV.

Preparation of antisera with R-MuLV for the immunofluorescence studies: Rabbit antisera to R-MuLV were produced by 8 weekly injections of purified R-MuLV isolated from virus-infected

JLS-V9 cells (CHOPRA and SHIBLEY 1967; HODGE et al. 1974; van GRIENSVEN et al. 1974). After absorption *in vitro* with lyophilized BALB/c mouse spleen and liver tissue, the antisera gave positive precipitation reactions in double-diffusion assays with purified R-MuLV and negative reactions with fetal calf serum and BALB/c mouse serum. These antisera proved to be effective up to and including the 1/640 dilution for specific indirect immunofluorescence. Non-infected BALB/c mouse tissues gave no fluorescence with rabbit antisera to R-MuLV.

Fluorescence antibody technique: Fluorescein-labelled goat antisera to rabbit Ig (lot 7-274, Nordic Diagnostic, Tilburg, The Netherlands) were used in a 1/50 dilution. Uteri of pregnant mice were removed and immediately frozen in liquid nitrogen. Serial sections (5 μm thick) were dried and fixed in acetone. After washing with PBS, sections were covered with a 1/5 dilution of rabbit antisera to R-MuLV and incubated for 30 minutes in a humidified chamber, washed with three changes of PBS, covered with anti-sera conjugate, incubated for 30 minutes, and washed again with three changes of PBS. Finally, the sections were mounted in glycerol-PBS and examined in a Leitz Orthoplan fluorescence microscope with an oil immersion objective, x 54.

XC test: The XC cell line, which derived from a Rous sarcoma virus-infected rat tumor, was used in assays to demonstrate the presence of murine leukemia virus genomes in the co-cultures. According to the method of KLEMENT et al. (1969), 50,000 XC cells were seeded in quadruplicate on petri dishes (Ø 3 cm) in Eagle's minimum essential medium (MEM) suplemented with 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (Flow Laboratories, Irvine, Scotland). One day later, 50,000 trypsinized embryo cells were added. Cells were grown at 37°C in a 100% humidified atmosphere with 5% CO_2. Four days later, the contents of the petri dishes were washed twice with PBS, fixed in methanol, and stained with 4% buffered Giemsa stain (Merck Chemical Division, Darmstadt, West Germany). The formation of polykaryons indicates the presence of murine leukemia virus genomes.

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CHAPTER 4

MORPHOLOGY OF R-MuLV INFECTION IN MICE

> In this era of molecular biology, the hematoxylin and eosin minded pathologist dealing with an archeologic matter such as fixed and colored tumor specimens, could himself appear as a fixed human specimen deserving nothing but a sympathetic glance from the audience.
> *L. Fiore-Donati*, 1973, Bibl. haemat. No: 39.

Introduction: After the isolation of a new laboratory strain of murine leukemia virus (R-MuLV) by RAUSCHER (1962), the nature and pathogenesis of R-MuLV-induced infection were studied intensively (ZEIGEL and RAUSCHER 1964; BRODSKY et al. 1967; OKUNEWICK and PHILIPS 1973). It is generally accepted that in mice R-MuLV causes a neoplastic disease which has been characterized as erythroleukemia (GRUNDMANN and ZEIGEL 1968) and is always fatal (RAUSCHER 1962; SIEGLER and RICH 1966; TÓTH et al. 1973; LOZZIO et al. 1974). GRUNDMANN and ZEIGEL (1968) noticed "blasts" in the liver, spleen, and bone marrow as early as 3 days after ip injection of R-MuLV, and pointed out that R-MuLV infection of mice could be related to Di Guglielmo's erythroleukemia in man.

Although BRODSKI et al. (1968) studied the effect of R-MuLV on thrombopoiesis starting 48 hours after injection, and others, including RAUSCHER (1962), ZEIGEL and RAUSCHER (1964), BRODSKY et al. (1967), and SEIDEL (1968) investigated tissue

changes as early as the third day after infection (GRUNDMANN and SEIDEL 1968), the periods characterizing R-MuLV infection were not defined on the basis of morphological criteria.

A few investigators, however, came to somewhat divergent conclusions on the nature and pathogenesis, which they saw as hyperplastic or auto-immune reactions rather than neoplastic disease (SIEGEL and MORTON 1970; BENTVELZEN 1972; COX and KEAST 1973; PROFFITT et al. 1973). BRODSKY and KAHN (1969) concluded that altered erythropoiesis was not a neoplastic manifestation but was caused by increased osmotic fragility, autohemolyis, and a direct effect of the R-MuLV on the red blood cell membrane.

The present investigation was undertaken to aquire more information about morphological changes during an R-MuLV-induced infection of BALB/c mice. The questions raised were: What morphological changes occur in the early involvement of organs and cells? Is it possible to determine the periods of the infection on the basis of the morphological changes in organs and cells? What is the nature of the R-MuLV-induced infection in mice?

Experimental design: The first experiment was designed to determine by light and electron microscopy the morphological changes occurring in the liver, spleen, bone marrow, blood, mesentery, and lymph nodes within 3 days of R-MuLV infection. Twenty-eight BALB/c mice divided into 7 groups of animals each were injected intraperitoneally (ip) with 4 mg of the virus. Samples were taken 2, 4, 8, 16, 48, and 72 hours after virus injection. Laparotomy was carried out under Nembutal anesthesia (0.15 ml/100 g body weight). For liver perfusion, the portal vein was cannulated with a 16-gauge needle, after which the loose ligature around the portal vessels was tightened and approximately 3 ml fixative solution introduced. From the simultaneously cut inferior caval vein, 0.2 ml venous blood was aspirated and immediately mixed with a cooled 1.5% glutaraldehyde. Four pieces of perfused liver were brought into a similar solution. Blood and liver samples were prepared for electron microscopy. At the same time, pieces of the spleen, sternum, mesentery, and the mesenteric lymph node were fixed in neutral formalin and prepared for light microscopy. The controls were four mice injected ip with 0.2 ml Hanks' balanced salt solution (HBSS).

The second experiment was designed to determine the numeri-

cal alterations in the components of the peripheral blood during the first 3 days after R-MuLV infection. Twenty-four mice were injected ip with 0.1 ml crude virus preparation, 24 mice with 2 mg R-MuLV isolated from spleen tissue, and 24 mice with 2 mg R-MuLV isolated from plasma. The control group consisted of 24 mice, 12 of which received 0.1 ml 10% sterile albumin ip and the others 0.1 ml of a 1% latex suspension (particles 1.1 μm in diameter; Polysciences, Inc., Warrington, U.K.). Samples were taken, as in the first experiment, 2, 4, 8, 16, 24, 48, and 72 hours after infection. Erythrocytes, leucocytes, thrombocytes, and reticulocytes were counted, and hemoglobin and hematocrit values were determined.

The third experiment was designed to determine the organic and cellular changes in relation to the virus dose at the light-microscopical level. Thirty mice were injected ip with 4 mg R-MuLV, 30 with 0.4 mg R-MuLV, and 30 with 0.04 mg R-MuLV isolated from spleen tissues, and 7, 12, 19, and 26 days after injection of virus 6 mice from each group were killed by cervical dislocation. The survival time of the 6 remaining mice in each group was recorded. Eight mice given 0.2 ml HBSS ip served as the control group. Specimens of skin, peritoneum, abdominal muscle, liver, spleen, gall-bladder, pancreas, mesentery, kidney, adrenal gland, esophagus, stomach, ileum with Peyer's patch, appendix, descending colon, uterus, fallopian tube, ovary, bladder, parotid gland, submandibular gland, trachea, lung, thyroid gland, heart, thymus, sternum, and mesentric and renal lymph nodes were collected, fixed in 3% glutaraldehyde, and embedded in Epon. Postfixation in osmium was omitted. Sections cut 1 μm thick were stained with H & A after removal of the Epon matrix according to LANE and EUROPA (1965). In addition, brains *in toto*, a lumbar segment of spinal cord, eyes, and the petrous part of temporal bone and femurs were fixed in neutral formalin, and 5 μm thick H & A sections prepared. Before fixation of the femurs for histological investigation, imprints of bone marrow were made on glass slides and stained according to May-Grünwald-Giemsa.

Results: The first histological changes were observed light-microscopically in the liver as early as 8 hours after an ip injection of virus. Some of the lining cells of the liver sinusoids were found to be prominent (Fig. 1). At each successive interval these cells gradually became more swollen and proliferated (Figs. 2, 3, 4). On the third day, disseminated obliteration of many sinusoids was produced

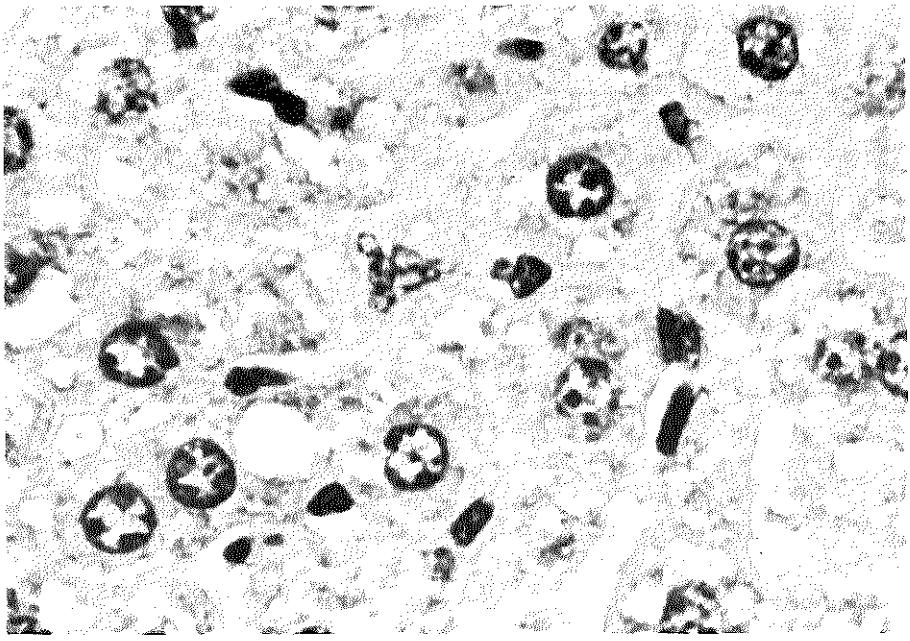


Figure 4-1. Liver tissue of a BALB/c mouse 8 hours after ip injection of 4 mg R-MuLV. Note several prominent sinus lining cells with little cytoplasm. The oval nuclei show a coarse granular and compact structure. Hepatocytes are normal and possess round vesicular nuclei. H & A. x 600.

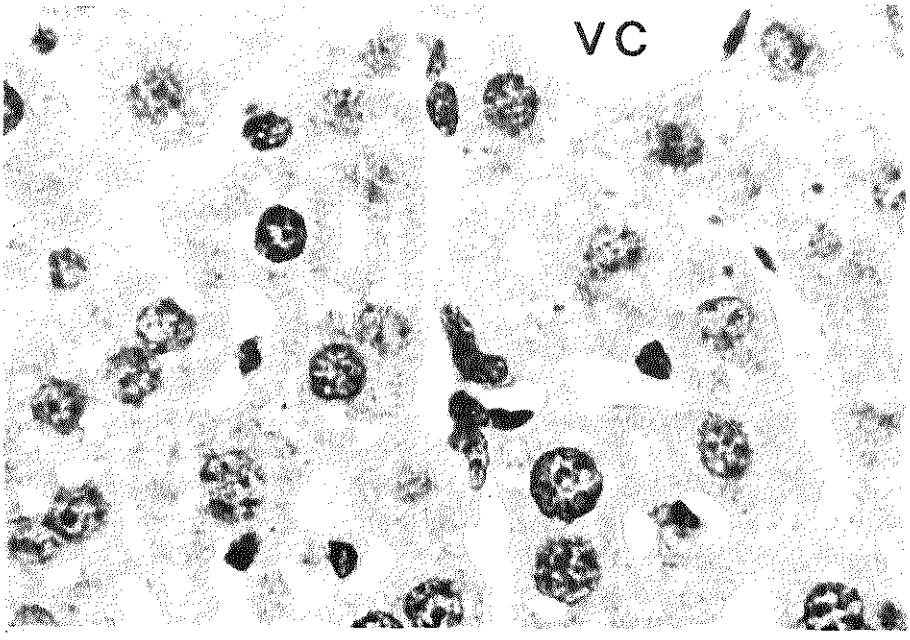


Figure 4-2. Liver tissue of a BALB/c mouse 16 hours after ip injection of 4 mg R-MuLV. The cells lining the liver sinusoids are now slightly swollen, and the chromatin pattern of the nuclei is granular but less compact. The sinusoids are open. VC: vena centralis. H & A. x 600.

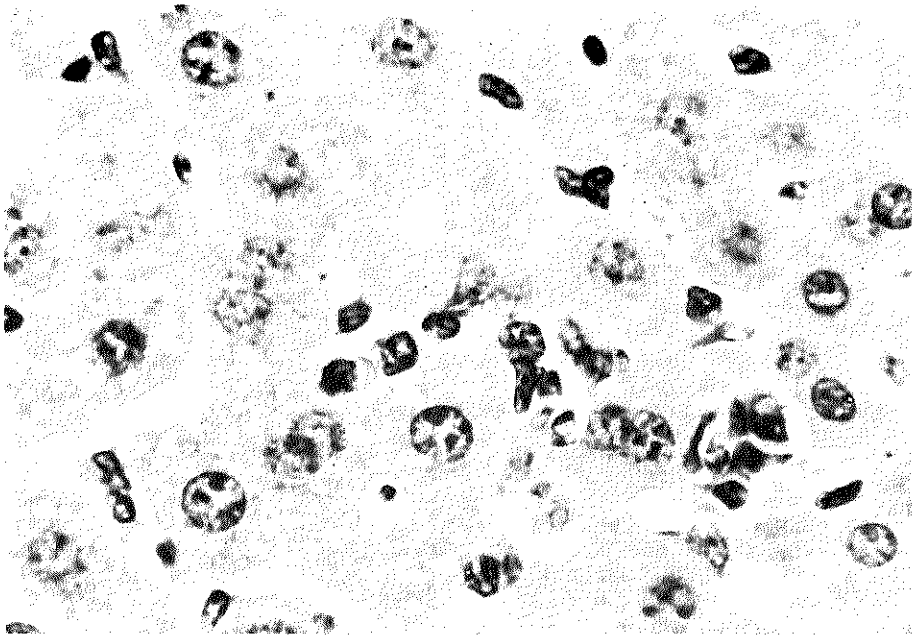


Figure 4-3. Liver tissue of a BALB/c mouse 48 hours after ip injection of 4 mg R-MuLV. Note small intrasinusoidal aggregations of swollen sinus lining cells. H & A. x 600.

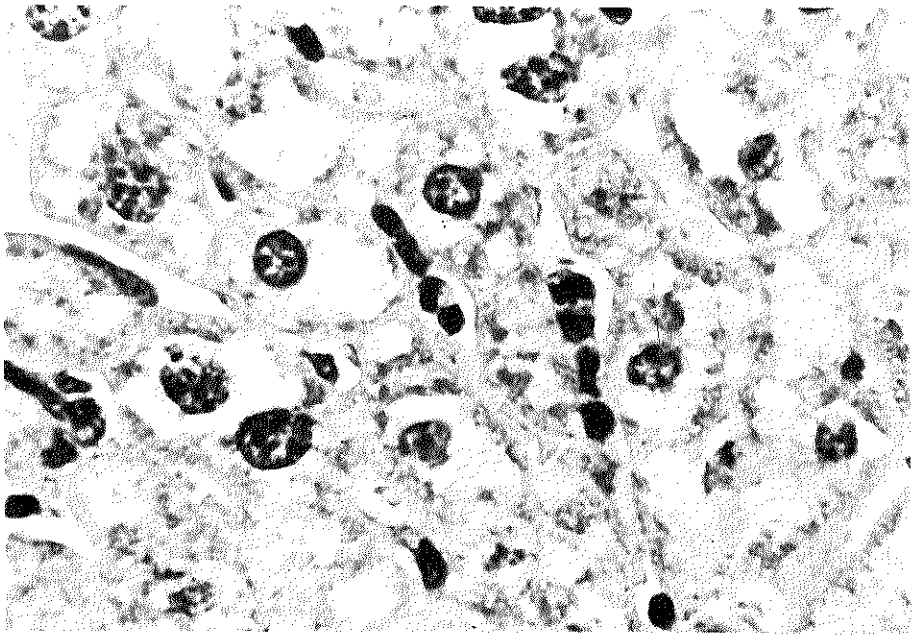


Figure 4-4. Liver tissue of a BALB/c mouse 48 hours after ip injection of 4 mg R-MuLV. Note mitotic proliferation of sinus lining cells. H & A. x 600.

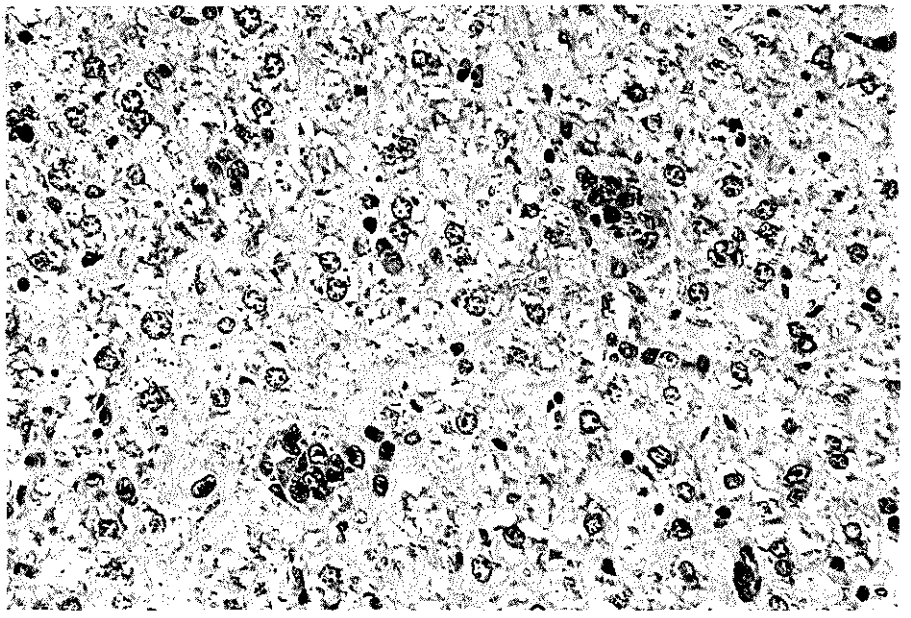


Figure 4-5. Live tissue of a BALB/c mouse 72 hours after ip injection of 4 mg R-MuLV. Note multicentric intrasinusoidal aggregations (arrow) of distictly swollen round to oval cells with vesicular nuclei. The chromatin pattern is granular and even nucleoli are visible. H & A. x 380.

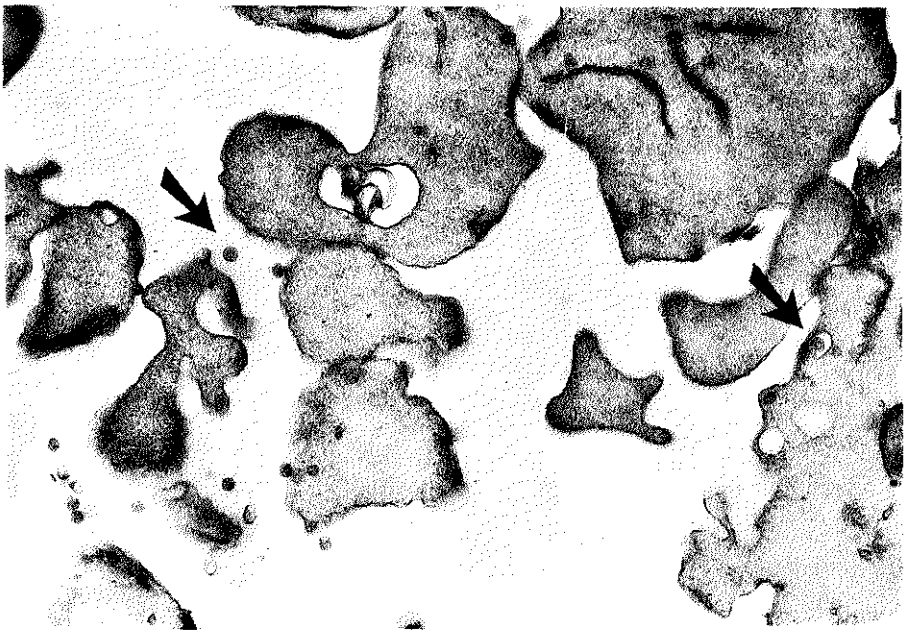


Figure 4-6. Thrombocytes in the liver sinusoids 8 hours after ip injection of R-MuLV. Mature C-type virus particles (arrows) are seen within or adjacent to the thrombocytes. Uranyl acetate. x 17,680.

by these dictinct, acidophilic, enlarged cells. The multicentric progression of this process gave the liver tissue a patchy appearance (Fig. 5). In these intrasinusoidal foci, deeply acidophilically stained shrunken necrotic cells were found for the first time on the third day of the R-MuLV infection. The first cellular alterations in the spleen and bone marrow were also observed on the third day, including increased cellularity, congestion of the sinusoids, and small aggregations of proliferating immature hemopoietic cells in the red pulp. The mesentery and mesenteric lymph nodes showed no light-microscopically visible structural changes during the first three days of the R-MuLV infection.

Eight hours after an ip virus injection, electron-microscopical analysis of the liver tissue showed C-type virus particles within or adjacent to the thrombocytes in the sinusoids (Figs. 6 and 7) and in the vacuoles of the swollen lining cells. From then on until the 72nd hour of the infection, we were unable to find any virus in these cells. From the third day on, budding virus particles were found in the cells that formed the intrasinusoidal foci (Fig. 8). At this time, too, free mature C-type virions reappeared in the intracellular space of these cellular foci in the liver sinusoids.

Electron-microscopical studies of the blood samples collected after the same intervals gave similar results. At 8 hours we found C-type virions in the thrombocytes and free viral particles in the plasma. Between 8 and 72 hours, no virions were seen. At 72 hours, the re-appearance of C-type virions in the plasma was observed.

Tissues from mice injected ip with HBSS showed normal pictures for the liver, spleen, bone marrow, peripheral blood, mesentery, and mesenteric lymph nodes in both light and electron microscopy. We found no endogenous C-type virus particles in our control material.

In the second experiment the only pathognomonic numerical alteration found in the blood samples was a change in the number of thrombocytes during the initial stage of the disease, particularly a sharp decrease starting 8 hours after an ip virus injection, regardless of the source of isolated R-MuLV. The thrombocytopenia persisted at more or less the same level during all of the ensueing stages of the disease, viz. $650 \pm 200 \times 10^3/mm^3$ as against $1.187 \pm 98 \times 10^3/mm^3$ for control BALB/c mice. During this stage the hemoglobin and hematocrit values did not show any significant alteration. The

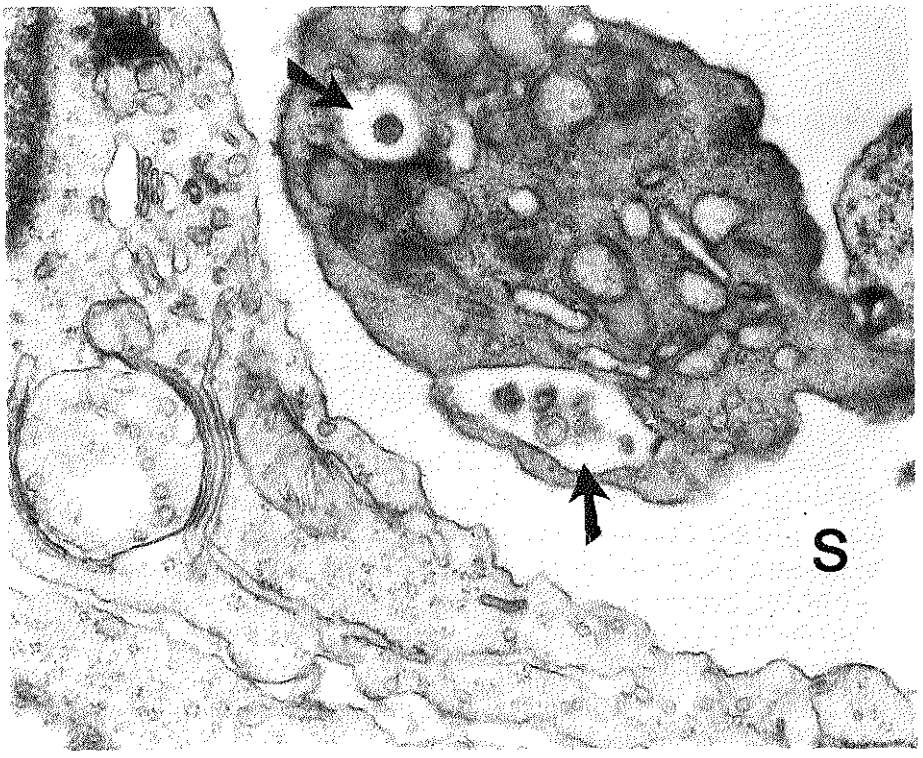


Figure 4-7. A thrombocyte in a liver sinusoid (S) carries mature C-type virus particles. Specimen from the liver of a BALB/c mouse, 8 hours after ip injection of 4 mg R-MuLV. Uranyl acetate. x 35,200.

control group showed no numerical alteration in the blood components after ip injection of albumin or the latex suspension.

In the third experiment the intervals chosen for the study of R-MuLV infection were 7, 12, 19, and 26 days after ip injection of the virus. The macroscopical and microscopical findings for each of the organs studied will be described below in relation to these intervals.

