GENETIC RESISTANCE TO BONE MARROW TRANSPLANTATION

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aan JEANNETTE

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Science is not a system of certain, or well-established, statements; nor is it a system which steadily advances towards a state of finality. Our science is not knowledge: it can never claim to have attained truth, or even a substitute for it, such as probability.

Karl R. Popper (1959)

GENERAL INTRODUCTION

Hemopoietic stem cells

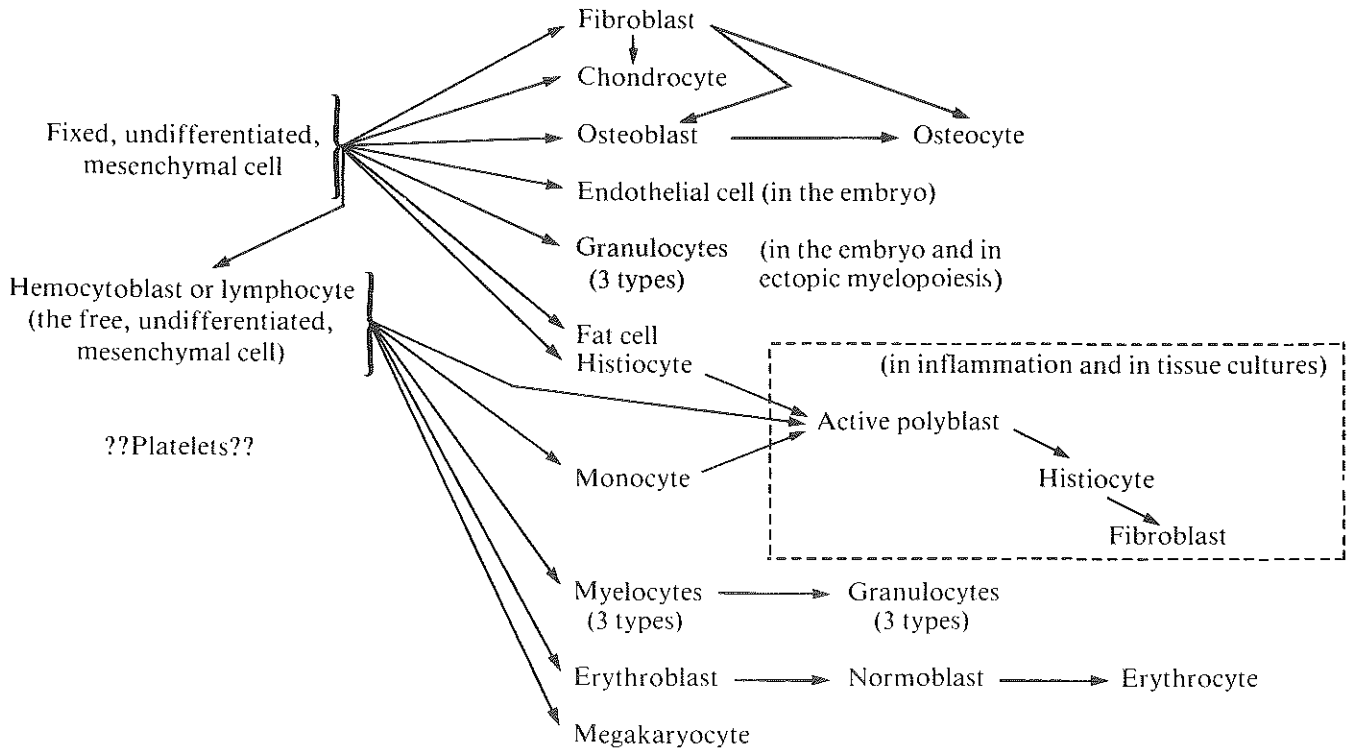
The homeostasis of several tissues is maintained by a process of proliferation and differentiation to replace the cells which are constantly lost. The most undifferentiated cells of a certain tissue, capable of proliferation and differentiation, are stem cells.

Stem cells have "the capacity for extensive proliferation, resulting in renewal of its own kind as well as giving rise to fully differentiated cells" (Caffrey-Tyler and Everett, 1966). In multicellular animals various types of stem cells can exist in a more or less differentiated form. In the most primitive multicellular organisms, the parazoa, any cell type is capable of proliferation and differentiation into the different cell types which form the organism. In these organisms each cell can be considered as a stem cell.

Among higher classified animals, Planarians (Platyhelminthes) are known for their enormous capacity to regenerate. These flatworms carry an omnipotent stem cell called neoblast. Different techniques were used to prove that neoblast population forms a stem cell pool which can differentiate into any cell type of the animal in case of a spontaneous fissure of the animal or a healing process after a trauma (cf. Brøndsted, 1969). Recent radiobiological studies produced suggestive evidence that the survival of planarians after irradiation is dependent on the presence of one single cell population (Lange, 1968a, b). This evidence is in favour of the neoblast concept, but could also be in favour of the concept which attributes the regeneration to dedifferentiation of differentiated cells.

In vertebrates various types of stem cells have been found. Cell renewal systems in these animals are generally restricted to tissues with relatively short living cells. Cell renewal systems are also found in other tissues and have a function in repair of local injuries. This latter type of repair seldom results in remodeling of the original form of the injured tissue. Among the vertebrates only the Urodele amphibians (Caudata) show a complete regeneration of an injured or amputated limb or tail. Most authors assume that this regeneration is not dependent on stem cells, but on dedifferentiation of differentiated cells (cf. de Both, 1969). The most active cell renewal systems are those which produce cells, that have a relatively short life time when compared with the life span of the individual. Examples of these cell renewal systems are among others the hemopoietic system, the epithelia of the skin, the gastro-intestinal tract and the urogenital system.

The concept of the hemopoietic cell renewal system has been a matter of discussion for many years. Different polyphyletic, dualist and trialist, and monophyletic or unitarian theories have been proposed. The polyphyletic theories assume the existence of two or three different types of hemopoietic



Schematic representation of the interrelationship between the cells of the blood and the connective tissue in mammals. The area within the dotted line indicates the changes which take place in inflammation. (Unitarian theory of blood and connective tissue formation. After Maximow, 1931).

stem cells. The monophyletic or unitarian concept assumes the existence of a single pluri-potent stem cell. Studies using chromosome markers, which will be discussed later, were performed in the last two decades and provided evidence that the monophyletic or unitarian theory as originally proposed by Maximow (1931) is probably the most acceptable one. The modern monophyletic theory implies the existence of a pluri-potent stem cell capable of migration which gives rise to the different blood cells: neutrophilic, eosinophilic and basophilic granulocytes, mononuclear phagocytes, thrombocytes, erythrocytes, T and B lymphocytes.

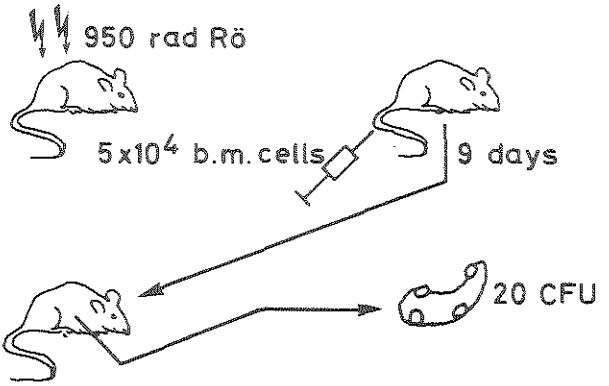
Bone marrow transplantation

The unfortunate development of atomic weapons initiated an extensive research on effects of radiation on organisms of practically any kind. The important aims of this research were the development of treatments for prevention and repair of radiation induced injuries.

Already in 1949 Jacobson et al. reported that the injection of a mouse spleen homogenate could prevent that mice died from a lethal dose of radiation. He attributed this effect to a humoral factor. In the early fifties more and more evidence became available that not a humoral factor was responsible for recovery of irradiated animals. It appeared that the recovery was due to the repopulation of the hemopoietic organs by the transplanted spleen or bone marrow cells. Three different groups revealed the existence of a hemopoieses of donor origin in irradiated animals treated with spleen or bone marrow cell suspensions. Vos et al. (1956) used three different techniques to prove the cellular basis of the radiation protection by a bone marrow graft: 1. serological identification of erythrocytes, a technique which permitted the authors to ascertain the donor origin of erythrocytes in the host; 2. a histochemical assay, capable of discrimination between rat and mouse granulocytes; 3. an experiment which suggested the presence of donor mouse bone marrow in the host, because the marrow of the grafted host was more efficient in preventing mortality of lethal irradiated mice isologous with the donor than of mice not isologous with the donor. The histochemical assay has been used by Nowell et al. (1956) also. Even more convincing evidence was obtained with chromosome analysis, which had been performed on hemopoietic cells of mice grafted with bone marrow from mice carrying the chromosome marker T_6 or with rat bone marrow (Ford et al., 1956). This technique permitted the authors to discriminate between donor and host cells on basis of differences in the karyogram. Lethally irradiated animals, which were treated with bone marrow, were designated ''radiation chimeras'' (Ford et al., 1956) according to Anderson

et al.'s (1951) definition of a chimera (Homer, 700 B.C.) being "an organism whose cells derive from two or more distinct zygote lineages". The radiation chimera offered tremendous experimental possibilities for experimental hematology, immunology, clinical hematology and oncology.

The first known clinical use of the newly acquired knowledge concerned the treatment of the victims of the so called Vinca accident (Mathé et al., 1959), an accident with a nuclear device in Jugoslavia.



Schematic representation of the spleen colony assay. The cells which give rise to a colony are designated as CFU.

An important contribution to the use of bone marrow transplantation in experimental hematology has been made by Till and McCulloch in 1961. They reported that the transplantation of small numbers of mouse bone marrow cells into irradiated mice resulted in the formation of colonies visible on the surface of the spleen. The number of spleen colonies was shown to be directly related to the number of bone marrow cells injected. The origin of such a colony was called a colony forming unit, CFU. Histological studies revealed that besides macroscopically visible colonies also microscopical colonies existed in the spleen. The colonies consisted of differentiated erythroid, myeloid or megakaryocytic cells or of a mixture of these. The spleen colony technique provided a tool for a quantitative study of the hemopoietic cell renewal system. A spleen colony has been shown to develop from one single colony forming cell (Till and McCulloch, 1961; McCulloch and Till, 1962). Studies using chromosome markers provided further evidence for the single cell origin of mixed spleen colonies (Wu et al., 1967; Fowler et al., 1967; Chen and Schooley, 1968). In animals recovered from a nearly lethal dose of irradiation various types of differentiating cells all carrying the same characteristic chromosomal abnormality were found in bone marrow, spleen, thymus and lymph nodes (Barnes et al., 1959). These results present very strong evidence for a common stem cell for the myeloid, erythroid and lymphoid series (cf. Micklem and Loutit, 1966). Bone marrow

transplantation can therefore be described as the transplantation of a complete hemopoietic cell renewal system consisting of hemopoietic stem cells and progenitor cells.

The study, in normal animals as well as in experimental situations, of the behaviour of the CFU, being a measure of the hemopoietic stem cell, is of importance since the complete cell renewal system is based on the proliferation and differentiation of the hemopoietic stem cells. The morphology of the hemopoietic stem cell remained obscure. Several authors described cells which were proposed to be hemopoietic stem cells. A promising attempt was made by van Bekkum et al. (1971). They studied the ultrastructural morphology of bone marrow cells in preparations, in which stem cells had been highly concentrated. A cell type with distinct morphological characteristics was proposed as the candidate for the hemopoietic stem cell.

The spleen colony assay, the technique which permits to measure the number of CFU in a cell suspension, determines indirectly the number of hemopoietic stem cells. The ratio of CFU: stem cells has been a subject of many studies. The percentage of bone marrow cells capable of forming a spleen colony present in an i.v. injected cell suspension, which form in fact a macroscopically visible colony on the spleen, has been estimated to lie between 3.7 and 5 (Matioli, Niewisch and Vogel, 1968; Lahiri, Keizer and van Putten, 1970).

In addition to the determination of the CFU two other **in vivo** techniques have been developed to determine hemopoietic proliferation: 1. ^{59}Fe incorporation into erythroid cells provides a technique to determine erythroid regeneration. It appeared that a good correlation exists between the number of hemopoietic cells transplanted and the blood iron incorporation (Hodgson, 1962; Blackett, Roylance and Adams, 1964), 2. Mobilisation of neutrophils into the peripheral blood by endotoxin. The technique measures the myeloid repopulation. The number of neutrophils in the peripheral blood shows a good correlation with the number of hemopoietic cells transplanted (Hellman and Grate, 1967).

Furthermore an **in vitro** technique became avaible for the determination of the number of myeloid and macrophage progenitor cells. Progenitor cells are defined as cells which have little or no self-replicative ability, they are restricted to one or two lines of differentiation and are sensitive to specific regulatory factors which are thought not to act on stem cells (Metcalf and Moore, 1971). The **in vitro** technique, which permits determination of progenitor cells, has been introduced independently by Pluznik and Sachs (1965) and Bradley and Metcalf (1966). The technique consisted of cultivation of hemopoietic cells in semi-solid agar. Proliferation of **in vitro** colony forming cells was induced by the addition of a colony stimulating factor to the culture. Suggestive evidence was obtained, that **in vitro** colony forming

cells can grow out to discrete colonies of different size. Myeloid, macrophage and mixed colonies were obtained. Extensive studies made it most unlikely, that the **in vitro** colony forming cells are identical with the spleen colony forming cells (Wu et al., 1968; Worton, McCulloch and Till, 1969; Haskill, McNeill and Moore, 1970). This can be considered as evidence that the **in vitro** colony forming cells are in fact differentiated progenitor cells, which are derived from the hemopoietic stem cell and limited to one or two lines of differentiation. Further evidence for the differentiated status of the **in vitro** colony forming cell was reported by van der Engh and Golub (1974), who showed that the **in vivo** CFU and the **in vitro** colony forming cell possesses different antigenic membrane markers.

Bone marrow transplantation has also been an important tool for experimental immunologists. The functions and characteristics of T and B lymphocytes were and are studied using lethal irradiation, to eliminate host origin lymphocytes, and subsequent transplantation with hemopoietic and/or lymphoid cells.

In man bone marrow transplantation has offered new possibilities for the treatment of a number of hemopoietic and immunological disorders. Intrinsic defects of the hemopoietic stem cell such as certain types of aplastic anaemia and combined immune deficiency can be treated by the administration of bone marrow from a healthy donor to the patient. Bone marrow transplantation can also extend the chemotherapy of oncological disorders since aplasia of the bone marrow due to the therapy can be restored by the injection of donor bone marrow.

Evidence for the existence of more than one hemopoietic stem cell in man is the situation in patients with a chronic myeloid leukemia. The Philadelphia chromosome, an abnormal chromosome, has been observed in myeloid, erythroid and megakaryocytic cells, but not in P.H.A.-stimulated cultures of lymphocytes (Whang et al., 1963; cf. Boggs, 1974). These observations would suggest the existence of a lymphoid stem cell. It appeared that the situation in man is different from that in mice since mice carry a pluri-potent hemopoietic stem cell as has been reported before. However, the long-lived nature of P.H.A. transformable lymphocytes could explain the observations, since it is possible that insufficient time has elapsed for the development of the chromosomal aberration in the stem cells and the replacement of the long-lived lymphocytes by new formed lymphocytes which carry the chromosomal abnormality (Metcalf and Moore, 1971; Boggs, 1974). So far no data have been reported which permit a decision with respect to the controversial issue of the existence of one or more hemopoietic stem cells in man.

A serious complication of allogeneic bone marrow transplantation is the

occurrence of graft-versus-host disease. This is an immunological reaction of lymphoid cells of donor origin, directed against the tissues of the recipient. A second complication can be the absence of a take of the graft leading to the death of the patient. The non take in certain cases can be due to the phenomenon of "Genetic Resistance" to bone marrow transplantation (Trentin, Rauchwerger and Gallagher, 1973). This phenomenon is the subject of study of this thesis.

The hemopoietic microenvironment

Under normal conditions the localisation of hemopoiesis in most adult mammals is limited to the bone marrow. Only in a few species as the mouse hemopoiesis is also observed in the spleen of healthy adults. During the ontogeny, hemopoiesis is found in the yolk sac, the mesenchymal tissues, the liver, the spleen and the bone marrow successively. The fact that the hemopoiesis is only localized in certain organs indicates, that besides humoral factors a certain environment is necessary to sustain hemopoiesis. The specific affinity of the hemopoietic organs, spleen and bone marrow for stem cells, is clearly demonstrated by the preference of i.v. injected bone marrow CFU to home in these organs.

Microscopical studies of colonies in spleen and bone marrow of the mouse obtained after irradiation and transplantation revealed that the ratio of the number erythroid: myeloid colonies was different in these organs. In the spleen a ratio of 3: 1 and in the bone marrow 1 : 2 has been found (Curry and Trentin, 1967; Wolf and Trentin, 1968). Trentin and coworkers concluded, that for each line of differentiation specific niches exist which induce hemopoietic proliferation and direct the line of differentiation. Such a niche has been postulated to be a "Hemopoietic Inductive Microenvironment, H.I.M." (Curry, Trentin and Wolf, 1967). This theory implies that the spleen and the bone marrow are subdivided into a variety of microenvironmental areas, niches, each inducing a single type of differentiation of the multipotent hemopoietic stem cell.

Interesting results were obtained when a plug of bone marrow of an irradiated mouse was implanted into the spleen of a second irradiated mouse which was subsequently injected with a bone marrow cell suspension. The colonies in the bone marrow implant showed an erythroid: myeloid ratio similar to the ratio in bone marrow in situ. The colonies which extended across the junction of the bone marrow and spleen showed in the spleen mostly an erythroid differentiation pattern, whereas in the bone marrow part a predominantly myeloid differentiation pattern was found (Wolf and Trentin, 1968). These experiments showed that the spleen and bone

marrow stroma have an inductive influence on the differentiation pattern of stem cells (Wolf and Trentin, 1968).

Further evidence for the existence of a microenvironment was obtained from studies using Steel mice. These mice carry a genetically determined anaemia (Bennett, 1956). The anaemia has been found not to be an intrinsic stem cell defect. Stem cells of Steel mice with genotype SL/SL^d transplanted into wild type recipient mice produce normal numbers of spleen colonies and give rise to a normal hemopoiesis in the radiation chimera. However, irradiated SL/SL^d mice given bone marrow cells of wild type mice showed almost no growth of CFU in the spleen (Sutherland, Till and McCulloch, 1970). These experiments suggested, that the genetically determined anaemia of SL/SL^d genotype mice was due to a defect of the hemopoietic microenvironment or to an absence of appropriate amounts of humoral factors. Parabioses of anaemic Steel mice with wild type mice did not lead to an increased growth of CFU in the anaemic partner neither to a depressed growth in the wild type partner (McCulloch et al., 1965). These results indicated that the anaemia of Steel mice is not due to a defect in the humoral regulation of the erythropoiesis. Other environmental factors, steric or humoral and acting over a very short range, seemed therefore to be responsible for the anaemia in Steel mice. The normal neutrophil count (Bennett, 1956) and the normal numbers of anti-SRBC plaque forming cells (Mekori and Philips, 1969) suggested, that the erythroid H.I.M. was effected in Steel mice.

Wolf (1974) studied in SL/SL^d mice the stromal control over the "four phases of erythropoiesis": 1. CFU lodgment. 2. commitment of stem cells to the erythroid differentiation. 3. proliferation of stem cells and committed precursor cells and 4. further differentiation of the precursor cells and their descendants. The lodgment of CFU was shown to be similar in SL/SL^d mice as in wild type mice. The commitment and proliferation were strongly reduced in the SL/SL^d mice. Wolf (1974) suggested that the differentiation into more mature forms was not under stromal control. Several authors proposed that central macrophages have an important role in the control of differentiation of more mature erythroid cells. Their proposal was based on the observation that a relationship seemed to exist between central macrophages and surrounding erythroblasts in erythroid islets in the yolk sac (Sorenson, 1961), bone marrow (Bessis and Breton-Gorius, 1956) and the spleen (Orlic, Gordon and Rhodin, 1965). De Vries (personal communication) observed similar erythroid islets with erythroblasts in different stages of differentiation around a central macrophage in the bone marrow of children with regenerating marrow after chemotherapy.

Further evidence for the influence of the microenvironment on stem cells has been obtained with experiments with hypertransfused mice.

The number of myeloid colonies was unaffected in such mice, whereas the number of erythroid spleen colonies showed a decrease (Curry et al., 1967). The erythroid colonies were replaced by cell nests of undifferentiated erythropoietin sensitive cells. These colonies did not become myeloid. The absence of a sufficient level of erythropoietin, affecting the early differentiating erythroid cells, caused reduction of erythroid proliferation. This demonstrated that definite areas are reserved for each line of differentiation. The evidence for the existence of a hemopoietic inductive microenvironment in the hemopoietic organs indicates the presence of a "structure", which is neither morphologically nor otherwise defined. McCulloch et al. (1973) designated the stromal elements responsible for the "cellular communication" i.e. induction of differentiation and proliferation of hemopoietic stem cells, "managerial" cells. This cellular communication was postulated to be based either on a cell-to-cell contact or on an intermediary acting at very short range.

The cellular basis of the microenvironment has been the subject of many studies. Knospe, Blom and Crosby (1966) reported that a secondary aplasia occurred in locally irradiated bone marrow, 2 to 6 months after irradiation with a dose of 2,000 - 10,000 rad. This secondary aplasia was shown to be correlated with the absence of the sinusoidal microcirculation. Hemopoietic regeneration has been found to be correlated with the regeneration of the sinusoidal microcirculation. The experiments showed that 1. stromal cells do not migrate from distant sites (Knospe, Blom and Crosby, 1968; Patt and Maloney, 1970); 2. the stromal cells are not macrophages since their precursors do circulate. The apparent initial radioresistance of stromal elements was assumed to be due to their low turnover rate (Knospe et al., 1966).

Finally it can be summarized that cells of the hemopoietic organs form the environment which directs the differentiation and proliferation of stem cells and progenitor cells. The nature of the interactions between the "managerial" cells and the hemopoietic cells remains obscure.

Genetic resistance to bone marrow transplantation

McCulloch and Till (1963) reported "a repression of colony forming ability of C57BL hemopoietic cells transplanted into non-isologous hosts". C57BL bone marrow, spleen and fetal liver cells were shown to form less spleen colonies in irradiated F1 (C3H × C57BL) than in C57BL or C3H mice, whereas C3H parental marrow cells formed similar colony numbers in C3H, C57BL and F1 hybrid mice. An immunological reaction of the host against donor cells was rejected as a possibility. The lethal irradiation dose was expected to abolish any immunological reaction. Moreover a

classical immune reaction seemed most unlikely since no evidence had been published before that hybrid mice could mount an immune reaction against their parents.

Cudkowicz and Stimpfling (1964) described a similar phenomenon and designated it "hybrid resistance". C57BL bone marrow cells showed a markedly reduced ability to repopulate the spleens of lethally irradiated hybrids of C57BL mice. The authors assayed the repopulation of the spleen at day 5 after transplantation with a radio-isotope incorporation technique. The uptake in the spleen of ^{131}IUdR, a thymidine analogue, was measured and expressed as a percentage of the radio-activity injected into each animal (Cudkowicz et al., 1964). A good correlation between the number of isogeneic grafted bone marrow cells and the ^{131}IUdR uptake was shown. Experiments with backcross progeny mice as recipients suggested strongly that the deficient growth of C57BL bone marrow cells in hybrid mice was associated with heterozygosity at the H_{-2} locus or at an independent H_{-2} linked locus.

Besides hybrid resistance, the graft reversal phenomenon (Popp, 1961, 1964) can form a serious complication in transplantation of C57BL bone marrow cells. Graft reversal is a process of regression of the transplant after repopulation of the irradiated recipient. Experiments with bone marrow from F2(C57BL × 101) donors transplanted into F1 hybrid mice revealed that hybrid resistance segregated independently from the graft reversal phenomenon (Popp and Cudkowicz, 1965).

