IONIZING RADIATION AND THE THYMUS

Effects of whole-body irradiation with fast fission neutrons and X-rays on the murine thymus

Ioniserende straling en de thymus
Effecten van totale lichaamsbestraling met snelle splijtingsneutronen en röntgenstraling op de thymus van de muis

PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de geneeskunde
aan de Erasmus Universiteit Rotterdam,
op gezag van de Rector Magnificus
Prof. Dr. A.H.G. Rinnooy Kan
en volgens besluit van het college van decanen.
De openbare verdediging zal plaatsvinden op
woensdag 1 oktober 1986 om 14.00 uur

DOOR

René Huiskamp

geboren te Apeldoorn

PROMOTIECOMMISSIE

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Dit proefschrift werd bewerkt binnen de afdeling Radiobiologie van het Energieonderzoek Centrum Nederland te Petten.

aan Christien

CONTENTS

ABBREVIATIONS

A	Average fluorescence intensity channel number
APC	Antigen Presenting Cell
D_0	The absorbed dose of ionizing radiation required to reduce a surviving fraction of stem cells with a factor 1/e or 0.37.
Gy	Gray, unit of absorbed dose 1 Gy = 1 J/kg = 100 rad
IDC	Inter-Digitating Cell
LET_∞	Linear Energy Transfer of the ionizing particles, the total energy transfer per unit of track length, expressed in keV per micro-meter.
MHC	Major Histocompatibility Complex
p	Peak channel number
R	Röntgen, unit of exposure for X-rays and gamma rays. One R is equal to 2.58×10^{-4} Coulomb per kilogram air. An exposure of one R corresponds with an absorbed dose equal to 0.0087 Gy in air and to about 0.0096 Gy in soft tissue.
RBE	Relative Biological Effectiveness, in this study equal to D_R/D_N, where D_R and D_N represent the absorbed doses of X-rays and neutrons, respectively, which cause the same biological effect.
TdT	Terminal deoxynucleotidyl Transferase
TNC	Thymic Nurse Cell

PREFACE - VOORWOORD

Op deze plaats wil ik iedereen bedanken die op enige manier betrokken was bij het tot standkomen van dit proefschrift.

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CHAPTER I

GENERAL INTRODUCTION

Irradiation with ionizing radiation as used in many radiotherapeutic procedures, can cause damage to organs vital to the organism. In this thesis, the effects of radiation on the thymus are investigated. In the first part of this chapter, general aspects of ionizing radiation are discussed. The second part deals with the thymus, an organ essential for the establishment of the immune defence mechanism, and gives a summary of the effects of irradiation on the lymphoid and stromal cells of the thymus.

I.1. IONIZING RADIATION

Ionizing radiation is a term used for those types of radiation that give rise, directly or indirectly, to ionizations when they interact with matter. Ionizing radiations comprise electromagnetic radiation, such as gamma- and X-rays but also particulate or corpuscular radiation, such as alpha particles, beta particles, protons and neutrons. All types of ionizing radiation react with molecules by causing either ionization or excitation. In case of ionization, an orbital electron is ejected from the molecule, resulting in the formation of an ion pair. In case of excitation, an electron is raised to a higher energy level. These excited or ionized molecules generate radicals which are electrically neutral and have an unpaired electron. These radicals are highly reactive with other molecules and may have an effect on living cells, especially when the radiation induced chemical alterations involve molecules or structures of great importance to the cell function and viability, i.e. the DNA. Since living cells consist for 80-90% of water, radiolysis of water is the most important source of free radicals in a cell. The radicals $H^{\cdot}$, $OH^{\cdot}$ and the hydrated electron e^{-}_{aq} are most commonly formed by radiolysis. These radicals react with each other and with organic and inorganic molecules. For instance in the reaction $R(H) + OH^{\cdot} \rightarrow R^{\cdot} + H_2O$, $R^{\cdot}$ can recombine with an $H^{\cdot}$ which means that the damage is not permanent. However, fixation of

damage can occur through "dimerization", $R^{\cdot} + R^{\cdot} \rightarrow R_2$, through "cross-linking", $R1^{\cdot} + R2^{\cdot} \rightarrow R1R2$, or through "oxidation", $R^{\cdot} + O_2 \rightarrow RO_2^{\cdot}$. Ultimately, through combination and recombination all radicals disappear.

Ionizing radiation can be directly ionizing i.e. alpha particles or indirectly ionizing i.e. neutrons and gamma- or X-rays. The directly ionizing particles are charged and, by virtue of their kinetic energy, possess the energy to produce ionizations along their track as a result of energy imparted to orbital electrons via electrical forces between the charged particles. The alpha particles have a great ionizing power but their large size results in very little penetrating power. The indirectly ionizing radiation types are electrically neutral and penetrate fairly well into tissue. However, the way of energy transfer into the irradiated tissue is different for gamma- or X-rays and neutrons. Gamma- as well as X-rays ionize tissues by ejection of directly ionizing fast electrons from the orbits of the atom whereas neutrons transfer their energy through collisions with mainly the nuclei of atoms. With fission neutrons, this results in ejection of positive recoil protons which are directly ionizing. The ionization density along the track of recoil protons is much higher than that of fast electrons. These differences in ionization density of radiation types can be expressed by the mean track-average LET_{∞} values. LET is an acronym for Linear Energy Transfer and is the rate of energy transfer per unit of track length in a medium. The track-average LET_{∞} of recoil protons produced by 1 MeV fast fission neutrons is equal to 57 keV per micron in water, whereas the mean track-average LET_{∞} of fast electrons produced by 300 kVp X-rays is about 3 keV per micron in water (Davids et al., 1969). These differences in LET values for X-rays and neutrons account for the observed differences of biological effectiveness after irradiation with these two types of irradiation (Davids, 1972; 1973). In order to compare the biological effectiveness of the two types of irradiation, the concept Relative Biological Effectiveness (RBE) has been introduced. The RBE of fast fission neutrons for a specific biological effect is defined as the ratio of the absorbed dose of a

reference radiation, i.e. 300 kVp X-rays, to the absorbed dose of neutrons which produces the same specific effect.

Neutron RBE determinations, as a function of the irradiation conditions, neutron energy spectrum, and biological endpoint, are relevant for radiation protection, fast neutron radiotherapy and protective treatment after accidental exposure with fast neutrons. Comparison of neutron RBE data obtained with fast neutrons of various mean energies shows that those for fast fission neutrons of 1 MeV mean energy are highest for each effect category investigated (Broerse & Barendsen, 1973). Therefore RBE data of fast fission neutron of 1 MeV mean energy can be regarded as the upper limit for neutron RBE data for each effect category.

In the Low Flux Reactor of the Netherlands Energy Research Foundation (ECN) at Petten, fission neutrons are produced by fission of uranium-235 (^{235}U) in a converter plate, which is exposed to thermal neutrons emitted from the core of the thermal reactor. The design of the neutron exposure facility, tissue dosimetry and neutron spectrometry have been described elsewhere (Davids, 1969). The use of small animals like the mouse as an experimental model for radiation research has the advantage that the dose-distribution after bilateral exposure of the animal is almost uniform and that several animals (e.g. 40 mice) can be irradiated simultaneously (fig. 1).

Using this exposure facility, RBE values for a number of tissues of an inbred CBA mouse strain have been obtained. De Ruiter-Bootsma and co-workers (1976) reported a neutron RBE value equal to 4.1 for a surviving fraction of spermatogonial stem cells at the 1% survival level. For gastric stem cell killing, an RBE equal to 3.7 was obtained (Kingma-ter Haar, 1982). These two RBE values are higher than those for jejunal crypt stem cell killing (RBE, 3.3) and hemopoietic stem cell killing (RBE, 2.4), reported by Davids (1973). These differences in RBE values for the various tissues are considered to be due to differences in the intrinsic radiosensitivity of the clonogenic cells in the tissues concerned (Broerse & Barendsen, 1973).