On the 7th day the spleens of all infected mice were at least twice as large as those of the control group. This enlargement was due to hyperplasia of hemopoietic cells, particularly under the capsule and along the septa, and also to heavy congestion. The liver showed intrasinusoidal foci consisting mostly of undifferentiated cells that had increased in number and size. In both the liver and the spleen we observed several shrunken necrotic cells in the proliferated cellular foci, and in many instances the remnants of the pyknotic nuclear mass of these cells differed in shape and size. The bone mar-

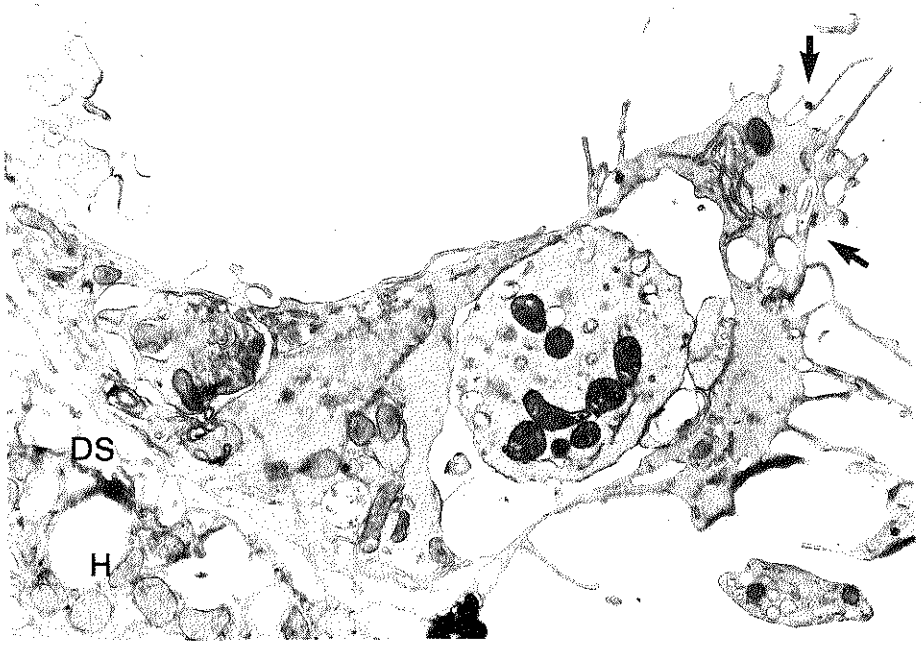


Figure 4-8. Liver tissue of a BALB/c mouse 72 hours after ip injection of 4 mg R-MuLV. H: hepatocyte, DS: Disse's space. The centrally situated, swollen triangular cell with phagocytic vacuoles is highly suggestive of a Kupffer cell. Arrows indicate budding virus particles on the cell surface. Uranyl acetate. x 11,800.

row, thymus, and all of the other organs studied, showed no light-microscopical changes at this stage. Ten-fold and hundred-fold dilution of the ip injected virus (0.4 mg and 0.04 mg R-MuLV) had no apparent effect on the development of histological changes in organs.

On the 12th and 19th days of R-MuLV infection the intensity of the above-mentioned changes had increased. We observed large, hyperplastic cellular aggregations in the liver sinusoids (Fig. 9) and in the red pulp of the spleens. Hyperplasia of proerythroblasts was a dominant feature, in association with an increase in the number of megakaryocytes. Hemopoietic cells, i.e., proerythroblasts, continued to show marked proliferation, which resulted in rapid enlargement of the spleen. On the 19th day the spleens were at least 10 times larger than normal and showed hemorrhagic and necrotic areas leading in some cases to acute death of the animal from intraperitoneal bleeding. The surfaces of the spleen and liver were finely nodular and the color variegated. On the 12th and 19th days the bone marrow too showed numerous similar aggregations of proliferating hyperplastic proerythroblasts. The capillary network of the bone marrow and the

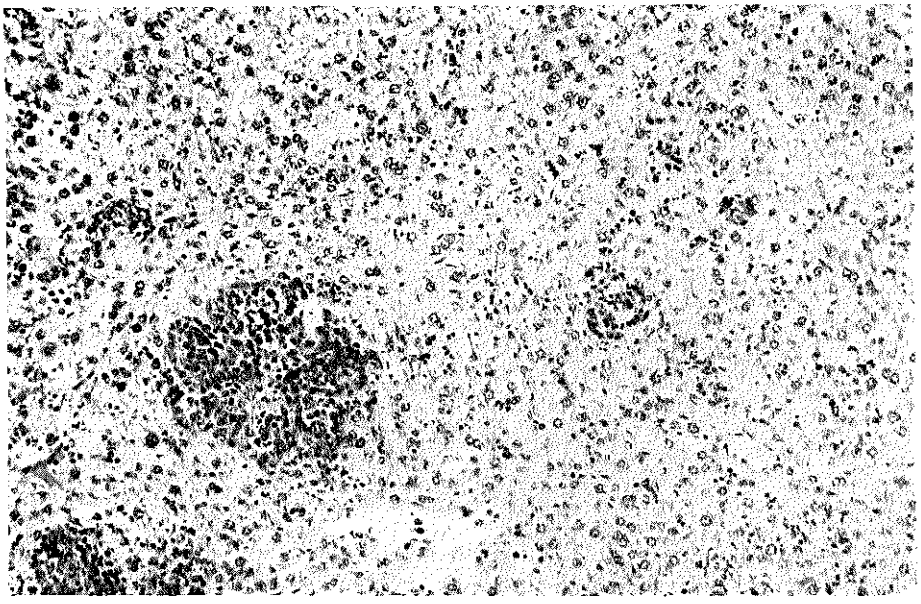


Figure 4-9. Liver tissue of a BALB/c mouse 12 days after ip injection of 4 mg R-MuLV. Hyperplastic cellular foci differing in diameter. H & A. x 40.

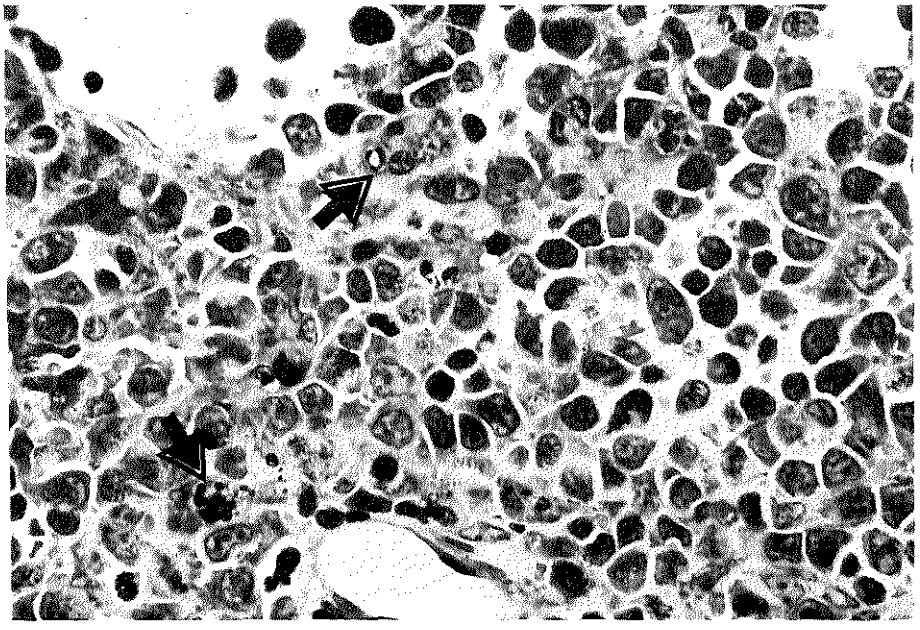


Figure 4-10. Spleen tissue of a BALB/c mouse on the 19th day of R-MuLV infection. Several immature hemopoietic cells are present. The sinusoids are dilated, and the wall of one (upper left) has ruptured. Some blasts are seen in the lumen. Note apoptotic bodies in the macrophages (arrows). H & A. x 440.

huge spleens was not adequate for the rapid structural changes due to the hyperplasia seen on the 19th day of infection, and thin-walled sinusoids ruptured. This permitted the escape of cells, mostly blasts, into the circulating blood (Fig. 10). The thymus and lymph nodes showed hyperplastic lymphoid follicles with enlarged and activated germinal centers. The lymphoid apparatus of the gastrointestinal tract was also hypertrophic. The spleen, liver, and bone marrow showed shrunken necrotic cells like those seen after the preceding intervals i.e., 3 and 7 days after viral infection. Small, acidophilic bodies were disseminated between the cells of hyperplastic hemopoietic foci. Phagocytic clearance of these necrotic cells and acidophilic bodies by macrophages and neighboring hyperplastic cells was regularly observed.

Ten-fold dilution of ip injected R-MuLV did not influence either the occurrence of histological and structural changes or the intensity of the process. Hundred-fold dilution of the virus preparation affected the intensity of the proliferative process.

The other organs under study showed no alteration except capillary congestion, and all specimens from normal control mice did not show any cytologic and histologic changes comparable to those described above.

On the 26th day of R-MuLV infection the enlargement of the spleen had increased to 30 to 50 times (Fig. 11) and the organ was spongy and very fragile. At this time, lymphoid follicles could not be distinguished in the white pulp of the spleen. Necrotic and hemorrhagic areas were frequently present. The histological structure of the spleen tissue was less pleomorphic in this stage. The main cellular component was immature hemopoietic cells. Large, often polygonal cells showed a relatively small amount of basophilic cytoplasm and large vesicular nuclei with prominent nucleoli. Many mitotic figures were present.

The spleen and liver imprints showed many more mitotic figures than were seen in the histological preparations. Cytologically, these slightly polymorphic cells were identified as proerythroblasts. They were, however, larger than their normal counterpart in the control mice.

Intermediate and late normoblasts were also found, but in smaller numbers. Progressive hemoglobinization of the cytoplasm and a distinct nuclear-cytoplasmic asynchrony was observed (Fig. 12a).

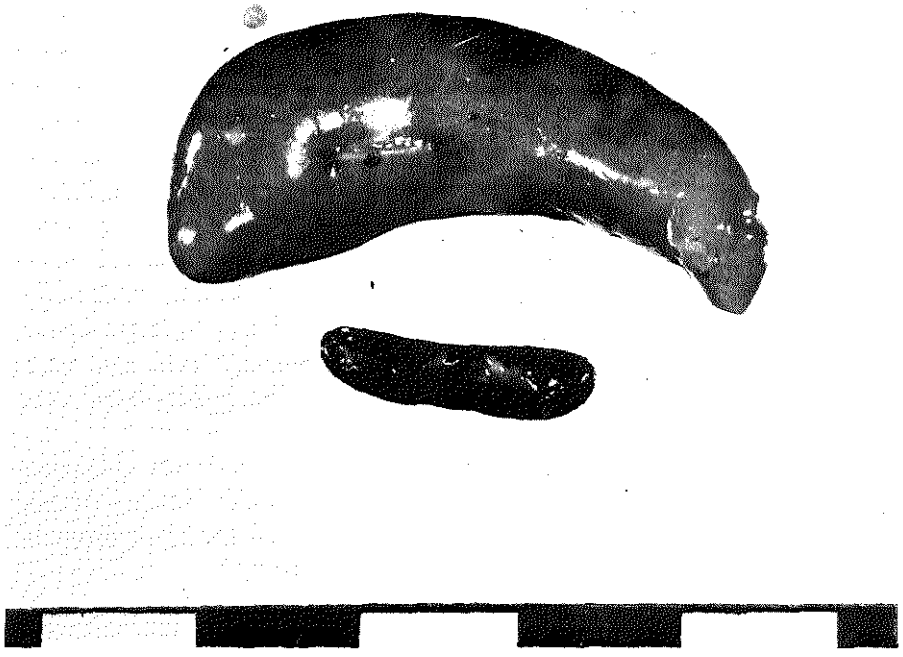


Figure 4-11. Spleen of a BALB/c mouse on the 25th day of R-MuLV infection. The nodular surface is multicolored. Compare this huge spleen with that of normal mouse. (Guage indicates 1 cm). x 2.

The cytoplasm of proerythroblasts and normoblasts was usually stained with PAS. This diffuse PAS positivity was never observed in normal erythroid cells of control mice. The reticulin network of the spleen had almost completely disappeared. New reticulin formation was never observed. The macrophages were scattered and often contained phagocytic particles such as membrane-bound nuclear remnants, which were called apoptotic bodies by KERR et al. (1972). We also observed many shrunken necrotic cells with a characteristic nuclear and cytoplasmic configuration. These cells were identified as apoptotic cells (Fig. 13). This mode of cell death, known as apoptosis, will be discussed in Chapter 5.

The liver was also strongly and increasingly involved in the process on the 26th day. The liver parenchyme was severely atrophied and almost all sinusoids were obliterated by excessively proliferated cells which were morphologically similar to those described above (Fig. 12a and b). The reticulin network of the liver parenchyme was

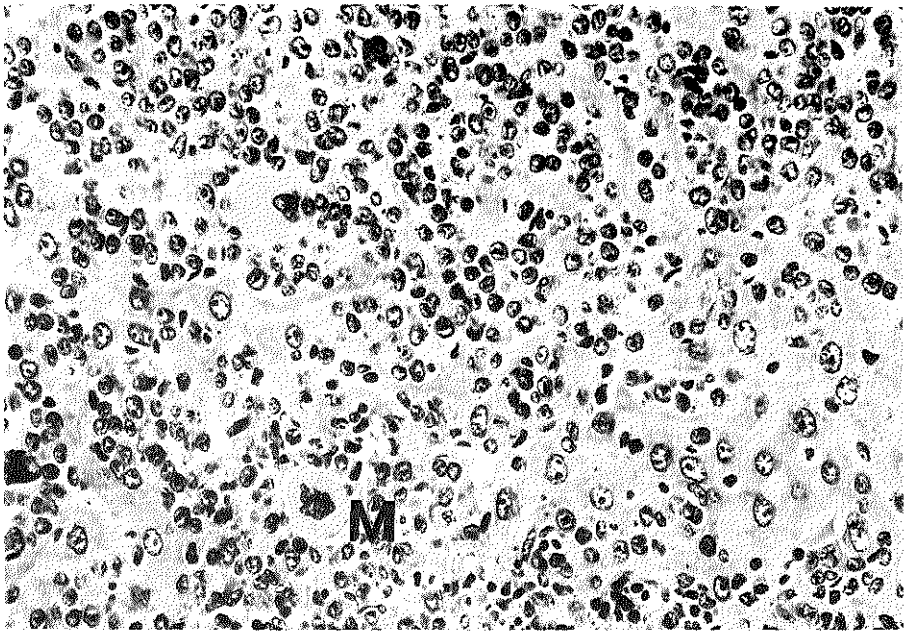


Figure 4-12a. Liver tissue of a BALB/c mouse on the 26th day of R-MuLV infection. The initially multicentric intrasinusoidal foci are now confluent. Hemopoietic cells, mostly immature, are present throughout the parenchyme. Cellular polymorphism and limited maturation are evident. M-megakaryocyte. H & A. x 380.

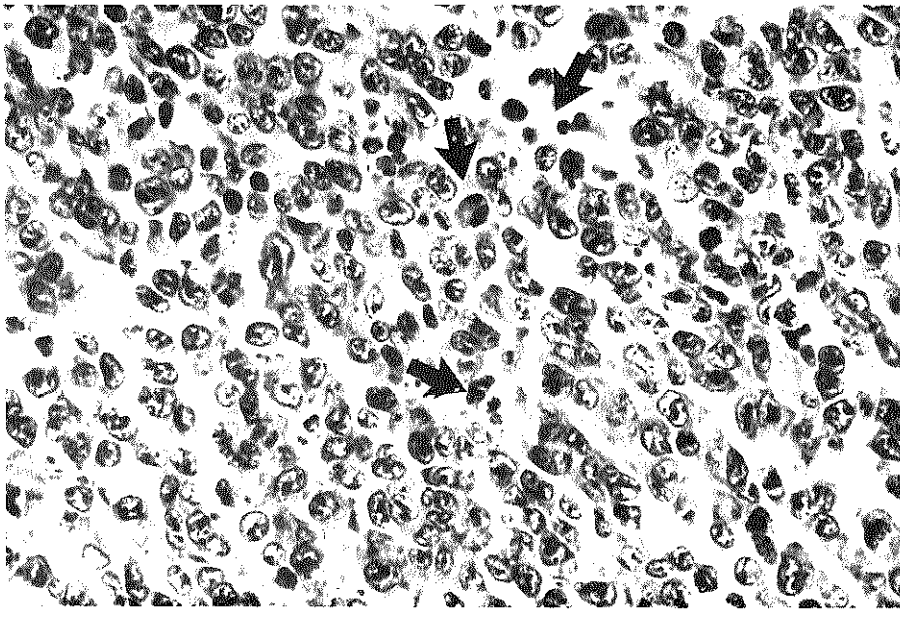


Figure 4-12b. Spleen tissue of the same mouse as in Fig. 12a. Predominantly immature hemopoietic cells, the Rauscher cells, are the main component. There are also several apoptotic cells and bodies (arrows). H & A. x 420.

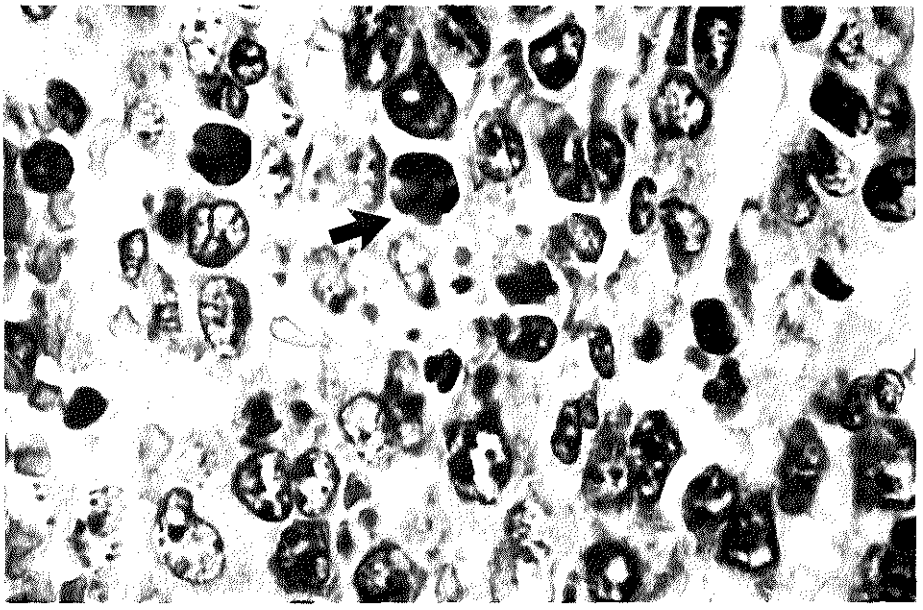


Figure 4-13. Detail of the same spleen tissue as in Fig. 12b. Shrunken and deeply stained cells contain nuclear fragments differing in shape and size. Apoptotic bodies and small cytoplasmic and nuclear remnants are disseminated between cells. Arrows indicate apoptotic bodies. H & A. x 800.

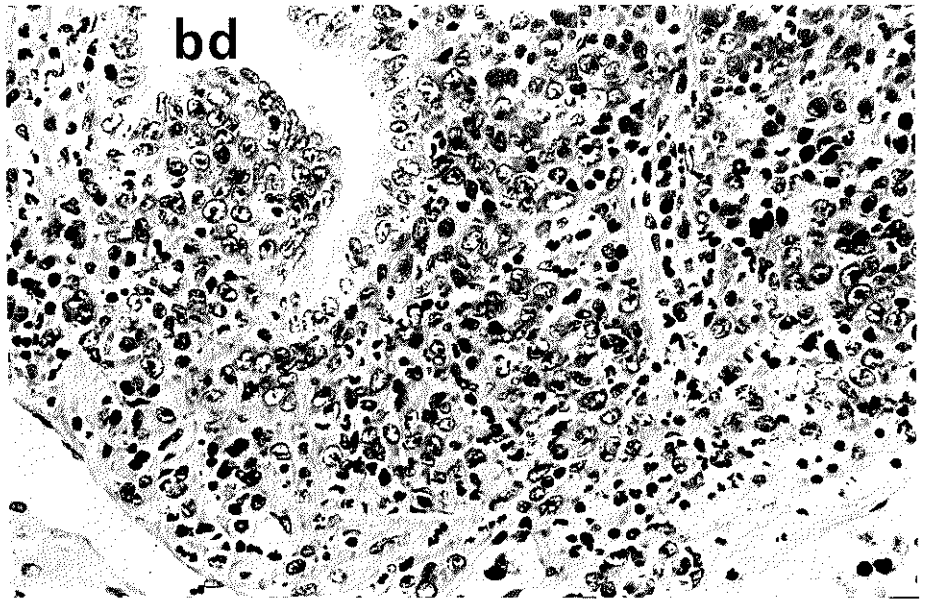


Figure 4-14. A bile duct (bd) and portal area on the 26th day of R-MuLV infection. The wall of the bile duct is partially infiltrated by Rauscher cells. H & A. x 380.

collapsed in the atrophied areas, and new formation of reticulin network by proliferating cells in the sinusoids was not observed. The wall of many sinusoids was ruptured and infiltration of the parenchyme was widespread. The portal tracts in the liver often showed expansive growth of the newly formed cells. Invasion of the walls of bile ducts (Fig. 14) and blood vessels (Fig. 15a and b) was almost a rule. Numerous apoptotic cells and apoptotic bodies were also found in the liver in exponentially increasing numbers from the third day on throughout the infection. In both the liver and the spleen the megakaryocytes were found intermingled with the newly formed tissue.

The liver imprints showed large, basophilic proerythroblasts and normoblasts with limited maturation. These cells often showed many mitotic figures. Cytoplasmic protrusions, comparable to the budding and blebbing described in peripheral lymphocytes of R-MuLV infected mice (HUNT and SIEGEL 1967), were present in the proerythroblasts.

The large, polygonal, basophilic cells morphologically resembling proerythroblasts, were identified as Rauscher cells in this stage of disease. They showed, however, somewhat different characteristics than normal proerythroblasts, as already mentioned. In the bone marrow we found groups of Rauscher cells adjacent to normal and activated hemopoietic tissue. Rauscher cells also occurred rather often in the dilated capillaries of the bone marrow, in the hepatic and mesenteric blood vessels, and in the capillaries of several of the other tissues studied. The imprints of bone marrow showed Rauscher cells with blebs and buds and many mitotic figures. These features were not observed in the imprints deriving from control mice.

The cortical sinus of many lymph nodes, especially the mesenteric and retroperitoneal nodes, were partially obliterated by Rauscher cells (Fig. 16a and b). Some cortical areas were even infiltrated by them. Similar perivascular infiltrations were also observed in the kidney, thymus, skeletal muscles, lung (Fig. 17a and b), and mesentery. The largest aggregates of infiltrating cells were found in the mesenteries. Similar infiltration was not observed in the specimens from control mice.

The animals given 4 mg virus ip died approximately 30 days after the infection, whereas those which received 0.4 mg virus survived for almost 40 days. The mean survival period of the mice

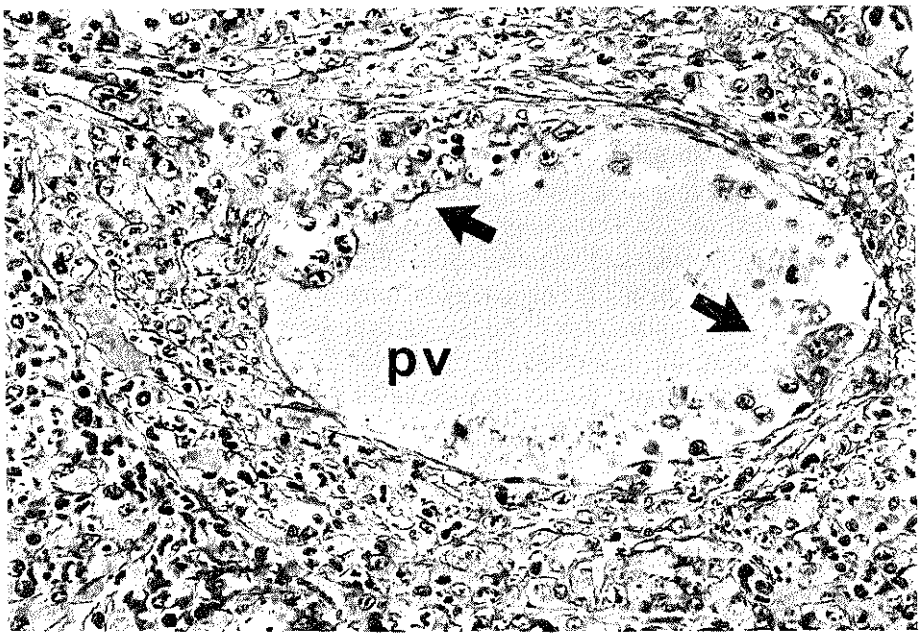


Figure 4-15a. Infiltration of the portal area and invasion of the wall of a dilated branch of portal vein (pv) by Rauscher cells. Note the bourgeonments (arrows) in the lumen. H & A. x 380.