Trentin et al. (1973) proposed the common designation "genetic resistance to bone marrow transplantation" for hybrid resistance, allogeneic resistance (Cudkowicz and Bennett, 1971 a) and a third type of resistance, xenogeneic resistance (Rauchwerger, Gallagher and Trentin, 1973 a). Trentin's group showed that xenogeneic resistance in a rat to mouse model as described by Goodman and Shinpock (1968) had the same characteristics as allogeneic and hybrid resistance (Rauchwerger et al., 1973 a, b, 1976; Gallagher et al., 1973). Rauchwerger et al. (1973 b) reported the interesting observation that 10^7 Lewis rat bone marrow cells transplanted into F1 (C57BL/6 × A) mice caused a confluent growth of colonies in the femoral bone marrow, whereas no spleen colonies were observed in the same mice. It was noticed that, although resistance occurred in the femoral bone marrow, it was weaker than in the spleen.

After the phenomenon of genetic resistance to a bone marrow graft had been discovered, many experimental hematologists have made efforts to elucidate the nature of the phenomenon.

Goodman and Wheeler (1966) performed experiments to determine whether the stroma of the parental donor implanted under the kidney capsule of F1 hybrid mice formed a more suitable environment for the transplanted

parental bone marrow cells in case of hybrid resistance, a phenomenon, which they designated "Poor Growth phenomenon". Slices of donor spleen were transplanted under the kidney capsule of F1 hybrid mice, which were lethally irradiated and grafted 19 or 21 days later. The transplantation of slices of parental spleen resulted in an enhanced growth of the C57BL parental bone marrow graft in the F1 hybrid recipient. The growth of the graft was measured with the ^{59}Fe uptake technique, which permitted a good measurement of the growth of the graft in the spleen. Only slices with viable spleen cells were active (Goodman and Wheeler, 1968 a). Further experiments were designed to reveal whether "stromal or immunopotent cells" were responsible for enhancement of the growth of the marrow graft. It appeared that spleen cells and lymph node cells of donor origin also induced a significant weakening of the resistance when injected 7 days before lethal irradiation and transplantation of F1 hybrid mice with C57BL bone marrow cells (Goodman and Wheeler, 1966, 1968 a, b). Therefore the conclusion was drawn, that immunopotent and not stromal cells were responsible for the weakening effect of the transplanted spleen grafts on the resistance. Subsequent studies revealed that the simultaneous injection of bone marrow and thymocytes of the same donor also enhanced the splenic growth of a parental (Goodman and Shinpock, 1968), allogeneic or xenogeneic bone marrow graft (Goodman and Shinpock, 1972) in a resistant transplantation combination. It has been shown that parental thymocytes were effective even when administered 1 to 2 days before or after the C57BL marrow transplantation. It appeared that the donor type thymocytes had to be intact cells, although high proportions of irradiated thymocytes were shown to be active also.

Later studies with chromosome markers revealed that the cells of the bone marrow donor were responsible for the repopulation of the spleen and not cells of the thymus donor (Dehamer and Goodman, 1973). The graft-versus-host activity of the thymocytes was shown not to be responsible for the growth-enhancing effect since donor thymocytes, specifically tolerant to the marrow recipient, were effective in weakening the resistance also. No special class of thymocytes responsible for the weakening of the resistance could be separated by density centrifugation (Pritchard, Shinpock and Goodman, 1975). The addition of thymocytes did also enhance the spleen colony formation of bone marrow cells in a transplantation combination subject to genetic resistance (Goodman and Grubbs, 1970; Kozlov, Kolesnikova and Meilikhov, 1974).

Goodman, Burch and Basford (1972) considered that "by offering an experimental situation in which marrow growth, ordinarily poor, can be greatly enhanced, P → F1 transplantation provides a sensitive and productive system for studying control of hemopoiesis". Goodman and collaborators proposed several theories to explain the enhancing effect of the treatment with donor cells on the growth of a marrow graft subject to genetic resistance.

The involvement of a humoral factor seemed unlikely since thymocytes implanted i.p. in millipore diffusion chambers did not affect the parental marrow grafts (Goodman and Shinpock, 1972). A cell-to-cell interaction between thymocytes or spleen cells and the bone marrow cells responsible for the enhancement of the growth of the marrow graft has been proposed. However, a short range humoral factor produced by the thymocytes remained also possible (Goodman and Shinpock, 1972). Furthermore it has been proposed that thymocytes could alter the surface of donor marrow cells, so that they can become attached at sites in the spleen. Alternatively Goodman and coworkers proposed that the thymocytes could settle in the spleen and provide attachment sites for marrow cells isologous with them. It has also been proposed that local factors in the non isologous hosts lengthen the cell cycle of the parental bone marrow cells, resulting in a slower repopulation of the hemopoietic organs of the irradiated recipient (Dehamer and Goodman, 1973).

Cudkowicz and Bennett (1971 a, b) reported a number of characteristics of hybrid resistance and allogeneic resistance: 1. the resistance does not appear in infant mice before 3 weeks of age. 2. adult thymectomy, irradiation and reconstitution with bone marrow does not affect the resistance. 3. the resistance is transferable with the bone marrow i.e. that in a radiation chimera the same resistance occurs as in the original marrow donor. The latter results were not confirmed by Goodman and Wheeler (1968 a) who reported that no resistance to C57BL/6 bone marrow cells occurred in F1 (C57BL/6 × DBA/2)→C57BL/6 radiation chimeras. 4. the administration of Corynebacterium parvum induced a time-related weakening of the resistance with an optimum at day 7 after injection. 5. sublethal pre-irradiation also resulted in a time-related weakening of the resistance. 6. injection of recipient mice with cyclophosphamide before irradiation and transplantation resulted in a significant weakening of the resistance. The latter results confirmed data of Sensenbrenner and Santos (1969), who showed that cyclophosphamide and busulphan conditioning of the recipient resulted in a normal colony formation of isogeneic as well as of non isogeneic transplanted bone marrow whether or not this was subject to genetic resistance.

Hybrid resistance and allogeneic resistance seemed to be a similar phenomenon (Cudkowicz and Bennett, 1971 b). The phenomenon of resistance to a bone marrow graft was described as "a peculiar type of reaction", because it did not require proliferation of lymphoid cells and it was tissue specific, thymus independent and regulated by genetic factors, which apparently do not affect the fate of other solid grafts (Cudkowicz and Bennett, 1971 a).

Cudkowicz and Bennett (1971 b) proposed a cytotoxic cell responsible for the disappearance of the grafted hemopoietic cells during the first 2 to

3 days after transplantation. From the data obtained with cyclophosphamide treatment and sublethal pre-irradiation, they concluded that the cytotoxic cells have a relatively short live time. The fact that the resistance was transferable with bone marrow indicated that the killer cells or their progenitors were transferable also. The abrogation of the resistance in the spleen induced by the administration of silica and carragheenan, which are macrophage toxic agents, suggested that macrophages might be the effector cells of the resistance in the light of the cytotoxic cell theory (Lotzová and Cudkowicz, 1973 b, 1974). B lymphocytes were proposed to be responsible for the specific recognition aspect of the phenomenon. The abrogating effect of the macrophage toxic agent silica was shown to be dependent on lysis of macrophages, since in mice treated with poly-2-vinylpyridine N-oxide, an agent which prevents the macrophage lysis induced by silica particles, the resistance was not abrogated.

Bennett (1973) showed that the effector cell of genetic resistance was dependent on the bone marrow for its differentiation. This conclusion was deduced from experiments with mice of which the bone marrow was depleted. The depletion of the bone marrow was induced by injections with the bone-seeking isotope ^{89}Sr. Such treated mice showed no resistance to a bone marrow graft in the spleen. The author showed that T and B cell functions were intact in ^{89}Sr treated mice. Therefore it has been concluded that the effector cells, designated "M" cells, to indicate their marrow dependence, are not identical with T or B cells. In the context of the proposal of Lotzová and Cudkowicz (1974) concerning non thymus dependent lymphocytes cooperating with macrophages, the "M" cell could represent both cell types.

Recently an **in vitro** assay in which spleen cells of F1 hybrid mice can generate cytotoxic activity against parental cells has been described by Shearer and Cudkowicz (1975). Spleen cells from hybrid mice had been co-cultured for 5 days with irradiated parental cells and tested subsequently for the presence of cytotoxic cells. ^{51}Cr labeled ascites tumor cells sharing the $H\text{-}_2$ type of the parental stimulator cells were added to the culture and the ^{51}Cr release, resulting from cytotoxicity, was measured. Parent F1 combinations which showed F1 antiparent activity **in vitro** were the same as those, which demonstrated genetic resistance to a parental bone marrow transplant. The pretreatment of F1 hybrid mice with parental spleen cells, a treatment which induced an abrogation of genetic resistance in the spleen, also appeared to abrogate the cytotoxic reaction **in vitro**. Spleen cells of juvenile mice which showed no resistance **in vivo** did not show cytotoxicity **in vitro** either. The authors concluded that although the effector cells of the **in vitro** procedure might not be the same as those **in vivo**, both are directed against the same hybrid histocompatibility gene products.

An other approach to the study of the nature of genetic resistance to a bone marrow transplant was provided by Till, Wilson and McCulloch (1970). They reported the surprising enhancing effect of the injection of horse anti-mouse thymocytes serum in hybrid recipients on the colony formation of C57BL bone marrow cells in these mice. They stated that the weakening effect of horse anti-mouse thymocytes serum on genetic resistance was not attributable to the common immunosuppression. The antiserum was proposed to have an effect on the mechanism responsible for the normal regulation of stem cell functions in the hemopoietic system. Further studies revealed that antisera against $H\text{-}2$ and non $H\text{-}2$ alloantigens could also induce an abrogation of the resistance in the spleen (Gregory, McCulloch and Till, 1971, 1972). The authors postulated that the antibodies in the antisera reacted with surface antigens which were suspected to have "cognition reaction" functions (Gregory et al., 1972; McCulloch et al., 1973). These antigens have been proposed to be involved in the cellular communication between stromal "managerial" cells on one hand and hemopoietic stem cells and progenitor cells on the other hand. This cellular communication, as it has been suggested, seemed to be similar to the inductive and regulation functions of the HIM.

The occurrence of genetic resistance in animals other than the mouse has been reported recently. Van Bekkum and coworkers observed both allogeneic and hybrid resistance in rats (personal communication). Furthermore the existence of genetic resistance has been suggested to occur in dogs (Bacharoff et al., 1973). Vriesendorp, Zürcher and Van Bekkum (1975) and Weiden et al. (1976) reported results which also indicated that genetic resistance occurs in dogs. Littermates with identical serological defined (S.D.) and lymphocyte defined (L.D.) histocompatibility antigens showed a good take of a bone marrow graft, whereas dogs, which are not identical for S.D. and L.D. antigens, rarely showed take of the graft.

The relationship between the degree of genetic resistance to a bone marrow transplant and the number of hemopoietic cells necessary to obtain survival of the grafted animals, has been subject of only very few studies. Rauchwerger et al. (1973 b) showed that the survival of Fl (C57BL/6 × A) mice after a lethal irradiation and transplantation with bone marrow cells of Lewis rats was directly correlated with the number of microscopic colonies in the bone marrow, while at these bone marrow doses no spleen colonies were observed. However, they did not show data of the survival of "non resistant" mouse strains, although in the latter mice confluent growth in the bone marrow had been obtained within 8 days after transplantation at ten times lower dosages of rat bone marrow (Rauchwerger et al., 1972). Recently Vriezendorp et al. (1976) reported a dissimilarity between the spleen colony

formation and survival in mice. They found in parent-to-F1 combinations, independent of the occurrence of genetic resistance in these combinations, no correlation between the spleen colony formation and survival. In allogeneic and xenogeneic combinations a positive correlation between spleen colony formation and survival was noticed. In our group attempts have also been made to correlate the occurrence and degree of genetic resistance to a bone marrow transplant and the bone marrow dose necessary to obtain 20 days survival (data not shown). Our results were inconsistent. It seemed that the expression of the resistance, which varies continuously within certain limits, between separate experiments in our transplantation combinations, was the cause of our failure. However, the data obtained, indicated that for low bone marrow doses the survival correlated better with the repopulation of the bone marrow than with the repopulation of the spleen.

The relevance of the phenomenon for human bone marrow transplantation is uncertain yet. In man symptomatic treatment as thrombocyte transfusions and anti-microbial therapy is extensively used after transplantation. Therefore the survival of patients is less dependent on the immediate repopulation of the hemopoietic organs than in the usual mouse experiments, in which symptomatic treatment is not given. However, the ultimate survival of patients does depend on the take of the graft. The occurrence of so called graft rejection or non take of the graft has been reported in several cases in which an adequate depletion of the hemopoietic and lymphatic system was induced (Fefer et al., 1974; Storb et al., 1974). A resistance to the bone marrow graft similar to the phenomenon described for mice might be the cause of failure of the grafts in these cases. Considering the enormous variation in conditioning of patients prior to transplantation, in bone marrow doses used, and in symptomatic treatment applied, it is likely that genetic resistance to the bone marrow transplant is hidden behind other causes of non take of the graft and graft rejection. However, it can be expected that when optimal treatments are given, genetic resistance might still become a serious complication in human bone marrow transplantation. Such complications could in addition to the complication of the GVH disease demand for an accurate matching of donor and host for human histocompatibility antigens, since it appears from data obtained in the mouse that the loci for histocompatibility antigens are closely linked with the loci for the so called hybrid-histocompatibility antigens, which will be discussed later.

From a teleological point of view it appears most unlikely that genetic resistance to bone marrow tranplantation is the normal biological role of the phenomenon.

The important analogy between genetic resistance to bone marrow trans-

plantation and the so called "hybrid effect" might indicate the presence of a surveillance mechanism directed at the elimination of maligant tumor cells carrying new antigens. The relative poor growth of certain parental lymphatic tumors in Fl hybrids (Snell, 1958) was designated as "hybrid effect" or "syngeneic preference" (Hellström, Hellström and Haughton, 1964). The hybrid effect was only observed, when low doses of tumor cells were transplanted. Hellström (1963) showed that the hybrid effect was correlated with the presence of certain H_{-2} antigens on the surface of the tumor cells.

Cudkowicz et al. (1972) also described similarities between the genetic control, which will be discussed later, of hybrid resistance to DBA/2 normal bone marrow cells and to grafts of leukemic cells. Furthermore, Bennett and coworkers showed, that treatment with ^{89}Sr of mice resistent to Friend leukemia virus rendered these mice susceptable to Friend leukemia virus. Normal immunological causes explaining the phenomenon could be excluded. They suggested, that the "M" cell exerts surveillance by rejecting leukemic cells (Kumar, Bennett and Eckner, 1974; Kumar and Bennett, 1976; Kumar, Caruso and Bennett, 1976).

Recent data indicated that there also exists a similarity between the suppression of allogeneic lymphomas and the occurrence of genetic resistance to bone marrow transplantation in a number of strain combinations (Bonmasser and Cudkowicz, 1976). The authors reported that poor growth of lymphomas occurred in the spleen, but not in the liver of these heavily irradiated mice. The latter data suggests that the resistance to lymphoma cells is restricted to the lymphatic and hemopoietic organs. This information seems to be in favour of the theory which explains genetic resistance as an inappropriate microenvironment not conductive to the outgrowth of the transplanted cells.

The genetic control of resistance to bone marrow transplantation has been studied by several groups. In mice genetic resistance to a bone marrow graft occurs only in donor-host combinations, which differ at the major histocompatibility complex (cf. Cudkowicz and Lotzová, 1973). The loci of donor and host, which determine the occurrence of genetic resistance are closely linked with the major histocompatibility complex and have been designated as hybrid-histocompatibility (Hh) loci. Therefore these loci belong to linkage group IX, which is located on chromosome 17 (Klein, 1971). The resistance of the hybrid mice against bone marrow cells of a parent has been explained as to be a reaction of the hybrid, in which the allele inherited from that parent is silent, against the parental Hh antigen. A study on the recombination between the loci which determine the resistance to a parental bone marrow graft and $H\text{-}2D$ in various (F1 $\times$ parent) backcross

mice revealed four different recombination percentages indicating the presence of four Hh loci (Lotzová and Cudkowicz, 1971, 1972, 1973 a ; Cudkowicz and Lotzová, 1973). The presence of four Hh loci has not been confirmed by progeny tests in order to demonstrate that the recombinations were not phenotypic variants, but real genotypic variants (Lotzová and Cudkowicz, 1973 a). Bennett (1972) reported data that were interpreted as indicating that homozygosity of the donor at the Hh loci is a prerequisite for genetic resistance independent of the zygosity of the recipient. In order to explain the rare exceptions, that a homozygous graft was accepted and a heterozygous graft was resisted, he proposed the existence of a closely linked ''switch'' gene, which controlled the expressivity of Hh specificity of the allele on the same chromosome. Recently Lotzová, Gallagher and Trentin (1975 b) showed that xenogeneic resistance, rat-to-mouse, is controlled by two independent autosomal genes of the recipient.

The information presented so far, on the genetic ''laws'' of resistance to a bone marrow graft, show that these ''laws'' are different from those which apply for graft-versus-host reactions and skin transplants.

INTRODUCTION AND DISCUSSION OF THE EXPERIMENTAL WORK

The nature of the phenomenon of genetic resistance to bone marrow transplantation is the main subject of the experimental work described in this thesis.

In **appendix paper I** it has been described that C57BL bone marrow cells, transplanted into F1 (C57BL × CBA) hybrid recipient mice, show a reduced colony formation which appeared to be about 1/10 of the isogeneic colony formation. These data indicated that the transplantation combination C57BL → F1 (C57BL × CBA) was useful for studies of genetic resistance. The repopulation of CFU in spleen and femoral bone marrow of irradiated F1 (C57BL × CBA) mice transplanted with bone marrow from isogeneic F1 (C57BL × CBA) mice and from parental C57BL mice has been studied. This study seemed worthwhile since the repopulation of the hemopoietic organs depends on the proliferation of hemopoietic stem cells. It appeared that the multiplication of CFU started later in the transplantation combination C57BL → F1 than in the isogeneic combination F1 → F1. The initial decrease of the CFU numbers in the spleen was prolonged to 72 h for C57BL bone marrow CFU, whereas for isogeneic F1 hybrid bone marrow CFU the initial decrease lasted about 24 h. The slope of the CFU multiplication phase in the spleen appeared to be similar for both transplantation combinations. The latter results confirmed earlier reported data of McCulloch, Gregory and Till (1973). They described that the multiplication phase of the growth curve of the semi-isogeneic transplanted bone marrow CFU in the spleen showed a shift to the right of about 8 days. The same authors showed that the curve, which represented the repopulation of the spleen with CFU-c, the **in vitro** colony forming cell, displayed a similar shift. Further experiments have been performed to study the prolonged initial decrease of CFU in the spleen. It has been studied whether this prolonged decrease could be caused by a higher proportion of resting, non cycling CFU in the spleen during the first 3 days after transplantation. Thymidine cytocide experiments showed that in the spleen the fraction of CFU in S-phase of the cell cycle was at 2 h as well as at 24 h similar for F1 and C57BL bone marrow CFU transplanted into F1 hybrid recipient mice. This indicated that other factors than a difference in fraction of proliferating CFU were responsible for the prolonged initial decrease of the CFU numbers. The shape of the initial dip in the growth curve was similar for both transplantation combinations, which suggests that the cause of the dip might be the same for semi-allogeneic and isogeneic transplanted CFU.

So far the occurrence in the bone marrow of genetic resistance to a bone marrow transplant had only been described for xenogeneic resistance in a rat to mouse model (Rauchwerger, Gallagher and Trentin, 1973 b). Therefore we studied the growth of C57BL bone marrow CFU in the femoral bone marrow of F1 hybrid recipient mice. The CFU growth curve showed a longer

lag phase, when compared with F1 bone marrow CFU transplanted into F1 hybrid mice. The multiplication of the transplanted CFU started 48 h later than in the isogeneic combination. The slope of the curve of the multiplication phase was similar for both transplantation combinations. These results resembled very much the data obtained for the CFU growth curve in the spleen. The data showed that hybrid resistance occurs in the femoral bone marrow also.

Splenectomy has been performed to study the role of the spleen in the phenomenon of the resistance to the repopulation of the bone marrow. An absence of the resistance in the femoral bone marrow was observed at 6 months but not at 2 months after splenectomy. This might suggest that a long lived cellular factor of splenic origin was responsible for the phenomenon. More recent observations, not included in the appendix papers, showed that other results were obtained with the combination C57BL→ F1 (C57BL × DBA/2). The origin of "splenic" cells responsible for the resistance was studied with neonatally splenectomized mice also. These experiments showed that in neonatally splenectomized mice an enhanced growth of the isogeneic bone marrow CFU was found in the femoral bone marrow. This applied for F1 (C57BL × CBA) and F1(C57BL× DBA/2) mice. The enhancement equalled a factor of about 2. A significantly higher enhancement was found for C57BL bone marrow CFU transplanted into neonatally splenectomized F1 (C57BL × CBA) mice. Similar experiments showed that in neonatally splenectomized F1 (C57BL × DBA/2) mice no enhancement of the growth of the C57BL CFU was found. The latter results suggested that the data obtained with splenectomized F1 (C57BL × CBA) mice might not be representative for the phenomenon of genetic resistance to a bone marrow transplant.

It can be concluded that genetic resistance occurs in the spleen and the bone marrow. The repression of colony numbers seemed to be higher (equalling a factor ± 10) than the reduction in CFU numbers (factor ± 4). These data made it possible to study the characteristics of the phenomenon of genetic resistance with substances which can weaken the resistance.

In **appendix paper II** a study of the influence of endotoxin from Salmonella typhosa, Freund's complete adjuvant and alloantiserum, C3H anti C57BL, on genetic resistance has been described.

It has been shown that endotoxin from Salmonella typhosa, a B lymphocyte stimulant (Dresser and Philips, 1973), in doses of 10, 100 and 500 µg induced a time-related enhancement of the colony formation of C57BL bone marrow cells in F1 hybrid mice. Freund's complete adjuvant, a T lymphocyte stimulant (Allison, 1973) induced similar results. In these experiments the colony forming ability of F1 bone marrow cells has been used as a control. The isogeneic colony formation was not significantly

affected by the treatments. A study of the influence of endotoxin and Freund's complete adjuvant on the growth of C57BL bone marrow CFU in spleen and femur of F1 hybrid mice revealed that the weakening effect was only observed in the spleen. The resistance to the transplant in the femoral bone marrow was not affected by the administration of the agents. Cudkowicz and Bennett (1971 a, b) showed that treatment of the recipient 7-18 days prior to transplantation with heat killed Corynebacterium parvum enhanced the growth in the spleen of a bone marrow graft subject to genetic resistance. They reported that this agent had no significant enhancing effect on the repopulation of the spleen with isogeneic bone marrow cells. Since our experiments showed that the weakening of the resistance, induced by endotoxin and Freund's complete adjuvant, was restricted to the spleen, experiments have been performed to investigate whether the influence of alloantiserum, capable of inducing a weakening of the resistance (Gregory, McCulloch and Till, 1971, 1972) affects the resistance in the spleen only or also in the bone marrow. It appeared that C3H anti C57BL serum injected into F1 (C57BL × CBA) hybrid recipient mice before transplantation induced an enhancement of the colony formation of C57BL bone marrow cells. The growth of C57BL bone marrow CFU in the spleen was also enhanced. However, no significant enhancing effect has been observed in the femoral bone marrow. The latter data did not support the suggestion of Gregory, McCulloch and Till (1972), that the antibodies in the alloantiserum were directed against surface antigens with "cognition reaction" functions on managerial cells. Our results would indicate that managerial cells have different "cognition reaction" antigens in spleen and femur while excerting the same functions, which complicates the earlier mentioned theory very much.

