EUR 484.e

ASSOCIATION EURATOM - TNO
European Atomic Energy Community
and
Stichting voor Toegepast Natuurwetenschappelijk Onderzoek

BONE MARROW TRANSPLANTATION AFTER WHOLE-BODY IRRADIATION

AN EXPERIMENTAL STUDY IN THE RAT

by

H. BALNER (EURATOM)

1963

Contract No 004-59-12 BIAN

LEGAL NOTICE

This report can be obtained, at the price of Belgian Francs 150,–, from : PRESSES ACADEMIQUES EUROPEENNES - 98, Chaussée de Charleroi - Brussels 6.

Please remit payments :

— to BANQUE DE LA SOCIETE GENERALE (Agence Ma Campagne) - Brussels - account No 964.558,

— to BELGIAN AMERICAN BANK AND TRUST COMPANY - New York - account No 121.86,

— to LLOYDS BANK (Foreign) Ltd. - 10, Moorgate London E.C.2,

giving the reference : "EUR 484.e - Bone Marrow Transplantation after Whole-Body Irradiation - An experimental Study in the Rat".

Printed by Levisson N.V., The Hague.
Brussels, February 1964.

EUR 484.e

BONE MARROW TRANSPLANTATION AFTER WHOLE-BODY IRRADIATION — An experimental study in the rat by H. BALNER.

Association : European Atomic Energy Community (EURATOM) and Stichting voor Toegepast Natuurwetenschappelijk Onderzoek (TNO)
Contract No 004-59-12 BIAN
Brussels, February 1964 - 133 pages - 49 figures.

1. Rats were treated with homologous bone marrow following high sublethal and lethal radiation doses. The majority of the animals survived beyond 4 weeks after irradiation with a functioning donor-type hemopoietic tissue.

There was no evidence of a so-called "median-lethal-dose-effect" (MLD-effect).

2. Secondary disease occurred during the second and third month following irradiation and homologous bone marrow therapy. Morbidity and mortality were variable but generally rather low, respectively about 30 and 15 %. The clinical and pathological findings were reminiscent of those described for mice with secondary disease except that colitis and diarrhea were not observed.

EUR 484.e

BONE MARROW TRANSPLANTATION AFTER WHOLE-BODY IRRADIATION — An experimental study in the rat by H. BALNER.

Association : European Atomic Energy Community (EURATOM) and Stichting voor Toegepast Natuurwetenschappelijk Onderzoek (TNO)
Contract No 004-59-12 BIAN
Brussels, February 1964 - 133 pages - 49 figures.

1. Rats were treated with homologous bone marrow following high sublethal and lethal radiation doses. The majority of the animals survived beyond 4 weeks after irradiation with a functioning donor-type hemopoietic tissue.

There was no evidence of a so-called "median-lethal-dose-effect" (MLD-effect).

2. Secondary disease occurred during the second and third month following irradiation and homologous bone marrow therapy. Morbidity and mortality were variable but generally rather low, respectively about 30 and 15 %. The clinical and pathological findings were reminiscent of those described for mice with secondary disease except that colitis and diarrhea were not observed.

EUR 484.e

BONE MARROW TRANSPLANTATION AFTER WHOLE-BODY IRRADIATION — An experimental study in the rat by H. BALNER.

Association : European Atomic Energy Community (EURATOM) and Stichting voor Toegepast Natuurwetenschappelijk Onderzoek (TNO)
Contract No 004-59-12 BIAN
Brussels, February 1964 - 133 pages - 49 figures.

1. Rats were treated with homologous bone marrow following high sublethal and lethal radiation doses. The majority of the animals survived beyond 4 weeks after irradiation with a functioning donor-type hemopoietic tissue.

There was no evidence of a so-called "median-lethal-dose-effect" (MLD-effect).

2. Secondary disease occurred during the second and third month following irradiation and homologous bone marrow therapy. Morbidity and mortality were variable but generally rather low, respectively about 30 and 15 %. The clinical and pathological findings were reminiscent of those described for mice with secondary disease except that colitis and diarrhea were not observed.

3. In a limited number of animals temporary chimerism was shown to be associated with persistence of specific tolerance towards the antigens of the former bone marrow graft.

4. The rat chimeras showed a considerable reduction of longevity as compared with controls. Cataract was consistently found, nephrosclerosis frequently encountered. Infectious complications were comparatively rare except in the urogenital tract. The incidence of benign and malignant tumours was high.

5. Clinical applications of homologous and autologous bone marrow therapy have been briefly reviewed and some of the problems, encountered both in experimental and clinical transplantation of hemopoietic tissues, have been discussed. The usefulness but also the limitations of the information gathered from experiments on animals were exposed.

3. In a limited number of animals temporary chimerism was shown to be associated with persistence of specific tolerance towards the antigens of the former bone marrow graft.

4. The rat chimeras showed a considerable reduction of longevity as compared with controls. Cataract was consistently found, nephrosclerosis frequently encountered. Infectious complications were comparatively rare except in the urogenital tract. The incidence of benign and malignant tumours was high.

5. Clinical applications of homologous and autologous bone marrow therapy have been briefly reviewed and some of the problems, encountered both in experimental and clinical transplantation of hemopoietic tissues, have been discussed. The usefulness but also the limitations of the information gathered from experiments on animals were exposed.

3. In a limited number of animals temporary chimerism was shown to be associated with persistence of specific tolerance towards the antigens of the former bone marrow graft.

4. The rat chimeras showed a considerable reduction of longevity as compared with controls. Cataract was consistently found, nephrosclerosis frequently encountered. Infectious complications were comparatively rare except in the urogenital tract. The incidence of benign and malignant tumours was high.

5. Clinical applications of homologous and autologous bone marrow therapy have been briefly reviewed and some of the problems, encountered both in experimental and clinical transplantation of hemopoietic tissues, have been discussed. The usefulness but also the limitations of the information gathered from experiments on animals were exposed.

EUR 484.e

ASSOCIATION EURATOM - TNO

European Atomic Energy Community

and

Stichting voor Toegepast Natuurwetenschappelijk Onderzoek

BONE MARROW TRANSPLANTATION AFTER WHOLE-BODY IRRADIATION

AN EXPERIMENTAL STUDY IN THE RAT

H. BALNER (EURATOM)

1963

Contract No 004-59-12 BIAN

This work represents a thesis for a doctoral degree at the University of Amsterdam

The experiments were performed at the Radiobiological Institute of the Health Organization T.N.O., Rijswijk, The Netherlands.

I would like to express my gratitude towards the following persons:

Dr. D. W. van Bekkum, the director of the Institute, who was of great help in the planning of this work and the preparation of the manuscript.

Dr. M. J. de Vries, the Institute's pathologist, who let me benefit from his experience in the diagnosis of animal tumours and his vast knowledge of the pathology of secondary disease.

Prof. Dr. C. A. Wagenvoort and Dr. L. M. van Putten for the critical reading of the manuscript.

Mr. H. Dersjant for invaluable technical assistance.

Dr. D. van der Waay, Miss W. M. Th. Zimmerman and Miss A. de Witte for technical services (bloodcultures and typing of erythrocytes).

I am also indebted to Mrs. T. Kooistra and Mr. J. Ph. de Kler for their skillful assistance.

CONTENTS

CHAPTER IV

COMPLICATIONS AFTER THE ADMINISTRATION OF HOMOLOGOUS CELLS IN THE WAG→BROFO COMBINATION

CHAPTER V

CONSEQUENCES OF IRRADIATION AND ADMINISTRATION OF HOMOLOGOUS CELLS IN OTHER STAIN COMBINATIONS

CHAPTER VI

HOMOGRAFT REACTIVITY AND SPECIFIC TOLERANCE IN CHIMERAS AND REVERSALS

CHAPTER VII

page

LATE RADIATION EFFECTS AND TUMOUR INCIDENCE IN RAT CHIMERAS

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER I

INTRODUCTION

Experimental bone marrow therapy after irradiation

Numerous investigations during the last decade have established that death due to the destruction of an animal's hemopoietic tissue by total-body irradiation * can be prevented by the intravenous injection of hemopoietic cells (16, 54, 105). Without treatment, these animals develop the so-called **bone marrow syndrome,** which is characterized by death during the second week after irradiation with pancytopenia and hemorrhages, often associated with bacteremia. The radiation dose range which induces this syndrome and after which bone marrow therapy may still be effective, varies somewhat for different species. In general, there is a rather narrow margin between lethal radiation doses causing mortality due to the bone marrow syndrome (approximately 600-1200 r) and doses causing irreparable damage to the intestinal tract as well. The latter mode of death, attributed to the **intestinal syndrome,** usually occurs within 5 days following doses of more than 1200 r and is not remediable by bone marrow therapy. Radiation doses above 10,000 r may damage the central nervous system and cause death within hours. However, in the present work we shall only be concerned with the first modality, namely death due to the bone marrow syndrome and more specifically with its prevention by bone marrow transplantation and the direct consequences of such therapy.

A bone marrow donor can be genetically identical with the irradiated host. The bone marrow graft is called autologous if it was taken from the same individual or isologous if from an animal of the same inbred strain or from an identical twin (as in humans). Intravenously injected bone marrow cells genetically identical with the irradiated recipient, have been shown to be capable of repopulating the depleted marrow of lethally irradiated individuals of many species, including man (55, 99, 80, 121, 60, 61, 107, 108). Primary mortality is thereby prevented and complications of an immunological nature are not to be expected.

Foreign bone marrow grafts can be homologous, if taken from donors of the same species or heterologous if the donor belonged to another species. It has been shown in recent years, that homologous and in some instances even heterologous bone marrow can replace the depleted hemopoietic tissue of heavily irradiated animals, provided the host's immunological reactivity had been sufficiently depressed. Though greater numbers of foreign bone marrow cells are needed than autologous or isologous cells to save lethally irradiated animals, a "take" of the foreign marrow can nevertheless frequently be obtained and primary mortality due to the bone marrow syndrome reduced (114, 95, 10, 179, 151, 185, 17, 60).

---

* In this context **total-body irradiation** means irradiation by high voltage X-rays or γ-rays, administered uninterruptedly at a high dose rate and with a homogeneous distribution throughout the body.

Subsequently however, usually a few weeks after this initial recovery, a number of complications may occur, leading to delayed morbidity and mortality. These complications have first been described in mice and have been called **secondary or homologous disease** (11, 192, 22, 56). They are mainly attributable to an immunological reaction of the graft against the host (see below). Later, similar complications were also described for other species and in a few cases of irradiated humans treated with homologous bone marrow (162, 61, 131, 126). Unfortunately it soon became clear, that secondary disease was a far more serious complication in primates than in small mammals such as mice and rabbits. It is in fact still the main stumbling block for a more widespread application of marrow transplantation in the clinic. Nevertheless, homologous bone marrow therapy of irradiated humans is presently being attempted, especially in cases where no alternative therapy is available (67, 96).

A number of procedures have been advocated to promote regeneration of heavily irradiated bone marrow in experimental animals by other means than the injection of viable hemopoietic cells (172, 178, 119). By omitting the transfusion of living cells, the risk of secondary disease is avoided. However, these "stimulans" have a rather limited range of effectiveness and must sometimes be administered before irradiation. Their future clinical usefulness as a substitute for bone marrow therapy seems very unlikely.

The possible clinical applications of bone marrow therapy

The hazards of homologous bone marrow grafts in irradiated humans are still such that, for the time being, indications are comparatively few and cases for such therapy must be very carefully selected. Autologous bone marrow, which does not cause secondary disease, is being used whenever possible (108, 98, 73) and improvements of marrow preservation techniques may even widen the scope of such therapy.

The clinical circumstances under which homologous bone marrow therapy would presently be considered include the following:

1) ACCIDENTAL WHOLE-BODY IRRADIATION

Accidents involving whole-body irradiation have occurred in recent years and in view of the rapid expansion of applied nuclear energy, an increased incidence of similar accidents may unfortunately be expected. Homologous bone marrow therapy has been applied in some of these cases (130). Evaluation of the effectiveness of such therapy however is difficult since the exact radiation dose and distribution will seldom be known and untreated controls will not be available.

A controversy still exists about the advisability of homologous bone marrow therapy in most cases of accidental whole-body irradiation. If the radiation dose is known to have been supralethal, a take of homologous bone marrow, even if temporary, might save the patient's life and the risk of secondary disease can obviously be taken. If on the other hand, it is not known whether the dose was 100 % lethal, the indication for such therapy becomes far more difficult. An initial observation period with rigorous preventive and conservative therapeutic measures may therefore be indicated in all cases of accidental whole-body irradiation. But when should homologous bone marrow therapy be administered under these circumstances? Given too soon it would expose the patient to an unnecessary risk of secondary disease, while

conservative therapy might have tided him over the dangerous period of pancytopenia until spontaneous regeneration of his own hemopoietic and lymphatic tissues occurred. Waiting too long, on the other hand, might jeopardize the effectiveness of a marrow graft. In animals, increasing the interval between irradiation and grafting beyond 3 days has been shown to reduce sharply a graft's therapeutic value (16, 157, 202). Nevertheless, Mathé (67) advocates a period of conservative observation in all such cases; patients are rigorously isolated in aseptic rooms (sterilized food, personnel in sterile cloths etc.) while the choice of therapy (platelet transfusions, selected antibiotics etc.) is guided by the clinical picture. Only if such therapy proves insufficient ("decompensation"), will homologous bone marrow be administered.

An additional hazard in such cases may be the so-called **"median lethal dose effect"** (MLD-effect)*. It has been shown experimentally that homologous bone marrow given after high, but sublethal radiation doses, can have a deleterious rather than a beneficial effect in mice; in other words, mortality of animals treated with homologous bone marrow can be higher than in untreated irradiated controls (189, 47, 194). Though this MLD-effect, as yet unexplained, has so far only been found in certain mouse strain combinations, it may nevertheless be an unnecessary risk to take in patients that stand a good chance to recover with conservative therapy only.

Autologous bone marrow infusions would obviously be the therapy of choice in all such cases. It is already practised in cases where heavy whole-body irradiation is to be expected within a short period (see below) while it seems that marrow preservation techniques may soon reach a state of perfection that would allow all individuals potentially exposed to radiation hazards (exploration of space for instance), to dispose of sufficient viable autologous bone marrow at any time.

2) THE TREATMENT OF MALIGNANCIES

There are neoplasms for which irradiation of the whole or large parts of the body may be required. These include malignancies of the hemopoietic and the lymphatic tissues and possibly also non-systemic neoplasms that are radiosensitive and metastasize widely. Treatment of such systemic neoplasms, even if highly radiosensitive, may require lethal or supralethal doses of irradiation or radiomimetic cytotoxic drugs. Although it is realized, based on the experience in mice (12, 207, 128, 43), that chances to obtain complete eradication of such tumours are negligible, reasonable remissions have been attained by this method in clinical trials. The hemopoietic system inevitably suffers and bone marrow injections, as replacement therapy, may be indicated. Only if the marrow space was free of tumour, or in cases with outspoken remissions, is autologous bone marrow therapy feasible and such therapy has proven to be beneficial in several instances (108, 96). In many cases however, unless an identical twin happens to be available, one will have to resort to homologous bone marrow.

Some investigators have already followed this line of treatment during recent years (129, 155).

---

* The **median lethal dose** range includes doses of total-body irradiation after which some, but not all the irradiated untreated animals die within a certain period of time. The lethality of a radiation dose (LD) is often indicated by the symbol **LD x/y** in which x stands for the percentage of animals dying within a certain number (y) of days after irradiation. Lethality is usually expressed as an LDx/30 since death occurring longer than 30 days after irradiation is not considered to be connected with the bone marrow syndrome.

The results of such clinical trials have been rather disappointing, again largely because of the histo-incompatibility between donor and host. If a "take" of a foreign marrow graft was obtained, symptoms highly reminiscent of secondary disease in monkeys developed within days or weeks, even if the marrow donor, usually a close kin, had been carefully chosen with regard to conventional blood group antigens. Remissions with useful prolongations of life were equivocal, at best. Methods to mitigate the severity of secondary disease have been proposed in animals (193, 23, 62, 186, 164, 127) but have not been found to be quite satisfactory and most of them have as yet not been tried under clinical conditions.

Some investigators have tried to utilize and enhance this very graft-versus-host activity therapeutically, combining it with radiation therapy in the treatment of mouse leukemia. By adding lymphoid cells to homologous bone marrow grafts or by pre-immunizing the donors against the future host, they hoped to suppress more thoroughly the regeneration of the irradiated tumour cells. These approaches, mostly applied against transplantable mouse leukemias (207, 128), are still very much in the experimental stage though clinical trials according to this pattern are in progress (126). The severity of secondary disease in primates as compared to mice, however, may be a serious obstacle, also in this respect.

3) ORGAN TRANSPLANTATION AND TOLERANCE

In many species, high doses of total-body irradiation or of radiomimetic drugs will permit a take of homologous bone marrow or a prolonged survival of transplanted foreign organs or tissues* (65, 187, 64, 132, 173, 69). In some species, such as the dog for instance, radiation would have to be supralethal to allow significantly prolonged survival of grafted homologous tissues (64, 77). In such cases, when lethal or supralethal doses are necessary, the depleted bone marrow would have to be replaced, preferably by injected autologous marrow taken from the individual before irradiation.

Several clinical cases of prolonged survival and proper functioning of grafted homologous kidneys following irradiation and/or treatment with radiomimetic drugs have been reported (138, 216, 109, 91). The radiation doses, with or without concomitant chemotherapy, usually did not reach levels at which marrow transfusion became absolutely necessary.

Generally speaking however, there is a limit to what can be achieved through non-specific suppression of the homograft reaction by simple total-body irradiation or administration of radiomimetic drugs. The survival time of orthotopically grafted homologous skin for example is only slightly prolonged by such procedures (65, 141). In other words, tolerance towards homologous tissue antigens is usually shortlived after temporary suppression of the immune response by irradiation. In recent years it has been found that specific tolerance to homologous tissue antigens could be induced experimentally in several ways (28, 217, 93, 117, 29, 188, 137). At the time of writing chimerism** still seems to be a strict condition for permanent takes

---

* Some grafted tissues, with little or no vascular supply such as the cornea and cartilage, as well as certain glandular tissues, form an exception to this rule, requiring no suppression of immune reactivity for a prolonged survival; there are also transplantation sites where the homograft rejection mechanism is particularly weak, as in the anterior chamber of the eye and the brain, or the cheek pouch of the hamster. Clinically, homograft reactivity may also be generally reduced under certain circumstances such as pregnancy, Hodgkin's disease, agammaglobulinemia and possibly uremia (215).

** A "chimera" is an individual, whose lympho- and hemopoietic system is partially or exclusively populated by proliferating foreign cells.

of grafted homologous tissue in experimental conditions unless histo-incompatibility between donor and host is low. This very circumstance makes total-body irradiation followed by transfusion of homologous hemopoietic cells an important issue in the field of organ transplantation (see chapter VI).
Radiation chimeras of all species so far investigated, permanently accept grafted tissue of the same genetic make-up as the transfused hemopoietic cells. These cells in fact also repopulate the host's lymphatic tissues, thereby "taking over" his immune apparatus and determining his homograft reactivity. However, permanent chimerism in adults, is only produced by heavy irradiation followed by a graft of hemopoietic cells. Clinical realization of permanent chimerism is still a virtual impossibility because of the inherent danger of secondary disease, as has already been pointed out.
Attempts to circumvent this serious complication have been numerous. The ideal solution would be to avoid the grafting of living cells altogether and to induce tolerance with purified tissue antigens. Many have attempted this approach (31, 94, 112). The results of some recent experiments were encouraging, but the degree of tolerance obtained with injections of purified antigens was partial at best and mostly occurred between mice of limited genetic disparity (34, 136). At this stage of laboratory experience the clinical possibilities of the method unfortunately still seem rather remote.
Others have tried to avoid the dangers of both lethal irradiation and of permanent chimerism, by giving comparatively low radiation doses or no radiation at all, and massive doses of viable homologous cells (123, 124, 90, 97, 125, 139, 74). This did probably not produce permanent chimerism, but nevertheless a reasonable degree of tolerance to grafted homologous skin in mice. The procedure may well be a useful and feasable alternative to simple total-body irradiation for obtaining better homograft tolerance, also in man.

The nature of secondary disease

The exact conditions for acceptance and continuous proliferation of a foreign marrow graft after irradiation, for its adaptation to the host's antigens or for the lack of such adaptation, the cause of graft-versus-host reactions, have been topics of intensive investigations during the last decade (11, 22, 192, 21, 50). Nearly all these experiments were done in mice. In this particular species, homologous and even heterologous bone marrow therapy has been successful and secondary complications comparatively mild. It was hoped, that somewhat similar possibilities would exist for bone marrow therapy in larger mammals. Unfortunately, as we know now, this was not the case. Lethally irradiated rabbits and hamsters showed reasonable primary and long-term survival if treated with homologous bone marrow, but irradiated dogs for instance, could only exceptionally be kept alive with similar treatment (77); in primates, secondary disease turned out to be nearly a prohibitive factor though "takes" of homologous marrow were easier to obtain than in dogs (61).
The pathogenesis of secondary disease has been thoroughly studied, again mostly in mice (24). Similar data, though less abundant than for mice, are available for rabbits and primates (162, 209). Delayed mortality after homologous or heterologous bone marrow therapy has also been described in a few other species, however without detailed information regarding the nature of these "late" deaths (174, 171, 186, 48).

It is now firmly established that secondary disease is caused by a graft-versus-host immune reaction in combination with long-term radiation effects and prolonged depression of the host's defenses against micro-organisms (16). The relative importance and the interrelationship of these three factors in determining the incidence and severity of the disease, are as yet not fully elucidated. Different symptoms and mortality rates were observed in the species that have been thoroughly investigated so far and these differences have been tentatively attributed to a number of variables:

1. the degree of histo-incompatibility between the homologous members of a species,
2. the sensitivity of certain target tissues to ionizing radiation or to the cellular or humoral antibodies developed by the graft and,
3. the relative number and the immunological activity of the lymphoid cells contained in the injected bone marrow suspension.

Comparison of the manifestations of secondary disease in various species

In view of the variable manifestations of secondary disease in the few species so far investigated, one must be very hesitant in assuming that results obtained with homologous bone marrow transfusion in one particular species will also apply to another. A brief comparison of the principal consequences of homologous bone marrow therapy in irradiated mice, rabbits and primates, will stress this point.

Mice given a sufficient number of homologous bone marrow cells can usually be kept alive and well for a number of weeks after lethal irradiation, even if the genetic disparity between donor and host is high. Several weeks after complete recovery, symptoms of secondary disease may develop. Wasting, skin lesions and a chronic type of colitis with diarrhea are usually observed. Morbidity and mortality are variable and largely depending on the host-donor combination.

Homologous bone marrow therapy of lethally irradiated primates takes a distinctly different course. Though functioning of the homologous bone marrow graft can often be demonstrated, complete clinical recovery of the animals during the first month following irradiation rarely occurs (61, 209). Usually, already during the first three weeks, a rapid and vicious secondary syndrome develops. The foremost pathological findings at autopsy include wide-spread denudation of the intestinal mucosa and typical lesions of the dermal epithelium. In a few human cases of homologous bone marrow therapy after lethal irradiation, the complications were unfortunately equally serious and highly reminiscent of those seen in monkeys with secondary disease (131, 126).

Rabbits suffering from secondary disease have not been reported to show skin lesions, which are common findings in primates and also in mice with secondary disease. In rabbits, on the other hand, the disease is characterized by a marked hemolytic anemia that has been shown to be an expression of the graft-versus-host reaction (162, 156). So far, immune hemolysis has not been demonstrated unequivocally in mice or in primates with secondary disease.

There is obviously a difference in time of onset, symptomatology and lethality of secondary disease, between monkeys and man on the one hand and mice and rabbits on the other. This difference has led to the assumption that lymphoid elements, which are considered to play a major role in the development of secondary disease, may be either more abundant or more

mature in primate marrow than in rodent marrow. Another hypothesis attributes the high lethality connected with secondary disease in primates to a particular sensitivity of their intestinal epithelium to the graft-versus-host immune reaction. Intestinal lesions in mice and rabbits with secondary disease do comprise crypt degeneration and inflammatory changes, but hardly ever the complete denudation of large parts of the mucosa typical for secondary disease in monkeys and man. It seems therefore, that though the number of target organs involved in secondary disease may be limited, their selective susceptibility to the graft-versus-host reaction may differ significantly from species to species.
Apart from the differences mentioned above there are also certain features of secondary disease which seem to occur in all species so far investigated. These include wasting, lymphoid atrophy and a rather typical cell death in certain parenchymal organs (56, 162, 208, 209). Their nature and relation to the graft-versus-host reaction will be elaborately dealt with in chapter IV.

Rationale and design of the performed experiments

It was important to investigate the possibilities and pitfalls of homologous bone marrow therapy, following lethal total-body irradiation in yet another species. The rat seemed the animal of choice for several reasons.

1. Few data were available in the literature regarding the immediate effectiveness of homologous bone marrow therapy following total-body irradiation of this widely used laboratory animal (80, 150, 118), while virtually no information was at hand concerned with possible secondary complications of such therapy. While this work was in progress, Dunjic (70) and Courtenay (58) reported about delayed deaths in irradiated rats treated with homologous bone marrow. However, their data were rather limited, especially with regard to the immunological nature of the disease and its pathogenesis.
2. Primary and long-term survival of lethally irradiated rats treated with homologous bone marrow had been very poor both in this laboratory (20) and elsewhere (118, 58). Only in cases of a rather close genetic relationship between donor and host, had a reasonable survival rate been obtained (80, 150).

The difficulty to obtain homologous rat chimeras was obviously in contrast with the positive experiences in mice. It was expected that a systematic study of homologous bone marrow transplantation in the irradiated rat would reveal some of the reasons for the apparent discrepancy. At the same time it was hoped that if reasons for the seemingly difficult establishment of homologous bone marrow grafts in irradiated rats were found, this might yield information somehow also applicable to certain clinical problems of homologous bone marrow therapy.
The underlying study is therefore an attempt to extend the existing knowledge regarding the possibilities of homologous bone marrow therapy in irradiated mammals. It was mainly concerned with the immediate (primary mortality), the delayed (secondary morbidity and mortality) and the long-term consequences (tumour incidence etc.) of lethal irradiation and homologous bone marrow therapy in the rat. Some of the experiments were deliberately patterned after experimental designs used in studies of bone marrow therapy in mice, rabbits, dogs or primates. It was the intention to compare results and, whenever possible, draw conclusions from observed similarities or discrepancies.

A few experiments were only indirectly linked with homologous bone marrow therapy and its consequences, for instance those dealing with persisting tolerance toward homologous transplantation antigens after disappearance of a homologous bone marrow graft. As has been pointed out already, homologous hemopoietic cells given intravenously, may play a role in the promotion of tolerance toward grafted homologous tissues or organs, as long as the use of purified tissue antigens does not yield more satisfactory results. The availability of chimeras and "reversals"* in the course of the present experiments led to an extension of the study into the field of immunological tolerance.
A fortunate coincidence was the availability of SPF (specific pathogen free) animals in this laboratory (Radiobiological Institute GO-TNO, Rijswijk Z.H., the Netherlands). The unique possibilities for radiation experiments offered by such rats, reasonably free of most of the common parasites and micro-organisms, will be discussed.

In **chapter III** the effect of high radiation doses (900-1000 r) on adult rats is first described. These animals did not receive bone marrow treatment. Mortality curves, symptoms, hematological and histological findings after serial killings or spontaneous deaths will be presented. So-called "early deaths" possibly associated with the presence of certain micro-organisms had to be differentiated from "normal" hemopoietic deaths during the second week. Furthermore, the optimal conditions for the take and continued functioning of a homologous bone marrow graft had to be determined. This included an appraisal of

1. the radiation dose necessary to obtain sufficient suppression of host immune reactivity to allow long-term takes of homologous bone marrow.
2. the number of homologous hemopoietic cells necessary for survival.
3. the speed of conversion of both red cells and nucleated cells from host-type to donor-type in these rat chimeras, and an assessment of the probability of immune hemolysis.
4. the effect of homologous bone marrow therapy after radiation doses in the „median lethal dose range" (see footnote p. 13).

Chapter IV deals with secondary disease, the delayed complications occurring in irradiated rats after the administration of homologous bone marrow. Factors influencing onset, incidence and severity of the disease were determined. Comparisons were made with other graft-versus-host reactions in the rat as well as with manifestations of secondary disease in other species. Acceleration and aggravation of this disease, the so-called "killing effect", produced by the addition of mature homologous lymphoid cells to a graft of hemopoietic cells, was also assayed and described.

Chapter V describes the effectiveness and consequences of the intravenous administration of homologous cells following lethal irradiation, for other strain combinations than those dealt with in chapter III and IV. Similarities and discrepancies will be presented and certain conclusions will be drawn from these observations.

Chapter VI is concerned with certain aspects of immune tolerance towards transplantation

---

* An animal that has reverted to host-type hemopoiesis after an initial take of a foreign bone marrow graft is called a **reversal.**

antigens. Radiation chimeras always accept skin grafts of the same genetic make-up as the bone marrow donor, "reversals" in general regain host-type immune reactivity (188, 221, 14, 160). Persistance of tolerance towards skin grafts of the genotype of the rejected marrow graft would implicate a kind of "memory" of the immune mechanism with regard to non-reactivity towards foreign transplantation antigens. This is an important clinical issue. The experiments described in chapter VI serve to obtain information on this kind of tolerance, a specific non-reactivity which seems not to be the consequence of permanent chimerism.

Chapter VII finally, is concerned with long-term effects of lethal total-body irradiation followed by bone marrow therapy. In view of the clinical importance of possible unfavourable late effects, all treated animals were kept for long-term observation. Time and probable causes of death were recorded and a histological classification of the many tumours occurring relatively soon after irradiation was attempted. Results will be discussed and compared with published findings for lethally irradiated rats surviving in parabiotic union with non-irradiated rats, as well as with long-term effects reported for lethally irradiated bone marrow treated mice.

CHAPTER II

MATERIALS AND METHODS

Animals

The rats were derived from two colonies maintained at this Institute. One was an inbred Wistar Albino strain (WAG/Rij) orginally obtained from the Glaxo Laboratories, England, and bred by brother x sister mating since 1953 at Rijswijk. Histocompatibility between the members of this colony was frequently tested and confirmed by reciprocal skin grafting. The other was a non-inbred large Wistar rat, obtained in 1955 from a commercial source. These rats, called "BROFO", have since been reared in this laboratory on a non-inbred basis. Genetic disparity between individual BROFO rats however is comparatively low. This was also repeatedly tested with reciprocal skin grafting, 60 % showing a normal rejection time of 10-12 days, the remaining 40 % having prolonged rejection times of 2-6 weeks but never permanent takes. Interstrain skin grafts, between WAG rats and BROFO rats, were consistently rejected within 10-12 days (see chapter VI).

Both strains were brought under specific pathogen free (SPF) conditions of breeding in 1960 by the rearing of ceasarian-derived young in isolated quarters. This procedure has resulted in a complete absence of ecto- and endoparasites and a substantial reduction, though not a complete absence of PPLO (pleuro-pneumonia-like organisms) infections.

The animals were kept in heat-resistant transparent plastic cages, 3 to 5 to a cage, on commercial pellets and tap water ad libitum. Cages were sterilized twice a week. Further details on post-irradiation sanitation as practiced in this laboratory, have been described before. (211).

Only young adult animals, 3-5 months of age, both male and female, were irradiated. Their weights ranged from 250-350 g for males and 200-250 g for females; they were randomized according to weight in each experiment.

The donors of bone marrow or spleen cells were 4-6 weeks old, weiging 80-110 g. Marrow of male donors was also used for female irradiated recipients after it had been found that sex-linked incompatibility did not exist and that the efficacy of bone marrow therapy and the incidence of complications were indentical for both sex combinations.

Irradiation

Rats were irradiated in groups of 10 in a circular perspex cage. Physical data of the irradiation were 250 kVp, 30 mA, HVL 2.15 mm Cu. The dose rate was 70 r p.m. at a focus-target distance of 67 cm (focus to midline of animals). The administered dose was monitored for each irradiation with a thimble ionization chamber mounted at the top of the cage. The mid-body dose was derived from measurements in a phantom. Maximal back scatter was employed.

Suspension of bone marrow and spleen

Bone marrow was obtained from femurs, sometimes also from the tibias of the donor animals. The bones were dissected, the proximal end cut off and the marrow content flushed out with Tyrode's solution. This preliminary suspension was filtered through a Nylon gauze to remove coarse particles and to obtain a homogeneous suspension of dissociated cells. The number of viable nucleated cells was then counted in a hemocytometer after addition of 9 volumes of 1 $^0/_{00}$ Trypan blue or 2 $^0/_{00}$ Eosin (the stained cells being considered non-viable).
Spleen cell suspensions were obtained by "teasing" the dissected spleens with a scalpel in Tyrode's solution. This suspension was then also filtered through a Nylon gauze. Counting and determination of cellular viability was done as described for the bone marrow suspensions. The cell suspensions were injected into the tail vein of the lightly anesthetized rats (ether) in a volume of approximately 1 ml, about 24 h. after irradiation, 48 h. after irradiation in one experimental group (group I).

Skin grafting technique

Only abdominal skin was grafted after it was found that back skin, especially of the male, was unsuitable for free full thickness grafting in both WAG and BROFO rats. Recipients were anesthetized with ether, the dorsal thoracic area shaved, covered with adhesive tape (Scotch no 471), and lifted in a fold to punch out 2 adjacent circular pieces of 1½ cm diameter. The grafts, punched similarly from abdominal skin and stripped of their panniculus with scissors, fitted snugly into the graft beds which maintained shape and position due to the surrounding tape. A dressing of impregnated gauze ("Carbonet", Smith and Nephew, England) and a fixing tape around the thorax were left on until the 8th day. With very few exceptions the graft healed in perfectly. Evaluation of graft survival was done macroscopically (hair growth, atrophy, edema, hemorrhages) and occasionally confirmed microscopically.
This technique allowed very rapid transplantation of up to 4 or more skin grafts per animal at a time. Ideal healing-in without the scar formation that is sometimes seen in sutured grafts, facilitated evaluation of the graft's fate. It is essentially a modification of Billingham's method of skin grafting in small mammals (26), with a special provision to secure a fixed position of the graft bed throughout the first 8 days. Somewhat similar skin grafting techniques have been used for mice (88, 41) but not for larger animals. Figures 31 to 34 depict several stages of the procedure.

Hematological techniques

Blood samples were taken from the tail. Erythrocytes were counted after dilution in Hayem's fluid, leucocytes were diluted in 2 % phosphoric acid in distilled water. Platelet counts were done in 3 % HCl cocaine plus 0.2 % NaCl, while reticulocytes were counted according to Seyfarth's technique in smears on brilliant-cresyl blue coated slides. Differential counts of the leucocytes were done from Giemsa-stained smears.

Identification of erythrocytes

Typing of erythrocytes was done with specific iso-antisera according to the technique of Gorer

and Mikulska (86). The degree of agglutination was expressed in arbitrary values ranging from O to 3+. Animals repeatedly displaying 2+ or 3+ agglutination with one type of antiserum and no agglutination (0) with the other antiserum were considered to have an erythropoietic system of the type against which the first serum was active. The iso-antisera were produced by repeated injections of WAG spleen cells into BROFO rats and vice versa. The injections were given at weekly intervals alternatively subcutaneously and intraperitoneally. Antisera were taken at least 2 weeks after the last immunizing injection and only used after the test on normal control cells had shown their efficacy.

Identification of nucleated cells

A modification of Gorer's cytotoxic test (87) was employed as has been described previously (8). Only cells from the peritoneal cavity were tested after it had been shown, in a number of chimeras, that lymph node cells always contained approximately the same proportion of host- and donor-type cells as did peritoneal cells; this was in accordance with previous findings for mouse radiation chimeras (8, 85).

Only one of the two iso-antisera, the WAG-anti-BROFO serum, was strong enough to be used in the cytotoxic test.

Peritoneal cells were harvested by injecting 20 ml Tyrode solution into the peritoneal cavity of superficially anesthetized (ether) rats. After gentle massaging of the abdomen the syringe was removed, the canule (gauge 12) left in situ. A sample of the peritoneal washing was easily obtained from the canule by slight pressure on the abdomen. One drop of this homogenous suspension of peritoneal macrophages and lymphocytes (containing approximately 20,000 cells) was incubated for ½ hour at 37°C with 1 drop of WAG-anti-BROFO serum (diluted 1 : 4) and 1 drop of complement (rabbit serum diluted 1 : 10). The cytotoxic effect was tested with Trypan blue (2 $^{0}/_{00}$) and recorded as the percentage dead (stained) cells. Cell suspensions of untreated BROFO rats serving as controls invariably showed 100 % mortality when so tested, while always less than 25 % of WAG-type cells stained with Trypan blue after similar treatment. Far less cytotoxic effect was obtained if complement was withheld or inactivated by heating.

Methods of blood culture

Blood obtained by heart puncture was cultured on blood agar plates. If growth of bacteria was present subcultures were performed for further determination.

Autopsies and histological techniques

A number of animals, both treated and controls, were killed serially but most autopsies were from animals that had been killed when moribund or had died spontaneously. Tissues were not taken for microscopic examination if postmortal autolysis was advanced. The tissues were fixed in 4 % buffered formaline. Paraffin sections of 7 μ were cut and routinely stained with hematoxylin and eosin.

Bone marrow, lymphatic tissue, lung, liver, kidneys, adrenals, ileum and colon were routinely examined, while other organs or tissues were examined if lesions were either seen at autopsy or expected.

Animals with tumours were sometimes sacrificed when it could be reasonably assumed that they would not live more than a few more days (see also chapter VII). The histological classification of tumours was not always possible after staining with eosin and hematoxylin. Special staining techniques were employed in such cases for the identification of reticulin and collagen fibers (Gomori, Azan, van Giesson), muscle (Mallory, Lendrum-Masson), osteoid and bone substances (PAS, Azan, van Giesson and Gomori) and mucoid substances (PAS and toluidine blue).

CHAPTER III

HOMOLOGOUS BONE MARROW THERAPY IN THE WAG→BROFO COMBINATION

INTRODUCTION

As has been pointed out already (see "Rationale and design of the performed experiments"), a variety of experimental data will be presented and discussed in the underlying chapter. The animals described in the first section (A) of the results are in fact the irradiated controls for rats that were irradiated and subsequently treated with homologous bone marrow. Primary survival as well as a number of complications occurring during the first month following irradiation and bone marrow treatment, will be described in section B of the results. The discussion treats the mentioned subjects more or less in the same order. Complications occurring mainly during the 2nd and 3rd month following irradiation and homologous bone marrow therapy, will be dealt with in chapters IV and V.