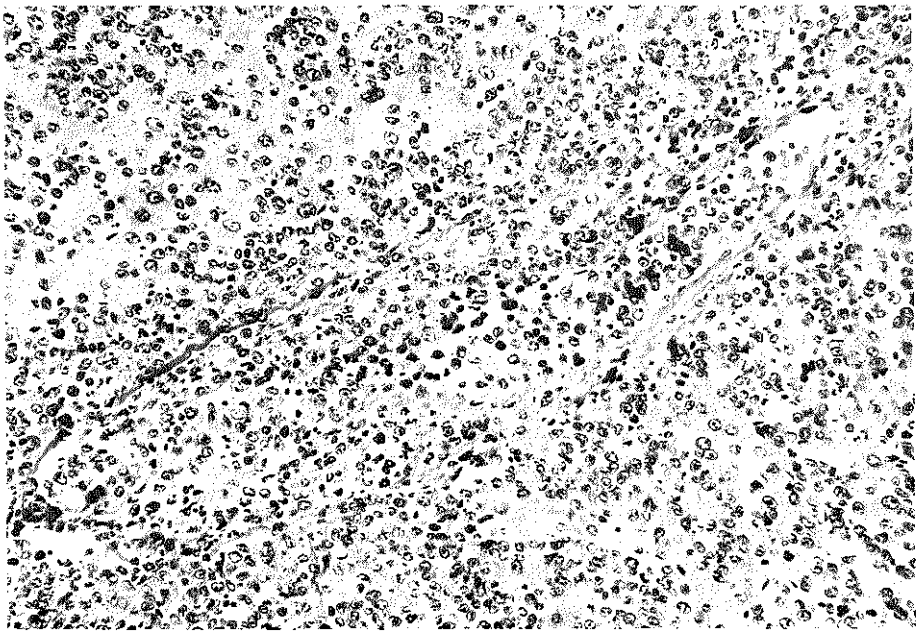


Figure 4-15b. Another section of the same liver tissue as in Fig. 15a. Note dilated and invaded central vein. The lumen is obliterated by a mass of neoplastic cells. The liver is also diffusely infiltrated. H & A. x 280.

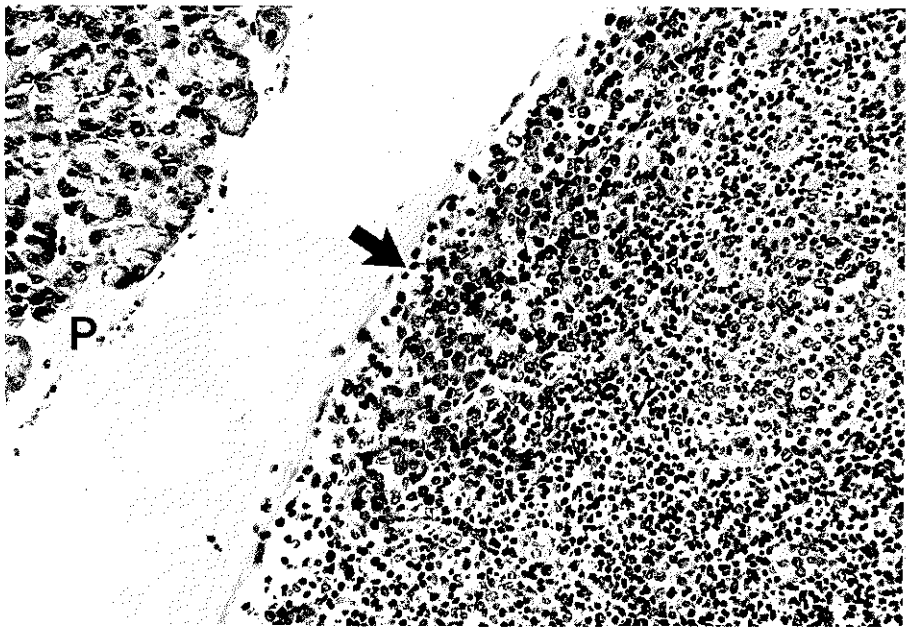


Figure 4-16a. Mesenteric lymph node of BALB/c mouse on the 26th day of R-MuLV infection. The cortex is infiltrated by neoplastic tissue, which is also present in the cortical sinus (arrow). P: pancreas. H & A. x 300.

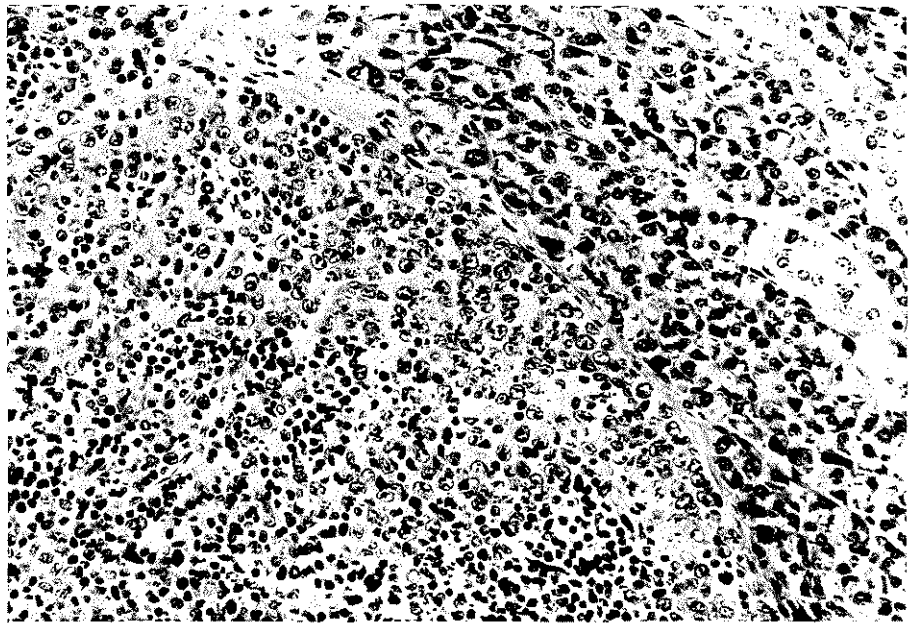
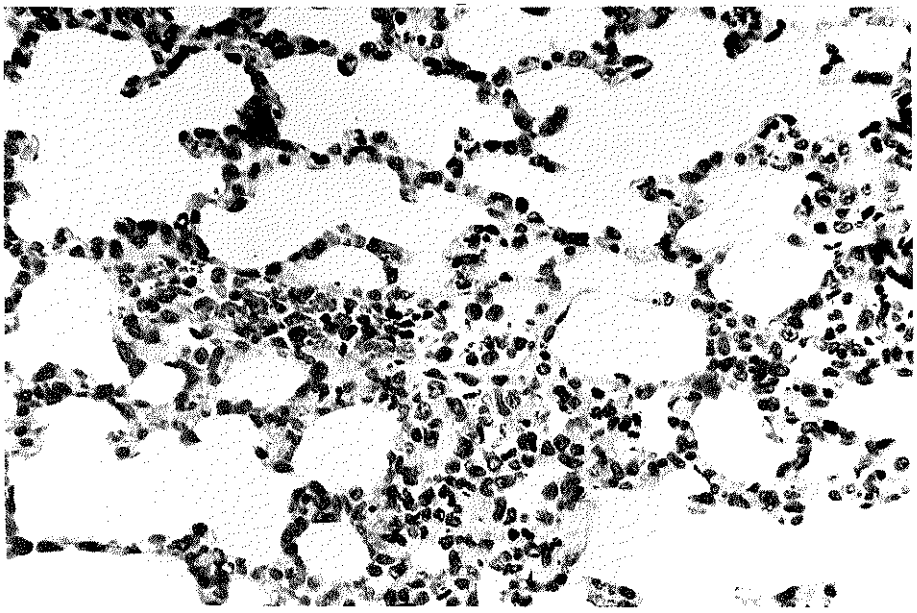


Figure 4-16b. A submandibular lymph node of the same animal. Infiltrating neoplastic cells are seen in the cortical sinus and cortex. H & A. x 320.



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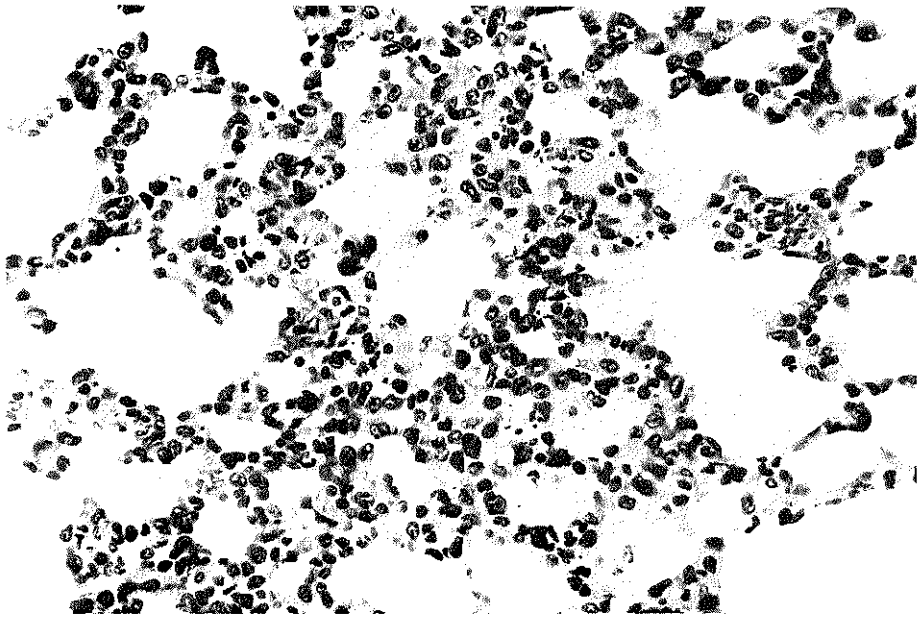

b

Figure 4-17a and b. Lung tissue of a BALB/c mouse on the 26th day of R-MuLV infection. Note the septa widened by neoplastic infiltration. The dilated capillaries contain many Rauscher cells. H & A. x 360.

given an ip injection of 0.04 mg virus was almost 60 days, but half of the mice in this group were dead on the 43rd day of the infection. The diminishing doses of ip injected R-MuLV played no role in the development of cellular changes, but the severity and course of the disease were found to be dose-dependent.

No solid tumor formation or infiltrations were seen in the other investigated tissues. No glomerular lesions were observed either, even in the kidneys infiltrated by Rauscher cells. Except for the meningeal capillary congestion and medullary edema, the brains were normal. The sections of vertebral lumbar bone and of the petrous part of temporal bone showed the same histological features as the bone marrow of the femur and sternum.

Discussion: The intraperitoneal injection of 4 mg R-MuLV into BALB/c mice causes an infection which runs a fatal course in approximately 30 days. This infection is an oncornavirus-induced neoplastic disease and is characterized by erythroleukemia (SIEGLER and RICH 1966) due to involvement of the bone marrow, spleen, and liver.

The abnormal cytological patterns, the expansive and infiltrative growth, and the invasion of the walls of blood vessels and bile ducts (Figs. 14 and 15) suggest, on histological grounds, that this viral infection causes a neoplastic disease in mice.

RAUSCHER (1962) noted in his original article that he could transplant modified cells from R-MuLV-infected mice with ease. The transplantable cell lines obtained from mice infected with R-MuLV have been established and used to determine the effects of chemotherapeutic agents on the malignant evolution (CHIRIGOS et al. (1963) or to produce transplantable tumors (DUC NGUYEN et al. 1967). The continuous transplantability is also consistent with the neoplastic nature of cells characterizing R-MuLV infection. De Both et al. (personal communication) also succeeded in transplanting hemopoietic tissue of infected mice to the other mouse strains in our laboratory.

On the basis of studies done with a virus preparation from original stock obtained from Rauscher, BOIRON et al. (1965) defined four stages in an R-MuLV infection of BALB/c mice:

a. A latent period, lasting between 10 to 35 days after ip injection of the virus.

b. Appearance of non-specific lesions, e.g. splenomegaly and myeloid hyperplasia, this period lasting 20 to 50 days.
c. The splenic and hepatic erythroblastosis stage.
d. The leukemic stage.

We distinguish three stages in the disease, i.e., a latent period, a hyperplastic period, and a neoplastic period.

The latent period, as generally defined in infectious disease, is the stage before the onset of symptoms, and in viral disease, before the first appearance of new virus particles. Our results show that the first budding virus particles appear from the cells in the liver sinusoids 72 hours after an ip injection of R-MuLV. The involvement of the spleen also coincided with the viral replication, i.e., at the end of 3rd day of the infection. Therefore, we define the first three days as the latent period of R-MuLV infection. The latent period is followed by a hyperplastic period corresponding to the second and third stages proposed by BOIRON et al. (1965), for which these authors mentioned splenomegaly, myeloid hyperplasia, and splenic and hepatic erythroblastosis. They also pointed out the absence of reticulin fibers in the newly formed tissues. We too found no reticulin network. This phenomenon argues against lymphomatous tumors.

The hyperplastic period extends, according to our morphological observations, from the end of the latent period to the 26th day of the infection.

The intervals we used (7, 12, 19, and 26 days after ip injection) are in agreement with those cited in the literature (SEIDEL 1968). When BOIRON et al. (1965) used lower doses of the virus, they had to lengthen the intervals. We also observed the prolongation of survival to 60 days when the virus dose was reduced. However, the dilution did not affect the duration of either the latent period or the hyperplastic period. The cytological changes were independent of the dose. At lower doses, however, the intensity of the symptoms was slightly decreased.

The last stage of the R-MuLV infection, which we call the neoplastic period, starts about 26 days after an ip virus injection. Taken together, the progressive and lethal evolution, the erythroleukemic picture of the peripheral blood, the histological structural alterations (especially of the hemopoietic organs), the infiltrative and invasive growth patterns of newly formed tissue, and the transplantability of the hemopoietic cells infected and modified by R-MuLV, all indicate

the neoplastic nature of this virus infection of mice. None of the measures taken to avoid the lethal evolution of the R-MuLV infection, such as blood transfusions, chemotherapy, and cortisone treatment, is successful (BRODSKY et al. 1968; SEIDEL 1972; LOZZIO et al. 1974). The splenomegaly, one of the parameters of the infection, is directly caused by the cellular proliferative effect of R-MuLV and is not a consequence of an increase in the red blood cell population (MARKOE and OKUNEWICK 1973; van GRIENSVEN et al. 1973). The blast crisis observed around the 19th day of the infection was due to the escape of cells into the circulation through ruptured capillaries of the hemopoietic organs.

It is generally accepted that a leukemia virus infection does not cause cell death. However, we observed apoptosis in all of the hemopoietic tissues involved in the process. DONALD et al. (1974) was the first to point out that R-MuLV causes individual cell death in a special way, i.e., apoptosis as described by KERR et al (1972). We invariably found apoptotic cells and apoptotic bodies scattered in the hemopoietic tissues in both the hyperplastic and the neoplastic periods, their numbers increasing as the infection progressed. Although we have not found any other description of the cytolotic effect of R-MuLV in the literature, we are convinced that R-MuLV can cause apoptosis (see Chapter 5).

Another significant feature of the latent period, is the thrombocytopenia with a sudden drop to a minimal level 8 hours after ip injection of R-MuLV. This finding is comparable to the results of BRODSKY et al. (1968). ISHIMOTO et al. (1971) also found significant thrombocytopenia in BALB/c mice infected with R-MuLV, and they related this phenomenon to the susceptibility of this mouse strain. In our experiments the occurrence of thrombocytopenia coincided with the first appearance of phagocytized virus particles in the sinus lining cells within or next to thrombocytes (Figs. 6 and 7).

RHIM et al. (1969) demonstrated that hamster embryo cells are transformed by R-MuLV *in vitro*. These authors also produced tumors by injecting the transformed embryo cells into hamsters subcutaneously. VIRELIZIER (1975) showed that macrophages are essential target cells for viral infection. REILLY and SCHLOSS (1971) thought that R-MuLV enters the primitive reticulum cell, an erythrocyte precursor, and replicates there during the cell's maturation to an erythrocyte. SILVESTRE et al. (1966) found R-MuLV

particles in the megakaryocytes from the fourth day onward and in all hemopoietic tissues after the 12th day of the infection. C-type virus particles, in both spleen and mesenteric lymph node germinal centers, were determined by HANNA et al. (1970) between 6 and 24 hours after an ip injection of R-MuLV. Preliminary studies carried out by van Griensven and by van Beek (unpublished observations) showed that ^{3}H-uridine-labelled R-MuLV appeared first in the liver of BALB/c mice 8 hours after ip injection and later in the spleen. These observations together with the results of our morphological studies in the latent period indicate that it is the cells of the reticuloendothelial system, whether in the liver or elsewhere, that are infected by R-MuLV.

BOIRON et al. (1965), SEIDEL (1968), and several other authors described multicentric and intrasinusoidal foci in the liver. Using ^{3}H-thymidine, SEIDEL (1968) found that the labelling index was about 85%, and he therefore concluded that these cells were proerythroblasts. The nidation of blood-borne hemopoietic stem cells in the liver sinusoids after the infection of R-MuLV is another possibility. Our investigations have shown that intrasinusoidal multicentric cellular proliferations are the earliest light-microscopical findings in R-MuLV infection.

The role of the thrombocytes in the pathogenesis of viral infections has been demonstrated (STERZ and WEISS 1973; SOTTNEK et al. 1975a, b), and in many respects R-MuLV infection offers an analog. The blood samples taken 8 and 16 hours after ip R-MuLV injection revealed that the thrombocytes functioned as carriers of virus particles, either on their surface or within the vacuoles (Figs. 6 and 7). It is not clear why only the thrombocytes carry the virions in the initial stage. In the later stages we found numbers of viral particles in the megakaryocytes and again within or on the thrombocytes. BRODSKY et al. (1967) demonstrated a direct effect of R-MuLV on thrombopoiesis. We suggest, therefore, that the thrombocytes play a role in the transport of R-MuLV, especially in the latent period, not solely by passive physical attachment but probably also as a result of their biological interaction with virions. Thrombocytopenia, which starts as early as 8 hours after an ip injection of R-MuLV, persists throughout the viral infection (BRODSKY et al. 1968).

MIYOSHI et al. (1967) noted glomerulopathy cause by R-MuLV. We could not find similar lesions in our material, or the

hematoxylin bodies described by these authors. It is possible that what they call hematoxylin bodies are apoptotic cells and apoptotic bodies. We indeed found some apoptotic cells and apopototic bodies in the kidneys infiltrated by Rauscher cells in the neoplastic period of the disease.

No sarcoma or carcinoma-like solid tumor formation was observed in the tissues and organs in our series. Except for the hemopoietic and reticular tissues of infected mice, all specimens showed the normal microscopical features seen in the tissues of the control mice.

The infection was progressive and lethal, as described by GROSS (1970) for murine leukemia virus infections in general, and without a single case of spontaneous recovery.

We do not consider the term erythroblastosis (BENTVELZEN 1972) appropriate for the disease caused by R-MuLV in mice. Erythroleukemia (SIEGLER and RICH 1966; LOZZIO et al. 1974) or, more generally, Rauscher disease (BOIRON et al. 1965) of mice, are preferable terms, since they avoid confusion about the nature of the infection.

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CHAPTER 5

EFFECTS OF R-MuLV INFECTION ON BALB/c MOUSE EMBRYOS AND APOPTOSIS

> . . . Without such cell death we would all be born with the webbed hands that the human fetus has at one stage.
> *Rick Gore. National Geographic*, 1976, 150: 355-395.

Introduction

After the analysis of the organic and cellular changes during R-MuLV infection of adult BALB/c mice, experiments were performed to determine the effects of the infection on mouse embryos *in utero*. In due course the cellular changes, including the cytopathogenic effects of R-MuLV, were studied light- and electron-microscopically in the embryonic and adult tissues of infected BALB/c mice.

The affinity of viruses to embryonic tissue is well known and has long been used for virus and vaccine production. In mammals, certain viral infections of the mother in the early phase of pregnancy result in either abortion (WIELENGA et al. 1961; TÖNDERY 1967) or fetal teratogenesis (ACKERMANN et al. 1974; HARRIS 1974) due to *in utero* infection of the progency. The direct relationship between the embryo and the maternal genital tract ceases with the formation of the placenta. Besides its circulatory and nutritional functions, the placenta acts as a physiological barrier protecting the fetus against injurious agents present on the maternal side. However, some toxins, vaccines (BOLOGNESI et al. 1973), and chemical car-

cinogens (CRAWFORD et al. 1972; DIWAN et al. 1974; NAPALKOV and ALEXANDROV 1974), as well as certain infectious biologic agents including viruses, can cross this pacental barrier (MARTEL et al. 1973; KILHAM and MARGOLIS 1974).

Viral infection of the embryo can occur in several ways:

1. Contamination with endogenous virus particles. Such particles, especially those of the oncogenic RNA viruses, are present in many strains of mice (VERNON et al. 1973; AARONSON and DUNN 1974). Both A- and C-type particles have been found in the blastocysts and egg cylinders of AKR mice (BICZYSKO et al. 1973; SOLTER et al. 1974), and endogenous virus particles have been regularly found in the two-cell embryonic stage of many mouse strains (CHASE and PIKO 1973; SOLTER et al. 1974). Embryos of BALB/c mice showed doughnut-shaped particles in the two-cell and pre-implantation stages (CALARGO and BROWN 1969), but no virus particles were seen in unfertilized ova or zygotes (BICZYSKO et al. 1973; SOLTER et al. 1974). Contamination probably accounts for these C-type virus particles, because they occur in the genital organs and could be transferred to embryos (FELDMAN and GROSS 1967).
2. Vertical transmission of virus, under which is understood the transmission of virus from the infected parent to the progency via the germinal cells, i.e., ova or spermatozoa. The vertical transmission of murine leukemia viruses has been demonstrated by several authors (GROSS 1951; JENSON et al. 1976). The virus may be able to infect ova and spermatozoa. The embryos formed from the infected germinal cells carry the genetic material of the virus. Spermatozoa can also be infected during their passage in the female genital tract.
3. Transmission via the mother's milk. The transmission of oncogenic viruses, especially the mouse mammary carcinoma virus (BITTNER 1936) and murine leukemia virus (LAW and MOLONEY 1961; BENTVELZEN 1968; SQUARTINI et al. 1973; SQUARTINI et al. 1974), via the mother's milk is known to lead to connatal virus infection. Since both milk samples collected from women belonging to families with a high breast-cancer rate (GROSS et al. 1950; LUNGER et al. 1964) and ultrathin sections of human breast-cancer tissue showed virus-like particles (DMOCHOWSKI et al. 1968), this mode of transmission of oncogenic viruses might also occur in man.
4. Horizontal transmission. The oncogenic viruses can be transmitted from an infected animal to another individual either directly

or via intermediary carriers (ROWE et al. 1958). Both natural and experimental horizontal transmission of murine leukemia virus have been demonstrated (GROSS 1970).

5. Transplacental transmission. This mode of transmission of oncogenic viruses has been shown to occur in many instances (IDA et al. 1966), but the authors did not consider *in utero* infection as a special form and included it under vertical transmission.

The transmission of murine leukemia virus strains has been studied by several investigators (GROSS 1951; LAW and MOLONEY 1961; MIRAND and MIRAND 1969; IDA et al. 1966). Transmission of the virus was demonstrated by several methods (MIRAND and MIRAND 1969; RICH et al. 1973; HARRIS 1974). Intra-uterine fetal death occurred in a high percentage of the cases and was ascribed to the effect of viral infection, but the histological features were not described. We therefore investigated the possible transmission of R-MuLV *in utero* and its effect on mouse embryos.

During organogenesis in embryos, cell death occurs as a normal physiological process. This is an inherited potential. Focal elimination of cells in developing embryonic tissues contributes to the genetically coded formation of whole organs and extremities. Morphologically, this phenomenon represents a special mode of cell death. The ultrastructural features of this process are similar to those of apoptosis. Apoptosis, first defined by KERR et al. (1972), either occurs inherently or is induced by several agents. Besides the already known exogenic factors such as noxious substances and toxins. R-MuLV was found to cause apoptosis (DONALD et al. 1974; UMAR and VAN GRIENSVEN 1976), although it had previously been considered non-cytocidal (MONTAGNIER 1970).

In our experiments, R-MuLV-induced apoptosis was studied light- and electron-microscopically in both neoplastic and embryonic tissues.

Experimental design

To investigate the transmission of R-MuLV and its effect on BALB/c mouse embryos, a number of experiments were performed. As shown schematically in Table 1, this investigation was based on two main groups of mice. The first group, comprising 24 mice

divided into three equal subgroups, was used for histological studies in the embryos of mothers given ip injection of 4 mg R-MuLV 12, 8, and 1 day before mating. The second group, consisting of 36 mice, was used mainly for XC testing and immunofluorescence studies to determine the presence of R-MuLV in the mouse embryos but also for histological investigation.

The mice of the second group were divided into three equal subgroups and injected ip with 2 mg R-MuLV 1 day before or 1 day or 4 days after conception. The number of animals in the various subgroups, the duration of the infection in each subgroup after ip injection of virus, and the studies performed are also shown in Table 1.

Table 1.

Design of the transmission experiments

Number of BALB/c mice	BEFORE MATING			AFTER MATING					Studies
	12 days	8 days	1 day	1 day	4 days	8 days	10 days	12 days	
8	4 mg					8 †			Histology
8		4 mg				4 †		4 †	Histology
8			4 mg			4 †		4 †	Histology
12			2 mg			6 †	6 †		XC test, immunology
12				2 mg		6 †	6 †		XC test, immunology
12					2 mg	6 †	6 †		XC test, immunology
8						8 †			Controls for XC test immunology, histology

R-MuLV, in a dose of 4 mg or 2 mg, was ip injected. † indicates number of autopsied BALB/c mice.

The embryos of 8 non-infected pregnant BALB/c mice served as a control group. The obtaining and preparation of mouse embryos for light microscopy, the XC test, and the immunofluorescence technique are described in Chapter 3. In a bio-assay, 8 BALB/c mice were inoculated ip with a cell suspension prepared from embryos infected *in utero* with R-MuLV, and autopsied 18 days later to determine the macro- and microscopical changes.