Endotoxin, Freund's complete adjuvant and alloantiserum are known to affect macrophages. It seemed therefore worthwhile to pay attention to the macrophage toxic agents silica and carragheenan, since these substances had been reported to have an weakening influence in the spleen on genetic resistance to a bone marrow transplant (Lotzová and Cudkowicz, 1973 b, 1974). In **appendix paper III** it has been reported that the latter substances administered to recipient mice did not affect genetic resistance in the femoral bone marrow. A significant migitation has been observed in the spleen. Histological studies revealed that the silica dose used did not cause a visible destruction of macrophages. The capacity of macrophages to phagocytize carbon particles was not significantly impaired. The silica particles have been observed in microscopical preparations of bone marrow and spleen. Therefore the absence of a weakening effect in the bone marrow cannot be explained by an absence of silica particles in the bone marrow. These results did not support the theory of Lotzová and Cudkowicz, (1974), that silica caused a depletion of macrophages being the effector cells of the

resistance. It appeared that there is an interesting coincidence between the capacities of some substances to induce lymphocyte trapping as well as to induce a weakening of genetic resistance to a bone marrow transplant in the spleen. Xenogeneic sera (Till et al., 1970), Freund's complete adjuvant, Corynebacterium parvum (Cudkowicz and Bennett, 1971 a, b), silica and carragheenan (Lotzová and Cudkowicz, 1973 b, 1974; Lotzová, Gallagher and Trentin, 1975 a) are known to be capable of inducing weakening of genetic resistance in the spleen. They are also known to be active in lymphocyte trapping (cf. Frost and Lance, 1973). Macrophages have been suggested to play a central role in this phenomenon. The absence of an effect of the agents mentioned above on the resistance in the bone marrow indicated that the agents do not directly affect the effector cells of genetic resistance. It has been proposed that the immunological processes, which are initiated by these substances in the spleen, affect besides the immunological microenvironment also the local hemopoietic microenvironment.

In **appendix paper III** the influence of the cytostatic agents cyclophosphamide (Cy), busulphan and vinblastin has been studied also. Cy had been reported to affect the hemopoietic microenvironment (Gregory, Fried, Knospe and Trobaugh, 1971; Fried et al., 1973; cf. Tavassoli, 1975). A weakening influence of Cy on genetic resistance had also been described (Sensenbrenner and Santos, 1969; Cudkowicz and Bennett, 1971 a, b). In our experiments F1 hybrid mice have been treated with Cy, busulphan or vinblastin 4 days prior to transplantation. The influence of these drugs on the growth of C57BL bone marrow CFU in spleen and femoral bone marrow has been studied in such pretreated mice. It appeared, that Cy induced an enhancement of the growth of C57BL bone marrow CFU in spleen and femoral bone marrow of F1 hybrid mice. A small enhancement for isogeneic transplanted F1 hybrid bone marrow CFU has been found but it was significantly lower. This indicated, that genetic resistance to C57BL bone marrow was weakened in the spleen and femoral bone marrow. Vinblastin and busulphan affected the resistance in the spleen only and not in the femoral bone marrow. These results indicated that the latter agents did not induce a depletion of effector cells, since these cells would be expected to be affected in the bone marrow also. Furthermore it has been concluded from these results that the weakening effect of Cy was not due to a killing of proliferating effector cells as suggested by Cudkowicz and Bennett (1971 a, b), since a similar sensitivity of the effector cells had been expected for the alkylating agent busulphan and possibly for vinblastin. The coincidence, that Cy caused a weakening of genetic resistance as well as it affects the hemopoietic microenvironment, formed circumstantial evidence, that the hemopoietic microenvironment is involved in the process of genetic resistance to a bone marrow transplant.

Appendix paper IV concerns the influence of irradiation, given several days prior transplantation, on genetic resistance to a bone marrow transplant and the growth of an isogeneic bone marrow graft. A significant weakening of genetic resistance to a bone marrow transplant was observed in the spleen and femoral bone marrow of F1 hybrid mice, which had been treated with a sublethal dose of irradiation 7 days prior to transplantation. It appeared that the growth of isogeneic bone marrow CFU was also enhanced in the spleen and bone marrow of such pretreated mice. Cudkowicz and Benett (1971 a, b) reported that a sublethal dose of irradiation of the recipient mice, given 6-37 days prior to transplantation induced a weakening in the spleen of genetic resistance to a bone marrow transplant. The influence of such a treatment on an isogeneic graft had been reported by Blackett and Hellman (1966). They measured the ^{59}Fe uptake in the peripheral blood at day 5 after transplantation. Mice pre-irradiated 7 days before transplantation showed a significant increase in ^{59}Fe uptake, when compared with not pre-irradiated mice. Our data showed that both the weakening of the resistance as well as the enhancement of the growth of the isogeneic transplant occurred in the bone marrow. These results resembled very much the results, which we obtained with F1 hybrid mice, that had been pretreated with Cy.

Furthermore local pre-irradiation experiments with F1 hybrid mice were performed. These experiments showed, that the enhancement of the CFU growth in the bone marrow was restricted to the irradiated part of the bone marrow. This indicated that the radiation induced local changes in the bone marrow cavity. It has been shown, that the 24 h seeding of isogeneic bone marrow CFU was not affected by the local irradiation. These results suggested, that the pre-irradiation induced changes in the local microenvironment in the pre-irradiated bone marrow which favoured the repopulation of the pre-irradiated bone marrow after transplantation.

The pre-irradiation of a single tibia of F1 hybrid mice has also been performed to test the "M" cell theory of Bennett (1973). Our data showed, that the irradiation of a single tibia with a sublethal dose caused a significant weakening of the resistance in the spleen. A depletion of progenitor "M" cells could not be responsible for this result, since the bone marrow of a single tibia forms only a very minor part of the total bone marrow of a mouse. The mechanism, which induced the weakening, observed in such pretreated mice, remained obscure. It resembled the weakening of the resistance observed in experimentally anaemic mice (Beran and Tribukait, 1974). We suggested, that a stimulus for proliferation could affect the microenvironment. Such a stimulation could possibly improve the inappropriate regulation of bone marrow cells subject to genetic resistance. The data presented supported the theories concerning the involvement of the

microenvironment in the phenomenon of genetic resistance to a bone marrow transplant. The data did not support the "M" cell theory (Bennett, 1973).

Appendix paper V describes experimental work which has been performed to study the mechanism of the weakening of genetic resistance, induced by administration of donor cells.

The enhancing effect of the addition of thymocytes of the donor to the bone marrow cells on the colony formation has been studied. The addition of 5×10^7 or 10^8 C57BL thymocytes to C57BL bone marrow cells transplanted into F1 hybrid mice abrogated the resistance. Löwenberg (1975) reported similar data for the colony formation of C57BL fetal liver cells, to which C57BL thymocytes were added, in F1(C57BL × CBA). This confirmed the data of Goodman and Grubbs (1970). It was shown that lower numbers of thymocytes did not induce a significant weakening of the resistance. Therefore we suggested that the effect of the number of thymocytes added to the bone marrow suspension and the colony forming ability of C57BL bone marrow cells in F1 hybrid recipient mice was not dependent on the ratio thymocytes: bone marrow cells. We considered that it seemed more likely that the colony forming ability of the C57BL bone marrow cells depended on the ratio of thymocytes: number of sites in the spleen, where a colony can form. According to this proposal the colony formation of C57BL bone marrow cells is optimal when all sites are filled up with C57BL thymocytes. The size of spleen colonies obtained at an optimal thymocyte dose was still smaller than the size of isogeneic C57BL → C57BL or F1 → F1 colonies.

The CFU growth curve in the spleen of C57BL bone marrow CFU transplanted simultaneously with an optimal dose of C57BL thymocytes into F1 hybrid mice was similar to the isogeneic F1 → F1 CFU growth curve. These data could not explain the smaller size of the C57BL spleen colonies. It suggested an impaired production of differentiated cells. The growth of C57BL bone marrow CFU in the femoral bone marrow of F1 hybrid mice was not significant enhanced by the addition of thymocytes of donor origin to the bone marrow graft. The latter result was in agreement with the suggestion of Goodman and Grubbs (1970), that weakening of the resistance induced by the addition of large doses of thymocytes of donor origin to the bone marrow transplant, is restricted to the spleen.

The treatment of F1 hybrid recipient mice with several injections of C57BL spleen cells at weekly intervals showed similar results. This treatment induced a weakening of the resistance in the spleen, but not in the femoral bone marrow. Two possible explanations for the weakening of the resistance induced by injections with donor C57BL spleen cells into the F1 hybrid recipient mice have been studied: 1. the presence of a C57BL hemopoiesis transplanted with the spleen cells induced a syngeneic soil bed on which

C57BL CFU grow as isogeneic transplanted CFU. 2. C57BL thymus derived lymphocytes caused the enhanced growth of C57BL CFU. The first explanation was studied with two techniques. The first technique concerned an attempt to induce a C57BL hemopoiesis in a part of the bone marrow. This approach seemed promising since Micklem et al. (1975) showed that it was possible to induce an isogeneic donor hemopoiesis in an irradiated limb by means of injection of hemopoietic cells after local irradiation. In our experiments irradiation of a tibia of F1 hybrid mice, followed by an injection with C57BL spleen cells, did not lead to a weakening of the resistance in the irradiated limb when tested 14 days after the treatment. The second technique used to study the influence of a C57BL hemopoiesis consisted of the measurement of C57BL CFU in spleen and bone marrow of F1 hybrid mice, which had been treated with C57BL spleen cells. No significant numbers of C57BL CFU were found in spleen or femur of such treated mice. In irradiated limbs of F1 hybrid mice treated with C57BL spleen cells no C57BL CFU has been found either. These results indicated that a selfsustaining C57BL hemopoiesis was not found in spleen and bone marrow of F1 hybrid mice treated with C57BL spleen cells. The first explanation seems therefore unlikely.

The second explanation of the weakening induced by the donor spleen cells postulated C57BL thymus derived lymphocytes to be involved. Therefore thymectomized, irradiated and fetal liver reconstituted C57BL mice were used as "B" spleen cell donors. These "B" spleen cells appeared to be also capable of inducing a weakening of the resistance in the spleens of F1 hybrid mice. This indicated that thymus derived lymphocytes could not be the only factor responsible for the weakening effect of the C57BL spleen cells. It can be summarized that other factors than a C57BL hemopoiesis and C57BL thymus derived lymphocytes can induce a weakening of the resistance to a C57BL bone marrow graft in the spleens of F1 hybrid mice. The results obtained with pretreatment with donor spleen cells could be explained in the light of the theorie, which concerns the involvement of the hemopoietic microenvironment, managerial cells, in the phenomenon of genetic resistance to a bone marrow transplant. The presence of C57BL cells, not being hemopoietic stem cells or thymus derived lymphocytes, in the spleen could form an isogeneic environment in F1 hybrid mice on which C57BL bone marrow CFU grow normally.

The results of appendix paper I-V have been evaluated in the context of the two main theories developed to explain the phenomenon of genetic resistance to a bone marrow transplant. This procedure was followed since we obtained no data which suggested another theory or made a simplification of the earlier mentioned theories possible. However, it has to be noticed

that both theories are based on a minimum of data. The theory concerning the cytotoxic cells responsible for the disappearance of the grafted hemopoietic cells was based on the observation that the grafted cells disappeared during the first three days after transplantation. Many data obtained later have been explained in the context of this theory. Recent observations of Shearer and Cudkowicz (1975), who showed that cells of F1 hybrid mice can react against cells of their parents, reinforce the cytotoxic cell theory. Lotzová and Cudkowicz (1974), suggested that the cytotoxic cells were macrophages. Since it appeared that macrophages on cellulose acetate membranes in the peritoneal cavity were not cytotoxic for grafted hemopoietic cells subject to genetic resistance (Kitamura et al., 1973), it was assumed that these macrophages are functionally distinct from the splenic and bone marrow macrophages. Our results obtained from silica treated F1 hybrid mice showed that we could not correlate any visible destruction of macrophages with the presence or absence of genetic resistance in spleen and bone marrow.

TABLE 1 Weakening of genetic resistance to C57BL bone marrow cells transplanted into F1 hybrid mice*.

Agents and treatments	Spleen	Femoral bone marrow
Cyclophosphamide	+	+
Pre-irradiation	+	+
Endotoxin	+	—
Freund's complete adjuvant	+	—
Allo-antiserum	+	—
Silica	+	—
Carragheenan	+	—
Vinblastin	+	—
Busulphan	+	—

* The growth of CFU has been determined.

However, it seems that the abrogation of genetic resistance by those substances which affect only the resistance in the spleen (Table 1) is correlated with processes in which macrophages are known to play a role. This seems to be not in favour of the cytotoxic cell theory, although it could be hypothesized that the cytotoxic cells are inhibited by the processes mentioned above.

The theory which explains the phenomenon as an "inappropriate regulation" of the grafted hemopoietic stem cells and progenitor cells is not based on any direct evidence. The fact that according to the classical

immunology no reaction could be expected in a lethally irradiated host without presensitization and certainly not in a F1 hybrid against parental antigens, has been considered as circumstancial evidence. Our data showed that both treatments which could abrogate the resistance in the spleen and bone marrow have been shown to affect the hemopoietic microenvironment.

Our data did not permit us to reject either one theory. It has to be remarked that both theories might not be adequate to explain the phenomenon of genetic resistance to a bone marrow graft. More experimental data are probably needed to explain the nature of the phenomenon.

SUMMARY

The hemopoietic homeostasis is maintained by replenishment of the differentiated hemopoietic cells, by a process of proliferation and differentiation of precursor cells and stem cells. In the literature conclusive evidence has been reported that true pluripotent hemopoietic stem cells occur in mice. The spleen colony technique made a quantitative measurement of these stem cells possible.

The hemopoietic microenvironment has been studied extensively. Careful studies on the differentiation pattern of hemopoietic colonies in spleen and bone marrow indicated that for each line of differentiation different niches exist which induce hemopoietic proliferation and differentiation. Such a niche has been called a Hemopoietic Inductive Microenvironment, HIM. Important information about the HIM has been obtained from studies with mice which carry a certain genetically determined anaemia, Steel mice. So far the nature and the morphology of the HIM has not been revealed.

The transplantation of murine bone marrow appeared to be complicated in certain donor host combinations. It has been observed that in such donor host combinations much more bone marrow cells were necessary to obtain a repopulation of the hemopoietic tissues similar to that after transplantation in an isogeneic combination. Two main theories have been developed to explain this phenomenon, which has been designated Genetic Resistance to bone marrow transplantation. The first theory described a cytotoxic cell, which is responsible for the disappearance of the grafted hemopoietic cells during the first days after transplantation. The second theory attributed the delayed repopulation of the hemopoietic organs to an inappropriate regulation of the hemopoietic stem cells and progenitor cells.

The experimental work concerned a study of the nature of the phenomenon of genetic resistance to a bone marrow transplant. Various methods have been used to affect the resistance in order to obtain information about its nature. In **appendix paper I** the growth kinetics of bone marrow CFU transplanted in a donor host combination subject to genetic resistance and in an isogeneic combination has been studied. This approach seemed worthwhile since the repopulation of the hemopoietic organs of irradiated and reconstituted animals depends on the proliferation and multiplication of hemopoietic stem cells. It has been found that the resistance occurs in spleen and femoral bone marrow. The onset of the multiplication phase of the CFU growth curve was in both organs about 48 hours delayed when compared with the isogeneic CFU growth curve. We showed that in the transplantation combination subject to genetic resistance this delay was not due to a lower fraction of proliferating CFU. Neonatally and to some extent adult splenectomy could abrogate the resistance in the femoral bone marrow in the donor host combination C57BL → F1 (C57BL × CBA), but not in the combination C57BL → F1 (C57BL × DBA/2).

In **appendix papers II and III** it has been shown that endotoxin, Freund's complete adjuvant, alloantiserum, silica and carragheenan administered to the F1 hybrid recipient mice could induce a weakening of genetic resistance to a parental bone marrow transplant in the spleen. The experiments also showed that the agents did not affect the resistance in the bone marrow significantly. A weakening of the resistance in both spleen and bone marrow would have been expected when the agents mentioned affected the effector cells of the resistance. Therefore it has been suggested that the effector cells were not directly affected by the agents. It has been proposed that macrophages in the spleen affected by the above mentioned substances are responsible for the weakening of the resistance in the spleen.

In **appendix papers III and IV** it has been reported that cyclophosphamide and irradiation treatment of the recipient mice prior to transplantation can induce a significant weakening of genetic resistance in spleen and femoral bone marrow. The sublethal pre-irradiation of a single tibia enhanced the growth of C57BL and F1 hybrid bone marrow CFU equally in the pre-irradiated marrow. Since the seeding of CFU was not affected by the pre-irradiation an influence of the irradiation on the local microenvironment has been postulated. Furthermore several authors reported that cyclophosphamide affects the hemopoietic microenvironment. Therefore we suggested that the hemopoietic microenvironment is involved in the phenomenon of genetic resistance to a bone marrow transplant.

It appeared that sublethal pre-irradiation of a single tibia induced a significant weakening of the resistance in the spleen. It has been concluded from these data that the resistance in the spleen is not dependent on an inflow from the bone marrow of progenitor effector cells of the resistance. Thus our data did not support the theory that marrow dependent effector cells are responsible for the phenomenon.

In **appendix paper V** the weakening of genetic resistance induced by treatment of the host with cells of the donor has been described. The addition of C57BL thymocytes to C57BL bone marrow cells enhanced the colony formation in F1 (C57BL × CBA) mice. The dose effect of the number of thymocytes added to the bone marrow cells on the colony formation has been studied. Since very high doses of thymocytes were necessary to induce an enhancement of the colony formation it has been suggested that the ratio thymocytes: number of sites where a colony can form, is the important factor. Injection of the F1 hybrid recipient with thymocytes or spleen cells affected only the resistance in the spleen significantly. It has been shown that thymus derived lymphocytes among the spleen cells are not per se responsible for the weakening induced with donor cells. Since the pretreatment of the host with spleen cells of the donor did not cause the establishment of a hemopoiesis of donor origin in the spleen of the recipient

mice, it appeared that such an isogeneic soil bed could not be responsible for the weakening of the resistance in the spleen. It has been attempted to induce a C57BL hemopoiesis in a part of the bone marrow of F1 hybrid mice. Mice were locally irradiated and injected with C57BL spleen cells. This treatment did not induce a C57BL hemopoiesis nor a weakening of the resistance to C57BL bone marrow cells in the irradiated bone marrow. Therefore the conclusion has been drawn that other cells than thymus derived lymphocytes and other factors than a donor type hemopoiesis were responsible for the weakening effect of donor spleen cells on genetic resistance. It seemed possible to explain the results in accordance with the theory concerning the inappropriate regulation of the grafted hemopoietic cells in the recipient. Other cells than thymus derived lymphocytes and a donor type hemopoiesis could have formed an isogeneic soil bed, in the spleens of pretreated F1 hybrid mice, on which C57BL CFU grow normally.

It has been concluded that the data reported in the **appendix papers** did not permit us to reject either the cytotoxic cell theory or the theory concerning the involvement of the hemopoietic microenvironment whereas for both theories only indirect evidence is at hand.

SAMENVATTING

Onder fysiologische omstandigheden worden binnen zekere grenzen constante aantallen van de verschillende typen bloedcellen gehandhaafd. De vorming van bloedcellen is dan in evenwicht met een voortdurende afbraak van bloedcellen. Uit een pluri-potente bloedvormende stamcel kunnen door proliferatie en differentiatie alle typen bloedcellen ontstaan. Bij muizen kan de aanwezigheid van deze stamcellen kwantitatief worden aangetoond met de zogenaamde milt kolonie techniek.

De bestudering van de verhoudingen tussen de aantallen myeloide en erythroide kolonies in de milt en het beenmerg van letaal bestraalde en met beenmerg getransplanteerde muizen onder verschillende omstandigheden heeft aanwijzingen geleverd voor de aanwezigheid van een zogenaamd micro-milieu in de bloedvormende organen. Dit micro-milieu bepaalt de differentiatie richting van de hemopoietische stamcel die zich daarin bevindt. Een dergelijk micro-milieu wordt aangeduid met de term hemopoietic inductive microenvironment (HIM). Experimenteel onderzoek, verricht aan zogenaamde Steel-muizen, die een genetisch bepaalde anaemie hebben, leverde aanvullende bewijzen voor de aanwezigheid van een dergelijk micro-milieu. De aard en morfologie van dit bloedvormende micro-milieu is nog geheel onbekend.

Beenmergtransplantatie bij muizen bleek in bepaalde donor-gastheer-combinaties minder goede resultaten te geven. Bij een dergelijke donor-gastheer-combinatie is meer beenmerg nodig om de bestraalde gastheer te bevolken met de bloedvormende cellen van de donor dan in een isologe combinatie. Dit verschijnsel wordt "genetische resistentie bij beenmergtransplantatie" genoemd. In de literatuur worden verschillende theorieën, die dit verschijnsel trachten te verklaren, beschreven waarvan de volgende twee de belangrijkste zijn. De eerste theorie wijt het verdwijnen van een deel van de getransplanteerde bloedvormende cellen aan cellen, die de getransplanteerde cellen opruimen. De tweede theorie verklaart het verschijnsel door aan te nemen, dat de regulatie door cellen van het micro-milieu van de differentiatie en proliferatie van de primitieve bloedvormende cellen in een dergelijke donor-gastheer-combinatie is gestoord. Verschillen in antigene determinanten tussen donor en gastheer zouden verantwoordelijk kunnen zijn voor dit proces. De experimenten, die in dit proefschrift zijn beschreven, werden verricht met het doel meer gegevens te verkrijgen over de eigenschappen van het verschijnsel "genetische resistentie bij beenmergtransplantatie".

In **publikatie I van de appendix** wordt een onderzoek beschreven van de repopulatie van bestraalde F1 (C57BL × CBA) muizen met beenmerg afkomstig van C57BL of F1 muizen. De resultaten toonden aan, dat de repopulatie van de F1 muizen met C57BL stamcellen, bepaald met de milt kolonie techniek en uitgedrukt als CFU, later plaatsvindt dan de repopulatie met

CFU afkomstig van F1 beenmerg. De toename van de CFU begon bij transplantatie met C57BL beenmerg 48 uur later dan bij transplantatie met F1 beenmerg. Het percentage CFU in de S-fase van de celcyclus bleek bij beide transplantatiecombinaties, C57Bl → F1 en F1 → F1, kort na transplantatie gelijk te zijn. Bij de donor-gastheer-combinatie C57Bl → F1 (C57Bl × CBA) bleek het verwijderen van de milt bij pasgeboren en bij volwassen gastheermuizen onder bepaalde omstandigheden de resistentie van het beenmerg tegen het transplantaat sterk te verminderen. Bij de transplantatie-combinatie C57BL → F1(C57BL × DBA/2) bleek daarentegen dat na het verwijderen van de milt de resistentie niet verminderde.

In de **publikaties II en III van de appendix** werd aangetoond, dat door het toedienen van endotoxine, Freund's compleet adjuvant, allo-antiserum, silica of carrageen aan F1(C57BL × CBA) gastheermuizen de resistentie in de milt sterk verminderde, terwijl een vermindering van de resistentie in het beenmerg niet werd gevonden. Deze resultaten wijzen erop, dat bovengenoemde agentia de mogelijke effectorcellen, cellen die verantwoordelijk zijn voor de resistentie, niet direkt beinvloeden, daar in dat geval ook een vermindering van de resistentie in het beenmerg zou worden verwacht. De resultaten met bovengenoemde stoffen, waarvan bekend is dat ze macrofagen beinvloeden, wijzen erop, dat macrofagen waarschijnlijk van betekenis zijn bij de vermindering van de resistentie in de milt.