RESULTS

A. **Effects of irradiation without bone marrow therapy**

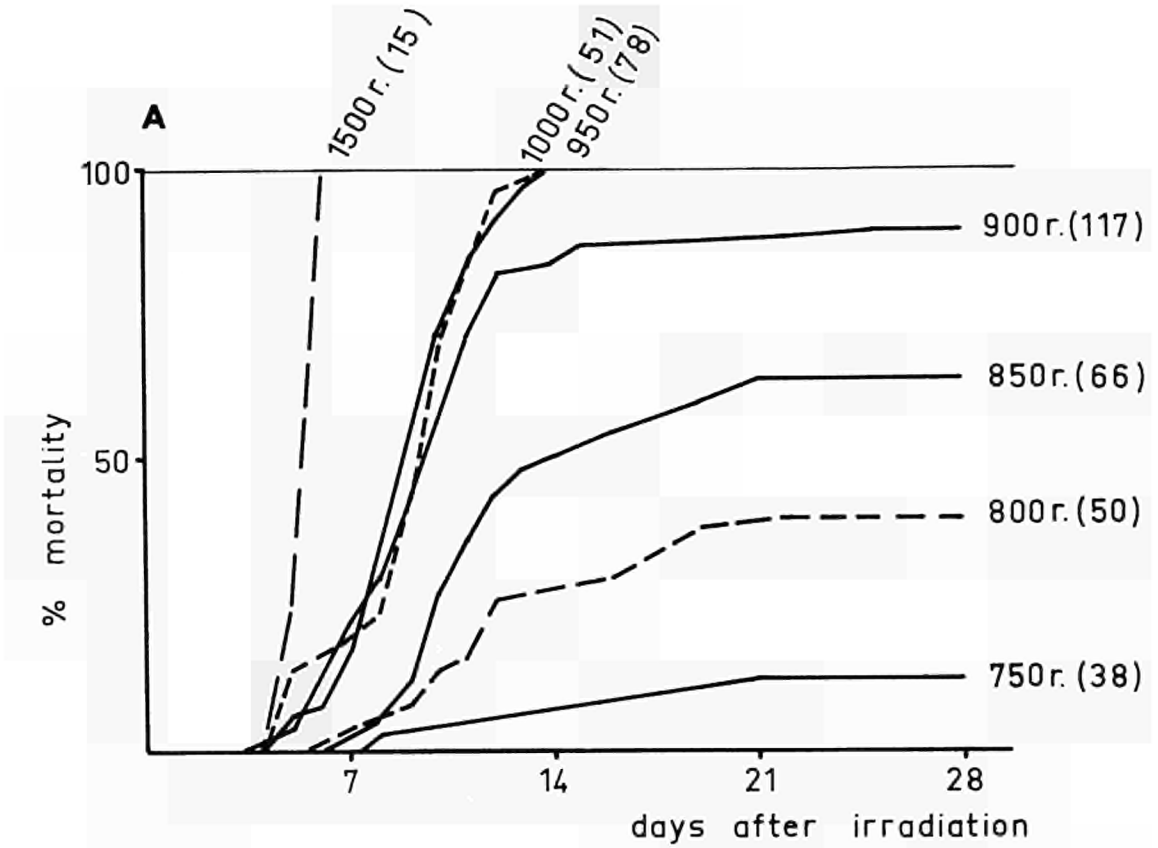
MORTALITY

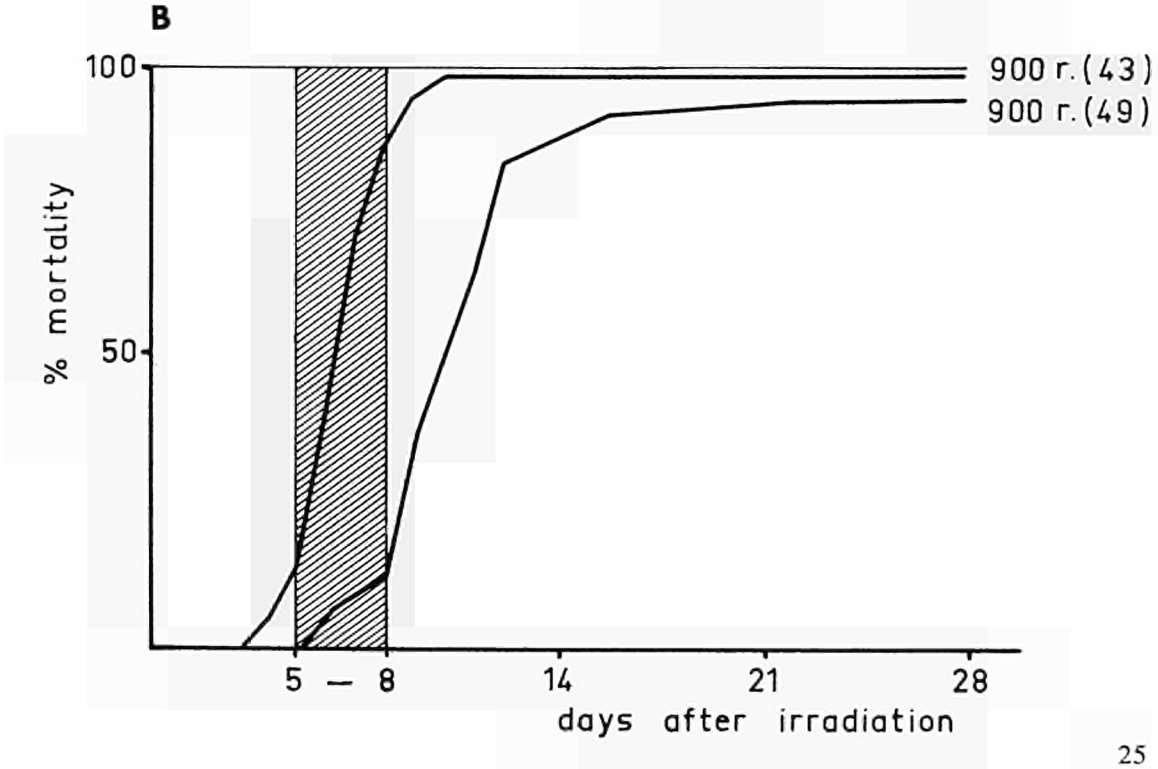
The mortality curves for BROFO rats after various radiation doses are depicted in text figure 1A. The curves represent pooled data from a number of experiments extending over a 9 months period. Though the animals were at all times bred and kept under standardized conditions, the survival times for various groups showed significant variations after identical radiation doses. Text figure 1B represents mortality curves for a radiation dose of 900 r. In this diagram experiments in which many deaths occurred from the 5th to the 8th day have been pooled separately and experiments with relatively few early deaths also. The early deaths were not caused by a typical intestinal syndrome (occurring at higher doses and causing death before the 6th day) nor by hemorrhagic diathesis, since wide-spread hemorrhages were not seen before the 9th day.

→

Text figure 1A: Mortality of adult BROFO rats after various doses of whole-body irradiation. The number of animals for each group of experiments is indicated in parentheses. About two thirds of the animals in each group were males, those that received 1500 r were all females.

Text figure 1B: Mortality of adult BROFO rats after a single dose of 900 r whole-body irradiation. Experiments showing a preponderance of early deaths (4 out of 11 experiments) and those showing mainly normal survival times (5 out of 11 experiments) have been pooled and charted separately. The remaining two experiments showed early as well as normal survival times and were not charted in this diagram.


A
1500 r. (15)
1000 r. (51)
950 r. (78)
100
50
% mortality
900 r. (117)
850 r. (66)
800 r. (50)
750 r. (38)
7
14
21
28
days after irradiation


B
100
50
% mortality
900 r. (43)
900 r. (49)
5 — 8
14
21
28
days after irradiation

SYMPTOMS

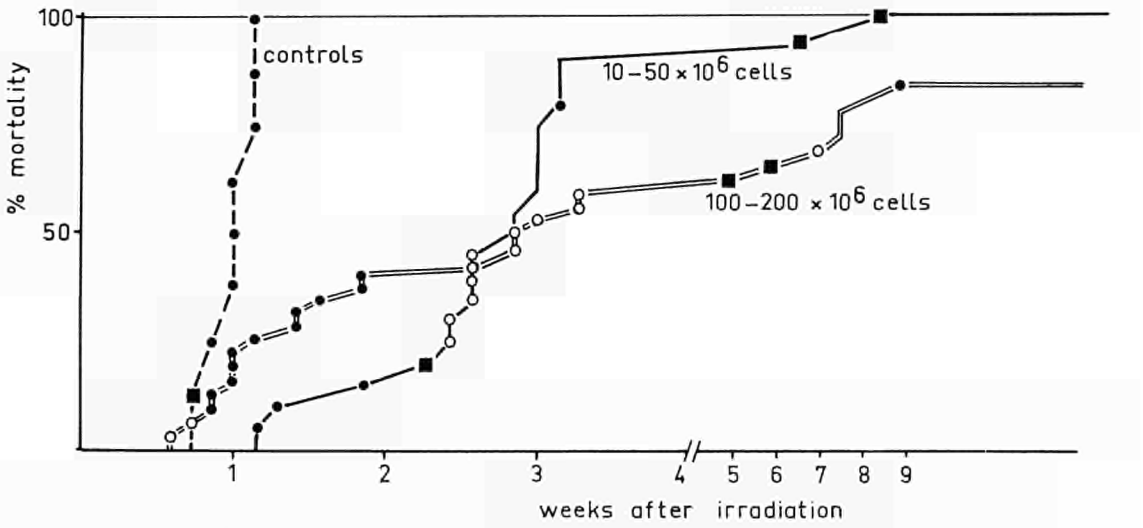
Following a single radiation dose of 900 to 1000 r the first symptoms were weight loss, and slightly blood-tinged secretions around nares and eyes. Diarrhea started around the 3rd day and did not last beyond the 5th or 6th day. Animals dying between the 5th and 8th day deteriorated rapidly but those not succumbing in that period regained their normal appearance and activity from the 4th day on and remained comparatively well until anemia developed around the 10th day. They usually died during the 2nd week with profuse hemorrhages.

HEMATOLOGY

Hematological data for BROFO rats having received a single dose of 900 r total-body irradiation are included in text figure 5 on page 31. These are values obtained for controls in an experiment in which homologous bone marrow and spleen cells were given after irradiation (see chapter IV). Leucocyte counts dropped to around zero very soon after irradiation and showed no recovery before death. The platelets showed a slightly reduced value on the 3rd day, had practically disappeared on the 7th day and there was only a negligable recovery towards the end of the 2nd week. The erythrocyte values, in spite of some early hemorrhages, showed a striking hemoconcentration during the 1st week. During the 2nd week however these counts fell below normal values, sometimes below 1 x 10^6/mm^3 in the terminal stages. No reticulocytes were seen on the 3rd day and very few had returned by the 10th day. Blood smears were made at all intervals but there were never enough leucocytes for dependable differentiation.

BACTERIOLOGY

Blood cultures were often done, especially in experiments with a high incidence of deaths



Text figure 2: Mortality and results of blood cultures for BROFO males given radiation only (8 controls, 900 r, radiation plus low doses of WAG bone marrow (20 animals, 900 r, 10-50 x 10^6 cells) and radiation plus an adequate dose of WAG bone marrow (32 animals, 900 r, 100-200 x 10^6 cells, experiment III c in text figure 3).

○ = sterile blood culture
● = blood culture positive for Pseudomonas aeruginosa
■ = blood culture positive for other bacteria

between the 5th and the 8th day after irradiation. In such experiments, the blood cultures were frequently found to be positive for Pseudomonas aeruginosa, not only in the irradiated controls but also in animals injected with homologous bone marrow cells that died during the early period (text figure 2). The available data suggest that the presence of this micro-organism predisposes irradiated animals for a relative early death though this conclusion is by no means certain. It is our impression, however, that the influence of Pseudomonas aeruginosa is definitely less disturbing in the present experiments than has been described for mice under similar conditions (211).

AUTOPSY

Gross autopsy findings of animals killed serially, revealed hemorrhages in the wall of the stomach, small and large intestines as well as in lymph nodes and Peyer's plaques as early as the 4th day but more extensively on the 6th day. The spleen and lymph nodes were atrophic. On the 4th day the stomach was distended due to delayed emptying and the ileum and colon were filled with liquid contents causing some distension too, especially of the coecum (this mechanical factor may explain the occurrence of some hemorrhages in the intestinal walls, several days before generalized hemorrhagic diathesis was evident). On the 8th day after irradiation most of these findings subsided, the gut and its contents resuming a normal macroscopic appearance. A severe hemorrhagic diathesis with multiple subcutaneous petechiae and massive hemorrhages in various organs was never seen before the 9th day, both in animals dying spontaneously and those killed serially.

Rats dying spontaneously between the 5th and 8th day were emaciated but rarely showed profuse hemorrhages, multiple abscesses or other macroscopic lesions as an immediate cause of death.

HISTOLOGY

Hemopoietic cells were virtually absent from the bone marrow on day 4, after radiation doses of 900 or 950 r. From day 6 on, small groups of myelocytes and erythroblasts reappeared in the marrow space, evidence that the potential of hemopoietic recovery had not been completely abolished. However, the output of mature blood cells, even after a radiation dose of 900 r, was hardly ever sufficient to keep the animals alive.

Complete lymphatic atrophy in the nodes and virtual absence of lymphatic tissue in the spleen was evident on day 4. After 900 r, the lowest dose investigated, an occasional rat showed very modest lymphopoietic activity as evidenced by the reappearance of some lymphoblasts and mature lymphocytes on day 6 and 8.

No histological lesions were seen in the livers of irradiated controls.

The intestinal epithelium showed extensive damage on the 3rd and 4th day, similar to lesions described by others for irradiated rats, mice and other mammals (167, 52): wide-spread cystic degeneration and desquamation of crypt cells was seen in the small as well as the large intestine. Recovery occurred rapidly from the 4th day on: many regenerating hyperplastic crypts were seen on day 4. On day 6 only a few degenerating crypts were found in the intestine though an appreciable number of desquamating crypts was still present in the colon on day 6. On the 8th day and therafter only an occasional isolated desquamating crypt was seen.

No radiation-induced damage of the skin was found in irradiated non-treated rats.

As will be demonstrated, the organs and tissues described in this section are those primarily

affected when complications occurred following the injection of homologous cells. A systematic histological study of other organs (such as testes, ovaria etc.) was not done since the main purpose of this part of the study was to differentiate between purely radiation-induced damage and histological changes eventually encountered in animals with secondary disease (see also introduction and discussion of chapter VII).

B. The therapeutic effect of homologous bone marrow

1. SURVIVAL DURING THE FIRST MONTH AFTER LETHAL IRRADIATION

Homologous bone marrow therapy and its possible complications were primarily studied in the combination WAG→BROFO, the inbred WAG rats being the marrow donors, the random-bred BROFO rats the irradiated recipients. However, other homologous combinations (BROFO→WAG and BROFO→BROFO) were also investigated as well as the isologous combination (WAG→WAG). The results of the latter two homologous combinations as well as of the isologous combination, will be presented in chapter V.

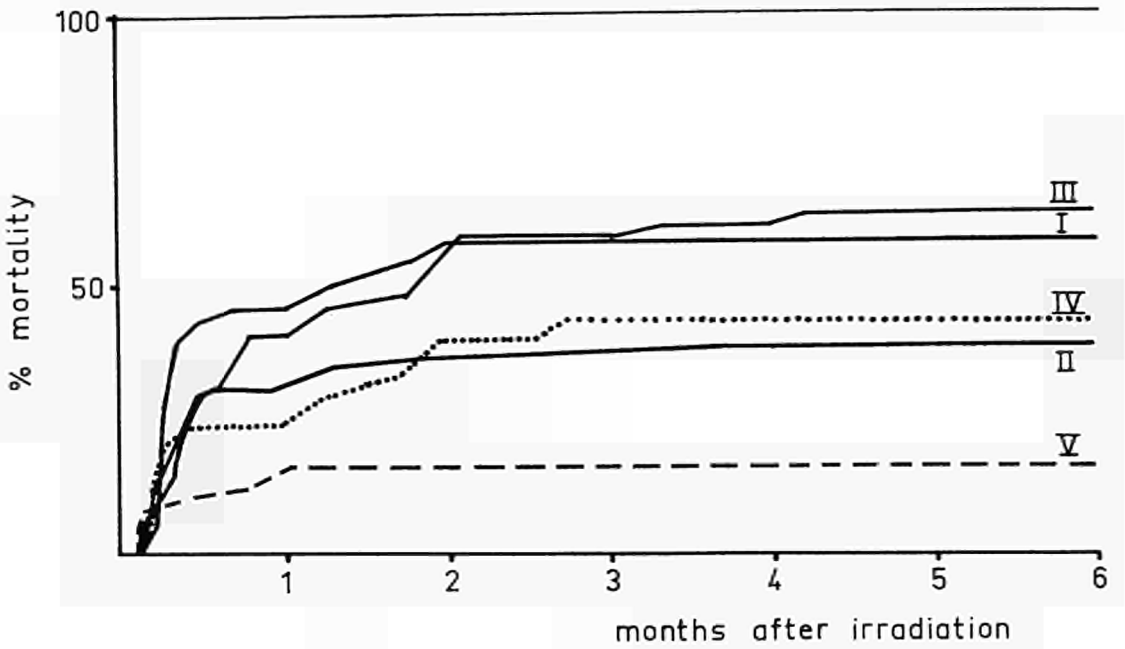
MORTALITY

Mortality curves for three groups of homologously treated BROFO rats are included in text figure 3 (I, II, III after respectively 1000, 950 and 900 r), each group representing pooled data for a number of identical but consecutive experiments. The curves demonstrate that i.v. administration of sufficient WAG bone marrow resulted in a fair number of 4 week and long-term survivors in all 3 groups. However, not all separate experiments of each group showed an identical incidence of early (first 2 weeks) and delayed mortality. Text figure 4 depicts mortality curves for 2 separate experiments of group III (WAG→BROFO, 900 r, 100-200 x 10^6 bone marrow cells), one having a particularly high incidence of primary and delayed mortality, the other, under identical experimental conditions a rather low mortality of either type. Many blood cultures were performed in the former experimental group, showing a high incidence of cultures positive for Pseudomonas aeruginosa in the treated animals as well as their controls dying during an early period (text figure 2).

The number of homologous bone marrow cells required to keep lethally irradiated BROFO rats alive varied between experiments. In general it can be stated that 50-100 x 10^6 cells resulted in 30-day survival of about 30 % of the animals while 200 x 10^6 were necessary to keep more than 50 % alive after 900 r (table I). In a few experiments a smaller number of cells proved to be effective. It should be noted that if 100 x 10^6 or fewer WAG bone marrow cells were given after 900 r, the graft did not always function permanently and some of the surviving animals were eventually found to have reverted to host-type hemopoiesis. Animals given more than 100 x 10^6 marrow cells after a radiation dose of 950 or 1000 r, invariably had a permanent donor-type erythropoietic system (table II and page 34).

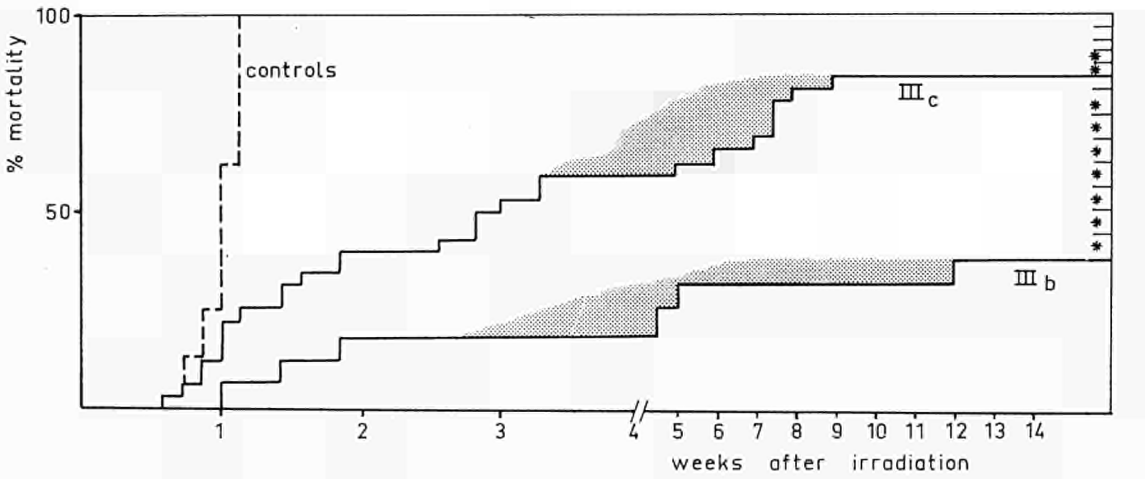
The sex of the recipients seemed to have no significant influence on the outcome of the experiments though it must be admitted that the number of females used in the experiments was much lower than that of males (text figure 3). No difference was ever observed between the effect of bone marrow grafts taken from male or female donors. The possible influence of age and body-weight of both recipients and donors was not elaborately investigated: only adult recipients, 3-5 months old, and very young donors, approximately 1 month of age, were used.

Preliminary experiments had shown a 24 to 48 hours interval between irradiation and bone



Radiation dose (r)	Experimental groups		Recipients ♂	Recipients ♀	Recipients total number	Recipients strain	Donors strain	Donors number of cells x 10^6	Donors interval irr.-b.m. in hours
1000	I	a	20						
		b	10	10	53	Brofo	WAG	100-300	48
		c	13						
950	II	a	10						
		b	18						
		c		19	61	Brofo	WAG	100-200	24
		d		14					
900	III	a	6						
		b	16		54	Brofo	WAG	100-200	24
		c	32						
900	IV	a	28						
		b	17		45	WAG	Brofo	100-400	24
900	V	a	5	7					
		b	14		51	WAG	WAG	20-40	24
		c	25						

Text figure 3: Mortality of rats treated with homologous or isologous bone marrow after various radiation doses. The table below the diagram provides data regarding host-donor combination, radiation dose, number of cells injected etc., for each experimental group.



Text figure 4: Mortality curves for 2 experiments of a group (experiments IIIb and IIIc). In both experiments BROFO males received 100-200 x 10^6 WAG bone marrow cells 24 hours after 900 r total-body irradiation. Shaded areas indicate approximate onset and duration of symptoms typical for secondary disease. Animals recovering after a period of similar symptoms, namely weight loss and skin lesions during the 2nd and 3rd month, are indicated by an asterisk, not by a shaded area (7 animals in experiment IIIb, 2 in experiment IIIc).

marrow therapy to be effective, while a delay beyond 48 hours reduced the efficacy of a bone marrow graft. This is in accordance with data obtained by Rogacheva (169) for isologous bone marrow therapy of irradiated rats.

HEMATOLOGY

Hematological data for a group of BROFO rats receiving 100 x 10^6 homologous bone marrow cells after 900 r are depicted in text figure 5. The depression of all cellular blood elements

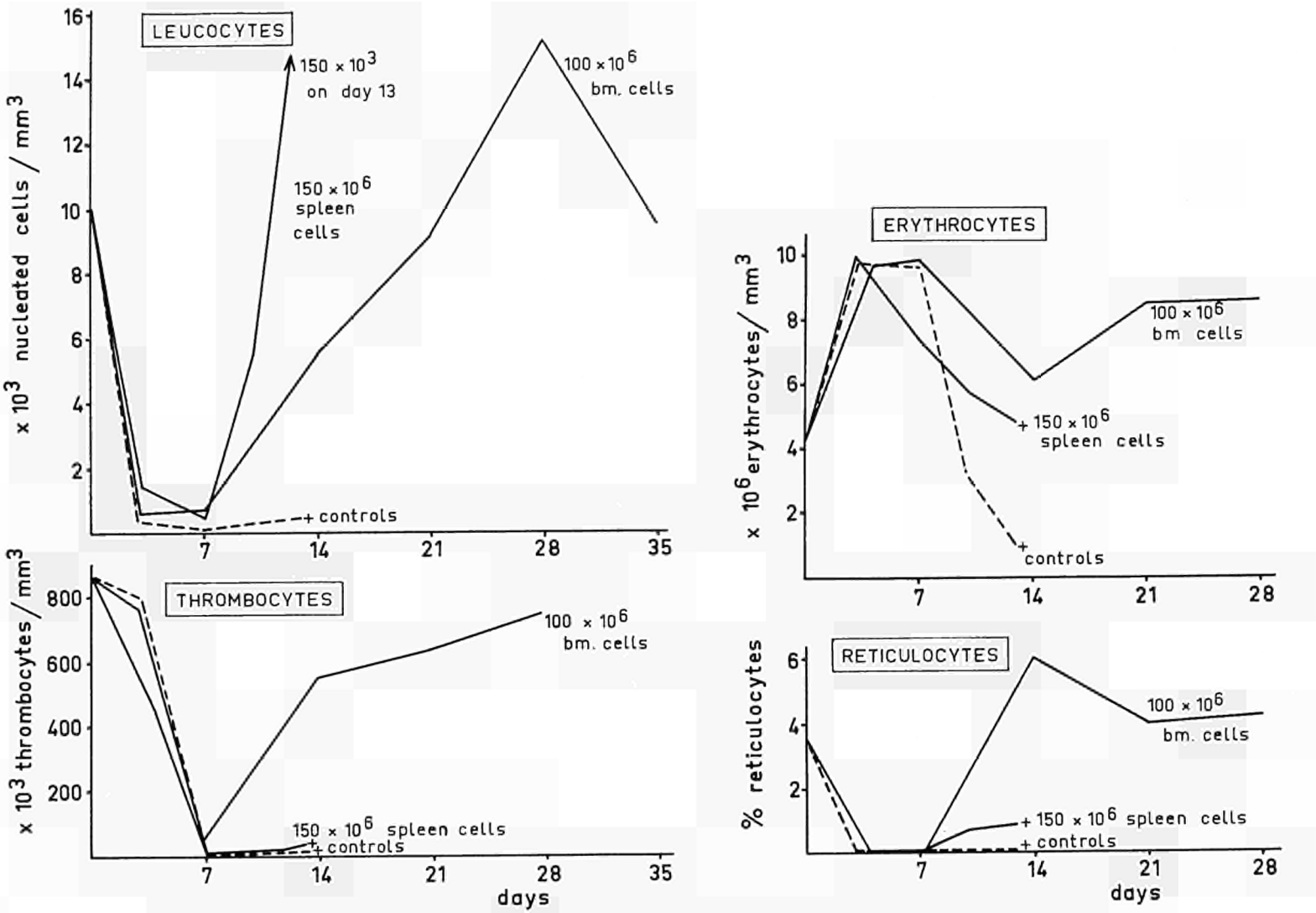
TABLE I

EFFECT OF THE NUMBER OF WAG BONE MARROW CELLS ON SURVIVAL AND DEVELOPMENT OF SECONDARY DISEASE IN BROFO RATS AFTER 900 R WHOLE BODY IRRADIATION *

		Survival		Secondary disease	
				number of animals	
number of b.m. cells x 10^6	number of animals	animals alive after irradiation (weeks) 2 w	4 w	showing symptoms of secondary disease **	dying of secondary disease
0	42	2	2		
10	10	7	0		
25	10	10	10	0/10	
40-50	49	24	16	3/16	3
100	59	27	20	6/20	6
200	66	43	39	20/39	7

* Pooled data from 8 separate experiments

** Out of number alive 4 weeks after irradiation



Text figure 5: Hematology of BROFO rats after irradiation only (controls) and of animals treated with 100 x 10^6 WAG bone marrow cells or 150 x 10^6 WAG spleen cells. Each point represents the average for 3-5 animals.

during the first week was identical with that of irradiated controls. The leucocytes rapidly recovered during the 2nd week. About 50 % of these were polymorphonuclear cells on day 14 after irradiation but this percentage dropped to below 20 % on day 21 and remained between 10 and 20 % during the 4th and 5th week. Platelets showed a recovery pattern parallel to that of leucocytes. There was obviously evidence of hemoconcentration during the first week; thereafter, a decrease of the number of erythrocytes occurred but the red cell counts remained high for several weeks. Recovery of reticulocytes reached a peak of 6 % at 2 weeks and returned to more or less normal values 3 weeks following the administration of the bone marrow.

SYMPTOMS AND AUTOPSY FINDINGS

Bone marrow treated animals not surviving the first 2 weeks after irradiation looked miserable and emaciated by the 5th day and usually died before the 10th day with edema and bloody crusts around the eyes but no apparent anemia and no diarrhea. Their blood cultures were frequently positive, mostly for Pseudomonas aeruginosa (text figure 2). Macroscopically, no obvious cause of death was seen at autopsy. The blood vessels were usually congested but there were no massive hemorrhages and no macroscopically visible localized or disseminated foci of infection.

Bone marrow treated animals that did not die during this early period regained a normal appearance by the 6th day when diarrhea had stopped and they started gaining weight again. The majority of these rats stayed well during the following 3 weeks. A number of them were serially killed. During the first 8 days the findings were not different from those in serially killed controls: limited hemorrhages of the gastric and intestinal wall, hemorrhages and atrophy of the lymphoid tissues, delayed emptying of the stomach and a bile-tinged liquid content in the small bowel up to the 5th day. If killed later than the 10th day, the spleen and lymph nodes had nearly regained their normal size and no macroscopic lesions were detectable except maybe traces of previous hemorrhages in lymph nodes and Peyer's plaques.

HISTOLOGY

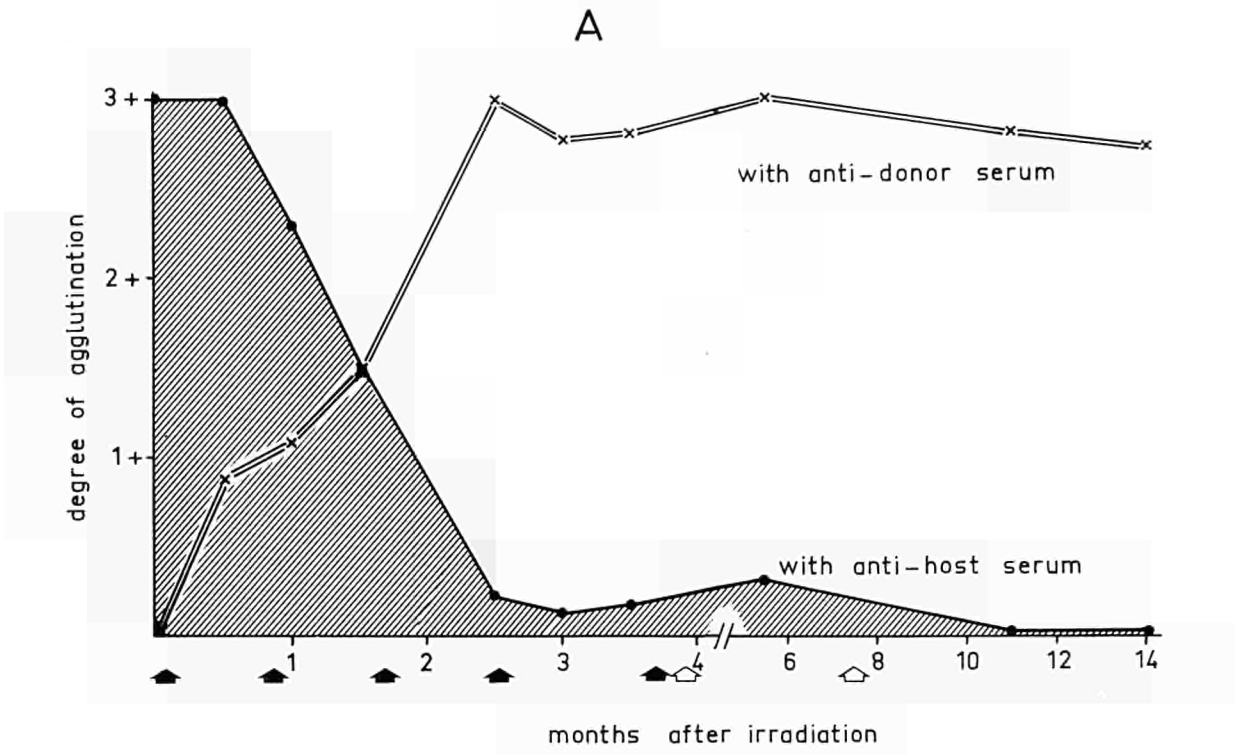
Bone marrow recovery in the homologously treated animals was first seen on day 6 and 8. Regeneration followed a typical histological pattern previously described for mice (208): patches of practically acellular marrow persisted amidst areas of normal cellularity.

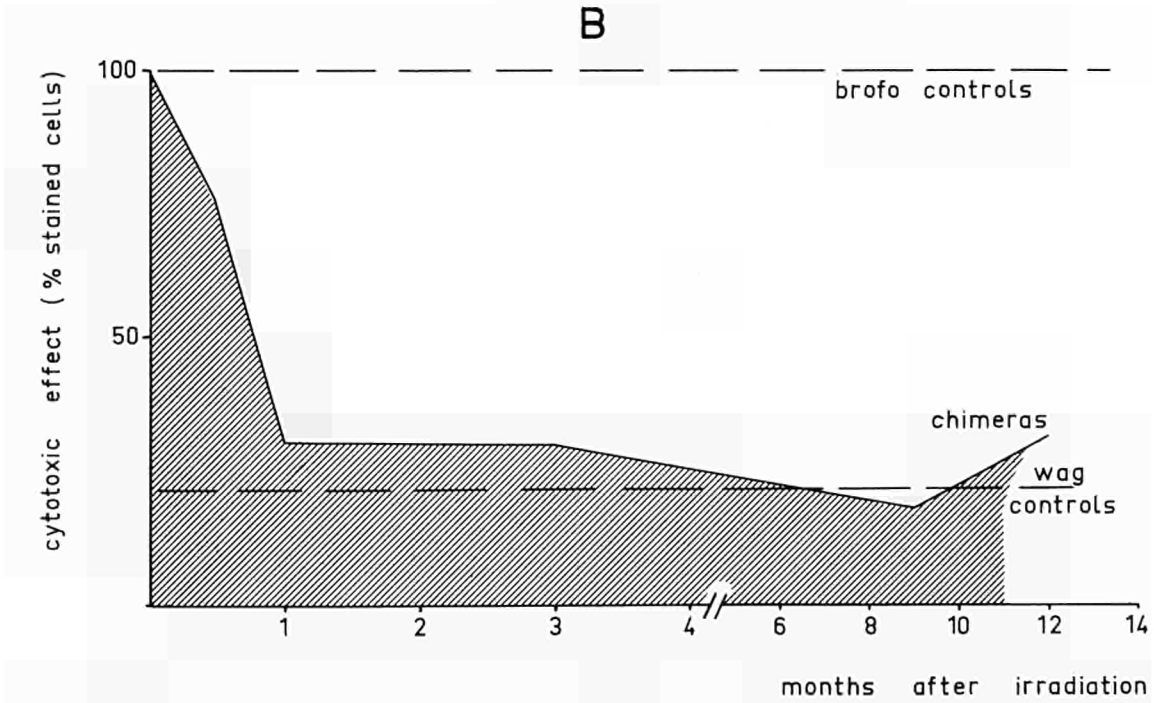
The lymphatic areas of spleen and lymph nodes were initially atrophic as in irradiated controls. Thereafter, slight regeneration was seen in a number of rats, although the lymphatic follicles remained severely depleted of lymphoid cells. Accumulation of erythrocytes in the lymphatic sinuses and extensive erythro-phagocytosis by macrophages which was seen until several weeks after irradiation, was possibly evidence of a bleeding tendency during the 2nd week. Megakaryocytic proliferation occurred but was slightly delayed as compared to other cell lines.

Early lesions of the intestines were on the whole similar to those found in controls though in 3 cases degenerating crypts were slightly more frequent on the 8th day after irradiation than in controls.

2. EVIDENCE FOR THE PRESENCE OF A FUNCTIONING DONOR-TYPE HEMOPOIETIC SYSTEM

Chimerism, or the "take" of a bone marrow graft, was assayed by serological typing of erythrocytes and of nucleated cells at various intervals after irradiation. Text figure 6A demonstrates





Text figure 6A: Determination of the chimeric state. BROFO males were given 200 x 10^6 WAG bone marrow cells 24 or 48 hours after a single dose of total-body irradiation (950-1000 r). The erythrocytes were serologically typed at various intervals, the degree of hemagglutination estimated semi-quantitatively as from 0 to 3+. Each point represents an average of values obtained from 4 to 13 animals. Donor-type skin was grafted at various intervals after irradiation (⬟) and was always kept indefinitely with perfect hair growth. A second skin graft of the same genetic type as the first was also invariably accepted (⌓).

Text figure 6B: Determination of the chimeric state. The cytotoxic effect of iso-antiserum (WAG-anti-BROFO) on peritoneal macrophages and lymphocytes of WAG→BROFO chimeras (900-950r, 100-200 x 10^6 bone marrow cells) expressed as the percentage of dead cells (stained with Trypan blue). Each value represents the average for 4-6 animals.

that following 950-1000 r and a graft of 200 x 10^6 homologous bone marrow cells, surviving animals had a mixed population of erythrocytes during the first 2-3 months. The data suggest that stable chimeras were subsequently obtained. A minor degree of agglutination with anti-host serum, seen in a few animals many months after irradiation, may have been caused by the continued presence of a small percentage of host-type erythrocytes but was more likely due to the inherent inaccuracy of the method.

Serological typing of nucleated cells of similar rat chimeras showed a faster replacement of host-type cells by donor-type cells. Text figure 6B demonstrates that a preponderance of donor-type macrophages and lymphocytes was present in peritoneal washings of chimeras one month after irradiation. Thereafter, virtually exclusively donor-type nucleated cells were demonstrable in stable chimeras in which erythropoiesis had also been shown to remain of the donor-type. Lymph node cells from some of these animals were also typed; this showed an identically low cytotoxicity of the anti-host serum suggesting a vast majority of donor-type lymphocytes, also in the lymph nodes.

Mixed populations of donor- and host-type cells were not seen during the later stages if the radiation dose had been 950 or 1000 r. After 900 r however, mixed populations of donor- and host-type erythrocytes were sometimes demonstrable 6 or more months after irradiation especially if low bone marrow doses had been given (table II). The nucleated cells of these animals were usually also of both donor and host origin. Sometimes total reversing to host-type hemopoiesis was seen. Such reversions occurred more frequently if the irradiation dose had been in the "median lethal dose" range (775-875 r, see page 36 and table III).

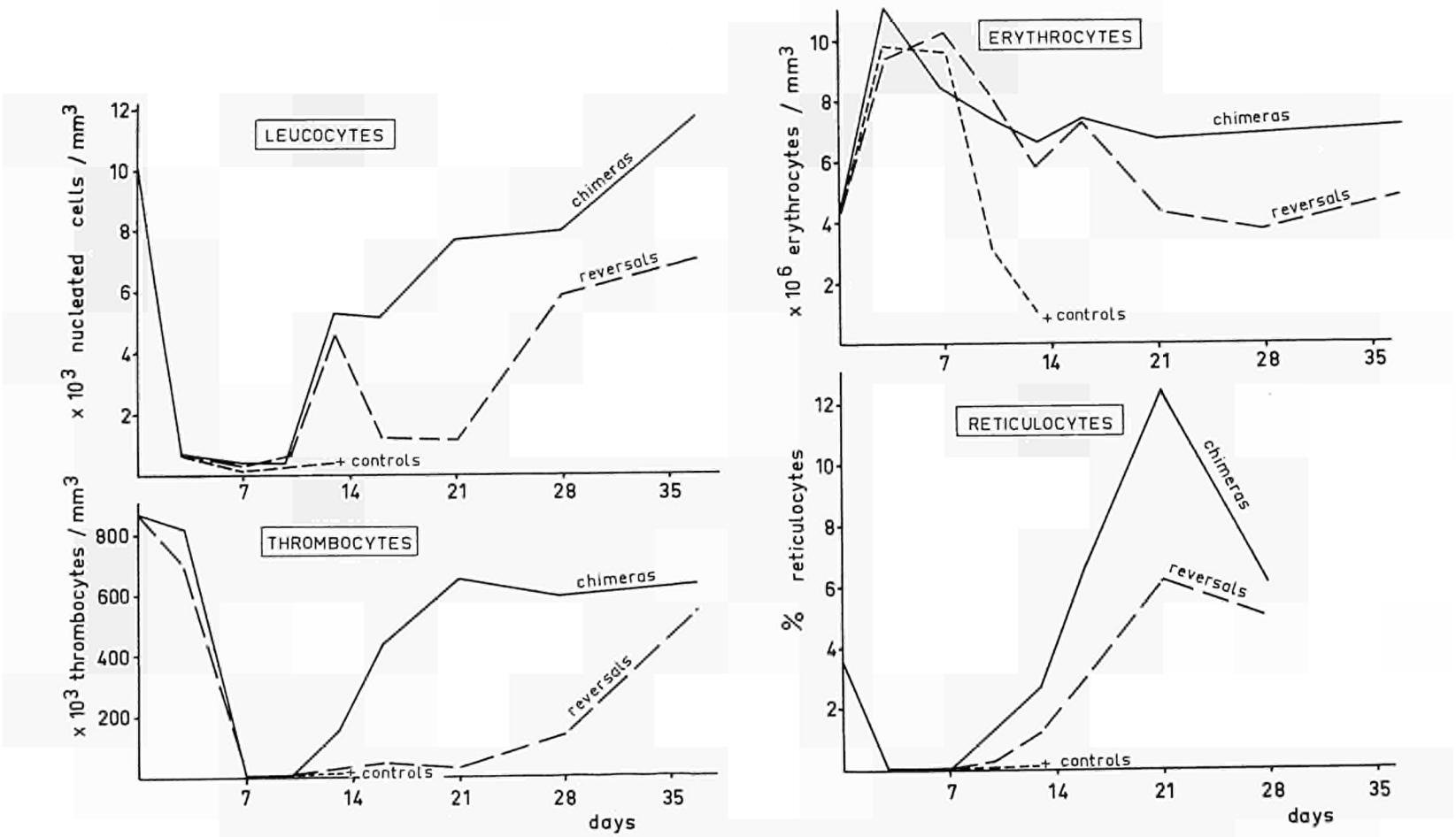
Implications of the prolonged presence of both types of hemopoietic systems in some of the chimeras, as well as some aspects of the immunological behaviour of total reversals, will be elaborately dealt with in chapter VI.

TABLE II

ORIGIN OF ERYTHROCYTES AFTER VARIOUS RADIATION DOSES AND TREATMENT WITH DIFFERENT NUMBERS OF HOMOLOGOUS BONE MARROW CELLS

X-ray dose	number of b.m. cells injected	hemagglutination performed at									number of total reversals (out of number of animals in group)
		1-2 months			5-6 months			8-12 months			
		donor	mixed	host	donor	mixed	host	donor	mixed	host	
950-1000	200-300	(20) 20*	60	20	(34) 88	12	-	(17) 100	-	-	0/58
900	200	(17) 12	88	-				(20) 80	20	-	0/32
	25-100				(34) 44	32	24				8/34
775-875	100	(21) 57	43	-	(19) 53	26	21	(24) 63	-	37	9/24

* percentage of animals showing indicated type(s) of erythrocytes out of total number of animals tested at each interval (this number is given in parenthesis for each group).



Text figure 7: Hematology of BROFO males after 900 r and i.v. administration of 25 x 10^6 WAG bone marrow cells. Chimerism was determined by serological typing of red cells 5 weeks after irradiation. Those indicated as "chimeras" in the figure were animals with predominantly donor-type red cells, the "reversals" had host-type erythrocytes only. Each point represents an average for 3-5 animals.