A light-microscopical study of apoptosis was performed during an R-MuLV infection in adult BALB/c mice. Of these mice, 24 were ip injected with 4 mg R-MuLV, 24 with 0.4 mg R-MuLV, and 24 with 0.04 mg R-MuLV; 8 mice given 0.2 ml HBSS ip were used as control group. Spleen, bone marrow, and liver tissues were studied, in particular to determine the morphological features of apoptosis at the light-microscopical level. The animals were autopsied on the 7th,

12th, 19th, and 26th days of the disease for determination of cytopathologic changes.

For electron microscopy, 20 mice were ip injected with 4 mg R-MuLV, and after 3, 6, 19, and 26 days, 5 mice were autopsied. Small pieces of perfused spleen and liver were immersed in the same fixative solution for at most 3 days and prepared as described in Chapter 3.

To determine and compare ultrastructural changes occurring in apoptosis of the mesodermal cells due to R-MuLV infection *in utero* before organogenesis, 24 pregnant BALB/c mice were ip injected with 4 mg R-MuLV isolated from spleen tissue and 12 pregnant BALB/c mice with 2 mg R-MuLV isolated from plasma. Pregnant uteri fixed by perfusion were dissected, and 120 embryos were collected in cooled fixative. The majority of these specimens were prepared for electron microscopy, the remainder for light microscopy. The control group consisted of the embryos of 4 non-infected mice.

Results

Half of the mice injected with R-MuLV 8 days and 1 day before mating were pregnant 8 days after mating. No signs of living embryos were found in either of these two subgroups 12 days after mating. Eight days after mating, none of the mice that had been injected 20 days before were pregnant.

Light-microscopical studies in serial sections of pregnant uteri showed that apoptotic cells were present in egg cylinders and in the embryos during subsequent developmental stages. The mesoderm appeared to be the main target of apoptosis and destruction.

Apoptotic cells, which were smaller than the surrounding embryonic cells, were usually spherical and had a homogenous, dark, acidophilic cytoplasm. The nucleus was generally pyknotic or fragmented, but the fragments, which varied in size and shape, were compact, hyperchromatic, and usually eccentrically situated at the periphery of the cells. The amniotic cavity contained apoptotic cells and granular, amorphous remnants of such cells (UMAR and VAN GRIENSVEN 1976). After the formation of mesoderm, apoptosis was seen exclusively in the mesoderm, which was of variable cellular density and even partially dissociated (Fig. 1). The ectoderm and

Figure 5-1. An 8-day old mouse embryo showing the onset of the formation of primitive organs 9 days after ip injection of the mother with 4 mg R-MuLV. Note the variation in cellularlity of the mesoderm (M) and the presence of numerous apoptotic cells, especially at the right. fg – foregut. H & A. x 120.

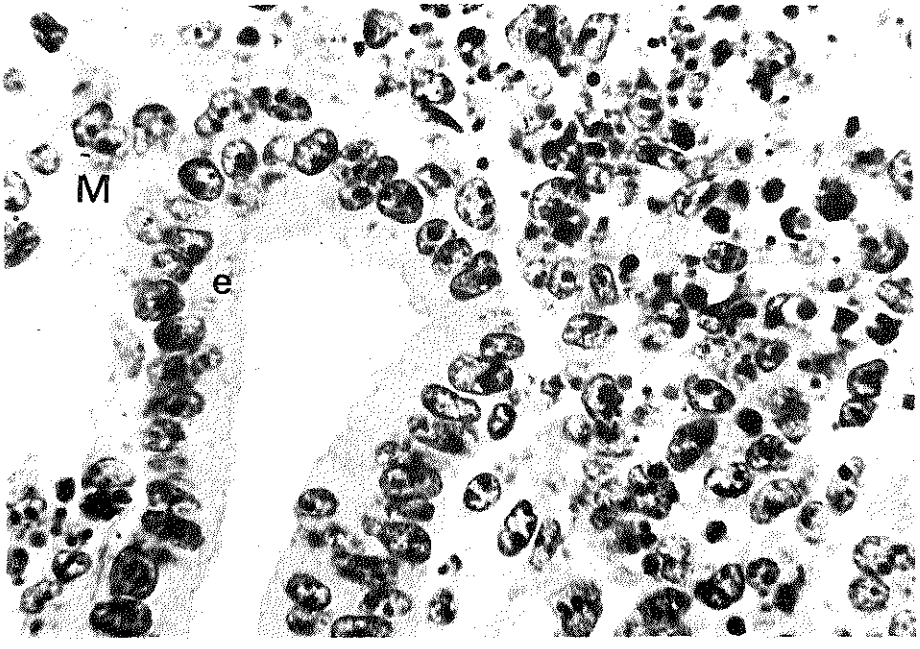

Figure 5-2. Higher magnification of Fig. 5-1. Note the mass of apoptotic cells in the mesoderm (M) and the cellular polymorphism. The entoderm (e) is intact. H & A. x 200.

entoderm were, however, normal in light microscopy (Fig. 2). With increasing duration of the R-MuLV infection, the number of apoptotic cells increased in the maternal spleen and liver tissue.

Results of immunofluorescence studies

To demonstrate the presence of R-MuLV antigens in the embryos exposed to infection *in utero*, the immunofluorescence studies were performed with spcific anti-R-MuLV sera. Independent of the developmental stage of the embryos, cytoplasmic indirect immunofluorescence was uniformly distributed in the embryos, but mesodermal and apoptotic cells with bright cytoplasm, sometimes with a granular patterns, were easily distinguished (Fig. 3). The nuclei showed no fluorescence. The cytoplasm of large trophoblastic cells, all the embryonic membranes, and the surface and glandular epithelium of the endometrium showed fluorescence in varying degrees. No fluorescence of decidua was seen.

Uteri and embryos from non-infected mothers were not fluorescent for antisera to R-MuLV.

Result of XC test

Trypsinized embryo cells from infected mothers co-cultivated with XC cells formed polykaryons over four nuclei. A considerable number of polykaryons, i.e., up to 3,500 per dish, were observed in co-cultures of XC cells and cells of embryos from mothers infected 4 days after determination of pregnancy. Fig. 4 shows two of these polykaryons.

Control dishes with embryos from non-infected mothers had up to 250 polykaryons per dish. The number of nuclei did not exceed four, however.

Light microscopy of apoptosis

Apoptosis was investigated in the lymphoreticular tissues, i.e., bone marrow, liver, spleen, and lymph nodes, of BALB/c mice in-

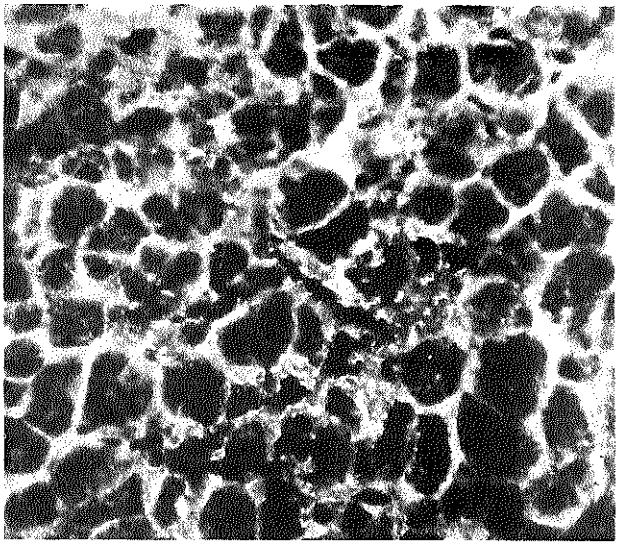

Figure 5-3. UV micrograph of mesoderm of an 8-day-old embryo, prepared with antiserum to R-MuLV for immunofluoresce 4 days after ip injection of the mother with 2 mg R-MuLV. Note the granular pattern of indirect intracytoplasmic immunofluorescence. x 270.

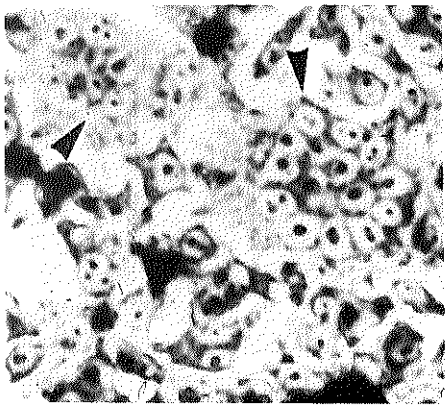

Figure 5-4. Micrograph of part of a plastic tissue-culture dish photographed through a phase-contrast microscope (objective x 25). Note the two polykaryons (arrows) in the co-culture of XC cells and cells from an embryo of a BALB/c mouse infected with 2 mg R-MuLV 7 days before the test.

fected with R-MuLV in the previous experiments. Apoptotic cells first appeared in the liver on the third day of the infection and was also observed 7, 12, 19, and 26 days after ip inoculation of virus. In these repeated experiments, the apoptotic cells and apoptotic bodies were randomly distributed between light-microscopically intact cells. The lower dose of R-MuLV did not affect the morphological appearance, but the number of apoptotic cells was slightly decreased. With increasing duration of the infection, the number of apoptotic cells increased. In the spleen of mice infected with 4 mg R-MuLV 26 days earlier, at least 4 apoptotic cells occurred in each microscopic field at a magnification of 787. However, no statistically significant correlation was found between the number of apoptotic cells and either the virus dose or the duration of the infection.

The lymphoreticular tissues of mice in the control group given HBSS, showed only tingible body macrophages (SWARTZENDRUBER and CONGDON 1963) in the germinal centers of the lymphoid follicles. No apoptotic cells were seen elsewhere.

Electron microscopy of apoptosis

After localization of the particular area with the light microscope, adjacent sections were made for electron microscopy to study the ultrastructural changes during apoptosis in both adult and embryonic tissues. The first detectable alteration in apoptosis was nuclear edema, followed by highly irregular configuration of the chromatin (Fig. 5). In the initial stage, some intracytoplasmic vacuolization also occurred in the endoplasmic reticulum. This stage was followed by nuclear dehydration and condensation of the chromatin. The compact nuclear mass had broken down into irregular fragments varying in shape and size (Fig. 6), and part of the nuclear envelope had disappeared. The cytoplasm showed a rough granular pattern, in some areas closely packed, but in general the endoplasmic reticulum was ruptured and disorganized. The mitochondria were grossly swollen and cristae had disappeared (Fig.6). The cell membrane had ruptured in many places. Immature and/or mature virus particles were frequently present next to or on the cell membrane (Figs. 7 and 8). Around these cells, morphologically normal cells or macrophages were always found. Most of the apoptotic cells in adult tissues had

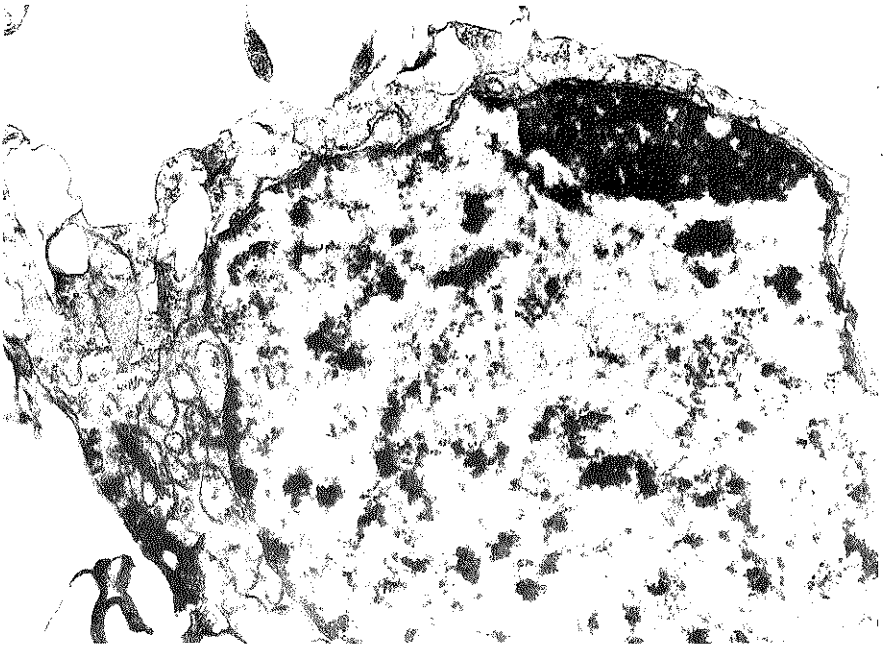

Figure 5-5. Mesodermal cell at the onset of apoptosis. The cell is somewhat shrunken. Note the pronounced nuclear edema and irregular chromatin clumps. The membranes are still intact. Uranyl acetate. x 63,200.

been phagocytized by macrophages, but in embryonic tissues phagocytosis of whole apoptotic cells was not observed. The next stage of the process was characterized by fragmentation of apoptotic cells into unequal parts, known as apoptotic bodies, consisting of membrane-bound nuclear remnants (Figs. 7 and 8).

Many apoptotic bodies, however, carried virions on the membrane. The apoptotic bodies were phagocytized by neighboring newly-formed cells and by mesodermal cells. The enzymatic disintegration of apoptotic bodies continued in the new host cells until there were only lamellar osmiophilic remnants (Figs. 7-9), which were ejected into the intercellular space (Fig. 10). We also observed immature virions and virus particles budding from macrophages (Fig. 8) and mesodermal cells phagocytizing apoptotic bodies (Fig. 11 a and b).

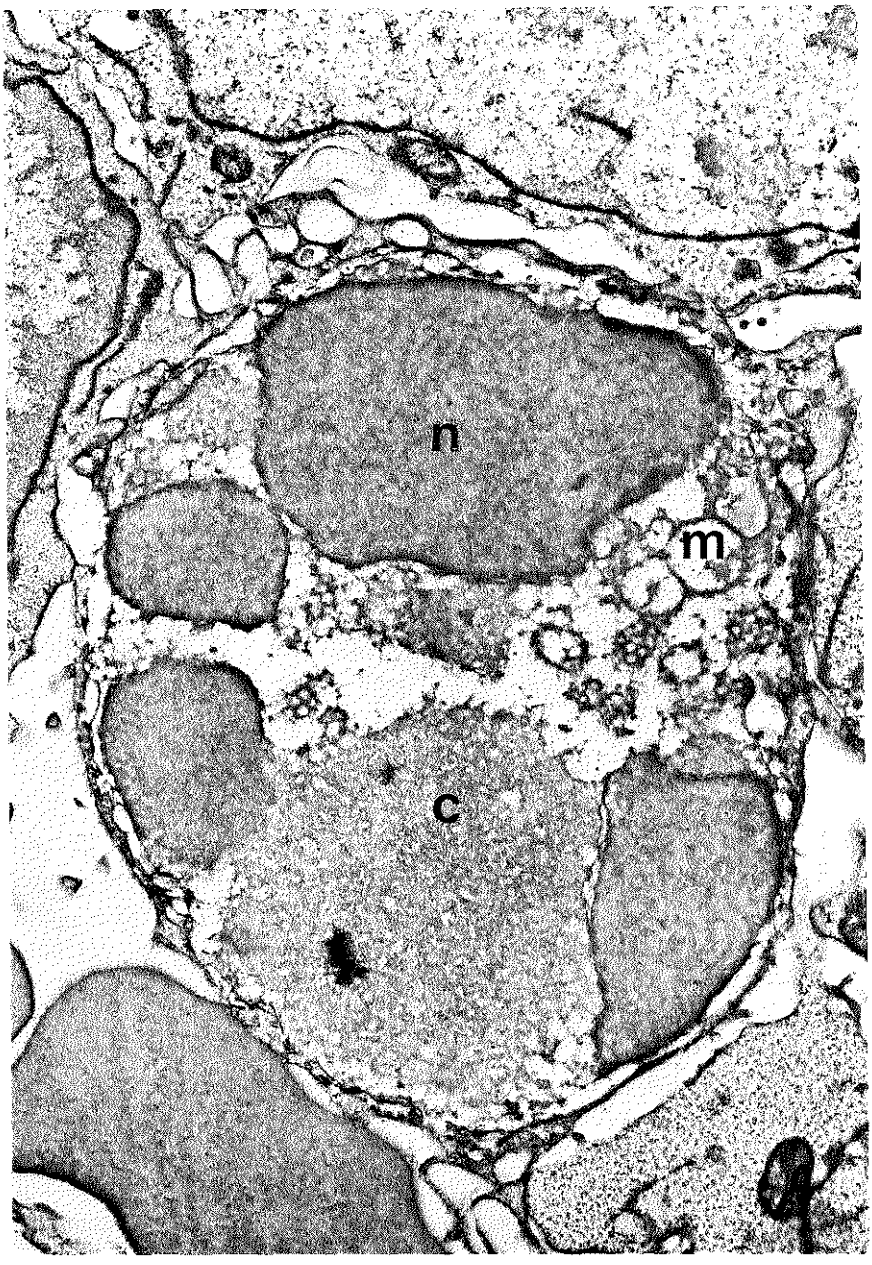

Figure 5-6. An apoptotic cell in the liver on the 19th day of an R-MuLV infection. The nucleus (n) is fragmented. The chromatin is dense. Some remnants of endoplasmic reticulum and mitochondria (m) are just faintly recognizable. Cell and nuclear membranes are ruptured in many places. Fragmentation of the cytoplasm (c) is almost complete. Uranyl acetate and lead citrate. x 17,920.

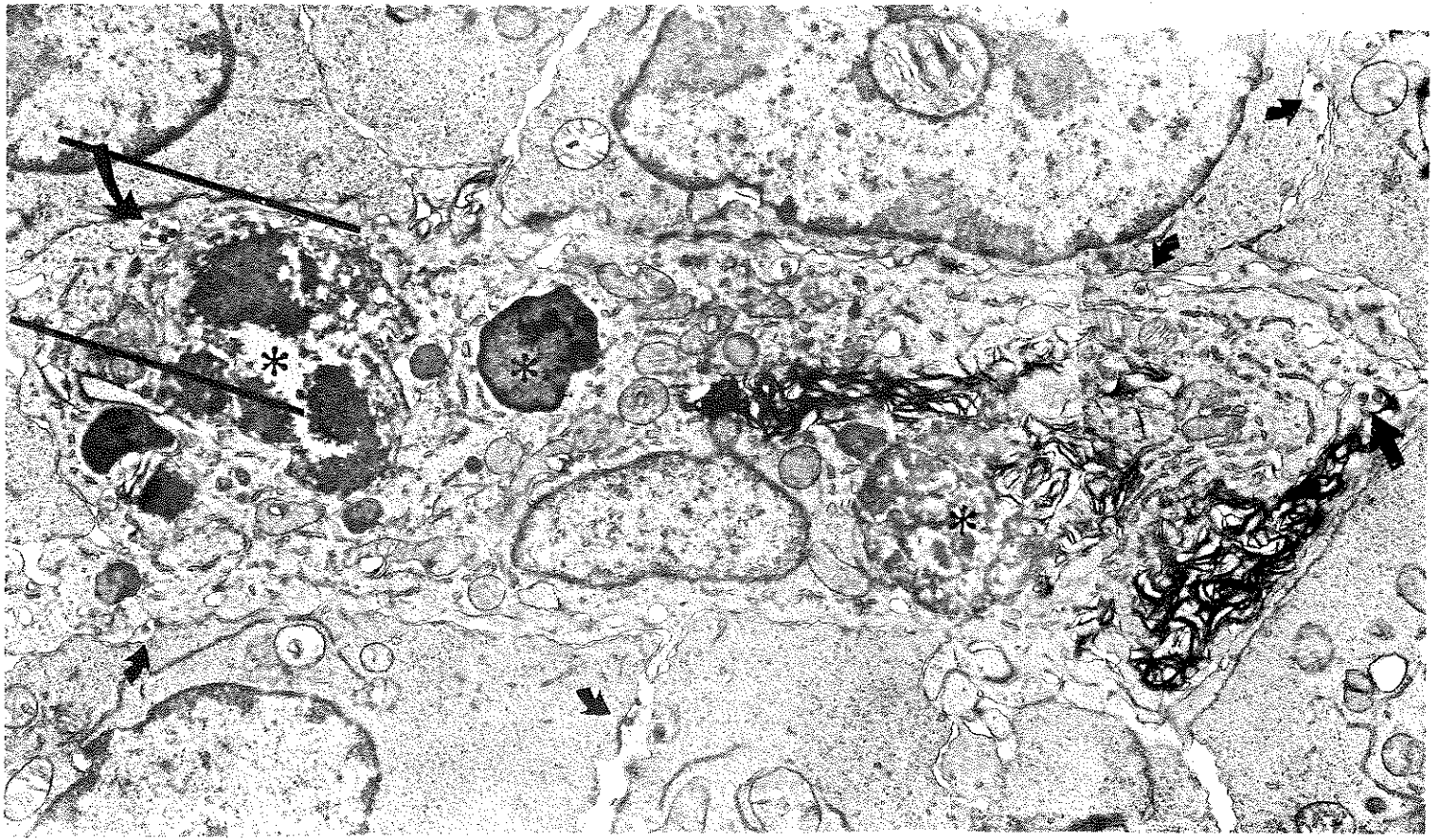

Figure 5-7. Apoptotic bodies (x) in a phagocytosing cell in the liver surrounded by Rauscher cells on the 26th day of infection. Virions can also be distinguished (arrows). Other parts of this cell show disintegration and degradation of apoptotic bodies. Uranyl acetate. x 14,600.

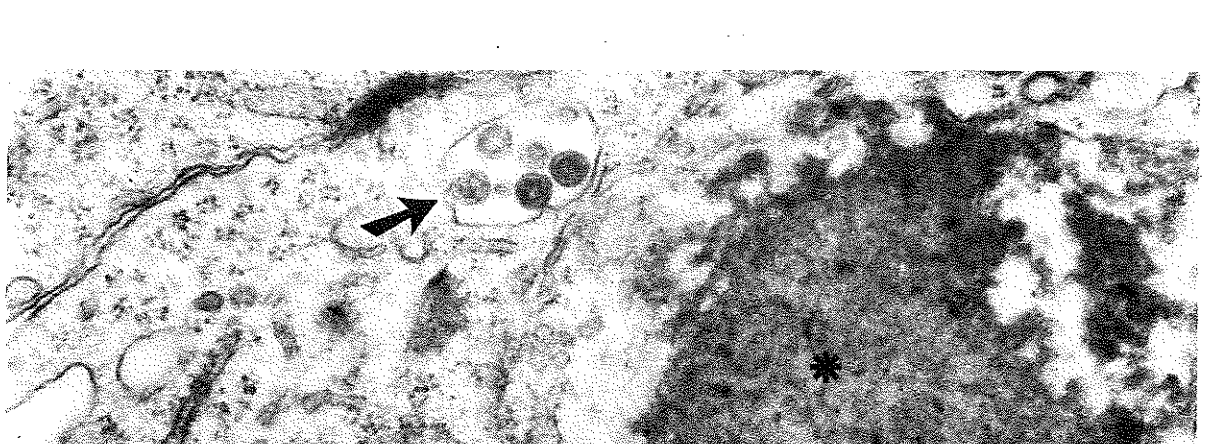

Figure 5-8. Magnification of area indicated in Fig. 5-7. Note the close relationship between apoptotic body and virus particles. Uranyl acetate. x 45,900.

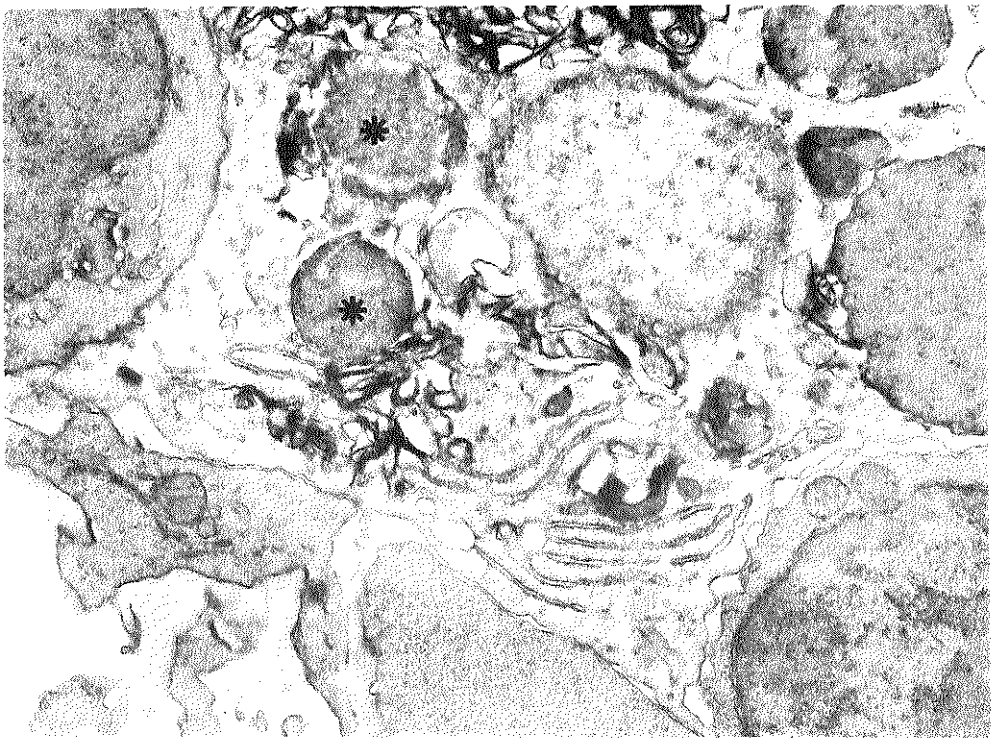

Figure 5-9. A phagocytic cell in the spleen, containing several apoptotic bodies (x) on the 26th day of an R-MuLV infection. Segregated, osmiophilic lamellar remnants of apoptotic bodies are ready to be discharged into the intercellular space. Uranyl acetate, x 17,000.