De **publikaties III en IV van de appendix** beschrijven de invloed van de behandeling van F1(C57BL × CBA) muizen met cyclophosphamide en bestraling met ioniserende straling op de resistentie van deze muizen tegen C57BL beenmerg. Deze behandelingen veroorzaakten een sterke vermindering van de resistentie in de milt en het beenmerg van de F1 muizen. Beide behandelingen verbeterden tevens de groei van een isoloog beenmergtransplantaat in beenmerg en milt. Met behulp van lokale bestraling van het beenmerg met ioniserende straling kon worden aangetoond, dat de groeibevordering van het transplantaat beperkt is tot het bestraalde beenmerg gedeelte. Tevens kon worden aangetoond, dat in het bestraalde beenmerg de toename van het aantal getransplanteerde CFU is vervroegd. Deze en andere resultaten duidden erop, dat bestraling van het beenmerg, enige dagen voor de letale bestraling, het micro-milieu in het beenmerg beinvloedt. Verschillende auteurs hebben beschreven, dat ook cyclophosphamide het micro-milieu in het beenmerg en in de milt beinvloedt. Deze gegevens gekombineerd met het feit, dat de behandeling van de gastheer met cyclophosphamide of bestraling de resistentie in het beenmerg en de milt sterk verminderde, zouden erop kunnen wijzen, dat het micro-milieu betrokken is bij het verschijnsel ''genetische resistentie bij beenmergtransplantatie''. Met partiele bestraling van het beenmerg kon verder worden aangetoond, dat de resistentie in de milt niet afhankelijk is van mogelijke uit het beenmerg

afkomstige effectorcellen.

De experimenten in **publikatie V van de appendix** beschrijven een onderzoek naar de aard van de vermindering van de genetische resistentie na de behandeling van de gastheer met miltcellen of thymuscellen van de donor. De resultaten toonden aan, dat het toevoegen van thymuscellen van de donor zowel als het tevoren behandelen van de gastheer met miltcellen van de donor, de resistentie alleen in de milt van de gastheer deed verminderen. Uit de waarneming, dat vergeleken met het aantal getransplanteerde beenmergcellen zeer hoge aantallen thymocyten nodig zijn voor een vermindering van de resistentie, werd geconcludeerd, dat waarschijnlijk een verzadiging van het micro-milieu met thymuscellen verantwoordelijk is voor de vermindering van de resistentie. Met behulp van partiele bestraling van het beenmerg gevolgd door een injectie met donor-miltcellen, werd getracht deze miltcellen te doen nestelen in het bestraalde deel van het beenmerg. Deze behandeling verminderde de resistentie in het bestraalde deel van het beenmerg niet. Daarnaast kon de aanwezigheid van donor-stamcellen niet worden aangetoond in de milt noch in het bestraalde en onbestraalde deel van het beenmerg. Deze resultaten toonden aan, dat in de met miltcellen behandelde F1 gastheer-muizen geen bloedvorming van donor oorsprong plaatsvond. Hieruit kon worden geconcludeerd, dat door de behandeling met miltcellen niet een C57BL bloedvormend milieu is ontstaan, waarin het C57BL beenmergtransplantaat beter kan groeien. Experimenten met "B" miltcellen, vrij van in de thymus gevormde lymfocyten, toonden aan, dat de in de thymus gevormde lymfocyten niet alleen verantwoordelijk kunnen zijn voor de vermindering van de resistentie in de milt. Deze resultaten wijzen erop, dat andere cellen uit de donormilt dan in de thymus gevormde lymfocyten of een bloedvorming van donor-oorsprong mogelijk een milieu in de milt vormen, waarin de donor beenmergcellen groeien zoals in een isologe combinatie.

Uit de resultaten is de conclusie getrokken, dat deze onvoldoende waren om één van beide theorieën, bedoeld om het verschijnsel "genetische resistentie bij beenmergtransplantatie" te verklaren, te verwerpen.

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NAWOORD

Op verzoek van de faculteit der Geneeskunde volgen hier enige persoonlijke gegevens.
Na een wat moeizame lagere school periode, die 7 jaar duurde, bezocht ik van 1960 tot 1965 het Christelijk Lyceum in Leiden, alwaar ik het eind-diploma H.B.S.-B behaalde.
Van 1965 tot 1971 studeerde ik biologie aan de Rijks Universiteit te Leiden. Bijzonder goede herinneringen heb ik aan de colleges van Prof. Dr. J. van der Vecht (diersystematiek), Prof. Dr. R. Hegnauer (plantensystematiek) en Prof. Dr. P. Dullemeyer (morfologie).
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APPENDIX

Ex omnibus partibus relucet totum

kardinaal Nicolaus von Cues
(1401-1464)

Cell Tissue Kinet. (1975) **8**, 111–118.

THE INFLUENCE OF GENETIC RESISTANCE ON CFU GROWTH KINETICS IN SPLEEN AND FEMUR

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ABSTRACT

An impaired colony formation of C57BL marrow cells transplanted into F_1 (C57BL × CBA) mice was observed. In accordance with the literature this phenomenon has been designated as 'genetic resistance'. Studies to elucidate the mechanism of the genetic resistance demonstrated that the multiplication phase of the CFU growth curve started in the semi-isogeneic combination about 48 hr later than in the isogeneic combination. In the spleen this resulted in a lower 'dip'. For the spleen as well as for the femur similar CFU doubling times were found during the multiplication phase when both transplantation combinations were compared. Furthermore the percentage of CFU in S-phase (assessed with the ^{3}H-TdR suicide technique) during the first days after transplantation were similar in both combinations. When the spleen was removed 5–6 months before irradiation and bone marrow transplantation was performed the growth curve of parental CFU in the femur was identical with the growth curve of isogeneic CFU (no delay was observed). These results are discussed and a few theories explaining the observations are proposed.

In some genetically determined donor–host combinations the development of a haemopoietic graft is inhibited. This phenomenon has been established by several authors using different techniques and in the literature a number of designations has been used. Trentin, Rauchwerger & Gallagher (1973) proposed the common designation 'genetic resistance'. The methods which can be used to assess the phenomenon are: determination of the number of CFU formed per 10^5 bone marrow cells ('CFU-repression', McCulloch & Till, 1963), the uptake of ^{59}Fe (Goodman & Shinpock, 1968) or ^{131}I-UdR (Cudkowicz & Stimpfling, 1964), by haemopoietic tissues.

The genetic basis of the phenomenon was thoroughly studied by Cudkowicz and co-workers (Cudkowicz & Stimpfling, 1964; Cudkowicz & Rossi, 1972; Cudkowicz *et al.*, 1972), who discovered that it is controlled by so called hybrid-histo-compatibility genes.

McCulloch & Till (1963, 1970) stated that the elucidation of the mechanism of

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'CFU-repression' may provide valuable information about the factors influencing the development of spleen colonies and the regulators of stem cell growth. Goodman, Burch & Basford (1972) were of the same opinion and put forward that an experimentally induced weakening of the resistance provides a good system for studying the control of haemopoiesis.

The authors considered it important to study the aberration of CFU kinetics after transplantation in a donor–host combination, which is subject to genetic resistance. In the present study growth curves of C57BL CFU transplanted into F_1(C57BL × CBA) were established and compared with isogeneic $F_1 \rightarrow F_1$ growth curves. The CFU kinetics are studied in both spleen and femur since Rauchwerger, Gallagher & Trentin (1973) observed a remarkable difference in colony formation in bone marrow and spleen after bone marrow transplantation in a resistant donor–host combination. Our results indicated that the long lag period in the kinetics of the CFU population in both spleen and femur was the cause of the poor growth. The data of McCulloch, Gregory & Till (1973) presented similar evidence that a delayed proliferation of CFU-s and CFU-c after transplantation of bone marrow was the cause of the impaired colony formation. To reveal a possible influence of stromal cells of splenic origin on the resistance in the marrow cavity, splenectomy of the recipient mice was carried out. The long turnover time of the stromal cells, which are of importance for haemopoiesis as shown by Knospe, Blom & Crosby (1966), made it necessary to perform splenectomy 5–6 months before irradiation and transplantation. In such splenectomized mice resistance could no longer be detected.

MATERIALS AND METHODS

Mice

Adult C57BL/Rij and F_1(C57BL/Rij × CBA/Rij) male mice, varying from 3 to 7 months old, were used. The C57BL/Rij were purchased from the Medical Biological Laboratory TNO, Rijswijk, and from the Reactor Center, Petten, The Netherlands. The F_1(C57BL/Rij × CBA/Rij) were bred at the Department of Animal Breeding of the Erasmus University, Rotterdam, The Netherlands.

Irradiation

The recipient F_1 mice received 950 rads and the recipient C57BL/Rij 800 rads whole body irradiation generated in a Philips Müller MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of the irradiation were: 250 kV (constant potential); 11 mA; added filtration of 1·0 mm Cu; irradiation was corrected for field inhomogeneity; focus–object distance 53 cm; animals were irradiated at a dose rate of 30–35 rad/min. Maximal backscatter was achieved by placing the cage on a layer of 11 cm hardboard. During irradiation the dose was measured with a Baldwin Ionex dosimeter. Radiation control mice died in 9–16 days.

Colony assay

The spleen colony assay of Till & McCulloch (1961) was used to measure the number of CFU. Cell suspensions of marrow and spleen were prepared, and diluted in a balanced salt solution (Mishell & Dutton, 1967). Cells were kept in melting ice till dilution and

injection. Cell counting was performed in a Coulter counter model B. Each suspension was administered to a group of ten to fifteen mice. The recipient mice were killed on day 8 after irradiation and the spleens were fixed in Telleyesniczky's solution. The colonies were counted using the low power objective of a stereo-microscope.

CFU growth curve

The growth curves of CFU in irradiated mice receiving marrow transplants were determined by the method of periodic sampling (McCulloch & Till, 1964). The primary recipients received 5×10^6 bone marrow cells. This dose made an accurate measurement possible. From the results it was concluded that this dose did not override resistance. The effect of overriding by huge donor cell doses is the subject of a further study. At various times after irradiation and bone marrow transplantation into a large number of recipient mice, groups of three or four animals were sacrificed and bone marrow and splenic cell suspensions were prepared. These suspensions were tested for their CFU content by injection of a known proportion of the suspension into irradiated recipients of the same strain as the original marrow donor. An estimate of the proportion to be injected was established by pilot experiments. The donor origin of C57BL CFU taken from F_1 chimaeras was ascertained regularly by specific antiserum treatment. The results indicated that no detectable numbers of host type CFU were present in the chimaeras.

Splenectomy

Splenectomy was performed 5–6 months before irradiation and transplantation. Less than 5% postoperative mortality was observed.

^{3}H-TdR suicide

Spleen cell suspensions were made by pooling cells from six animals. One half of the suspension, being 0·5 ml, was mixed with an equal volume of a solution of tritiated thymidine (^{3}H-TdR) (sp. act. 5 Ci/mmol, The Radiochemical Centre, Amersham) in B.S.S. at a concentration of 200 μCi/ml, so that the final concentration of tritiated thymidine was 100 μCi/ml. The other half of the suspension was incubated with an equal amount of cold thymidine. Both suspensions were incubated for 30 min at a temperature of 37°C with frequent agitation. At the end of the incubation period the cells were washed with B.S.S. and kept in melting ice until injection into recipient mice, which were isogeneic to the original marrow donor. The incubated cells were administered to two groups of fifteen recipient mice. The per cent of killing with ^{3}H-TdR was calculated as follows:

$$1 = \frac{\text{No. of CFU from } ^3\text{H-TdR incubated suspension}}{\text{No. of CFU from } ^1\text{H-TdR incubated suspension}} \times 100\%$$

RESULTS

Colony formation

The colony formation of C57BL bone marrow was studied after isogeneic as well as after semi-isogeneic (C57BL → F_1) transplantation. The semi-isogeneic colony formation was lower than the isogeneic (C57BL as well as F_1) colony formation. From Table 1 it

TABLE 1. Colony forming ability of isogeneic and semi-isogeneic transplanted marrow

Donor	Host	CFU/10^5 bone marrow cells
F_1 (C57BL × CBA)	F_1 (C57BL × CBA)	4·1 ± 0·5 (S.E.M.)
C57BL	C57BL	2·3 ± 0·4
C57BL	F_1 (C57BL × CBA)	0·3 ± 0·04

appears that the colony forming effectiveness of C57BL marrow transplanted to F_1 was about one tenth of the isogeneic colony formation. The size of the semi-isogeneic colonies was remarkably reduced when compared with the isogeneic colonies. These results agree with the findings of McCulloch & Till (1963), who described a similar repression of colony formation and justify the use of the transplantation combination C57BL → F_1 for our studies of genetic resistance.

CFU growth curves

CFU growth curves were established in spleen and femur after both isogeneic and semi-isogeneic transplantations. The splenic curves (Fig. 1) show after both transplantations a similar 2 hr seeding of about 15–20% of the original inoculum. The decrease phase which exists during the first 24 hr after isogeneic transplantation is prolonged after semi-isogeneic transplantation and exists for about 72 hr. The exponential growth phase which

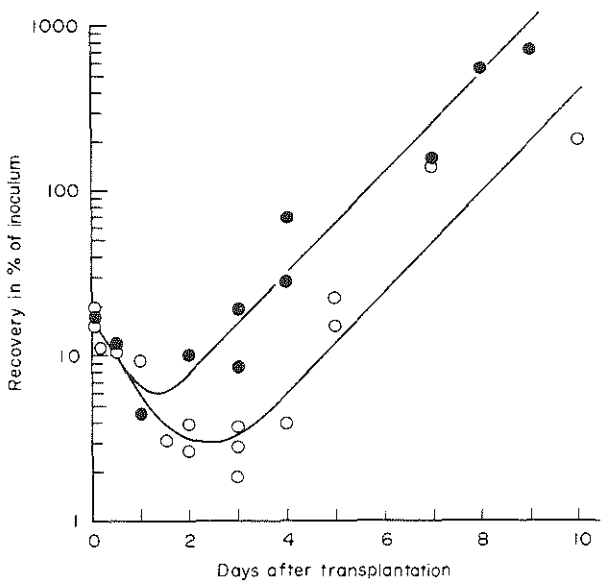

FIG. 1. CFU growth curves in spleen after semi-isogeneic and isogeneic transplantation of 5×10^6 bone marrow cells. Curves are drawn by eye. ○, C57BL → F_1(C57BL × CBA); ●, F_1(C57BL × CBA) → F_1(C57BL × CBA).

follows the decrease phase has the same characteristics in both transplantation combinations. The CFU doubling time is 22 hr for both curves. The difference between the two curves is the prolonged decrease period after semi-isogeneic transplantation which causes a very low minimum at 72 hr after transplantation. The femoral curves (Fig. 2) show a

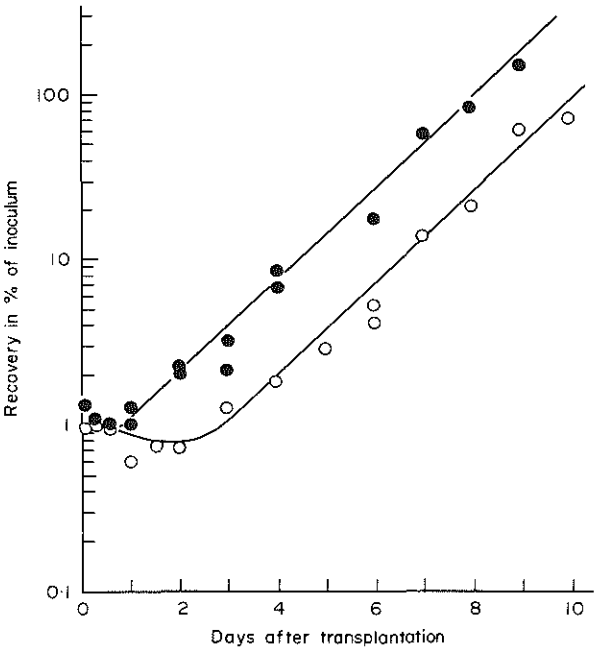

FIG. 2. CFU growth curves in femoral bone marrow after semi-isogeneic and isogeneic transplantation of 5×10^6 bone marrow cells. Curves are drawn by eye. ○, C57BL → F_1(C57BL × CBA); ●, F_1(C57BL × CBA) → F_1(C57BL × CBA).

pattern similar to the splenic curves. The 2 hr seeding and the exponential growth phase are similar; however, the lag period is prolonged after semi-isogeneic transplantation. The exponential growth phase starts about 48 hr later when the marrow is transplanted semi-isogeneically. A moderate decrease is observed during the prolonged lag period.

Splenectomized recipients

The femoral CFU growth curve of C57BL bone marrow transplanted to splenectomized F_1 mice shows no difference from the isogeneic curve ($F_1 \rightarrow F_1$), when we used the recipient mice 5–6 months after splenectomy (Fig. 3), hence the delay of the growth phase of semi-isogeneic cells was abolished by splenectomy. When splenectomy was performed 2 months before irradiation and transplantation the delay of the growth curve was similar to that in non-splenectomized mice (data are not shown in the graphs).

^{3}H-TdR suicide

The ^{3}H-TdR suicide technique was used to study the cell cycle kinetics of CFU after both isogeneic and semi-isogeneic transplantation in the spleen. The results (Table 2)

revealed no difference in cell cycle kinetics neither during the decrease period at 2 hr nor at day 2. The results indicate for the 2 hr data a ^{3}H-TdR suicide of about 20% for both isogeneic and semi-isogeneic transplanted marrow. For the suicide on day 2 a mean of about 40% was found for both transplantation combinations.

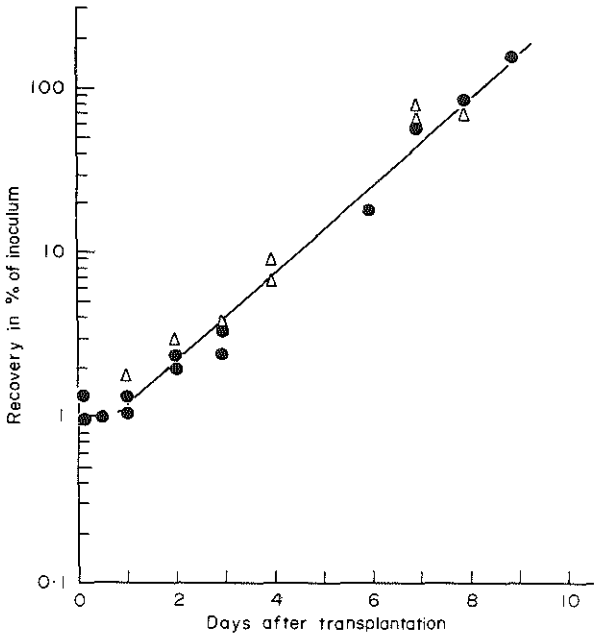

FIG. 3. CFU growth curves in femoral bone marrow after semi-isogeneic transplantation to splenectomized F_1 and after isogeneic transplantation to normal F_1 of 5×10^6 bone marrow cells. Curves are drawn by eye. △, C57BL → F_1(C57BL × CBA)Sx; ●, F_1(C57BL × CBA) → F_1(C57BL × CBA).

TABLE 2. Per cent of CFU recovered from spleen in S-phase killed by ^{3}H-TdR incubation

Time after transplantation of marrow	F_1(C57BL × CBA) → F_1(C57BL × CBA)	C57BL → F_1 (C57BL × CBA)
2 hr	17·8% Mean 21·8%	19·4% Mean 18·0%
	19·5	20·0
	23·2	18·7
	26·8	13·9
2 days	30·8% Mean 38·1%	38·6% Mean 39·9%
	45·0	46·0
	38·5	25·0
		50·0

DISCUSSION

The results we obtained demonstrate that the transplantation combination of C57BL → F_1 (C57BL × CBA) is suitable for studies of the phenomenon 'genetic resistance' (Trentin *et al.*, 1973). It could be shown that less colonies were formed in the semi-isogeneic combination than in the isogeneic combination.

Furthermore the size of the colonies in the semi-isogeneic combination was smaller. This indicates that not only the multiplication of CFU is inhibited, but also the proliferation of precursor cells and possibly even of more differentiated cells is restricted.

The shape of the CFU growth curves in both spleen and femur obtained after C57BL → F_1 as well as after F_1 → F_1 transplantation displays the pattern of the standard CFU growth curve, only the decrease phase in the splenic curve and the lag phase in the femoral curve are prolonged after semi-isogeneic transplantation. The onset of the exponential growth phase is delayed about 48 hr in both organs. McCulloch *et al.* (1973) showed that the CFU doubling time in the spleen during the exponential growth phase was similar after both transplantation combinations. Our results confirm their findings and extend them to the femoral CFU kinetics. It was shown that the splenic curves both exhibited a dip before the exponential growth phase started. The very low minimum reached was an interesting result: the shape of the dip was similar in both curves. This observation gives rise to the idea that the cause of the dip may be the same in both curves.

The cause of the decrease phase in isogeneic transplantation has been a matter of discussion among many authors. The migration of CFU (Kretchmar & Conover, 1969), a differentiation death of CFU (Lahiri, Keizer & van Putten, 1970), a higher sensitivity to the trauma of suspending the cells (Metcalf & Moore, 1971) were postulated to explain the initial decrease phase.

The results obtained with the ^{3}H-TdR suicide technique indicate that the fraction of CFU in cycle is similar after both transplantation combinations. It can be concluded that the delayed multiplication of P-CFU is not due to an initial resting phase of the transplanted P-CFU.

The data obtained with splenectomized F_1 recipients indicate that a splenic factor is responsible for the phenomenon 'genetic resistance'. Goodman & Grubbs (1970) reported that splenectomy carried out 2 weeks before irradiation and transplantation had no effect on genetic resistance when the ^{59}Fe uptake was measured. We have data which indicate that splenectomy performed 8 weeks before transplantation has no effect either.

The cause of the splenic factor is still obscure. If a 'managerial' cell (McCulloch & Till, 1970) exists, it will be very likely in our experimental situation that its origin is splenic, because splenectomy abolishes its effect in the femur. A humoral splenic factor responsible for genetic resistance is not very likely. If a humoral factor is responsible, a removal of the spleen should be followed by a rather fast disappearance of the resistance. The results of Bennett (1973) and of Kumar, Bennett & Eckner (1974) showed that the administration of ^{89}Sr causes a complete depletion of the marrow which goes with suppression of genetic resistance in the spleen. Bennett (1973) concludes from this 'bone marrow-ectomy' experiments that a cell originating in the marrow, the 'M' cell, is responsible for genetic resistance.

These data and conclusion seemed to be rather contradictory to our results concerning the abrogation of resistance caused by spleen-ectomy. The rather rapid onset of the suppression caused by ^{89}Sr administration could be caused by killing a population of cells originating in the marrow but it could also be caused by a mechanism similar to that causing

suppression after administration of *Corynebacterium parvum* (Cudkowicz & Bennett, 1971), endotoxin and complete Freunds adjuvants.

The influence of splenectomy, 'bone marrow-ectomy' and other treatments abrogating genetic resistance have to be studied in more detail before the factors responsible for the existence and disappearance of genetic resistance can be revealed.

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Weakening of Genetic Resistance

I. The Effect of Injection of Endotoxin, Freund's Complete Adjuvant and Alloantiserum

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A parent to F_1 transplantation combination was used to study the weakening effect of endotoxin, Freund's complete adjuvant and alloantiserum on genetic resistance. The relationship between time of treatment with endotoxin, *Salmonella typhosa*, and Freund's complete adjuvant, and their weakening effect was assessed by use of the spleen colony technique.

CFU growth studies revealed that both endotoxin and alloantiserum were capable of weakening genetic resistance in the spleen but were unable to induce weakening of the resistance in the femoral marrow cavity. These results led us to the conclusion that the agents might not have a direct effect on the effector cells of the resistance.

The weakening induced by endotoxin and alloantiserum seemed to be related to a certain immunological phenomenon in the spleen. In this phenomenon macrophages are likely to play a role since a number of agents capable of weakening resistance were known for their capacity to influence the mononuclear phagocytic system.