3. DELAYED REJECTION OF THE BONE MARROW GRAFT AND THE "MLD EFFECT"

In some experiments (text figure 2, table I) a number of bone marrow treated animals died during the 3rd and 4th week with clinical evidence of anemia. Autopsy of such animals revealed multiple subcutaneous petechiae and often massive hemorrhages in the subcutaneous tissue and many organs. Microscopically, bone marrow aplasia (figure 24) and complete atrophy of the lymphatic tissues were seen. Sometimes extramedullary hemopoiesis was present in the spleen, though evidently this did not provide an adequate volume of hemopoietic tissue to prevent death from hemopoietic failure in most cases. At times however, bone marrow treated animals that developed severe cytopenia during the 3rd and 4th week, did not die. In experiment IIIe for instance (depicted in text figure 7 and table I) only 25 x 10^6 homologous bone marrow cells had been given after a radiation dose of 900 r. An initial take of the graft was proven by the rise of the number of leucocytes on the 13th day. During the 3rd and 4th week, 4 out of these 10 animals developed anemia and leucopenia. When erythrocytes were serologically typed 5 weeks after irradiation, these 4 rats had exclusively host-type erythrocytes while the other 6 had predominantly donor-type cells ("reversals" and "chimeras" respectively, in text figure 7).

Table III demonstrates the results of the transfusion of WAG bone marrow into BROFO rats after single sublethal radiation doses, ranging from 775 to 875 r. Serological typing of erythrocytes was done at about 3 months intervals. If 100 x 10^6 WAG marrow cells were given after 775 r (approximately an LD $^{15}/_{30}$) a mixed population of red blood cells was found at 2 months, a preponderance of host-type cells after 6 and 9 months. Serological typing of peritoneal cells in animals surviving beyond 9 months after irradiation, confirmed the virtual absence of donor-type nucleated cells. Most of these animals were obviously reversals to host-type hemopoiesis. The group receiving 825 r (approximately an LD $^{40}/_{30}$) did show a preponderance of donor-type cells after 2 months, but subsequently a few of these animals also reverted to host-type hemopoiesis. No reversals were seen in the group receiving 875 r (LD $^{80}/_{30}$).

All groups showed a slight mortality rate but the presence of a homologous bone marrow graft did certainly not have a detrimental effect when functioning temporarily after 775 r and 825 r, while there can be no doubt that a beneficial effect was obtained after 875 r. Minor weight loss and skin lesions reminiscent of secondary disease were seen during the 2nd month in an occasional animal in the 825 and 875 r group, but all of these recovered completely, one as a reversal. The implications of these results will be discussed below.

DISCUSSION

The possible influence of infection on primary mortality.

The presented dose-mortality curves demonstrate that rats submitted to radiation doses of 900-1000 r without bone marrow therapy, died within about 2 weeks after irradiation. A limited number of the animals died on the 5th and 6th day after irradiation but virtually without diarrhea and the histology of the intestines of such animals showed advanced repair rather than the extensive damage that is seen in rats dying of the intestinal syndrome (184). Accordingly, these early deaths were not considered to be "intestinal deaths" but were tentatively

TABLE III

RESULTS OF SEROLOGICAL TYPING OF ERYTHROCYTES AND NUCLEATED CELLS OF BROFO RATS GIVEN 100 x 10^6 WAG BONE MARROW CELLS AFTER SUBLETHAL IRRADIATION

rad. dose	30-day survivors (out of total number)	2 months hemaggl. anti donor	2 months hemaggl. anti host	6 months hemaggl. anti donor	6 months hemaggl. anti host	9 months hemaggl. anti donor	9 months hemaggl. anti host	12 months peritoneal cells toxicity of anti host serum ■
775 r	18/20					-	3+*	100
						-	3+	died
						-	3+	90
						-	3+	100
						3+	-	died
		2+	2+	3+	+	3+	(+)	15
		2+	+	-	3+	-	3+	100
		2+	+	-	3+	-	3+	died
		2+	2+	2+	+	-	3+	80
825 r	17/20	+	2+	(+)	2+	-	3+	100
		2+	2+	-	3+	-	3+	100
		3+		2+	(+)	3+	-	
		3+	(+)	+	2+	3+	-	12
		2+		2+		3+	-	
		3+		3+		3+	-	
		2+		2+		2+	(+)	10
		3+		3+		3+	-	
		2+		+	2+	died		
875 r	15/20	3+		3+	(+)	3+	-	
		3+		3+	-	3+	-	
		3+		3+	-	3+	-	
		3+		3+	(+)	3+	-	
		3+		2+	(+)	3+	-	
		3+		3+	-	3+	-	
		3+		3+	-	3+	-	
		3+		3+	-	died		

* shading indicates predominance of host type cells

■ expressed as percentage dead cells (stained if tested with Trypan blue)

attributed to pre- or post-irradiation infection of the animals. This was suggested by the frequency of positive blood cultures (text figure 2) and the occurrence of septic foci in the liver of animals in those experiments where many individuals died during this early period (5th-8th day). However, the majority of the untreated rats died during the second week after irradiation of a typical bone marrow syndrome as evidenced by histological and hematological data.

Conard (53) mentioned the overlapping of the hemopoietic and intestinal syndrome in rats, i.e. the occurrence of typical intestinal death after radiation doses below an LD $^{100}/_{30}$. Although no such overlap was seen here, it was found that the margin between hemopoietic and intestinal death in the rats used, was indeed small. Following radiation doses of 1100-1200 r, not much above the doses used in the present experiments (900-1000 r), the majority of the animals died before the 7th day with symptoms and histological findings suggestive of the intestinal syndrome.

Unlike in mice, where the majority of lethally irradiated (LD $^{99}/_{30}$) individuals develop pancytopenia but die with septicemia before fatal hemorrhages occur (212), a fair number of our non-treated irradiated rats died during the 2nd week with no other findings than pancytopenia and massive hemorrhages. In experiments in which many of the non-treated animals lived long enough to develop such fatal hemorrhagic diathesis, blood samples taken from these highly anemic individuals were often sterile.

It seemed that the outcome of individual experiments, as far as survival rates of bone marrow treated rats were concerned, also depended to some extent on the presence of certain micro-organisms. Pseudomonas aeruginosa was frequently cultured from the heart blood of treated rats dying during the first 2 weeks, especially in experiments with few 30-day survivors*. Whether the presence of this micro-organism was indeed the reason for reduced survival times and survival rates of both treated animals and controls remains to be proven. Chances that an infection might have been introduced with the bone marrow suspension are negligible since the organisms cultured from treated animals and from their controls were usually identical (text figure 2).

The number of injected bone marrow cells necessary for survival

In view of the variable incidence of early mortality that was tentatively attributed to infectious complications, a precise determination of the minimal number of homologous bone marrow cells necessary for long-term survival of an irradiated host was difficult. It seemed that 50-100 x 10^6 bone marrow cells could be regarded as the minimal dose necessary for a significant degree of protection of adult rats after 900-1000 r, while 200 x 10^6 cells seemed to be the lowest dose for optimal protection (table I). These numbers are more or less in accordance with the estimated number of homologous bone marrow cells necessary for survival after lethal irradiation, extrapolated by van Bekkum from data of bone marrow therapy in a number of smaller and larger mammals (17).

As in all species in which this has been investigated, the number of homologous bone marrow

---

* Death related to the bone marrow syndrome does not occur beyond the 4th week; consequently, deaths occurring longer than 30 days after irradiation are considered "secondary"; see also footnote on page 13.

cells necessary to keep irradiated rats alive was found to be much larger than that of isologous cells (for precise data see chapter V). The most obvious explanation for this difference has been the assumption that the administered radiation dose did not completely depress the immunological reactivity of the host and that the remaining activity would suffice for the rejection of a small graft of homologous cells. This theory is supported by recent experiments by Wooles and Di Luzio (219) in mice; activation of reticulo-endothelial activity, which has been shown to enhance homograft reactivity and to maintain at least part of its enhanced activities after lethal irradiation (152, 7, 25, 5), caused rejection of an otherwise protecting graft of homologous or heterologous bone marrow, but did not influence the take of isologous marrow. Vos (205) however was unable to confirm these results, nor could he demonstrate that activation of the reticulo-endothelial system increased the number of homologous bone marrow cells necessary for survival of lethally irradiated mice.
Courtenay (58) recently demonstrated that if a supralethal radiation dose was administered at a low dose rate (approximately 1 r/min) 50-160 x 10^6 isologous bone marrow cells were able to save the rats while the same number of homologous cells was of no avail; insufficient suppression of immune reactivity by the protracted irradiation was believed to be the reason.
Naturally, the degree of genetic disparity between donor and host plays an important role. It is of interest, however, that takes of rat bone marrow (WAG) are easily obtained in mice, though it has not been possible so far to obtain a functioning graft of the same mouse strain bone marrow in irradiated WAG rats (17). At present, it is not known whether this difference is attributable to immunogenetic factors only. It could be reasoned, for instance, that the biochemical milieu might adversely affect the proliferating potential of a foreign bone marrow graft as compared with an isologous one. (15). This assumption, however, was not supported by results of experiments on non-irradiated mice. Initial survival and proliferation of transfused H^3-labeled, isologous or homologous bone marrow cells seemed independent of such a hypothetical milieu factor (6).

Temporary takes of homologous bone marrow grafts

Somewhat similar arguments as those put forward in the previous section may be used to explain the disappearance or "delayed rejection" of an established graft of homologous bone marrow. In the present experiments this was occasionally seen after radiation doses of 900 r or less, and more frequently if the graft had been a small one (text figure 2, table II). At radiation doses above 900 r and with grafts of at least 200 x 10^6 homologous bone marrow cells, rejection of an established graft did not occur. Some animals died after rejection of an apparently temporary graft, as many did in experiment IIIc after 900 r and grafts of 10-50 x 10^6 cells (text figure 2), others survived with their own slowly regenerating hemopoietic tissues (text figure 7).
A clear line of distinction between these last animals and those reversing to host-type hemopoiesis at a somewhat later stage is difficult to draw. Generally, one speaks of "delayed rejection" if a just established bone marrow graft ceases to function and the irradiated animal dies with a depleted marrow and pancytopenia. If however an established graft disappears at a later stage and the irradiated animal survives with its recuperated host-type hemopoiesis, the animal is called a reversal (see also p. 36). Reversing was thought to be consequence of the

re-establishment of host-type immune reactivity, which was held responsible for an actual rejection of the established donor-type bone marrow graft. More recently however, evidence obtained by several investigators suggested that replacement of donor-type by host-type hemopoiesis in reversals was probably not a matter of immunological rejection. Barnes et al (15) postulated that chimeras sometimes possessed two co-existing, mutually tolerant, hemopoietic systems; eventually, the one better adapted to the environment would outnumber and finally totally replace the other. They were able to demonstrate that reversing to host-type hemopoiesis was most likely not a matter of immunological rejection but rather one of a proliferative advantage of one hemopoietic system over the other. A more detailed analysis of these interesting phenomena will be presented in chapter VI.

The MLD-effect

The results suggested that administration of homologous bone marrow after sublethal radiation doses had, if anything, a beneficial effect on rats at all dose levels of irradiation investigated. In other words, this particular rat strain combination apparently did not display a so-called "median-lethal-dose phenomenon" (lack of beneficial effect of a homologous graft) nor a "median-lethal-dose effect" (MLD-effect), a detrimental influence of a homologous bone marrow graft after sublethal doses of irradiation.
The MLD-effect had been demonstrated in a few mouse strain combinations (189, 47, 194, 195). An explanation for the occurrance of this phenomenon seemed difficult. While early lethality or delayed complications following homologous bone marrow therapy of lethally and supra-lethally irradiated animals were readily attributed by most investigators to either inadequate takes of the grafts or to graft-versus-host reactions (see chapter I) no such relatively simple formulas could be found to explain the MLD-effect. Trentin (190) had observed that this effect only took place if the recipients could potentially reject a donor graft. This took care of the lack of bone marrow protection in such cases (the MLD phenomenon) but hardly explained the deleterious effect, the increased mortality observed in the MLD-effect. A toxic effect of the desintegrating donor-type cells or an unexplained interference by the graft with recovery of the host's hemopoietic tissue had to be postulated. Vos et al (202) implicated a host-versus-graft reaction and also stressed the importance of the antigenic difference between donor and host.
In view of the vast clinical importance of this subject (see also chapter I) and the not quite satisfactory explanations exposed above, explorations of the MLD-effect have continued. Several investigators obtained preliminary results indicating the absence of an MLD-effect in sublethally irradiated monkeys treated with homologous bone marrow (2, 165). There is even limited clinical evidence that homologous bone marrow therapy in sublethally irradiated humans may be useful rather than damaging; a "tiding-over" effect seemed to have been obtained by homologous bone marrow therapy in the victims of the Vinca accident (130).
In an effort to disclose the immunogenetic conditions for the occurrance of the MLD-effect, Uphoff recently undertook an elaborate study of homologous bone marrow therapy in sublethally irradiated mice, utilizing a multitude of host-donor combinations (195). Though a number of problems remained unsolved and a simple explanation for the phenomenon could still not be provided, it became clear that the MLD-effect occurred only in a very limited

number of mouse strain combinations and that it depended mainly on the genetic make-up of the host. The extreme cautiousness with regard to clinical application of homologous bone marrow therapy after sublethal total-body irradiation may therefore have been unwarranted.
Odell and Caldwell (151) had found an MLD-effect in a rat stain combination of rather low genetic disparity; however these experiments were done on a rather small scale and have not been confirmed since. The results of the present experiments certainly showed no such effect. A temporarily functioning graft probably tided the animals over the critical period of leuco- and thrombopenia and a beneficial rather than a detrimental effect was seen in this rat strain combination of proven high genetic disparity.

CHAPTER IV

COMPLICATIONS AFTER THE ADMINISTRATION OF HOMOLOGOUS CELLS IN THE WAG→BROFO COMBINATION

INTRODUCTION

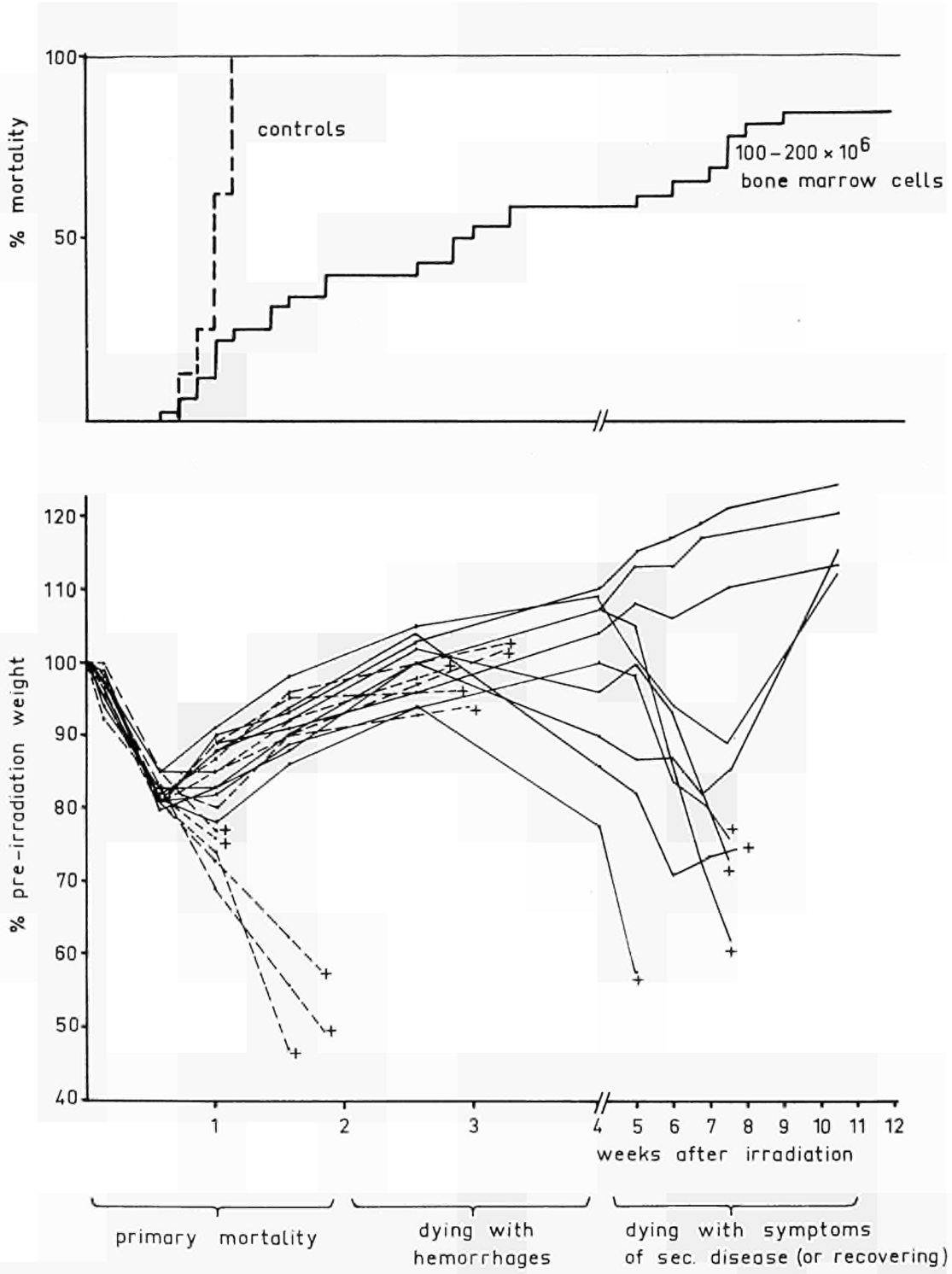
It is customary to speak of secondary disease if certain delayed complications occur in irradiated animals treated with foreign bone marrow. As has been pointed out in chapter I, these complications are now attributed to a number of factors, the foremost being the graft-versus-host reaction, which is almost certainly caused by the lymphoid elements contained in the injected bone marrow graft. The delay in the onset of secondary symptoms must probably be attributed to the time interval needed by the injected hemopoietic cells to produce sufficient numbers of mature, immunologically competent lymphocytes (see also p. 65). Consequently, if large numbers of mature lymphocytes are injected immediately after irradiation, with or without a concomitant bone marrow suspension, the graft-versus-host reaction occurs much sooner and usually takes a more fulminant course. Though it is essentially an accelerated type of secondary disease, it is often called the "killing effect" of foreign lymphoid cells and is regarded as a more or less separate entity. Here too, the results of chapter IV have been divided into two sections, one dealing with the complications after homologous bone marrow therapy only (A) the other with the "killing effect" of a mixture of mature lymphoid cells and hemopoietic cells (B). The lymphoid cells for this last procedure were obtained from the spleens of the donor animals.

RESULTS

A. **Secondary disease after homologous bone marrow therapy**

SYMPTOMS

Bone marrow treated animals surviving the critical period of the first 14 days gained weight and stayed well untill symptoms of secondary disease started to develop (the few animals dying during the 3rd and 4th week due to a "delayed rejection" of the graft were described in the preceding pages). The first symptom was invariably weight loss starting around the 4th week (text figure 8). Concomitantly, skin lesions developed in several areas: the face, mostly around the eyes (figure 1), the ventral parts of the jaw, neck and thorax and, most consistently, the dorsal aspect of the forelegs (figure 2-4) and the hind legs. Starting with erythema and slight depilation, especially of the dorsal skin of the paws, these lesions sometimes evolved within 1-2 weeks into moist areas completely devoid of hair with scattered excoriations and



Text figure 8: Weight curves of individual animals of experiment IIIc. BROFO males receiving 100-200 x 10^6 WAG bone marrow cells after 900 r. Each point represents the percentage of the individual's pre-irradiation body-weight. Rats dying during the 3rd and 4th week showed histologic evidence of bone marrow aplasia and had a body-weight comparable to that of long-term survivors.

Figure 1-3
Typical skin lesions of the face and the limbs occurring in BROFO rats 4-6 weeks following irradiation and homologous bone marrow treatment (900 r, 100-200 x 10^6 WAG cells).

Figure 4
Appearance of a homologously treated irradiated BROFO rat that died during the 5th week after 900 r total-body irradiation. Note emaciation, hunched back, skin lesions and inflammation of the eye lids.


1


2

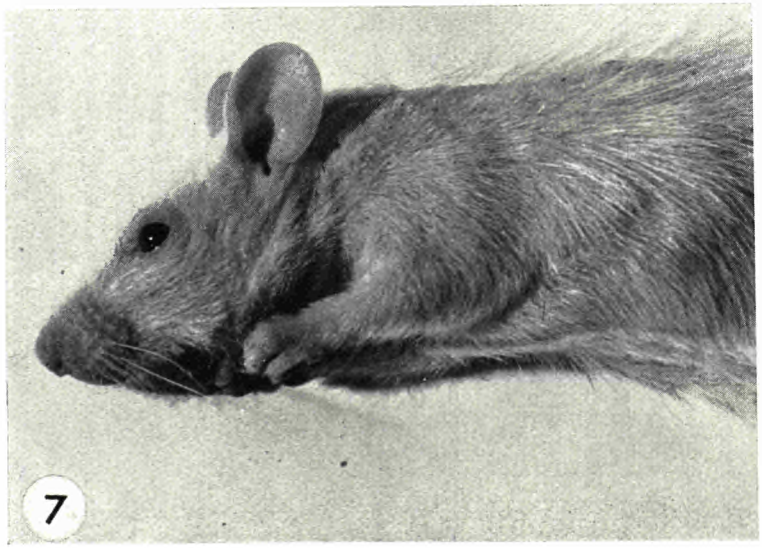

3


4

Figure 5-8
Extensive and severe skin lesions occurring in a homologously treated irradiated BROFO rat (900 r, 200 x 10^6 WAG bone marrow cells). Lesions were most outspoken during the 5th week post-irradiation as in figure 5 and 6; advanced recovery 2 weeks later is shown in figure 7 and 8.

5

6



7

8

ulcerations. The lesions were often limited to the face (typical mask-like appearance, figure 1) and the paws, but at times the ventral part of the body was also involved (figure 5, 6), occasionally even the skin of the entire body. The animals frequently became increasingly emaciated and developed the characteristic hunched back and typical gait previously described for mice with secondary disease and for runted rats (33) (figure 4). At no time was there diarrhea, nor obvious anemia though the latter may have been somewhat masked by hemo-concentration.

MORTALITY

Morbidity due to secondary disease was different in various experiments and mortality never exceeded 20 % of the total number of animals in any one experiment. We have not been able to detect a statistically significant correlation between incidence and severity of secondary disease and variables such as radiation dose and number of injected cells, though one got the impression that secondary disease occurred somewhat more frequently if large doses of WAG bone marrow had been given (table I).

In general there was a correlation between the severity of the symptoms and mortality: animals displaying extreme weight loss and wide-spread typical skin lesions usually succumbed to the disease. Only a small number of those with severe symptoms survived and recovered completely within 1 or 2 months (examples in text figure 8 and figures 5-8).

From a limited number of hematologic follow-ups and from the macroscopic appearance of the animals it was concluded that anemia was not part of the syndrome.

AUTOPSY

Autopsy of animals dying with the described symptoms during the 2nd and 3rd month hardly ever revealed a macroscopically evident cause of death. Emaciation was usually advanced. Skin lesions were sometimes severe and wide-spread and at times it was possible to wipe off the epidermal layer of the dorsal skin of the paws by gentle stroking. In other instances there were only mild, localized skin lesions. No petechiae or massive localized hemorrhages were observed and there was never clinical evidence of anemia as has been mentioned before. The size of the spleen and lymph nodes was reduced as compared with normal. The macroscopic appearance of the instestine, the liver and other organs was usually normal.

A small number of animals died during the period from the 2nd to the 6th month, also emaciated, but without skin lesions. In these instances a direct cause of death was found on several occasions, namely disseminated abscesses, mostly in the lungs. Deaths occurring more than 6months after irradiation were often due to tumours; these and other long-term observations will be described in chapter VII.

HISTOLOGY

The microscopic lesions found in marrow-treated animals dying spontaneously or killed with typical symptoms of the disease during the 2nd and 3rd month, are listed in table IV. The bone marrow was usually of normal or slightly subnormal cellularity. In one case advanced fibrosis of the marrow was found, though 100×10^6 cells had been given. This animal belonged to a group with a particularly high incidence of "delayed rejections" (text figure 2, experiment IIIc). In general, severe atrophy of the lymphatic tissues was present, sometimes associated with fibrosis (figure 9). Only in 2 cases was there rather extensive repopulation bij lymphoblasts and lymphocytes (figure 10). However, these 2 animals had been killed for comparison with

those dying spontaneously at a time when symptoms of the disease were still outspoken but obviously diminishing. Judging from their clinical appearance these animals might have survived.

In one case a characteristic type of liver necrosis was found. Only the liver cells were affected, while the supporting stroma remained intact (figure 13). A number of animals showed proliferation of histiocytic cells in the liver, occasionally increasing the space between the liver cell cords. Infiltration by lymphocytes and granulocytes was frequently observed periportally as well as within the liver lobules, and isolated necrotic liver cells were also seen in these cases. However, such lesions were most outspoken when lymphoid cells as well as hemopoietic cells had been injected (figure 14-16).

No severe intestinal changes were found in this group. Mild degeneration of a few isolated crypts was seen in 3 cases of secondary disease following homologous bone marrow therapy (table IV).

The affected areas of the skin showed a number of typical histologic features listed in table IV as "chronic dermatitis". An important feature of this dermatitis was the atrophy of hair follicles and sebaceous glands, starting with loss of follicular lumen, leaving cords of epithelial cells which progressively became thinner and eventually disappeared (figure 17-21). In the epidermis acanthosis was evident, with swelling and paleness of nuclei of the cells in the stratum spinosum. Dyskeratosis, parakeratosis and sometimes vacuolar degeneration of cells of the Malpighian layer and of basal cells were observed. Proliferation of fibroblasts and a mixed infiltrate of lymphoid cells and histiocytes surrounding the atrophic hair follicles was seen in the deeper layers of the corium and in the subcutis, but also between epithelial cells of the epidermis and the hair follicles. Fibrosis of the corium was common. Desquamation of large necrotic areas of epidermis with ulceration of the corium (figure 18), was not frequently seen in animals treated with bone marrow only, but was rather common in animals given spleen cells as well (table IV, figure 21).

Another typical lesion, also more frequently seen if lymphoid cells had been added, was the proliferation of reticular or histiocytic cells in the adipose tissue around lymph nodes and of the intestinal subserosa and mesentery. In some cases this proliferation extended into the muscular coat and the mucosa of the intestine, while it was occasionally found in the mesentery surrounding the pancreas. The fatty tissue seemed replaced by a dense infiltrate of mononuclear cells, presumably histiocytes and lymphocytes, some of which showed foamy vacuolation of the cytoplasm (figure 22). Plasma cells and eosinophils were also frequently evident in these infiltrates. Foreign body giant cells and macrophages filled with lipid substance were occasionally seen. Although necrotic fat cells were possibly present, we do not know whether fat cell necrosis is actually the cause of this peculiar reaction.

B. **Complications occurring after the administration of homologous lymphoid cells**

SYMPTOMS MORTALITY AND AUTOPSY FINDINGS

In a limited number of animals homologous spleen cells were given in addition to the bone marrow graft in the WAG→BROFO combination (10 animals, 900 r, 100 x 10^6 bone marrow + 150 x 10^6 spleen cells). This resulted in a higher incidence of secondary complications and higher delayed mortality (text figure 9) than when only homologous bone marrow had been

TABLE IV

HISTOLOGY OF SECONDARY DISEASE IN IRRADIATED BROFO RATS (900 r) INJECTED WITH HOMOLOGOUS CELLS

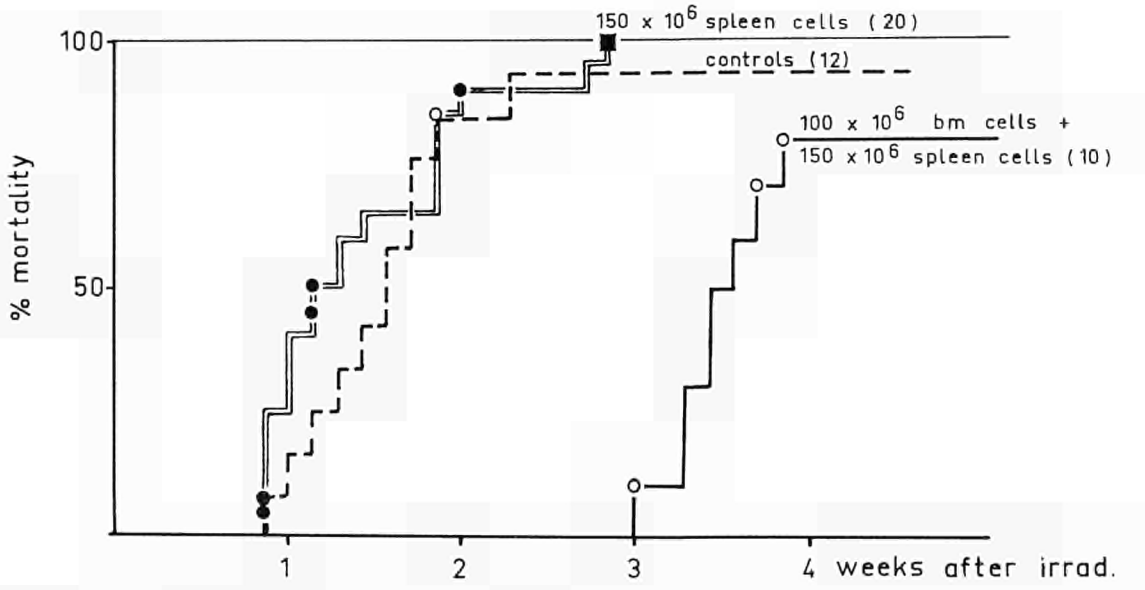
		WAG bone marrow 100-200 x 10^6											WAG marrow 100 x 10^6 + WAG spleen cells 150 x 10^6				WAG spleen cells 150 x 10^6							BROFO spleen cells 150 x 10^6					
Marrow	atrophy	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
	regeneration	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
Lymph. tissue	atrophy	-	-	+	+	+	+	+	+	±	0	0	±	+	+	+	-	-	±	±	±	+	+	-	-	-	±	-	
	regeneration	+	+	-	-	-	-	-	-	+	0	0	±	-	-	-	+	+	±	±	±	-	-	+	+	+	±	+	
Skin	necrosis	+	-	-	0	0	-	-	-	-	-	-	+	0	+	-	0	0	0	0	+	0	-	+	+	+	+	+	
	chron. dermatitis	+	+	+	0	0	-	+	+	±	+	±	+	0	+	+	0	0	0	0	+	0	+	-	+	+	-	+	
Colon lesions	sporadic	-	+	+	0	0	-	-	+	-	0	0	-	0	-	-	-	-	-	-	-	-	0	-	0	+	-	+	
	extensive	-	-	-	0	0	-	-	-	-	0	0	+	0	-	-	+	+	+	+	+	+	0	-	0	-	+	-	
Liver cell necrosis		-	-	-	-	-	-	+	0	-	0	-	+	0	-	-	-	±	+	±	+	-	-	-	-	-	-	-	
Septic foci		-	-	-	-	-	-	-	0	-	0	+	-	0	-	-	-	-	+	-	-	-	-	-	-	-	-	-	
Histiocytic reaction		-	+	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	
Blood culture		0	0	0	+	+	+	-	0	0	+	+	-	0	-	-	0	0	0	0	0	0	+	+	0	+	+	-	
Autopsy on day	killed	28	35												27					9	13					15	17		
	died spont.			35	36	38	47	49	57	59	61	63	21	24		29	7	7	9			14	19	15	15			17	

indications for positive findings are + or if less outspoken ± and the areas are shaded.

indications for negative findings are - and 0 if examination was omitted.

given. Clinical symptoms, particularly inanition and skin lesions, though somewhat more pronounced, were essentially similar to those described for bone marrow treated animals. However, the symptoms started during the 2nd instead of the 4th week and death occurred before the end of the 1st month after irradiation. Histological data for a few of these animals, dying during the 3rd and 4th week with typical symptoms, are included in table IV. As has already been pointed out, the reticulo-histiocytic reaction was more common in this group. Severe crypt degeneration in the colon was found in one case. Bone marrow regeneration and lymphatic atrophy were similar to that seen in animals treated with homologous bone marrow only.

If only spleen cells had been given, none of the animals survived (20 animals, 900 r, 150 x 10^6 spleen cells, text figure 9). They all died during the 2nd and 3rd week, approximately at the same time as the controls. However, the controls, except for the few dying before the 8th day, did not loose much weight during the first 10 days. They became anemic but continued to look relatively well in the final stages. The spleen-treated animals on the other hand, lost weight and looked miserable during the first week and became extremely emaciated terminally (text figure 10 and figure 27). Localized skin lesions including erythema, depilation, edema and scalyness (figure 28) developed during the 2nd week but never became as generalized as in the bone marrow treated animals.



Text figure 9: Mortality and blood cultures of BROFO rats given 150 x 10^6 WAG spleen cells after 900 r. The solid line represents a separate experiment in which both 100 x 10^6 bone marrow cells and 150 x 10^6 spleen cells had been given simultaneously. The number of animals in each group is indicated in parentheses.
○ = sterile blood culture
◍ = blood culture positive for Pseudomonas aeruginosa
▣ = blood culture positive for other bacteria

HEMATOLOGY

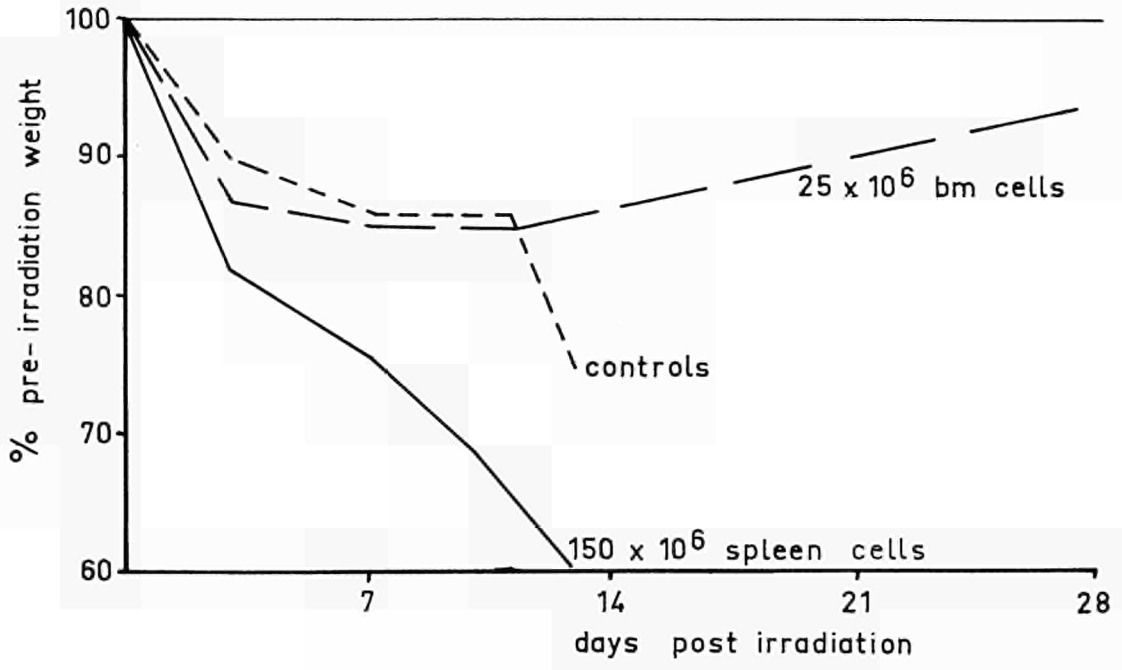
Hematological data for a representative group (150 x 10^6 WAG spleen cells following 900 r) were included in text figure 5. It is noteworthy that none of the investigated animals displayed anemia and that an extreme leucocytosis (about 80 % mononuclears, 20 % polymorphonuclear leucocytes) occurred in the terminal stages.

HISTOLOGY

As can be seen in table IV, BROFO rats dying after treatment with homologous spleen cells, and those given bone marrow cells as well, had many findings in common. Minor differences can probably be attributed to a time factor since the animals treated with spleen cells as well as bone marrow died somewhat later.

The bone marrow was severely hypocellular in animals treated with spleen cells only, dying before the 10th day. Only occasional foci of hemopoietic cells were found in a predominantly fatty marrow. The late marrow regeneration in the spleen-treated animals was reflected in the occurrance of profuse hemorrhages in the wall of the intestinal tract and in the adrenals. In one of the animals, localized hemorrhagic necrosis with bacterial invasion was found in the wall of the colon. Another animal in this group showed foci of septic necrosis in the spleen. Rats that died 13 days and later after injection of spleen cells only, or of spleen cells plus bone marrow, showed normal cellularity of the marrow space.

The spleen and lymph nodes of animals that died between the 7th and 14th day showed a variable degree of lymphopoietic activity in the follicles. In most cases an appreciable number



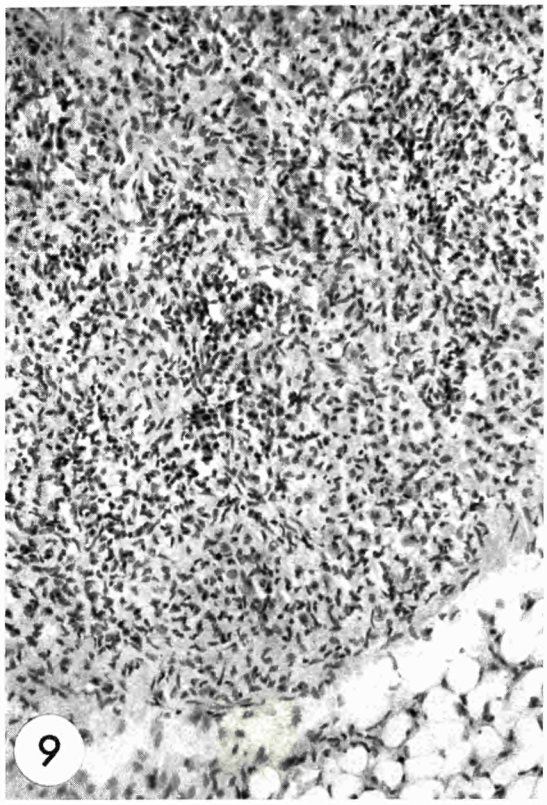
Text figure 10: Response of body-weight in male BROFO rats given WAG bone marrow or WAG spleen cells after a single dose of 900 r. Each point represents the average for 3-5 animals.