Discussion

In these experiments the mice with a 12-day-old R-MuLV infection at mating showed no sign of pregnancy 8 days after mating. Because R-MuLV infection leads to a greatly increased spleen volume as well as lordosis of the vertebral column, both mating and conception are impeded. Pregnancy could not be diagnosed from either vaginal plugs or slides showing decidual changes of the endometrial stroma cells.

When ovaries were examined for signs of pregnancy, numerous apoptotic cells were found between the follicular cells and in the antrum of Graafian follicles. If the cellular changes in apoptosis are

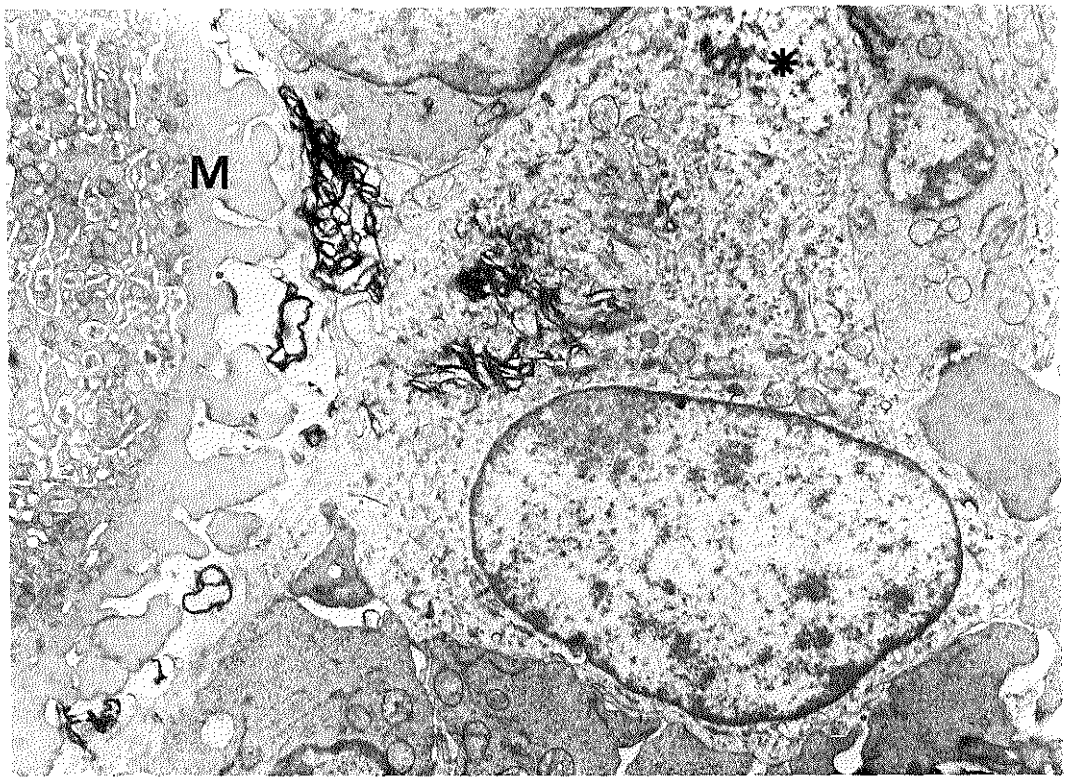

Figure 5-10. A phagocytic cell in the spleen on the 26th day of an R-MuLV infection. An almost totally disintegrated apoptotic body (x) is present. Lamellar osmiophilic structures are being discharged, and some can be seen in the intercellular space. There is also a megakaryocyte (M) with many virions.

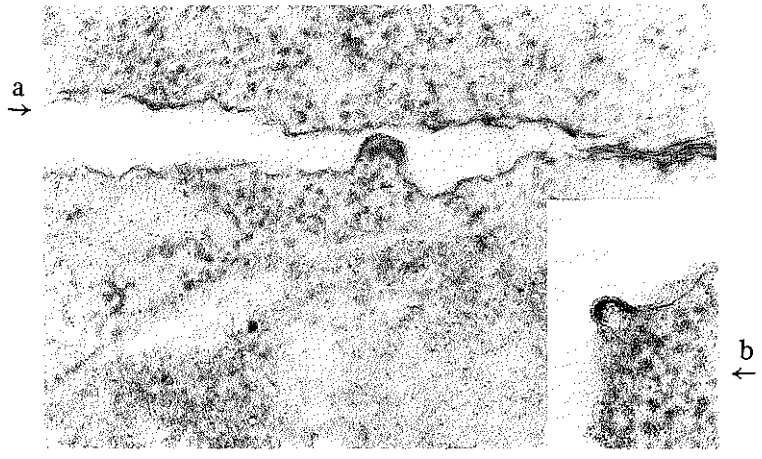

Figure 5-11 a, b. Two examples of virus particles budding on the mesodermal cells. Uranyl acetate and lead citrate. 11a: x 61,200; 11b: x 51,900.

specifically due to the effect of R-MuLV, as postulated by DONALD et al. (1974), it seems likely that the ova, too, will be infected *in situ* by the virus. The possibility that such ova can be fertilized is questionable. The ovaries of R-MuLV infected mice showed the same cellular changes 8 days and 1 day before mating, but half of the animals in these groups became pregnant.

In the genital tract, the possibility of R-MuLV contamination of fertilized eggs, morulae, or pre-implantation blastocysts also cannot be exluded (FELDMAN and GROSS 1967). In a study on the transmission of Moloney murine leukemia virus (M-MuLV) during embryogenesis and its effect on the litters after intravenous injection of this virus into pregnant A/LN mice, IDA et al. (1966) demonstrated that M-MuLV crossed the placental barrier and infected the embryos. After a latent period, the incidence of M-MuLV-induced leukemia in each litter was 25-100%. However, intrauterine fetal death attributable to M-MuLV also occurred in this study, but the authors did not describe the histological features of these embryos.

Organogenetic cell death is a general phenomenon in morphogenesis (SAUNDERS 1966; BALLARD and HOLT 1968; KRSTIC and PEXIEDER 1973). A few apoptotic cells were observed in normal embryonic tissue of non-infected control BALB/c mice in our experiments. The occurrence of a vast number of apoptotic cells in spleens from R-MuLV infected BALB/c mice has been described. The large numbers of apoptotic cells in embryos infected with R-MuLV provide further support for the hypothesis of DONALD et al. (1974).

Because apoptotic cells in embryos show strong fluorescence after exposure to specific antisera to R-MuLV, it seems likely that virus replication in these cells can lead to cell death. Before mesoderm formation the distribution of apoptotic cells in the embryos is haphazard, but afterward it is especially prevalent in the mesoderm. In some embryos the apoptotic cells lay adjacent to or were in contact with the entoderm and neural plate (Fig. 2), a localization that can be ascribed to simple migration of apoptotic cells.

Physiological changes, for instance tissue anoxia (TEDESCHI et al. 1956; KERR 1971) after R-MuLV infection, can also explain cell death in tissue, but in embryos anoxia could not be responsible for the individual cell death restricted to the mesoderm. Only agents with a specific topism, such as viruses, can produce this effect.

It could be argued that fetal antigens on embryo cells are

responsible for the increase in polykaryons in the XC test. This cannot be the case, however, because trypsinized embryo cells from non-infected mothers did not produce large numbers of polykaryons in the XC test. When trypsinized cells from embryos of R-MuLV infected mothers were injected ip into BALB/c mice (10^5 cells/mouse), the resulting splenomegaly and the microscopical findings provided proof of R-MuLV infection of the embryos. The histological material prepared 18 days after inoculation showed multicentric formation of blast cells in liver sinusoids and in the splenic red pulp. The injection of cell-free supernatants deriving from these trypsinized embryonic cells led to the same phenomena and hence to the same conclusion.

The presence of bright cytoplasmic immunofluorescence (YOSHIDA et al. 1966) in large trophoblastic cells and embryonic membranes and the positive XC test results obtained with embryo cells prove, together with the bio-assay findings, that R-MuLV infection of mouse embryos can occur *in utero*. Under our experimental conditions, the transmission of R-MuLV to the embryos could have occurred either by contamination in the genital tract or by vertical transmission via ova. In the mouse, the placenta develops about 8 days after fertilization and 4 days after implantation of the fertilized ova. Although we infected the mothers with R-MuLV intraperitoneally before placenta formation, transplacental transmission of the virus particles, which replicate continuously during the embryonal development, could have taken place later on.

The use of lower doses of virus for infection of the mother mouse might make it possible to investigate a teratogenic effect of R-MuLV (TÖNDERY 1969) and also the occurrence of connatal leukemia as a result of R-MuLV infection of embryos.

Apoptosis is a striking process of cellular change seen during the productive stage of infection. This term, which was coined by Cormack and was first applied by KERR et al. (1972), indicates two rather universal phenomena, i.e., cell death and phagocytosis, but combined in a special way. The apototic cells are usually isolated cells that always undergo the same series of morphologic changes; once the process starts, it evolves progressively until the cell dies and is phagocytized. The viral genetic information carried by the apoptotic cell may be re-used by the phagocytizing cell, and this can prolong the infection (TROWELL 1957).

It is known that apoptosis occurs under various physiological and pathological conditions and that endogenous, inherited, or exogenous factors can trigger it (KERR et al. 1972). The organogenetic cell death in the embryo is a model of apoptosis occurring under inherited, completely physiological conditions (SAUNDERS 1966), which explains our use of embryos that had not yet reached the stage of organogenesis.

It has been shown that R-MuLV is one of the etiologic factors in apoptosis of some of the infected cells (DONALD et al. 1974) and selectively of mesodermal cells (UMAR and VAN GRIENSVEN 1976). SZAKACS et al. (1968) also described cytoplasmolysis and nucleolysis due to the presence of murine leukemia virus.

In a retrospective comparative study of material in our archives, we found similar cellular alterations in sarcomatous neoplastic tissues induced by a Moloney murine sarcoma virus infection. This supports our conclusion that viral cellular infection can cause individual cell death in the form of apoptosis, as seen in R-MuLV infection, even although cytolytic or cytopathic effects of these viruses had not generally been expected.

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CHAPTER 6

TRANSPLANTABILITY OF R-MuLV-INFECTED CELLS
A methodological investigation

Introduction

Demonstration of the autonomous character of neoplastic tissues and cells requires not only histopathological criteria but propagation in tissue culture and transplantability in a new host (FOLEY and HANDLER 1957). For this purpose, genetically uniform laboratory animals, such as inbred mice or golden hamsters, are used (BILLINGHAM and SILVERS 1959).

The successful transplantation of mouse ova in the anterior chamber of the eye (RUNNER 1947) opened new possibilities for the study of transplantation (FAWCETT et al. 1949; ANDRUS et al. 1951). The transplantation of neoplastic cells in the abdominal cavity has provided ascites forms of transplantable tumors. Using human malignant cells, including leukemia cells, SOUTHAM et al. (1969a) demonstrated that the intrascapular fat pad, brain, and eyes also offer potential sites for heterotransplantation in newborn rats. Human cell lines derived from leukemia, Burkitt's lymphoma, and reticulum cell sarcoma have been grown subcutaneously in immunologically tolerant rats (SOUTHAM et al. 1969b).

The absence of lymphatic drainage in the meninges of the brain means that there is no easy access to lymphoid tissues for antigenic stimulation of transplants. The cheek pouches of the hamster also have no lymphatics, and this gives them too a priviliged status in transplantation experiments (SHEPRO et al. 1963; BILLINGHAM

and SILVERS 1964). LUTZ et al. (1951) and LEMON et al. (1952) demonstrated that the connective tissue of the cheek pouch wall of golden hamsters is a favorable site for transplantation studies on malignant tumors of both animal and human origin. Transplantation of human tumors into the cheek pouch of cortisone-conditioned golden hamsters resulted in the survival of a large number of the transplants (PATTERSON et al. 1954; HANDLER and FOLEY 1956). Cortisone treatment may alleviate inflammatory reactions (CHUTE et al. 1952). Non-neoplastic tissues reveal no definite evidence of growth in the cheek pouches although they sometimes persist for a long time without mitotic activity. Furthermore, the pouches can be observed after transplantation to determine the growth of the transplant, and they can be biopsied without loss of the animal.

It has been demonstrated morphologically that the final phase of an R-MuLV-induced infection in mice represents a neoplastic state (see Chapter 4). Hypothetically, the undifferentiated Rauscher cells in the hemopoietic organs could be neoplastic cells that have acquired the character of endless proliferation, properties of transplantability, and autonomous control mechanisms. If the postulated malignant transformation of cells by R-MuLV actually occurs, these neoplastic cells should be transplantable. Therefore, a series of preliminary experiments was designed to explore the possibility of transplantation of Rauscher cells in mice and golden hamsters.

Experimental design

Preliminary experiments were performed to develop a convenient method to study the transplantability of cells infected and transformed by R-MuLV. Almost all possible sites in mice were considered, and were evaluated after injection of suitable cell suspensions. In these experiments, hetero-transplantations were also performed in golden hamsters. The final experimental design is shown in Table 2. The isolation technique used for both normal and infected cells is described in Chapter 3. The cell suspension of the lymph-node material was made with the same method. Rat ureter carcinoma cells (RUC) were cultivated in serial passages, and transplantability of these neoplastic cells was tested by the supplier (Institute of Radio-

Table 1.

Design of transplantation experiments for methodological study done in BALB/c mice and golden hamsters

Transplantation site	Inocula	Concentration	Remarks	Observation period (days)
ant. chamber	normal spleen cells	10^4/0.02 ml	left eye inoculated	7
ant. chamber	leukemic spleen cells	10^4/0.02 ml	left eye inoculated	7
intracerebral	R-MuLV	0.4 mg	left hemisphere inoculated	7
intracerebral	normal spleen cells	10^4/0.02 ml	left hemisphere inoculated	7
intracerebral	leukemic spleen cells	10^4/0.02 ml	left hemisphere inoculated	7
intrapleural	leukemic spleen cells	10^4/0.2 ml	left side inoculated	7
intrapleural	R-MuLV	2 mg/0.1 ml	left side inoculated	7
cheek pouch	R-MuLV	2 mg/0.1 ml	left pouch injected	60
cheek pouch	leukemic spleen cells	10^3/0.1 ml	bilaterally inoculated*	60
cheek pouch	RUC cells	10^3/0.1 ml	bilaterally inoculated*	60
intraperitoneal	lymph node cells	10^4/0.2 ml		7
intraperitoneal	normal spleen cells	10^5/0.2 ml		7
intraperitoneal	leukemic spleen cells	10^5/0.2 ml		7
intraperitoneal	leukemic spleen cells	10^5/0.4 ml	incubated with R-MuLV antiserum, 10 minutes 37°C	7

* 2.5 mg cortisone acetate subcutaneously injected twice a week.

biology, TNO, Rijswijk, The Netherlands). *In vitro* propagation of these carcinoma cells was carried out in tissue-culture dishes before hetero-transplantations, as controls for the preliminary experiments. Suspensions of both R-MuLV-infected spleen cells and RUC cells were diluted in HBSS to 10^3 cells per 0.1 ml before inoculation into the cheek pouches of the hamsters. Cheek pouches were bilaterally inoculated with cell suspension under Nembutal anesthesia (0.15 ml/per 100 g body weight), and were checked daily for the presence of tumoral growth under the epithelium. A normal golden hamster serving as control was killed by ether asphyxia for histological study of the cheek pouch and visceral organs.

Independently, spleen cell suspensions (10^7 cells in 0.2 ml sterile HBSS) derived from BALB/c mice in the neoplastic period of R-MuLV infection, were inoculated under the cheek-pouch epithelium of 10 golden hamsters according to above-described method, by van Griensven in our laboratory.

Finally, 4 BALB/c mice were inoculated ip with spleen cell suspensions derived from mice in the neoplastic period of R-MuLV infection. Just before inoculation, 10^5 cells in 0.4 ml sterile HBSS

were incubated together with an adequate amount of specific antiserum to R-MuLV for 20 minutes at 37°C.

The observation period after transplantation was 7 days for mice and 60 days for golden hamsters. The organs on the left side were used for transplantation and those on the right side for comparative histology. Inoculation into the anterior chamber of the left eyes was performed under a stereomicroscope.

Both eyes of each mouse, both cheek pouches of hamsters cut into several pieces, the brain *in toto*, and the whole mesentery cut into four pieces, were fixed in Bouin's solution and sectioned serially (5 μm thick). Besides H & A, certain special stains were also applied.

Results and conclusions

Seven days after inoculation of 10^4 normal and R-MuLV-infected spleen cells in the anterior eye chamber of the mice, no histological signs of successful transplantation were observed in the serial sections. Microscopic structures of the inoculated left eyes showed the same histological appearance as those of the untreated right eyes. It was therefore concluded that the anterior chamber of the mouse eye is not a suitable site for the transplantation of spleen cells, whether normal or neoplastic, at least not at the chosen concentration and duration.

When normal spleen cells were inoculated intracerebrally, some necrotic cells and nuclear debris were found together with signs of moderate phagocytic activity. In some instances meningeal and cortical nidations were found 7 days after intracerebral inoculation of 10^4 R-MuLV-infected spleen cells into 4-week-old BALB/c mice. Proliferating cells in these transplantation foci (Fig. 1) were morphologically comparable to the inocula cells.

When 0.4 mg R-MuLV was injected intracerebrally into 4-week-old mice, the virus effects were the same as after ip injections. The liver of these animals showed multicentric intrasinusoidal proliferations and aggregations of cells, a constant feature in the liver 7 days after R-MuLV infection. Histologically, however, the cerebral tissue showed slight bleeding but few phagocytic cells. Proliferative or neoplastic changes were not observed.

It is concluded on this basis that cerebral tissue might serve as a

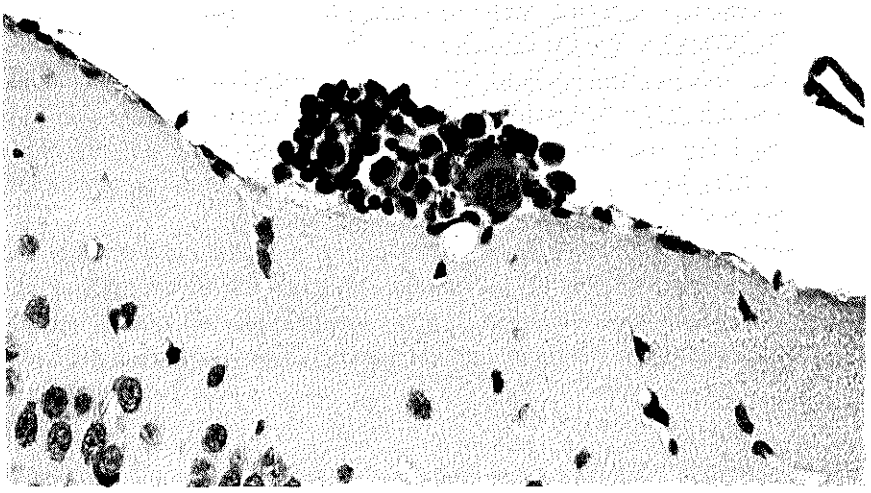

Figure 6.1. Micrograph of a section of the brain of a 4-week-old BALB/c mouse seven days after intracerebral inoculation of 10^4 R-MuLV-infected spleen cells. Note the supracortical nidation of proliferating hemopoietic cells. H & A. x 320.

transplantation site for neoplastic cells, but that more than 10^4 cells per inoculum should be injected. The number would have to be chosen with care, however, because a very large number of cells or a larger quantity of inocula can cause serious damage and can sometimes even be lethal. Although intracerebral inoculation of Rauscher cells was successful in some cases, it was not possible to avoid the injurious effects of this method and therefore other sites and less harmful methods were investigated.

Subepithelial injection of 2 mg R-MuLV into the hamster cheek pouch did not lead to infection, even after 2 months of exposure. The liver, spleen, and bone marrow of these hamsters showed normal histological features. Neither 10^3 RUC cells nor 10^3 R-MuLV-infected spleen cells inoculated into the cheek pouches of the cortisone-conditioned golden hamsters led to any tumoral growth during the 60-day period after hetero-transplantation. This negative result, which was contrary to our expectation, might be explained by the relatively low concentration of inoculated cells or the rather short control period. The low number of cells was chosen because some investigators had reported that in cortisone-conditioned hamsters, smaller quantities of neoplastic cells grow selectively at the trans-

plantation site (HANDLER and FOLEY 1965; FOLEY and HANDLER 1957).

Thus, it is clear that if cheek pouches are to be used as the transplantation site, either more than 10^3 cells/inoculum or more than two months of post-transplantation observation must be considered. The attempts made by van Griensven to transplant 10^7 neoplastic spleen cells into the cheek pouches of the hamsters also failed. The results of these preliminary experiments made it obvious that the cheek pouches of the golden hamster were not suitable sites for our purposes.

Intrapleural inoculation of 10^4 neoplastic spleen cells did not give any positive results, but on the 7th day the mice showed an infection similar to that seen 7 days after ip injection of the virus.

Intraperitoneal inoculation of 10^4 cells from mesenteric lymph nodes of BALB/c mice infected with R-MuLV did not lead to transplantation.

Intraperitoneal inoculation of both normal and neoplastic cells (10^5 cells in 0.2 ml HBSS) resulted in microscopically small mesenteric foci under the mesothelium (Figs. 2 and 3). The cells of these foci, some of them in mitosis (Fig. 4), were completely comparable morphologically to the inoculated cells. We considered these mesen-

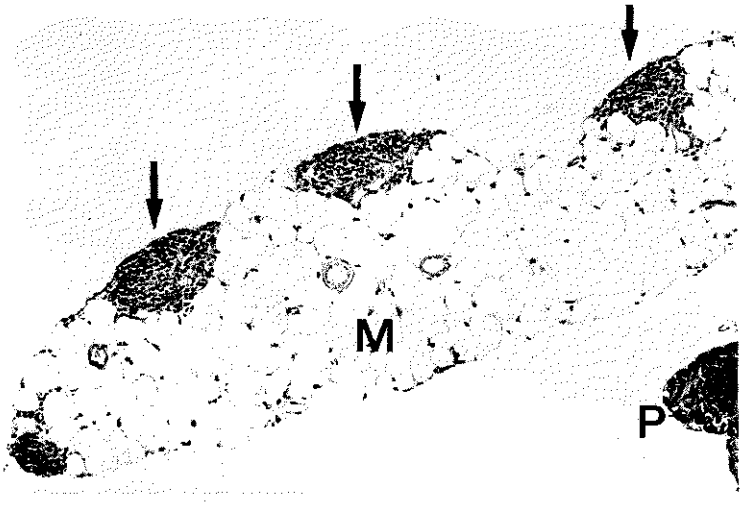

Figure 6.2. Micrograph of a section of the mesentery of a BALB/c mouse, 7 days after intraperitoneal inoculation of 10^5 syngeneic normal spleen cells. Note triangular shape of mesenteric hemopoietic colonies (arrows). M – mesentery. H & A. x 95.

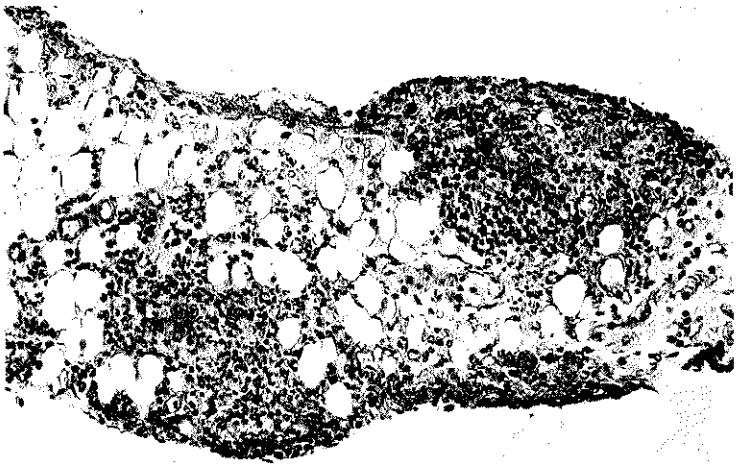

Figure 6.3. Micrograph of a section of the mesentery of a BALB/c mouse, 7 days after intraperitoneal inoculation of 10^5 R-MuLV-infected spleen cells. Note two mesenteric hemopoietic colonies growing between fat cells of the mesentery. H & A. x 130.

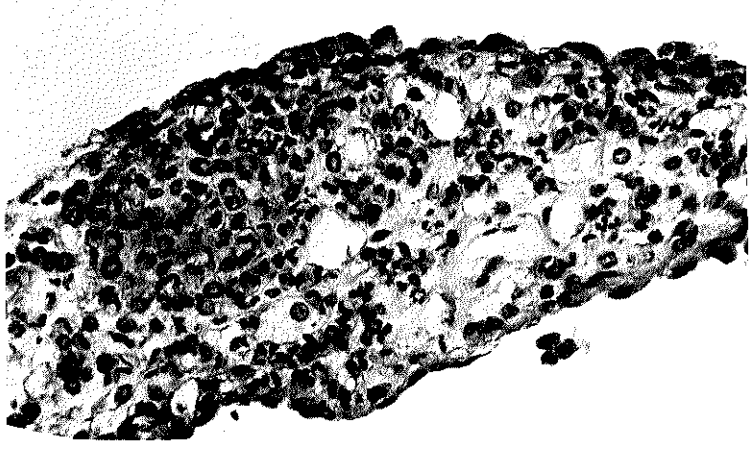

Figure 6.4. Micrograph of a section of the mesentery of the same animal as in Fig. 6.3. Note mostly immature erythroid cells and maturing myeloid cells. There are also several mitotic figures. Fat cells of the mesentery are almost totally replaced by the proliferating hemopoietic cells. Some of the mesothelial cells have disappeared. H & A. x 320.

teric foci to be the result of proliferation of the inoculated spleen cells. Since the inocula also contained a certain number of stem cells, however, the probability of clonal proliferation of stem cells is very high. Cells form both normal and neoplastic spleens showed the capacity for nidation and proliferation in the mesentery between fat cells, where they formed distinct and sometimes confluent foci. From this point on, these cellular foci in the mesentery were designated as mesenteric hemopoietic colonies (MHC) (UMAR and van GRIENSVEN 1977). The neoplastic state of the hemopoietic tissue from which the cells derived did not interfere with the formation of MHC. Surprisingly, no ascites forms were found after ip inoculation of neoplastic hemopoietic cells. MHC were never seen under the parietal peritoneum.