Key words: genetic resistance – endotoxin – Freund's complete adjuvant – CFU growth – colony formation

The defective growth of bone marrow transplanted after a lethal total body irradiation of the host in certain donor host combinations, hybrid resistance (Cudkowicz & Stimpfling 1964), allogeneic resistance and xenogeneic resistance, has been designated as 'Genetic resistance' by Trentin et al. (1973).

Weakening of the resistance with different agents has been used to study the characteristics of the phenomenon. Measurements of ^{125}IUdR uptake showed that administration of suspensions of heat-killed *Corynebacterium parvum* improved the development of the graft in the spleen (Cudkowicz & Bennett 1971a). Gregory et al. (1972) and McCulloch et al. (1973) showed that certain alloantisera are capable of weakening the resistance in the spleen. They measured an increased colony formation as well as an advanced multiplication of CFU in the spleen of the alloantiserum treated

recipient mice. These authors suggested that the antibodies of the alloantisera were reacting with surface antigens with 'cognition-reaction' functions of 'managerial cells'.

So far studies on weakening of hybrid and of allogeneic resistance were limited to splenic hemopoiesis. Since the spleen plays only a limited role in hemopoiesis we considered it worthwhile to study the influence of such treatments on both splenic and femoral bone marrow hemopoiesis. The splenic hemopoiesis was measured by means of spleen colony formation as well as CFU growth. Femoral bone marrow hemopoiesis was measured as CFU growth. A clear difference between the effects in spleen and femur was observed in our experiments. It was found that neither endotoxin nor alloantiserum were able to weaken the resistance in the femoral marrow cavity. Lotzova & Cudkowicz (1974) postulated a possible role of macrophages in the phenomenon of genetic resistance; the weakening effect of *Corynebacterium parvum*, endotoxin, Freund's complete adjuvant and antisera are discussed in the context of their postulation.

MATERIALS AND METHODS

Mice

Adult C57BL/Rij and F1 (C57BL/Rij × CBA/Rij) male mice varying from 3–7 months old were used. The C57BL/Rij mice were purchased from the Medical Biological Laboratory, TNO, Rijswijk, and from the Reactor Centre of The Netherlands, Petten, The Netherlands. The F1 (C57BL/Rij × CBA/Rij) mice were obtained from the Animal Breeding Centre of the Erasmus University, Rotterdam, The Netherlands.

Irradiation

Irradiation was performed using a Philips-Mueller MG 300 X-ray machine. Physical constants and other radiation details were described in a previous paper (Buurman et al. 1975). Recipient F1 mice received 950 rads and recipient C57BL/Rij mice 800 rads whole body irradiation. Radiation control mice (both treated and non-treated) died between 9 and 16 days after irradiation, on day 8 less than an average of 0,2 spleen colonies was found in such mice.

Spleen colony assay

The spleen colony technique was performed as described previously (Buurman et al. 1975). The enhancing effect of endotoxin and Freund's complete adjuvant was expressed as a ratio of the number of colonies derived from treated mice divided by the number of colonies derived from non-treated mice. The ratios and the 95 % confidence limits were calculated by random sampling of pairs of the number of colonies from spleens of both treated and control mice. The quotients of the random pairs were used for the calculations. Each point in the figures was calculated from data of two groups of 15 or more mice.

Injections

Endotoxin, lipopolysaccharide of *Salmonella typhosa* prepared by the method of Boivin (Difco Laboratories, Michigan, U.S.A.) was intravenously administered. Freund's complete adjuvant was intraperitoneally administered in a dose of 0.05 ml per mouse.

Alloantiserum was produced by six weekly injections of C3H/fA mice with C57BL/Rij spleen cells. The serum was administered in a dose of 0.5 ml intraperitoneally, 2 hours before irradiation. The C3H anti C57BL sera which have been demonstrated to be capable of abrogating genetic resistance in spleen colony formation experiments were used for CFU growth kinetics studies in spleen and marrow.

CFU growth kinetics

CFU growth was established 6 days after irradiation and transplantation of 5×10^6 bone marrow cells into lethally irradiated F1 mice. The donor origin of C57BL CFU taken from F1 chimeras was ascertained regularly by specific antiserum treatment before retransplantation to irradiated C57BL secondary hosts. The results in-

dicated that no detectable numbers of host type CFU were present in the chimeras. The determination of CFU growth was performed 6 days after transplantation. The 6 day interval was chosen because the CFU in both spleen and femur are then for 3 or more days in an exponential growth phase after both isogeneic and semi-isogeneic transplantation.

RESULTS

Colony formation

Endotoxin, a B lymphocyte stimulant (Dresser & Phillips 1973) was used to study weakening of the colony repression. The effect of endotoxin injections at various times before irradiation and transplantation of the bone marrow was studied in both isogeneic and semi-isogeneic colony formation. The effect of endotoxin was expressed as the ratio between colony formation in treated versus non-treated mice. The ratio was obtained by dividing the number of colonies in treated mice by the number of colonies in non-treated mice. The weakening of colony repression was found to be dependent on the time interval between endotoxin administration and transplantation (Figures 1, 2). This applied for doses of both 10 μg and 100 μg of endotoxin from *S. typhosa*. The effect was maximal when the recipient mice were treated 14–15 days before transplantation. The ratio was about 10 at that time. This value indicates a complete abrogation of the resistance, since the colony formation in the semi-isogeneic transplantation combination was about 1/10 of the normal isogeneic colony formation (Buurman et al. 1975). It was observed that, when the resistance was abrogated, spleen colonies in treated semi-isogeneic recipients were greater than in untreated mice. However, they seemed to be still smaller than those in the isogeneic combination. Furthermore it appears from Figures 1 and 2 that the isogeneic colony formation was hardly affected by the treatment. No significant effect was observed at any time after treatment.

Freund's complete adjuvant, intraperitoneally administered before irradiation and transplantation, was used to study the influence of a strong T lymphocyte stimulant (Allison 1973) on genetic resistance. Treatment with Freund's complete adjuvant resulted in a significant weakening of the colo-

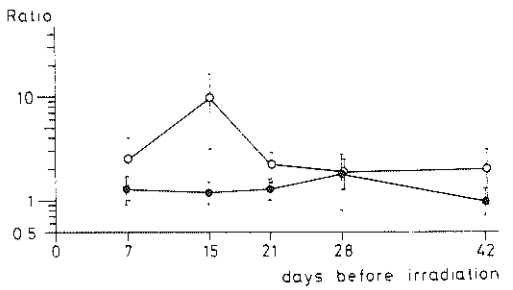

Figure 1. Enhancement of colony formation after intravenous administration of 10 μg *Salmonella typhosa* endotoxin.

$$\text{Ratio} = \frac{\text{no. colonies treated recipients}}{\text{no. colonies control recipients}}$$

○ C57BL bm → F1 (C57BL × CBA)
● F1 (C57BL × CBA) bm → F1 (C57BL × CBA)

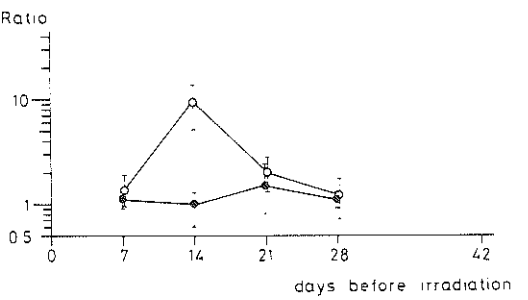

Figure 2. Enhancement of colony formation after intravenous administration of 100 μg *Salmonella typhosa* endotoxin.

$$\text{Ratio} = \frac{\text{no. colonies treated recipients}}{\text{no. colonies control recipients}}$$

○ C57BL bm → F1 (C57BL × CBA)
● F1 (C57BL × CBA) bm → F1 (C57BL × CBA)

TABLE 1

Effect of alloantiserum on colony forming ability of semi-isogeneic transplanted marrow

Donor	Host	Treatment	CFU/10^4
C57BL	F1 (C57BL × CBA)	none	0.3 ± 0.04
C57BL	F1 (C57BL × CBA)	0.5 ml C3H anti C57BL serum i.p.*	2.7 ± 0.5

* Injection 2 hours before irradiation and transplantation.

ny repression at day 7, 15 and 28 after treatment (Figure 3). Cudkowicz & Bennett (1971b) described a weakening of genetic resistance at 7–18 days after intravenous injection of heat-killed *Corynebacterium parvum*. In our experiments Freund's complete adjuvant also showed a prolonged effect. The results differ from the results obtained after endotoxin treatment, where the effect is limited to day 14–15. However, when we used doses as high as 500 μg endotoxin (data not shown), we obtained results similar to those of Cudkowicz & Bennett (1971b).

The abrogation of genetic resistance by means of alloantisera as described by Gregory et al. (1972) was also observed in our experiments. The C3H anti-C57BL sera which we tested caused a significant diminution of the resistance (Table 1). The size of the colonies seemed similar to the isogeneic C57BL → C57BL and F1 → F1 colonies.

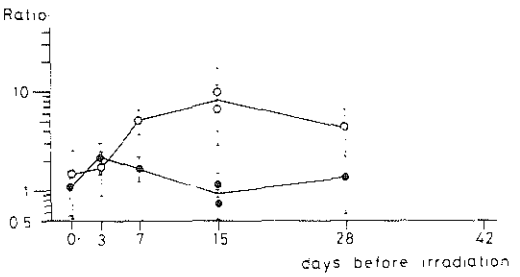

Figure 3. Enhancement of colony formation after intraperitoneal administration of 0.05 ml complete Freund's adjuvant.

$$\text{Ratio} = \frac{\text{no. colonies treated recipients}}{\text{no. colonies control recipients}}$$

○ C57BL bm → F1 (C57BL × CBA)

● F1 (C57BL × CBA) bm → F1 (C57BL × CBA)

CFU growth kinetics

The endotoxin-induced weakening of genetic resistance was measured by assessing the number of CFU in spleen and femur 6 days after intravenous administration of 5×10^6 C57BL/Rij marrow cells. Both splenic and femoral marrow CFU growth were measured in order to investigate whether the weakening was a general effect in marrow and spleen or only restricted to the spleen.

Table 2 shows the splenic and femoral CFU growth of isogeneic F1 and allogeneic C57BL marrow cells transplanted into control and treated F1 mice. The splenic CFU growth of C57BL marrow transplanted into resistant F1 mice was shown to be improved when endotoxin was administered to F1 mice 14–15 days before transplantation. The CFU growth of semi-isogeneic transplanted marrow in treated recipients never reached the same level as in the isogeneic F1 → F1 combination. The increase in CFU growth, however, was not observed

TABLE 2

CFU growth in isogeneic and semi-isogeneic conditions measured 6 days after transplantation of 5×10^6 marrow cells

Donor	Host	Treatment	No. CFU/spleen	No. CFU/femur
F1	F1	—	3700	650
			8950	540
			2850	720
			5080	468
			3960	296
C57BL	F1	—	463	92
			400	88
			280	59
			160	61
			170	44
C57BL	F1	Endotoxin	2275	48
		100 μg	2000	31
		i.v.*	1600	22
C57BL	F1	0.5 ml C3H	3100	150
		anti C57BL	2000	176
		serum i.p.*	2120	80
			2260	70

* Endotoxin was administered 15 days before transplantation.
Alloantiserum was administered 2 hours before irradiation.

in the femur. The treatment with endotoxin seemed to be deleterious to the femoral CFU growth. We can conclude from these data that endotoxin did weaken the resistance, as assessed by CFU growth in the spleen but not in the marrow.

The splenic CFU growth of C57BL marrow transplanted to F1 was greatly affected by alloantiserum treatment (Table 2). The splenic CFU growth did not reach the normal isogeneic level (Table 2), which is in accordance with the results of McCulloch et al. (1973). The number of C57BL CFU obtained from the femurs of antiserum-treated F1 mice was similar to that of the non-treated F1 mice. This indicated that no weakening of the resistance was induced in the marrow by alloantiserum treatment. The antiserum treatment was not deleterious to the marrow CFU growth. As to the discrepancy between the results in splenic and femoral bone marrow hemopoiesis it seems unlikely that the antibodies of the alloantisera are reacting with the antigens with 'cognition-reaction' functions of 'managerial cells' as proposed by McCulloch et al. (1973), since these 'managerial cells' are expected to be present in both spleen and femur.

DISCUSSION

Cudkowicz & Bennett (1971a) observed a weakening of genetic resistance after treat-

ment with the immunostimulant *Corynebacterium parvum*. From 7–21 days after treatment, an increased ^{125}IUdR uptake was detected in the spleen as compared with the non-treated mice. Our data obtained by treatment with Freund's complete adjuvant also show a prolonged weakening of the resistance over a period of 7–28 days after injection (Figure 3). We can conclude that the B lymphocyte stimulant, *Corynebacterium parvum,* as well as the T lymphocyte stimulant, Freund's complete adjuvant, which both strongly affect macrophages (Allison 1973), are able to induce weakening of the resistance.

The diminution of the resistance induced by both adjuvants in the doses used was extended over several weeks. Endotoxin treatment in doses of 10 μg and 100 μg caused a decrease of the resistance, which was restricted to a relatively short period (around the fourteenth day after injection). In a few pilot experiments (data not shown) it was observed that injection with a high dose of *S. typhosa* (500 μg) endotoxin induced prolonged weakening of the resistance from 7 to 21 days after treatment. This might indicate that at such a high dose the endotoxin has the same effect as Freund's adjuvant and *Corynebacterium parvum*.

It was concluded that in order to get crucial information about the weakening of genetic resistance, the hemopoietic repopulation has to be studied in both spleen and femur. It would be of interest to investigate if the decrease in resistance as observed in the spleen is related to certain changes in the lymphoid tissues. We observed that the treatment with endotoxin did not affect the resistance in the marrow cavity.

Treatment with alloantiserum gave similar results to those reported by Gregory et al. (1972) and McCulloch et al. (1973). Enhanced spleen colony formation, as well as improved CFU growth, in the spleen was observed; however, alloantisera did not influence CFU kinetics in the femur. The suggestion that antibodies in the serum were directed against surface antigens, which might have 'cognition reaction' functions (McCulloch et al. 1973), is not very likely because this would imply that the 'managerial cells' have different surface antigens in spleen and femur.

It appears that there is an interesting coincidence between the capacities of some substances to induce lymphocyte trapping as well as to weaken genetic resistance. Xenogeneic sera (Till et al. 1970), Freund's complete adjuvant, *Corynebacterium parvum* (Cudkowicz & Bennett 1971a), silica (Lotzova & Cudkowicz 1973, Lotzova & Cudkowicz 1974) and carragheenan (Lotzova & Cudkowicz 1973) are known to be substances capable of weakening genetic resistance; they are also known to be active in lymphocyte trapping (cf. Frost & Lance 1973). The latter authors suggested that macrophages play a central role in this trapping phenomenon. Lotzova & Cudkowicz (1974) put forward the suggestion that macrophages participate in the mechanism that underlies the phenomenon of genetic resistance. Their proposal was based on their results that the administration of Poly-2-vinylpyridine n-oxide (PVNO), a macrophage stabilizing agent, prevented the abrogation of resistance caused by silica and carragheenan. It has been reported that PVNO inhibits the killing of macrophages by silica and carragheenan (Rios & Simmons 1972). The lysis of macrophages was concluded to be the cause of the abrogation of resistance induced by silica and carragheenan. It is not clear whether agents

such as alloantisera, endotoxin, Freund's complete adjuvant and *Corynebacterium parvum* induce weakening by killing macrophages. These macrophages could be the effector cells of genetic resistance or cells which influence the effector cells. Since it was demonstrated that silica also causes the death of femoral macrophages (Pearsall & Weiser 1968) it is of interest to study whether it is capable of abrogating the resistance in the femoral marrow cavity too. If not, this would indicate that the effector cell is not killed. Our data do not indicate the identity of the effector cells of genetic resistance. It has been shown that the splenic environment is necessary for weakened resistance induced by endotoxin and alloantiserum. This could imply that the effector cells are only in the splenic environment capable of reacting to the treatments, or that the effector cells are subject to local stimulation by other cells in the spleen. This stimulation could be the result of a cell to cell contact or be mediated by humoral factors which are only effective over a very short range, due to local high concentrations of the humoral factor. Since the resistance is not reduced in the marrow cavity the stimulation apparently does not occur there. These hypotheses are being subjected to further study. Bennett (1973) proposed an 'M' cell, a marrow-dependent cell as effector cell of allograft rejection. Mice treated with ^{89}Sr did not reject bone marrow whereas control mice did so. The continuous inflow in the spleen of irradiated cells from the bone marrow will disturb the normal function of the lymphoid and mononuclear phagocytic cells in the spleen. This might have a weakening effect on the resistance in the spleen.

The abrogation of the resistance after sublethal irradiation might also be caused by the same process of loading the mononuclear phagocyte system with cell debris. The compensatory increase in hematopoietic tissue in the spleen due to ^{89}Sr administration was suggested to be regulated by a humoral mechanism (Fried et al. 1966). Such a humoral mechanism itself could have an influence on the resistance as has been shown in hypoxic mice which also show a weakened resistance in the spleen (Beran & Tribukait 1974). The influence of radiation and of hypoxia need further investigation to disentangle the process of graft rejection.

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Weakening of Genetic Resistance
II. The Effect of Injection of the Macrophage Toxic Agents Silica and Carragheenan and the Cytostatic Drugs Cyclophosphamide, Busulphan and Vinblastin

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Silica (4 mg/i.v.) and Carragheenan (20 mg/i.p.) were shown to be capable of weakening hybrid resistance in the spleen whereas no improved growth was observed in the femoral bone marrow. Histological data did not indicate that the agents in the doses used caused significant macrophage mortality. It was concluded that the weakening effect of the agents was not due to death of the effector cells but possibly to a stimulation of the mononuclear phagocytic system in the spleen.

Cyclophosphamide (250 mg/kg) induced a weakening of hybrid resistance which was observed both in the spleen and the femoral bone marrow. Experiments with the alkylating agent Busulphan and with Vinblastin showed that both agents were only capable of inducing a moderate stimulation of growth of the transplant in the spleen. A direct specific influence of cyclophosphamide on the microenvironment was proposed.

Xenogeneic resistance was studied by transplantation of rat bone marrow to mice. Silica and cyclophosphamide were shown to be capable of inducing a weakening of the resistance in the spleen. The development of the graft in the femoral bone marrow did not appear to be affected by the administration of silica or cyclophosphamide.

Key words: genetic resistance – silica – carragheenan – cyclophosphamide

In a previous paper (Buurman & van Bruggen 1975) it was shown that a weakening of genetic resistance to a bone marrow graft could be induced by immunologically active agents such as endotoxin, Freund's complete adjuvant and alloantiserum. It could be demonstrated that these agents enhanced the growth of CFU in the spleen whereas the CFU growth in the femoral bone marrow remained repressed. It was suggested that macrophages in the spleen may play a role in the process of weakening the resistance to repopulation after transplantation.

In addition to the agents mentioned

above, in the literature other agents have been reported to be capable of inducing a weakening of the resistance. Lotzová & Cudkowicz (1973, 1974) showed that the administration of the macrophage toxic agents, silica and carragheenan, induced an abrogation of the resistance in the spleen. They postulated that macrophages were the effector cells responsible for the death of the grafted hemopoietic cells. A second cell type was suggested to be involved in the process, a thymus independent lymphocyte responsible for the very specific recognition aspect of the phenomenon.

Other mechanisms responsible for the resistance to a bone marrow graft have been reported. McCulloch & Till (1970) and McCulloch et al. (1973) described a managerial cell theory. According to this theory managerial cells were involved in the regulation of normal proliferation and differentiation of hemopoietic cells via short range interactions. CFU repression or genetic resistance to a bone marrow graft was considered to be an inappropriate regulation due to differences in antigenic receptors of grafted hemopoietic cells and the managerial cells present in the hemopoietic organs of the host.

In the present paper two macrophage toxic agents, silica and carragheenan (Allison et al. 1966) were used to investigate the role of macrophages as possible effector cells. We studied the effects of pretreatment of recipient mice with the agents on CFU growth after transplantation of resistant marrow both in the spleen and the femoral bone marrow. Furthermore we used cyclophosphamide (Cy), busulphan and vinblastin in order to investigate whether we could gather information about the proliferative nature of the effector cells and the weakening induced by cytostatic agents. The weakening capacity of Cy and busulphan was first reported by Sensenbrenner & Santos (1969) who showed that mice conditioned for bone marrow transplantation by a combination of both drugs have a greatly diminished hybrid resistance or none at all when measured by spleen colony formation. Cudkowicz & Bennett (1971a, b) reported similar results for hybrid and allogeneic resistance when Cy was used. They suggested that the abrogation of the resistance, measured in the spleen as an enhanced ^{125}IUdR uptake, was possibly caused by a lethal effect of the drug on the effector cells, which were suggested to be killer cells of the grafted hemopoietic cells.

MATERIALS AND METHODS

Experimental animals

Adult C57BL/Rij, F1(C57BL/Rij x CBA/Rij) and F1(DBA/2 x C57BL/Rij) male mice 3–7 months of age were used.

The C57BL/Rij and F1(DBA/2 x C57BL/Rij) were purchased from the Medical Biological Laboratory, TNO, Rijswijk.

The F1(C57BL/Rij x CBA/Rij) mice were obtained from the Animal Breeding Centre of the Erasmus University, Rotterdam, The Netherlands.

Adult WAG/Rij male rats were used as marrow donors.

Irradiation

Irradiation was performed using a Philips-Mueller MG300 X-ray machine. Physical constants and other radiation details were described in a previous paper (Buurman et al. 1975). Recipient F1 (C57BL/Rij x CBA/Rij) mice received 950 rad, recipient C57BL/Rij 800 rad and recipient F1 (DBA/2 x C57BL/Rij) 850 rad whole body irradiation. Radiation control mice (treated with silica, carragheenan, or a cytostatic, as well as non-treated) died between 9 and 16 days after irradiation; on day 8 less than an average of 0.2 spleen colonies was found in such mice.

Injections

Silica, particles < 5 μm (kindly provided by Dr. Ing. M. Reisner, Steinkohlenbergbauverein, Essen G.F.R.) 4 mg/mouse, suspended in a B.S.S. (Mishell & Dutton 1967) was administered i.v. Carragheenan (Sigma, St. Louis), 20 mg/mouse, dissolved in B.S.S. was administered i.p. Both agents were injected 2 hours after irradiation and 2 hours prior to transplantation.

Cyclophosphamide (Asta, Brackwede), 250 mg/kg, Vinblastin (Lilly & Co., Indianapolis), 2 mg/kg, Busulphan (kindly provided by Wellcome, Amsterdam), 50 mg/kg were administered i.p. 4 days before irradiation and transplantation. Two thirds of the LD_{50} was selected as the drug dose.

CFU growth kinetics

CFU growth was established 6 days after irradiation and transplantation of 5×10^6 bone marrow cells into lethally irradiated recipient mice. The CFU growth of spleen and femur are then in the exponential phase (Buurman et al. 1975).

For each assay 4–5 primary recipient mice were used. Femurs and spleens were used for bone marrow and spleen cell suspensions. A known proportion was injected into 10–15 irradiated recipient mice syngeneic with the original marrow donor. Spleen colonies were counted 8 days after transplantation.