Figure 9
Severe athrophy and fibrosis of mesenteric lymph node of BROFO rat dying 6 weeks following irradiation (900 r) and administration of 100×10^6 WAG bone marrow cells. x 120

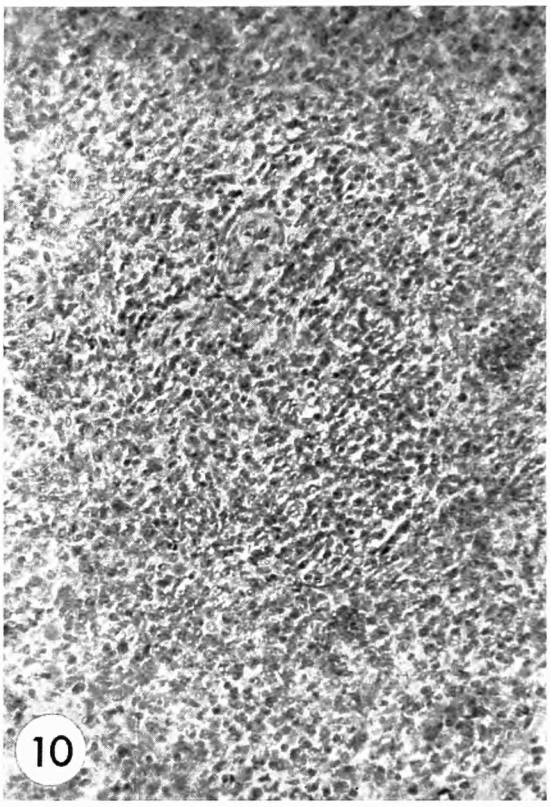
Figure 10
Beginning regeneration of lymphoid follicle in spleen of homologously treated rat (900 r, 50×10^6 bone marrow cells) killed 5 weeks following irradiation while recovering from secondary disease. x 190

Figure 11
Lymphopoietic activity in the cortex of mesenteric lymph node of irradiated BROFO rat dying 9 days after injection of 150×10^6 WAG spleen cells. x 190

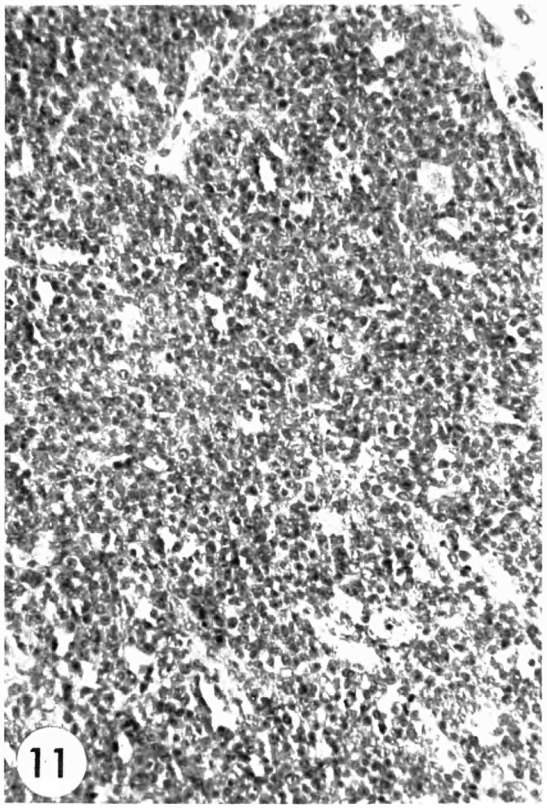
Figure 12
Lymph node of BROFO rat dying 3 weeks following irradiation and treatment with BROFO spleen cells (900 r, 150×10^6 spleen cells). Note pronounced lymphoblastic proliferation (mitoses), presence of plasmocytes and karyorrhexis. x 300



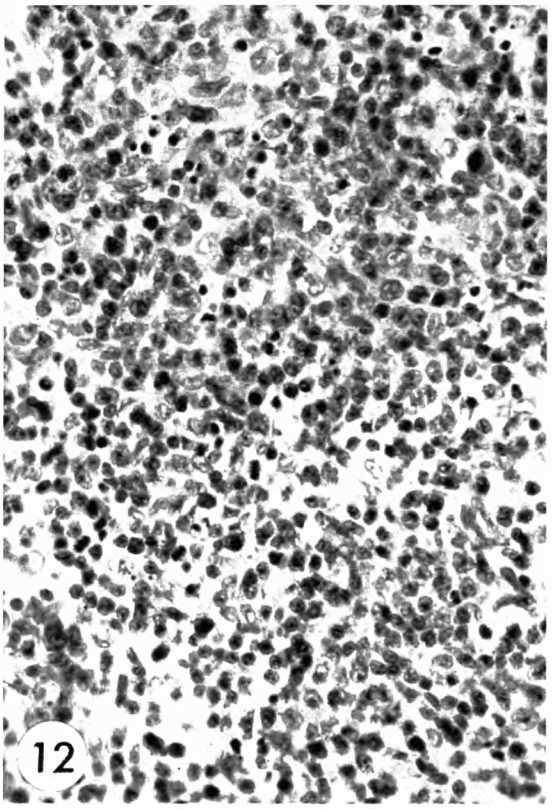
9



10



11



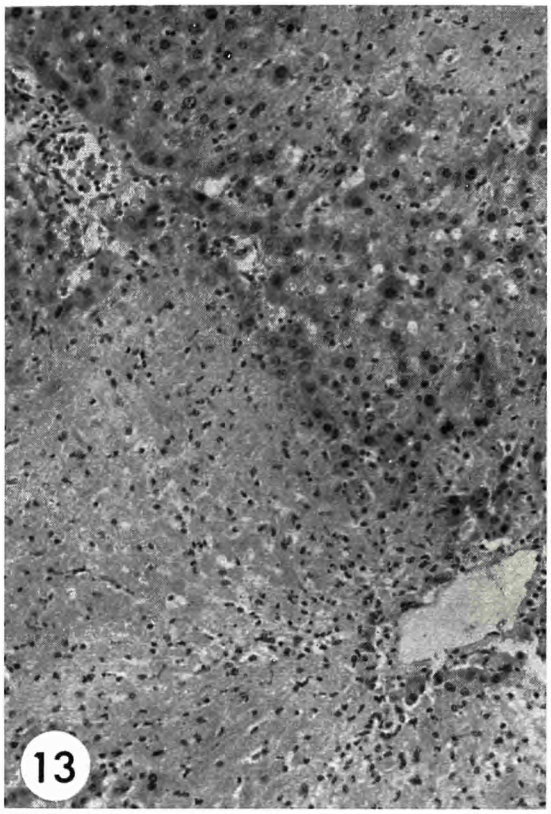
12

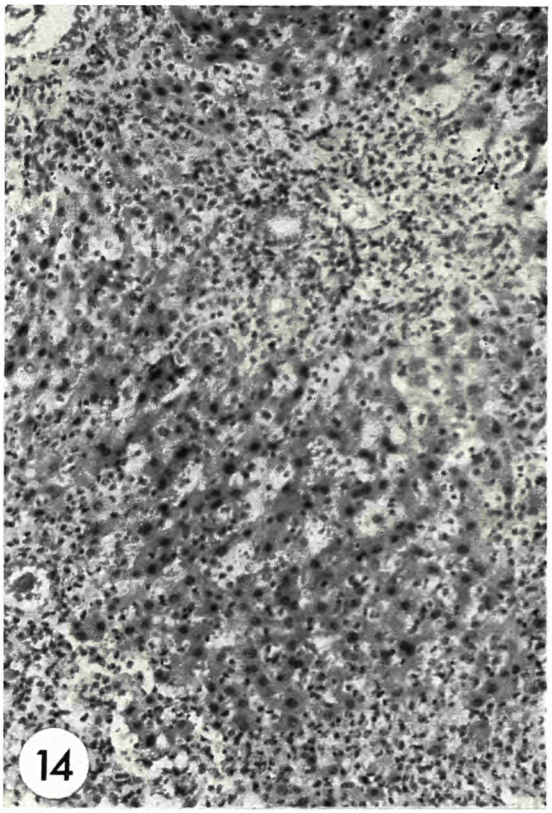
Figure 13
Liver necrosis in BROFO rat dying 6 weeks following irradiation and homologous bone marrow therapy (900 r, 100×10^6 WAG bone marrow cells). Note preservation of normal stroma pattern.

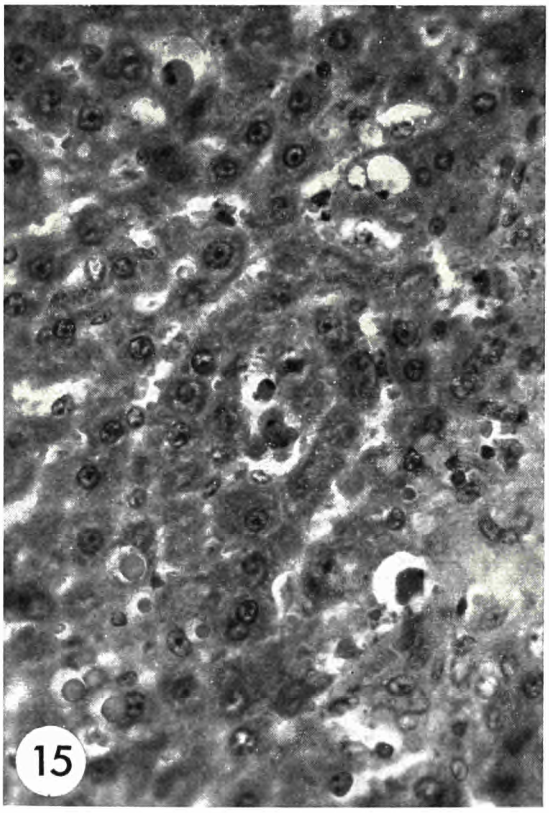
Figure 14
Periportal proliferation of histiocytic cells, extending between liver cell cords; irradiated BROFO rat dying 3 weeks following treatment with homologous bone marrow and spleen cells (900 r, 100×10^6 WAG bone marrow cells + 150×10^6 WAG spleen cells). x 120

Figure 15
Isolated, desintegrated liver cells in animal dying 9 days after irradiation and injection of 150×10^6 homologous spleen cells. x 300

Figure 16
Liver of rat dying 9 days after irradiation and injection of homologous spleen cells (900 r, 150×10^6 cells). Desintegration of an isolated parenchymal cell is seen at left, two liver cells in mitosis near the right border of micro-photograph. x 300


13


14


15

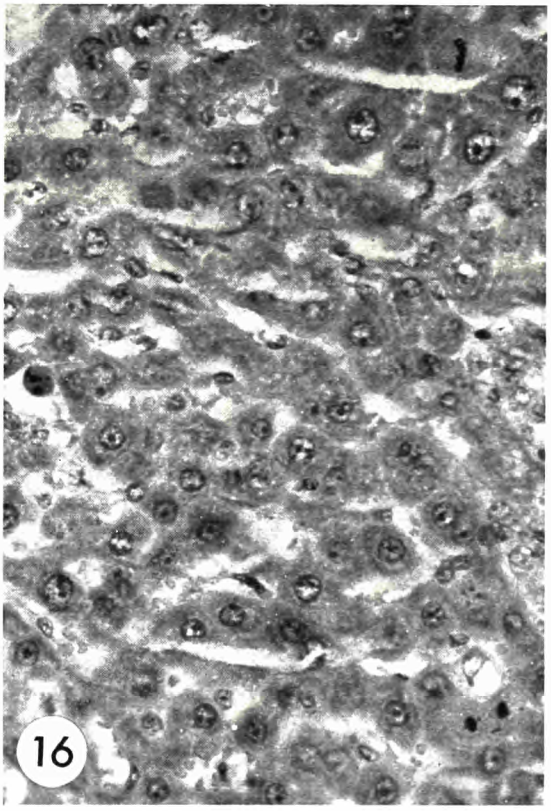

16

Figure 17
Advanced atrophy of hair follicles in the skin of rat dying 6 weeks after irradiation and treatment with homologous bone marrow (900 r, 100×10^6). x 190

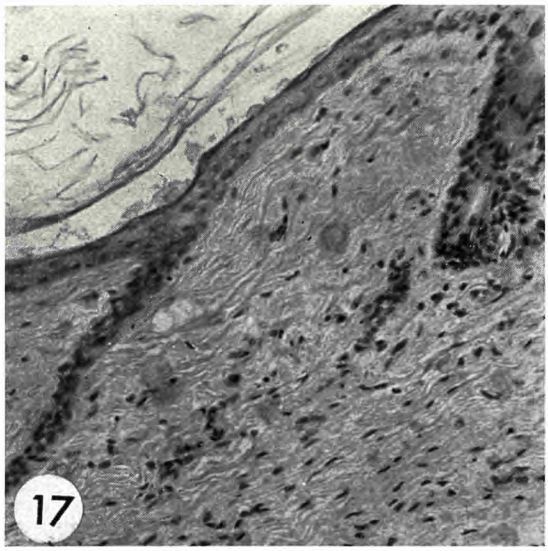
Figure 18
Necrosis and desquamation of the epidermis of another rat treated with homologous bone marrow (50×10^6 cells) after 900 r total-body irradiation. x 120

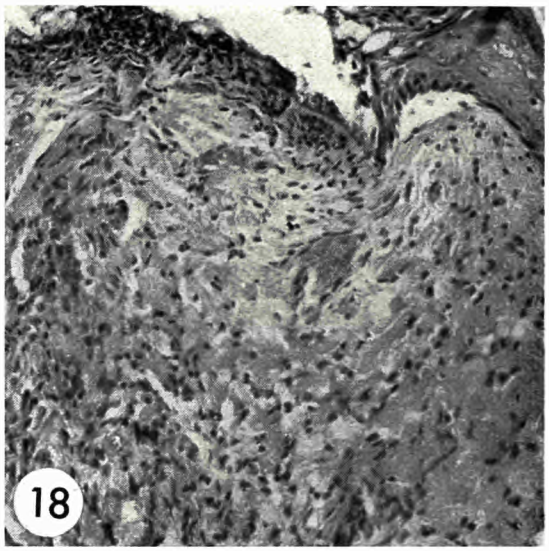
Figure 19
Skin of rat dying 3 weeks following irradiation and treatment with homologous bone marrow and spleen (900 r, 100×10^6 bone marrow cells, 150×10^6 spleen cells). Note acanthosis and follicular keratosis, lymphoid cell infiltration and cellular desintegration in hair follicles. x 190

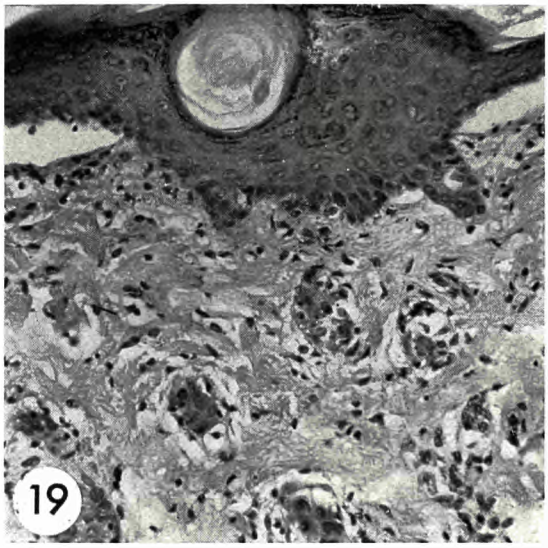
Figure 20
Skin of same rat as in figure 19. Note dyskeratosis in epidermis and mononuclear infiltration in superficial layer of the corium and in the epidermis. x 300

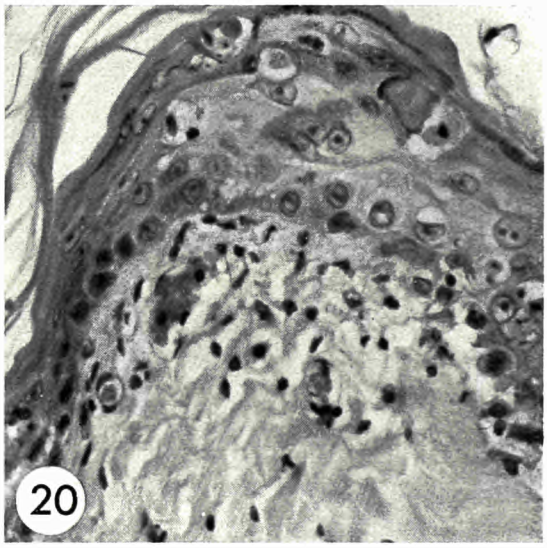
Figure 21
Extensive necrosis of the epidermis in irradiated rat treated with homologous bone marrow and spleen (900 r, 100×10^6 bone marrow cells, 150×10^6 spleen cells).

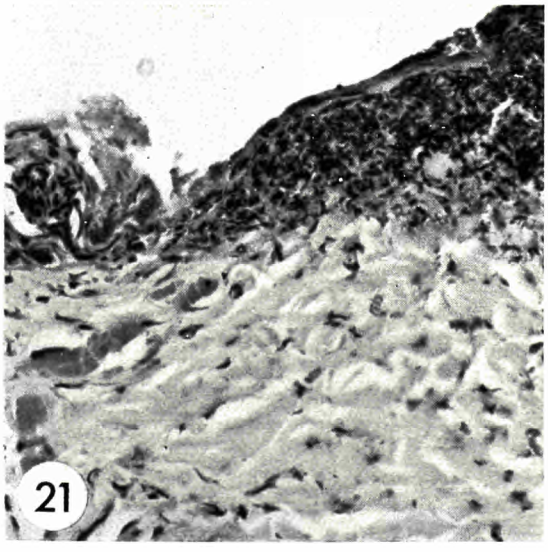
Figure 22
Infiltration by histiocytic and lymphoid cells in adipose tissue surrounding a mesenteric lymph node. Animal was killed 9 days following 900 r whole-body irradiation and administration of 150×10^6 homologous spleen cells. x 190


17


18


19


20


21

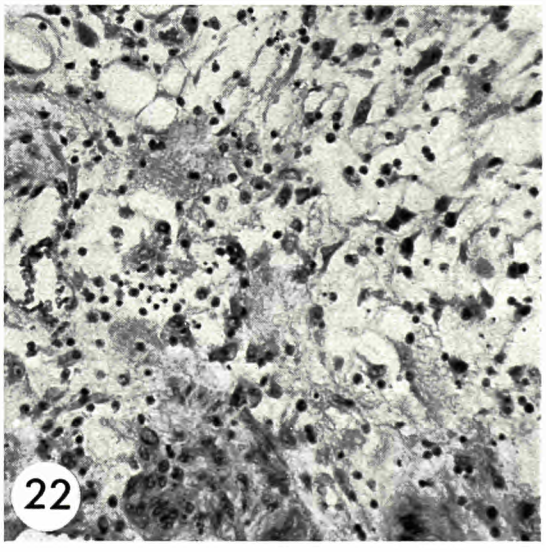
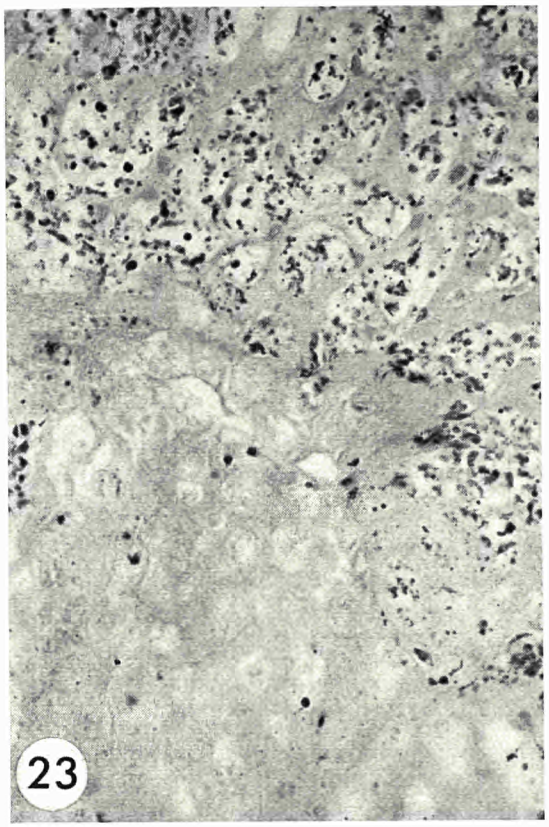

22

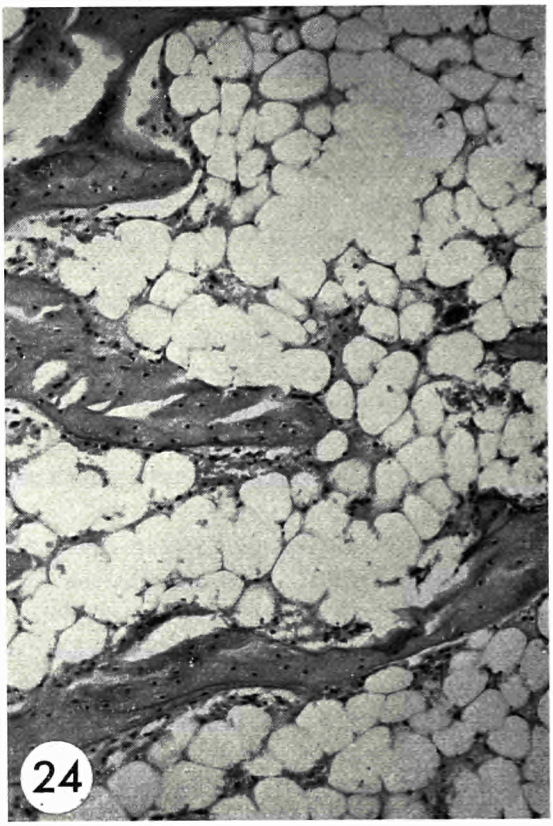
Figure 23
Focal septic necrosis in liver of rat dying 3 weeks after total-body irradiation and treatment with homologous bone marrow cells (900 r, 200 x 10^6 cells). Note complete loss of structural details and compare with figure 13. Clumps of bacteria were present. x 300

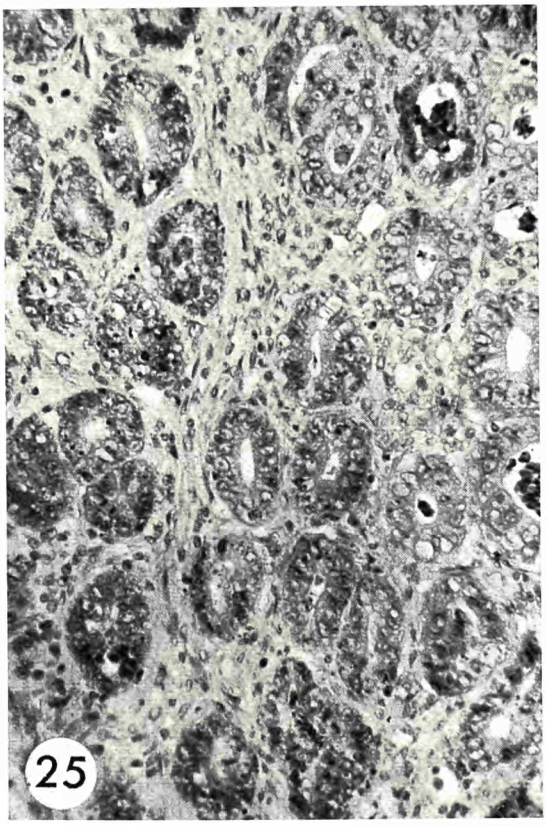
Figure 24
Aplasia of sternal marrow of rat dying 3 weeks after total-body irradiation and treatment with homologous bone marrow cells (900 r, 100 x 10^6 cells). x 120

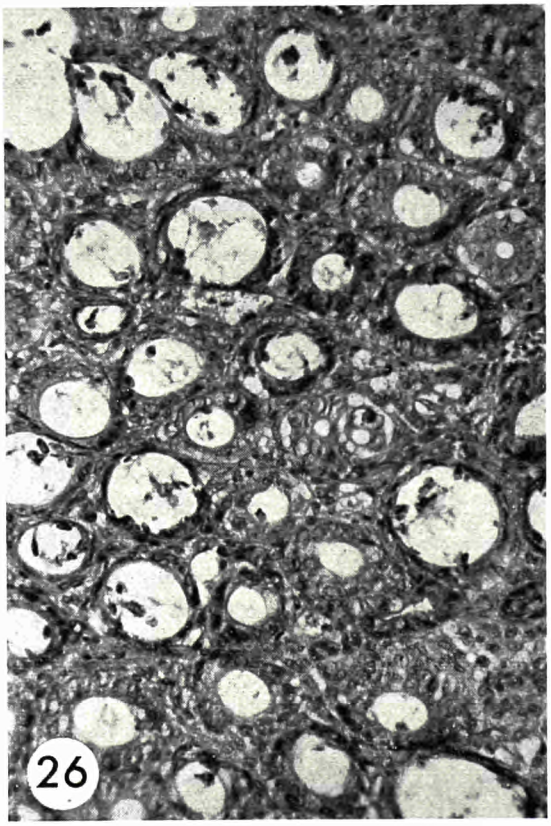
Figure 25
Karyorrhexis and desquamation of crypt cells in the colon of BROFO rat dying 9 days after irradiation (900 r) and injection of 150 x 10^6 WAG spleen cells. x 190

Figure 26
Massive desintegration and desquamation of crypt cells in the colon; many of the crypts have cystic appearance. This rat also died on the 9th day after similar treatment as under figure 25. x 190


23


24


25


26

of lymphoblasts was seen (figure 11). In some, the cortex of the lymph nodes was highly cellular, though desintegrating lymphoid cells were also seen. In animals dying beyond the 2nd week, the lymphatic tissues were atrophic. Interestingly enough, the administration of BROFO instead of WAG spleen cells, appeared to result in more constant and more vigorous proliferation of lymphoid cells in the spleen and nodes of irradiated BROFO rats (table IV, figure 12 and discussion of chapter V).

In all animals that died on day 7 and day 9, wide-spread lesions of the mucosa of the colon were present. These included diffuse karyorrhexis of crypt epithelium (figure 25) and desquamation of degenerated epithelial cells which gave many crypts a cyst-like appearance (figure 26). Denudation, the actual loss of crypts and surface epithelium was not encountered in these animals, and inflammatory infiltrates were not present in the lesions. On day 14 and thereafter, fewer rats had crypt lesions, and if present they were less severe than in the animals dying before the 14th day.

Severe skin lesions were present in the majority of spleen-treated rats dying later than the 9th day. In addition to the dermatosis described earlier, necrosis and desquamation of large areas of the epidermis occurred. The earliest changes probably took place in the hair follicles, their cells showing karyorrhexis and other degenerative changes (figure 19). Lymphoid cells were found to have invaded the follicles and the overlying epidermis. The epidermal lesions started with parakeratotic changes in the superficial cell layers and infiltration by neutrophil granulocytes, swelling of cells, increased cytoplasmic eosinophilia, as well as nuclear pyknosis and desintegration (figure 20). Total necrosis, which often also affected the superficial corium could ultimately be seen (figure 21).

In the liver, focal groups of degenerating parenchymal cells were present in many of the animals. Diffuse loss of liver cells was evidenced by an appreciable increase of liver cell mitoses (figure 16). In addition an increased number of cells of the sinusoidal lining and also foci of histiocytic cells in the portal tracts or in the liver lobules were noted. In one animal excessive proliferation of histiocytic cells had resulted in atrophy of liver cell cords (figure 14). Proliferation of reticular and histiocytic cells in mesenteric fat with infiltration by lymphoid and sometimes eosinophilic cells, as described earlier, was found in a few rats. A possible relation to fat necrosis has been mentioned before.

DISCUSSION

The complications occurring during the 2nd and 3rd month after irradiation and homologous bone marrow therapy in the rat, are certainly reminiscent of the secondary syndromes described for mice and rabbits (162, 208, 16). However, it will be noted that interesting and sometimes unexpected differences exist between the manifestations of secondary disease in the rat and those seen in other species. The most conspicuous difference is undoubtedly the virtual absence of intestinal lesions and of colitis and diarrhea in homologously treated rats, while these are prominent and nearly constant symptoms in mice, rabbits and primates with secondary disease.

Secondary disease in the rat

To be able to implicate a graft-versus-host reaction as an important factor in the pathogenesis

of delayed morbidity and mortality after homologous bone marrow therapy, some or all of the following criteria should be considered:

a) the existence of a chimeric state (demonstration of a functioning graft).

b) the characteristic lesions, especially those occurring at an early stage after irradiation, should be differentiated from those caused by radiation only.

c) similar symptoms and lesions should not occur if genetically compatible bone marrow cells are given instead of foreign cells.

d) the incidence and severity of the disease should be enhanceable by pre-immunization of the donor against the future host antigens and usually also by the addition of lymphoid cells to the bone marrow graft.

These conditions have been fulfilled in the present experiments, with the exception of the pre-immunization of the donor animals against the future hosts. A graft-versus-host reaction can therefore be assumed to be a major factor in the pathogenesis of the described disease in the rat.

Secondary complications occurring in irradiated rats after bone marrow therapy only and those after the concomitant administration of mature lymphoid cells have been treated separately in the "results" of this chapter. Rather than treating these findings separately also in this section, it was decided to discuss the various affected organs or tissues, one by one. Whenever certain lesions were found exclusively or predominantly as a consequence of the accelerated type of graft-versus-host reaction following the administration of homologous spleen cells, this will be specifically stated.

THE HEMOPOIETIC TISSUE

Rats succumbing after homologous bone marrow therapy with typical symptoms of secondary disease almost invariably showed a regenerated, actively proliferating marrow; only in one case was a partly depleted bone marrow noted. Hemorrhagic diathesis with major hemorrhages was very rare and there was never clinical evidence of anemia. Immune hemolysis, a prominent symptom of secondary disease in the rabbit, has been suspected in mice with secondary disease, but never proved (208, 134, 213). Several graft-versus-host reactions have been described in a number of species, in which selective hemolysis of the host's erythrocytes by a homologous cellular graft has been demonstrated (175, 102, 174, 159, 153), though the selectivity was not always confirmed by others (92, 78). The possibility of a certain degree of immune hemolysis in our rat chimeras cannot be ruled out as the elimination of labeled erythrocytes was not investigated. It is rather unlikely however, since frequent serological typing of red cells showed that the disappearance of host-type erythrocytes and their replacement by donor-type red cells usually took 2-3 months, also in most chimeras recovering from secondary disease. This would suggest a normal life span of the host's erythrocytes (163). Coombs' tests, direct and indirect, were done in a limited number of the rat chimeras but were never positive.

The clinical and histological findings in rats dying with pancytopenia after rejection of a temporarily functioning graft are quite different, so that confusion of this type of delayed death with that caused by the secondary syndrome is most unlikely. An acellular bone marrow, extreme anemia, little weight loss and absence of skin lesions were typical for the former kind of death which occurred during the 3rd and 4th week, not later.

Regeneration of the bone marrow was also quite advanced in secondary disease after injection

of spleen cells in combination with bone marrow (table IV). Only in those individuals dying very soon (7 or 9 days) after receiving homologous spleen cells without bone marrow, was the marrow space rather empty. The animals that lived somewhat longer, however, did show reasonable bone marrow regeneration and extensive hemopoiesis in the spleen. One animal treated with spleen cells and dying on the 9th day after irradiation, had developed hemorrhagic diathesis parallel with severe symptoms of an accelerated secondary syndrome.

THE LYMPHATIC TISSUE

Regeneration of the lymphatic tissue was slow in rats treated with homologous bone marrow only, similar to what has been shown for chimeras of other species. The delay in onset, and the rather chronic character of secondary disease after bone marrow treatment might be attributed to this slow repopulation of the chimera's lymphatic tissue by the cells which are generally believed to be responsible for the graft-versus-host reaction (203).

A delayed type of graft-versus-host reaction developed in about 30 % of the animals. The lymphatic tissues of those with severe symptoms of secondary disease were depleted at autopsy, except in two individuals that had been killed while recovering from the disease (table IV). These two animals showed actively proliferating lymphatic follicles in the lymph nodes and in the spleen and it is tempting to assume that a degree of tolerance of the graft towards the host had belatedly developed in these two cases (see also chapter V).

BROFO rats receiving WAG lymphoid cell suspensions with or without bone marrow, invariably showed fast repopulation of the lymphatic tissues during the first 10 days, but a partial involution of the lymphatic follicles was usually seen when they succumbed with a severe type of graft-versus-host reaction during the 2nd to 4th week. Similar observations have been made in mice and monkeys where initial proliferation of lymphoid cells in irradiated, homologously treated hosts had been observed, followed by a rather sudden involution of the lymphatic tissues (206, 209 and discussion of chapter V).

THE GASTRO-INTESTINAL TRACT

The absence of severe intestinal lesions in secondary disease of the rat, except when large numbers of mature lymphoid cells were given, was an unexpected finding. Even in the latter case, denudation of the mucosa of the colon, as observed in mice and monkeys, did not occur. Rats have generally been found to have a particularly radiosensitive intestinal tract (80), the immediate damage of its epithelial lining caused by radiation being at least as extensive, if not more so, than that seen in mice, rabbits and primates. Moreover, the radiation dose necessary to cause the complete elimination of hemopoietic tissue in the rat, approaches the dose causing death of the intestinal syndrome, leaving a rather narrow margin between the two modes of death when compared with other species. Nevertheless, rats treated with homologous bone marrow and dying with symptoms of secondary disease during the 2nd and 3rd month showed surprisingly few histological lesions of the intestinal tract. Sporadic crypt degeneration and karyorrhexis of crypt epithelium was seen in the colon but neither leucocytic infiltration, nor clinical evidence of diarrhea as in mice with secondary disease, was ever found. Only if homologous lymphoid cells had been injected, with or without a concomitant bone marrow graft, did frequent and extensive degenerative lesions in the colon occur. These were comparable to those seen in mice, though not quite as severe as those reported for bone marrow-treated monkeys with secondary disease. Denudation of large areas of the intestinal mucosa

was never encountered in these rats, not even in the fast and invariably lethal syndrome seen in irradiated BROFO rats after the injection of 150 x 10^6 WAG spleen cells.
No satisfactory explanation for the absence of diarrhea and for the mildness of the intestinal lesions in secondary disease of the rat can as yet be given. It may be speculated that the susceptibility of the intestinal epithelium of the rat to an immunological attack by the graft may be low compared with that of the mouse or primate. The frequent lesions of the epidermis in rats with secondary disease are certainly suggestive of an immunological activity of the graft against other epithelium of the host.
The occurrence of colitis associated with mucous secretions and diarrhea in secondary disease might also be influenced by the types and multitude of micro-organisms present in the gut of the experimental animal before irradiation. The intestinal flora would then provide the inflammatory element superimposed upon anatomical lesions and hemorrhages induced by radiation and presumably by the subsequent graft-versus-host reaction. Hypothetically, the use of SPF rats ("specific pathogen free", see Materials and Methods), devoid of several of the usual commensals and parasites, might explain the absence of secondary bacterial infection and diarrhea in these rats with secondary disease even when, following the injection of homologous lymphoid cells, rather extensive crypt lesions of the colon could be demonstrated histologically.

THE EPIDERMIS

Skin lesions were nearly always seen in WAG→BROFO chimeras with secondary disease. If treated with bone marrow only, larger areas of the body surface were involved than in the spleen-treated groups, possibly because more time was available for the lesions to develop. On the other hand, the protracted secondary disease after bone marrow treatment hardly ever produced complete necrosis of the epidermis as seen in animals treated with spleen cell suspensions. The chronic dermatitis of the bone marrow treated animals, though involving a larger area of the body surface, seemed reversible and rather benign. Several animals with lesions covering more than 50 % of the entire body surface, were able to recover within 2 weeks (figure 5-8).
No explanation can be given for the peculiar localization of the skin lesions nor for the mechanism which causes them. Though the immunological activity of the graft is very likely involved (16), a direct interaction between cellular or humoral antibody and the affected epidermis has as yet not been demonstrated (see chapter VIII). It seems quite unlikely that radiation damage as such plays a role in the pathogenesis of this symptom. Skin lesions of very similar character have been described in other graft-versus-host reactions in the rat, where radiation had played no part whatsoever (181). Gowans et al (89) described typical skin lesions in unirradiated 11 week old F_1 hybrid* rats after the injection of parent-type thoracic duct lymphocytes. The resulting fatal disease had much in common with the accelerated secondary disease seen in the spleen-treated irradiated rats presented here.
Skin lesions, histologically identical with those seen in the bone marrow treated rats of the present experiments, have been observed in mice and primates with secondary disease (208, 209). Not found in those other species were the ulceration and necrosis of the epidermis and dermal appendages, rather constantly occurring in BROFO rats treated with homologous

* An F_1 hybrid, the cross between 2 inbred strains, contains transplantation antigens of both parent strains and can therefore not reject cellular transplants from either parent; the grafted cells however will react against the antigens of the other parent in the F_1 hybrid.

spleen cell suspensions. The histology in the latter cases was reminiscent of the appearance of homologous skin transplants in the course of their rejection.

OTHER ORGANS AND TISSUES

Liver lesions, strikingly similar to those found in monkey and mouse chimeras with secondary disease were observed. In most cases these implied the death of isolated liver cells, compensated by liver cell proliferation as evidenced by an increased number of mitoses of liver cells. It has been suggested that such liver cell death could be a direct effect of cytotoxic antibodies interacting with host-antigens.

The significance of the histiocytic cell proliferation as described in a previous section, is difficult to evaluate. Rather than being directly related to the graft-versus-host reaction it may merely be the host's response to cell destruction taking place in certain organs and tissues, probably as a consequence of the graft-versus-host reaction. It would obviously be of interest to determine whether the histiocytic cells involved in this reaction are of host- or of donor-origin.

CHAPTER V

CONSEQUENCES OF IRRADIATION AND ADMINISTRATION OF HOMOLOGOUS CELLS IN OTHER STRAIN COMBINATIONS

INTRODUCTION

It is known that homologous bone marrow therapy of irradiated mice produces quite variable secondary morbidity and mortality in different strain combinations. Even if one single strain combination is used, as for instance the C57BL and CBA strains, secondary disease may be very severe if CBA mice are the irradiated recipients and C57BL the donors, while the reverse combination produces a rather mild form of secondary disease (21). Since genetic disparity between donor and host had obviously remained the same, the reason for the difference must be due to other factors. The genetically determined immunological reactivity of the lymphoid elements contained in the bone marrow graft, the ability of the graft to develop tolerance towards the antigenic environment, but also the susceptibility of the host to various effects of the graft-versus-host reaction may play a role.

For more than one reason it seemed therefore worth-while, to investigate the effects of bone marrow therapy between the available rat strains, using as many combinations as possible. For one thing, it was important, to obtain isologously treated control animals next to those contracting secondary disease after the administration of homologous bone marrow. This was not very well possible in the WAG→BROFO combination. Isologous bone marrow is not available for irradiated BROFO rats since these rats are not inbred. Obviously, isologously treated controls were obtainable in the reverse, the BROFO→WAG combination. Another reason why it seemed desirable to obtain a group of bone marrow treated WAG rats was the importance of being able to compare the late effects in this strain with those seen in BROFO rats. The use of WAG rats as irradiated hosts also made it possible to exclude an influence of the graft-versus-host reaction on the late effects, by treating them either with isologous or homologous bone marrow.

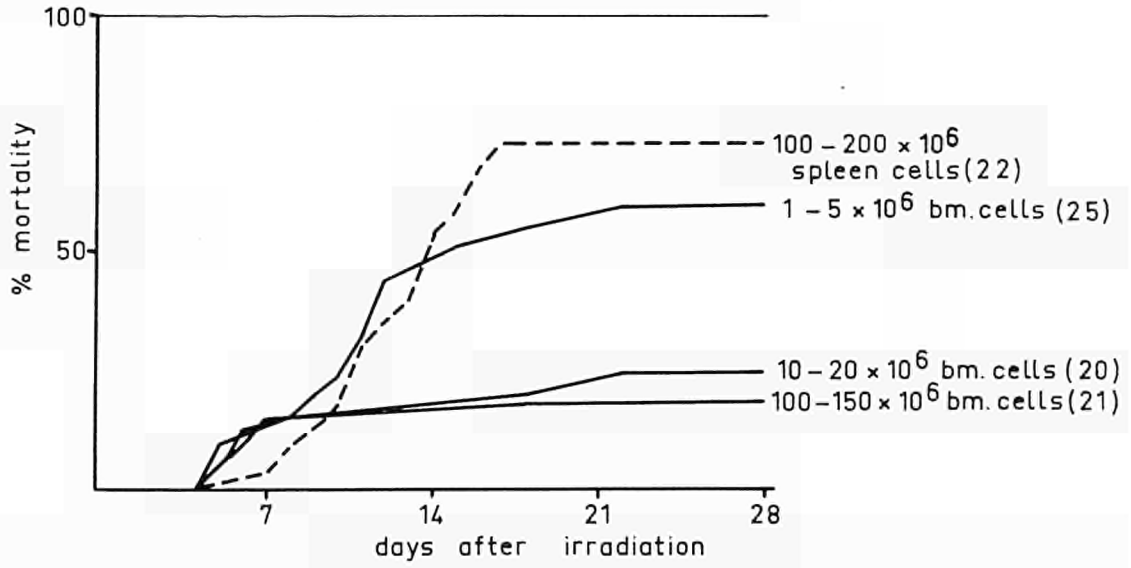
RESULTS

Pooled bone marrow from 20 or more BROFO rats was used to protect irradiated WAG and BROFO rats in a limited number of experiments. The efficacy of these grafts to protect the irradiated animals against the bone marrow syndrome as well as the subsequent incidence of early and late complications were recorded.

The BROFO→WAG combination

Text figure 3 (experimental group IV) demonstrated that, in the BROFO→WAG combination, both early and delayed mortality were comparable to the average for the more frequently used WAG→BROFO combination (groups I, II and III). One significant difference however was noted in the clinical manifestations of secondary disease. Skin lesions characteristic for the secondary syndrome in BROFO rats were never seen in WAG recipients. Apart from that, there was not much difference as far as symptoms of secondary disease were concerned; weight loss usually lead to severe emaciation, while there was no evidence of diarrhea nor of anemia. The limited number of autopsies performed on animals of this group dying during the 2nd and 3rd month, revealed a picture, except for the skin lesions, similar to that seen in WAG→ BROFO chimeras dying with symptoms of secondary disease during that same period. A comparatively high incidence of affections of the lungs, often suggestive of PPLO infection, was seen in irradiated WAG rats, irrespective of the genotype of the protecting bone marrow. PPLO-, or other types of bronchopneumonia were rarely seen in WAG→BROFO chimeras succumbing during the first few months. This was in accordance with observations, made previously in this laboratory, that unirradiated BROFO rats are less susceptible to bronchopneumonia than WAG rats (20). Differences between bone marrow treated WAG and BROFO rats concerning complications occurring beyond the third month after irradiation will be described and discussed in chapter VII.

No titration for the minimal number of bone marrow cells necessary for survival was done in the BROFO→WAG combination. Typing of erythrocytes was performed in only one group and the chimeric state of these rats (possessing erythrocytes exclusively of BROFO origin) was demonstrated at 3 as well as at 12 months after irradiation.



Text figure 11: Mortality of BROFO rats given various numbers of BROFO bone marrow cells (24 hours after a single radiation dose of 1000 r). The interrupted line represents irradiated BROFO rats given 100-200 x 10^6 BROFO spleen cells after 1000 r.

An intravenous injection of 100-150 x 10^6 BROFO spleen cells into irradiated WAG rats (only 8 animals; not included in the diagrams) had a uniform killing effect within 2 weeks after irradiation. The animals died after a period of weight loss and extreme irritability (figure 29, 30), similar to the consequences of an injection of WAG spleen cells into irradiated BROFO rats (figures 27, 28). Skin lesions, however, were virtually absent in the former combination.