The neoplastic cells, which can carry numbers of virions, did not cause R-MuLV infection in recipient mice within the 7-day observation period, because the number of infectious virus particles injected together with cells in inocula was negligible.

Next, 10^5 neoplastic spleen cells were incubated with rabbit antiserum to R-MuLV at 37°C for 20 minutes and inoculated ip in 4 BALB/c mice. R-MuLV antigens on the cell surface and probably also intracytoplasmic antigenic factors were stabilized and even inactivated by the incubation with specific antiserum. Cytolysis was not observed after 20 minutes of incubation with specific antiserum. Intraperitoneal inoculation of these incubated spleen cells led to the formation of MHC comparable to those occurring after ip transplantation of normal or neoplastic spleen cells without incubation with antiserum.

On the basis of these findings it was concluded that, at least in BALB/c mice, the mesentery with its adipose and reticular structures is a suitable site for the transplantation of normal or neoplastic hemopoietic cells.

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CHAPTER 7

MESENTERIC HEMOPOIETIC COLONIES

7.1. Introduction

In healthy adult mice hemopoiesis takes place in the bone marrow and spleen (DUNN 1954; SCHERMER 1967). When these organs undergo radiation damage, an intravenous injection of syngeneic bone marrow or spleen cells can lead to recovery by re-population of the hemopoietic system (LINDSEY et al. 1955; MAKINODAN et al. 1958). TILL and McCULLOCH (1961) showed that in mice recovery after irradiation is effected by the formation of hemopoietic colonies in the spleen. These colonies become easily visible with naked eye after fixation of the organ in Bouin's solution. Each of the spleen colonies is considered to be the product of the proliferation of a single stem cell present in the inoculum (BESSIS 1973).

It has also been shown that intraperitoneal transplantation of spleen tissue (HALEY et al. 1975) and of bone marrow and spleen cells has a protective effect in lethally irradiated mice (LORENZ and CONGDON 1954a, 1954b; van BEKKUM et al. 1956; CONGDON 1959), but the morphological evolution of this process was not described.

When syngeneic normal and neoplastic hemopoietic cells were inoculated ip in BALB/c mice, they induced the formation of functionally active mesenteric hemopoietic colonies (see Chapter 6). In the first set of the present experiments (7.2.) we obtained mesenteric hemopoietic colonies (MHC) from syngeneic normal spleen and bone marrow cells in BALB/c mice. We then investigated the occur-

rence and protective effect of MHC in the lethally irradiated BALB/c mice, using syngeneic normal and leukemic hemopoietic cells.

The occurrence and the protective effect of MHC against radiation damage in lethally irradiated BALB/c mice raised the question of whether the occurrence of MHC is a general phenomenin in mice and perhaps even in higher species. In the second set of transplantation experiments the occurrence and protective effect of MHC were studied in six other mouse strains after lethal irradiation, to find out whether MHC formation is a general phenomenon in mice (7.3.).

During these experiments we found small cellular aggregations consisting mainly of macrophages, mesothelial cells, lymphocytes, and a few mast cells, which we call mesenteric pseudocolonies. These pseudocolonies were also found in mice after lethal irradiation. Since they closely resemble the early MHC in both shape and localization, the occurrence and cellular composition of these pseudocolonies were investigated in the third set of experiments (7.4).

Finally, to study the effect of R-MuLV infection, mesenteric hemopoietic colonies, and lethal irradiation on the occurrence and cellular composition of the mesenteric pseudocolonies, the fourth set of experiments was carried out (7.5).

In addition, the occurrence of mesenteric hemopoietic colonies was investigated further in lethally irradiated *Macaca* monkeys after ip inoculation of their own bone marrow cells. This study is described in Chapter 8.

Experimental design, results, and conclusions

7.2. The first set of transplantation experiments, with 3 categories.

In total, 280 specific pathogen-free female BALB/c mice aged 8 weeks and weighing 20-24 g were used. Cell suspensions in HBSS were prepared from the femurs and spleens of 6 syngeneic donor mice according to DICKE et al. (1971). Nucleated cells were counted in a Bürker chamber.

Category 1:

One hundred and fifty mice were divided into 5 equal groups and given an ip inoculation of 10^5, 10^6, 10^7 spleen cells, 5 x 10^5 bone marrow cells, or 0.2 ml HBSS. Half of the animals of each group were killed 7 days and the other half 14 days after inoculation (see Table 7.1).

Table 7.1.

Design of experiments of category 1

Number of non-irradiated BALB/c mice	Inocula
30	10^5 spleen cells
30	10^6 spleen cells
30	10^7 spleen cells
30	5 x 10^5 bone marrow cells
30	0.2 ml HBSS

Cellular foci were present in the mesentery of all non-irradiated BALB/c recipients, 7 and 14 days after ip inoculation of normal syngeneic bone marrow cells and spleen cells. These triangular foci of hemopoietic cells were located among fat cells of the mesentery under the mesothelium. The MHC were composed of cells histologically comparable to the inoculated cells, and counts of 200 randomly selected microscopical fields showed on average 2 mitotic figures per field (objective x 40). Differentiation and maturation of inoculated hemopoietic cells were observed in all foci. Frequently, megakaryocytes were seen close to small groups of proerythroblasts (UMAR and van GRIENSVEN 1977a). All MHC contained a few lymphocytes and macrophages, the content of the latter usually including hemosiderin pigment. Besides MHC, there were so-called mesenteric pseudocolonies, which were composed mainly of macrophages and lymphocytes and showed no increase in size or histological changes in the cellular content 14 days after ip inoculation of hemopoietic cells. The term mesenteric pseudocolonies was given to these cellular aggregations because of their close resemblance in shape and localization to the MHC, especially during the early period of MHC formation.

On the 14th day, the MHC were slightly larger than those of occurring on the 7th day, but no appreciable increase in their number was observed.

The various concentrations of spleen cells in the inocula had no histological effect on the formation of MHC.

It was concluded that ip inoculated hemopoietic cells from both spleen and bone marrow induce the formation of MHC in non-irradiated BALB/c mice.

Category 2:

Ninety mice divided into 3 equal groups were given total body irradiation (40 rad/min) with a Philips-Müller roentgen generator. This lethal exposure permits development of less than 0.2 spontaneous colonies/spleen. Within 2 hours, 30 mice were inoculated ip with 10^6 spleen cells and 30 mice with 5×10^5 bone marrow cells; the other 30 mice were untreated. On the 5th, 10th, and 15 days after the inoculation 8 mice in each group were killed, the remaining animals being retained for survival determination (see Table 7.2).

Table 7.2.

Design of experiments of category 2

Number of irradiated BALB/c mice	Irradiation dose (rad)	Inocula	Number of mice autopsied on day			
			5	10	15	at death
30	730	5×10^5 bone marrow cells	8	8	8	6
30	730	10^6 spleen cells	8	8	8	6
30	730	0.2 ml HBSS	8	8	8	6

Mesenteries from lethally irradiated mice showed similar foci of proliferating cells after ip inoculation of normal syngeneic bone marrow cells and spleen cells. The number and size of the MHC increased with time, and confluence was seen as early as the 10th day after cellular inoculation. This feature was only observed in lethally irradiated mice. The spleen and bone marrow were depleted of hemopoietic cells after lethal irradiation, and bone marrow hemopoiesis did not recover within 15 days after ip inoculation of hemopietic cells. The spleen, however, showed macroscopically identifiable colo-

nies by the 15th day. The number of these spleen colonies varied from 8 to 43 in individual mice. Proliferating hemopoietic cells were found histologically in these spleen colonies. Erythropoietic and myelopoietic cells were present, and some of the colonies also contained megakaryocytes.

The MHC in mice in this group showed a mixed cellular population (Fig. 1). Erythroid, myeloid, and megakaryocytic cells were found in all MHC. On average, 4 mitotic figures per field (objective x 40) were found in counts of 200 randomly selected fields on the 15th day after ip inoculation of both spleen and bone marrow cells. The origin of the hemopoietic cells did not influence the occurrence of MHC or have any apparent effect on their number.

The mean survival period of 12 lethally irradiated BALB/c mice after ip inoculation of normal syngeneic hemopoietic cells was 80 days. At the time of death, the bone marrow and spleen of these

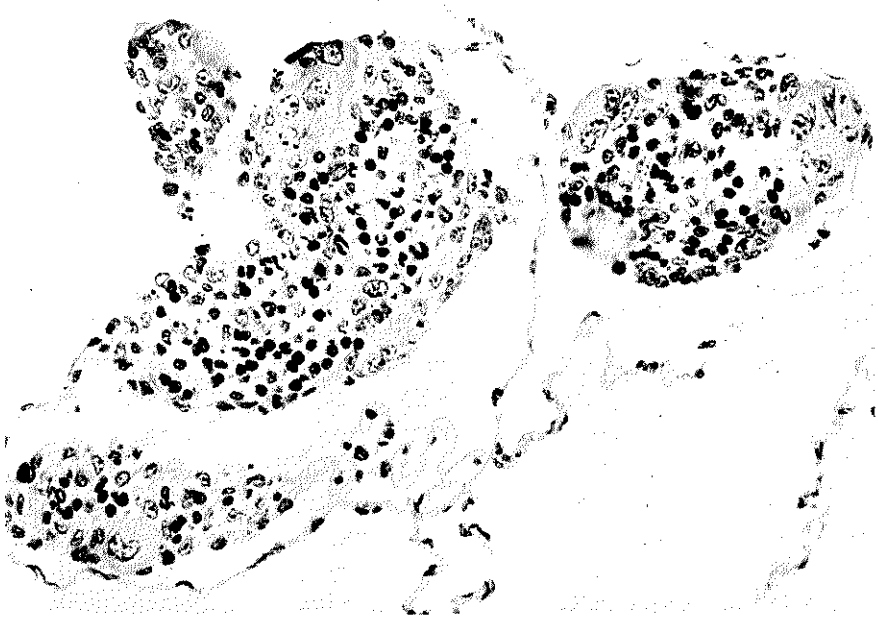

Figure 7.1. Mesentery of a non-irradiated BALB/c mouse 10 days after ip inoculation of 10^6 syngeneic normal spleen cells. Note mesenteric hemopoietic colonies showing megakaryocytes and erythropoietic and myelopoietic cells. H & A. x 500.

mice were almost completely re-populated by functioning hemopoietic cells.

In the control mice injected ip with HBSS, we found only mesenteric pseudocolonies. All of the lethally irradiated mice not injected with cell suspensions died between 10 and 30 days after irradiation with loss of weight and cerebral and intestinal syndromes essentially attributable to thrombocytopenic bleeding. None of these animals developed MHC or spleen colonies spontaneously. The lymph nodes and thymus of the lethally irradiated mice became atrophic during the 15-day observation period.

It was concluded that the MHC can also occur in lethally irradiated BALB/c mice after ip inoculation of normal syngeneic hemopoietic cells. Hemopoietic spleen colonies too can be induced in this way. MHC and spleen colonies function together in the protection of mice against radiation damage to hemopoietic system.

Category 3:

Using leukemic spleen cells, we obtained MHC in the mesentery of non-irradiated BALB/c mice. The occurrence of MHC in 40 lethally irradiated BALB/c mice was investigated after ip inoculation of both spleen and bone marrow cells deriving from mice in the neoplastic period of R-MuLV infection.

Leukemia was induced in BALB/c mice by ip injection of 4 mg purified R-MuLV. The leukemic mice, which showed all of the morphological signs of the disease (BOIRON et al. 1965; van GRIENSVEN et al. 1974), were killed 26 days after the injection, and neoplastic spleen and bone marrow cells were collected.

Twenty lethally irradiated mice in each group received ip inoculations of cell suspensions deriving from leukemic mice (either 10^6 bone marrow cells or 10^7 spleen cells). Ten days after the inoculation, these mice were killed (see Table 7.3) and macroscopic spleen colonies were counted after immersion of the organ for 2 hours in Bouin's solution. The mesentery was cut out into four pieces before being placed in the fixative solution. Paraffin-embedded 5 μm thick serial sections were stained with hematoxylin-azophloxin. Material from the spleen, liver, peritoneum, thymus, inguinal and mesenteric lymph nodes, and sternum was also studied histologically.

Table 7.3.

Design of experiments of category 3

Number of irradiated BALB/c mice	Irradiation dose (rad)	Inocula from R-MuLV infected BALB/c mice	Autopsied on the 10th day
20	730	10^6 bone marrow cells	20
20	730	10^7 spleen cells	20

MHC produced by inoculation of cell suspensions from leukemic mice grew very rapidly in the lethally irradiated BALB/c mice, and soon became confluent. Counts of 200 randomly selected microscopic fields (objective x 40) gave an average of 6 mitotic figures in each field. The MHC could not, however, be seen macroscopically. The histological appearance of the MHC in this group of mice was microscopically comparable to that of colonies produced by the inoculation of normal syngeneic hemopoietic cells except for the presence of apoptic cells, which were seen only in these MHC and not in those occurring in the above-described experiment.

The MHC almost always showed a mixture of erythroid, myeloid, and megakaryocytic hemopoietic cells in various stages of maturation. The maturation of the erythropoietic cells was, however, retarded, and as a result the MHC in this group contained distinctly more immature cells than those in the other groups. Histologically, proerythroblasts were found to predominate.

The spleen showed several hemopoeitic colonies detectable with the naked eye (Fig. 2). The three developmental stages of erythroid

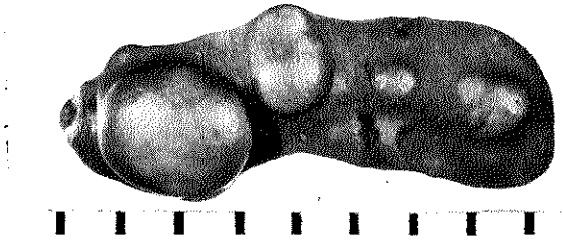

Figure 7.2. Spleen of a lethally irradiated BALB/c mouse 10 days after ip inoculation of 10^7 spleen cells deriving from leukemic mice. Note the colonies varying in size and shape. Scale indicates millimeters.

spleen colonies described by van BEEK et al. (1976) were distinguished histologically. Most of the spleen colonies showed mixture of hemopoietic cells, but a few colonies were of purely erythropoietic. Disseminated apoptotic cells were found in all spleen colonies.

The mesenteric pseudocolonies were invariably found in this group as well.

In the liver we found some small intrasinusoidal cellular foci that could be interpreted as the initial stage of R-MuLV infection. The peritoneum was free of any kind of cellular nidation. The lymph nodes and thymus were atrophic. The bone marrow of the sternum was hemorrhagic and showed no signs of re-population 10 days after lethal irradiation and ip inoculation of neoplastic hemopoietic cells. However, a marked reticulocytosis was found in the peripheral blood samples taken just before the mice were killed on the 10th day.

It was concluded that neoplastic hemopoietic cells of mice were able to form MHC in lethally irradiated BALB/c mice. The occurrence of hemopoietic spleen colonies was accepted as an indicator of the presence of stem cells in the inocula. It was found that the apoptotic cells observed in both the MHC and hemopoeitic spleen colonies were the result of the R-MuLV infection, which was transferred to the new host.

7.3. The second set of transplantation experiments

These experiments were designed to show whether MHC formation is a general phenomenon in mice. Six other strains of mice, selected from the list of STAATS (1972), were used. Recipients, aged 6 to 8 weeks, received lethal total body irradiation. Irradiation doses leading to the development of less than 0.2 spontaneous colonies per spleen in non-transplanted control mice, were given in accordance with previously established values in our laboratory. Table 7.4 shows the strains and the lethal dose of total body irradiation applied.

Suspensions of normal syngeneic bone marrow cells in HBSS were prepared from the femurs of two donors. Inocula containing 10^7 bone marrow cells in 1 ml were given ip to 8 recipients 2 hours after lethal irradiation. Five and 10 days after the inoculation, 4

Table 7.4.

Design of the second set of transplantation experiments

Mouse strains	Sex	Irradation dose (rad)	Autopsied after 5 days	Autopsied after 10 days	E/M* ratio after 5 days	E/M* ratio after 10 days
CBA	f	875	4	4	25/27	59/41
C57BL	f	825	4	4	20/80	56/44
CPB-Q	f	900	4	4	30/70	61/39
Swiss	f	900	4	4	24/76	60/40
C3H	m	875	4	4	21/79	57/43
F_1(C57BLxCBA)	m	975	4	4	18/82	55/45

*E/M = erythroid/myeloid

mice in each group were killed by cervical dislocation. The mesentery and spleen were studied macro- and microscopically as described above.

Results of the second set of experiments:

In all of the lethally irradiated mice of the indicated six strains, the occurrence of MHC was demonstrated histologically 5 and 10 days after ip inoculation of normal syngeneic bone marrow cells (UMAR and van GRIENSVEN 1977b). Cells of MHC, which lay between fat cells of the mesentery, showed many mitotic figures. Counts of 200 microscopic fields (objective x 40) showed an average of 3 mitotic figures in CBA mice and 4 mitotic figures in C3H mice per field. Erythroid, myeloid, and megakaryocytic differentiation and various stages of maturation were seen in these MHC in all six strains (Figs. 3 and 4), but mixed cellular nature of these MHC was a prominent feature. The ratio of erythroid to myeloid cells in the 5-day-old MHC was predominantly in favour of myeloid cells (E/M = 23/77), whereas in 10-day-old MHC this ratio was reversed (E/M = 58/42). Only the C3H mouse strain showed pure megakaryocytic colonies in both the mesentery and the spleen (Fig. 5).

The mesenteries of all six mouse strains regularly showed mesenteric pseudocolonies as well as MHC.

The number of hemopoietic spleen colonies was distinctly increased on the 10th day after the inoculation, although it ranged from an average of 4 colonies in C3H mice to 17 colonies in CBA

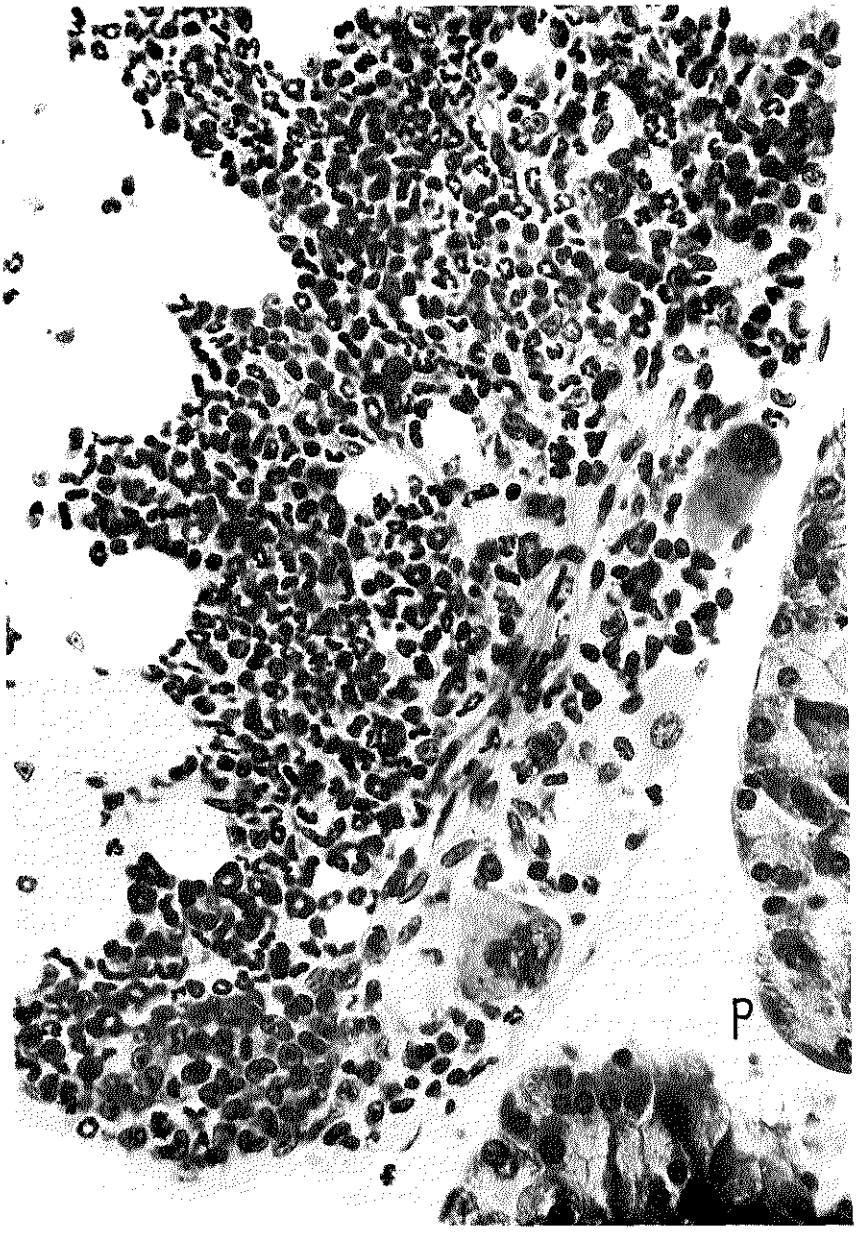

Figure 7.3. Mesentery of a lethally irradiated C57BL mouse showing a mesenteric hemopoietic colony 5 days after ip inoculation of 10^7 normal syngeneic bone marrow cells. Note the hemopoietic cells showing various degrees of maturation and differentiation. P – Pancreas. H & A. x 380.

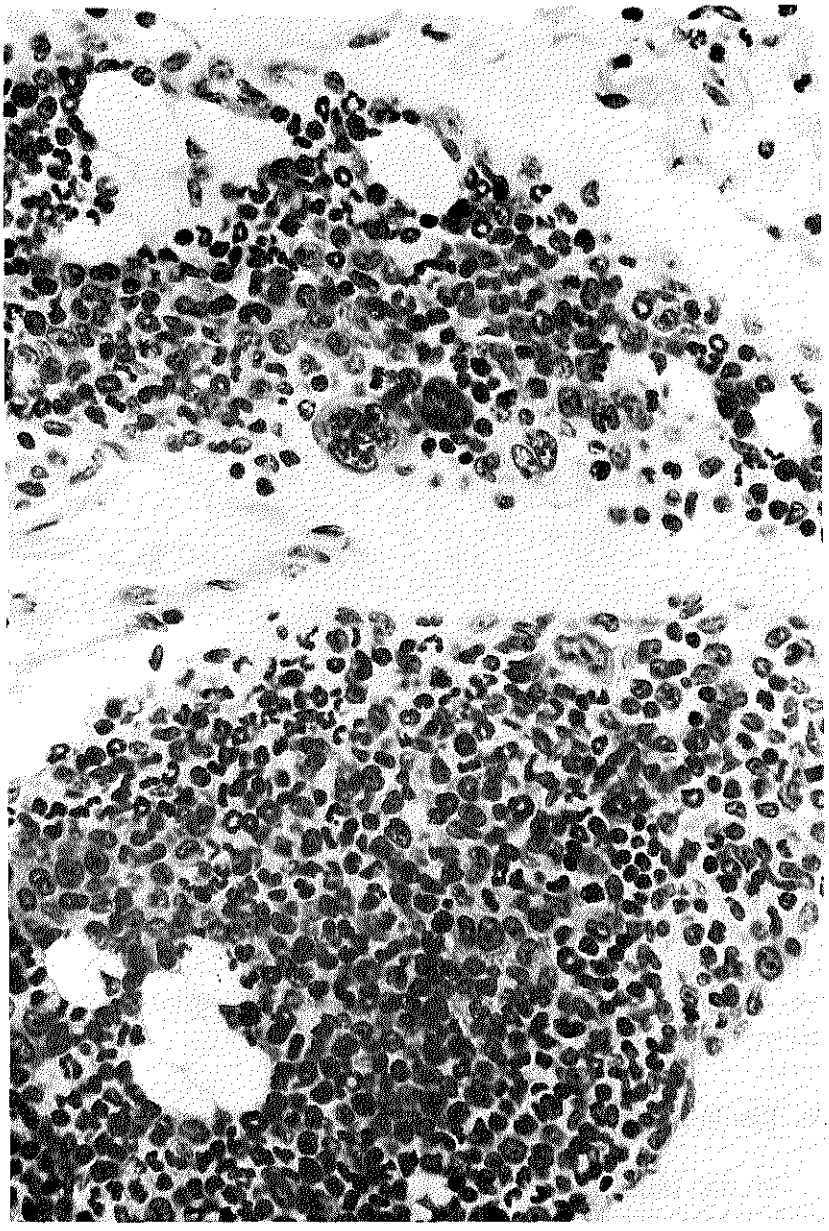

Figure 7.4. Mesentery of a lethally irradiated CBA mouse showing a mesenteric hemopoietic colony 10 days after ip inoculation of 10^7 normal syngeneic bone marrow cells. Note the mixed nature of hemopoietic cells, and also the many mitotic figures. H & A. x 380.