Estimation of ^{125}IUdR uptake by the transplanted marrow

The estimation of the growth of a bone marrow transplant by means of the measurement of the ^{125}IUdR uptake several days after transplantation has been shown to be an accurate method (Cudkowicz & Stimpling 1964). The ^{125}IUdR uptake was established 6 days after irradiation and transplantation of 10^7 rat bone marrow cells into lethally irradiated recipient mice. Recipient mice received 1 μC of ^{125}IUdR i.p. ^{125}IUdR with a specific activity of 100 mCi/mg was purchased from the Radiochemical Centre, Amersham, England. No stable iodine was given beforehand. Animals were sacrificed 6 hours after injection of ^{125}IUdR and spleens and femurs were placed in a test tube. Radioactivity was measured in a Nuclear Chicago well-type scintillation counter. Radiation control mice both treated and non-treated were used to obtain information about the background incorporation in the marrow injected recipients. The ^{125}IUdR uptake in spleen or femur was expressed as a percentage of the total radioactivity injected into the animal. It was assumed that the uptake resulted almost entirely from incorporation of ^{125}IUdR into DNA. For each assay five recipient mice were used. The means of the percentages of uptake in ten femurs and five spleens are given.

Preparation for histological examination

For light microscopy perfusion fixation was carried out on anesthesized mice. The tissues were embedded in Epon. 2 μm thick sections were cut. Technical details were described in a previous paper from our laboratory (van Ewijk et al. 1974).

RESULTS

Hybrid resistance

Silica and carragheenan. Carragheenan administrated 2 hours prior to the transplantation resulted in a decrease of the resistance in the spleen, which was reflected by the increase in CFU numbers obtained from the spleens of mice treated in this way (Table 1). A similar influence of carragheenan on the resistance was shown by Lotzová & Cudkowicz (1973). Moreover, our results revealed that the resistance in the femoral bone marrow was not significantly influenced by carragheenan treatment (Table 1).

The weakening effect on the resistance caused by carragheenan seemed to be restricted to the spleen. Silica, another macrophage killing agent, administered 2 hours before transplantation produced similar results (Table 1). The absence of a weakening effect in the marrow could be due to an ineffective destruction of macrophages by both agents in the femoral bone marrow. Histology of the marrow (data not shown)

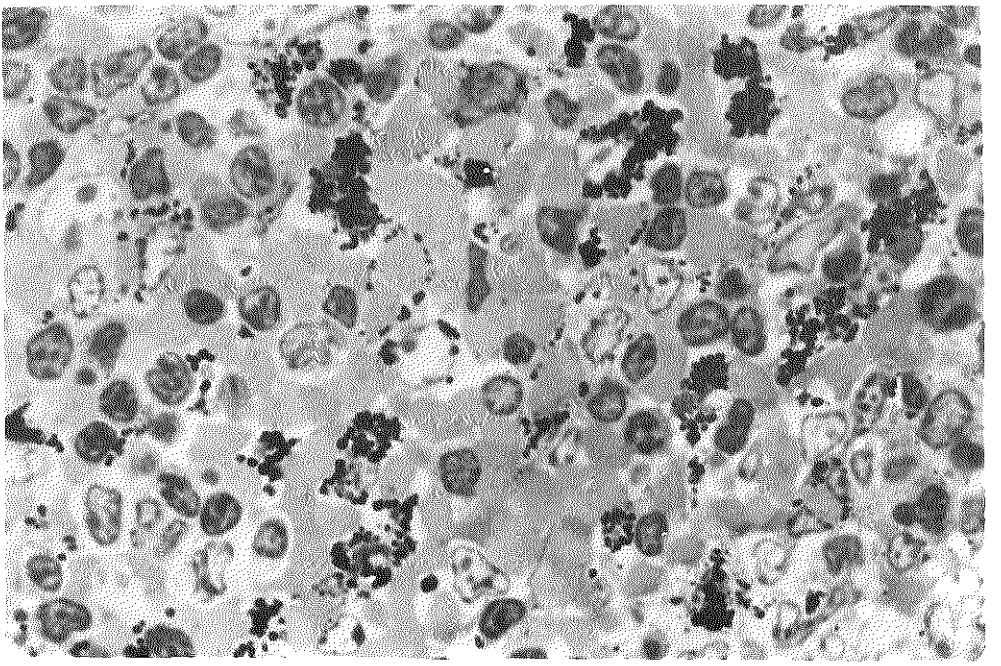

Figure 1. Section of the spleen of a mouse sacrificed 2 hours after silica injection and 30 min after carbon treatment. Note the apparent uptake of carbon particles in mononuclear phagocytes of the red pulp. (Magnification 1200 ×)

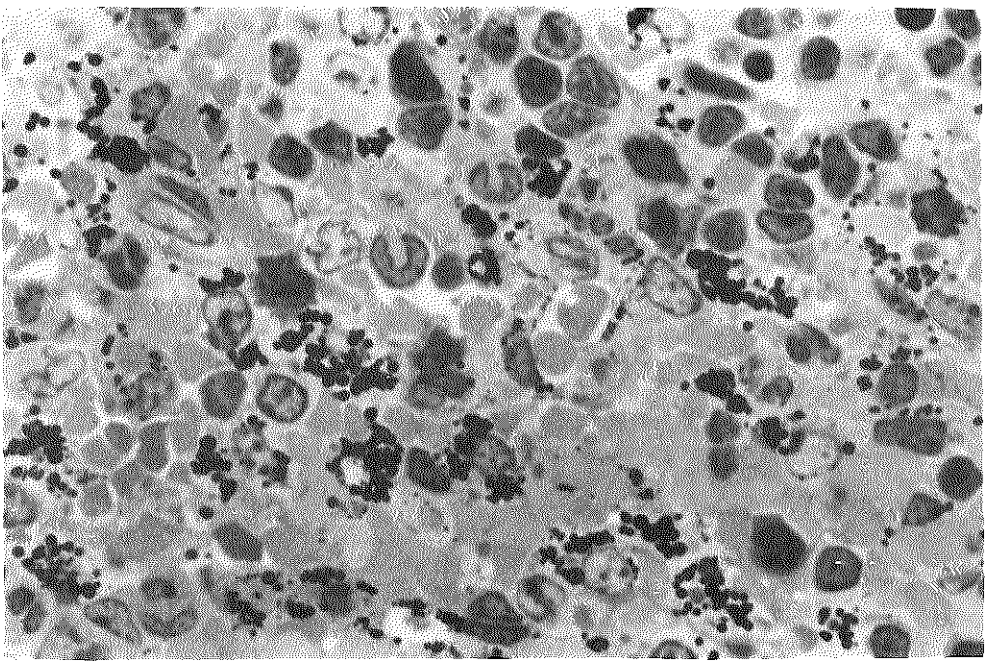

Figure 2. Section of the spleen of a mouse sacrificed 24 hours after silica injection and 30 min after carbon treatment. Note that the carbon particles are found again in the mononuclear phagocytes of the red pulp. (Magnification 1200 ×)

TABLE 1

CFU growth in semi-isogeneic condition measured 6 days after transplantation of 5×10^6 bone marrow cells

Donor	Host	Treatment	No. CFU/femur*		No. CFU/spleen	
C57BL	F1(C57BL × CBA)	silica 4 mg, i.v.	183		2460	
			146	mean	1850	mean
			41	105	2100	3078
			49		5900	
C57BL	F1(C57BL × CBA)	carragheenan 20 mg, i.p.	141		1900	
			134	mean	2325	mean
			63	113	2260	2162
C57BL	F1(C57BL × CBA)	control	85		565	
			88	mean	680	mean
			66	98	1240	1016
			152		1580	
C57BL	F1(DBA/2 × C57BL)	silica 4 mg, i.v.	180	mean	1600	mean
			305	243	3400	2500
C57BL	F1(DBA/2 × C57BL)	control	220	mean	1280	mean
			115	168	1680	1480

* Each suspension was prepared from 4–5 primary recipient mice.
CFU were assayed in 10–15 recipient mice.

and of the spleen (Figures 1, 2) of silica treated mice revealed a normal carbon uptake in macrophages at 2 h and 24 h after silica injection. Thus silica administration did not inhibit the carbon uptake of mononuclear phagocytes at 2 h and 24 h after injection. Carbon particles were administered 30 min before the animals were sacrificed. In the spleens and femurs of silica injected mice a minimal macrophage destruction was observed at 24 h and 48 h after injection. These results indicated that the silica dose used was not high enough to cause a marked depletion of macrophages. The silica dose, 4 mg/mouse, caused about 10 % early mortality of the irradiated and transplanted mice. Higher doses caused a significantly higher mortality, 10 mg/mouse caused 90 % mortality (data not shown). The cause of the mortality was not investigated.

Cytostatic agents: Cyclophosphamide, busulphan and vinblastin. We tried to investigate the mode of action of Cy on the resistance in the spleen and femoral bone marrow by using two other cytostatic agents. One being active against both cycling and non-cycling cells (busulphan) and the other being active against cycling cells only (vinblastin). We found that Cy weakened the resistance in both spleen and femoral bone marrow (Table 2). The effect of Cy on the isogeneic CFU growth was minor when compared with the effect on the semi-isogeneic CFU growth.

TABLE 2

CFU growth in isogeneic and semi-isogeneic conditions measured 6 days after transplantation of 5×10^6 bone marrow cells

Donor	Host	Treatment	No. CFU/femur*		No. CFU/spleen*	
C57BL	F1(C57BL × CBA)	cyclophosphamide 250 mg/kg, i.p.	470 185 445 340 250	mean 338	5575 2325 1750 3075 4250	mean 3395
C57BL	F1(C57BL × CBA)	busulphan 50 mg/kg, i.p.	155 130 60 115 90	mean 110	1100 2175 1225 1500 1375	mean 1475
C57BL	F1(C57BL × CBA)	vinblastin 2 mg/kg, i.p.	78 75 170 138 103	mean 113	1220 1680 3050 2375 1850	mean 2035
C57BL	F1(C57BL × CBA)	control	110 66 88 46 92	mean 80	1240 960 680 550 1080	mean 902
F1	F1	Cy	610 540 620 590	mean 590	3960 3480 4320 4840	mean 4150
F1	F1	control	468 296 395 332	mean 372	5080 3960 4520 2560	mean 4030

* Each suspension was prepared from 4–5 primary recipient mice. CFU were assayed in 10–15 recipient mice.

Busulphan and vinblastin affected the resistance only in a very moderate way in the spleen, whereas no clear effect was observed in the femoral bone marrow (Table 2). The alkylating agents Cy and busulphan act in a similar way and are both toxic for cycling cells in all phases of the cell cycle. Vinblastin is only active in the mitotic phase and therefore specifically toxic for fast cycling cells (Bruce et al. 1966). We cannot explain the capacity of Cy to induce abrogation of the resistance in both

TABLE 3

*Repopulation of femoral bone marrow and spleen measured (with the ^{125}IUdR uptake method) 6 days after transplantation of 10^7 rat bone marrow cells into mice**

Exp. no.	Recipient mouse	Treatment	Femur		Spleen	
			% uptake in marrow injected animals	% uptake in non marrow injected animals	% uptake in marrow injected animals	% uptake in irradiated non marrow injected animals
1	Fl(DBA/2 x C57BL)	Cy	0.27	0.21	1.24	0.05
	Fl(DBA/2 x C57BL)	silica	0.23	0.14	1.29	0.20
	Fl(DBA/2 x C57BL)	control	0.16	0.08	0.13	0.05
2	Fl(DBA/2 x C57BL)	Cy	0.24	0.14	1.85	0.02
	Fl(DBA/2 x C57BL)	silica	0.29	0.22	1.22	0.32
	Fl(DBA/2 x C57BL)	control	0.21	0.09	0.14	0.02
3	Fl(DBA/2 x C57BL)	Cy	0.6	0.7	1.2	0.2
	Fl(DBA/2 x C57BL)	silica	0.2	0.4	1.2	0.2
	Fl(DBA/2 x C57BL)	control	0.2	0.2	0.3	0.1
	C3H	control	0.5	0.6	1.7	0.4
4	Fl(DBA/2 x C57BL)	Cy	0.14	0.15	0.36	0.03
	Fl(DBA/2 x C57BL)	silica	0.06	0.05	0.21	0.04
	Fl(DBA/2 x C57BL)	control	0.03	0.01	0.08	0.01
	C3H	Cy	0.15	0.10	0.50	0.08
	C3H	control	0.07	0.13	0.38	0.09
5	Fl(DBA/2 x C57BL)	Cy	0.06	0.09	0.39	0.01
	Fl(DBA/2 x C57BL)	silica	0.06	0.02	0.32	0.04
	Fl(DBA/2 x C57BL)	control	0.04	0.03	0.10	0.02
	C3H	control	0.08	0.07	0.48	0.05

* Each figure represents the average of 5 recipient mice.

spleen and femoral bone marrow by the cytostatic action of the drug, because identical results would be expected for busulphan and possibly for vinblastin, unless we assume that the effector cells are less sensitive to busulphan at the dose used (two thirds LD_{50}). It was not possible to test higher doses since mice treated with higher doses did not survive the lethal irradiation long enough to perform the necessary experiments.

Xenogeneic resistance

Silica and cyclophosphamide. In addition to hybrid resistance, xenogeneic resistance (Rauchwerger et al. 1973) was investigated by comparison of the weakening effect of silica and Cy on both phenomena. The uptake of ^{125}IUdR in spleen and bone marrow 6 days after transplantation was used to measure the growth of the transplant.

Silica treatment resulted in a significantly higher ^{125}IUdR uptake in the spleen (Table

3). No significantly higher uptake was established in the femoral bone marrow. The ^{125}IUdR uptake of spleens of the silica treated Fl(DBA/2 x C57BL) mice was still lower than that of C3H mice which were used as controls, since they showed no resistance against a rat bone marrow transplant in the spleen (Table 3). We therefore conclude that silica has a similar effect on xenogeneic resistance as on hybrid resistance. In both cases the development of the transplant is ameliorated in the spleen whereas no effect was seen in the femoral bone marrow (Tables 1 and 3).

The results obtained with Cy treatment were less consistent when hybrid resistance and xenogeneic resistance were compared. The administration of Cy improved the splenic repopulation but did not improve the repopulation of the femoral bone marrow with xenogeneic hemopoietic cells (Table 3), whereas Cy induced a weakening effect on hybrid resistance in both the spleen and femoral bone marrow (Table 2).

DISCUSSION

Silica and carragheenan were shown to be capable of inducing a weakening of genetic resistance in the spleen whereas no significant weakening was observed in the femoral bone marrow. We observed that the administration of silica and carragheenan in the doses used did not lead to a significant visible destruction of macrophages. The latter result is contradictory to the proposal of Lotzová & Cudkowicz (1974) that the weakening induced by these agents was due to destruction of macrophages which were, according to their postulation, the killer cells. Only if we assume that the effector macrophages belong to a special class of macrophages present in very low numbers, compared with other splenic and bone marrow macrophages, would the killing of these cells by the agents silica and carragheenan not be observed. The assumption leaves unsolved, however, the problem that no weakening was observed in the femoral bone marrow. In the event of a specific mortality of effector macrophages, a weakening effect of the agents would be expected to be found in the femoral bone marrow also. Since we found no weakening of the resistance in the bone marrow the assumption seems to be unlikely. We suggest therefore that the weakening effect of the agents mentioned on the resistance in the spleen cannot be caused by the killing of effector cells.

The specific site of action of silica and carragheenan is the same as that of the specific weakening of resistance caused by the immunological active agents endotoxin, alloantiserum and Freund's complete adjuvant (Buurman & van Bruggen 1975), viz. the spleen. Nor did these agents induce a weakening of the resistance in the femoral bone marrow. We have reported the interesting coincidence between the capacities of these substances, including silica and carragheenan, to induce lymphocyte trapping, a phenomenon in which macrophages are suggested to play a central role (Frost & Lance 1973), and their capacity to induce weakening of the resistance to a bone marrow graft in the spleen. We suggest that these agents may affect in some way the membrane structure of cells in the spleen and lymph nodes, possibly via short range stimuli produced by macrophages which are triggered by the agents. The observation that a specific weakening effect is found only in the spleen could be explained if we assume that the hemopoietic microenvironment and the immunological micro-

environment which are probably functionally related, might react similarly to the short range stimuli triggered by the agents. In this postulation the microenvironment plays a central role in the phenomenon of genetic resistance to a bone marrow graft in accordance with McCulloch et al.'s (1970, 1973) theory concerning the managerial cells.

The results of the experiments with the cytostatic drugs Cy, busulphan and vinblastin indicated that the weakening effect of these agents was not uniform. Only Cy induced a significant weakening in the femoral bone marrow. Furthermore the weakening effect of Cy on the splenic hemopoiesis was significantly stronger than that of busulphan or vinblastin. The fact that vinblastin and busulphan induced no weakening of the resistance in the femoral bone marrow suggested that the weakening of the resistance induced in the spleen by these agents was not due to a depletion of effector cells, since these cells would be expected to be present in the bone marrow also. Since Cy and busulphan are both alkylating agents, a specific cytotoxic effect of Cy on effector cells remains unlikely.

The Cy-induced enhancement of the isogeneic CFU growth both in the spleen and the femoral bone marrow is in agreement with the results of Gregory et al. (1971) and Fried et al. (1973). As Cy did not affect the slope of the CFU growth curve the latter author suggested an influence of Cy on the microenvironment. Such a direct effect of Cy on the microenvironment might also explain the Cy-induced weakening of genetic resistance. In the light of McCulloch's theory this would imply that a microenvironment with an inappropriate regulation of the grafted hemopoietic cells is affected in such a way that the regulation becomes more appropriate for hemopoietic cells which are subject to genetic resistance.

Since Cy did not affect xenogeneic resistance in the femoral bone marrow it appears that the results which we obtained with rat bone marrow cells infused into Cy treated and lethally irradiated resistant mice are in contrast to the data obtained with semi-isogeneically transplanted mouse bone marrow. We do not have an explanation for this discrepancy so far. We can conclude that our results do not support the postulation that the mechanism responsible for xenogeneic resistance to a bone marrow graft is based upon an active destruction of the grafted hemopoietic cells by a special class of killer cells. We consider that our results are additional data in favor of McCulloch's theory concerning the hemopoietic microenvironment being involved in the phenomenon of resistance to a bone marrow graft. The data of Lengerova et al. (1973) who used 'modulated' bone marrow cells reinforce the concept that certain host type 'self' antigens play a critical role in the regulation of hemopoietic cells after transplantation. Bone marrow of hybrid mice was made 'modulated' by treatment with antibodies directed against one of the parents. These antibodies were produced in mice of the other parent strain. Such modulated bone marrow cells expressed only the phenotype of one parent. The colony formation of these modulated F_1 bone marrow cells was similar to the colony formation of the bone marrow of the expressed phenotype.

These results are in agreement with the managerial cell concept, that a good accordance of antigenic receptors between hemopoietic cells on the one hand and the cells forming the microenvironment, managerial cells, on the other hand would be

necessary for an appropriate regulation of the hemopoiesis, whereas resistance against a bone marrow graft is the result of an inappropriate regulation due to differences in antigeneic receptors of host and donor cells.

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EFFECTS OF PRE-IRRADIATION ON ISOGENEIC AND SEMI-ISOGENEIC CFU GROWTH: A STUDY ON GENETIC RESISTANCE

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ABSTRACT

The genetic resistance to a parental bone marrow transplant as demonstrated, when transplantation was performed early after irradiation, failed to occur if the interval between irradiation and transplantation was increased to 4 days. A similar radiation induced weakening of genetic resistance to a parental bone marrow graft in spleen and bone marrow could be demonstrated in mice, which had been irradiated with a sublethal dose at 7 days prior to the lethal irradiation and transplantation. The pre-irradiation of the recipient with a sublethal dose induced an enhancement of the growth in spleen and bone marrow of isogeneic transplanted CFU. The pre-irradiation of a single tibia also resulted in a significant weakening of the resistance in the spleen. The experiments with partial body pre-irradiation suggested a local effect of the pre-irradiation, but it could be shown that the enhanced CFU growth is not caused by an enhanced seeding of CFU in pre-irradiated bone marrow. The role of microenvironment in the phenomenon of genetic resistance is discussed.

INTRODUCTION

The decreased growth of a bone marrow transplant in certain donor host combinations was first reported by McCulloch & Till (1963). The fact that a lethal dose of irradiation does not immediately result in an abrogation of this phenomenon as well as the fact that no presensitization is required indicate that this is not based on a classical immunological process.

Cudkowicz & Bennett (1971a) found that a sublethal irradiation, given several days before the lethal irradiation and transplantation, enhanced the growth of a bone marrow graft subject to genetic resistance to a bone marrow transplant. This growth was measured as the splenic ^{125}I-UdR uptake. The ^{125}I-UdR uptake by an isogeneic bone marrow graft was not affected by the sublethal pre-irradiation. However, Blackett & Hellman (1966) found that a sublethal irradiation 6 days before lethal irradiation and transplantation with isogeneic bone marrow induced a twofold increase of the ^{59}Fe uptake in the peripheral blood. A

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weakening of the resistance to a bone marrow transplant was found, when the interval between the sublethal pre-irradiation and the lethal irradiation ranged from 4 days to 2 months. An optimal effect was observed with an interval of 14 days (Cudkowicz & Bennett, 1971a). The radiation induced weakening of genetic resistance to a bone marrow transplant in the spleen was observed in a number of parent-to-F_1 (Cudkowicz & Bennett, 1971b; Cudkowicz & Rossi, 1972; Lotzová & Cudkowicz, 1971, 1972, 1973) and allogeneic combinations (Cudkowicz & Bennett, 1971a; Lotzová & Cudkowicz, 1973) as well as in a xenogeneic combination (data to be published), which indicated that this phenomenon is a characteristic of genetic resistance (Trentin, Rauchwerger & Gallagher, 1973). The results of the radiation nduced weakening led Cudkowicz & Bennett (1971b) to the conclusion that the effector cells of the resistance belong to a cell renewal system. The origin of the effector cells was studied by Bennett (1973). A complete and persistent depletion of the bone marrow of mice was obtained by injecting these animals with the bone seeking isotope ^{89}Sr. In mice treated in such a way no resistance to a bone marrow transplant was observed in the spleen, when the lethal irradiation was given at 17, 28 or 56 days after injection of the isotope. A normal seeding of erythropoietic progenitor cells, normal numbers of anti-SRBC sensitive units, which give rise to anti-SRBC PFC as well as a normal content of allo-antigen sensitive units of the mouse lymphoid tissues were observed in such ^{89}Sr pretreated mice. Bennett drew the conclusion that the effector cells of the resistance are marrow dependent for their differentiation and designated the effector cells 'M' cells to distinguish them from B and T cells, which have been shown to be not dependent on the marrow for their differentiation. Since in adult thymectomized and reconstituted mice (Cudkowicz & Bennett, 1971a) and in neonatally thymectomized mice (unpublished data) normal resistance was observed, it appeared that T cells are not likely to play a role in the phenomenon of genetic resistance.

In the present paper the effect of irradiation, given several days prior to transplantation with isogeneic or parental bone marrow subject to genetic resistance to a bone marrow transplant, was studied.

MATERIALS AND METHODS

Experimental animals

Adult C57BL/Rij and F_1(C57BL/Rij × CBA/Rij) male mice varying from 3 to 7 months old were used. The C57BL/Rij mice were purchased from the Medical Biological Laboratory, TNO, Rijswijk, The Netherlands. The F_1(C57BL/Rij × CBA/Rij) mice were obtained from the Animal Breeding Centre of the Erasmus University, Rotterdam, The Netherlands.

Irradiation

Irradiation was performed using a Philips-Mueller MG300 X-ray machine. Physical constants and other radiation details were as described previously (Buurman, van Bruggen & Vos, 1975). Recipient F_1(C57BL/Rij × CBA/Rij) mice received 950 rads and recipient C57BL/Rij 800 rads total body irradiation. Radiation control mice died between 9 and 16 days after irradiation, on day 8 an average of less than 0·2 colonies per spleen was found in such mice. Partial body irradiation was carried out using a lead shielding under (to shield from backscatter) and over the shielded area of the mouse, which was slightly anaesthetized by nembutal 0·4 µg/g mouse (Abott, Brussels) to restrain the animal. All control animals were treated at the same time as the irradiated animals with a similar dose of nembutal.