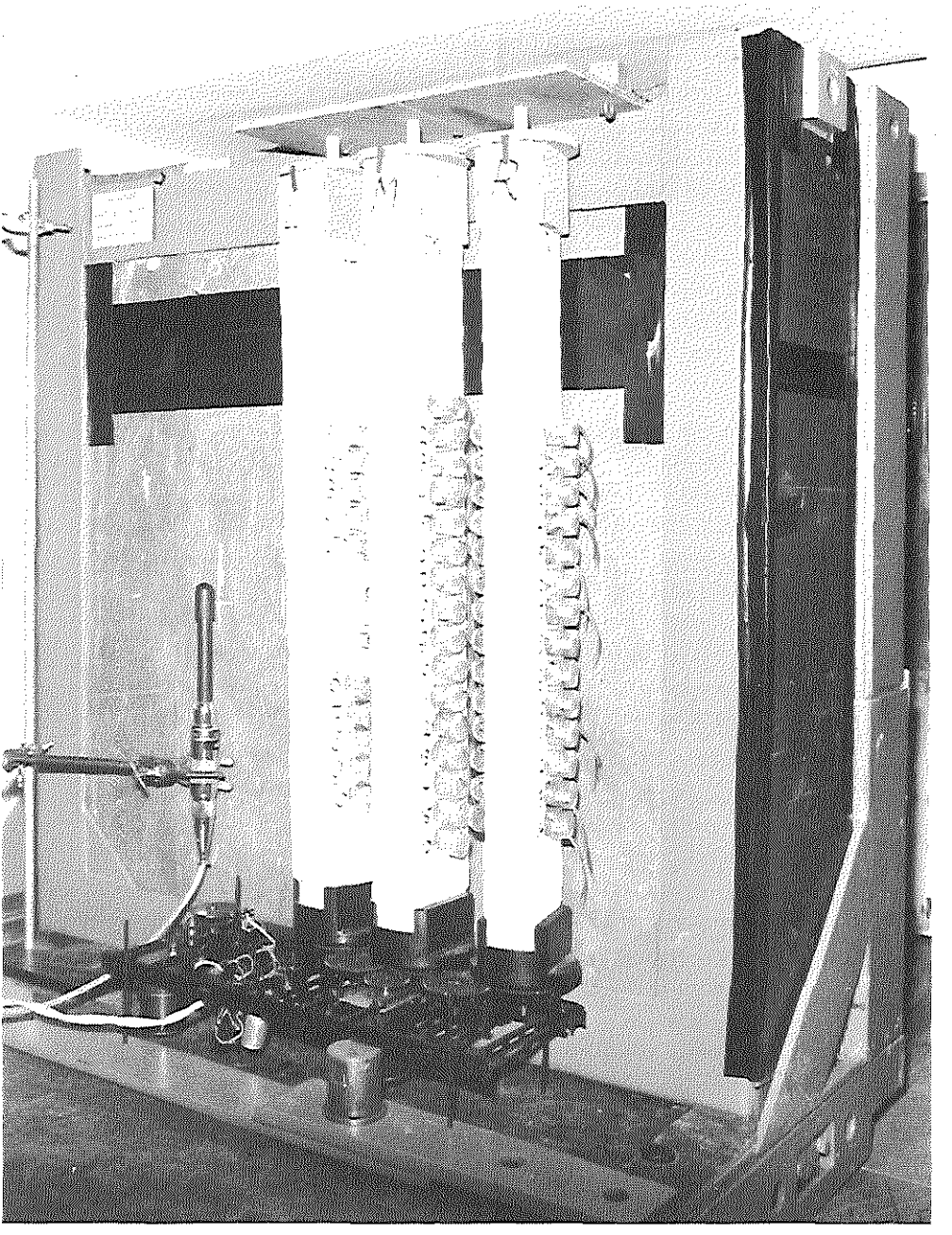

Fig. 1. Fast neutron exposure facility for mice in the Low Flux Reactor (LFR)

Another aspect in which high LET radiation, i.e. neutrons, differs from low LET radiation, i.e. gamma- and X-rays, is the so-called oxygen effect. This oxygen effect is illustrated by the fact that a higher dose of X-rays is required to reduce the survival of cells to a certain level in hypoxic than in oxygenated conditions. The ratio of these two doses is termed oxygen enhancement factor or Oxygen Enhancement Ratio (OER). The OER is in general 2.5-3.0 for X-rays and decreases with radiation types with higher LET values (Barendsen, 1968). With irradiation sources with a very high LET value (> 160 KeV per micron), the OER equals 1. Therefore, radiation with a high LET could be extremely useful for irradiation of tumors which are partly hypoxic.

A further radiobiological difference exist between fission neutrons and X-rays. After neutron irradiation no accumulation and repair of sublethal damage occurs (Davids, 1973; de Ruiter-Bootsma, 1976; Kingma-ter Haar, 1982).

In this thesis, effects of radiation on the thymus are investigated. The biological effectiveness of fast fission neutrons is compared with that of 300 kVp X-rays. The biological effects which were investigated all pertain to the thymus of the mouse. As an introduction, the following parts of this chapter will give a description of the mouse thymus.

I.2. The thymus

The thymus is essential for the establishment of the immune defence mechanism. It is considered as a primary lymphoid organ. The functions of the thymus include the reception of pre-T cells, the maturation and/or selection of antigen-specific T cells, the induction of tolerance to "self" antigens, and the selective release of such cells to the periphery. T cells can act as effector cells in the cellular immune response and as regulatory cells in both the humoral and the cellular immune response (Miller & Osaba, 1967, Cantor & Weissman, 1976; Stutman, 1978).

I.2.1. Architecture of the thymus

During ontogeny of the mouse, the thymic stroma arises from contributions of the third pharyngeal pouch and third branchial cleft (Cordier & Haumont, 1980). Thus, the epithelium of the mouse thymus originates from both ectoderm and endoderm. Though there is general agreement that endoderm buds into the surrounding mesenchyme which originates from the neural crest, the ectodermal contribution to the formation of the thymus is still controversial (LeDouarin et al., 1984). Already early in ontogeny (from day 13 onwards), the two major domains in the thymus, i.e. cortex and medulla can be identified as separate regions (van Vliet et al., 1985). During the course of ontogeny, these areas are seeded by lymphoid precursors and antigen presenting cells (APC), both originating from pluripotent stem cells in the yolk sac, fetal liver or bone marrow (Bartlett & Pyke, 1982; Boersma, 1983; Owen & Jenkinson, 1984). Gradually, both lymphoid and stromal components further expand and the thymic anlage assumes the architecture of the adult thymus.

The architecture of the adult thymus and the identification of various lymphoid and stromal cell types have extensively been studied (Hoshino, 1963; Hwang et al., 1974; van Ewijk, 1984; van de Wijngaert et al., 1984; van Vliet et al., 1984a,b). In the mouse, the thymus consists of two separate lobes each showing a cortical and medullary area. The cellular composition of both areas in the thymus is schematically illustrated in Fig.2. Stromal cells in the cortex are mainly epithelial cells. These cells have long thin processes which attach each other by means of desmosomes. Thus, these epithelial-reticular cells form a fine stromal meshwork in the cortex. In contrast, medullary epithelial cells do not show these long processes. These cells are closely packed and more spindle-shaped.

Special lympho-epithelial complexes, termed thymic nurse cells (TNC) have been demonstrated in thymic cell suspensions (Wekerle et al., 1980; van Vliet et al., 1984b) and in situ (van de Wijngaert et al., 1983). TNC are complexes of a single epithelial cell which completely envelopes a number of intact thymocytes. The TNC have been located in the subcapsular and outer cortical areas (Wekerle et al., 1980; Kyewski et al., 1982).

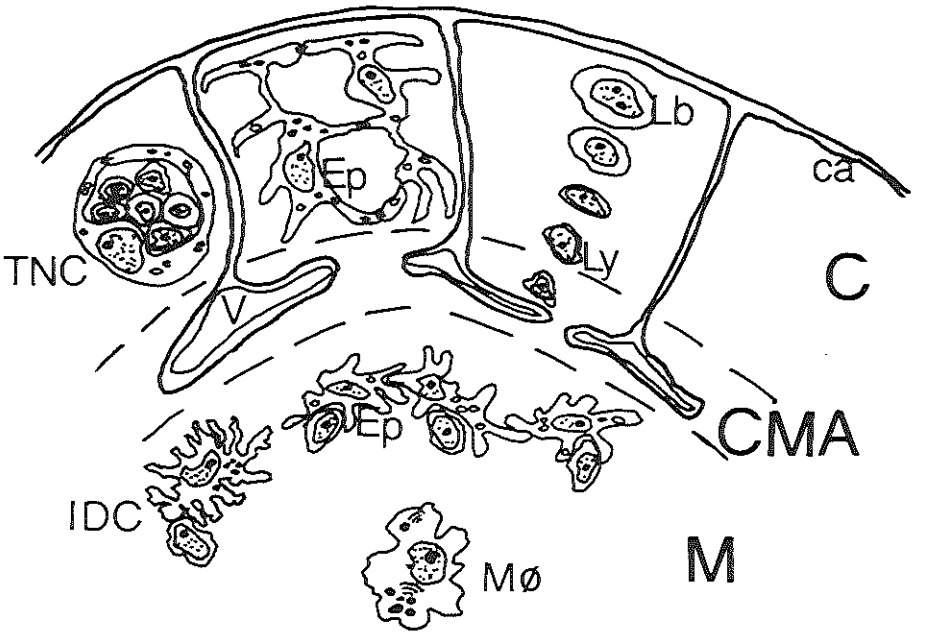

Fig. 2. Illustration of the cellular composition of the thymus, ca: capsule, C: cortex, CMA: cortico-medullary area; Ep: epithial cell; IDC: interdigitating cell; Lb: lymphoblast; Ly: lymphocyte; M: medulla; Mø: macrophage; TNC: thymic nurse cell; v: blood vessel.