The BROFO→BROFO combination

The efficacy of bone marrow therapy was also tested within the BROFO strain. Genetic closeness between non-littermate members of this colony had been proven by reciprocal skin grafting (see chapter VI) and was also reflected in the results of bone marrow therapy as presented here. Text figure 11 demonstrates that less than 5 x 10^6 cells of pooled BROFO bone marrow had a distinctly beneficial effect while secondary morbidity or mortality was never observed. This result, resembling the findings for the isologous combination (see below) suggests a low genetic disparity within the BROFO strain.
However, the administration of 100-200 x 10^6 pooled BROFO spleen cells, with or without additional bone marrow, resulted in an accelerated type of secondary disease in irradiated BROFO rats, very similar to that observed in irradiated BROFO rats given WAG spleen cells. While the latter combination resulted in 100 % mortality (text figure 9) the BROFO→BROFO combination showed a slightly lower mortality of about 70 % (text figure 11). In general, macroscopic and microscopic autopsy findings were similar for both combinations with only one significant difference: the accelerated death caused by BROFO lymphoid cells in irradiated BROFO rats, was not associated with lymphatic atrophy as in the WAG→BROFO combination. On the contrary, extensive proliferation of lymphoid cells was observed in the BROFO→BROFO combination (table IV, figure 12). The possible reasons for this difference will be discussed below.

The WAG→WAG combination

Finally, for several reasons already mentioned in the introduction of this chapter, bone marrow therapy was also assayed in the isologous combination. Group V, also depicted in text figure 3, represents irradiated WAG males treated with a comparatively large dose of WAG bone marrow. Previous titration of the bone marrow dose necessary to keep lethally irradiated WAG rats alive had shown a beneficial effect of less than 1 x 10^6 viable cells (22). The injection of more than 100 x 10^6 isologous spleen cells provided protection against near-lethal irradiation while secondary morbidity or mortality was never observed. The radiation dose of 900 r, which was an LD_{90} for male BROFO rats (text figure 1) caused a slightly higher mortality of approximately 95 % in WAG males not treated with hemopoietic cells. Extensive dose-mortality curves, as presented for BROFO rats, are not yet available for the WAG strain.

DISCUSSION

The results reported in this chapter provide evidence that the severity of secondary compli-

cations in the irradiated bone marrow treated rat largely depended on the genetic disparity between host and donor, as had previously been shown for mice. The symptomatology of the disease, however, may also have depended on an, as yet unexplained, different susceptibility of certain target organs to the graft-versus-host reaction. This was born out in the present experiments by the finding that BROFO→WAG chimeras dying during the 2nd and 3rd month post-irradiation, did not show macroscopic skin lesions, while a typical dermatitis was nearly always seen in irradiated BROFO rats dying in that same period after treatment with WAG marrow. The absence of skin lesions, which are a typical symptom of secondary disease in most species so far investigated (see chapter I), brought up the question whether these WAG rats were actually dying as a consequence of secondary disease or due to causes unrelated to the graft-versus-host reaction. The fact that WAG rats given WAG bone marrow (group V, text figure 3), with an occasional exception, dit not die during that particular post-irradiation period, while BROFO spleen cells injected into irradiated WAG rats did cause a typical accelerated type of fatal secondary disease, also virtually without macroscopic skin lesions, made the graft-versus-host reaction a very likely cause of secondary mortality in WAG rats treated with BROFO bone marrow.

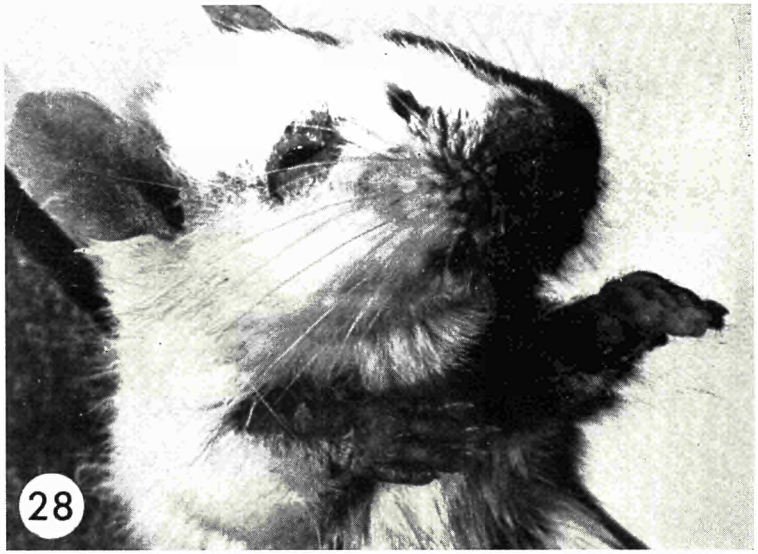
According to the literature, the occurrance of skin lesions seems to be variable also in other graft-versus-host reactions. The runting syndrome in rats, caused by the injection of viable homologous bone marrow or lymphoid cells into newborn animals, has been reported to show a variable incidence of skin lesions. Typical dermatitis was reported for some strain combinations (106, 33) while it was absent or virtually absent in others (218, 3, 147). Kren et al (106) noted that dermatitis in runt disease of rats was observed only in a few strain combinations and that its occurrance seemed an exception rather than the rule. These investigators also proved the immunological nature of such skin lesions by demonstrating that skin grafts, genetically identical with the injected cells and transplanted shortly after birth, remained perfectly intact amidst the badly affected host skin, throughout the runting period. This certainly ruled out a metabolic disorder as the cause for the dermatitis.

Irradiated BROFO rats protected with pooled BROFO bone marrow apparently did not develop secondary disease at all. This is not surprising in view of the fact that genetic disparity between BROFO rats was low. According to current theories about the development of tolerance in radiation chimeras, lymphatic precursor cells contained in the injected bone marrow suspension, can develop specific tolerance towards host-type antigens while proliferating in the presence of an overwhelming amount of those antigens (see also chapter VI). It seems reasonable to assume that genetic compatibility will facilitate this process of adaptation, so that BROFO cells were more likely to become tolerant towards BROFO antigens than were WAG cells under otherwise identical circumstances. Consequently, secondary disease following bone marrow therapy sometimes developed in the WAG→BROFO-, but never in the BROFO→BROFO combination.
Mature BROFO lymphoid cells, less likely to develop tolerance towards the antigenic environment of the host, did often have a lethal effect on irradiated BROFO rats. The effect was again somewhat less outspoken than the consistently lethal effect of an injection of WAG spleen cells (text figures 9 and 11). Nevertheless, severe graft-versus-host reactions could apparently occur also in the BROFO→BROFO combination. It seems reasonable to assume,

Figure 27-28
The "killing effect" of homologous lymphoid cells; typical appearance of BROFO rats dying 2 weeks after irradiation and administration of WAG-type spleen cells (900 r, 150 x 10^6 cells). Note emaciation as well as edema, erythema and hair loss of the skin in circumscribed areas.

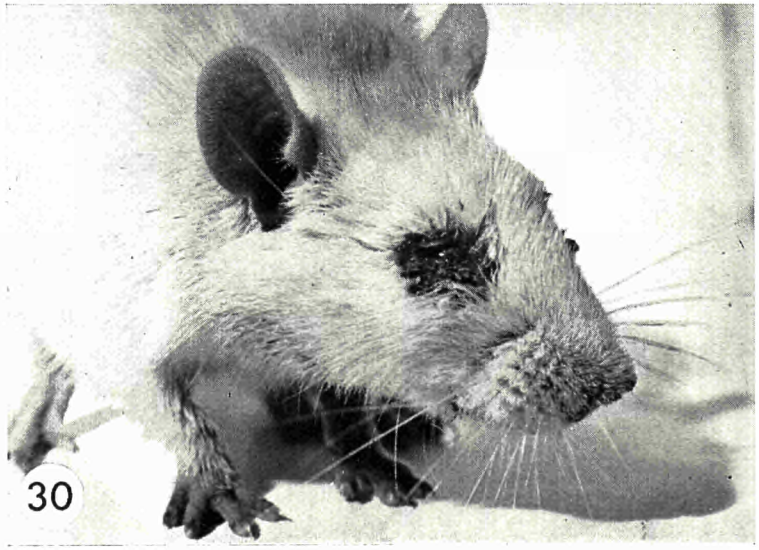
figure 29-30
Moribund WAG rat 10 days after a high sublethal radiation dose and administration of BROFO-type spleen cells. Note miserable appearance, hunched back and inflammation of eyes but scarcity of skin lesions compared to the reverse strain combination (figure 27-28).

27



28

29



30

that the absence of manifest secondary disease in the bone marrow treated animals in the BROFO→BROFO combination, was a result of rather easily acquired specific tolerance of the grafted cells towards host-type antigens.

If lymphoid cells are injected into an irradiated animal genetically incompatible with the donor, lymphoid proliferation in spleen and nodes of the host is often followed by lymphatic atrophy in these organs. Such proliferation and subsequent atrophy was indeed observed in irradiated BROFO rats given WAG spleen cells but not if BROFO spleen cells had been given. Figure 12 and table IV demonstrate that extensive proliferation of lymphoid cells was observed in the nodes and spleen of several of these animals when autopsied. Similar lymphoid proliferation was seen in the nodes and the spleen of irradiated monkeys dying of a severe graft-versus-host reaction soon after the injection of homologous bone marrow (209). Initial proliferation of donor-type lymphoid elements is obviously not an exception in severe secondary disease following the injection of lymphoid cells or of bone marrow containing a rather large proportion of lymphoid precursor cells (as is believed to be the case in primate marrow). It may depend on the time of death of the host whether the lymphoid organs are still found in a proliferative stage or are already atrophic. However, since BROFO rats treated with BROFO spleen cells died at approximately the same time and, except for the skin lesions, with a similar symptomatology as those treated with WAG spleen cells, the genetic relationship between donor and host may also account for the variable aspect of the lymphoid tissues at autopsy. Whether spleen cells from one BROFO donor or a pooled suspension from several donors had been injected did not seem to make a difference in this respect.

Another puzzling observation was the low incidence of "early" or primary deaths in bone marrow treated irradiated animals in the more compatible strain combinations. In both, the WAG→WAG as well as the BROFO→BROFO combination, mortality during the first 10 days post-irradiation was rather low. This early mortality occurring in irradiated rats in spite of the administration of an adequate number of bone marrow cells, has been elaborately discussed in chapter III and was tentatively attributed to infections. Low early mortality was seen in an occasional group of irradiated rats in the WAG→BROFO or BROFO→WAG combination. But as a rule, early mortality in these "incompatible" combinations amounted to something like 20-40 % of the total number of rats in each single experiment.
The most likely explanation for the different early mortality in irradiated animals treated with compatible or incompatible bone marrow cells would seem to be the immediate therapeutic effect against early infectious complications of the more mature lymphocytes contained in the marrow graft. Such an effect would subsequently be enhanced by a presumably better proliferative capacity of the compatible bone marrow. Survival and proliferative capacity of labeled mouse bone marrow cells however, did not seem to be influenced by the antigenicity of the new environment within the first few days after transfusion (6).

CHAPTER VI

HOMOGRAFT REACTIVITY AND SPECIFIC TOLERANCE IN CHIMERAS AND REVERSALS

INTRODUCTION

Orthotopic free skin grafting is accepted as the most convenient and practical method to test homograft reactivity. The technique is relatively simple and macroscopic evaluation of the degree of reactivity is possible and does not require special skills as do many other techniques in the field of immunology.

In the course of the present experiments transplantation of donor-type skin was done to demonstrate chimerism in lethally irradiated rats treated with homologous bone marrow. Radiation chimeras are known to be specifically tolerant towards skin grafts of the same genetic make-up as the bone marrow donor (117, 188, 221). After reversing to host-type hemopoiesis however, animals have been shown to regain host-type immune reactivity (221, 14, 160).

In a number of rats that were reversals according to serological typing of erythrocytes (see chapter III), donor-type skin grafts were nevertheless permanently accepted. This was an unexpected finding and subsequently all sublethally irradiated bone marrow treated animals, being potential reversals, were tested for the genotype of their erythrocytes and nucleated peritoneal cells. Those that had completely (or nearly completely) reverted to host-type hemopoiesis were repeatedly challenged with donor-type skin grafts. The results will be presented below.

Specific tolerance towards transplantation antigens

The observation of persistant tolerance in a few proven reversals was important in view of the still controversial issue regarding the maintenance of specific tolerance towards transplantation antigens independent of a permanent chimeric state.

Specific tolerance induced in newborns by injecting foreign cells into the immature animals posed more or less the same question. A continued presence of donor-type cells was observed in those animals when adult, but it was not considered an absolute condition for the persistence of tolerance (32). When Trentin and Session (191) demonstrated that the degree of specific tolerance in such animals, when adult, correlated with the proportion of donor-type cells in the host's lymphatic tissues, the question seemed to have been settled. Apparantly tolerance could not persist unless the specific antigen was present in large quantity. In other words, the immune system did not possess a "memory". Van Bekkum (19) also supported this hypothesis by

demonstrating, in a different experimental set-up, that tolerance in a population of specifically tolerant lymphoid cells could not persist in the absolute absence of the particular antigen.
However, Michie et al (140) and later Doria (68), both working with animals made specifically tolerant at birth by the injection of living cells, found that the great majority of the immunologically competent cells of the tolerant hosts, when adult, were of host origin. Evidently, though chimerism was not excluded, the concept of a "take over" of the immune system by donor-type cells, as had been proven for specifically tolerant radiation chimeras, was abandoned by these authors.
The question whether chimerism is indispensable for the induction and maintenance of tolerance towards strong transplantation antigens was obviously not solved. Attempts to induce specific tolerance towards transplantation antigens with non-living material had been unsuccessful except when very low antigenic differences were involved (34). Medawar (136) however, recently succeeded in obtaining a degree of tolerance across strong histocompatibility barriers in mice, by injecting large amounts of purified antigens; this has renewed the hope that tolerance towards transplantation antigens may eventually be obtained while avoiding the hazards of the transplantation of living cells and thereby of chimerism.
The advantages of persistent tolerance in reversals (sublethally irradiated), as compared to chimeras (lethally irradiated), would be a reduction of radiation injury and, at the same time, the avoidance of permanent chimerism. Several attempts in this direction have been reported for mice. After partial success with celomic parabiosis between adult mice, a degree of tolerance was obtained by injecting large numbers of spleen cells into adult homologous mice or cells from F_1 hybrids into animals of the parent strain (123, 124, 97, 125, 139); sublethal or no irradiation had been administrated to the hosts in these experiments but only a few specific strain combinations of rather low genetic disparity had been used. Fefer and Davis (74) following more or less the same approach, injected spleen cell suspensions into homologous mice after sublethal irradiation. In all these experiments a degree of tolerance was obtained only if no strong histocompatibility barriers had to be overcome, or if the CBA-A strain combination was used which, though differing at the H-2 locus,* lends itself exceptionally well for the induction of tolerance (27). In some of these experiments chimerism was shown to persist even after comparatively low radiation doses (139), in others chimerism was not specifically ruled out by serological typing of the hemopoietic and lymphatic cells of the tolerant hosts.
Such testing for chimerism was rigorously performed in the present study, involving rat strains of distinct genetic disparity. The rather unexpected finding that a number of proven reversals did show persistent total or partial tolerance towards donor-type skin grafts will be discussed in the light of the controversy regarding the persistence of tolerance independent of chimerism.

RESULTS

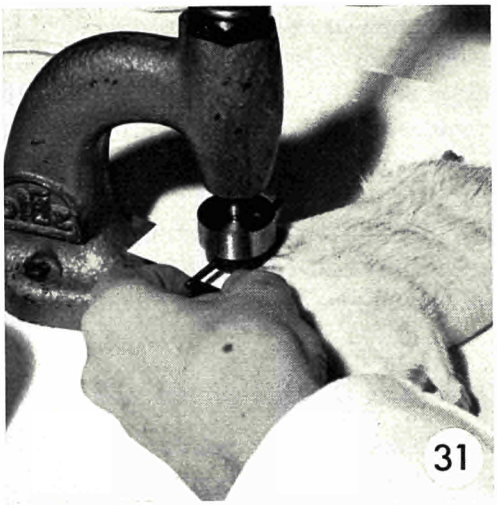
Autologous skin grafts and transplants between members of an inbred strain are, if ingrowth and vascularization are adequate, always permanently accepted. Rejection of skin grafts

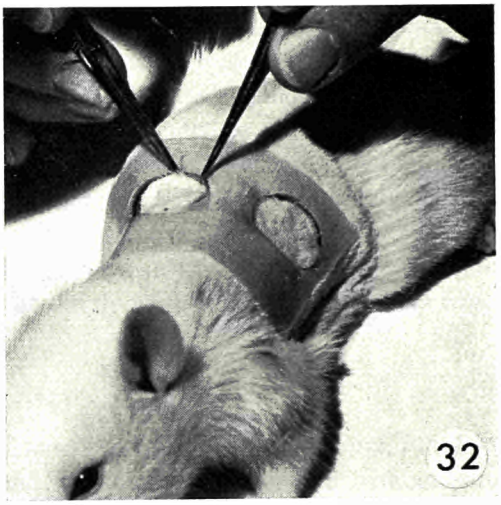
* For information regarding the significance of histocompatibility antigens consult references (180, 195a).

Figure 31-34
Various stages of the skin grafting technique as described in chapter II, (Materials and Methods). Note perfect healing-in of the grafts after removal of the tape on the 10th day (figure 34).

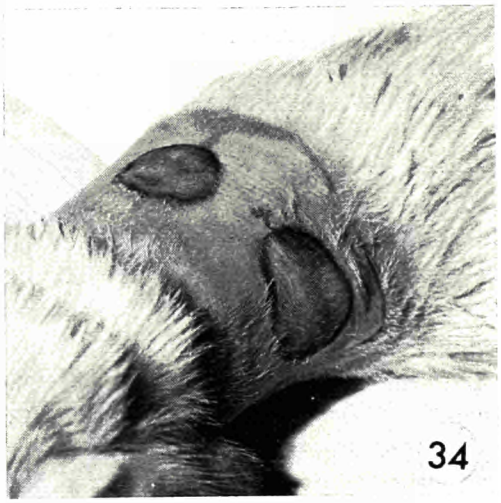
Figure 35
Tolerance of an old WAG—BROFO chimera towards donor-type skin. On the left a viable WAG skin graft, on the right a rejected 3rd party BROFO transplant.

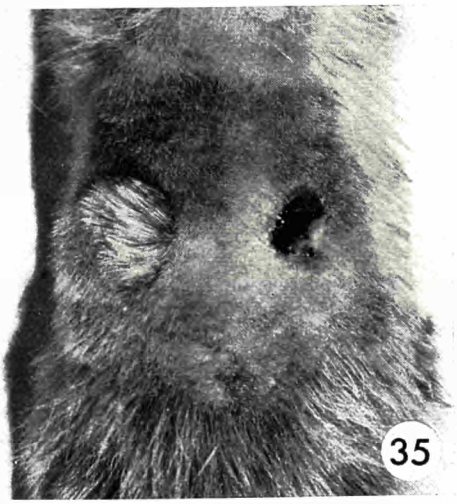
Figure 36
Persistant tolerance of a „reversal" towards donor-type skin. A first donor-type graft transplanted early (upper L) and another one transplanted after reversing to host-type hemopoiesis (lower L) were both permanently accepted. Autografts are seen on the right.


31


32


33


34


35


36

between homologous members of most species takes about 8-14 days. If, after rejection of a first transplant of skin or other tissue, an animal receives a second skin graft of identical genetic make-up as the first, it will be rejected in an accelerated fashion, usually within 5-8 days. This is called a "second set" reaction and can be attributed to the immunizing effect of the tranplantation antigens of the first graft (135). If, on the other hand, a homologous skin graft is not rejected within 12 days, the rejection mechanism is obviously impaired. This can be due to a non-specific depression of the immune system, as for instance after total-body irradiation or administration of radiomimetic drugs, or to specific depression of homograft reactivity which will be called specific tolerance or simply tolerance in the following pages.

Reciprocal skin grafting in WAG and BROFO rats

Various methods of skin grafting in small animals have been described. In general, full thickness free skin grafts in small rodents such as mice and hamsters can be applied without suturing. Larger animals often require better fixation of the graft in its bed, while "split thickness" skin is often preferable to full thickness grafts to assure an adequate vascular supply. To avoid the time-consuming procedure of suturing the graft in its bed, a different method of skin grafting for rats was employed. This technique has been extensively described in chapter II (figure 31-34).

Histo-incompatibility between BROFO and WAG rats was high as evidenced by skin grafting experiments performed on unirradiated adult animals. Table V shows that reciprocal skin grafts were always rejected within 12 days. This suggests a degree of histo-incompatibility between BROFO and WAG rats comparable to that encountered between mouse strains differing at the H-2 locus. The table also demonstrates that the survival time of grafted WAG skin on irradiated BROFO rats was hardly influenced by radiation as such, even when grafting was done within a week following 500 r of total-body irradiation. The intravenous injection of viable or killed homologous cells following such low radiation doses had, if anything, an immunizing effect.* Genetic closeness between non-littermate members of the non-inbred BROFO strain was expressed in the slow rejection of reciprocal skin grafts in approximately 40 % of these animals. The majority of reciprocal skin transplants within the BROFO strain, however, showed normal rejection times of 10-12 days.

Tolerance of permanent chimeras towards donor-type skin grafts

Table VI depicts the results of skin grafting in rat radiation chimeras. Isologous (WAG→WAG) chimeras rejected genetically unrelated BROFO skin (3rd party) normally within 12 days

---

* These and a few other pertinent experiments were done to dispose of **"enhancement"** as a possible mechanism in the reduced homograft reactivity observed in some of the reversals of the present study. Enhancement was defined by Kaliss (100) as promotion of progressive growth of tumour homografts by antibody. Several authors have considered enhancement as a possible explanation for the prolonged survival of skin homografts following the administration of the particular homologous antigens under certain conditions (35, 30, 133, 146). For detailed information about the mechanism of enhancement, one of the many papers or a review article dealing with this intricate and still controversial subject should be consulted (100, 145).

TABLE V

RESULTS OF SKIN GRAFTING IN VARIOUS COMBINATIONS BETWEEN INBRED WAG AND NON-INBRED BROFO RATS

genotype of skin donor	genotype of host	pretreatment of host: irradiation	pretreatment of host: donor-type cells injected i.v. after irradiation	pretreatment of host: time before skin grafting	number of animals	rejection time: in days	rejection time: in comparison with normal
WAG	WAG	-	-	-	25	∞	
WAG	BROFO	-	-	-	25	10-12	
		500 r	-	1 week	10	10-13	normal
		500 r	100×10^6 bm cells	"	10	< 10	accelerated
		500 r	100×10^6 bm cells killed with 5000 r	"	10	< 10	accelerated
		850-900 r	-	1-4 months	8	10-12	normal
BROFO (various donors)	BROFO	-	-	-	25	10-12 (60%)	normal
						14-42 (40%)	prolonged
BROFO (various donors)	WAG	-	-	-	8	10-12	

except if grafting was done right after irradiation. BROFO→BROFO chimeras rejected foreign WAG skin (3rd party) normally, but were permanently tolerant towards BROFO skin grafts taken from various individual BROFO donors. Pooled BROFO bone marrow (from 20 donors) apparently contained a sufficient number of transplantation antigens to confer tolerance towards numerous individual BROFO skin grafts in these experiments. If pooled BROFO marrow was given to irradiated WAG rats, permanent tolerance towards "other" BROFO rats was evident if grafting was done up to a few weeks after irradiation. However, another group of animals, transplanted 6 months after irradiation, did not show such permanent tolerance, though erythropoiesis of these animals was still of the BROFO-type.

Results are more clearcut in the case of WAG→BROFO chimeras, the most common donor-host combination in the present experiments. Survival of grafted (donor-type) WAG skin was always permanent if the recipient was a chimera, proven by serological typing of erythrocytes. Skin grafts from individual BROFO rats, genetically not identical with either donor or host, showed variable rejection times, more or less as in the case of BROFO skin grafted on untreated BROFO rats (figure 35).

Rejection or survival of skin grafts was usually assessed macroscopically, and occasionally confirmed microscopically. Permanently accepted skin grafts showed normal vascularization, colour and hairgrowth from the very beginning. Microscopically, the epidermis was of normal thickness with well-developed actively proliferating hair follicles and there was no evidence of cellular infiltration in the corium. Slow rejection was sometimes seen in WAG→BROFO chimeras given a (3d party) BROFO skin graft, in cases of temporarily suppressed reactivity as in WAG→WAG chimeras grafted (homologous skin) soon after irradiation as well as in cases of partial tolerance in reversals (see below). It was characterized by paleness and shrinking

TABLE VI

RESULTS OF SKIN GRAFTING PERFORMED ON ISOLOGOUS AND HOMOLOGOUS RAT RADIATION CHIMERAS (900-1000 r)

genotype of bone marrow donor	genotype of irradiated host	skin transplant	time of skin grafting (after irradiation and bone marrow transfusion)	number of animals	rejection time in days	rejection time in relation to normal (in that particular combination)
WAG	WAG	BROFO	1 day	10	20-35	prolonged
		(3d party)	4 weeks	7	10-12	normal
WAG	BROFO	WAG	1 day – 6 months	60	∞	tolerant*
		BROFO	3 months	10	variable <12 to ∞	slightly prolonged
BROFO	BROFO	BROFO	6 months	5	∞	tolerant
		WAG	4 weeks	5	10-12	normal
		(3d party)	6 months	5	10-12	normal
BROFO	WAG	BROFO	1 day	5	∞	tolerant
			8 weeks	5	∞	tolerant
			6 months	9	10-14	normal

* unless reversing to host-type hemopoiesis had taken place.

of the graft, while hair growth, if present, was initially scarce and later totally absent. Microscopically there was acanthosis of the epidermis, athrophy and later absence of hair follicles but mononuclear infiltration as seen in normal homograft rejections was never abundant.

Persistence of tolerance towards donor-type skin grafts in reversals

As has been mentioned in previous chapters, reversion to host-type hemopoiesis occasionally occurred after an initial take of homologous bone marrow. Such reversing was particularly frequent after 775 r, approximately an $LD^{10}/_{30}$. A homologous bone marrow graft of 100 x 10^6 cells, was nevertheless initially accepted after such radiation doses. If the radiation dose was reduced much further however, immune reactivity was apparently not sufficiently suppressed to allow even an initial take of transfused marrow (table V, 500 r + 100 x 10^6 homologous bone marrow cells). Immunity rather than tolerance towards skin grafts of the same genotype as the injected marrow was the consequence.

Table VII presents the results of skin grafting in a number of reversals.* The hosts were BROFO rats that had been given sublethal radiation doses (775-900 r) and rather small grafts of WAG bone marrow (25-100 x 10^6 cells). The majority of the animals so treated, except after a radiation dose of 775 r, did not revert to host-type hemopoiesis. Being permanent chimeras, they invariably accepted donor-type skin, grafted at any time, and were not included in the table.

Many of the reversals shown in this table were still chimeras when typed 2-3 months after irradiation, but most of them showed a preponderance of host-type erythrocytes 5-6 months

* The group of 4 animals showing only host-type erythropoiesis already during the 2nd month after 900 r and 25 x 10^6 homologous cells, had nevertheless been shown to have an initially functioning marrow graft (text figure 7, chapter III).

TABLE VII

TOLERANCE TOWARD DONOR-TYPE SKIN GRAFTS IN WAG→BROFO CHIMERAS AFTER REVERSING TO HOST-TYPE HEMOPOIESIS

BROFO rats (radiation dose and number of WAG bm cells injected)	genotype of erythrocytes (E) and peritoneal cells (P) (time of typing in months after irr.)								period in which WAG skin was grafted (months after irr.)			
	2-3		5-6			9-10						
	E		E		P	E		P				
	WAG	BR	WAG	BR	BR	WAG	BR	BR	1-3	4-6	6-8	8-12
775-825 r 100 x 10^6						-	3+	100			●	✖
						-	3+				Ö	died
						-	3+	80			Ö	Ö
						-	3+	100			●	✖
	2+	+	-	3+		-	3+	100	Ö		Ö	Ö
	2+	+	-	3+		-	3+		Ö		●	died
	2+	2+	2+	+		-	3+	90	Ö		◎	◎
	+	2+	(+)	2+		-	3+	100	◎		◎	◎
	2+	2+	-	3+		-	3+	100	Ö		●	●
900 r 100 x 10^6			-	3+	100					●		✖
			-	3+	100					●	✖	✖
			-	3+	100					Ö	Ö	Ö
			-	3+	99					●		●
900 r 25 x 10^6	-	3+	-	3+	100					◎	Ö	
	-	3+	(+)	3+	100					◎	◎	
	-	3+	-	3+	100					Ö	Ö	
	-	3+	-	3+	100					●	✖	

E = erythrocytes tested with anti-WAG (WAG) and anti-BROFO (BR) sera; degree of agglutination (- to 3+) determines genotype.

P = peritoneal lymphocytes and macrophages tested with anti-BROFO serum + complement; cytotoxic effect (expressed as percentage dead cells) determines genotype.

Ö = graft permanently accepted with abundant hair growth ◎ = no hair growth, rejection time 3-12 weeks

● = normal pattern of homograft rejection (10-12 days) ✖ = accelerated rejection (<10 days)

after irradiation and thereafter. Typing of peritoneal nucleated cells demonstrated that a parallelism nearly always existed between the genotype of erythropoiesis and leucopoiesis.
Grafting of donor-type (WAG) skin at an early stage after irradiation showed that permanent takes were obtained except in one case in which very slow rejection of the skin graft took place; this animal had been particularly fast at reverting to host-type hemopoiesis. All other early skin grafts remained intact with abundant hair growth until the animal's death, independent of whether a second or third WAG skin transplant was eventually rejected or accepted.
Many of the animals were grafted and regrafted with WAG skin at later stages, when rejection of the original WAG bone marrow graft, as evidenced by serological typing, was complete or virtually complete. At that time, several individuals rejected WAG skin grafts normally within 10-12 days, some rejected them slowly, but others accepted WAG skin permanently (figure 36). The "slow" rejection took 3-12 weeks and was reminiscent of the slow rejection pattern of male skin isografts by female mice of certain strains (72, 220). If WAG skin grafted 6 or 8 months after irradiation was tolerated by a reversal, a second or third WAG skin transplanted several months later, was always again permanently accepted. Skin grafts still present from previous occasions never changed their appearance. The majority of the animals showing normal rejection times of a first WAG skin graft, rejected a subsequent WAG graft in an accelerated fashion, comparable to a "second set" rejection of homografts between non-irradiated animals.
The specificity of the tolerance towards donor-type skin grafts shown by several of the reversals was assayed by transplanting BROFO skin, the only available "3rd party". This was not an ideal 3rd party in view of the genetic closeness of the members of the BROFO strain. Nevertheless, the variable rejection pattern shown by all reversals was virtually the same as that of normal non-littermate BROFO rats. Besides, there was no difference in rejection time of "3rd party" BROFO skin between tolerant and non-tolerant reversals. Non-reactivity could therefore be presumed to be specific with regard to WAG antigens.

DISCUSSION

In the present study many of the rat radiation chimeras rejected donor-type skin grafts after reversing to host-type hemopoiesis; a number of the reversals however showed persistence of tolerance or at least reduced reactivity towards the former donor-type antigens if tested by repeated skin grafting. This was of interest in view of the still unanswered question whether tolerance towards transplantation antigens can exist independent of a chimeric state.
It has been repeatedly shown that the donor-type cells of radiation chimeras can develop specific tolerance towards host-type antigens (49, 18, 204). The reverse, tolerance of host-type cells towards antigens of the donor, both in radiation chimeras and in animals made tolerant by the injection of homologous cells at birth, was assumed on the basis of circumstantial evidence, though it was difficult to prove (14, 158, 140, 125, 68). But it is in fact this type of tolerance, namely non-reactivity of host cells towards donor-type antigens, that was tested in the reversals of the present experiments.
It is fair to assume that a degree of mutual tolerance exists, at least temporarily, between two proliferating hemopoietic systems in animals that sustain a mixed population of donor- and host-type cells for a long period. Prolonged co-existence of donor- and host-type hemopoiesis

was repeatedly seen in the present experiments, mostly after sublethal radiation doses and comparatively small bone marrow grafts (table II, chapter III). Such co-existence had previously been described for mouse radiation chimeras and for rat chimeras between strains of low genetic disparity (210, 150). Barnes et al (15) and Popp (158) found suggestive evidence that the replacement of an established marrow graft in radiation chimeras by the surviving portion of the host's own hemopoietic tissue, need not be a matter of active rejection by the recovering host-type immune system. Transpopulation* experiments had indicated that reversing to host-type hemopoiesis may simply be a matter of better environmental adaptation of the host-type precursor cells, giving them a proliferative advantage over similar donor-type cells in the long run. Reactivity of the host's immune system against donor-type antigens was therefore not believed to be a prerequisite for reversing.

In the present experiments donor-type skin, grafted during the early period after irradiation, was permanently accepted, except in one case in which reversing to host-type hemopoiesis had taken place rather soon after irradiation (table VII). The continued survival of these early skin grafts, even after the disappearance of donor-type cells from the blood and peritoneal cavity did not prove persistence of specific tolerance. It has repeatedly been shown that a homograft, once established, can withstand rather vigorous immune reactivity against it (214). However, when donor-type skin was grafted and accepted long after the disappearance of donor-type cells from the blood and peritoneal cavity, as was actually the case in some of the reversals (table VII), the possibility of persistent specific tolerance in the absence of the donor-type cells (and presumably of donor-type antigen) had to be considered.

It may be argued that the methods of cell typing were not accurate enough to exclude the presence of a relatively small number of donor-type cells. This may be so as far as typing of erythrocytes is concerned; small numbers of donor-type erythrocytes might indeed have gone unnoticed. As far as the cytotoxic test is concerned however, one can be fairly sure that 100 % mortality caused by incubation with anti-BROFO serum virtually excludes the presence of nucleated cells of WAG origin. Even small admixtures of WAG cells could be readily detected. Peritoneal cell suspensions from permanent WAG→BROFO chimeras rarely showed more than 10-20 % mortality if incubated with anti-BROFO serum; this is about the non-specific mortality of any cell population. The two reversals of table VII, showing 80 and 90 % mortality of the peritoneal cells after incubation with anti-BROFO serum, obviously still contained some WAG-type nucleated cells, but probably a small quantity if the presence of excess donor-type antigen is the criterium (see below).

Before discussing the possible mechanisms involved in tolerance or reduced reactivity, it should be recalled that about 50 % of the reversals were not tolerant when tested with donor-type skin grafts after reversing to host-type hemopoiesis. Of the remaining animals, some showed partial, and a few absolute tolerance towards donor-type skin grafts and this state of reduced reactivity seemed permanent. In view of what is known from the literature (221, 14, 160, 4), the immunological behaviour of the non-tolerant animals was what could have been expected, while the tolerant or partially tolerant reversals would be the exception. In the following paragraphs it will be attempted to reconcile the immunological behaviour of either group with the principal current theories regarding induction and maintenance of tolerance.

---

* Transpopulation means injecting host-type hemopoietic cells into an established chimera.

Theories on the induction of tolerance

The mechanism of immunological tolerance has been the subject of numerous studies in recent years. One of the issues was, whether or not tolerance towards transplantation antigens, once it had been induced, could be retained without the continuous presence of large amounts of the antigen, in other words, whether the lymphatic system possessed something like a "memory" for specific tolerance. Two basically different hypotheses on the induction of tolerance have been proposed and both have been used to explain certain aspects of immune non-reactivity.

The **first theory** postulates an irreversible, hereditary change of the cells of the immune apparatus. If a population of potentially reactive cells, while still in an immature stage, is exposed to large quantities of a certain antigen, this population may become specifically non-reactive or tolerant towards that antigen. One may think of a genetic transformation or of a selective elimination of cell lines that are potentially reactive against that antigen, in the sense of Burnet's clonal selection theories (44, 45). Tolerance should be long lasting or permanent if such a selective elimination or a hereditary change had occurred. In other words, the immune system would have a "memory", while continuous presence of the antigen in the environment should not be essential.

The **second theory** puts emphasis on the continuous presence of large quantities of antigen, both for the induction and the continuation of specific tolerance. It is based on results of numerous experiments in which specific non-reactivity was obtained in animals by administering large amounts of non-living antigens by various routes and at various stages of maturity (177). Maintenance of tolerance in such cases often depends on the presence of a relative excess of antigen at all times. Immaturity of the individual or the cell population facilitates the induction of tolerance also according to this theory while the elimination of certain cell lines or a genetic alteration of the immunological "stem cells" is not required. Hence, as the antigen becomes less available, tolerance should gradually diminish or disappear.

The possible explanation of persistant tolerance in reversals

Loss of specific tolerance parallel with the disappearance of the donor-type bone marrow graft apparently occurred in 8 out of 17 animals with initially two co-existing hemopoietic systems. This could rather easily be explained by the **second theory,** allowing for the disappearance of total or partial tolerance when the availability of specific antigen diminished. The permanent presence of an "early" donor-type skin graft did not influence the outcome in one way or another; such grafts could therefore not be regarded as a continuing source of donor-type antigen. Return of normal reactivity was furthermore proven by the fact that non-tolerant individuals were sensitizable by donor-type skin grafts as evidenced by accelerated rejections of subsequent transplants of identical genetic make-up (table VII).

Results obtained by van Bekkum, using a somewhat different experimental design, would be in agreement with this reasoning. The "memory" of an immune system was tested by transplanting lymphoid cells, taken from permanent mouse radiation chimeras, into irradiated animals isologous with these transplanted cells. Specific tolerance towards the antigens of the previous host was gradually lost in the absence of those antigens in the new host (19).

Adhering to this second theory, which makes persistence of tolerance dependent on the presence of excess antigen, the immunological behaviour of the other reversals, those that remained partially or totally tolerant, becomes more difficult to explain. One might reason that mature tolerant lymphoid cells, already present before reversing, could by themselves, perpetuate the tolerant state for quite somè time. In this context it should be recalled that lymphocytes probably responsible for these immune reactions, are likely to have a long lifespan (154, 59, 114) and that even mature lymphocytes may be able to divide (111). A long time might therefore elapse before lymphoid cells that arose and became tolerant while donor antigen was abundant, would be completely replaced by new crops of non-tolerant lymphocytes, stemming from cells that arose and matured afterwards, uninfluenced by donor-type antigen.

However, there was little experimental evidence that the tolerant reversals eventually regained reactivity against donor-type antigens at all. On the contrary, repeated skin transplantations proved this specific tolerance to be stable for many months after irradiation (table VII). This would actually be in favour of the **first theory**, postulating an irreversible change of the immune reactivity of the lymphoid system in these cases. Since a reversal's lymphoid cells are very likely derived from heavily irradiated surviving host-type stem cells (15), it is conceivable that, while proliferating and maturing in the presence of an overwhelming amount of donor-type antigen, an elimination of reactive cell clones had taken place in the sense of Burnet's original theories (44). Such a change would express itself as permanent total or partial tolerance, independent of the continuous presence of donor-type antigens. That actually seemed to be the case in the limited number of WAG→BROFO chimeras that became total reversals but nevertheless maintained a reduced reactivity towards the former donor-type antigens.

Zaalberg et al (222), unlike van Bekkum (19), indeed assumed that a specifically tolerant population of lymphoid cells could maintain tolerance in the absence of the particular antigen. Such lymphoid cell suspensions, taken from mouse radiation chimeras, did sometimes loose this specific tolerance when transferred to an irradiated host devoid of the antigens of the former host. However, this was not attributed to a "loss of memory" by the lymphoid population as such, but to a kind of replenishment by non-tolerant lymphatic cells derived from the concomitantly injected bone marrow (from the same chimeras). If prior thymectomy of the new host had been performed to interfere with the production of (new and uninfluenced) immunologically competent cells by the bone marrow (142, 81), the "memory" for the specific tolerance remained virtually intact (223).

Evidence rather against maintenance of tolerance in the absence of excess antigen comes from several investigators who reduced existing tolerance by irradiating (sublethally) adult mice or rats, that had been made tolerant at birth towards a variety of antigens, including transplantation antigens (148, 120, 75, 182). The completeness of tolerance initially obtained, the nature of the stem cells repopulating the depleted hemopoietic and lymphatic tissues, as well as the amount and the kind of antigen present during the recovery phase, seemed critical factors. Denhardt and Owen on the other hand, reported that sublethal irradiation did not abolish tolerance towards BSA (bovine serum albumen) in rabbits (66).

However, none of the investigations mentioned in the preceding paragraphs had an experimental design quite similar to the one presented in this work and a strict parallel between the respective results can therefore not be drawn.

Concluding, it can only be stated that tolerance towards homologous transplantation antigens

was seemingly maintained in a limited number of rats in the absence or virtual absence of the particular antigen. Neither of the two principal theories on the mechanism of induction and maintenance of tolerance lends itself well to explain all the facets of the obtained results. Nevertheless, the fact that a proven temporary graft of homologous hemopoietic cells after sublethal total-body irradiation sometimes resulted in a degree of tolerance, should be interesting to those working in the field of organ transplantation. Manick et al (122) had already tried to improve the transplantability of homologous kidneys in dogs by prior irradiation of the animal and administration of hemopoietic cells from the same donor. The findings of the present study, added to somewhat similar positive evidence already available for mice, might promote clinical trials in this direction.