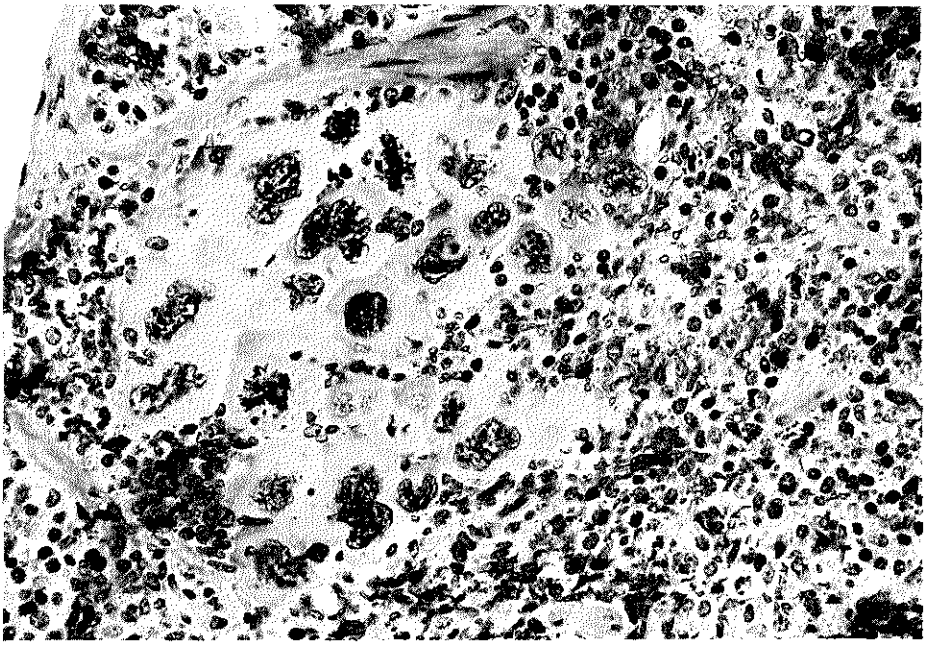

Figure 7.5. Spleen of a lethally irradiated C3H mouse 10 days after ip inoculation of 10^7 normal syngeneic bone marrow cells. Note the pure megakaryocytopoietic colony on the left, between the septa. H & A. x 360.

mice. The spleen colonies were histologically comparable to those seen after intravenous induction in lethally irradiated mice (CURRY and TRENTIN 1967). Complete re-population of the spleens did not occur within the observation period of 10 days.

We therefore assume that the formation of MHC is a general phenomenon in mice, and that the murine mesentery acts as a microenvironment for extramedullary hemopoiesis (TRENTIN 1970). Stem cell mobilization from MHC occurs and is expressed in the formation of hemopietic spleen colonies.

7.4. The third set of experiments

In the third set of experiments, the cellular composition of pseudocolonies in the mesentery of mice was studied. The details of these experiments are indicated in Table 7.5.

Mouse peritoneal macrophages can be labelled with carbon particles by injection of a colloidal carbon suspension into the ab-

Table 7.5.

Design of the third set of experiments

Number of mice	Treatment	Studies
4 BALB/c	HBSS	Histology of the mesentery
4 Swiss	HBSS	Histology of the mesentery
4 BALB/c	20 mg carbon	Autopsied 24 hrs after carbon injection
	ip	Histology of the mesentery and RES*
4 Swiss	20 mg carbon	Autopsied 24 hrs after carbon injection
	ip	Histology of the mesentery and RES

*RES = Reticuloendothelial system, including liver, spleen, and lymph nodes.

dominal cavity. We used this method to differentiate between the cellular components of the mesenteric pseudocolonies of mice. After dialysis of a carbon suspension containing 100 mg of carbon per milliliter (Günther Wagner, Hannover, West Germany, batch no. C11-1431a) against water to remove the phenol (COTRAN 1965), 0.2 ml of the colloidal suspension containing 20 mg carbon particles was injected ip into 4 BALB/c and 4 Swiss mice. The animals were killed 24 hours after the injection, and the mesentery, liver, spleen, bone marrow, and mesenteric lymph node were studied histologically. Four untreated BALB/c mice and 4 untreated Swiss mice served as controls.

Results of the third set of experiments:

In BALB/c and Swiss mice after ip injection of HBSS or colloidal carbon suspension, serial sections showed the presence of mesenteric pseudocolonies. In all instances these pseudocolonies lay just beneath the mesothelium and were identifiable by their triangular shape. These mesenteric pseudocolonies were composed of mesothelial cells, macrophages, lymphocytes, and a few mast cells (Figs. 6 and 7). No mitotic figures were seen in the uniformly polygonal, acidophilic mesothelial cells in the pseudocolonies. The only similarity between these pseudocolonies and early MHC was the localization and shape. Hemopoietic cells were never found in the mesenteric pseudocolonies. The cellular components of pseudocolonies showed no significant difference between the two strains

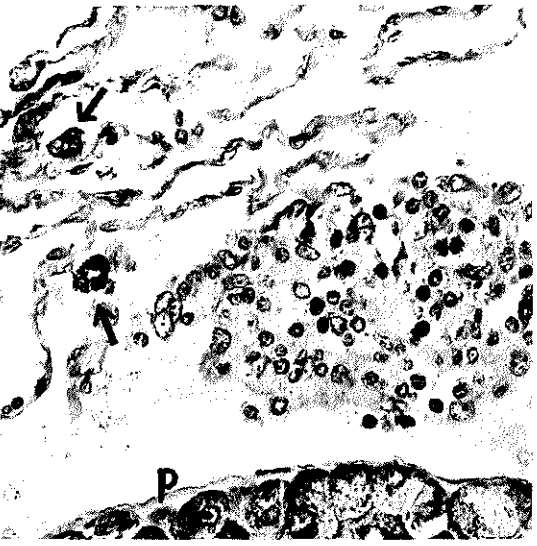

Figure 7.6. A pseudocolony of the mesentery after ip injection of HBSS into non-irradiated BALB/c mouse. Groups of mesothelial cells among which lie macrophages and lymphocytes. Mast cells (arrows); P – pancreas. H & A. x 380.

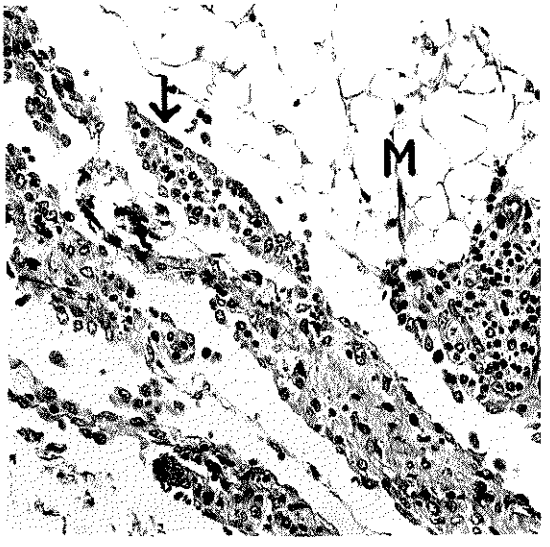

Figure 7.7. A pseudocolony formed by proliferated mesothelial cells (arrows) after ip injection of HBSS into non-irradiated BALB/c mouse. M – mesentery. H & A. x 235.

studied or within individual mice. After ip injection of the carbon suspension, there was no noteworthy alteration in the number, form, or cellular composition.

After ip injection of a colloidal carbon suspension, many macrophages loaded with carbon particles were found but the mesothelial cells were still recognizable as the main component of the mesenteric pseudocolonies (Fig. 8). However, some mesothelial surface cells showed a low degree of phagocytic activity against carbon particles.

The cellular components of mesenteric pseudocolonies consist, in order of decreasing numbers, of mesothelial cells, macrophages, lymphocytes, and mast cells. The macrophages can be loaded by the injection of a colloidal carbon suspension. Lethal irradiation results in the disappearance of lymphocytes from these non-hemopoietic cellular aggregations of the mesentery.

We therefore assume that the mesenteric pseudocolonies are a rather constant histologic feature in mice, and that peritoneal macrophages (DAEMS and BREDEROO 1971) might originate from mesenteric pseudocolonies.

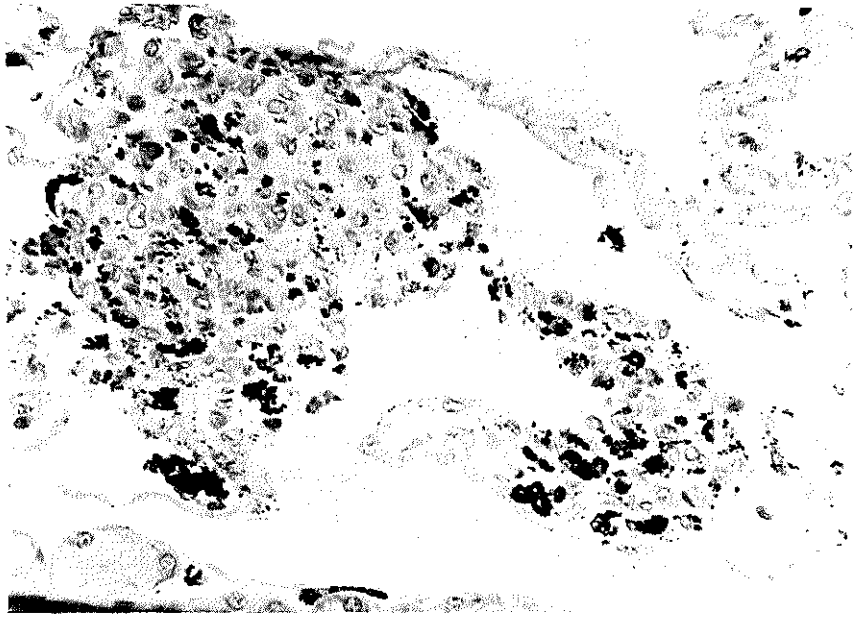

Figure 7.8. Two mesenteric pseudocolonies containing macrophages loaded with carbon particles. Some of the mesothelial cells also contain carbon particles, but fewer than the macrophages. H & A. x 380.

7.5. The fourth set of experiments

The fourth set of experiments were designed to study the effect of the formation of MHC, and of R-MuLV infection on the occurrence and cellular components of the mesenteric pseudocolonies. Table 7.6 shows the details of these experiments.

Thirty male BALB/c mice were infected ip with 4 mg R-MuLV, and on the 19th day were given 750 rad lethal total body irradiation before the inoculation of 10^7 normal bone marrow cells. Twelve days later, 4 of these mice were given an ip injection of 20 mg carbon suspension and on the following day they were killed for analysis, mainly of the mesenteries. The remaining 26 mice were used to determine the survival time. Controls were 10 male BALB/c mice given 750 rad lethal total body irradiation. The survival time was determined and the mesenteries were studied at the time of death. In addition, 10 male BALB/c mice were injected ip with 4 mg R-MuLV and the mesenteries were studied at the time of death.

Table 7.6.

Design of the fourth set of experiments

Number of BALB/c mice	Treatment	Studies
30	4 mg R-MuLV ip	Only 4 mice received carbon suspension
	750 rad lethal dose on the 19th day	Determination of survival in 26 mice
	10^7 bone marrow cells ip on the 19th day	
	20 mg carbon suspension 12 days later	
10	750 rad lethal dose	Survival time and histology of mesentery
10	4 mg R-MuLV	Histology of mesentery at death

Results of the fourth set of experiments:

The 10 lethally irradiated (750 rad) male BALB/c mice died, with thrombocytopenic bleeding, between 9 and 18 days after radiation. Histological investigation of the mesenteries showed an extremely atrophic adipose tissue with tiny mesothelial cells. Mesenteric pseudocolonies of these mice were distinctly smaller than those of non-irradiated mice. The number of lymphocytes was greatly reduced, and the other cellular components were all present.

The 10 BALB/c mice infected ip with 4 mg R-MuLV, died 27 to 34 days after injection of the virus. The mesenteries showed both pseudocolonies and neoplastic infiltrations. The R-MuLV infection did not affect either the cellular composition or the size of the mesenteric pseudocolonies.

All of the 30 BALB/c mice had palpable spleens on the 19th day of R-MuLV infection before the administration of lethal total body irradiation (750 rad) and an ip inoculation of 10^7 normal syngeneic bone marrow cells on the same day. Twenty-six of these mice survived between 84 and 97 days after lethal irradiation, whereas in our previous experiments mice given a lethal dose of radiation did not survive more than a month. Histologically, the mesenteries showed confluent MHC and also mesenteric pseudocolonies. The MHC were, in general, of a mixed cellular type and showed many mitotic figures. Iron-pigment-loaded macrophages were also found in the MHC.

The spleens of these mice weighed at most 600 mg and were completely re-populated by functioning hemopoietic cells, but the spleen colonies with their characteristic growth pattern were still macro- and microscopically recognizable. Many iron-pigment-loaded macrophages were found throughout the spleen. Fibrous scar tissue and signs of old hemorrhages wers still present even three months after irradiation.

The bone marrow was also re-populated by hemopoietic cells.

In the 4 mg R-MuLV infected BALB/c mice lethally irradiated and injected ip with colloidal carbon suspension 12 days after ip inoculation of bone marrow cells, histological study of the mesenteries revealed many confluent MHC and disseminated pseudocolonies. In the MHC a few macrophages contained carbon particles, whereas the pseudocolonies showed many macrophages loaded with carbon particles (Fig. 9). The spleens of these mice showed macro-

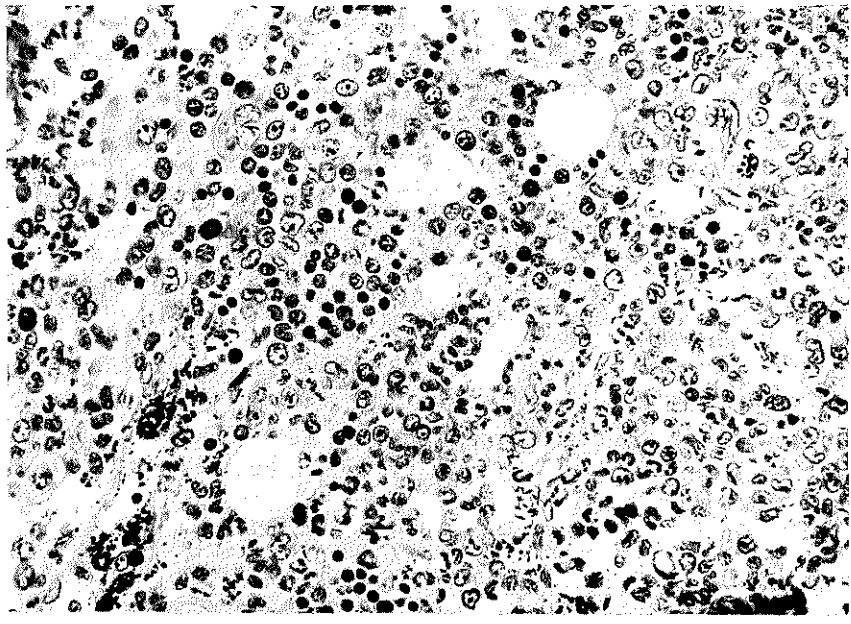

Figure 7.9. Mesentery of a BALB/c mouse treated as follows: ip injection of 4 mg R-MuLV, 19 days later lethal irradiation and ip inoculation of 10^7 normal syngeneic bone marrow cells, 4 days thereafter ip injection of 20 mg carbon suspension. Note the mesenteric hemopoietic colony of mixed cellular composition and the presence of considerably fewer macrophages containing carbon particles than in Fig. 7.8.

phages with both iron pigment and carbon particles. Several hemopoietic spleen colonies were also found.

It was therefore concluded that the presence of MHC and R-MuLV infection did not affect the persistence or the cellular composition of the mesenteric pseudocolonies. MHC functioned in the recovery and maintenance of the hemopoeitic system in mice previously infected with R-MuLV and lethally irradiated on the 19th day of infection.

7.6. Discussion

All mice inoculated ip with normal or neoplastic syngeneic hemopoietic cell suspensions developed mesenteric hemopoietic colonies, also those given lethal irradiation before transplantation. Although the clonal origin of MHC was not separately investigated, the reproducible histological and functional patterns strongly suggest that these colonies are clonal in nature (MITCHINSON 1956; MEKORI and FELDMAN 1965).

It may be postulated that MHC contain proliferating stem cells, e.g. CFU-S (METCALF and MOORE 1971), that are capable of migration to and colonization of the spleen (McCULLOCH et al. 1974; HASHTORPE and HODGSON 1974). Our findings indicate that lethally irradiated mice given an adequate number of hemopoietic cells intraperitoneally, can survive. This provides supporting evidence for the hypothesis regarding cellular radiation protection (VOS et al. 1956). The spontaneous occurrence of MHC was never observed in either normal or lethally irradiated mice. The MHC must therefore have originated from ip inoculated hemopoietic cells. Since the inocula contained a certain number of stem cells, it is clear that these cells can reach the mesentery, where they can proliferate and form MHC.

The mesenteric stroma with its adipose and reticular structure may function as an inductive micro-environment for hemopoietic stem cells. Furthermore, this stroma is rich in capillaries and macrophages. These essential features are also present in bone marrow. On the basis of the present findings, stem cell proliferation and clonal growth in the mesentery of mice seems to us to explain the formation of MHC. This is why we propose that these mesenteric colonies,

which to the best of our knowledge have not been described before, be called mesenteric hemopoietic colonies, or MHC.

Adipose tissue of mice proved to be a suitable site for hemopoietic tissue transplantations (AGGIO et al. 1974; WARNER and KRUEGER 1975; HALEY et al. 1975), and the subcutaneous capillary-rich adipose tissue of the hamster cheek pouch has been successfully used for the transplantation of bone marrow cells (McCUSKEY et al. 1975). The mesentery is a new addition to this series.

The numerous mitotic figures observed in the MHC of lethally irradiated mice account for their rapid expansion and early confluent growth. The observation that MHC growth is not as rapid in non-irradiated mice suggests involvement of a feedback mechanism.

The murine MHC, which are invariably microscopic in size, cannot be mistaken for accessory spleens. The latter were seldom observed in our experimental series. Accessory spleens are larger than MHC and are always encapsulated in fibrous tissue. MHC grow between fat cells of the mesentery and tends to become confluent very soon.

Because of the small volume of the MHC, attempts to determine their absolute numbers by counts in serial sections led to standard deviations from the mean that were too large for statistical purposes. For acceptable quantitative results, the counting method will have to be improved.

The induction of MHC in lethally irradiated mice after R-MuLV infection led to an unexpectedly long survival time and a total repopulation of the diseased hemopoietic system. This findings seemed to offer a therapeutic approach to neoplastic diseases, for instance murine leukemia. To investigate the applicability of this finding, we attempted to produce MHC in lethally irradiated monkeys. The results of these experiments made it clear that the induction of MHC is also possible in higher species (see Chapter 8).

MHC originating from the hemopietic cells of mice in the neoplastic period of R-MuLV infection were found to be functional and produced hemopoietic spleen colonies. Both MHC and hemopoietic spleen colonies showed numerous apoptotic cells. It has been shown that apoptosis can also be a result of R-MuLV infection. We therefore concluded that in our material apoptosis was a consequence of R-MuLV infection, most probably of stem cells. Apoptotic cells were never observed in the MHC induced by ip inoculation of normal

syngeneic hemopoietic cells or in the spleen colonies originating from these MHC. The morphological characteristics of spleen colonies arising from MHC induced by ip inoculation of neoplastic hemopoietic cells, were similar to those described by van BEEK et al. (1976).

Neither the R-MuLV infection nor the neoplastic state of the donor mice interfered with the formation and persistence of MHC and spleen colonies or their protective activity against radiation damage.

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CHAPTER 8

OCCURRENCE OF MESENTERIC HEMOPOIETIC COLONIES IN *CYNOMOLGUS* MONKEYS AFTER TRANSPLANTATION OF NORMAL AUTOGENEIC BONE MARROW CELLS

Introduction

Regeneration of the hemopoietic system of rodents after irradiation can be induced by the transplantation of viable hemopoietic cells (LINDSEY et al. 1955; MAKINODAN et al. 1958). TILL and McCULLOCH (1961) showed that the recovery is effected by the formation of hemopoietic colonies in the spleen. The intraperitoneal transplantation of spleen tissue (HALEY et al. 1975) and bone marrow or spleen cells was reported to have a protective effect in lethally irradiated mice (LORENZ and CONGDON 1954; van BEKKUM et al. 1959; CONGDON 1959), but the process of regeneration itself was not described.

Recently, we demonstrated that the intraperitoneal injection of syngeneic hemopoietic cells into lethally irradiated BALB/c mice leads to the formation of mesenteric hemopoietic colonies (UMAR and van GRIENSVEN 1977a). These studies were extended to include six more strains of mice, all of which showed the same response (UMAR and van GRIENSVEN 1977b).

Recovery from radiation damage occurs by the formation in the mesentery of actively proliferating hemopoietic colonies of erythroid, myeloid, megakaryocytic, or mixed origin. Ten days after transplantation, the colony growth leads to confluency and reticulocytosis is observed. Fifteen days after transplantation, large spleen colonies are found. Ultimately, the mice recover completely.

Rodents, however, show a somewhat different immunologic reaction to the transplantation of hemopoietic cells than the response seen in humans. This raised the question of whether primates, which are more closely related to man, show the same response to intraperitoneal transplantation. If that were the case, this particular route of transplantation would be worthwhile investigating, since it could be considered for application in patients.

The purpose of this last study was to find out whether lethally irradiated monkeys can form mesenteric hemopoietic colonies after the intraperitoneal transplantation of bone marrow cells.

Material and methods

In this study use was made of *Cynomolgus* monkeys (*Macaca fascicularis*). For details concerning the methods (maintenance care, preparatory procedures, irradiation, and transplantation), see Chapter 3.

Results

Cynomolgus monkeys subjected to total body irradiation (800 r) and ip injected with their own bone marrow cells deriving from one femur, invariably suffered from thrombocytopenia, which developed within 72 hours after lethal irradiation and continued progressively. The thrombocytopenia resulted in bleeding of the serous and mucous surfaces. In three cases heavy intestinal, nasal, and pulmonal bleeding preceded spontaneous death.

As can be seen from Table 8.1, all monkeys that survived between seven and sixteen days after lethal irradiation and ip injection of autogeneic bone marrow cells, showed MHC microscopically. One monkey died 24 hours after the operation with massive bleeding and without signs of MHC formation. In the liver and mesentery of this monkey well-capsulated, shrunken parasites were found.

Some of the hemopoietic colonies in the mesentery were macroscopically recognizable, since they had reached a size of 6 mm and showed a blueish-red color. Histologically, MHC were identified in almost all sections (Figs. 1, 2, 3 and 4). Most of the MHC were

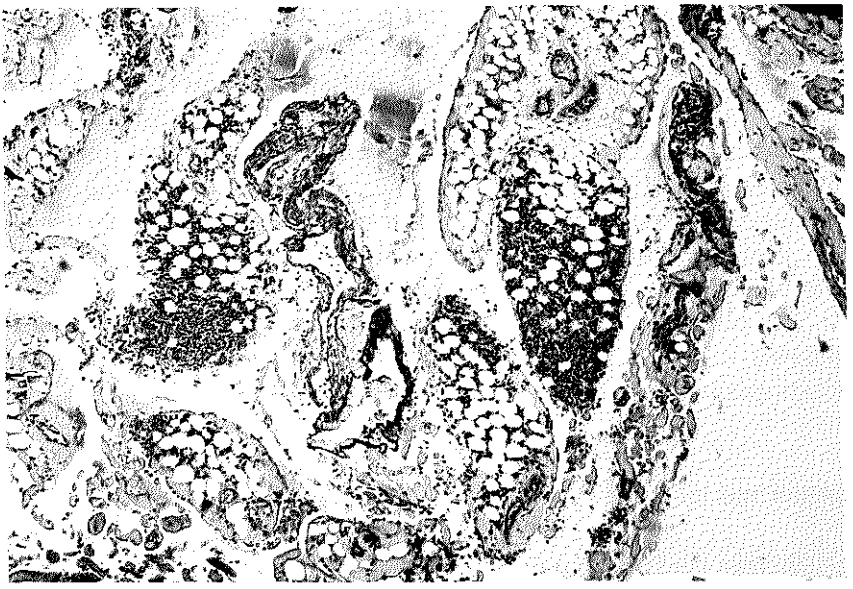

Figure 8.1. Mesentery of lethally irradiated *Macaca fascicularis* monkey. Mesenteric hemopoietic colonies present 12 days after intraperitoneal inoculation of autogeneic bone marrow cells. H & A. x 60.

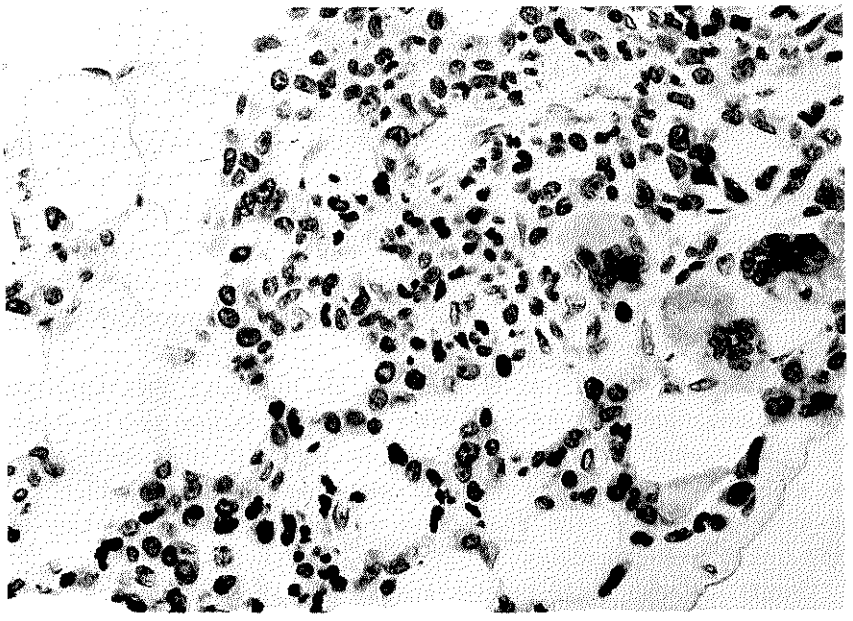

Figure 8.2. Mesentery of *Macaca fascicularis* monkey 15 days after lethal irradiation and intraperitoneal inoculation of autogeneic bone marrow cells. Micrograph showing details of mixed mesenteric hemopoietic colony. H & A. x 360.

found to have a mixed population, showing all types of hemopoietic cells in various proportions. However, some pure erythroid or myeloid colonies were also observed. A small number of similar foci were seen in the omentum of two monkeys. In all MHC iron pigment was present in abundance. The hemopoietic cells, which are the main component of the colonies, showed numerous mitotic figures.