Pre-irradiation on isogeneic and semi-isogeneic CFU growth

CFU growth curve

The growth curve of CFU in irradiated F_1(C57BL/Rij × CBA/Rij) mice, which received 5×10^6 bone marrow cells of F_1 hybrid or C57BL origin, was determined by the method of periodic sampling and retransplantation to mice isogeneic with the original bone marrow donor as described previously (Buurman *et al.*, 1975). A known proportion of pooled spleens of four primary recipient mice was injected into ten to fifteen irradiated secondary recipient mice. Spleens were fixed and colonies counted 8 days after transplantation.

CFU growth kinetics

CFU numbers were measured after irradiation and transplantation of 1×10^6 or 5×10^6 bone marrow cells into lethally irradiated recipient mice by retransplantation into isogeneic recipient mice. In most experiments the number of CFU was determined at 6, 7 or 8 days after transplantation. The growth of CFU in spleen and femur is then in an exponential phase (Buurman *et al.*, 1975). Each assay was performed with the pooled spleens, femurs or tibiae of three, four or five primary recipient mice. A known proportion of each suspension was injected into ten to fifteen irradiated secondary recipient mice, which were isogeneic with the original bone marrow donor.

RESULTS

Lethal total body irradiation 4 days prior to transplantation

The degree of resistance to a parental bone marrow graft 4 days after irradiation was studied by following CFU numbers in the spleen of F_1 hybrid recipients. Isogeneic F_1 hybrid and parental C57BL bone marrow was injected into F_1 hybrid mice, which had been irradiated 4 days prior to transplantation. The results obtained (Fig. 1) showed that isogeneic

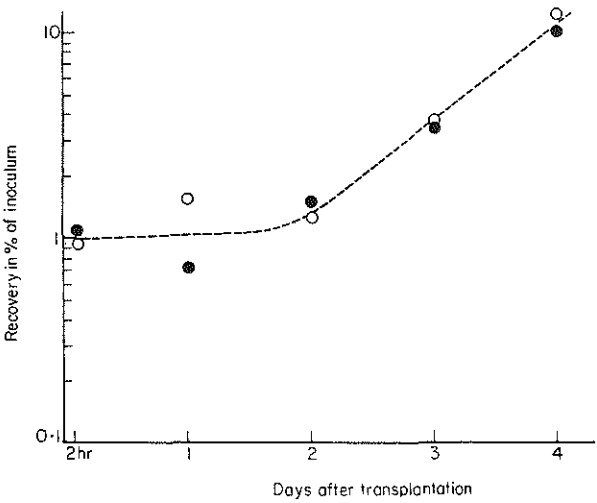

FIG. 1. CFU growth curves in spleen after semi-isogeneic and isogeneic transplantation of 5×10^6 bone marrow cells into F_1 mice which were lethally irradiated 4 days prior to transplantation. Each dot represents the mean of two or three different experiments. The curve is drawn by eye. ○, C57BL → F_1 (C57BL × CBA); ●, F_1 (C57BL × CBA) → F_1 (C57BL × CBA).

and semi-isogeneic transplanted CFU followed similar growth curves. The initial seeding efficiency as well as the exponential growth rate were similar. The lag period, that lasted 2 days after transplantation, had also been observed by Lahiri, Keizer & van Putten (1970), who studied the growth of isogeneic transplanted CFU in mice, which had been irradiated 3 days prior to transplantation. We showed earlier (Buurman *et al.*, 1975) that at day 4 after a transplantation, performed immediately after irradiation, the exponential growth phase of isogeneic and of semi-isogeneic transplanted CFU displays similar slopes.

Sublethal total body pre-irradiation

The effect of sublethal total body irradiation of the recipient 7 days prior to irradiation and transplantation was studied. The CFU numbers were determined in spleen and femoral bone marrow 6 or 8 days after transplantation. Our results revealed that a pre-irradiation dose of 400 rads, given to the F_1 hybrid recipients, had an enhancing effect on the growth of isogeneic transplanted CFU in the spleen (ratio 2·2) and femoral bone marrow (ratio 2·4) (Table 1). The enhancing effect of the sublethal pre-irradiation, given to the F_1 hybrid recipients, on the growth of parental bone marrow CFU, was considerably higher. For spleen a ratio of 5·4 and for femoral bone marrow a ratio of 4·9 was found (Table 2). These results indicated that pre-irradiation induced a weakening of the resistance in the spleen and the femoral bone marrow while it also enhanced the growth of isogeneic CFU.

TABLE 1. CFU growth of isogeneic transplanted bone marrow in sublethally pre-irradiated F_1 hybrid mice*

	No. CFU/femur				No. CFU/spleen			
Exp.	Pre-irradiated mice	Control mice	Ratio†		Pre-irradiated mice	Control mice	Ratio†	
1	240	110	2·2		6650	2240	3·0	
2	125	33	3·8	Mean	1950	950	2·1	Mean
3	160	92	1·7	2·4	4400	2350	1·9	2·2
4	1825	675	2·7	SEM	—	10500	—	SEM
5	1006	644	1·6	0·4	10000	6062	1·6	0·3

* In experiments 1, 2 and 3 CFU numbers were determined 6 days and in experiments 4 and 5, 8 days after transplantation of 5×10^6 bone marrow cells. The pre-irradiation dose of 400 rads was given 7 days before transplantation. Each experiment consisted of an experimental and a control group of four primary recipient mice, which received samples of the same bone marrow cell suspension.

† The ratio gives the quotient of the number of CFU found in pre-irradiated mice divided by the number found in control mice.

Pre-irradiation of a single tibia of F_1 hybrid mice

The irradiation of a single tibia of the F_1 hybrid recipient 7 days before lethal irradiation and transplantation resulted in an enhanced CFU growth in the irradiated tibia when compared with the non-irradiated tibia as well as with tibiae of control mice (Table 3). The increased CFU numbers found in the irradiated tibia suggested that a local mechanism is responsible for the enhancement induced by the pre-irradiation. The enhancing effect on the CFU growth of the radiation dose of 500 rads on the exposed tibia is less than the enhancing effect induced by the dose of 400 rads used for total body irradiation. The radiation protection induced by nembutal (Keizer & van Putten, 1976) could possibly explain this discrepancy.

Pre-irradiation on isogeneic and semi-isogeneic CFU growth

TABLE 2. CFU growth of semi-isogeneic transplanted bone marrow in sublethally pre-irradiated F_1 hybrid mice*

Donor	Host	Treatment	No. CFU/femur		No. CFU/spleen	
C57BL	F_1	400 rads 7 days before transplantation	300 150 390 780 280 330 270	Mean 357	2460 3120 2960 4080 1450 1550 4300	Mean 2845
C57BL	F_1	Not pre-irradiated	65 75 78 50 97	Mean 73	470 400 380 570 820	Mean 528
		Ratio†		4·9		5·4

* CFU growth was established 6 days after transplantation of 5×10^6 bone marrow cells. Each assay consisted of four primary recipient mice.

† The ratio gives the quotient of the number of CFU found in pre-irradiated mice divided by the number found in not pre-irradiated mice.

The growth of parental CFU in the spleen is significantly ($P < 0{\cdot}02$) higher in F_1 hybrid recipients with a pre-irradiated tibia. Since we did not find such an effect for the isogeneic CFU we can draw the conclusion that a specific weakening of genetic resistance occurred in the spleens of these F_1 hybrid mice. The slightly stronger enhancement of CFU growth found in irradiated tibiae of mice grafted with parental bone marrow, when compared with mice grafted with isogeneic bone marrow, could indicate a small but not significant decrease of the resistance to the parental bone marrow in the irradiated tibiae. Control animals not injected with bone marrow showed less than 0·5 CFU/spleen which indicated that the increased CFU numbers found were not due to an endogenous growth.

Pre-irradiation of F_1 hybrid mice with a single tibia shielded

The growth of parental CFU in the spleen was strongly enhanced by the pre-irradiation (ratio 13·0), whereas only a low increase in CFU growth was observed for the isogeneic transplanted CFU (ratio 2·1) (Table 4). The resistance to the parental bone marrow in the spleen was clearly weakened by the treatment. These results are in agreement with results obtained with total body pre-irradiation (Tables 1 and 2).

The enhanced CFU growth observed in the irradiated tibia seemed to be non specific, because it occurred for isogeneic (ratio 1·8) and semi-isogeneic transplanted CFU (ratio 1·6). The irradiation of the bone marrow seemed to create a better environment for the proliferation of isogeneic and semi-isogeneic transplanted CFU.

In the non-irradiated tibia the growth of the semi-isogeneic transplanted CFU was enhanced, when compared with the CFU growth in the tibia of control mice (ratio 2·1, SEM 0·6), the isogeneic CFU growth showed a lower increase (ratio 1·3, SEM 0·1). This difference did not indicate a significant weakening effect of the treatment on the resistance to a parental bone marrow graft in the non-irradiated tibia.

TABLE 3. CFU growth measured in mice after pre-irradiation of a single tibia*

Donor	Recipient	Bone marrow dose	Irradiated tibia	Non-irradiated tibia	Ratio†		Tibiae of control mice	Spleen of irradiated mice	Spleen of control mice	Ratio†	
C57BL	F_1	5×10^6	212	68	3·1		51	2560	1420	1·7	
		—	276	92	3·0	Mean	73	2060	680	3·0	Mean
		—	184	192	1·0	2·1	181	3800	1580	2·4	2·6
		—	144	104	1·4	SEM	73	4833	1960	2·5	SEM
		—	300	288	1·0	0·4	137	2340	650	3·6	0·3
		1×10^6	60	19	3·2		15	—	—	—	
F_1	F_1	1×10^6	310	150	2·1		98	1800	2000	0·9	
		—	—	92	—	Mean	74	1400	1440	1·0	Mean
		—	156	92	1·7	1·6	92	1880	1720	1·1	1·1
		—	160	116	1·4	SEM	116	1140	1160	1·4	SEM
		—	195	160	1·2	0·2	88	1675	1350	1·2	0·1

* Each experiment consisted of an experimental and a control group of three or four primary recipient mice, which received samples of the same bone marrow suspension. The local pre-irradiation dose of 500 rads was given 7 days before transplantation. CFU growth was measured 7 days after transplantation.

† The ratio gives the quotient of the number of CFU found in the pre-irradiated mice divided by the number found in the control mice. The data for the tibiae of control mice give the means of the left and right tibiae determined independently.

TABLE 4. CFU growth in mice after pre-irradiation with a single shielded tibia*

Donor	Recipient	Bone marrow dose	Irradiated tibia	Non-irradiated tibia	Ratio†		Tibiae of control mice	Spleen of irradiated mice	Spleen of control mice	Ratio†	
C57BL	F_1	1×10^6	90	46	2·0	Mean	27	720	80	9·0	Mean
		—	135	76	1·8	1·6	72	2240	235	9·5	13·0
		—	80	82	1·0	SEM	22	1660	70	23·7	SEM
		—	99	60	1·7	0·2	30	1320	135	9·8	3·6
F_1	F_1	1×10^6	290	125	2·3	Mean	73	3300	1845	1·8	Mean
		—	290	115	2·3	1·8	128	2850	1950	1·5	2·1
		—	360	128	1·4	SEM	88	3360	1975	1·7	SEM
		—	184	140	1·3	0·2	116	3640	1160	3·1	0·3
		—	210	110	1·9		88	3400	1350	2·5	

* Each experiment consisted of an experimental and a control group of three or four primary recipient mice which received samples of the same bone marrow suspension. The pre-irradiation dose of 500 rads was given 7 days before transplantation.

† The ratio gives the quotient of the number CFU found in the pre-irradiated mice divided by the number found in the control mice. The data for the tibiae of control mice give the means of the left and right tibiae determined independently.

Seeding of CFU in pre-irradiated and control tibiae

The 24 hr seeding of isogeneic F_1 bone marrow was measured in the irradiated and non-irradiated tibiae of mice, which had been irradiated 7 days prior to transplantation with one tibia shielded. No enhanced 24 hr seeding was found (ratio 1·0) (Table 5). These data suggested that the enhanced isogeneic CFU growth measured 7 days after transplantation was probably caused by a shortened lag period.

TABLE 5. Seading 24 hr after transplantation of isogeneic bone marrow, $F_1 \rightarrow F_1$, in tibiae of pre-irradiated mice of which a tibia was shielded*

No. CFU/pre-irradiated tibia	No. CFU/non-irradiated tibia	Ratio	
9·5	9·2	1·0	Mean
9·6	10·5	0·9	1·0
15·8	16·1	1·0	SEM
12·8	10·3	1·2	0·1

* Each experiment consisted of four primary recipient mice which received samples of the same bone marrow suspension. The pre-irradiation dose of 500 rads was given 7 days before transplantation.

DISCUSSION

The similarity between the CFU growth curves in the spleen obtained for isogeneic and semi-isogeneic transplanted CFU grafted 4 days after a lethal total body irradiation, suggested that genetic resistance to a bone marrow graft disappeared within 4 days after irradiation. Furthermore the present data indicated that the disappearance of the resistance to the parental bone marrow graft, as appears from the onset of the multiplication of CFU at day 3 after a transplantation performed early after irradiation (Buurman *et al.*, 1975), was not due to a modification of the resistance induced by the donor cells themselves.

In view of the theories concerning effector cells (killer cells), responsible for the resistance (Cudkowicz & Bennett, 1971a; Bennett, 1973; Lotzová & Cudkowicz, 1974) a radiation induced depletion of these effector cells, belonging to a rapid cycling cell renewal system, could explain the radiation induced abrogation of the resistance. Cudkowicz & Bennett (1971b) and Buurman *et al.* (1975) reported that the disappearance of the transplanted haemopoietic cells persisted during the first 2–3 days after transplantation. An interphase death of effector cells seemed therefore unlikely.

McCulloch & Till (1970) proposed an inappropriate cell mediated regulation of haemopoietic stem cells and committed precursor cells to explain the phenomenon of genetic resistance. A second explanation for the abrogation of the resistance, which we observed at day 4 after irradiation, could be derived from their theory. This explanation is based on the assumption that a radiation induced mechanism triggered by the very strong demand for haemopoietic cells might affect managerial cells (McCulloch, Gregory & Till, 1973) responsible for cell mediated regulation, resulting in an improved capacity of these cells to interact with resistant bone marrow cells. We suggest that a similar mechanism could be responsible for the hypoxia induced weakening of the resistance reported by Beran & Tribukait (1974). The results of the experiments with a sublethal total body irradiation of the

recipient, 7 days prior to the lethal irradiation and transplantation, a procedure which also induced an abrogation of the resistance in the spleen and bone marrow, did not offer additional information which could help us to explain the radiation induced weakening of the resistance in the spleen.

Experiments with sublethal partial body pre-irradiation were performed to obtain further information about the irradiation induced weakening of the resistance. The bone marrow in a tibia represents only a very minor part of the total bone marrow. A radiation induced destruction of the marrow in a single tibia would very unlikely result in a depletion of effector cells in the spleen, which leads to a weakening of the resistance in the spleen. We can conclude that our results do not support Bennett's (1973) hypothesis concerning 'M' cells, marrow dependent effector cells, which was deduced from experiments with ^{89}Sr pretreated mice deprived of a normal bone marrow.

The sublethal total body pre-irradiation of the F_1 hybrid recipient 7 days prior to transplantation with isogeneic F_1 hybrid bone marrow resulted in an enhanced growth of CFU in spleen and femoral bone marrow. These results were similar to the data obtained by Blackett & Hellman (1966). They explained the enhanced growth by assuming that the pre-irradiated recipient mice have a greater than normal stimulus for proliferation early after the lethal irradiation. The data of Lahiri & van Putten (1972) showed, however, that the number of CFU in S phase of the cell cycle were already increased within 2 hr after transplantation. The earlier stimulation for proliferation in the context of the assumption of Blackett & Hellman cannot cause therefore an earlier onset of the proliferation of CFU. We suggest that the period, during which the CFU loss due to differentiation is larger than the increase in CFU numbers due to proliferation, is shortened in pre-irradiated mice. The results of our seeding experiments supported this suggestion, because it was shown that the increased CFU numbers found in pre-irradiated recipients, were not caused by an enhanced seeding of CFU.

The experiments with partial body pre-irradiation showed that the enhanced CFU growth was restricted to the irradiated bone marrow. The process of CFU differentiation and multiplication has been proved therefore to be also regulated by local factors. Managerial cells should be responsible for these factors.

The results of the CFU growth of isogeneic and semi-isogeneic CFU transplanted into sublethally pre-irradiated F_1 hybrid recipient mice showed a strong similarity with the results, which had been obtained with cyclophosphamide pre-treatment. Cy pre-treatment induced an enhanced growth of isogeneic transplanted CFU in the femoral bone marrow (Fried *et al.*, 1973) and spleen (Gregory *et al.*, 1971). It also caused a weakening of genetic resistance to a bone marrow transplant in the spleen and the femoral bone marrow (Sensenbrenner & Santos, 1969; Buurman & van Bruggen, 1976). Fried *et al.* (1973) suggested that the enhancement of isogeneic CFU growth induced by cyclophosphamide was modulated by the number of mature myeloid elements present in the microenvironment in the bone marrow. An explanation which does not fit very well into our data, since in our experiments almost normal numbers of mature myeloid elements were present in the sublethally irradiated tibiae and femurs (unpublished data). Neither the enhanced growth of the isogeneic transplanted CFU nor the weakening of the resistance to the parental bone marrow is explained, however. The role of cells other than mature myeloid cells in the microenvironment is expected.

The hypothesis that an inappropriate regulation of the transplanted haemopoietic cells by the microenvironment, i.e. managerial cells (McCulloch & Till, 1970), is the cause of genetic

resistance to a bone marrow transplant was confirmed by Matioli, Niewisch & Ashley (1973). They showed that the stem cell decay rate in the adult liver, an organ without a stem cell self-renewing microenvironment, was similar with the stem cell decay in the spleen in resistant combinations.

Our results showed that the enhancing effect of a sublethal pre-irradiation is a local phenomenon, which is probably regulated by changes in the microenvironment and not by humoral factors. The role of the microenvironment in the phenomenon of genetic resistance to a bone marrow transplant could not be proved. The theory concerning 'M' cells was not confirmed by our experiments. The experiments described in this paper did not permit us to reject the theory concerning the involvement of effector killer cells or the theory concerning the involvement of the microenvironment in the process of genetic resistance to a bone marrow transplant.

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A STUDY OF THE MECHANISM OF THE WEAKENING OF GENETIC RESISTANCE TO A BONE MARROW TRANSPLANT INDUCED BY DONOR THYMOCYTES AND SPLEEN CELLS

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SUMMARY

The colony forming ability in F1 hybrid mice of parental bone marrow cells to which graded doses of parental thymocytes were added, was studied. The growth of C57BL bone marrow CFU transplanted simultaneously with C57BL thymocytes into F1 hybrid mice was significantly enhanced in the spleen but not in the bone marrow. Similarly it was shown that pretreatment of F1 hybrid mice with C57BL spleen cells also resulted in weakening of genetic resistance in the spleen but not in the bone marrow. No evidence could be obtained that the weakening of the resistance was caused by a donor type hemopoiesis or by T cells transplanted with the injected C57BL spleen cells.

The results are discussed in the light of the current theories on the resistance to a bone marrow transplant.

INTRODUCTION

Grafts of murine hemopoietic cells in heavily irradiated mice grow deficiently in certain donor host combinations. This phenomenon has been designated "Genetic Resistance" to a bone marrow graft (23). It is genetically specific, since it occurs only in certain donor-host combinations (3, 4, 5). However, it cannot readily be explained by classical immunology (3,4).

This paper reports a study of the influence of a treatment of the recipient with donor cells on the development of a bone marrow graft of the same donor subject to genetic resistance. Goodman and Wheeler (11, 12) showed that treatment of recipient mice with parental spleen cells 7 days or more before the lethal irradiation and transplantation enhanced the growth of a parental bone marrow graft. This effect was shown to be strain specific, i.e. only spleen cells of the donor strain were effective. It appeared that

spleen and lymph node cells were effective. Furthermore it was demonstrated that only viable cells could induce a weakening of the resistance to the bone marrow graft. A comparable weakening of the resistance could be obtained with donor thymocytes when these cells had been injected one day prior to, simultaneously with or one day after irradiation and transplantation (6, 8, 9, 10, 13). In this experimental set up, however, spleen cells (12) and thymocytes (21) killed by irradiation were also effective although only in very high doses.

In splenectomized mice no reduction of the resistance to the bone marrow graft was observed when donor thymocytes were added to the parental bone marrow cells, since no enhancement of the ^{59}Fe uptake was measured in the peripheral blood of these mice (8). It has been suggested therefore that the spleen is the site of action of the weakening of the resistance induced by the parental thymocytes.

We demonstrated before, that after transplantation of bone marrow cells subject to genetic resistance a delayed onset of the multiplication phase of the CFU growth occurred in the spleen and the femoral bone marrow (2). This indicated that genetic resistance occurs in the spleen as well as in the bone marrow. The present experiments have been performed to study the influence of the administration of donor cells on the growth of parental C57BL CFU in spleen and bone marrow of F1 hybrid mice. Pretreatment of recipient mice with donor spleen cells, as well as the addition of donor thymocytes to the bone marrow graft, have been used in these experiments. We also studied the effect of the number of donor thymocytes on the colony formation of C57BL bone marrow cells in the spleen of F1 hybrid recipient mice.

Two possible explanations for the weakening of the resistance to the bone marrow graft induced by a pretreatment with donor spleen cells have been investigated. The first explanation is that the presence of a donor type hemopoiesis in the F1 recipient at the time of transplantation results in enhanced growth of the bone marrow transplant, by formation of a syngeneic environment for the grafted cells. The other explanation would be that thymus derived lymphocytes have a specific role in forming an isogeneic environment in the spleens of the F1 hybrid mice, pretreated with parental spleen cells.

MATERIALS AND METHODS

Mice

Adult C57BL/Rij, CBA/Rij and F1(C57BL/Rij × CBA/Rij) male mice varying in age from 1 to 7 months were used. The C57BL/Rij and CBA/Rij mice were purchased from the Medical Biological Laboratory, TNO, Rijswijk, and from the Reactor Centre of the Netherlands, Petten, The Netherlands. The F1(C57BL/Rij × CBA/Rij) mice were obtained from the Animal Breeding Centre of the Erasmus University, Rotterdam, The Netherlands.

Irradiation

Irradiation was performed using a Philips-Mueller MG 300 X-ray machine. Physical constants and other radiation details have been described in a previous paper (2). Recipient F1(C57BL/Rij × CBA/Rij) mice received 950 rad and recipient C57BL/Rij 800 rad total body irradiation. Radiation control mice died between 9 and 16 days after irradiation, on day 8 less than an average of 0.1 colonies per spleen was found in such mice.

Partial body irradiation was carried out using a lead shielding under (to shield from backscatter) and over the shielded area of the mouse, which was slightly anaesthetized by nembutal 0.4 µg/g mouse (Abott, Brussels) to restrain the animal. All control animals were treated at the same time with a similar dose of nembutal.

Spleen Colony assay

The spleen colony assay of Till & McCulloch (22) was used to measure the number of CFU. Cell suspensions of bone marrow and spleen were prepared. Cell counting was performed in a Coulter counter model B. Each suspension was administered to a group of ten to fifteen mice. The mice were killed on day 8 after irradiation and the spleens were fixed in Telleyesniczky's solution. The colonies were counted using the low power objective of a stereo-microscope.

In order to eliminate F1 hybrid CFU in some experiments immune C57BL recipient mice were used. C57BL mice were immunized against F1(C57BL × CBA) cells by 2 weekly i.p. injections with 1/5th of a CBA spleen. These mice were used as recipients for the spleen colony assay 7 days after the second injection.