Other non-lymphoid cells in the thymus are mononuclear phagocytes such as macrophages located both in the cortex as well as in the medulla (Beller & Unanue, 1980; Duijvestein et al., 1982) and interdigitating cells (IDC) which exclusively reside in the medulla (Duijvestein et al., 1982). Furthermore, fibroblasts are present in the thymic capsule and medulla (van Vliet et al., 1986).

The thymus also contains areas which are devoid of stromal cells (Rouse et al., 1979; van Ewijk, 1984). These areas are randomly distributed in both cortex and medulla. Their function has not been identified, yet.

The vascularization of the thymus occurs via small arterioles which enter the thymus through septa and penetrate as far as the cortico-medullary area. From this region capillaries arise which supply the cortex and the subcapsular area, and then curve back into the lobe. These vessels merge into large postcapillary venules in the cortico-medullary area and in the medulla. The medulla is supplied by capillaries which are directly derived from the arterioles in the cortico-medullary area. In the cortex, the presence of tight junctions on endothelial cells of the vessel walls indicates a poor passage of macromolecules whereas the walls of the postcapillary venules in the medulla do allow passage of macromolecules (Raviola & Karnovski, 1972). Furthermore, close association of cortical capillaries and epithelial cells and the localization of macrophages along them, also prevents access of antigen to the cortex. These observations indicate the existance of a "blood-thymus barrier" in the cortex against circulating substances in the cortex. However, recent observations dispute the existence of the so-called blood-thymus barrier since after injection of monoclonal anti-I-A antibodies in neonatal mice, these antibodies were found to be present in the thymic cortex (Kruisbeek et al., in preparation). In the medulla and the cortico-medullary area, lymphocytes are freely exposed to blood-borne substances. Moreover, in these areas cell passages in or out the thymus have been observed (Raviola & Karnovski, 1972).

I.2.2. T cell differentation

I.2.2.1. Cell surface antigens on T cells (Table I)

In mice, the thymus is seeded by lymphoid precursor cells originating from the bone marrow (Ford et al., 1966; Jones-Villeneuve et al., 1980). These so-called "prothymocytes" enter the thymus in the subcapsular area as well as in the medulla. Using embryonic chicken-quail chimeras, Jotereau and LeDouarin (1982) provided the first evidence that thymus precursor cells migrate into the cortex and the medulla. Recently, analysis of sections of the irradiated thymus repopulated with mixtures of Thy-1.1 and Thy-1.2 marked cells indicated also that thymic precursor cells are already committed to a cortical and medullary stream (Ezine et al., 1984). Additional analysis of frozen sections

of irradiation chimeras where Thy-1 allelic differences were monitored in the course of bone marrow-derived repopulation has also indicated that there are two sites, subcapsular and medullary, for cellular entrance into the thymus (Ceredig & Schreyer, 1984). However, in the latter study, it appeared that cells that enter the subcapsular site may expand more rapidly in number than cells which enter the medulla.

The phenotype of the thymus precursor cells in mice is presumably $H\text{-}2K^{+}$; they lack all T cell differentiation antigens (Ritter, 1978; Kamarck & Gottlieb, 1977; Goldschneider et al., 1982). During differentiation, thymic precursor cells loose or acquire differentiation markers on their cell surface. The most common cell surface antigens of murine T cells are listed in Table I. The most prominent T cell markers such as TL, Thy-1, T-200, Lyt-1, Lyt-2, MT-4 and MEL-14 are briefly discussed below.

The TL antigen is an early differentiation antigen which in a number of TL^{+} mouse strains is found only on immature thymocytes and not on peripheral T cells (Cantor & Boyse, 1977; Old & Stockert, 1977). Some TL^{-} mouse strains can express TL antigens anomalously on thymic lymphoma cells (Boyse & Old, 1969).

The Thy-1 antigen on murine T cells is generally regarded as a pan T cell marker, i.e. is expressed on virtually all T cells. However, Thy-1 is also expressed on murine CFU-s as well as myeloid progenitor cells (Schrader et al., 1982). Furthermore, Thy-1 is expressed on skin connective tissue (Morris & Ritter, 1980) and on nervous tissue (Reif & Allen, 1964).

In contrast, the T-200 antigen, or leucocyte common antigen is exclusively expressed on hemopoietic cells (Trowbridge, 1978; Ledbetter & Herzenberg, 1979, Ralph & Berridge, 1984).

The Lyt antigens are the first antigens on T cells that were shown to be related with distinct functional T cell subpopulations (Cantor & Boyse, 1975a,b; 1977). Thus, helper effects of T cells on humoral responses were associated with $Lyt\text{-}1^{+},2^{-}$ cells whereas the $Lyt\text{-}1^{-},2^{+}$ T cells were associated with suppressor effects and cytotoxicity. However, studies using flow cytofluorometry indicate that the Lyt-1 anti-

TABLE I

Markers of murine T cells

Marker	Percentage positive thymocytes	References
H-2K	41-68	Scollay et al., 1980 Lepault et al., 1983
H-2D	80-96	Scollay et al., 1980
I-A	34-43	Scollay et al., 1980 Lepault et al., 1983
TL, 1,2,3	63-85	Old & Stockert, 1977 Shen et al., 1982
TL 3	69-74	Scollay et al., 1980 Lepault et al., 1983
TL 5	65	Flaherty et al., 1977
Thy-1	96-100	Ledbetter et al., 1980 van Ewijk et al., 1981 Lepault et al., 1983
T-200	97	van Ewijk et al., 1981 Lepault et al., 1983
B14	70-80	Sidman et al., 1983
ThB	40-60	Stout et al., 1975 Lepault et al., 1983
Lyt-1	97	Ledbetter et al., 1980 van Ewijk et al., 1981
Lyt-2,3	81,82	Ledbetter et al., 1980 van Ewijk et al., 1981
L3T4	87	Dialynas et al., 1983 Scollay&Shortman, 1985
MEL-14	3	Gallatin et al., 1983 Reichert et al., 1984
B2A2	96.5	Scollay&Shortman, 1985
LFA-1	97	Kaufman et al., 1982
PNA	85-88	Reisner et al., 1976 Chervenak&Cohen, 1982
SBL	1	Raedler et al., 1983
TdT	85	Kung et al., 1975

gen is expressed on almost all T cells but in quantitatively different levels (Mathieson et al., 1979; Ledbetter, 1980; van Ewijk et al., 1981; Mathieson & Fowlkes, 1984). In addition, some B cell lymphomas (Lanier et al., 1981) and a number of normal B cells (Manohar et al., 1982) have shown to express the Lyt-1 antigen. Therefore the Lyt-1 antigen can no longer be used as an absolute marker of T cell subpopulations or T cells. More recently, a new monoclonal antibody (GK 1.5) has been developed by Dialynas et al. (1983) which detects a cell surface molecule designated L3T4, the murine equivalent of the human LEU 3/T4 antigen (Dialynas et al., 1983; Ceredig et al., 1983b). Another monoclonal antibody (H129-19) which detects a similar molecule designated "mouse T4" (MT-4) with a similar reactivity pattern, has been generated and characterized by Pierres et al. (1984). Summarized, functional T cells can be divided in Lyt-2^+, MT-4^- cells (cytotoxic/suppressor cells) and Lyt-2^-, MT-4^+ cells (helper/inducer cells) (Dialynas et al., 1983; Pierres et al., 1984).

Gallatin and co-workers (1983) developed a monoclonal antibody which detects an antigen called MEL-14. This antigen is associated with a "homing" receptor on peripheral lymphocytes for peripheral lymph nodes (Gallatin et al., 1983, 1986).

Pretreatment of T cells with MEL-14 antibodies prevents the adherence of T cells to postcapillary high endothelial venules, which mediate the entry of T cells into the lymph node parenchyma. MEL-14 is expressed on only 3 percent of the thymocytes and can be observed on cortical as well as medullary cells (Reichert et al., 1984; van Ewijk, 1984).

I.2.2.2. Thymocyte subpopulations and their relationships

With the use of a number of these T cell markers, functional as well as immature subpopulations can be distinguished in the thymus (see Mathieson et al., 1979; Ledbetter et al., 1979; van Ewijk et al., 1981; Dialynas et al., 1983; Scollay & Shortman, 1983, 1985; Scollay, 1983; Mathieson & Fowlkes, 1984). The frequency, size and localization of these thymocyte subpopulations are listed in Tabel II. Within the cortex, it is assumed that the typical maturation lineage is formed by

subpopulations 6, 7, 2, and 1 respectively involving both acquisition of T cell specific markers and a concomittant decrease in cell size. Such a sequence has indeed been demonstrated during ontogeny (van Ewijk et al., 1982; Ceredig et al., 1983a) and in the regenerating thymus after cortisone treatment (van Ewijk et al., 1981). However, the relationship between the cortical cells and the medullary subpopulations is as yet unclear and a number of models dealing with this problem have been proposed (reviewed by Mathieson, 1982; Scollay, 1983; Mathieson & Fowlkes, 1984; Scollay & Shortman, 1985).