CHAPTER VII

LATE RADIATION EFFECTS AND TUMOUR INCIDENCE IN RAT CHIMERAS

INTRODUCTION

A number of publications can be found in the literature dealing with the late effects of sublethal irradiation in rats protected by chemical agents or by temporary parabiosis (79, 40, 104, 36, 110). Little is known however, regarding the ultimate fate of rat radiation chimeras, though a number of investigators have studied the late effects of lethal irradiation in mice protected by shielding of limbs or injections of hemopoietic cells (51, 149, 13). It was obviously opportune to keep the chimeras of the present study for long-term observation, to determine such late effects also in bone marrow protected irradiated rats. The development of degenerative lesions, occurrance of infectious complications, duration of life-span and the tumour incidence were recorded.

Numerous studies have been concerned with the late effects of ionizing radiation on animals, especially with regard to life-shortening and carcinogenesis (82, 42, 196, 199, 37, 61). Controversies still exist as to the basic mechanism for either phenomenon. It is obvious that shortening of the life-span by irradiation can hardly be treated quite separately from tumour induction, since the latter, in many instances, determines or influences longevity. The subject becomes very controversial if the so-called "aging effect" of irradiation is introduced and held partly responsible for the early and seemingly increased incidence of neoplasms in irradiated animals. In recent years, the generalization that irradiation accelerates the normal aging processes (46, 83, 113) has been amended or practically abandoned by many investigators (201, 132, 57, 1, 200). Serial killings of irradiated mice for instance, in experimental designs representing typical examples of "radiation accelerated aging" did not reveal a shortened latency period with an unaltered incidence for several of the pathological findings that are predominantly seen during normal senescence (1).

These new insights regarding the late effects of irradiation are important. In the experiments reported here, the principal late effects were a reduced life-span and a high tumour incidence. Regardless of the exact mechanism of either, one will have to speculate whether and to what extent, one parameter may be influenced by the other. Most investigators studying tumour incidence after whole-body irradiation in mice and rats, have also had to contemplate a possible relationship between life-shortening and a carcinogenic effect of irradiation. Their results and conclusions will also be discussed below. The term "accelerated aging" was used by several of these authors when a reduced life-span was observed associated with a number of pathological findings that are usually seen in older animals.

RESULTS

Animals included in this study of late effects were mostly rats from experimental groups depicted in text figure 3 as well as from a few other smaller groups, similarly irradiated and treated with bone marrow. The bone marrow treated, lethally irradiated rats, surviving beyond the period of secondary disease, gained weight normally and remained indistinguishable from non-irradiated controls up to the 7th month after irradiation. From then on, the general appearance and activity of the animals, the incidence of infections and degenerative lesions and, most conspicuously, the tumour incidence, showed a striking difference with non-irradiated controls, while longevity was sharply reduced.

The control group consisted of 71 BROFO rats, 29 males and 42 females; non-irradiated WAG rats of comparable age were not available. Control animals were maintained under identical conditions and in the same air-conditioned quarters as the irradiated rats. All animals were inspected and the findings recorded about once every two weeks. Those with large tumours or in poor general condition were checked daily or every other day. Nevertheless a number of animals was not detected immediately after death and escaped classification as to probable cause of death, because of cannibalism and/or autolysis. It was therefore eventually decided to sacrifice very sick animals, especially those with large neoplasms, when life expectancy according to the experience gained, could not be more than a few days.

Nearly all experimental animals (151 out of 159) had died at the time of writing; the few still alive, were in poor physical shape and several had visible tumours. Only 9 out of the 71 control animals were dead by this time. The others, more than two years old, are being kept for further observation to obtain final data on longevity, infectious and degenerative complications as well as the final incidence of spontaneous tumours in BROFO rats.

Degenerative changes and infectious complications

The outward appearance of the chimeras started to deteriorate around the 8th month after irradiation, when the animals were 10-12 months of age. Cataract was always one of the first visible changes and was consistently found. All irradiated animals eventually developed full cataracts and could be presumed to be blind by the 12th month after irradiation. There were virtually no variations in time of onset and in severity of these lens opacities between rats given various radiation doses and there was no sex difference either. BROFO rats seemed to show a somewhat earlier development of this lesion than did WAG rats.

The developing cataract, deteriorating fur and reduced general activity lent these animals, hardly a year old, a precocious look of senility. About 5 % of the long-term survivors developed characteristic lung disorders with "sneezing" as a prominent symptom. This syndrome, not necessarily fatal, occurred cagewise and was most likely due to PPLO (pleuropneumonia-like organisms) infection. The agent is difficult to retrieve and this was not attempted. However, when animals with obvious symptoms of PPLO infection came to autopsy, histological findings suggestive of this disease were usually demonstrable.

A considerable number of animals died, mostly in the period from 6-15 months post-irradiation, showing no obvious immediate cause of death. In most of these cases the animals had been

miserable and emaciated for a number of weeks and finally died or were sacrificed when moribund. If massive inflammatory lesions were found and histologically confirmed in the lungs, the liver, the urogenital tract or elsewhere, these cases were classified as "infectious deaths" (table IX). Blood cultures or cultures taken from such lesions were sometimes positive for one or several of the common micro-organisms (Proteus morgagni, Enterococci, Streptococci etc.). Some autopsies did not reveal any macroscopic or microscopic cause of death ("undetermined" in table IX). If, as was sometimes the case, such unidentified deaths occurred cagewise, bacterial or possibly viral infections might have been causative also in these cases. A combination of degenerative and infectious lesions as a possible cause of death had to be considered as well (see below).

Infectious complications of the urogenital tract were frequent, especially in male WAG rats (text figures 14 and 15). Huge tumorous masses in the area of the prostate and seminal vesicles were often associated with dilatation of the bladder, clinically characterized by urine retention or incontinence. Histologically these lesions showed suppurative and necrotizing inflammations of the prostate gland, seminal vesicles and other tissues around the neck of the bladder. In those animals in which the prostatic structure was preserved, hyperplasia of the glandular epithelium was evident (figure 44). At times, such infections in the lower urogenital tract were combined with suppurative pyelitis or pyelo-nephritis and these were in turn sometimes superimposed upon sclerotic lesions of the kidney.

Nephrosclerosis* is a well-documented finding in irradiated animals (110, 149, 199, 57). It was frequently encountered in the present experiments, sometimes in animals hardly a year old and with increasing frequency in irradiated rats surviving beyond a year. Its extent ranged from slight thickening of Bowman's capsule and the glomerular basal membranes, only demonstrable microscopically, to very extensive, grossly visible lesions. At autopsy such kidneys were usually found to be shrunken, with a granular appearance, while cysts could often be seen macroscopically. Microscopically the familiar fibrosis and hyalinization of glomeruli, atrophy or cystic dilatation of tubules, as well as arteriolar sclerosis and interstitial fibrosis were seen. Infiltration by lymphocytes and occasionally by plasma cells was a not infrequent complication and it was at times impossible to decide whether sclerosis was the basic lesion with chronic pyelonephritis superimposed on it, or vice versa. The possible role of uremia, a likely consequence of severe nephrosclerosis, as a contributing factor in the immediate cause of death in many of the animals, will be discussed.

Fatty degeneration of circumscribed areas of the liver parenchyma (figure 43) was seen occasionally in animals dying during the second year after irradiation. These lesions, if extensive, might also have contributed to the immediate death of the animals. Because of its low incidence however, this lesion was not separately charted in table IX. The fatty infiltration of the adrenal cortex will be discussed when tumorous lesions of the adrenals are described. The importance and possible indirect effects of this complication have been elaborately reviewed by Lamson et al (110).

Since the primary interest of this follow-up study was the determination of tumour incidence

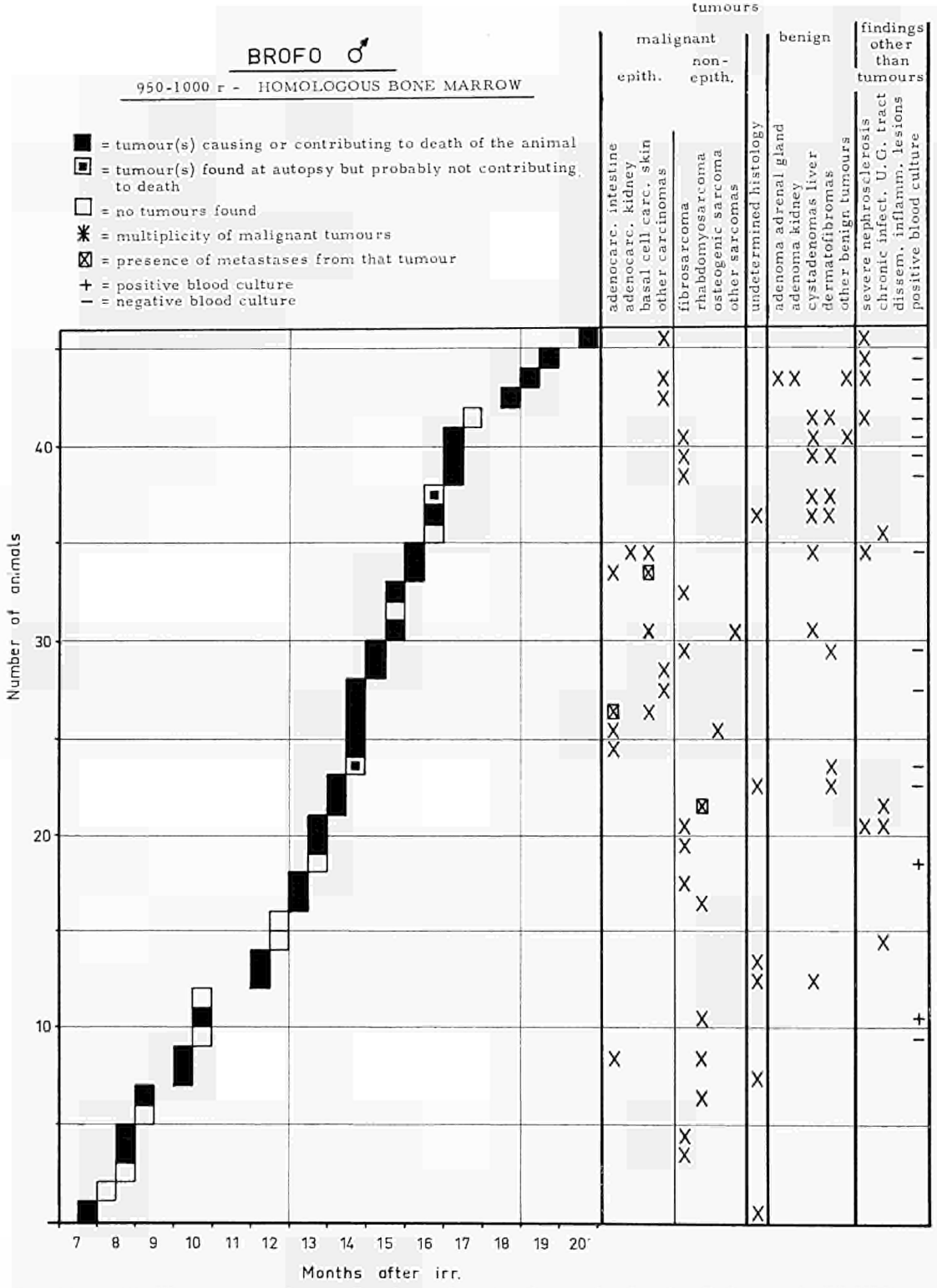
---

* Identical pathological findings as those described in this paragraph have been reported for the kidneys of total-body irradiated animals. The rather general term "nephrosclerosis" was consistently employed to describe these lesions.

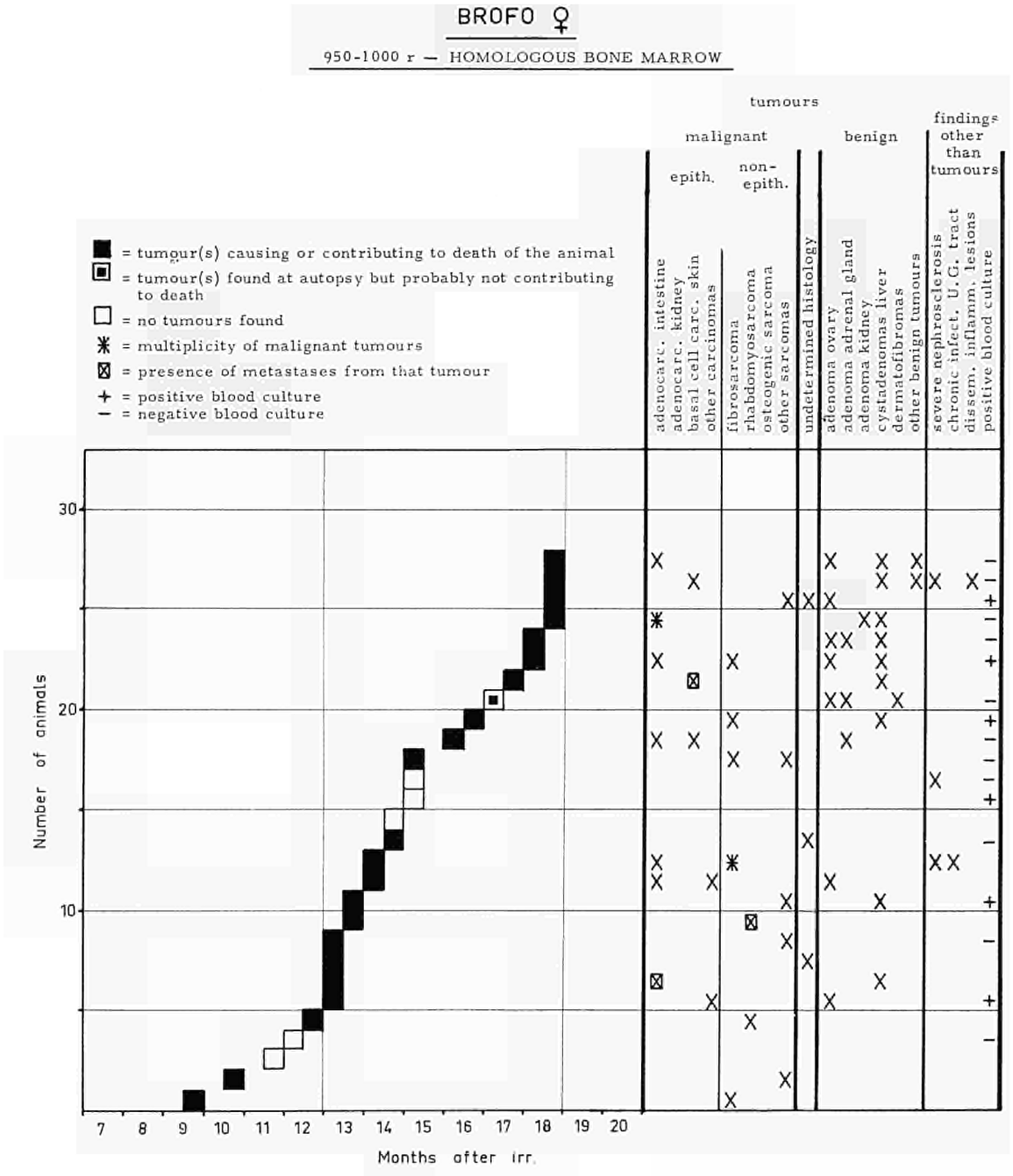
and the time and immediate cause of death rather than an extensive investigation of all possible degenerative changes induced by lethal total-body irradiation, a systematic search for lesions in other organs was omitted. Histological examination of organs such as the pancreas, testis, ovary etc. was usually only performed if lesions possibly connected with a disease or with death of an animal were seen at autopsy or were expected. The central nervous system was only examined when neurological disorders had been observed. The bone marrow, lymphatic tissue, lung, liver and the intestines were routinely examined histologically, the kidneys and adrenals in most of the cases.
At the time of writing, when nearly all experimental animals had died spontaneously, the majority of the non-irradiated controls were still healthy and vigorous with a thick shiny fur; very few had clinical symptoms of obvious lung infections (PPLO or other). Cataract was not seen. One female had died with an extensive urogenital infection, one other with a neurological disorder for which no cause was found. Out of 43 female rats, 4 had developed fibroadenomas of the mamma (figure 39) and one a fibroma; these animals had to be sacrificed when the essentially benign tumours became extremely large (see below). One of the animals, two years of age, had a squamous cell carcinoma originating in the skin of the face. None of the control animals dying spontaneously or sacrificed with large tumours, was less than 18 months old (table VIII). Mild nephrosclerosis was seen in two non-irradiated controls, both at the age of approximately two years.

Incidence and classification of tumours

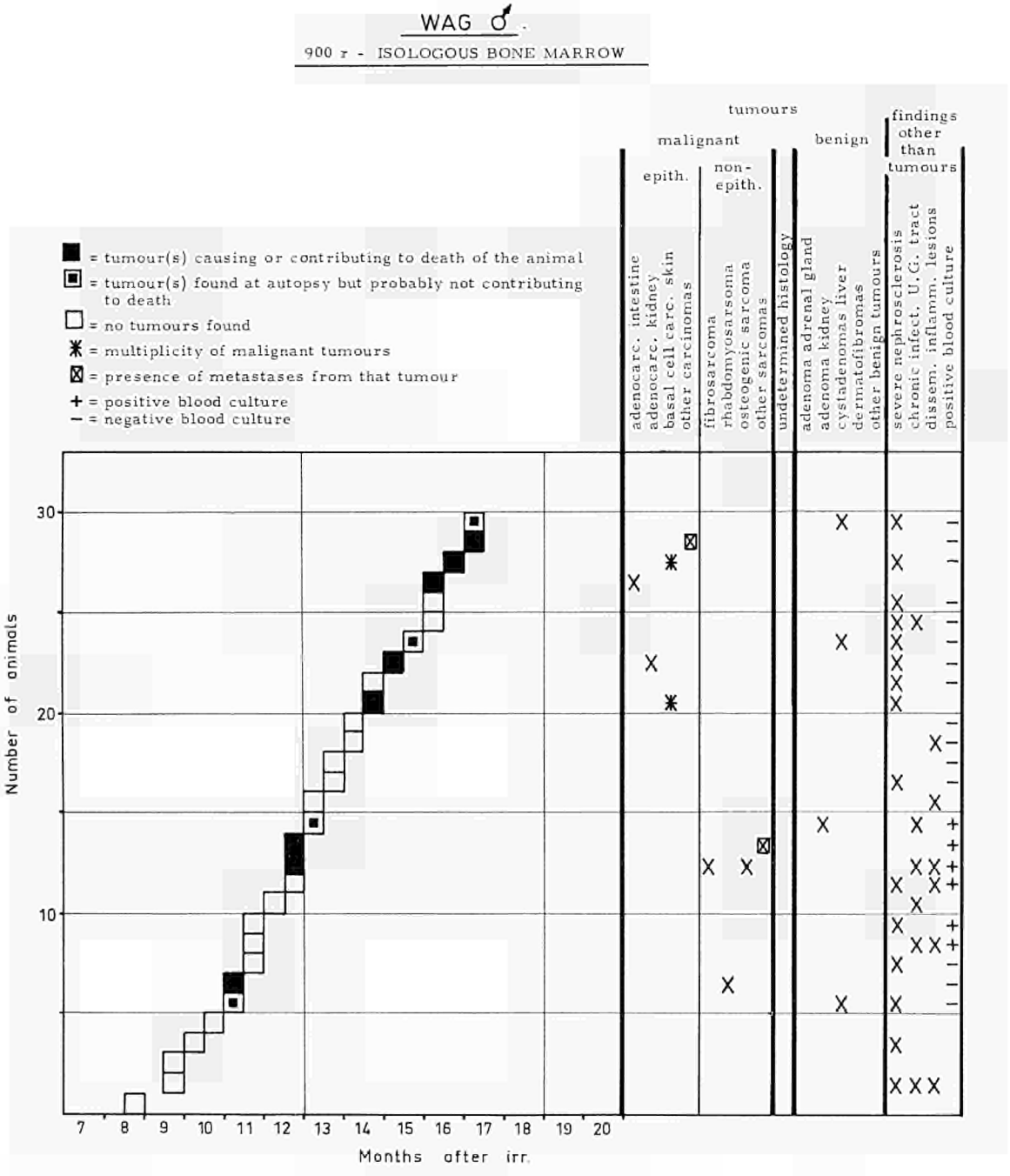
The time course of late mortality for various pooled experimental groups is depicted in text figures 12 to 15. These diagrams show tumour incidence, the probability of tumours as the immediate cause of death, presence of metastases and finally the number of different tumours per animal.
Text figure 12 records these data for the available BROFO males given 950-1000 r irradiation and a protective dose of homologous bone marrow. Many BROFO rats given lower radiation doses (775-900 r) plus homologous bone marrow protection were still alive at the time of writing and their cumulative tumour incidence is not yet presented here; preliminary data indicate however, that no significant difference existed so far between these animals and those given 950-1000 r with regard to time of appearance, histological types, multiplicity and metastasizing of the tumours. Data for similarly treated BROFO females (950-1000 r plus homologous bone marrow) are presented in the diagram of text figure 13. Not all the animals of this group were dead at the time of writing. However, since these females are of particular interest for comparison with irradiated males and with non-irradiated BROFO females, the diagram of their long-term mortality, though still incomplete, is presented. No such diagram is given for male or female controls since so few had hitherto died, but these animals were included in tables VIII and IX, while detailed information with regard to their autopsy findings are presented in the text.
Long-term mortality for irradiated WAG rats is depicted in two separate diagrams, one for animals protected with isologous bone marrow (text figure 14, 3 of the 33 animals were still alive), the other for those treated with homologous bone marrow (text figure 15). Separate charting was done to exclude a possible influence of the genetic origin of the bone marrow



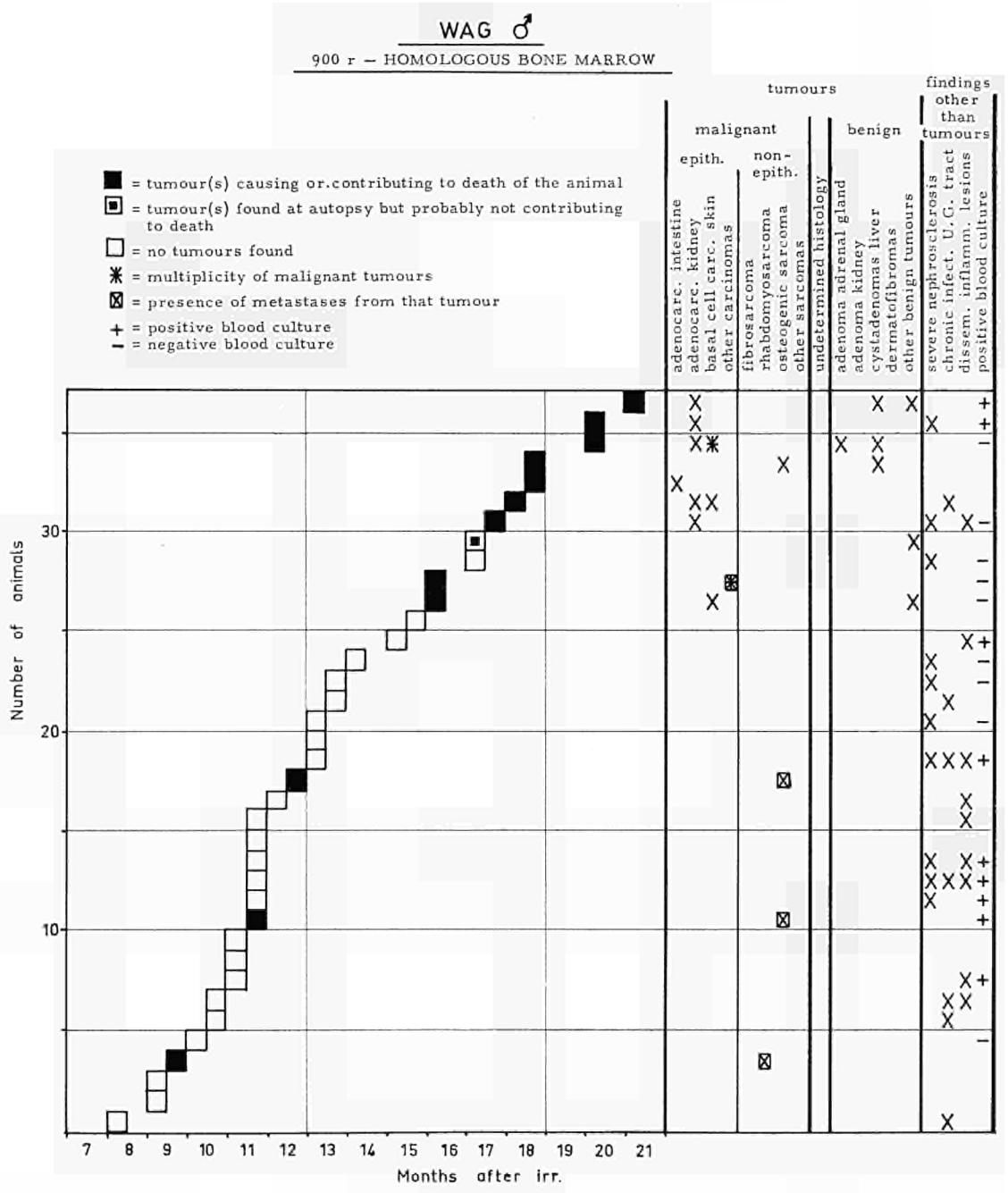
Text figure 12: Time course of mortality from the seventh month after irradiation; male BROFO rats treated with homologous bone marrow.



Text figure 13: Time course of mortality from the seventh month after irradiation; female BROFO rats treated with homologous bone marrow.



Text figure 14: Time course of mortality from the seventh month after irradiation; male WAG rats treated with isologous bone marrow.



Text figure 15: Time course of mortality from the seventh month after irradiation; male WAG rats treated with homologous bone marrow.

graft on the final incidence of tumours and other long-term complications, however unlikely that may be.
All these diagrams record the time of death with or without tumours, but not the time at which the tumours, if present, first appeared. This interval, from the time of detection of outwardly visible tumours to the final succumbing of an animal, was variable but hardly ever shorter than 6 weeks or longer than 4 months. Animals with basal cell carcinomas of the skin (fig. 37) were often followed for a long time before death occurred. These sometimes ulcerating tumours usually did not affect the animal's well-being for prolonged periods. Histologically benign tumours such as cystadenomas of the liver, renal or adrenal adenomas and certainly the dermatofibromas were not likely a direct cause of death of an animal. Other essentially benign tumours, such as the mammary fibroadenomas, could grow so large that the animals had to be sacrificed; these cases were registered as tumour deaths. Large fibrosarcomas, mostly of the subcutaneous tissue, were registered as the cause of death even if vital organs were not infiltrated; in these cases it may not only have been the size of the tumours but possibly also indirect effects that contributed to the emaciated state of the host. Unless a more plausible immediate cause was found at autopsy, such deaths were attributed to the tumour even though its role may have been indirect. It should be emphasized that the determination of the immediate cause of death was often difficult and in some instances arbitrary as will also be exposed in the discussion.

The histological classification of tumours in the present study may require some comment. Certain rules had to be followed to designate a growth as a tumour or not, and at times the choice between benign or malignant was difficult, maybe even arbitrary. In the following paragraphs some of the more problematic types of tumorous lesions as well as a number of benign or malignant tumours are discussed.
Typical fibromas of the **skin or subcutaneous tissue** were rare in the present study. Many of the irradiated animals however showed multiple whitish and rather solid, intracutaneous tumours. These tumours, consisting largely of relatively acellular hyalinized collagen, were usually not more than 1-2 cm in diameter. They were called dermatofibromas in analogy with histologically rather similar tumours seen in clinical pathology. The frequently occurring large solid fibrous tumours, mostly arising in the subcutaneous or retroperitoneal tissues, were termed fibrosarcomas. Although these tumours were not found to metastasize and usually did not show macroscopic infiltration of the surroundings, they were nevertheless classified as malignant tumours on the basis of cellular pleiomorphism, frequency of mitoses and microscopic evidence of infiltration of surrounding tissue at the edges, occurring in most cases.
The label "tumour" was at times difficult to apply in cases of adenomatous lesions of the **adrenal marrow**. Small islands of large cells of uniform morphology and with rather basophilic cytoplasm were frequently seen in the marrow of the adrenals of old irradiated animals; but only if a well-delineated area of such cells, measuring several mm in diameter was present, was this labeled a tumour (adenoma or phaeochromocytoma). Usually a number of mitoses was seen in these tumours. One cannot be sure however, whether these lesions represented autonomous growth or hyperplasia.
Another tumorous lesion, however not classified as a tumour, was the well-documented so-called "nodular hyperplasia" of the **adrenal cortex** (110, 57). It was a very regular finding in

the adrenals of the irradiated rats. Many transitional forms between simple fatty infiltration of not sharply demarcated areas of the cortex and the rather sharply delineated "nodules" of vacuolated cortical cells were encountered (figure 41).
Adenomatous tumours of the cortex of the **kidneys** were somewhat arbitrarily designated as adenoma or adenocarcinoma ("hypernephroma") on the basis of absence or presence of cellular pleiomorphism and the number of mitoses (figure 42). None of these tumours was encapsulated but unequivocal infiltration into the adjoining tissue was usually not seen, nor were metastases found. It should be mentioned that in 3 of the tumours classified as renal carcinomas (table VIII and text figures 14 and 15) a definite choice between adenoma and carcinoma was impossible.
Classification of the tumours of the **liver** was relatively simple; there were the frequently occurring cystadenomas clearly originating from hyperplastic bile ducts; their size ranged from small lesions, discovered only microscopically, to large cystic blebs covering up to two thirds of the liver tissue. Hepatomas, tumours of liver cells, were found in only two instances. They too were classified as benign tumours.
Papillomatous tumours of the **gastro-intestinal tract** were all classified as adenocarcinomas (figure 38) even though in a few instances no actual infiltration into the muscularis of the intestinal wall nor metastatic dissemination was found in the sections. Cytologically and histologically however, these tumours were identical with those showing widespread infiltration and metastases, since cellular pleiomorphism, atypia, anaplasia and large numbers of mitoses were present. The tendency of the intestinal crypts of the rat to proliferate in preformed planes between muscular bundles of the intestinal wall, thereby simulating infiltration, was taken into account when diagnosing actual infiltration by such neoplasms.
Identification of **ovarian tumours** found in the present study followed the classification previously applied for mice by others from this laboratory (166). Of the 4 common types, only those derived from granulosa cells were encountered. Transitional forms between pure granulosa cell tumours and "luteomas" were seen but tubular adenomas, so frequently seen in mice, were not encountered and neither were cystadenomas found.

Comparison of mortality rate and tumour spectrum for the various groups of rats

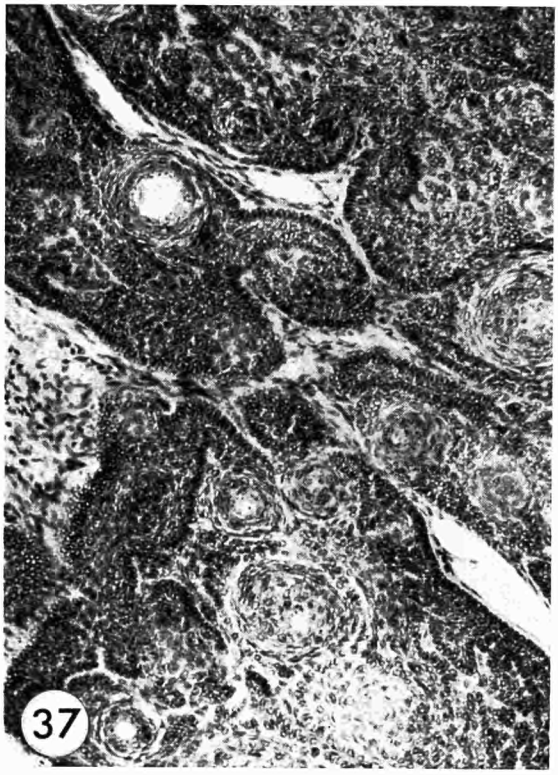
Text figure 12 demonstrates that all irradiated BROFO males succumbed in the period from the 7th to the 20th month, the majority with tumours that either caused, or contributed to the immediate death of the animals. Although the relatively small number of animals precludes an accurate statistical evaluation, the data suggest that certain tumour types tended to occur soon after irradiation, others relatively late. Rhabdomyosarcomas (figure 47), for instance, appeared rather early (this was confirmed in other irradiated groups, some not included in the present survey) while basal cell carcinomas and the benign dermatofibromas arose mostly during the second year after irradiation. The frequently occurring fibrosarcomas (figure 45) of the subcutaneous tissue were found at any time after the 6th month. Though there was certainly no statistically valid evidence that certain tumours frequently occurred simultaneously, the available data suggest a tendency for some tumours, possibly those subject to hormonal influences, to occur simultaneously more often than would be expected by chance (text figure 13). So-called nodular hyperplasia of the adrenal cortex was a frequent finding in older

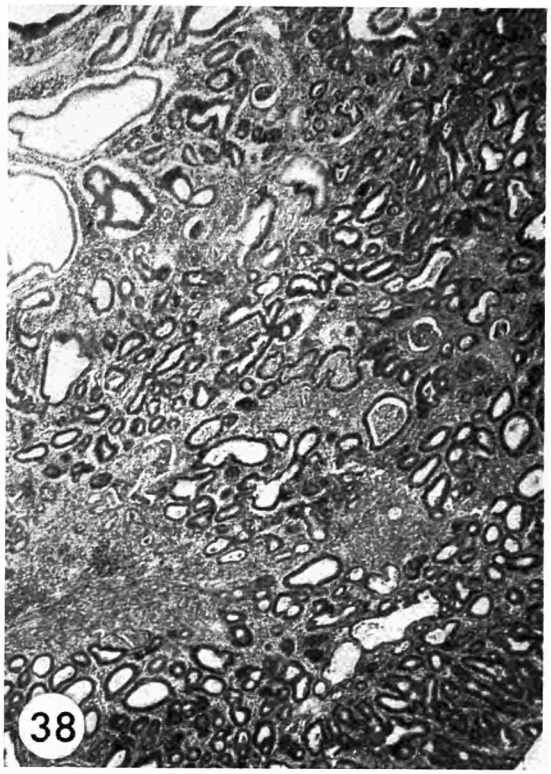
Figure 37
Basal cell carcinoma of the epidermis in Wag male rat, 14 months after irradiation and isologous bone marrow therapy. Note abortive formation of hair follicles. x 120

Figure 38
Polypoid adenocarcinoma of the rectum in BROFO female rat, 16 months after irradiation and homologous bone marrow therapy. x 30

Figure 39
Fibroadenoma of the mammary gland in female BROFO control rat, 24 months of age. x 30

Figure 40
Papillary adenocarcinoma of the mammary gland in a female BROFO rat, 14 months after irradiation and homologous bone marrow therapy. x 30


37


38


39

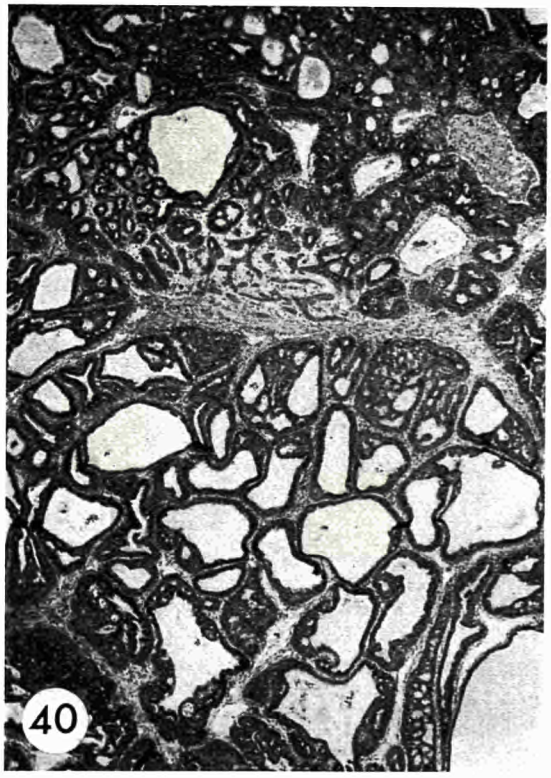
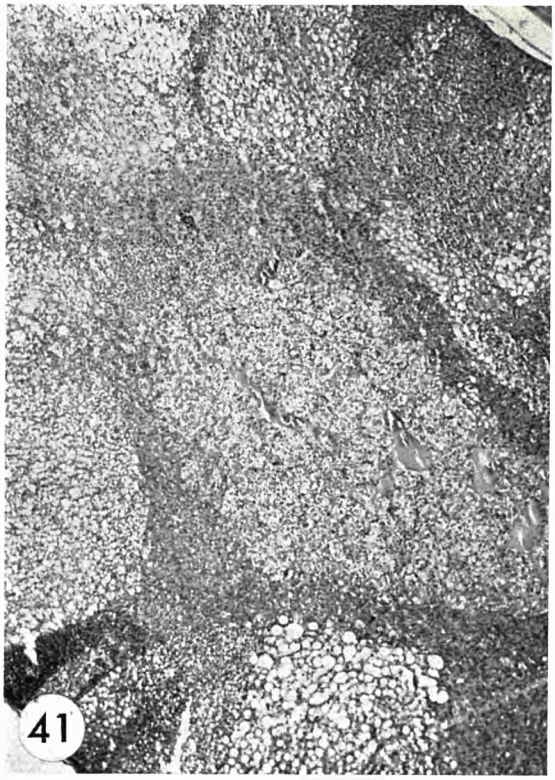

40

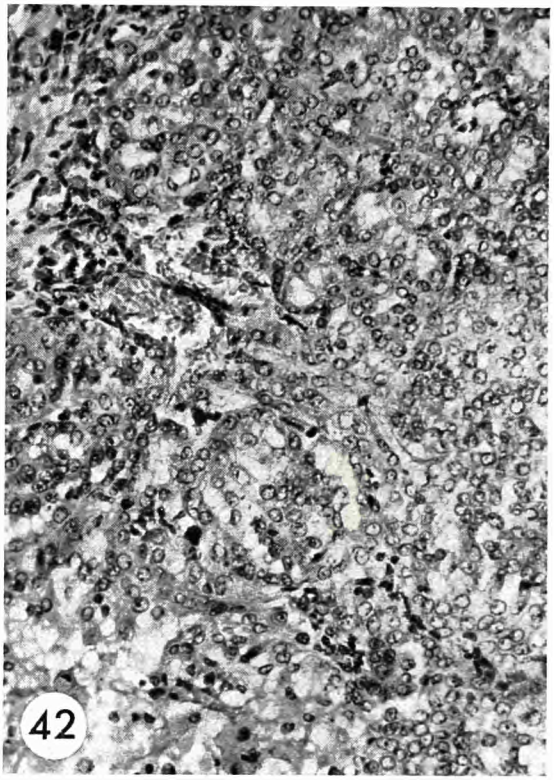
Figure 41
Fatty alterations of the adrenal cortex, sometimes called "nodular hyperplasia", was found in the majority of rats surviving beyond one year after irradiation. x 30

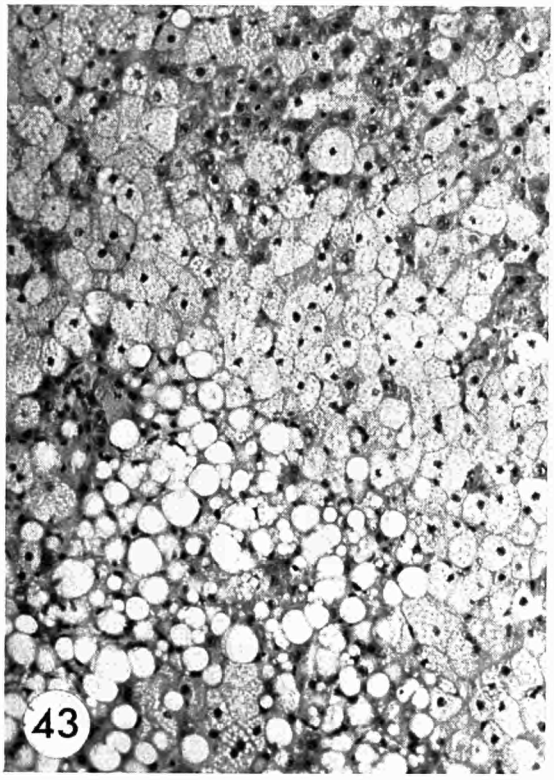
Figure 42
Adenocarcinoma of the renal cortex in BROFO male rat 16 months after irradiation and homologous bone marrow therapy. Note necrotic area in the left lower corner of photo-micrograph. x 190

Figure 43
Focus of fatty infiltration in the liver as seen in several of the older irradiated rats. x 120