In two monkeys autopsied 7 and 15 days after the ip injection of hemopoietic cells, the stroma of the mesentery showed fibroblastic proliferation and thickening as well as partially calcified, newly formed osteoid. The osteoid islands were surrounded by large, cuboidal osteoblasts. Hemopoietic tissue was present between the irregularly arranged bone trabeculae.

The mesenteric pseudocolonies consisting mainly of macrophages and lymphocytes, which had been observed in mice (UMAR and van GRIENSVEN 1977a), were not found in the monkeys. However, disseminated lymphocytes, macrophages, and mast cells occurred in all mesentery sections. No proliferation or aggregation of mesothelial cells was seen.

Spontaneous extramedullary hemopoiesis was not observed in the mesentery of the normal control or the non-transplanted but lethally irradiated monkeys. Splenic or hepatic hemopoiesis was not

Table 8.1.

Mesenteric hemopoietic colonies in *Macaca fascicularis* monkeys after lethal irradiation and ip transplantation of autogeneic bone marrow cells

Monkey number	Irradiation dose (rad)	Intraperitoneal transplantation	Survival (days)	MHC formation	Remarks
1	–	–		–	control
2	800	–	12	–	control
3	800	+	1	–	parasite infection
4	800	+	15	+	
5	800	+	7	+	
6	800	+	14	+	
7	800	+	11	+	
8	800	+	12	+	
9	800	+	12	+	
10	800	+	12	+	

seen in any of the monkeys either. Thymus, lymph nodes, and spleen were invariably atrophic after radiation. The bone marrow tissue collected from the sternum of lethally irradiated monkeys 15 days after transplantation did not show recovery of hemopoiesis.

Discussion

This last study was performed to determine whether mesenteric hemopoietic colonies would develop in primates after lethal whole body irradiation and intraperioneal transplantation of bone marrow cells.

Macaca fascicularis monkeys weighing at most 3 kg received a total body irradiation of 800 rad according to MÜLLER-BERAT et al. (1966). The experiment was carried out in the Radiobiological Institute, T.N.O., Rijswijk, The Netherlands, according to standard procedures (van BEKKUM et al. 1967). Clinical, hematological, and pathological studies performed in control monkeys of the indicated weight invariably showed total destruction of lymphoreticular tissue at the administered radiation dose without necrosis of intestinal mucosa or other undesirable site effects of supralethal irradiation (MÜLLER-BERAT et al. 1966). MERRITT et al. (1972) reported values in close agreement with ours, i.e., 812 rad for *Macaca mulatta* monkeys.

In this study we transplanted autogeneic bone marrow cells to avoid the graft-versus-host disease. The aspirated content of one femur, estimated to contain at least 10^8 living bone marrow cells, is considered large enough to permit successful recovery from radiation damage when injected intravenously (van BEKKUM et al. 1969). In all animals thrombocytopenia occurred soon after lethal irradiation and sometimes caused manifest and serious bleeding. This loss of blood aggravated the monkey's clinical condition, but because we intended to study the formation of mesenteric hemopoietic colonies rather than survival time, we did not apply transfusion of blood or thrombocytes.

The mesentery of all monkeys that survived irradiation and transplantation for more than a week, showed hemopoietic colonies. Mesentery studied during the formation of MHC in mice and monkeys has a structure comparable to some extent to that of normal

bone marrow. Both bone marrow and mesentery are rich in capillaries, and have fat cells and macrophages and a reticular stroma in which the cells are embedded. On the basis of these morphologic properties, mesentery may be assumed to show more or less the same micro-environmental features as bone marrow does.

Almost without exception, the mesenteric hemopoietic colonies induced in mice and also in monkeys showed macrophages loaded with iron pigment. These macrophages lay rather centrally in the erythroid foci. In pure myeloid colonies most of them were found underneath the mesothelium. The source of this iron pigment was undoubtedly phagocytized erythrocytes. The occurrence of a certain number of erythrocytes in aspirated bone marrow is unavoidable, but the presence of erythrocytes in the transplants permits the direct recycling and re-use of iron pigment needed for erythropoiesis. In mice surviving longer than two months after transplantation, the macrophages no longer contained iron pigment. Transplanted non-hemopoietic cells are expected to disappear as a result of phagocytic activity of resident macrophages. Two of our monkeys showed osteoid islands in mesenteric tissue, even with the formation of lamellar bone trabeculae covered by osteoblasts (Fig. 3). The origin of these osteoblasts and newly formed bone tissue is not yet clear. COUTIERE (1975) reported that bone marrow cells can induce bone formation. There are, however, other possibilities, for instance the presence of bone fragments and of osteoblasts in the inocula. Furthermore, mesenteric tissue itself may contain cells that start to differentiate after stimulation by a bone marrow transplant.

The histological and cellular characteristics of MHC in monkeys are similar to those in mice. However, we could demonstrate the migration and determine the numbers of CFU-S in mice from the presence of spleen colonies, whereas in monkeys this was impossible. Consequently, the proliferation and migration of stem cells in monkeys from MHC to the original site of hemopoiesis, i.e., the bone marrow, remains uncertain.

The process of regeneration of the hemopoietic system seems to be slower in monkeys than in mice, in which case the repopulation of bone marrow would need a longer survival time in the former species. Under our conditions, repopulation of bone marrow was not demonstrated histologically within 16 days. Because hemopoietic reconsti-

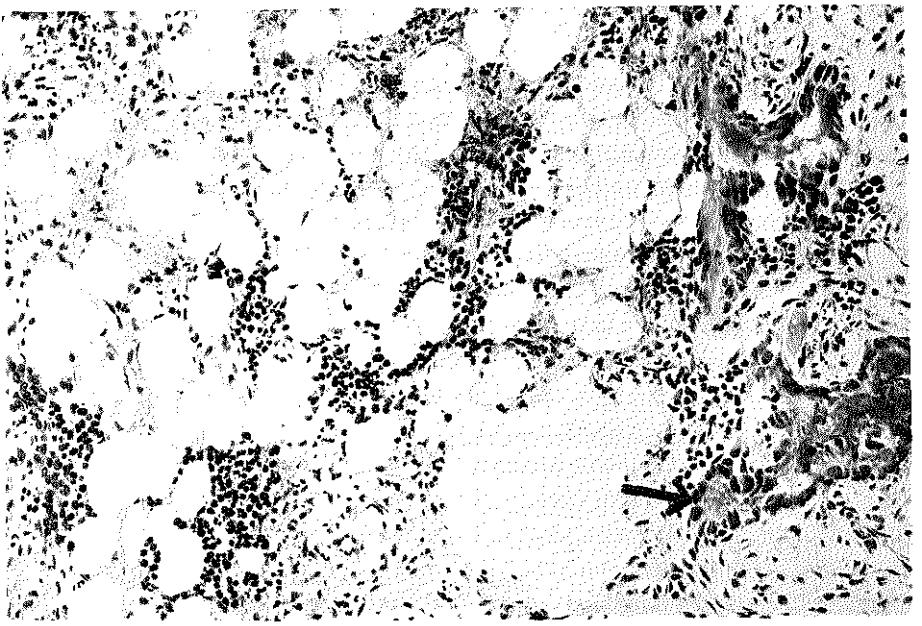

Figure 8.3. Mesentery of *Macaca fascicularis* monkey 7 days after letnal irradiation and intraperitoneal inoculation of autogeneic bone marrow cells. Note lamellar bone trabeculae (arrow). H & A. x 150.

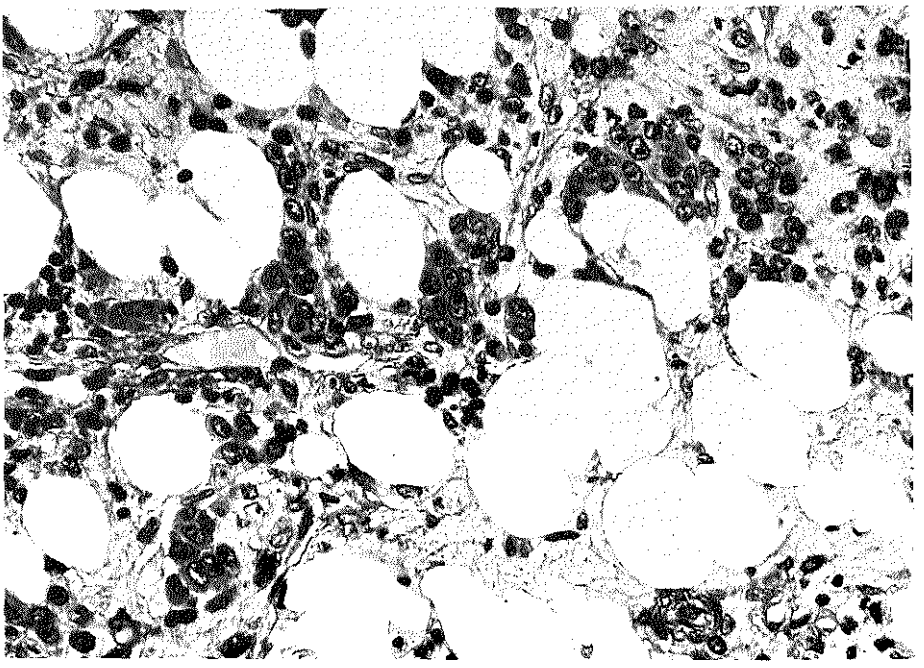

Figure 8.4. Mesentery of *Macaca fascicularis* monkey 12 days after lethal irradiation and intraperitoneal inoculation of autogeneic bone marrow cells. Note small erythroid islands in the growing hemopoietic colony. H & A. x 380.

tution is a time-dependent process, new experiments including thrombocyte transfusion to prevent early death from thrombocytopenic bleeding should be carried out so that stem cell mobilization and subsequent recovery of the bone marrow can be followed over a longer period.

Intraperitoneal transplantation of bone marrow cells leading to the formation of mesenteric hemopoietic colonies might then be found to be a useful procedure in the management of transplantation in primates.

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SUMMARY

Rauscher murine leukemia virus (R-MuLV) is an oncogenic biological agent and has been thought to induce a neoplastic disease in mice. The present studies concerned mainly morphological aspects of the R-MuLV infection.

R-MuLV ip injected into mice causes a three-phase lethal infection within about 30 days.

Thrombocytopenia and phagocytosis of viral particles and thrombocytes by Kupffer cells occur in the 8th hour of infection. From this point on, formation of intrasinusoidal, multicentric foci becomes manifest and continues throughout the infection. The first phase of the infection, the latent period, ends 72 hours after ip injection of the virus with the onset of viral replication. The beginning of the second phase, the hyperplastic period, is characterized by the involvement of the spleen and bone marrow and the first occurrence of a form of individual cell death caused by R-MuLV, called apoptosis.

During the hyperplastic period the process intensifies. Proliferating hemopoietic cells form confluent islands in the spleen, bone marrow, and liver in this period. From the 19th day on, undifferentiated hemopoietic cells and many erythroblasts are seen in the blood stream and the frequency of apoptosis increases. The spleen enlarges rapidly.

From the 26th day on, neoplastic infiltration of the lung, kidney, mesentery, muscles, and lymph nodes occur. Invasion of the walls of blood vessels and bile ducts can also be seen. Besides this infiltrative and invasive character of the newly formed hemopoietic tissue, cellular and nuclear polymorphism together with many atypical mitotic figures provide morphological evidence of the neoplastic nature of this third and final phase of Rauscher disease.

The neoplastic period starts on the 26th day after ip injection of R-MuLV and lasts until the death of the animal. When the dose of virus is reduced, the neoplastic period is prolonged.

R-MuLV infection of pregnant mice causes apoptosis in the embryos, especially in the mesoderm, before organogenesis. The apoptosis observed in these investigations was an unexpected finding, since the R-MuLV has hitherto been accepted as non-cytocidal. Light- and electron-microscopical descriptions of apoptosis both in neoplastic tissues and in R-MuLV-infected embryos are presented. Apoptotic cells and their membrane-bound nuclear remnants, called apoptotic bodies, are phagocytized by neighboring cells or by macrophages, which suggests that any viral genetic information they may carry is re-used.

Among the many possible sites evaluated for transplantation of hemopoietic cells infected and/or transformed by R-MuLV, the mesentery of mice proved to be highly suitable for this purpose. The intraperitoneally inoculated hemopoietic cells, from both normal and infected mice, form mesenteric hemopoietic colonies (MHC). In addition, the presence of mesenteric pseudocolonies was observed and their cellular composition was analyzed. MHC also developed in the lethally irradiated BALB/c mice, and six other strains investigated after ip inoculation of normal syngeneic hemopoietic cells. The migration of stem cells from MHC was demonstrated by the occurrence of hemopoietic spleen colonies and re-population of spleen and bone marrow in lethally irradiated mice.

The formation of MHC in mice and *Macaca* monkeys warrants further study to evaluate the applicability of this finding to man.

SAMENVATTING

Het Rauscher Murine Leukemia Virus (R-MuLV), een tumorverwekkend, biologisch actief materiaal, wordt verondersteld in muizen neoplasmen te kunnen induceren.

Intraperitoneale injectie van dit virus in muizen veroorzaakt binnen ongeveer dertig dagen een dodelijk verlopende infectie waarin drie fasen zijn te onderkenen.

Trombocytopenie en phagocytose van virus deeltjes of met virus beladen thrombocyten door Kupffer-cellen vindt plaats in het achtste uur na het begin van de infectie. Vanaf dit moment werd de vorming van intrasinusoidale, multicentrische celhaarden waarneembaar, welke aanwezig bleven gedurende de gehele infectieperiode. Deze fase van de infectie, de latente periode, eindigt ongeveer 72 uur na de intraperitoneale virus inoculatie met de start van virus replicatie.

Het begin van de tweede fase, de hyperplastische periode, wordt gekarakteriseerd door massieve celproliferatie en het voorkomen van een speciale vorm van celdood (apoptosis), die, in de bij de infectie betrokken organen als beenmerg, lever en milt, optreedt.

Prolifererende haemopoietische cellen vormen in de derde fase celeilanden in de milt, beenmerg en lever die geleidelijk samenvloeien. De milt en lever nemen sterk in omvang toe. De frequentie van de apoptosis eveneens. Vanaf de 19e dag na de infectie verschijnen veel ongedifferentieerde haemopoietische cellen en erythroblasten in de bloedbaan.

Vanaf de 26e dag na de infectie verschijnen neoplastische infiltraten in spieren, longen, nieren en in mesenterium en de lymphklieren.

Penetratie in de bloedvaatwanden en galgangen kan ook worden waargenomen. Behalve dit invasieve en infiltrerende karakter van het nieuw gevormde haemopoietische weefsel, verschaffen zowel het po-

lymorphisme van de cellen en de kernen plus de vele atypische mitose figuren, morfologische argumenten voor het neoplastische karakter van deze derde, tevens laatste fase van de ziekte van Rauscher. De neoplastische periode begint op ongeveer de 26e dag na de intraperitoneal injectie van het R-MuLV en duurt tot de dood van het dier. Als de dosis van het intraperitoneaal ingespoten virus wordt gereduceerd duurt de neoplastische periode langer.

R-MuLV infectie van zwangere muizen veroorzaakt apoptosis in de embryos, met name in het mesoderm dat voor de organogenese aanwezig is en waaruit het haemopoietisch weefsel gevormd wordt.

De in deze studie waargenomen apoptosis was onverwacht omdat tot nu toe werd aanvaard, dat een R-MuLV infectie geen celdood veroorzaakt.

In deze studie zijn zowel de met behulp van de licht–microscoop als de electronen-microscoop, waargenomen apoptotische veranderingen in het neoplastische weefsel en met R-MuLV geïnfecteerde embryonale weefsels, beschreven. De apoptotische, membraan omgeven kernresten, de z.g. apoptotische lichaampjes, worden door de in de omgeving aanwezige cellen of door de aanwezige macrophagen, opgenomen hetgeen doet vermoeden dat het van virus afkomstige genetische materiaal dat deze celresten zou kunnen bevatten, nogmaals wordt gebruikt.

Onderzoek naar de mogelijke plaatsen, die geschikt zouden zijn voor transplantaten van al dan niet geïnfecteerde bloedvormende cellen, toonde aan, dat het mesenterium van de muis hiertoe uiterst geschikt was.

Intraperitoneale inoculatie van bloedvormende cellen van zowel gezonde als van virus-geïnfecteerde muizen vormen in het mesenterium bloedvormende kolonies (de zgn. mesenteriale haemopoietische kolonies = MHC). Behalve de aanwezigheid van deze mesenteriale bloedvormende kolonies werd ook de samenstelling van de in deze kolonies aanwezige cellen geanalyseerd. MHC bleken zich ook te ontwikkelen in lethaal bestraalde BALB/c muizen alsmede in nog zes andere muizenstammen, na intraperitoneale inoculatie van normale syngenetische bloedvormende cellen.

Migratie van stamcellen vanuit MHC kon worden aangetoond door dé aanwezigheid van bloedvormende kolonies in de milt en de herbevolking van milt en beenmerg in lethaal bestraalde muizen. Behalve de vorming van MHC in muizen verschaft de waargenomen

vorming van MHC's in apen (*Macaca fascicularis*) de basis voor verdere studie van de mogelijke toepasbaarheid van deze bevindingen bij de mens.

ÖZET

Bu çalışma Rotterdam Erasmus Üniversitesi Tıp Fakültesinde, Klinik Patoloji ve Eksperimental Patoloji departmanlarında gerçekleştirilmiştir.

Rauscher murine leukemia virüsü (R-MuLV) bir onkojenik biolojik ajandır ve farelerde neoplastik bir hastalığa sebeb olur. R-MuLV intraperitoneal olarak farelere enjekte edildiğinde yaklaşık 30 gün içerisinde ölümle sonuçlanan üç devreli bir hastalık doğurur.

Trombositopeni ve virus partiküllerinin ve trombositlerin Kupffer hücreleri tarafından fagosite edilişi enfeksiyonun 8. ci saatında tesbit edilir. Bundan sonra, karaciğer sinüslerinde hücresel toplanmalar başlar. Bu mültisentrik intrasinüsoidal adacıklar hastalığın sonuna kadar giderek artarlar. Hastalığın ilk devresi – latent peryodu – virüs enjeksiyonundan 72 saat sonra sona erer. Bu anda ilk defa virüs replikasyonu başlar. Hastalığın ikinci devresi – hiperplastik peryodu – kemik iliği ve dalağın semptomatik ve morfolojik değişiklikleriyle karakterlenir. Bu peryodda ayrıca özel bir "tek hücre ölümü" şekli de artan bir sayıda tesbit edilir. Bu hücre ölümü morfolojik özellikleri nedeniyle "Apoptosis" diye adlandırılmıştır.

Hiperplastik peryodda hastalık tablosu süratle gelişir. Devamlı çoğalan hemopoietik hücreler dalak, karaciğer ve kemik iliğinde birbirleriyle kavuşan adacıkları yaparlar. Hastalığın 19. cu gününden başlayarak perifer kanda endifferansiye hemopoietik hücreler ve pek çok eritroblastlar görülür. Dalağın hacmi süratle artar.

Hastalığın 26.cı gününden itibaren akciğerlerde, böbreklerde, kaslarda, mezanterde ve lenf düğümlerinde neoplastik enfiltrasyonlar görülmeye başlar. Damar duvarlarının ve entrahepatik safra kanallarının envazyonuna da sıklıkla rastlanır. Enfiltratif ve envazif özelliklerinin yanı sıra, yeni teşekkül etmiş hemopoietik doku hücreleri büyüklük, şekil ve boyanma farkları gösterirler. Atipik mitotik figür-

lere bol miktarda rastlanır. Bu morfolojik bulgular hastalığın üçüncü ve son devresinin neoplastik natürlü oluşunu doğrulamaktadırlar. Bizim deneylerimizde 4 mg R-MuLV enjekte edilen farelerde neoplastik peryod hastalığın 26.cı gününde başlar ve deney hayvanının ölümüyle sonlanır. Eğer virüs dozu dilüe edilirse neoplastik peryodun uzadığı görülür.

R-MuLV enfeksiyonu anneden embryoya geçer. Özellikle mezodermde, organogenesisden önce apoptosise sebeb olur. R-MuLV infeksiyonu, şimdiye kadar inanılmış olan klâsik görüşün aksine, bazı hücrelerde "cytocidal" etki yapmaktadır. Apoptosis, gerek neoplastik dokularda gerekse *in utero* enfekte olmuş embryolarda, ışık ve elektron mikroskopuyla bu çalışma sırasında etüd edilmiştir. Apoptotik hücreler ve onların nükleer kalıntıları - ki bunlara apoptotik cisimcikler adı da verilir- makrofajlar veya komşu tümoral hücreler tarafından fagosite edilirler. Bu şekilde taşıdıkları viral genetik material tekrar enfeksiyonun devamı için kullanılmaktadır.

R-MuLV ile enfekte olmuş hemopoietik hücreler fare mezanterine transplante edilebilmiştir. Kontrol hemopoietik hücreler de ayni şekilde mezanterde proliferasyon göstermektedirler. Çoğalan hücreler "Mesenteric Hemopoietic Colonies" (MHC) meydana getirir ve bu koloniler letal irradiye edilmiş farelerde protektif olarak çalışırlar. MHC literatürde ilk defa otör tarafından tarif edilmiştir. Ayrıca psödokolonilerin sellüler kompozisyonları bu çalışma sırasında araştırılmıştır.

MHC, BALB/c farelerinden başka, diğer ayrı 6 cins fare gurubunda ve *Macaca fascicularis* maymunlarında da letal irradiyasyondan sonra fonksiyonel olarak endükte edilebilmiştir. Koloni doğuran kan ana hücreleri "stem cells", letal irradiye edilmiş farelerde MHC' lerden dalak ve kemik iliğine göç etmektedirler. Dalağa giden ana hücreler orada da hemopoietik dalak kolonilerini yaparlar. Ana kan yapıcı hücrelerin migrasyonu atrofiye olmuş hemopoietik sistemin, özellikle dalak ve kemik iliğinin, repopülasyonunu başlatır.

Fare ve maymunlarda MHC endüksiyonu ve bunların protektif fonksiyonları, bu bulgunun tıbba tatbikinin araştırılmasına ümid verici bir ışık tutmaktadır.

CURRICULUM VITAE

	Mustafa Halit UMAR, geboren 19.1.1943 in Akhisar in Turkije.
1949-1957	Lager en Middelbaar Onderwijs te Akhisar.
1957-1960	Lyceum te Balıkesir.
1960	Einddiploma Lyceum B met facultatief Latijn en Engels.
1960-1966	Studie aan de Medische Faculteit te İzmir.
1962	Doctoraal examen I.
1964	Doctoraal examen II.
1965-1966	Assistent-arts, in dienst van de afdeling Pathologische Anatomie van de Medische Faculteit, İzmir.
28.6.1966	Artsexamen, dat het recht verleende tot het uitoefenen van de geneeskunde in Turkije.
30.6.1966	Begin opleiding tot Patholoog-anatoom aan de afdeling Pathologie van de Medische Faculteit te İzmir.
1.9.1966-18.11.1966	Vervulling militaire dienstplicht.
18.11.1966	Diploma militaire arts.
18.11.1966-31.8.1968	Schoolarts en leraar Anatomie en Physiologie aan de militaire kaderopleiding te İzmir.
31.8.1968-1.4.1969	Huisarts te Torbalı, İzmir.
14.4.1969	Na overleg en op advies van Prof. Dr. H.E. Schornagel en Prof. Dr. M.J. de Vries van de afdeling Pathologische Anatomie, Medische Faculteit te Rotterdam werd een aanvang gemaakt met de opleiding tot Patholoog-anatoom in het Westeinde Ziekenhuis te Den Haag onder leiding van Dr. W.H. Minder. (B-opleiding)

1.6.1971-31.5.1973	Vervolg van de opleiding bij de afdeling Pathologische Anatomie van de Medische Faculteit, Rotterdam onder leiding van Prof. Dr. G. Wielenga.
20.6.1973	Werd mij door de Minister van Volksgezondheid en Milieuhygiene de bevoegdheid verleend tot het uitoefenen van de geneeskunde in Nederland.
17.7.1973	Inschrijving als Patholoog-anatoom in het Specialisten Register.
1.6.1973-1.9.1974	Werkzaam als Chef de Laboratoire in het Pathologisch Laboratorium van het Westeinde Ziekenhuis te Den Haag onder leiding van Dr. W.H. Minder.
1.9.1974-31.12.1976	Werkzaam als Patholoog-anatoom in de rang van wetenschappelijk hoofdmedewerker aan het Pathologisch Laboratorium van de Medische Faculteit te Rotterdam, alwaar onder leiding van Prof. Dr. G. Wielenga een proefschrift werd bewerkt.
1.1.1977-tot heden	Werkzaam als Patholoog-anatoom in associatief verband in het Pathologische Laboratorium van het St. Clara Ziekenhuis te Rotterdam.