In the first model, proposed by Cantor & Boyse (1977), both functional medullary subpopulations are believed to be derived from a common immature Lyt-1$^+$, Lyt-2$^+$ cortical precursor cell. This model sug-

TABLE II

Thymocyte subpopulations

Phenotype	Size in the thymus	Frequency	Localization
1. Thy-1$^+$, T-200$^+$, Lyt-1$^+$, Lyt-2$^+$, L3T4$^+$	small	60-70	thymic cortex
2. Thy-1$^+$, T-200$^+$, Lyt-1$^+$ Lyt-2$^+$, L3T4$^+$	large	15	thymic cortex
3. Thy-1$^+$, T-200$^+$, Lyt-1$^+$, Lyt-2$^-$, L3T4$^+$	medium	8-13	thymic medulla
4. Thy-1$^+$, T-200$^+$, Lyt-1 dull Lyt-2$^+$, L3T4$^-$	medium	5	thymic medulla
5. Thy-1$^-$, T-200$^-$, Lyt-1$^-$ Lyt-2$^-$, L3T4$^-$	large	1	thymic medulla
6. Thy-1$^-$, T-200^{-or+}, Lyt-1$^-$ or dull, Lyt-2$^-$, L3T4$^-$	large	1	thymic subcapsular and outer cortex
7. Thy-1$^+$, T-200^{-or+}, Lyt-1$^-$ or dull, Lyt-2$^-$, L3T4	large	1	thymic capsular and outer cortex

gests that during maturation cortical cells loose either Lyt-1 or Lyt-2 antigens. This model was based on autoradiographic studies of Weissman (1973) showing that some medullary cells were derived from subcapsular cortical cells. However, in contrast with the first model it has been shown that during ontogeny, Lyt-1^+ cells appear before cells bearing the Lyt-2 antigens (Mathieson et al., 1981; van Ewijk et al., 1982). Furthermore, Lyt-1^+ cells can differentiate into Lyt-1^+, 2^+ in vitro (Ceredig et al., 1983c).

The second model proposes that prothymocytes are already committed into two lineages that populate independently the cortex and the medulla (Shortman & Jackson, 1974). In this model, cortical cells are "dead-end" cells which cannot leave the thymus whereas the medulla is believed to be the sole source of both functional T cell subpopulations.

A third model has been proposed by Scollay (1983) and Mathieson (1984). They have suggested that the functional subpopulations may develop independently in the cortex and the medulla. One subpopulation, the T helper cells (Scollay, 1983), would develop in the cortex and a selected limited number would migrate into the medulla. Cytotoxic and suppressor cells would develop directly in the medulla. Thus, this model combines the previous described models.

Recently, Scollay and Shortman (1985) analyzed the early stages of T lymphocyte development in the thymus cortex and the medulla. The results of this study suggest that the separate developmental streams of cortical and medullary thymocytes may be traced back to common precursor cells in the subcapsular region of the cortex. This indicates the existence of a single early branch point for cortical and medullary maturation. This concept of two distinct streams from a single early branch point does reconcile the earlier disputed views concerning the origin of cortical and medullary thymocytes. A fourth alternative has been proposed by Reichert et al. (1984). In their model, the cortex is considered to be the major contributor of thymus emigrants. The rare cortical MEL-14^+ cells, which are larger in size, have a mature phenotype and are enriched for cytotoxic T lymphocyte precursors. They have been implicated to be the cells that leave the thymus (Fink et al., 1985). In addition, it was proposed that cortex and medulla both contribute to the peripheral T cell pool but produce different classes of

migrants. In this context, Reichert et al., (1984) and van Ewijk (1984) suggested that in the cortical sterile environment "virgin" T cells are generated, whereas the antigen-accessible medulla is the site for antigen-driven T cell expansion.

I.2.3. Stromal cell markers

Stromal cells, i.e. non-lymphoid cells, in the thymus can be detected with a variety of monoclonal antibodies (Table III).

TABLE III

Monoclonal antibodies directed against determinants on thymic stromal cells

Monoclonal antibody	Reacts with/ target antigen	Reference
10-216	$I\text{-}A^k$	Oi et al., 1978
13/4.R5	$I\text{-}E^k$	Hämmerling et al., 1979
11-4.1	$H\text{-}2K^k$	Oi et al., 1978
B22-249.R.1	$H\text{-}2D^b$	Hämmerling et al., 1979
ER-TR1	$Ia^{k,d,q,s}$	van Vliet et al., 1984a
ER-TR2	$Ia^{k,s,r}$	van Vliet et al., 1984a
ER-TR3	$Ia^{k,b,d,q,r,}$	van Vliet et al., 1984a
ER-TR4	cortical epithelial cells	van Vliet et al., 1984a
ER-TR5	medullary epithelial cells	van Vliet et al., 1984a
ER-TR6	medullary interdigitating cells	van Vliet et al., 1984a
ER-TR7	reticular fibroblasts	van Vliet et al., 1984a
M1/70	Mac-1	Springer et al., 1979
M3/38	Mac-2	Ho & Springer, 1982

Using antibodies specific for class I (H-2K/D) and class II (I-A, I-E) major histocompatibility complex (MHC) antigens, it has been shown that I-A, I-E and H-2K/D are mainly expressed on stromal cells in the thymus as well as on medullary thymocytes (Rouse et al., 1979; van Ewijk et al., 1980; van Ewijk, 1984). H-2D, however, is also expressed at low density on cortical thymocytes and in particular on subcapsular lymphoblasts (van Ewijk, 1984). Both class I and class II MHC antigens are expressed on the medullary stroma in a confluent staining pattern associated with bone marrow-derived IDC and medullary thymocytes. In contrast, only class II antigens are detectable on cortical stromal cells, i.e. epithelial cells, in a reticular pattern whereas some class I determinants are detectable only faintly, if at all, in the cortex.

With the use of the recently developed ER-TR monoclonal antibody series (van Vliet et al, 1984) discrete environments can be distinguished. The following subpopulations can be identified (van Vliet et al., 1984a,b; van Ewijk, 1984):

1. ER-TR4+, Ia+, ER-TR5-	cortical epithelial cells, including TNC.
2. ER-TR4-, Ia+, ER-TR5+	medullary epithelial cells.
3. ER-TR4-, Ia+, ER-TR5+	medullary epithelial cells
4. ER-TR4-, Ia+, ER-TR5-, ER-TR6+	medullary IDC.
5. ER-TR7+	reticular fibroblasts in cortex and medulla
6. ER-TR6+	macrophages.

Monoclonal antibodies directed to the Mac-1 and Mac-2 antigens, were initially reported to be specific for cells of the murine mononuclear phagocyte system. Mac-1 is regarded as a pan macrophage marker (Springer et al., 1979) and is expressed on scattered cells throughout the thymus (van Ewijk, 1984). A number of Mac-1^+ cells are also found in the cortex, especially in the vicinity of blood vessels and under the thymic capsule. Mac-2 antigens, specific for antigen-activated macrophages (Ho & Springer, 1982) are expressed at very low levels on cells in the medulla (van Ewijk, 1984).

I.2.4. Lympho-stromal interaction

Numerous in vivo and in vitro experiments indicate that the thymic stromal cells are involved in the differentiation and maturation of T cells. A number of hormone-like substances such as thymosin or "facteur thymique sérique", secreted by stromal cells, have been implicated to have effects on T cell differentiation (reviewed by Kruisbeek, 1979 and Trainin et al., 1983). However, conclusive evidence concerning the effects of thymic hormones is still lacking. A number of observations suggest that the thymic stroma produces a chemotactic factor, i.e. a soluble substance which attracts prothymocytes (Cohen & Fairchild, 1979, Pyke & Bach, 1979, Jotereau et al., 1980). Recently, Champion and co-workers (1986) detected chemotactic activity in conditioned media from avian embryonic thymic epithelium in culture. Two fractions containing chemotactic peptides with molecular weights of 4000 and 1000 daltons were considered to be responsible for the thymic recruitment of the first hemopoietic precursors and the renewal of these precursors during adult life.