Figure 44
Typical example of an inflammatory lesion of the lower urogenital tract as it frequently occurred in irradiated WAG males: purulent or necrotizing prostatitis with absces formation depicted in the lower part and papillary proliferation of glandular epithelium in the upper part of the photo-micrograph. x 30


41


42


43

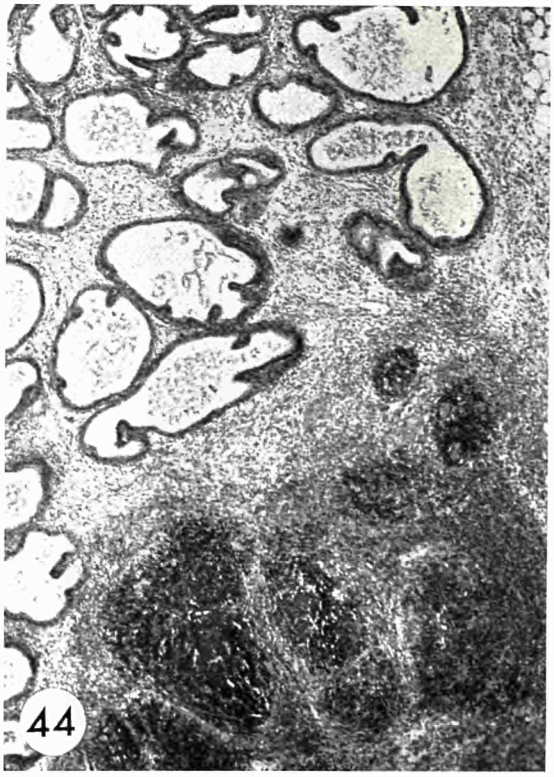

44

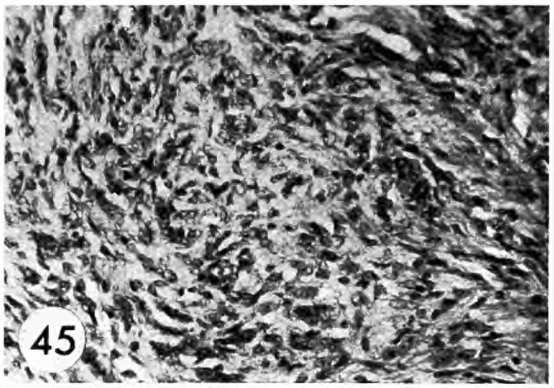
Figure 45
Subcutaneous fibrosarcoma as frequently seen in BROFO rats, 6-18 months after irradiation. Note great cellularity and relative scarcity of collagen fibers. x 190

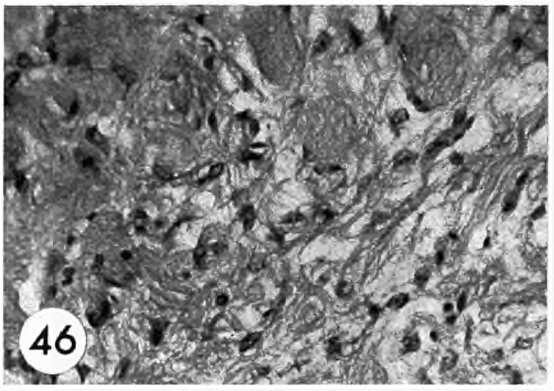
Figure 46
Subcutaneous fibrosarcoma showing an abundance of collagen fibers. This relatively acellular type of fibrosarcoma was less frequently found than the type depicted in figure 45. x 190

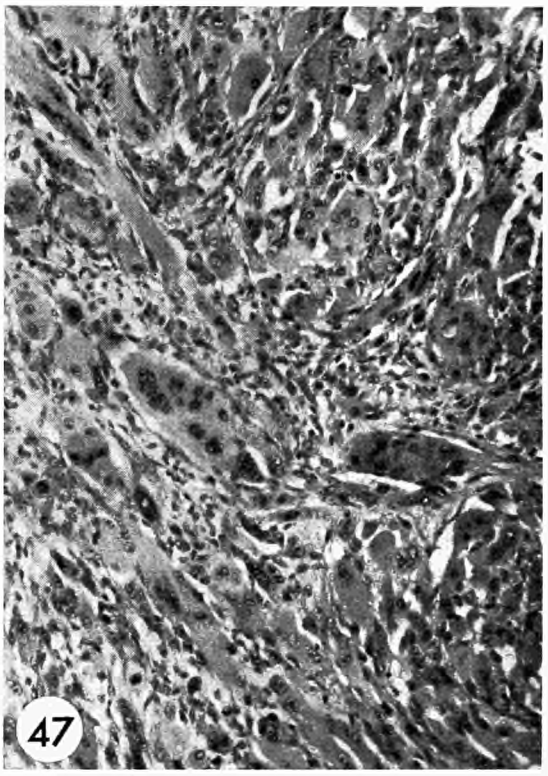
Figure 47
Rhabdomyosarcoma of the subcutaneous tissue, a type of tumour found relatively soon after irradiation (from the 6th to the 14th month). Metastases in lungs and lymph nodes from such tumours, if present, also showed the long, straplike myoblasts, staining strongly positive for myofibrils. x 120

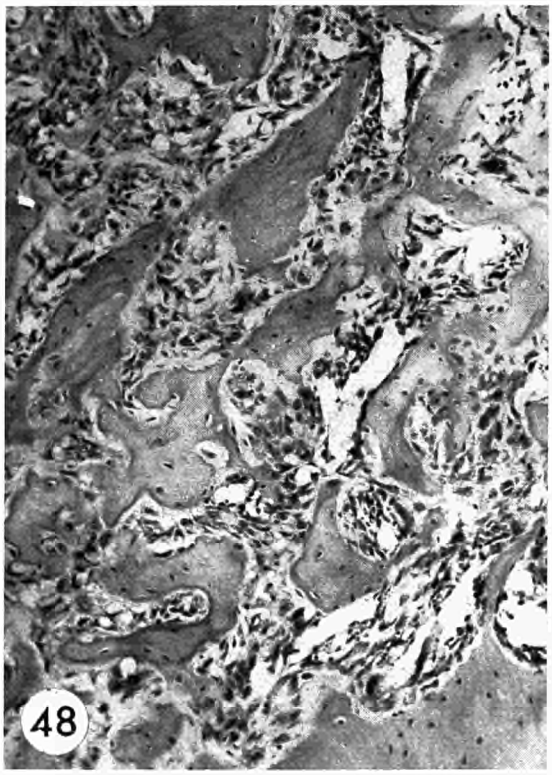
Figure 48
Osteosarcoma originating in the sternum of a male WAG rat, 12 months after irradiation and isologous bone marrow therapy. The tumour had eroded the cortex and invaded the surrounding muscle. x 120

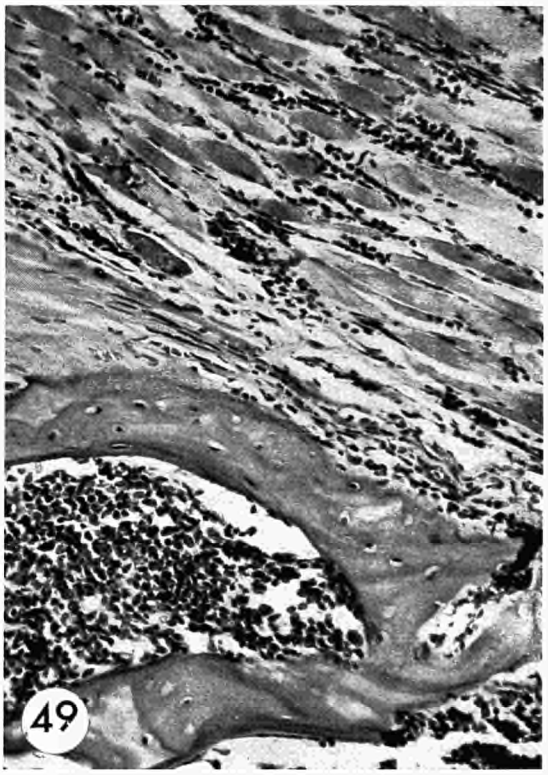
Figure 49
Lymphatic leukemia in an isologous WAG chimera 12 months after irradiation. The photo-micrograph shows infiltration of the marrow space of the sternum and surrounding muscle with leukemic cells. x 190


45


46


47


48


49

chimeras but was rarely found before the second year after irradiation. As has been pointed out, this lesion was not regarded as neoplastic; it is not listed in the diagrams and tables.

Text figure 13 presents similar data for irradiated BROFO females also treated with homologous bone marrow. In general there was little difference in mortality rate and tumour incidence between males and females. The slightly delayed mortality of females as compared to males is probably not statistically significant. Types and origin of the tumours did not show striking differences between the sexes though there seemed to be certain trends: the incidence of fibrosarcomas appeared somewhat lower in females and the majority of these tumours did not arise in the subcutaneous tissues as in males, but rather in the retroperitoneal fibrous tissues. Rhabdomyosarcomas again appeared rather early, all carcinomas rather late.

However, a significantly different tumour distribution and incidence is noted when the data for irradiated WAG rats are studied in text figures 14 and 15. Whether protected with isologous or homologous bone marrow, the tumour incidence seemed significantly lower than that of BROFO rats, male or female. Infectious disorders, on the other hand, were more frequent in WAG rats. It could be argued that WAG rats did not live long enough to produce a tumour incidence as high as that for BROFO rats but, in general, life expectancy of both WAG groups was similar to that of the BROFO rats. The tumour incidence did indeed become much higher from the 15th month on, equaling that of BROFO rats; this indicated that it could indeed have been equally high, had not most of the WAG rats died from intercurrent disease. The difference in tumour frequency between irradiated WAG and BROFO rats would thus be reduced to one of time of occurrance rather than of susceptibility (see discussion). As for histological types, there was a comparatively high incidence of metastasizing osteogenic sarcomas (figure 48) and of renal tumours in irradiated WAG males, while subcutaneous fibrosarcomas, so common in BROFO rats, were rather rare. The few rhabdomyosarcomas again appeared early in life, the basal cell and other carcinomas late.
The small difference in radiation dose (900 r versus 950-1000 r respectively) cannot possibly account for the significant difference in tumour incidence between WAG and BROFO rats. This was born out by data accumulated for groups of BROFO males given lower radiation doses (775-900 r) who, so far, showed a tumour incidence and distribution virtually identical with that of BROFO males given the somewhat higher doses (950-1000 r).

A separate diagram for the initial mortality rate and tumour incidence of non-irradiated BROFO controls will not be given. Only one of the males had so far died: an interesting kidney lesion was seen which is sometimes present in humans suffering from diabetes mellitus (the Armanni-Ebstein syndrome), in which cells of the renal tubules are found to be loaded with glycogen. Six of the female BROFO controls were dead with tumours; four of these tumours were histologically benign mammary fibroadenomas, one was a large subcutaneous fibroma and one a malignant tumour of the skin, a squamous cell carcinoma. The five benign tumours had grown so large that the animals had to be sacrificed. Evidence of malignancy was not found in these huge tumorous masses. The two female control animals dead without tumours, have been discussed in the previous section.
A breakdown of all the tumour data presented in text figures 12-15 as to frequency, period of occurrance, localization and histological characteristics, is given in table VIII. Distribution of

TABLE VIII

INCIDENCE AND (HISTOLOGICAL) TYPES OF TUMOURS FOUND AT AUTOPSY DURING VARIOUS POST-IRRADIATION PERIODS (IN MONTHS)

			irradiated									controls*					
			BROFO ♂ (46)			BROFO ♀ (33)			WAG ♂ (70)			BROFO ♂ (29)			BROFO ♀ (43)		
			6-12	12-18	18-21	6-12	12-18	18-21	6-12	12-18	18-21	6-12	12-18	18-21	6-12	12-18	18-21
malignant	epithelial	carcinoma intestine	1	4			7			2							
		" mamma					1										
		" kidney		1	1					3	3						
		" pancreas		1			1										
		" testis			1					1							
		" prostate								1							
		" basal cells skin		4			3			5	1						
		" other origin		2													1
	non-epithelial	fibrosarcoma	2	8		1	4		1								
		rhabdomyosarcoma	3	2		1	1		2								
		osteogenic "		1					3	1							
		sarcoma of other origin		1		1	4										
		leukemia (lymphatic)							1								
benign	epithelial	adenoma lung			1												
		" kidney			1		1			1	1						
		" adrenal gland			1		3				1						
		" ovary					7										
		fibroadenoma mamma					2									2	2
		cystadenoma parotis		1													
		" " liver	1	7			9		1	3	2						
		hepatoma								1	1						
		epidermal cyst								1							
	non-ep.	dermatofibroma		7			1										
		fibroma		1													1
number of animals dying during a certain period (post-irradiation)		with one or more malignant tumours	3	19	2	3	16	-	6	11	3	-	-	-	-	-	1
		with one or more benign tumours	1	11	1	-	14	-	1	6	2	-	-	-	-	2	3
		with a tumour of undetermined histology	4	1	-	-	3	-	-	-	-	-	-	-	-	-	-
		with benign or malignant tumours (proportion and percentages)	9/16 56%	23/27 85%	3/3	3/5	20/23 87%	-	7/32 22%	15/32 47%	-	-	-	-	-	2/3	4/5

* the intervals for the non-irradiated controls were calculated from the time they were 4 months old.

TABLE IX

PROBABLE CAUSES OF DEATH

death mainly attributed to	principal findings at autopsy	irradiated			controls*	
		BROFO ♂	BROFO ♀	WAG ♂	BROFO ♂	BROFO ♀
tumour	tumour only	26	15	10		6
	tumour plus severe nephrosclerosis and/or infections	7 — 33	7 — 22	10 — 20	0	— 6
uremia	severe nephrosclerosis only	1	1	12		
	severe nephrosclerosis + inflammatory lesions	- — 1	- — 1	8 — 20	0	0
infection	localized or disseminated inflammatory lesions	1	-	13		1
	only positive blood culture	1 — 2	1 — 1	- — 13	0	- — 1
undetermined		10	4	14	1**	1
	number of animals dead out of total	46/46	28/33	67/70	1/29	8/43

* data are given up to 21 months after the time of irradiation. The controls were then a little over 2 years of age.

** the findings for this animal are described in the text.

benign and malignant tumours over the various experimental groups is also presented in this table. Table IX finally records, somewhat arbitrarily, the probable causes of death of the animals.

DISCUSSION

The main purpose of this follow-up study was to determine the fate of large groups of lethally irradiated bone marrow protected rats. The time and, whenever possible, cause of death were recorded and particular attention was paid to the incidence of benign and malignant tumour growths. As could be expected from the results of other investigators who had made similar long-term observations on sublethally and lethally irradiated rats protected by other means than bone marrow grafts, longevity of the animals was sharply reduced and the incidence of certain degenerative changes and, above all, of tumours was high.

There is a vast body of experimental data on the carcinogenic effect of ionizing radiation in general and the subject has been extensively reviewed in the literature (42, 84). As for the effect of total-body irradiation on tumour incidence in mammals, most of the early observations have been made on mice, first on animals surviving sublethal radiation given in single, protracted or fractionated doses (82, 198), later also in mice surviving a single, supralethal radiation dose after shielding of limbs or injection of hemopoietic cells (51, 149). The effect

of irradiation on bone marrow protected animals was a reduction of life-span and an increased incidence of tumours that were normally rare while leukemia and lymphomas, occurring rather frequently in some of the mouse strains, were reduced in number (83). Decreased longevity and a shift in the causes of death was stressed by these authors while tumour incidence was linked to so-called "accelerated aging" (see introduction of this chapter).

The effect of lethal total body irradiation on rats was first studied in animals kept alive by temporary parabiosis or by chemical protection. Brecher et al (40) found that in 9 out of 32 surviving female Sprague Dawley rats various types of tumours appeared within a year after irradiation, several of which were normally not seen in rats allowed to live out their normal life-span (63, 168). Similarly, Finerty et al (79) found several neoplasms of uncommon histological types in some 30 female Holtzman rats also surviving due to temporary parabiotic union with non-irradiated animals. When these latter were followed until their natural death, no tumours had developed. The early appearance, high incidence and uncommon histological types of the observed tumours led both groups of authors to the conclusion that the neoplasms were actually induced by irradiation and not merely an expression of shortened latency or hastened senescence. However, in view of the more recent theories mentioned in the introduction of this chapter, the difference between these alternatives may be simply one of terminology. Irradiation may simply reduce the period of latency for tumour induction which is normally longer than the animal's life-span, to one that is shorter than it. This would eliminate the difference between the two modalities.

Koletsky and Gustafson (104) came to a similar conclusion as did Finerty et al., based on data from sublethally irradiated (660 r) male Wistar rats. They regarded total-body irradiation as a highly potent carcinogenic agent, causing its effect through either direct or indirect action. The multiplicity of tumours in many older irradiated animals led to a theory of a systemic factor, a "tumour diathesis", brought about by hypothetical radiation-induced alterations in the internal environment, favourable to tumour growth.

Lamson et al (110) however reached quite different conclusions. Their lethally irradiated rats were protected by low oxygen tension during irradiation and the impression was that tumours in the irradiated animals appeared sooner but not necessarily in greater numbers than in controls. Unlike the three groups of investigators mentioned in the previous paragraphs, who sometimes kept separated parabiotic partners with a limited life-span as controls, Lamson and collaborators kept normal control animals until their natural deaths. Observing an incidence and variety of tumours in their oldest controls nearly comparable to that of irradiated animals, they claimed, as did Upton and Furth (198), that the seemingly different incidence of neoplasia was simply the consequence of a shift in the time of occurrance. Tumours other than of the ovary, and possibly of the lung, the breast and the liver, were not believed to have been specifically increased by irradiation.

The "controversy" can obviously be reduced to the following question: do the many tumours arising so soon after total-body irradiation of an animal also occur, at a later stage, in non-irradiated control animals? Some of the investigators apparently think they do not (which would make those tumours "radiation induced" in the original sense), others think that they would occur anyway. Rosen et al (170) did not find renal tumours in controls while many occurred in their irradiated rats. They rightly argued that a comparable incidence of renal

tumours also in controls, would be statistically inevitable, provided the animals survived long enough. But such reasoning has obviously little practical value in view of the actual life-span of laboratory animals. Besides, for the health physicist and all those concerned with the clinical aspects of whole-body irradiation, the immediate consequences are the important ones. The question whether an individual would eventually have developed a similar tumour incidence had he lived out his normal life-span, becomes purely academic in this context.

The tumour spectrum of irradiated BROFO and WAG rats.

COMPARISON WITH RESULTS OBTAINED BY OTHER INVESTIGATORS FOR DIFFERENT RAT STRAINS.

The incidence and classification of spontaneously occurring tumours in rats is not as well known as that for mice. From a number of studies concerned with the occurrance of spontaneous tumours in many different rat strains it was concluded, however, that the incidence is comparatively low (63, 168). As in mice, the mammary fibroadenoma is the most common benign tumour and of the malignant tumours sarcomas outnumber the carcinomas. This seems also true for the non-irradiated control animals of the present study though the frequency of mammary fibroadenomas in female BROFO rats was, already at this time, significantly higher than any percentage of spontaneous tumour incidence reported in the literature. Only one malignant tumour had as yet developed in the control animals. The final incidence of spontaneously occurring tumours depends to some extent on the life expectancy of the experimental animal and in this respect SPF breeding of the animals, as was the case in the present study, again offers particular advantages. Many a control animal of a conventional colony might by this time have been dead of bronchopneumonia or another intercurrent infection, thereby excluding itself as a potential tumour bearer.
In spite of the fact that, at the time of writing, the majority of the control rats was still alive, it seems reasonable to assume that irradiation, by whatever mechanism, had increased or precipitated the incidence of benign and malignant tumours. In view of what has been reported about the spontaneous tumour incidence in rats, it seems rather unlikely that the control animals of the present experiments (even though they are SPF bred with a high life expectancy) would still develop the multitude and variety of malignant tumours seen in the irradiated animals. Obviously, such a possibility cannot be excluded, though.

A decreased frequency of some tumours that are normally found and an increase of tumours not spontaneously encountered, has been reported previously as a consequence of total-body irradiation in mice (83). Nowell and Cole (149) stressed the occurrance of ovarian tumours and various carcinomas in neutron-irradiated mice while, at the same time, the incidence of leukemias and lymphomas was reduced in a strain with a high spontaneous incidence of such neoplasms. In lethally irradiated mice protected with injected hemopoietic cells this latter "therapeutic" effect was even more outspoken (51). A similar trend can already be detected in the present experiments, when the incidence of mammary tumours in experimental animals (low) and controls (high, so far) are compared. Lamson et al (110) had also found a shift in the incidence of certain tumour types occurring in irradiated rats and their controls; they suggested a hormonal inbalance in the irradiated animals, indirectly promoting tumour growth in several organs. The frequently occurring changes in the histological appearance of the adrenal cortex in old irradiated rats were implicated as the link in this mechanism.

The virtual absence of leukemias and lymphomas in the present study is not surprising. Already in 1953, Kaplan and Brown (101) had described the protective effect of the injection of hemopoietic cells against radiation-induced mouse lymphomas.
Total-body irradiation is known to produce a variable incidence of leukemias in rats (104, 110, 116) though not much is known about the susceptibility of different rat strains in this respect. In the present study, only one case of leukemia (lymphatic) was found amongst the many very variable tumours in the irradiated rats. One explanation may be the circumstance that virtually all animals were permanent chimeras, their hemopoietic and lymphatic system being composed of donor-type cells that had never been irradiated. Other explanations for such "therapeutic effect" of proliferating hemopoietic cells against radiation-induced leukemia have been suggested (51). Significantly, the one leukemia found here, occurred in an isologous chimera that had received 900 r of irradiation, a just sublethal dose; consequently the tumour might have arisen from surviving host-type cells. Whenever pieces of other tumours, induced in homologous chimeras, were grafted, the tumours "took" if transplanted into members of the host strain, not in animals genetically identical with the bone marrow donor. Parenthetically it should be mentioned that leukemia and lymphomas developing in mouse radiation chimeras have been reported to be genetically either of the host- or donor-type.

The time of appearance of most malignancies in the present study was rather similar to that reported by others. Significant differences, however, were noticed in the frequency of certain histological types encountered. Some differences may be due to the fact that in the present study, a number of organs were not routinely examined histologically but only if lesions were grossly visible or could be expected. The fact that our irradiated rats had been protected with bone marrow might account for the virtual absence of leukemias and lymphomas, as has been mentioned before.
Another reason for the somewhat different tumour spectrum reported by others, might be a variable susceptibility to tumour induction of the various strains employed. Most studies mentioned above, also report data on strains of Wistar rats, but it can be seen from table VIII and text figures 12-15 that even WAG and BROFO rats, both derived from Wistar stock, showed marked differences, not only in the tumour incidence during the first 15 months after irradiation, but to some extent also in the histological types of the tumours encountered. Studies by Dunning (71) and Sydnor et al (183) may be relevant in this context. These authors observed significantly different responses by various rat strains to a number of carcinogens other than irradiation.

The absence of intestinal neoplasms in the irradiated Wistar rats in Lamson's and Koletsky's studies is certainly puzzling in view of the rather high incidence of such tumours found here for WAG and BROFO rats. It would be tempting to speculate, that damage caused by the graft-versus-host reaction in rats protected by homologous bone marrow might somehow enhance X-ray carcinogenesis. However, this type of tumour also occurred in isologously treated WAG rats (one of the 5 animals with tumours had an intestinal adenocarcinoma (text figure 14)). Also the pathogen-free (SPF) breeding of the animals is not likely to be of importance in this respect. Brecher et al (40) did find several intestinal carcinomas in a small group of irradiated Sprague-Dawley rats that were certainly not SPF bred. Besides, with an altered intestinal flora, containing fewer pathogens, one would hardly expect a lower intestinal tumour

incidence (see below), unless an effect by certain bacteria on the local oxygen tension is considered a possibility (149).

The question of pre-existent inflammatory or degenerative lesions as a potentiating factor in X-ray carcinogenesis should also be considered. If chronic irritation associated with cellular proliferation enhances the carcinogenic effect of irradiation, one would expect a rather high incidence of tumours in those locations where inflammations or degenerative changes most frequently occur. The high frequency of renal carcinomas from the 16th to the 20th month after irradiation in the homologously treated group of WAG rats would support such reasoning. A particularly high incidence of severe nephrosclerosis (frequently combined with pyelonephritis) seemed associated with a high incidence of renal tumours at a later stage (text figure 15). However, the isologously treated WAG rats only produced one malignant renal tumour in spite of a comparable incidence of early severe nephrosclerosis with concomitant urogenital infections. Since the genotype of the injected bone marrow can certainly not explain this difference, a relation between sclerotic and inflammatory changes in the kidneys and renal carcinomas cannot be assumed. Rosen et al (170) came to similar conclusions with regard to the renal carcinomas in their irradiated Sprague Dawley rats; non-irradiated controls of an advanced age often showed comparable nephrosclerosis with extensive scarring, but renal tumours never occurred.
In discussing the occurrance of intestinal tumours in irradiated rats, Brecher et al (40) also ruled out infectious lesions as a contributing factor in X-ray carcinogenesis; they found no inflammations at the site of the tumours. In accordance with these authors, a preference of tumour localization for sites of inflammation was not found in the present experiments.

Other factors contributing to death of the animals.

INFECTIONS AND DEGENERATIVE LESIONS.

Long-term complications other than tumour formation, have been given relatively little attention in the present work and will not be elaborately discussed. As has been mentioned, a systematic study of such long-term effects was not intended. Degenerative and infectious complications were mainly of interest when contributing to the immediate death of the animals. Unfortunately, an immediate cause of death was not always found, both in animals succumbing with and without tumours. Positive blood cultures and histological evidence of septic foci in the liver, lung or elsewhere, sometimes provided a clue and such animals were then listed under "infectious deaths" in table IX.
The extent of the sclerotic lesions in the kidneys of many of the irradiated rats dying relatively young with no other obvious cause of death, suggests the possibility of uremia as a major factor. It may have been the immediate cause of death in the majority of WAG rats dying within the first 15 months after irradiation (text figure 14 and 15). Several investigators have pointed to this possibility and have associated such severe nephrosclerosis with extensive cardiovascular changes in the animals and the likelihood of clinical uremia (110).
In other cases, no indication whatsoever was found as to the possible cause of death or, as occurred rather frequently, congested or partly infarcted lungs were seen at autopsy for which no obvious reason was detected. A far more meticulous clinical follow-up, including regular hematological surveys, determinations of blood pressure and blood urea etc., would probably

have been required to determine the cause of death in some of the cases that are now classified as "undetermined".

In spite of the incompleteness of the data, a tentative classification of the possible causes of death has been presented in table IX. If an animal carried one or more malignant tumours but had nephrosclerosis and inflammatory lesions as well, it was arbitrarily decided to attribute such deaths to the tumours. The implications of the high incidence of tumours and renal complications relatively soon after total-body irradiation and bone marrow therapy will be briefly discussed in chapter VIII.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

The presented experimental work, mainly dealing with bone marrow therapy of the irradiated rat, can be conveniently divided into a number of separate subjects.

1) **Primary survival** after irradiation and complications occurring during the 1st month post-irradiation (chapter III and part of chapter V).
2) **The secondary complications** mainly occurring during the 2nd and 3rd month post-irradiation (chapter IV and part of chapter V).
3) **The immune reactivity** of permanent and temporary chimeras and information concerning the development and maintenance of specific immunological tolerance towards transplantation antigens (chapter VI).
4) **The late effects** of high total-body irradiation (chapter VII).

Additional comments and certain conclusions will be presented in the following pages for each subject separately. Since the problems of immune reactivity and tolerance have been elaborately discussed in chapter VI, no additional comments or conclusions will be given here.

Survival after total-body irradiation

The results presented in chapter III clearly demonstrate that hemopoietic death in rats can be prevented by the intravenous administration of homologous bone marrow. A considerable number of animals died during the first 8 days after irradiation in spite of the injection of an adequate number of bone marrow cells. Such early mortality occurred both after high sublethal (900 r) and after lethal irradiation doses (950-1000 r) and was not attributable to irreparable damage of the intestinal tract (the intestinal syndrome). There was some evidence that infection played a role in those early deaths. The fact that isologous bone marrow therapy produced a somewhat lower rate of early mortality (as discussed in chapter V) may have been due to a better immediate therapeutic effect and a proliferative advantage of the more compatible grafted bone marrow cells.

Histocompatibility also played a role as far as the **number of grafted bone marrow cells** necessary for survival was concerned. Similar to what had been shown previously for certain other species, the effective number of homologous cells was about 20 times as high as that for an isologous bone marrow graft. This difference was consistently found and has been attributed to residual reactivity of the host's immune system even after high doses of total-body irradiation. This assumption has been difficult to prove though, and other factors may be involved as well.

The earliest complication after initial survival was the so-called **"delayed rejection"** of an established graft resulting in (delayed) hemopoietic death during the 3rd and 4th week after irradiation. This occurred mainly if the homologous bone marrow graft had been relatively small (less than 100 x 10^6 cells). Again, it is not quite clear why such rejection of an established graft belatedly occurs. If this is due to recovery of the host-type immune system it is difficult to understand why concomitant recovery of hemopoiesis should be inadequate to keep the animal alive. If an animal does not die under these circumstances the event is referred to as early reversing to host-type hemopoiesis rather than to delayed rejection of the graft; it was indeed seen in several instances after high (but just sublethal) irradiation doses and small bone marrow grafts (text figure 7). Usually, **reversing to host-type hemopoiesis** occurred later, from the 2nd to the 5th month after irradiation. Late reversing has also been attributed to recovering host-type immune reactivity. Lately however, there have been reports indicating that reversing need not necessarily be the result of an immunological rejection mechanism. A kind of proliferative competition between the two hemopoietic tissues has been proposed (15). This may in the long run lead to a gradual replacement of those cells that are less adapted to the environment. Prolonged co-existence of the two homologous hemopoietic systems implies mutual tolerance and some of the consequences and possibilities of this type of tolerance have been explored in chapter VI.

Lethal irradiation followed by more than 100 x 10^6 homologous bone marrow cells did as a rule not result in delayed rejection of the graft or reversing, but led to **permanent chimerism.** In general it took 2-3 months until all host-type erythrocytes were replaced by donor-type red cells, which fact (amongst other data) suggested that selective immune hemolysis of host-type erythrocytes did not occur in rat chimeras. Conversion of leukopoiesis from host- to donor-type was considerably faster.

Sublethal radiation doses followed by homologous bone marrow therapy did not lead to increased mortality (the "MLD-effect") which has been found to occur in certain mouse strain combinations (see chapter III). Such an **MLD-effect** had also been reported for rats by some investigators (151) but could not be confirmed in the present study.

Secondary complications

The occurrence of secondary disease in irradiated rats treated with homologous bone marrow as well as the accelerated type of graft-versus-host reaction following the injection of homologous lymphoid cells was described in chapter IV and V. Morbidity due to secondary disease in bone marrow treated rats was moderate; mortality was rather low, hardly ever exceeding 20 % of the total number of animals. The symptomatology was nearly uniform for each strain combination, generally starting with weight loss and skin lesions, except in WAG hosts where skin lesions were negligible even after administration of homologous lymphoid cells. The animals deteriorated rapidly and often died in an emaciated state during the 2nd or 3rd month after irradiation and homologous bone marrow therapy. As has been pointed out already, the virtual absence of intestinal lesions and diarrhea in fatal secondary disease of bone marrow treated rats was surprising. Even when homologous lymphoid cells had been administered and histological lesions of the intestinal epithelium were evident, diarrhea was absent. A possible

connection between this peculiarity and the probability of a reduced intestinal flora in SPF-bred rats has been suggested (discussion in chapter V).
In the following pages a short review will be given of the available evidence for an interaction in secondary disease between donor cells or the antibody produced by them, with the affected host-type cells or tissues. Finally the manifestations of secondary disease that may contribute to the death of the animals will be analyzed.

Possible interaction between cellular or humoral antibody and the affected host tissues in secondary disease

Numerous experiments have been carried out to assess the role of lymphoid cells or of humoral antibody in the production of symptoms in secondary disease and in other graft-versus-host reactions (175, 76, 102, 89, 161). Whether these hosts are newborns, F_1 hybrids, or animals that were irradiated or treated with radiomimetic drugs, a sometimes fatal syndrome can be caused by cellular grafts in such immunologically "defenseless" hosts. The mechanisms of interaction between antibody (humoral or cellular) and the target tissues in graft-versus-host reactions is still largely unknown (176). Attempts to demonstrate direct interaction of humoral anti-host antibody with host-type cells in chimeras have so far been successful for red blood cells only. The presence of donor-type γ-globuline on the surface of host red cells (143), and the elution of specific anti-host antibodies from red cells of chimeras (159, 153) can be considered as direct evidence of such interaction, while numerous studies on immune hemolysis in various homologous and heterologous graft-versus-host situations have provided most convincing indirect evidence (175, 102, 174, 156). Other investigators have reported though, that hemolysis in certain graft-versus-host reactions, such as of parent spleen cells injected into irradiated F_1 hybrid mice, may affect donor and host red cells indiscriminately (92), or that random bleeding rather than selective destruction of host erythrocytes may be the major cause of anemia in un-irradiated F_1 rats given parent-type spleen cells (78). Immune hemolysis or other evidence of antibody - host erythrocyte interaction was not demonstrable in irradiated mice with secondary disease following the injection of heterologous bone marrow or lymphoid cells (213). The rats with secondary disease, in the present experiments, had no clinical evidence of anemia and direct and indirect Coombs' tests performed on the erythrocytes of a limited number of animals, were negative.
Several attempts have been made to identify the histological type of lymphoid cell responsible for the immune attack on host tissues and to follow these cells to the site of interaction with their hypothetical target organs or tissues. Porter (161) demonstrated that the small lymphocyte is instrumental in causing fatal runting in newborn rats, while Gowans et al (89) traced labeled parent-type lymphocytes after i.v. injection into unirradiated F_1 rats. These investigators reached the conclusion that the small lymphocytes are most likely responsable for the initiation of a fatal graft-versus-host reaction. Porter also demonstrated that small lymphocytes, settling in the lymphatic tissues, could develop into pyroninophilic cells. However, with the exclusion of the lymphatic tissues where lymphocytes settle and proliferate, neither experiment could demonstrate a direct interaction between the injected cells and host tissues obviously affected by the graft-versus-host reaction, such as the skin for instance. Gowans did find labeled large lymphocytes in the stroma of the intestinal villi 24 hours after their injection into F_1 rats, but

did not implicate this cell type in the fatal outcome of the graft-versus-host reaction. Surprisingly few of these large labeled lymphocytes were found in the lymphatic tissue.
De Vries (206) linked the damage of intestinal epithelium and the more restricted degeneration of cells in other tissues, seen in irradiated mice several days after injection of homologous lymph node cells, to the cytotoxic action of the injected cells: large numbers of mononuclear cells, very likely of donor-type, were encountered in and around the intestinal lesions found in this accelerated type of secondary disease. However, in an attempt to trace tritum-labeled spleen and lymph node cells to this specific target tissue, under similar experimental conditions, we have not been successful so far: after initial random distribution in many organs, labeled large and small lymphocytes were found nearly exclusively in the lymphatic tissues at 1, 2, 4 and 8 days after intravenous injection (9). No other specific target areas were evident, but it must be admitted that few small lymphocytes had been initially labeled in the donor cell suspension so that a considerable portion of the injected cells, presumably responsible for the killing effect, may have gone unnoticed.

Factors contributing to death in secondary disease of the rat.

Weight loss has been found in most fatal graft-versus-host reactions and was a consistent finding also in the rats of the present experiments dying of secondary disease. No satisfactory explanation for its occurrance can as yet be given. The affected rats showed no obvious functional disturbance and only minor histological lesions of the intestine. It seems therefore rather unlikely that fatal wasting should be attributable merely to malfunctioning of the gastro-intestinal tract. This aspect was not specifically studied in the present experiments but it has been reported by Congdon and Urso (56) that mice dying of secondary disease consume normal amounts of food. McRea (134) however, demonstrated that wasting of irradiated F_1 mice injected with parental cells, could largely be attributed to a decreased food intake. Mechanical factors such as ulcerations of the pharynx and the base of the tongue that may contribute to the so-called oral death of animals subjected to very high X-ray doses, were not demonstrable in the present experiments. Kaplan and Rosston (102), in describing wasting disease in F_1 mice given parental spleen cells, speculated that inanition, lymphoid atrophy and body fat atrophy may be linked to stress and adrenal hyperfunction and demonstrated that adrenalectomy indeed reduces the susceptibility of F_1 mice to this fatal wasting disease (103).

Irradiated rats treated with homologous spleen cells did, as a rule, show rather severe lesions of the intestinal epithelium which might very well have contributed to the animals' deaths. Such intestinal lesions have also been seen in irradiated mice treated with homologous lymphoid cells and, far more conspicuously, in (homologous) bone-marrow treated irradiated monkeys. There seems to be no doubt that such severe lesions of the alimentary tract may, directly or indirectly, cause the death of the animals.

The skin lesions, encountered in most of the rats dying with secondary disease in the WAG→BROFO combination, can hardly be a direct cause of death in those cases where only localized dermatitis with hair loss and minor ulcerations were present. In some cases however, when large moist areas of dermatitis combined with localized epidermal necrosis were seen, the

lesions may have contributed to the animals' death through protein or electrolyte loss or complicating infections.

Lymphoid atrophy, a constant and important finding in secondary disease of bone marrow treated mice and rabbits, and its possible role in secondary mortality has been widely discussed in the literature. In irradiated animals treated with homologous bone marrow only, the donor-type lymphatic tissue is very slow at regenerating and unless a degree of tolerance towards host antigens develops, it may remain atrophic, leaving the chimera with an inadequate defense mechanism against bacterial invasion. Some investigators have postulated that certain of the lesions found in mice with typical secondary disease may be a consequence of the crippled defense system rather than a more direct effect of the graft-versus-host reaction (115). Whether or not this assumption will be substantiated in the near future, there seems to be no doubt that lymphoid atrophy should be considered as an important indirect factor contributing to secondary mortality.
A possible detrimental effect of large amounts of cellular débris derived from the destruction of lymphoid tissues has been discussed in chapter III (see section on the MLD-effect). Lymph nodes of BROFO rats treated with WAG spleen cells, initially showing vast lymphoid proliferation, indeed became atrophic during the 2nd week. The cause for such rather sudden involution and the reasons for its detrimental effect, if any, are as yet not known. Boyse (38) postulated a so-called "allergic death" for donor-type lymphoid cells reacting with host-type antigens, in a comparable situation in mice.
It is questionable wether the limited destruction of parenchymal cells in certain organs contributes to death in secondary disease of the rat.

Late effects after total-body irradiation

The majority of the irradiated bone marrow treated animals survived both the early period of bone marrow aplasia as well as the graft-versus-host reaction (secondary disease) during the second and third month. They were kept for long-term observation and, as it turned out, nearly all experimental animals were dead 21 months after irradiation. The findings at autopsy were generally in accordance with those reported by other investigators for irradiated rats protected by other means than bone marrow transplantation.
Many of the irradiated rats developed certain typical degenerative lesions during these 21 months and the incidence of benign and malignant tumours was particularly high. The most frequent causes of death, as far as these could be determined, were malignant growths and severe sclerotic lesions in the kidneys, or a combination of the two. Bronchopneumonias, disseminated inflammatory lesions or septicemia may have also contributed to death in a limited number of animals. Significant differences between WAG and BROFO rats as far as the incidence of early severe nephrosclerosis, infections of the urogenital tract and the frequency and time of occurrance of certain tumour types are concerned, have been pointed out in chapter VII.
The problems one encounters when trying to establish that a particular lesion or tumour seen in total-body irradiated animals, is specifically "radiation-induced", have been stressed in the discussion of chapter VII. The assumption that whole-body irradiation causes "accelerated

aging" had produced a comparatively simple theory regarding the early and frequent appearance of certain lesions, including tumour growth; it implied a shift in the time of appearance of all consequences of "old age", apparently also of neoplasms. However, it has been shown in recent years that reduced longevity in total-body irradiated animals cannot be attributed merely to an earlier occurrance of changes that presumably cause or contribute to death in old age. The latent period for certain changes believed to be characteristic for "aging", was reduced for some but not for others, while the incidence of such lesions in irradiated animals was not always consistent with the idea of premature senescence (201, 1). Explanations for the carcinogenic effect of total-body irradiation have consequently been altered and have become even more complex and controversial. An evaluation of the merits of various current theories on the mechanism of X-ray carcinogenesis in general, is considered beyond the scope of this work.

Comparison of the tumour incidence in irradiated animals with that seen in appropriate controls, is obviously essential. Unfortunately, the majority of the control animals had not yet died at the time of writing and it may take another year before the final incidence of benign and malignant tumours in non-irradiated BROFO rats will be known. The advantage of using SPF ("specific pathogen free') rats is evident in this respect. A low incidence of bronchopneumonia and other infectious complications in such rats no doubt increases their life-span as compared to conventional control animals. The reduced lethality due to intercurrent infectious disease is likely to reveal, eventually, a higher incidence and wider spectrum of tumours and degenerative lesions, making comparison with that occuring in irradiated animals more meaningful.