CFU growth curve

The growth curve of bone marrow CFU transplanted into irradiated F1(C57BL/Rij × CBA/Rij) mice was determined by the method of periodic sampling. Bone marrow or spleen cells of the primary recipients were retransplanted into secondary recipient mice isogeneic with the original bone marrow donor as described previously (2). Colonies were counted on the spleens of these secondary recipient mice.

CFU growth kinetics

CFU numbers were measured 7 days after irradiation and transplantation of 1×10^6 C57BL bone marrow cells into lethally irradiated F1 hybrid mice by retransplantation of bone marrow and spleen cells of these mice into lethally irradiated secondary recipient mice isogeneic with the original donors. The growth of CFU in spleen and femur is in an exponential phase at that time (2).

Thymocytes and "B" spleen cells

Thymuses of 5 - 6 weeks old C57BL/Rij mice were removed and cell suspensions prepared. The cells were washed twice and injected i.v. simultaneously with the bone marrow. "B" spleen cell suspensions were obtained from spleens of thymectomized irradiated and fetal liver reconstituted mice. Thymectomy was performed according to the method of Miller (20) at an age of 5 weeks. The animals were irradiated and reconstituted with 5×10^6 isogeneic fetal liver cells of 16 - 18 days old embryos, 2 weeks after surgery. Spleens of these animals were used at 4 - 8 weeks after transplantation to prepare the "B" spleen cell suspension. It has been shown that spleens of such treated mice contain very few thymus derived lymphocytes (7, 20). The mediastinum of these mice was grossly inspected for thymic remnants before the spleen was used. Spleen cell suspensions were made from 3 or more mice.

RESULTS

Spleen colony formation of C57BL bone marrow cells transplanted simultaneously with C57BL thymocytes into F1 hybrid mice.

The dose-effect relationship between the number of thymocytes and their enhancing effect on the colony formation of parental marrow cells was studied. The enhancing effect has been expressed as a ratio of the number of colonies obtained with the bone marrow plus thymocytes divided by the number of colonies obtained from the bone marrow only. The results (Fig. 1) indicated that for the amount of bone marrow cells used in these experiments (4×10^4 - 1×10^5) more than 10^7 thymocytes were needed to evoke a significant weakening of the resistance. The data demonstrated that doses of 5×10^7 and 10^8 thymocytes abrogated the resistance, since the ratio obtained with these doses equalled a factor of about 10 being the reduction of the colony formation due to genetic resistance in this donor host combination (2) as has been determined before. We observed that the size of the spleen colonies did increase upon addition of 10^8 thymocytes, but was still distinctly smaller than the size of isogeneic C57BL → C57BL or F1 → F1 colonies.

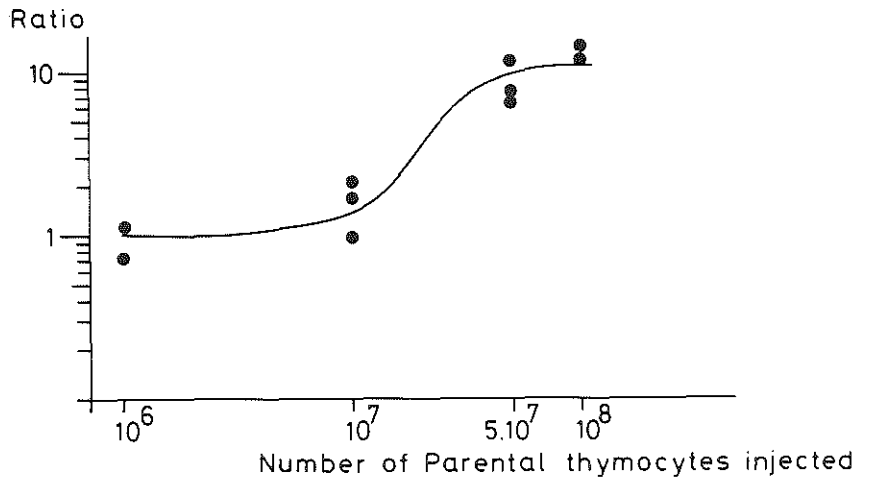

Fig. 1. The enhancement of the colony formation of C57BL bone marrow in F1 hybrid mice resulting from the addition of C57BL thymocytes.

$$\text{Ratio} = \frac{\text{no. of colonies obtained from bone marrow with thymocytes added.}}{\text{no. of colonies obtained from bone marrow.}}$$

Bone marrow doses of 4×10^4 - 10^5 cells were used.

CFU growth of C57BL bone marrow cells transplanted simultaneously with 5×10^7 C57BL thymocytes.

In order to determine whether the enhancing effect of the addition of C57BL

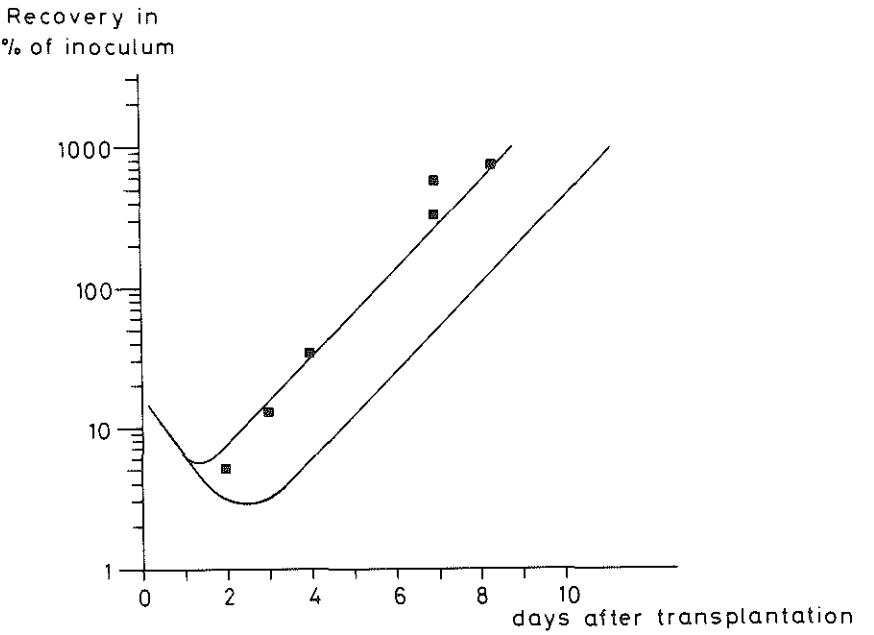

Fig. 2. The growth of C57BL bone marrow CFU in the spleen of F1(C57BL × CBA) mice, which were transplanted with C57BL bone marrow to which C57BL thymocytes were added. Each assay consisted of 4 primary recipient mice.
■ 5×10^6 C57BL bone marrow cells + 5×10^7 C57BL thymocytes.
The drawn line represents earlier reported data (2).
The upper line represents F1 bone marrow transplanted to F1.
The lower line represents C57BL bone marrow transplanted to F1.

thymocytes on the growth of the C57BL bone marrow cells transplanted into F1 hybrid mice, occurred not only in the spleen but also in the femoral bone marrow, the growth of CFU in these hemopoietic organs was studied. 5×10^6 C57BL bone marrow cells to which 5×10^7 thymocytes had been added, were transplanted into F1 hybrid mice. The dose of 5×10^7 thymocytes has been chosen, because previous experiments showed that this dose abrogated the resistance. The growth of C57BL bone marrow CFU in the spleen of pretreated F1 hybrid mice (Fig. 2) was similar to the growth of isogeneic CFU in the spleen of control F1 hybrid mice. The slope of the exponential multiplication phase was not affected whereas the onset of the multiplication phase was clearly moved forward in time, as compared to the semi-isogeneic CFU growth curve. The growth of CFU in the femoral bone marrow (Fig. 3) did not indicate an abrogation of the resistance to the parental bone marrow in this organ. The exponential multiplication

phase of the CFU growth curve seemed similar to the multiplication phase of the semi-isogeneic C57BL → F1 CFU growth curve.

The data obtained showed that the addition of donor thymocytes to a parental marrow graft resulted in a weakening of the resistance in the spleen, whereas the resistance in the femoral bone marrow was not affected significantly.

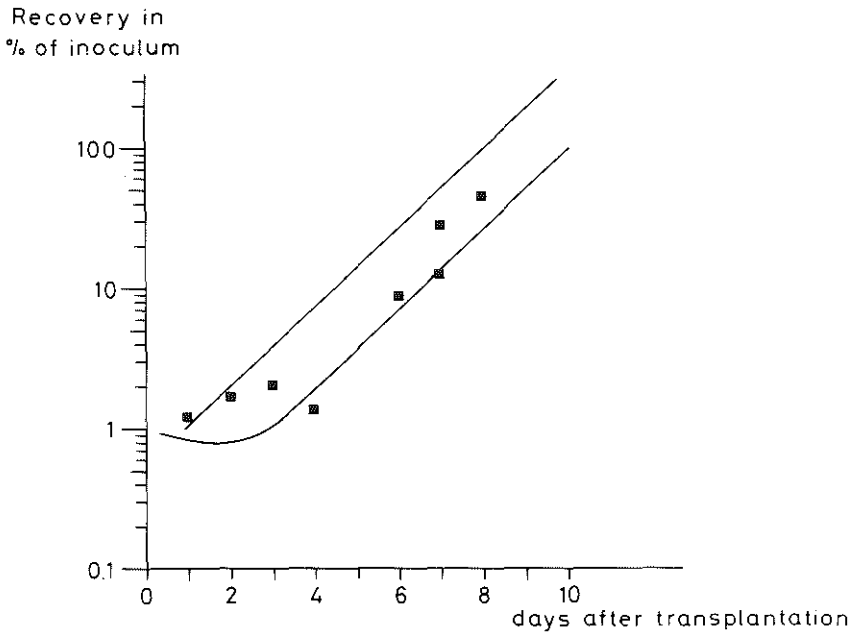

Fig. 3. The growth of C57BL bone marrow CFU in the femoral bone marrow of F1(C57BL × CBA) mice, which were transplanted with C57BL bone marrow to which C57BL thymocytes were added.
Each assay consisted of 4 primary recipient mice.
■ 5×10^6 C57BL bone marrow cells + 5×10^7 C57BL thymocytes.
The drawn lines represent earlier data of the CFU growth in the femoral bone marrow (2).
The upper line represents F1 bone marrow transplanted to F1.
The lower line represents C57BL bone marrow transplanted to F1.

Pretreatment of F1 hybrid recipient mice with C57BL spleen cells.

It has been shown by ^{59}Fe incorporation studies, that pretreatment of F1 hybrid mice with donor spleen cells enhanced the growth of a parental bone marrow graft subject to genetic resistance (10, 11). We investigated the effect of pretreatment of F1 hybrid recipient mice with donor spleen cells on the growth of CFU in spleen and femur after transplantation of C57BL bone marrow cells and lethal irradiation. The F1 hybrid mice were

TABLE 1. Growth of C57BL CFU measured in lethally irradiated F1 hybrid mice pretreated with C57BL spleen cells*.

No. of injections with C57BL spleen cells	CFU per spleen		CFU per femur	
	treated mice	control mice	treated mice	control mice
3	2100	765	46	43
3	2700	1070	31	76
3	1750	200	67	44
2	775	95	8	94
2	450	55	38	60
2	475	200	8	8

* 2×10^7 C57BL spleen cells were i.p. administered at weekly intervals.

The irradiation took place two weeks after the last injection.

Each experiment consisted of an experimental and a control group of 3 or 4 primary recipient mice which received samples of the same bone marrow suspension.

The number of CFU was determined 7 days after transplantation.

TABLE 2. Growth of C57BL CFU measured in lethally irradiated F1 hybrid mice which were partially pre-irradiated and pre-treated with spleen cells*.

		CFU per spleen		CFU per tibia		
				treated mice		control mice
Exp.	Type of cells injected	treated mice	control mice	irradiated tibia	normal tibia	both tibiae
1	C57BL spleen cells	1050	240	22	20	34
2	,, ,, ,,	800	75	63	11	15
3	,, ,, ,,	710	195	9	12	50
4	,, ,, ,,	1725	65	13	10	21
5	,, ,, ,,	1250	300	60	35	65
6	,, ,, ,,	1075	60	9	12	2
7	F1 spleen cells	430	520	62	40	62
8	,, ,, ,,	520	500	98	56	16
9	,, ,, ,,	65	65	57	15	12
10	,, ,, ,,	80	45	10	20	11

* In each experiment 2×10^7 spleen cells were i.v. administered 24 h after partial irradiation.

The transplantation took place two weeks after the pretreatment with spleen cells.

Each experiment consisted of an experimental and a control group of 3 or 4 primary recipient mice which received samples of the same marrow suspension.

The number of CFU was determined 7 days after transplantation.

pretreated with 2 or 3 i.p. injections of 2×10^7 C57BL spleen cells, given at weekly intervals. The irradiation and transplantation was carried out 2 weeks after the last injection. Control groups of non-pretreated F1 hybrid mice of the same age were used in these experiments. The numbers of CFU were determined in spleen and femoral bone marrow. The results showed a significant increase of the splenic CFU growth in the pretreated mice (Table 1). The CFU growth in the femoral bone marrow was not affected by the pretreatment.

It can be concluded that the pretreatment with donor spleen cells resulted in a weakening of the resistance in the spleen, whereas no weakening was observed in the bone marrow.

A possible explanation for the limitation to the spleen of the weakening of genetic resistance caused by the addition of thymocytes to the graft or by the pretreatment with spleen cells, may be that these cells seed preferentially to the spleen. To enhance seeding of donor cells to the bone marrow, the following experiment was carried out. A single tibia of F1 hybrid mice was irradiated with a dose of 500 rad and 24 h later those mice were treated with a single i.v. injection with 2×10^7 F1 or C57BL spleen cells. This procedure seemed promising since it has been shown that upon partial irradiation of the bone marrow the seeding of the subsequently injected hemopoietic cells in the irradiated part of the bone marrow was greatly enhanced (19). The transplantation with C57BL bone marrow took place two weeks after the partial irradiation. For experiments with partially irradiated mice injected with F1 or C57BL spleen cells, non-irradiated F1 mice of the same age were used as controls. The growth of C57BL bone marrow CFU in spleen and tibiae was determined as before. The mice, which were pretreated with F1 spleen cells did not show an enhanced growth of CFU in the spleen or in the tibiae (Table 2). The pretreatment with C57BL spleen cells resulted in an enhanced growth of C57BL CFU in the spleen. The growth of C57BL CFU in the irradiated as well as in the non-irradiated tibiae was not significantly affected.

Determination of C57BL CFU in normal F1 hybrid mice pretreated with C57BL spleen cells

Experiments were performed in order to test the assumption that the presence of a donor type hemopoiesis caused by treatment with C57BL spleen cells would enable the donor bone marrow transplant to settle more easily in F1 hybrid mice. The incidence of C57BL CFU was used as a parameter for the presence of a C57BL hemopoiesis in pretreated F1 hybrid mice. In order to prevent colony formation of F1 hybrid origin, the C57BL recipient mice used in these experiments, were immunized against

TABLE 3. Determination of C57BL CFU present in normal F1 hybrid mice which were pretreated with C57BL spleen cells.*

Exp.	Treatment	No. C57BL CFU per spleen	No. C57BL CFU per femur
1	3 inj. C57BL spleen cells	3	0
	non treated F1	10	n.d.
2	2 inj. C57BL spleen cells	0	n.d.
3	1 inj. C57BL spleen cells	7	9
4	2 inj. C57BL "B" spleen cells	0	0
	non treated F1	0	0

* The presence of C57BL CFU was measured in F1 hybrid mice 2 weeks after the last injection with 2×10^7 C57BL spleen cells. One tenth of a spleen and 1/5th of the bone marrow cells of a femur were injected into lethally irradiated C57BL mice, which were immunized against CBA mice and therefore immune against F1 hybrid cells.

In each experiment 4 F1 mice were tested.

CBA antigens. In such mice no colonies of F1(C57BL × CBA) origin should be formed. However, a small number of CFU per organ has been found sometimes; this is probably due to the fact, that a low percentage of the injected CFU of F1 origin was not killed by the host. In comparison to the number of C57BL CFU injected into the F1 hybrid mice practically no C57BL CFU were found in the spleens or femurs of pretreated F1 hybrid mice (Table 3).

In order to investigate whether the irradiation of a tibia of F1 hybrid mice and the subsequent i.v. injection of C57BL spleen cells enhanced the seeding of C57BL hemopoietic cells in the bone marrow of the F1 hybrid mice, as described in the previous section, C57BL CFU were determined in the irradiated as well as in the non irradiated tibiae of such mice. The results suggested, that no selfsustaining C57BL hemopoiesis occurred in the bone marrow of F1 hybrid mice, whether one tibia was irradiated or not (Table 4).

TABLE 4. Determination of C57BL CFU present in normal F1 hybrid mice which were partially pre-irradiated and pretreated with C57BL spleen cells*.

Exp.	No. CFU per irradiated tibia	No. CFU per irradiated tibia
1	0	2
2	10	2
3	5	5
4	4	3

* A single tibia was irradiated with 500 rad and 24 h later 2×10^7 C57BL spleen cells were i.v. injected. The presence of C57BL CFU was measured 2 weeks after the injection. One half of a tibia was injected into lethally irradiated C57BL mice, which were immunized against CBA mice and therefore immune against F1 hybrid cells.

In each experiment 4 F1 mice were tested.

Pretreatment of F1 hybrid recipient mice with C57BL "B" spleen cells

The weakening of genetic resistance by pretreatment with donor spleen cells may be caused by thymus derived lymphocytes present in the spleen cell suspension. Therefore F1 hybrid mice were pretreated with 2 or 3 i.p. injections of 2×10^7 C57BL "B" spleen cells prepared from thymectomized C57BL mice, reconstituted with isogeneic fetal liver cells. As discussed in the methods section these spleen cells are virtually free of thymus derived lymphocytes. The injections were given at weekly intervals. Irradiation and transplantation were carried out 2 weeks after the last injection. The growth of C57BL CFU in the spleen of F1 hybrid mice treated with "B" spleen cells was significantly increased when compared with control mice (Table 5). The growth of C57BL CFU in the femoral bone marrow was not enhanced in these mice. These results demonstrated that thymus derived lymphocytes of the donor are not an indispensible factor in the weakening of the resistance in the spleen. In the latter experiments, cells other than thymus derived lymphocytes must have induced the weakening of the resistance to a bone marrow transplant in the spleen.

TABLE 5. Growth of C57BL CFU measured in lethally irradiated F1 hybrid mice pretreated with C57BL "B" spleen cells*.

No. of injections with C57BL "B" spleen cells	CFU per spleen		CFU per femur	
	treated mice	control mice	treated mice	control mice
2	275	55	0	4
2	325	140	1	7
2	320	53	5	15
3	825	60	6	19
3	925	275	14	19

* 2×10^7 "B" spleen cells of C57BL mice were i.p. administered at weekly intervals.

The irradiation took place two weeks after the last injection.

Each experiment consisted of an experimental and a control group of 3 or 4 primary recipient mice which received samples of the same bone marrow suspension.

The number of CFU was determined 7 days after transplantation.

DISCUSSION

The addition of C57BL thymocytes to C57BL bone marrow cells greatly enhanced the colony formation in F1 hybrid recipient mice. This effect of thymocytes is specific for a transplantation combination subject to genetic resistance (8). The addition of very high numbers of thymocytes to bone marrow cells, which were transplanted into syngeneic hosts, enhanced the spleen colony formation with factors ranging from 1.5-1.9 (data not shown). Similar results were obtained by Lord and Schofield (14) and Löwenberg (15).

The very high dose of parental thymocytes necessary to obtain a complete weakening of the resistance for both lower (4×10^4 - 10^5) and higher (5×10^6) bone marrow doses, indicated that the number of thymocytes injected, is the determining factor in the abrogation of genetic resistance. This result suggests, that the ratio of donor thymocytes: number of sites where a colony can form is important. The thymocytes could form a syngeneic "soil bed" for the bone marrow cells subject to genetic resistance.

The addition of C57BL thymocytes to C57BL bone marrow cells, which were transplanted into F1 hybrid mice resulted in a strongly enhanced growth of CFU in the spleen. The fact that the growth of C57BL bone marrow CFU under these conditions is similar to the growth of the isogeneic transplanted F1 bone marrow CFU does not imply that the hemopoiesis equals that of an isogeneic graft. The smaller size of the spleen colonies, which we observed when 5×10^7 or 10^8 C57BL thymocytes were added to the bone marrow cells, indicated that this might not be the case. The effective hemopoiesis is difficult to assess, however. The absence of a significant enhancing effect of the thymocytes on the growth of CFU in the femoral bone marrow indicated that the effect of the thymocytes is localized in the spleen. These results seem to be in agreement with the data reported earlier by Goodman and Grubbs (8). They showed that the addition of thymocytes to parental bone marrow cells did not enhance the growth of the graft in splenectomized F1 hybrid mice.

Our results with pretreatment of F1 hybrid mice with donor spleen cells were comparable with the results, which we obtained by addition of thymocytes to the bone marrow graft. Also in these experiments the resistance was only effectively weakened in the spleen. We suggest that pretreatment with donor spleen cells as well as the addition of thymocytes to the bone marrow cells could induce a syngeneic environment, which could provide a fertile "soil bed" for the C57BL bone marrow cells. By irradiating a part of the bone marrow and subsequent injection with C57BL spleen cells, we tried to enhance a possible C57BL environment in the bone

marrow of F1 hybrid mice injected with C57BL spleen cells. Since no improvement of the growth of C57BL CFU was found in the irradiated part of the bone marrow, no enhancement of a possible C57BL environment could be demonstrated. Two possible origins of such a syngeneic environment were studied:

1. The C57BL hemopoietic cells among the transplanted C57BL spleen cells could form a syngeneic "soil bed" on which C57BL CFU, injected after irradiation then grew as under isogeneic conditions. The experiments carried out to test this possibility showed that no C57BL CFU derived from a selfsustaining C57BL hemopoiesis could be demonstrated in the spleen of pretreated F1 hybrid mice, whereas a significant weakening of the resistance to the bone marrow graft was found in the spleen. Experiments performed with the aim of establishing a C57BL hemopoiesis in the bone marrow of F1 hybrid mice were not succesful. No C57BL CFU were found in the pre-irradiated tibiae of mice treated with C57BL spleen cells. These results are in disagreement with the results obtained with isogeneic bone marrow by Micklem et al. (19), who showed that isogeneic bone marrow has a significant preference for the irradiated bones. Our results indicated that a mechanism exists, which prevents C57BL CFU from surviving in the irradiated as well as in the non irradiated part of F1 hybrid mice.

2. Thymus derived lymphocytes of C57BL origin could induce a C57BL environment in F1 hybrid mice. Our experiments showed that "B" spleen cells were effective in weakening the resistance in F1 hybrid mice. It was concluded that cells other than T cells can play an important role in the process of weakening the resistance in the spleen of F1 hybrid mice. It is likely that C57BL cells other than thymus derived lymphocytes or those derived from a selfsustaining C57BL hemopoiesis, are present in the spleens of pretreated F1 hybrid mice. Th fact, that the resistance is only weakened in the spleen, could be explained by assuming that the injected cells lodge preferentially in the spleen and that their concentration in the spleen is high enough to form a syngeneic "soil bed" for most of the injected stem cells.

Two main theories designed to explain the phenomenon of the resistance to a bone marrow transplant are suggested in the literature. The first proposes an inappropriate cell mediated regulation of the transplanted hemopoietic stem cells and committed precursor cells (17,18). The second proposes that specific cytotoxic cells are responsible for the disappearance of the transplanted stem cells (1, 3, 4, 16). This cytotoxic cell was suggested to be a macrophage, which cooperates with a non-thymus dependent lymphocyte responsible for the recognition of the foreign cells. Our postulate, that cells of donor origin present in high concentrations in the splenic

hemopoietic microenvironment, could provide a syngeneic environment, which improves the inappropriate regulation by the F1 microenvironment, supports the first theory.

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