On the other hand, direct cell-cell contact of thymic stroma with thymocytes is required for certain steps in T cell differentiation (reviewed by Stutman, 1978). These lympho-stromal interactions have been particularly demonstrated in so-called "thymic nurse cells" (TNC). TNC are in vitro isolated epithelial cells enclosing upto 50 thymocytes (Wekerle & Ketelsen, 1980; Kyewski & Kaplan, 1982; van Vliet et al., 1984b). Besides TNC, so-called "thymocyte rosettes" have been isolated in vitro. These "thymocyte rosettes" are complexes of thymocotyes with macrophages of medullary dendritic cells (Kyewski et al., 1982; Kyewski et al., 1984; Fink et al., 1984). In these rosettes are cytotoxic T lymphocyte precursors, that are indistinguishable from mature peripheral T cells by parameters of self tolerance, alloreactivity, H-2 restriction, and stringency of self H-2 preference (Fink et al., 1984; Kyewski et al., 1984). These lympho-stromal complexes isolated in vitro, may represent the in vivo association of thymocytes and thymic stromal cells (Fink et al., 1984; van Vliet et al., 1984b).

These studies, as well as other functional studies, implicate that

MHC antigens expressed on stromal cells are involved in the aquisition of MHC restriction specificity, the imposition of self tolerance, and the selection of the developing T cells (Zinkernagel et al., 1978; Fink & Bevan, 1978; Wagner et al., 1981; Kruisbeek et al., 1981; Sharrow et al., 1981; Singer et al., 1982).

The instructive role of the thymic stroma is indicated by a number of observations:

1. During ontogeny, the initial expression of MHC antigens on thymic stromal cells correlates with the onset of proliferation of prothymocytes in the embryonic thymus (Jenkinson et al., 1980);
2. Furthermore, the acquisition of MHC antigens on stromal cells in the embryonic thymus preceeds the expression of the T cell differentiation antigens Lyt-1 and Lyt-2 on thymocytes (Jenkinson et al., 1980; van Ewijk et al., 1982);
3. Another morphological indication for the role of MHC antigens in T cell differentiation can be observed in nude mice. These mice which are T cell deficient, have a thymic rudiment which differs markedly in MHC expression from the normal developed thymus since class I but not class II is expressed on its epithelial cells (Jenkinson et al., 1981; van Vliet et al., 1985);
4. In aged mice, the Ia expression on the cortical epithelial cells decreases (Farr & Sidman, 1984) and is accompanied by decreases in thymus weight and cellularity, and a reduced capacity to promote T cell differentiation (Hirokawa & Makinodan, 1975).

Besides the above reported morphological studies, a number of elegant functional studies have directly proven that class II antigens can influence T cell differentiation. Sprent (1980) demonstrated that blocking of Ia antigens on stromal cells through in vivo administration of monoclonal antibodies against I-A/E determinants interfered with the activation of Ia-restricted T cells in the spleen. Kruisbeek et al. (1983) showed that multiple injections of purified monoclonal anti-I-A antibodies in neonatal mice interfered with the generation of immunocompetent Ia-restricted T helper cells. Thus, these data argue that masking of MHC determinants on stromal cells by injection of anti-class

II antibodies, prevents the recognition of these determinants by the developing T helper cells and, as a consequence, interferes with the clonal amplification of these cells. Recently, Kruisbeek and co-workers confirmed this hypothesis and showed that neonatal anti-Ia antibody treatment results in reduced Ia-antigen expression in the thymus, especially in the medulla, and in the absence of thymic and splenic $L3T4^{+}$, $Lyt\text{-}2^{-}$ (helper) T cells (Kruisbeek et al., 1985). These animals also showed a reduced medullary compartment. These data strongly implicate that the class II medullary stromal cells play an important role in the generation of functional T helper cells.

Numerous functional studies reported in the literature, emphasize an instructive role of the thymic stroma with respect ot restriction imposed upon T cells. T cells recognize foreign antigens only in association with polymorphic gene products of the MHC (Zinkernagel and Doherty, 1975; Fink & Bevan, 1978; Katz et al., 1978; Zinkernagel, 1978; Sprent, 1980; Kindred, 1980, 1981; Wagner et al., 1981; Kruisbeek et al., 1981; Sharrow et al., 1981; Singer et al., 1982). The MHC determinants involved appeared to be similar to those expressed by the "host environment", i.e. the thymus, in which the T cells matured. There is, however, controversy on the role of class I and class II microenvironments in the thymus in the process of imposing H-2-restriction.

Studies with:

a. $F_1 \rightarrow F_1$ radiation bone marrow chimeras that were thymectomized and grafted with a parental thymus (Fink & Bevan, 1978; Zinkernagel et al., 1978);

b. $F_1 \rightarrow$ Parent radiation bone marrow chimeras (Zinkernagel et al, 1978);

c. Parent $\rightarrow F_1$ radiation bone marrow chimeras (von Boehmer & Haas, 1976; Pfizenmaier et al., 1976; Zinkernagel, 1976) and

d. Fully allogeneic A $\rightarrow$ B chimeras (Kruisbeek et al., 1982) suggest that the thymus dictates the self-class I restricted repertoire of mature cytotoxic T cells. In contrast, studies with allogeneic thymus engrafted nude mice suggested that the thymus is not the site that determines the class I restricted repertoire (Kindred, 1978; Zinker-

nagel, 1980). Recent studies with fully allogeneic chimeras or allogeneic thymus engrafted nude mice revealed the existence of an intra- en extrathymic differentiation pathway for class I restricted T cells of which the "self" recognition expressed by helper T cells which are class II restricted, is solely determined by the intrathymic environment (Singer et al., 1981, 1982). Kast and co-workers (1984) recently extended these studies and examined besides the MHC restiction specificity of T cells also the immune response (Ir) gene-controlled responsiveness. They showed that the thymus dictates the MHC specificity and Ir gene phenotype of class II restricted T cells but not of class I restricted T cells.

The question can be raised which stromal cell type imposes MHC restriction to the differentiating T cells. The critical cell in the thymus responsible for determining the class II restriction of the developing T cells might be a bone marrow-derived antigen presenting cell (APC) or dendritic cell (Longo & Schwartz, 1980; Longo & Davis, 1983). Using radiation bone marrow chimeras, these authors investigated the donor or host origin of thymic APC and the class II restriction specificity of the T cells. They demonstrated that with increasing dose of irradiation used to establish the radiation chimeras, host APC are more quickly depleted and replaced by donor-type APC. In conventional chimeras, irradiated with 925 R*, the thymic APC, two weeks after irradiation, are still of host-type origin and T cells in these mice are restricted to antigen recognition in the context of host H-2 gene products. At higher irradiation doses (1200 R) used to establish chimerism, these host-type APC are depleted and replaced by donor-type APC. T cells in these mice recognized donor H-2 gene products as self. Recently, Longo et al. (1985) confirmed their earlier findings in nude mice reconstituted with thymuses of radiation-induced bone marrow chimeras and showed that the T cell class II restriction of nude mouse recipients is determined at least in part by the phenotype of the bone

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* When exposure unit R is used in this thesis, the quoted references report doses in units R. One R corresponds with an absorbed dose equal to 0.0087 Gy in air and to about 0.0096 Gy in soft tissue.

marrow derived APC in the chimeric thymus. However, using chimeras established with deoxyguanosine treated thymus grafts, Lo and Sprent (1986) showed recently that the epithelial cells in the thymus are responsible for imprinting the H-2-restricted specificity of T helper cells. The induction of tolerance to "self" antigens was mediated by the macrophages and dendritic cells in the thymus.

In summary, these experiments show that the MHC antigens expressed on the thymic stromal cells determines at least in part the specificity of the self restriction of mature T cells and are essential in the generation of tolerance to "self" antigens.

I.3. Ionizing Radiation and the Thymus

I.3.1. Effects of irradiation on lymphoid cells in the thymus

The effects of ionizing radiation on the cellularity of the murine thymus have been extensively studied (Blomgren & Révész, 1968; Takada et al., 1969; 1971; Blomgren, 1971; Sharp & Thomas, 1975; Kadish & Basch, 1975; Hiesche & Révész, 1979). Following whole-body doses of X-irradiation, including doses within the lethal range, the regeneration of the thymus follows a biphasic pattern. After the initial decrease in thymus weight and cellularity until Day 5, the thymus of the surviving animals reaches a peak size at about 14 days post-irradiation. Subsequently, there is a second decline in thymus weight and cellularity to a nadir on Day 21 followed by a final recovery at Day 40 post-irradiation. The initial decrease of thymus weight and cellularity is due to interphase death of thymocytes and an induced cessation of mitosis (Takada et al., 1969). The following regeneration has been attributed to proliferation of intrathymic surviving radioresistant precursor cells (Kadish & Basch, 1975; Hiesche & Révész, 1979; Sharrow et al., 1983) which are not directly derived from the bone marrow derived stem cells. The second involution of the thymus can be explained by a limited proliferative capacity and exhaustion of this pool of radioresistant precursor cells (Blomgren & Révész, 1968; Dukor et al., 1965) together with an impaired production of thymus precursor cells in the bone marrow due to

radiation damage (Takada et al., 1969; Blomgren, 1971; Boersma et al., 1981). The ultimate recovery of the thymus is due to a replenishment of the prothymocyte pool in the recovered bone marrow (Takada et al., 1969; Hiesche & Révész, 1979; Coggle, 1981).