In spite of the restrictions imposed by the absence of a terminated survival curve for the controls, certain conclusions can nevertheless be drawn already at this stage. The irradiated rats had a considerably reduced lifespan, independent of the genotype of the protecting bone marrow graft. There was a high incidence and a multitude of histological types of tumours, most of which appeared within the first year after irradiation. Susceptibility for the early development of severe nephrosclerosis and probably also the occurrance of certain tumour types seemed different for WAG and BROFO rats. These observations are grossly in accordance with the late effects reported for bone marrow protected lethally irradiated mice.

The occurrence of life-threatening complications relatively early after high total-body irradiation, obviously calls for the greatest caution in the clinical application of total-body irradiation for other than strictly life-saving purposes. In many instances radiomimetic drugs or a combination of such drugs with low doses of total-body irradiation have been used to suppress immune reactivity before the transplantation of homologous kidneys, instead of total-body irradiation only. Less is known about possible late detrimental effects of the treatment with high doses of radiomimetic drugs though there are indications that such treatment produces long-term effects rather similar to those seen after total-body irradiation, also as far as the carcinogenic effect is concerned (197).

SUMMARY

1. Rats were treated with **homologous bone marrow** following high sublethal and lethal radiation doses. The majority of the animals survived beyond 4 weeks after irradiation with a functioning donor-type hemopoietic tissue. The experimental groups showed variable mortality during the first 10 post-irradiation days, ranging from 20-40 %. This early mortality was possibly due to infectious complications.
A few animals died with pancytopenia following so-called "delayed rejection" of the graft during the 3rd and 4th week.
The minimal dose to obtain an effective graft of homologous bone marrow was approximately 50×10^6 cells.
There was no evidence of a so-called "median-lethal-dose-effect" ((MLD-effect).

2. Secondary disease occurred during the second and third month following irradiation and homologous bone marrow therapy. Morbidity and mortality were variable but generally rather low, respectively about 30 and 15 %.
The clinical and pathological findings were reminiscent of those described for mice with secondary disease except that colitis and diarrhea were not observed while only minor histological lesions of the intestinal epithelium were found in secondary disease of the rat.
A concomitant injection of homologous lymphoid cells produced a vicious, accelerated type of secondary disease, usually killing the hosts within 2-3 weeks. Though extensive intestinal lesions were found histologically in these cases, there was again no clinical evidence of colitis or diarrhea.

3. In a limited number of animals temporary chimerism was shown to be associated with **persistence of specific tolerance** towards the antigens of the former bone marrow graft. Usually, such reversing to host-type hemopoiesis runs parallel with recovery of host-type immune reactivity. This was also the case in about 50 % of the "reversals" of the present study; the others however remained wholly or partially tolerant when tested with donor-type skin grafts at a later stage. These results have been presented and discussed in the light of the controversy regarding the possibility of tolerance towards transplantation antigens independent of chimerism, i.e. in the absence of excess specific antigen.

4. Rat chimeras surviving beyond 3 months after irradiation remained well until the 7th month after which a number of characteristic lesions started to develop. There was a considerable **reduction of longevity** as compared with controls. Virtually all (151 out of 159) experimental animals included in this long-term study were dead 21 months after irradiation while only a few of the controls (9 out of 71) were dead by this time.

Cataract was consistently found, while nephrosclerosis was a frequently encountered degenerative lesion. Infectious complications were comparatively rare except for those in the urogenital tract where inflammatory lesions were often seen.
The incidence of benign and malignant **tumours** was high. The earliest tumours occurred approximately 7 months after irradiation. The majority of the irradiated rats of one of the strains (BROFO) died with malignant tumours as a very likely cause of death. Most chimeras derived from the other strain (WAG) also died during the first year after irradiation, but rarely with tumours; they frequently showed severe sclerotic changes in both kidneys. So far, few control animals had died; only one had a malignant tumour while severe nephrosclerosis was never found.

5. Clinical applications of homologous and autologous bone marrow therapy have been briefly reviewed and some of the problems, encountered both in experimental and clinical transplantation of hemopoietic tissues, have been discussed. The usefulness but also the limitations of the information gathered from experiments on small and large mammals, for guidance in clinical bone marrow therapy, were exposed.
The subject of organ transplantation was introduced in view of the role still played by whole-body irradiation and transplantation of foreign hemopoietic cells, also in that field.

SAMENVATTING

Gedurende de afgelopen tien jaren is een groot aantal experimentele gegevens omtrent beenmergtransplantatie na bestraling verkregen. Het is mogelijk gebleken, het door bestraling van het gehele lichaam vernielde beenmerg van proefdieren door een relatief klein aantal intraveneus toegediende vreemde beenmergcellen te vervangen. Dit in de nieuwe gastheer prolifererende donorbeenmerg bleek in staat te zijn de sterfte ten gevolge van beenmergaplasie (het z.g. beenmerg-syndroom) te voorkomen.
De succesvolle resultaten van de transplantatie van homoloog beenmerg bij lethaal bestraalde muizen en konijnen, hebben er toe bijgedragen dat de verwachtingen voor de klinische toepassingsmogelijkheden aanvankelijk hoog waren gespannen. Het bleek echter al spoedig dat een aantal ernstige complicaties kan optreden. Een belangrijk percentage van de met homoloog beenmerg behandelde lethaal bestraalde muizen en konijnen ging na een aanvankelijk herstel, enkele weken later aan z.g. secundaire ziekte te gronde; bij lethaal bestraalde honden bleek het „aanslaan" van vreemd beenmerg moeilijker dan op grond van de bij muizen bereikte resultaten kon worden verwacht. Bij apen bleek tenslotte dat, hoewel homoloog beenmerg relatief gemakkelijk kon aanslaan, de secundaire ziekte dusdanig ernstige vormen aannam dat nauwelijks van een initieel herstel van de bestraalde dieren kon worden gesproken.

Secundaire ziekte is eerst bij muizen grondig bestudeerd, later ook bij konijnen en apen. Op grond van talrijke onderzoekingen kan het als vaststaand worden beschouwd dat deze vooral bij primaten zeer ernstige ziekte, voornamelijk een gevolg is van een immunologische reactie van de ingespoten donorcellen (die ook het lymphatische systeem van de gastheer vervangen) tegen de antigenen van de gastheer, de z.g. „graft-versus-host" reactie. De gegevens die thans omtrent de beperkte klinische toepassingen van homologe beenmergtherapie beschikbaar zijn, wijzen erop dat secundaire ziekte bij de mens veel overeenkomst vertoont met de ziekte bij apen en voorlopig een ernstig obstakel vormt voor een meer uitgebreide klinische toepassing van beenmergtransplantatie.

Het welslagen van homologe beenmergtherapie bleek dus, bij de enkele diersoorten waarbij dit tot nu toe is bestudeerd, van een aantal deels bekende, deels nog onbekende factoren afhankelijk te zijn. Het leek derhalve aangewezen, homologe beenmergtherapie bij nog een andere diersoort te bestuderen. De rat was bij uitstek geschikt voor een dergelijk onderzoek. Homologe beenmergtherapie bij de rat was zowel in dit laboratorium als elders toegepast doch bleek ineffectief tenzij donor- en gastheerdier nauw met elkaar verwant waren. Bovendien was vrijwel niets bekend omtrent voorkomen, pathogenese en pathologie van secundaire ziekte bij deze diersoort. Daarom werd een systematische studie van homologe beenmergtherapie bij bestraalde ratten ondernomen. Bijzondere aandacht werd geschonken aan de voorwaarden voor het „aanslaan" van het vreemde beenmerg, het ontstaan en verloop van vroege complicaties (o.a.

secundaire ziekte) en aan het uiteindelijk lot van de overlevende dieren. Hierbij werd speciaal op het voorkomen van benigne en maligne tumoren gelet.
Het onderzoek was dus een poging om de kennis omtrent de mogelijkheden en de gevaren van homologe beenmergtherapie bij bestraalde zoogdieren uit te breiden, bestaande opvattingen omtrent het ontstaan van bepaalde complicaties te toetsen, en zo mogelijk informaties in te winnen die voor klinische toepassing van belang zouden kunnen zijn.

De resultaten kunnen als volgt worden samengevat:

1. Het bleek mogelijk de meerderheid van lethaal bestraalde ratten met intraveneus toegediende suspensies van homoloog beenmerg in leven te houden. Vrijwel alle overlevende dieren hadden een functionerend hemopoietisch apparaat van het donortype, waren dus permanente „chimeren".
   De **mortaliteit** gedurende de eerste maand na bestraling was variabel voor de verschillende experimentele groepen. Een bepaald percentage (20-40 %) van de behandelde dieren stierf gedurende de eerste tien dagen ondanks een adequate dosis vreemd beenmerg, waarschijnlijk ten gevolge van fulminante infectieuze complicaties. Een beperkt aantal andere dieren die de beginperiode overleefden, vertoonde een z.g. „vertraagde afstoting", waarbij een aanvankelijk functionerend beenmergtransplantaat gedurende de 3e of 4e week te gronde gaat en de dieren alsnog aan pancytopenie overlijden.
   De **minimale dosis homoloog beenmerg** benodigd voor overleving bleek ongeveer 50 x 10^6 beenmergcellen te zijn.
   Er waren geen aanwijzingen voor een **„Median Lethal Dose" effect.** Een dergelijk effect was na behandeling van bepaalde muizenstammen met vreemd beenmerg aangetoond; het houdt in dat homoloog beenmerg i.p.v. therapeutisch te werken, de mortaliteit van sublethaal bestraalde dieren verhoogt vergeleken met die voor onbehandelde controles.

2. **Secundaire ziekte** na homologe beenmergtherapie trad tijdens de tweede en derde maand op. Morbiditeit en mortaliteit t.g.v. deze complicatie waren variabel maar in het algemeen vrij laag, respectievelijk ongeveer 30 en 15 %. De ziekteverschijnselen en histologische bevindingen geleken op die welke bij muizen zijn beschreven. Colitis en diarrhoea, typisch voor secundaire ziekte bij muizen, werden bij ratten echter niet gevonden. Het wordt mogelijk geacht dat dit verband houdt met de speciale fokmethode die voor deze ratten werd gebezigd, het z.g. SPF- of „specific pathogen free" breeding, waardoor het aantal in de proefdieren aantoonbare parasieten en pathogene bacterien wordt gereduceerd.
   Intraveneuze toediening van homologe miltcelsuspensies na lethale bestraling veroorzaakte een ernstiger vorm van secundaire ziekte waarbij de meerderheid der dieren binnen twee à drie weken extreem geëmacieerd en vaak met ernstige huidaandoeningen te gronde ging. Hoewel hierbij uitgebreide histologische afwijkingen van het darmslijmvlies aanwezig bleken, werd ook bij dit ernstige ziektebeeld geen colitis en/of diarrhoea gevonden.

3. Stralingschimeren hebben de eigenschap huidtransplantaten van eenzelfde genetisch type als het toegediende beenmerg, te accepteren. Deze **tolerantie t.o.v. donor-type weefsel-**

antigenen berust op een volledige overname van het immunologische systeem van de chimeer door lymphatische cellen van het donortype. De literatuur vermeldt verder dat indien de donorcellen weer door gastheer-type cellen worden vervangen (dit komt meest na sublethale bestraling voor en deze dieren worden aangeduid met de Engelse term „reversal") dit meestal gepaard gaat met een terugkeer van de oorspronkelijke imuunreactiviteit van de gastheer. Huidtransplantaten van het donortype worden dus in het algemeen door een „reversal" niet meer geaccepteerd.
Een aantal van de sublethaal bestraalde, homoloog behandelde ratten bleek echter ook geruime tijd na volledige terugkeer tot een gastheer-type hemopoietisch systeem, tolerant of verminderd reactief te zijn voor donor-type huidtransplantaten, ook indien deze lang na het verdwijnen van het vreemde beenmerg werden getransplanteerd. Dergelijke specifiek gereduceerde reactiviteit, die noch lethale bestraling noch permanent chimerisme vereist en bij volwassen dieren geïnduceerd kan worden, zou van belang kunnen zijn voor weefsel- en orgaantransplantatie bij de mens. Inductie van specifieke tolerantie d.m.v. permanent chimerisme lijkt voor primaten, wegens de ernstige secundaire complicaties, voorlopig immers onuitvoerbaar.
De verkregen resultaten werden besproken in verband met de vraag of een immuunsysteem een eenmaal geïnduceerde specifieke tolerantie voor homologe transplantatie-antigenen zou kunnen handhaven ondanks de afwezigheid van de betreffende antigenen, dus onafhankelijk van persisterend chimerisme.

4. Vrijwel alle rat-chimeren die de eerste drie maanden overleefden bleven in uitstekende conditie tot omstreeks de zevende maand. Nadien ontstond echter spoedig een aantal **degeneratieve afwijkingen,** vooral cataract en nephrosclerose, en nam het aantal **tumoren** snel toe. Deze en waarschijnlijk ook andere afwijkingen leidden tot een aanzienlijke levensduurverkorting van de bestraalde dieren zodat 21 maanden na de bestraling 151 van 159 experimentele dieren waren overleden, tegen een slechts gering aantal (9 van de 71) van de even oude controles.
Er bestonden enige verschillen tussen de twee gebruikte rattenstammen betreffende de frequentie waarmee sommige aandoeningen, met name nephrosclerose en tumoren, voorkwamen. Het genotype van het beschermende beenmerg scheen in de hier beschreven proeven geen invloed te hebben op de late effecten van lethale bestraling.
Er werd ook aandacht besteed aan de vraag of aan het schijnbaar sterk carcinogene effect van bestraling (m.a.w. het grote aantal vroegtijdig na de bestraling optredende tumoren) een absolute vermeerdering van het aantal tumoren ten grondslag ligt of slechts een verkorting van hun respectievelijke latente perioden. Het vraagstuk van de z.g. „versnelde veroudering" als mogelijk gevolg van totale lichaamsbestraling kwam hierbij tevens ter sprake.

5. In het eerste hoofdstuk was een korte beschouwing opgenomen over de tegenwoordige toepassingsmogelijkheden van autologe en homologe beenmergtherapie na accidentele of therapeutische bestraling van de mens. De bruikbaarheid voor eventuele klinische toepassing, maar ook de soms beperkte waarde van gegevens, die door middel van dierexperimenten op deze gebieden kunnen worden verkregen, werd belicht.

REFERENCES

1. ALEXANDER, P. and D. I. Connell, in Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation, Ed. R. J. C. Harris, Academic Press, London, New York, 1963, p. 277.
2. AMBRUS, C. M. et al., Proc. Symp. Bone Marrow Therapy and Protection in Irradiated Primates, Radiobiological Institute TNO, Rijswijk, Netherlands, 1962, p. 27.
3. ANDERSON, N. F., E. J. Delorme and M. F. A. Woodruff, Transpl. Bull., 7, 93, 1960.
4. ARGYRIS, B. F., Transpl. Bull., 30, 140, 1962.
5. BALNER, H., L. J. Old and D. A. Clarke, Radiation Res., 15, 836, 1961.
6. BALNER, H., E. B. Simmel and D. A. Clarke, Transpl. Bull., 29, 55, 1962.
7. BALNER, H., L. J. Old and D. A. Clarke, Proc. Soc. Exptl. Biol. and Med. 109, 58, 1962.
8. BALNER, H., Transplantation I, 217, 1963.
9. BALNER, H. and H. Dersjant, (in preparation).
10. BARNES, D. W. H. and J. F. Loutit, Nucleonics, 12, no. 5, 68, 1954.
11. BARNES, D. W. H. and J. F. Loutit, in Spleen protection: The cellular hypothesis. Radiobiology Symposium 1954, p. 134, 1954.
12. BARNES, D. W. H. and J. F. Loutit, Brit. J. Haematol., 3, 241, 1957.
13. BARNES, D. W. H., J. F. Loutit and D. R. Westgarth, Gerontologia, 3, 137, 1959.
14. BARNES, D. W. H., C. E. Ford, S. M. Gray and J. F. Loutit, in Biological Problems of Grafting, Blackwell Scientific Publications, Oxford, 12, 1959, p. 274.
15. BARNES, D. W. H., C. E. Ford, S. M. Gray and J. F. Loutit, in Progress in Nuclear Energy, Series VI, vol. 2, Pergamon Press, 1959.
16. BEKKUM, D. W. van, in Mechanisms in Radiobiology, vol. II, chapter 5, Academic Press, New York-London, 1960, p. 297.
17. BEKKUM, D. W. van, Proc. 7th Congr. europ. Soc. Haemat., London 1959, p. 947, 1960.
18. BEKKUM, D. W. van, in Mechanisms of Immunological Tolerance, Publishing House Czech. Acad. Sci., Prague, 1962, 385.
19. BEKKUM, D. W. van, Transplantation, I, 39, 1963.
20. BEKKUM, D. W. van, (personal communications)
21. BEKKUM, D. W. van, L. M. van Putten and M. J. de Vries, Ann. New York Acad. Sci., 99, 53, 1962.
22. BEKKUM, D. W. van and O. Vos, J. Cell. and Comp. Physiol, 50, suppl. 1, 139, 1957.
23. BEKKUM, D. W. van and O. Vos, Int. J. Rad. Biol., 3, 173, 1961.
24. BEKKUM, D. W. van, O. Vos and W. W. H. Weyzen, J. Nat. Cancer Inst., 23, 75, 1959.
25. BENACERRAF, B., E. Kivy-Rosenberg, M. M. Sebestyen, B. W. Zweifach, J. Exptl. Med., 110, 49, 1959.
26. BILLINGHAM, R.E., in Transplantation of Tissues and Cells, chapter I, Ed. R. E. Billingham and W. K. Silvers, The Wistar Institute Press, Philadelphia, 1961, p. 1.
27. BILLINGHAM, R. E., (personal communication).
28. BILLINGHAM, R. E., L. Brent and P. B. Medawar, Nature, 172, 603, 1953.
29. BILLINGHAM, R. E. L., Brent and P. B. Medawar, Phil. Trans. Roy. Soc. London, 239, 357, 1956.
30. BILLINGHAM, R. E., L. Brent, and P. B. Medawar, Transpl. Bull., 3, 84, 1956.
31. BILLINGHAM, R. E., L. Brent and P. B. Medawar, Transpl. Bull., 5, 377, 1958.

32. BILLINGHAM, R. E. et al., Transpl. Bull., 5, 80, 1958.

33. BILLINGHAM, R. E., V. Defendi, W. K. Silvers and D. Steinmuller, J. Nat. Cancer Inst., 28, 365, 1962.

34. BILLINGHAM, R. E. and W. K. Silvers, J. Immunol., 85, 14, 1960.

35. BILLINGHAM, R. E. and E. M. Sparrow, J. Embryol. and Exptl. Morph., 3, 265, 1955.

36. BINHAMMER, R. T., J. C. Finerty, M. Schneider and A. W. B. Cunningham, Radiation Res., 6, 339, 1957.

37. BOND, V. P., E. P. Cronkite, S. W. Lippincott and C. J. Shellabarger, Radiation Res., 12, 276, 1960.

38. BOYSE, E. A., Immunology, 2, 170, 1959.

39. BOYSE, E. A., L. J. Old and G. Thomas, Transpl. Bull., 29, 63, 1962.

40. BRECHER, G., E. P. Cronkite and J. H. Peers, J. Nat. Cancer Inst., 14, 159, 1953.

41. BROWN, A. M., J. Animal Techn. Assoc., XIV, 11, 1963.

42. BRUES, A. M., Radiation Res., 3, 272, 1955.

43. BURCHENAL, J. H., H. F. Oettgen, E. A. D. Halmberg, S. C. Hemphill and J. A. Reppert, Cancer Res., 20, 425, 1960.

44. BURNET, F. M. and F. Fenner, in Production of Antibodies, 2nd edition, Mc. Millan, Melbourne, 1949.

45. BURNET, F. M., in The Clonal Selection Theory of Acquired Immunity, Cambridge University, 1959.

46. CASARETT, G. W., Accelaration of aging by ionizing radiation, University of Rochester, Atomic Energy Project Report UR-492, 1957.

47. COHEN, J. A., O. Vos and D. W. van Bekkum, in Advances in Radiobiology, Oliver and Boyd Ltd., Edinburgh, 1957, p. 134.

48. COLE, L. J. and E. L. Alpen, Blood, 21, 373, 1963.

49. COLE, L. J. and W. E. Davis, Proc. Nat. Acad. Sci. U.S., 47, 594, 1961.

50. COLE, L. J. and W. E. Davis, Transpl. Bull., 29, 79, 1962.

51. COLE, L. J., P. C. Nowell and M. E. Ellis, J. Natl. Cancer Inst., 17, 435, 1956.

52. CONARD, R. A., Radiation Res., 5, 167, 1956.

53. CONARD, R. A., E. P. Cronkite, G. Brecher and C. P. A. Stroma, J. Appl. Physiol., 9, 227, 1956.

54. CONGDON, C. C., Amer. Rev. Med., 13, 203, 1962.

55. CONGDON, C. C., D. E. Uphoff and E. J. Lorenz, J. Nat. Cancer Inst., 13, 73, 1952.

56. CONGDON, C. C. and I. S. Urso, Amer. J. Pathol., 33, 749, 1957.

57. COTTIER, H., in Strahlenbedingte Lebensverkürzung, Springer Verlag, Berlin, Göttingen, Heidelberg, 1961.

58. COURTENAY, V. D., Brit. Radiol., 36, 440, 1963.

59. CRONKITE, E. P., V. P. Bond, T. M. Fliedner and S. A. Killmann, in Hemopoiesis, CIBA Found. Symsium, Churchill, London, 1960, p. 68 and p. 70.

60. CROUCH, B. G. and R. R. Overman, Blood, 17, 444, 1961.

61. CROUCH, B. G., L. M. van Putten, D. W. van Bekkum and M. J. de Vries, J. Nat. Cancer Inst., 27, 53, 1961.

62. CUDKOWICZ, G., Proc. Soc. Exptl. Biol. and Med., 107, 821, 1961.

63. CURTIS, M. R., F. D. Bullock and W. F. Dunning, Amer. J. Cancer, 15, 67, 1931.

64. DEMPSTER, W. J., Brit. J. Surgery, 40, 447, 1953.

65. DEMPSTER, W. J., B. Lennox and J. W. Boag, Brit. J. Exptl. Pathol., 31, 670, 1950.

66. DENHARDT, D. T. and R. D. Owen, Transpl. Bull., 7, 394, 1960.

. GENERAL DISCUSSION ON BONE MARROW TRANSPLANTATION IN MONKEYS. In Proc. Symp. Bone Marrow Therapy and Protection in Irradiated Primates, Radiobiological Institute TNO, Rijswijk, Netherlands, 1962, p. 153.

. DORIA, G., Nat. Acad. Sci., 49, 281, 1963.

. DUNJIC, A., Nature, 194, 493, 1962.

. DUNJIC, A., Nature, 198, 603, 1963.

. DUNNING, W. F., M. R. Curtis and M. E. Madsen, Cancer Res., 7, 134, 1947.

. EICHWALD, E. J. and C. R. Silmser, Transpl. Bull. 2, 148, 1955.

. FEDER, B. H., Blood, 21, 773, 1963.

. FEFER, A. and W. C. Davis, Transplantation, I, 75, 1963.

. FEFER, A. and G. J. V. Nossal, Transpl. Bull., 29, 73, 1962.

. FELDMAN, M. and D. Jaffe, J. Nat. Cancer Inst., 21, 697, 1958.

. FERREBEE, J. W. and D. T. Thomas, Radiology, 75, 1, 1960.

. FIELD, E. O., M. G. Rinsler, and J. E. Gibbs, Brit. J. Exptl. Pathol., 43, 320, 1962.

. FINERTY, J. C., R. T. Binhammer, M. Schneider and A. W. B. Cunningham, J. Nat. Cancer Inst., 14, 149, 1953.

. FISHLER, M. C., L. J. Cole, V. P. Bond and W. L. Milne, Amer. J. Physiol., 177, 236, 1954.

. FORD, C. E. and H. S. Micklem, Lancet, February 1963, p. 359.

. FURTH, J. and O. B. Furth, Amer. J. Cancer, 28, 54, 1936.

. FURTH, J., A. C. Upton and A. W. Kimball, Radiation Res. Suppl. 1, 243, 1959.

. GLÜCKSMANN, O., in Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation, Ed. R. J. C. Harris, Academic Press, London, New York, 1963, p. 121.

. GOODMAN, J. W., Blood, (in press), 1963.

. GORER, P. A. and Z. B. Mikulska, Cancer Res., 14, 651, 1954.

. GORER, P. A. and P. O'Gorman, Transpl. Bull., 3, 142, 1956.

. GOTTFRIED, B. and M. Padnos, Transpl. Bull., 6, 427, 1959.

. GOWANS, J. L., B. M. Gesner and D. D. Mc. Gregor, in Biological Activity of the Leucocyte, CIBA Foundation Study Group no. 10, 1961, p. 32.

. GUTTMANN, R. D. and J. B. Aust, Nature, 192, 564, 1961.

. HAMBURGER, J., J. Vaysse, J. Crosnier, J. Auvert and J. Dormant, Ann. N. Y. Acad. Sci, 99, 808, 1962.

. HARRISS, E., C. Currie, J. P. Kriss and H. S. Kaplan, J. Exptl. Med., 113, 1095, 1961.

. HASEK, M. and T. Hraba, Nature, 175, 764, 1955.

. HASKOVA, V. and J. Majer, Folia Biologica, 5, 1, 1959.

. HILFINGER, M. F., H. Ferguson and P. A. Rumen-Schneider, J. Lab. Clin. Med., 42, 581, 1953.

. HILL, J. M. and E. Loeb, Proc. Symp. Bone Marrow Therapy and Protection in Irradiated Primates, Radiobiological Institute TNO, Rijswijk, Netherlands, 1962, p. 321.

. HOWARD, J. G., D. Michie and M. F. A. Woodruff, in Transplantation, CIBA Foundation Symposium, London, 1962, p. 138.

. HUMBLE, J. G., Proc. Symp. Bone marrow Therapy and Protection in Irradiated Primates. Radiobiological Institute TNO, Rijswijk, Netherlands, 1962, p. 309.

. JACOBSON, L. O., E. K. Marks and E. O. Gaston, in Radiobiology Symposium 1954, Ed. Z. M. Bacq. Academic Press, New York, 1955, p. 122.

100. KALISS, N., Cancer Res., 18, 992, 1958.

101. KAPLAN, H. S., M. B. Brown and J. Paull, J. Nat. Cancer Inst., 14, 303, 1953.

102. KAPLAN, H. S. and B. H. Rosston, Stanford Med. Bull., 17, 77, 1959.

103. KAPLAN, H. S. and B. H. Rosston, Transpl. Bull., 6, 107, 1959.

104. KOLETSKY, S. and G. E. Gustafson, Cancer Res. 25, 100, 1955.

105. KOLLER, P. C., A. J. S. Davies and S. M. A. Doak, in Advances in Cancer Research, vol. 6, Academic Press, New York, 1961, p. 181.

106. KREN, V., A. Braun, O. Stark, R. Kraus, B. Frenze and R. Brolicka, Folia Biologica, 8, 341, 1962.

107. KURNICK, N. B., in Diagnosis and treatment of acute radiation injury, World Health Organization, Geneva, 1961, p. 309.

108. KURNICK, N. B., Proc. Symp. Bone Marrow Therapy and Protection in arridiated Primates, Radioblological Institute TNO, Rijswijk, Netherlands, 1962, p. 289.

109. KÜSS, R. et al, Rev. franc. ét. Clin. et Biol., VII, 1028, 1962.

110. LAMSON, B. G., R. A. Meek and L. R. Bennett, A. M. A. Arch. of Pathol., 64, 505, 1957.

111. LEBLOND, C. P. and G. Sainte-Marie, in Hemopoiesis, CIBA Foundation Symposium, Churchill, London, 1960, p. 152.

112. LEJEUNE, G., in Homogreffes cutanées, section V, Ed. Arscia, Brussels, 1962, p. 155.

113. LINDOP, P. and J. Rotblad, Gerontologia, 3, 122, 1959.

114. LITTLE, J. R., G. Brecher, T. R. Bradley and S. Rose, Blood, 19, 236, 1962.

114a. LORENTZ, E., C. C. Congdon and D. Uphoff, Radiology, 58, 863, 1952.

115. LOUTIT, J. F. and H. S. Micklem, Brit J. Exptl. Pathol., XLIII., 77, 1962.

116. LYNCH, Th. J. and W. C. Moloney, Blood, 21, 764, 1963.

117. MAIN, J. M. and R. T. Prehn, J. Nat. Cancer Inst. 15, 1023, 1955.

118. MAISIN, H., in Contribution a l'étude du Syndrome Médullaire après irradiation. Ed. Arscia, Brussels, 1959, p. 128.

119. MAISIN, J., P. Dumont and A. Dunjic, Nature, 186, 487, 1960.

120. MÄKELÄ, O. and G. J. V. Nossal, J. Immunol., 88, 613, 1962.

121. MANNIK, J. A., H. L. Lochte, C. A. Ashley, E. D. Thomas and J. W. Ferrebee, Blood, 15, 255, 1959.

122. MANNIK, J. A., H. L. Lochte, C. A. Ashley, E. D. Thomas and J. W. Ferrebee, Surgery, 46, 821, 1959.

123. MARIANI, T., C. Martinez, J. M. Smith and R. A. Good, Ann. N. Y. Acad. Sci., 87, 93, 1960.

124. MARTINEZ, C., F. Shapiro and R. A. Good, in Mechanisms of Immunological Tolerance, Publishing House Czech. Acad. Sci., Prague, 1962.

125. MARTINEZ, C., J. M. Smith and R. A. Good, Transpl. Bull., 30, 123, 1962.

126. MATHÉ, G., in Diagnosis and Treatment of Acute Radiation Injury, World Health Organization, Geneva, 1961, p. 191.

127. MATHÉ, G., J. L. Amiel, L. Schwarzenberg and A. M. Mery, C. R. Acad. Sci. (Paris), 255, 2863, 1962.

128. MATHÉ, G. and J. Bernard, Rev. franc. ét Clin. et Biol., 4, 442, 1959.

129. MATHÉ, G. et al, Rev. franc. ét. Clin. et Biol., 4, 675, 1959.

130. MATHÉ, G. et al, Rev. franc. ét. Clin et Biol., 4, 226, 1959.

131. MATHÉ, G. et al, Rev. hémat. 15, 115, 1960.

132. MAXWELL, R. E. and J. K. Weston, Fed. Proc., 15, 457, 1956.

. McKHANN, C. F. and J. H. J. Barrian, J. Immunol., 86, 345, 1961.
. McRAE, J., in Studies of the Graft Host Interaction in the Mouse, UCRL-9448, Thesis, 1960.
. MEDAWAR, P. B., J. Anat. Lond., 78, 176, 1944.
. MEDAWAR, P. B., Transplantation, I, 21, 1963.
. MEDAWAR, P. B. and M. F. A. Woodruff, Immunology, I, 27, 1958.
. MERRILL, J. P., J. E. Murray, J. H. Harrison, E. A. Friedman, J. D. Dealy and G. J. Dammin, New Engl. J. Med., 262, 1251, 1960.
. MICHIE, D., and M. F. A. Woodruff, Proc. Roy. Soc. B 156, 280, 1962.
. MICHIE, D., M. F. A. Woodruff and I. Zeiss, Immunology, 4, 413, 1961.
. MICKLEM, H. S., Brit. J. Radiol., 36, 457, 1963.
. MILLER, J. F. A. P., Ann. N. Y. Acad. Sci., 99, 340, 1962.
. MITCHISON, N. A., Transpl. Bull., 5, 66, 1958.
. MOLE, R. H. and A. M. Thomas, Int. J. Rad. Biol., 36, 457, 1963.
. MÖLLER, G., J. Nat. Cancer Inst., 30, 1153, 1963.
. NELSON, D. S., Brit. J. Exptl. Pathol., 43, 1, 1962.
. NISBET, N. W., B. F. Heslop and I. M. Zeiss, Brit. J. Exptl. Pathol., 41, 443, 1960.
. NOSSAL, G. J. V., A. Fefer and O. Mäkelä, in Mechanisms of Immunological Tolerance, Publishing House Czech. Acad. Sci., Prague, 1962, p. 157.
. NOWELL, P. C. and L. J. Cole, Radiation Res., 11, 545, 1959.
. ODELL, T. T., F. G. Tausche, D. L. Lindsley and R. D. Owen, Ann. N. Y. Acad. Sci., 64, 811, 1957.
. ODELL, T. T. and B. C. Caldwell, J. Nat. Cancer Inst., 20, 851, 1958.
. OLD, L. J., D. A. Clarke and B. Benacerraf, Nature, 184, 291, 1959.
. OLINER, H., R. Schwartz, and W. Dameshek, Blood, 17, 20, 1961.
. OTTESEN, J., Acta Physiol. Scandinavica, 41, 325, 1957.
. PEGG, D. E., J. G. Humble and K. A. Newton, Brit. J. Cancer, XVI, no. 3, 417, 1962.
. PIOMELLI, S. and M. S. Brooke, Ann. N. Y. Acad. Sci., 87, 472, 1960.
. PIOMELLI, S. and M. S. Brooke, Blood, 17, 579, 1961.
. POPP, R. A., J. Nat. Cancer Inst., 26, 629, 1961.
. PORTER, K. A., Ann. N. Y. Acad. Sci., 87, 391, 1960.
. PORTER, K. A., in Diagnosis and treatment of Acute Radiation Injury, World Health Organization, Geneva, 1961, p. 329.
. PORTER, K. A. and E. H. Cooper, J. Exptl. Med., 115, 997, 1962.
. PORTER, K. A. and J. E. Murray, J. Nat. Cancer Inst., 20, 189, 1958.
. PUTTEN, L. M. van, Blood, 13, 789, 1958.
. PUTTEN, L. M. van, Transpl. Bull., 29, 421, 1962.
. PUTTEN, L. M. van, R. R. Overman, D. W. van Bekkum and J. R. Newsome, Discussion in Proc. Symp. Bone Marrow Therapy and Protection in Irradiated Primates, Radiobiological Institute TNO, Rijswijk, Netherlands, 1962, p. 80-85.
. PUTTEN, L. M. van and M. J. de Vries, J. Nat. Cancer Inst., 28, 587, 1962.
. QUASTLER, H., Lanzl, E. F., M. E. Kellen and J. W. Osborne, Amer. J. Physiol., 164, 546, 1951.

168. RATCLIFFE, H. L., Amer. J. Pathol., 16, 237, 1940.

169. ROGACHEVA, S. A., in Biological Effects of Radiation and Problems of Radioactive Isotope Distribution. AEC-tr. -5265, Moscow, p. 43, 1961.

170. ROSEN, V. J., T. J. Castanera, D. J. Kimeldorf, and D. C. Jones, Amer. J. Pathol., 38, 359, 1961.

171. ROSENTHAL, S. A., L. G. Harber, R. L. Baer and W. Friedman, Radiation Res., 13, 496, 1960.

172. ROSS, O. A. et al., Fed. Proc., 14, 418, 1955.

173. SCHWARTZ, R., W. Dameshek and J. Donovan, J. Clin. Invest., 39, 952, 1960.

174. SHAW, D. A. and H. Vermund, Proc. Nat. Acad. Sci., 45, 23, 1959.

175. SIMONSEN, M., Acta path. and microbiol. Scand., 40, 480, 1957.

176. SIMONSEN, M., in Mechanism of Cell and Tissue Damage Produced by Immune Reactions. Benno Schwabe and Co., Basel/Stuttgart, 1961, p. 233.

177. SMITH, R. T., in Advances in Immunology, Ed. W. H. Taliaferro, J. H. Humphrey, vol. I, 1961, p. 67.

178. SMITH, W. W., I. M. Alderman, and R. E. Gillespie, Amer. J. Physiol., 192, 549, 1958.

179. SMITH, W. W., R. S. Marsten, L. Gonshery, I. M. Alderman and H. J. Ruth, Amer. J. Physiol., 183, 98, 1955.

180. SNELL, G. D., J. Genetics, 49, 87, 1948.

181. STASTNY, P. and M. Ziff, Ann. N. Y. Acad. Sci., 99, 663, 1962.

182. STONE, W. H. and R. D. Owen, Transplantation I, 107, 1963.

183. SYDNOR, K. L., O. Butenandt, F. P. Brillantes and C. Higgins, J. Natl. Cancer Inst., 29, 805, 1962.

184. TAKETA, S. T., M. N. Swift, and V. P. Bond, Amer. J. Physiol., 196, 987, 1959.

185. THOMAS, E. D., C. A. Ashley, H. L. Lochte, Jr., A. Jaretzki, O. D. Sahler and J. W. Ferrebee, Blood, 14, 720, 1959.

186. THOMAS, E. D., J. A. Collins, E. C. Hermans Jr., and J. W. Ferrebee, Blood, 19, 217, 1962.

187. TOOLAN, H. W., Proc. Soc. Exptl. Biol and Med., 77, 572, 1951.

188. TRENTIN, J. J., Proc. Soc. Exptl. Biol. and Med., 92, 688, 1956.

189. TRENTIN, J. J., Proc. Soc. Exptl. Biol. and Med., 93, 98, 1956.

190. TRENTIN, J. J., J. Nat. Cancer Inst., 22, 219, 1959.

191. TRENTIN, J. J., and J. Session, Transpl. Bull., 29, 148, 1961.

192. UPHOFF, D. E., J. Nat. Cancer Inst., 19, 123, 1957.

193. UPHOFF, D. E., Proc. Soc. Exptl. Biol. and Med., 99, 651, 1958.

194. UPHOFF, D. E., Radiation. Res., 12, 482, 1960.

195. UPHOFF, D. E., J. Nat. Cancer Inst., 30, 1115 and 1143, 1963.

195a. UPHOFF, D. E. and L. W. Law, J. Nat Cancer Inst., 20, 617, 1958.

196. UPTON, A. C., J. Gerontology, 12, 306, 1957.

197. UPTON, A. C., J. W. Conklin, T. P. McDonald and K. W. Christenberry, in Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation, Ed. R. J. C. Harris, Academic Press, London, New York, 1963, p. 171.

198. UPTON, A. C. and J. Furth, J. Nat. Cancer Inst., 15, 1005, 1955.

199. UPTON, A. C., A. W. Kimball, J. Furth and K. W. Christenberry, Cancer Res., 20, 1, 1960.

200. UPTON, A. C., M. A. Kastenbaum and J. W. Conklin, in Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation, Ed. R. J. C. Harris, Academic Press, London, New York, 1963, p. 285.

VERZAR, F., Gerontologia, 3, 163, 1959.

VOS, O., B. G. Crouch and D. W. van Bekkum, Int. J. Rad. Biol., 3, 337, 1961.

VOS, O., M. J. de Vries, J. C. Collenteur and D. W. van Bekkum, J. Nat. Cancer Inst., 23, 53, 1959.

VOS, O. and W. W. H. Weyzen, Transpl. Bull., 30, 117, 1962.

VOS, O., (personal communications), 1963.

VRIES, M. J. de, Transpl. Bull., 30, 13, 1962.

VRIES, M. J. de, and O. Vos, J. Nat. Cancer Inst., 21, 1117, 1958.

VRIES, M. J. de and O. Vos, J. Nat. Cancer Inst., 23, 1403, 1959.

VRIES, M. J. de, B. G. Crouch, L. M. van Putten and D. W. van Bekkum, J. Nat. Cancer Inst., 27, 67, 1961.

WELLING, W., O. Vos, W. W. H. Weyzen and D. W. van Bekkum, Int. J. Rad. Biol., 1, 143, 1959.

WENSINCK, F., D. W. van Bekkum and H. Renaud, Radiation Res., 7, 491, 1957.

WENSINCK, F. and H. Renaud, Brit. J. Exptl. Pathol., 38, 483, 1957.

WEYZEN, W. W. H., De Identifikatie van het Immunologisch Apparaat van Heterologe Stralingschimeren, Thesis, Leyden, 1963.

WOODRUFF, M. F. A., in Biological Problems of Grafting, Blackwell Scientific Publications, Oxford, 1959, p. 83.

WOODRUFF, M. F. A., in The Transplantation of Tissues and Organs, chapter IV, C. C. Thomas, Publisher, Springfield, U.S.A., 1960. p. 51.

WOODRUFF, M. F. A., Proc. Roy Soc. 54^{II}, 1150, 1961.

WOODRUFF, M. F. A. and L. O. Simpson, Brit. J. Exptl. Pathol., 36, 494, 1955.

WOODRUFF, M. F. A. and E. M. Sparrow, Transpl. Bull., 4, 157, 1957.

WOOLES, W. R. and N. R. Di Luzio, Amer. J. Physiol., 203, 404, 1962.

ZAALBERG, O. B., Transplantatie en aan het Geslacht Gebonden Weefselincompatibiliteit, Thesis, Leyden, 1960.

ZAALBERG, O. B., O. Vos and D. W. van Bekkum, Nature, 180, 238, 1957.

ZAALBERG, O. B., W. W. H. Weyzen and O. Vos, Nature, 197, 300, 1963.

ZAALBERG, O. B., Paper read at the Joint Meeting of the Netherlands Radiobiological Society and the British Association for Radiation Research at Scheveningen, May 1963.
(abstract in press, Int. J. Rad. Biol.).

Printed by: N.V. Drukkerij Levisson, The Hague (Netherlands)