The initial rapid decrease in thymus weight and cellularity after whole-body irradiation indicates that the lymphoid compartment of the thymus is extremely sensitive to radiation. Morphological alterations are already evident after whole-body exposures as small as 0.05 Gy (Anderson et al., 1977). Several studies have characterized the radiosensitivity of the lymphoid cells in the thymus. Cortical thymocytes have a D_0 value, i.e. the radiation dose that reduces the cell population by a factor e to 37 percent of its initial size, equal to about 50 R (Trowell, 1961; Sato & Sakka, 1969; Kadish & Basch, 1975). Medullary thymocytes were reported to have a D_0 value of about 200 R (Trowell, 1961). Sato & Sakka (1969) and Sharp & Watkins (1981) reported a D_0 value of 1.20 to 1.35 Gy for the small cell population in the thymus but these estimates represent a composite value for cortical and medullary thymocytes.

Thymocytes during the initial phase of regeneration have a D_0 value of about 0.70-0.80 cGy (Kadish & Basch, 1975; Sharp & Watkins, 1981). However, the intrathymic precursor cells from which these cells originate, might be more radioresistant (Sharp & Thomas, 1974, 1975; Kadish & Basch, 1975). A small (less than 8 percent) thymocyte population, presumably localized in the medulla, has been reported to be very radioresistant with a D_0 value of about 450 R (Sato & Sakka, 1969).

I.3.2. Effects of irradiation on stromal cells in the thymus

There is, at present, no completely acceptable method of quantitative assessment of the cell survival of the non-lymphoid cells of the thymus, but a number of indirect approaches have been employed to gain insight in this problem.

In a semiquantitative histopathological evaluation of damage to epithelial cells at various intervals after lethal doses of whole-body irradiation, it was found that thymic epithelium was relative sensitive

to irradiaton and that its recovery was severly impaired after an exposure to doses of 1100 R and higher (van Bekkum, 1967).

Using primary cell cultures of thymic tissue, Sharp and Watkins (1981) measured the survival and the proliferative capacity of thymic stromal cells in culture following X-irradiation. The vast majority of non-lymphoid cells in primary cell cultures of the mouse thymus were macrophages. The survival curve for these cells was characterized by a D_0 value equal to 1.25 Gy. Using similar techniques, D_0 values equal to 3.00 Gy and 3.20 Gy have been obtained for rat fibroblasts and dog thymic epithelial cells, respectively.

The same authors studied also the capacity of the thymic microenvironment to support the repopulation by thymocyte precursor cells in vivo. In these experiments, mice received 8.0 Gy whole-body X-irradiation and additional doses were given to the thymus only, up to a total thymus dose of 13.0 Gy. The authors assumed that the thymic microenvironment is fully able to support the proliferation of thymic precursor cells at doses up to 7.0 to 8.0 Gy. The resulting "survival" curve for doses above 8.0 Gy, had a D_0 value of about 1.80 Gy and was considered as a composite value for several stromal cell populations within the thymic microenvironment that support T cell differentiation. However, this approach does not exclude radiation effects on the thymic precursor cells and therefore the reported D_0 value cannot be regarded as an exact estimate of the radiosensitivity of the thymic microenvironment.

Recently, Hirokawa and Sado (1984) reported on the regeneration and T cell differentiation supporting function of the thymic stroma after irradiation. In their experiments, mice were thymectomized and grafted with a neonatal thymus which had been X-irradiated prior to transplantation. An exponentional decrease was observed in graft size and the T cell supporting function of the graft at doses of 600 R and higher. Additional graded doses of irradiation given only to the thymus of adult mice previously exposed to 1000 R X-rays and reconstituted with syngeneic bone marrow cells, had a less pronounced effect on the T cell supporting function of the thymus. In a third system, the thymus

of neonatal mice was locally exposed to graded doses of X-rays. In this system it was shown that the regenerative potential of these thymuses was radioresistant. Even a local irradiation of the thymus with 2000 R had no effects on immunological parameters such as Con A, PHA, and anti-sheep red blood cell responses. Comparing the results of these experiments, Hirokawa and Sado (1984) concluded that the T cell supporting function of the thymus was highly radioresistant while its regenerative potential was radiosensitive.

Using also *in situ* irradiation of the thymus, Davis and Cole (1969) reported an eventually recovered thymic function after exposure up to 2500 R X-rays. Thus, although there are no direct measurements of thymic stromal cell survival, *in vitro* measurements and a number of indirect approaches concerning thymic function, indicate that the thymic microenvironment is highly radioresistant.

REFERENCES

Anderson, R.E., G.B. Olson, J.R. Autry, J.L. Howarth, G.M. Troup and P.H. Bartels. Radiosensitivity of T and B Lymphocytes. IV. Effect of whole-body irradiation upon various lymphoid tissues and numbers of recirculating lymphocytes. J. Immunol. 118, 1191 (1977).

Barendsen, G.W. Responses of cultured cells, tumours and normal tissues to radiations of different linear energy transfer. In Current Topics in Radiation Research (M. Ebert and A. Howard, Eds.), Vol. 4, 293, North-Holland, Amsterdam, 1968.

Bartlett, P.F. and K.W. Pyke. Evidence for extrinsic origin of Ia positive cells in embryonic murine thymus. Adv. Exp. Med. Biol. 149, 375 (1982).

Bekkum, D.W. van. Immunological and hematological aspects of recovery: Implications for therapeutic use of bone marrow grafts. In: Recovery and Repair Mechanisms in Radiobiology. Brookhaven Symposia in Biology, Upton, Brookhaven National Laboratory 20, 190 (1968).

Beller, D.J. and E.R. Unanue. Ia antigens and antigen-presenting function of thymic marcophages. J. Immunol. 124, 1433 (1980).

Blomgren, H. The role of bone marrow of X-irradiated mice in thymic recovery. Cell Tissue Kinet. 4, 443 (1971).

Blomgren, H. and L. Révész. The effect of bone marrow protection on the cellular composition of the irradiated mouse thymus. Exp. Cell Res. 53, 261 (1968).

Boehmer, H. von and W. Haas. Cytotoxic T lymphocytes recognize allogeneic tolerated TNP-conjugated cells. Nature 261, 139 (1976).

Boersma, W.J.A. Prothymocytes in mouse fetal liver. Thymus 5, 419 (1983).

Boersma, W., I. Betel, R. Daculsi and G. van der Westen. Post-irradiation thymocyte regeneration after bone marrow transplantation. I. Regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet. 14, 179 (1981).

Boyse, E.A. and L.J. Old. Some aspects of normal and abnormal cell surface genetics. Ann. Rev. Genet. 3, 269 (1969).

Broerse, J.J. and G.W. Barendsen. Relative biological effectiveness of fast neutrons for effects on normal tissues. Curr. Top. Radiat. Q. 8, 305 (1973).

Cantor, H. and E.A. Boyse. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is a differentiative process independent of antigen. J. Exp. Med. 141, 1375 (1975a).

Cantor, H. and E.A. Boyse. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperating between subclasses of Ly^+ cells in the generation of killer activity. J. Exp. Med. 141,

1930 (1975b).

Cantor, H. and E.A. Boyse. Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev. 33, 105 (1977).

Cantor, H. and I.L. Weissman. Development and function of subpopulations of thymocytes and T lymphocytes. Prog. Allergy 20, 1 (1976).

Ceredig, R. and M. Schreyer. Immunohistochemical localization of host and donor-derived cells in the regenerating thymus of radiation bone marrow chimeras. Thymus 6, 15 (1984).

Ceredig, R., H.R. MacDonald and E.J. Jenkinson. Flow-cytometric analysis of mous thymus development in vivo and in vitro. Eur. J. Immunol. 13, 185 (1983a).

Ceredig, R., D.P. Dialynas, F.W. Fitch and H.R. MacDonald. Precursors of T cell growth factor producing cells in the thymus: Ontogeny, frequency and quantitative recovery in a subpopulation of phenotypically mature thymocytes defined by monoclonal antibody GK1-5. J. Exp. Med. 158, 1654 (1983b).

Ceredig, R., R.P. Sekaly and H.R. MacDonald. Differentiation in vitro of Lyt-2^+ thymocytes from embryonic Lyt-2-precursors. Nature 303, 248 (1983c).

Champion, S., B.A. Imhof, P. Savagner, and J.P. Thiery. The embryonic thymus produces chemotactic peptides involved in the homing of hemopoietic precursors. Cell 44, 781 (1986).

Chervenak, R. and J.J. Cohen. Peanut lectin binding as a marker for activated T lineage lymphocytes. Thymus 4, 61 (1982).

Coggle, J.E. The absence of late effects of radiation on the cellularity of the mouse thymus. Int. J. Radiat. Biol. 40, 229 (1981).

Cohen, J.J. and S.S. Fairchild. Thymic control of proliferation of T cell precursors in bone marrow. Proc. Natl. Acad. Sci. USA 76, 6587 (1979).

Cordier, A.C. and S.M. Haumont. Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice. Am. J. Anat. 157, 227 (1980).

Dialynas, D.P., D.B. Wilde, P. Marrack, A. Pierres, K.A. Wall, W. Havran, G. Otlen, M.R. Loken, M. Pierres, J. Kappler and F.W. Fitch. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK 1-5: expression of L3T4a by functional T cell clones appears to correlate primarily with class 11 MHC antigen-reactivity. Immunol. Rev. 74, 29 (1983).

Davids, J.A.G. Application of a fast-neutron facility in an Argonaut reactor for radiobiological research. In Radiobiological Applications of Neutron Irradiation, p. 27, International Atomic Energy Agency, Vienna, 1972.

Davids, J.A.G. Acute effects of 1-MeV fast neutrons on the haematopoietec tissues, intestinal epithelium and gastric epithelium in mice. In Advances in Radiation Research (J.F. Duplan and A. Chapiro, Eds.), vol. 2, p. 565, Gordon & Breach, New York, 1973.

Davids, J.A.G., A.P.J. Mos and A. de Oude. Fast-neutron facility for biological exposures in an Argonaut reactor: design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14, 573 (1969).

Davis, W.F. and C.J. Cole. Retarded immunological recovery in sublethally X-irradiated mice by additional thymic exposure. Reversal with injected marrow cells. Proc. Soc. Exp. Biol. Med. 130, 1336 (1969).

Dukor, P., J.F.A.P. Miller, W. House and V. Allman. Regeneration of thymus grafts. I. Histological and cytological aspects.

Transplantation 3, 639 (1965).

LeDouarin, N.M., F. Dieterlen-Lièvre and P.D. Oliver. Ontogeny of primary lymphoid organs and lymphoid stem cells. Am. J. Anat. 170, 261 (1984).

Duijvestijn, A.M., T. Sminia, Y.G. Köhler, E.M. Janse and E.C.M. Hoefsmit. The rat thymus microenvironment: a morphological and functional characterization. Adv. Exp. Med. Biol. 149, 441 (1982).

Ewijk, W. van. Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. Am. J. Anat. 170, 311 (1984).

Ewijk, W. van, P.L. van Soest and G.J. van den Engh. Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T-200. J. Immunol. 127, 2594 (1981).

Ewijk, W. van, E.J. Jenkinson and J.J.T. Owen. Detection of Thy-1, T-200, Lyt-1 and Lyt-2 bearing cells in the developing lymphoid organs of the mouse embryo in vivo and in vitro. Eur. J. Immunol. 12, 262 (1982.

Ewijk, W. van, R.V. Rouse and I.L. Weissman. Distribution of H-2 micro-environments in the mouse thymus. Immunoelectronmicroscopic identification of I-A and H-2K bearing cells. J. Histochem. Cytochem. 28, 1089 (1980).

Ezine, S., I.L. Weissman and R.V. Rouse. Bone marrow cells give rise to distinct cell clones within the thymus. Nature 309, 629 (1984).

Farr, A.G. and C.L. Sidman. Reduced expression of Ia antigens by thymic epithelial cells of aged mice. J. Immunol. 133, 98 (1984).

Fink, P.J. and M.J. Bevan. H-2 antigens of the thymus determine lymphocyte specificity. J. Exp. Med. 148, 766 (1978).

Fink, P.J., I.L. Weissman, H.S. Kaplan and B.A. Kyewski. The immuno-competence of murine stromal cell-associated thymocytes. J. Immunol. 132, 2266 (1984).

Fink, P.J., W.M. Gallatin, R.A. Reichert, E.C. Butcher and I.L. Weissman. Homing receptor-bearing thymocytes, an immunocompetent cortical sub-population. Nature 313, 233 (1985).

Flaherty, L., K. Sullivan and D. Zimmerman. The Tla locus: a new allele and antigenic specificity. J. Immunol. 119, 571 (1977).

Ford, C.E., H.S. Micklem, E.P. Evans, J.G. Gray and D.A. Ogden. The inflow of bone marrow cells to the thymus: studies with part-body irradiated mice injected with chromosome-marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci. 129, 283 (1966).

Gallatin, W.J., I.L. Weissman and E.C. Butcher. A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304, 30 (1983).

Gallatin, M., T.P.St. John, M. Siegelman, R. Reichert, E.C. Butcher and I.L. Weissman. Lymphocyte homing receptors. Cell 44, 673 (1986).

Goldschneider, U., K. Shortman, D. McPhee, S. Linthicum, G. Mitchell, F. Battye and F.J. Bollum. Identification of subsets of proliferating low Thy-1 cells in thymus cortex and medulla. Cell. Immunol. 69, 59 (1982).

Hiesche, K.D. and L. Révész. Effect of cortisone and X-irradiation on cellular depletion and regeneration between thymus lymphocyte precursors in the bomne marrow and in the thymus. Pathol. Res. Pract. 164, 157 (1979).

Hirokawa, K. and T.Makinodan. Thymic involution: effect on T cell differentiation. J. Immunol. 114, 1659 (1975).

Hirokawa, K. and T. Sado. Radiation effects on regeneration and T cell-inducing function of the thymus. Cellular Immunol. 84, 372 (1984).

Ho, M. and T.A. Springer. Mac-2, a novel 32,000 M mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. J. Immunol. 129, 1221 (1982).

Hoshino, T. Electronmicroscopic studies of the epithelial reticular cells of the mouse thymus. Z. Zellforschung 59, 513 (1963).

Hwang, W.S., T.Y. Ho, S.C. Luk and G.T. Simon. Ultrastructure of the rat thymus. A transmission, scanning electronmicroscope, and morphometric study. Lab. Invest. 31, 473 (1974).

Jenkinson, E.J., J.J.T. Owen and R. Aspinall. Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus. Nature 284, 177 (1980).

Jenkinson, E.J., W. van Ewijk and J.J.T. Owen. Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice. J. Exp. Med. 153, 280 (1982).

Jones-Villeneuve, E.V., J.J. Rusthoven, R.G. Miller and R.A. Philips. Differentiation of Thy-1 bearing cells from progenitor in long-term bone marrow cultures. J. Immunol. 124, 596 (1980).

Jotereau, F.V. and N.M. LeDouarin. Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. J. Immunol. 129, 1869 (1982).

Kadish, J.L. and R.S. Basch. Thymic regeneration after lethal irradiation: Evidence for an intra-thymic radioresistant T cell precursor. J. Immuno. 114, 452 (1975).

Kamarck, M.W. and P.D. Gottlieb. Expression of thymocyte surface

allo-antigens in the fetal mouse thymus in vivo and in organ culture. J. Immunol. 119, 407 (1977).

Kast, W.M., L.P. de Waal and C.J.M. Melief. Thymus dictates major histocompatibility complex (MHC) specificity and immune response gene phenotype of class II MHC-restricted T cells but not of class I MHC-restricted T cells. J. Exp. Med. 160, 1752 (1984).

Katz, D.H., B.J. Skidmore, L.P. Katz and C.A. Bogowitz. Adaptive differentiation of murine lymphocytes. I. Both T and B lymphocytes differentiating in F1 → parental chimeras manifest preferential cooperative activity for partner lymphocytes derived from the same parental strain type corresponding to the chimeric host. J. Exp. Med. 148, 727 (1978).

Kaufmann, Y., P. Golstein, M. Pierres, T.A. Springer and Z. Eshbar. LFA-1 but not Lyt-2 is associated with killing activity of cytotoxic T lymphocyte hybridomas. Nature 300, 357 (1982).

Kindred, B. Functional activity of T cells which differentiate from nude mouse precursors in a congenic or allogeneic thymus graft. Immunol. Rev. 46, 60 (1978).

Kindred, B. Lymphocytes which differentiate in an allogeneic thymus. III. Parental T cells which differentiate in an allogeneic or semiallogeneic thymus help only B cells of their own genotype. Cell. Immunol. 51, 64 (1980).

Kindred, B. A comparison of H-2 restriction of helper function using T cells from radiation chimeras and nuce mice bearing thymus grafts. Cell. Immunol. 63, 228 (1981).

Kingma-ter Haar, J.M. Effects of fission neutrons and X-rays on the epithelium of the mouse stomach. Thesis, State University, Utrecht 1982.

Kruisbeek, A.M. Thymic factors and T cell maturation in vitro: a

comparison of the effects of thymic epithelial cultures with thymic extracts and thymus dependent serum factors. Thymus 1, 163 (1979).

Kruisbeek, A.M., S.O. Sharrow, B.J. Mathieson and A. Singer. The H-2 phenotype of the thymus dictates the self-specificity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. J. Immunol. 127, 2168 (1981).

Kruisbeek, A.M., K.S. Hathcock, R.J. Hodes and A. Singer. T cell from fully H-2 allogeneic (A → B) radiation bone marrow chimeras are functionally competent and host restricted but are alloreactive against hybrid Ia determintants expressed on (A x B) F_1 cells. J. Exp. Med. 155, 2864 (1982).

Kruisbeek, A.M., S.O. Sharrow and A. Singer. Differences in the MHC-restricted self-recognition repertoire of intra-thymic and extra-thymic cytotoxic T lymphocyte precursors. J. Immunol. 130, 1027 (1983a).

Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer and J.J. Mond. Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia-antibodies interferes with differentiation of I-restricted T cells but not K/D-restricted T cells. J. Exp. Med. 157, 1932 (1983b).

Kruisbeek, A.M. J.J. Mond, B.J. Fowlkes, S. Bridges and D.L. Longo. In vivo treatment of neonatal mice with anti-Ia antibodies: development of the $L3T4^+Lyt\text{-}2^-$ T cell subset is dependent on thymic Ia bearing antigen-presenting cell function. J. Exp. Med. 161, 1029 (1985).

Kung, P.C., A.E. Silverstone, R.P. McCaffrey and D. Baltimore. Murine TdT: cellular distribution and response to cortisone. J. Exp. Med. 141, 855 (1975).

Kyewski, B.A. and H.S. Kaplan. Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. J. Immunol. 128, 2287 (1982).

Kyewski, B.A., R.V. Rouse and H.S. Kaplan. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow derived stromal cells in the mouse thymus. Proc. Natl. Acad. Sci. USA 70, 5646 (1982).

Kyewski, B.A., M. Travis and H.S. Kaplan. Intra-thymic lymphopoiesis: stromal cell-associated proliferation of T cells is independent of lymphocyte genotype. J. Immunol. 133, 1111 (1984).

Lanier, L.L., N.L. Warner, J.A. Ledbetter and L.A. Herzenberg. Expression of Lyt-1 antigen on certain murine B cell lymphomas. J. Exp. Med. 153, 998 (1981).

Ledbetter, J.A. and L.A. Herzenberg. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63 (1979).

Ledbetter, J.A., R.V. Rouse, H. S. Micklem and L.A. Herzenberg. T cell subsets defined by expression of Lyt-1, 2, 3 and Thy-1 antigens. J. Exp. Med. 152, 280 (1980).

Lepault, F., R.L. Coffman and I.L. Weissman. Characteristics of thymus-homing bone marrow cells. J. Immunol. 131, 64 (1983).

Lo, D. and J. Sprent. Identity of cells that imprint H-2 restricted T-cell specificity in the thymus. Nature 319, 672 (1986).

Longo, D.L. and R.H. Schwarz. T cell specificity for H-2 and Ir gene phenotype correlates with the phenotype of thymic antigen-presenting cells. Nature 287, 44 (1980).

Longo, D.L. and M.L. Davis. Early appearance of donor-type antigen presenting cells in the thymuses of 1200 R radiation-induced bone marrow chimeras correlates with self-recognition of donor I region gene products. J. Immunol. 130, 2525 (1983).

Longo, D.L., A.M. Kruisbeek, M.L. Davis and L.A. Matis. Bone marrow-derived thymic antigen-presenting cells determine self-recognition of Ia-restricted Tlymphocytes. Proc. Natl. Acad. Sci. USA 82, 5900 (1985).

Manohar, V., E. Brown, W.M. Leiserson and T.M. Chused. Expression of Lyt-1 by a subset of B lymphocytes. J. Immunol. 129, 532 (1982).

Mathieson, B.J. A re-viewing of thymocyte differentiation. In: A. Bernard and L. Boumsell (eds.), Human Leucocyte Markers detected by monoclonal antibodies. Springer Verlag, Berlin, p. 654, 1984.

Mathieson, B.J. and B.J. Fowlkes. Cell surface expression on thymocytes: development and phenotypic differentiation of intrathymic subsets. Immunol. Rev. 82, 141 (1984).

Mathieson, B.J., S.O. Sharrow, P.S. Campbell and R. Asofsky. A Lyt differentiated thymocyte subpopulation detected by flow microfluorometry. Nature 277, 478 (1979).

Miller, J.F.A.P. and D. Osoba. Current concepts of the immunological functions of the thymus. Phys. Rev. 47, 437 (1967).

Oi, V.T., P.P. Jone, J.W. Goding, L.A. Herzenberg and L.A. Herzenberg. Properties of monoclonal antibodies to mouse Ig allotypes. Curr. Top. Microbiol. Immunobiol. 81, 115 (1975).

Old, L.J. and E. Stockert. Immunogenetics of cell surface antigens of mouse leukemia. Ann. Rev. Genet. 11, 127 (1977).

Owen, J.J.T. and E.J. Jenkinson. Early events in T lymphocyte genesis in the fetal thymus. Amer. J. Anat. 170, 301 (1984).

Pierres, A., P. Naquet, A. van Agthoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A.M. Schmitt-Verhulst and M. Pierres. Rat anti-mouse T4 monoclonal antibody (H129.19) inhibits the proliferation of Ia-reactive T cell clones and delineates two phenotypically distinct

(T4+, Lyt2,3- and T4-, Lyt2,3+) subsets among anti-Ia cytolytic T cell clones. J. Immunol. 132, 2775 (1984).

Pyke, K.W. and J.F. Bach. The in vitro migration of murine fetal liver cells to thymic rudiments. Eur. J. Immunol. 9, 317 (1979).

Raedler, A., E. Raedler, K.U. Scholz, R. Arndt and H.G. Thiele. The intrathymic microenvironment: expression of lectin receptors and lectin-like molecules of differentiation antigens and MHC gene products. Thymus 5, 311 (1983).

Ralph, S.J. and M.V. Berridge. Expression of antigens of the "T-200" family of glycoproteins on hemopoietic stemm cells: evidence that thymocyte cell lineage antigens are represented on "T-200". J. Immunol. 132, 2510 (1984).

Raviola, E. and M.J. Karnovsky. Evidence for a blood-thymus barrier using electronopaque tracers. J. Exp. Med. 136, 466 (1972).

Reichert, W.A., W.M. Gallatin, E.C. Butcher and I.L. Weissman. A homing receptor bearing cortical thymocyte subset: implications for thymus cell migration and the nature of cortisone-resistant thymocytes. Cell 38, 89 (1984).

Reif, A.E. and J.M.V. Allen. The AKR thymic antigen and its distribution in leukaemias and nervous tissues. J. Exp. Med. 120, 413 (1964).

Reisner, Y., M. Linker-Israeli and N. Sharon. Separation of mouse thymocytes into two subpopulations by the use of peanut agglutinin. Cell. Immunol. 25, 129 (1976).

Ritter, M.A. Embryonic mouse thymus development: stem cell entry and differentiation. Immunol. 34, 69 (1977).

Rouse, R.V., W. van Ewijk, P.P. Jones, and I.L. Weissman. Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122, 2508 (1979).

Ruiter-Bootsma, A.L. de, M.F. Kramer, D.G. de Rooij and J.A.G. Davids. Response of stem cells in the mouse testis to fission neutrons of 1 MeV mean energy and 300 kV X rays. Methodology, dose-response studies, relative biological effectiveness. Radiat. Res. 67, 56 (1976).

Sato, C. and M. Sakka. Cell population analysis of radiation damage and recovery of thymus and lymph node in mice. Radiat. Res. 38, 204 (1969).

Scollay, R. Intrathymic events in the differentiation of T lymphocytes: a continuing enigma. Immunol. Today 4, 282 (1983).

Scollay, R. and K. Shortman. Thymocyte subpopulations: an experimental review, including flow cytometric cross-correlations between the major murine thymocyte markers. Thymus 5, 245 (1983).

Scollay, R. and K. Shortman. Identification of early stages of T lymphocyte development in the thymus cortex and medulla. J. Immunol. 134, 3632 (1985).

Scollay, R. and K. Shortman. Identification of early stages of T lymphocyte development in the thymus cortex and medulla. J. Immunol. 134, 3632 (1985).

Scollay, R., S. Jacobs, L. Jerabek, E. Butcher and I. Weissman. T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to MHC region antigens. J. Immunol. 124, 2845 (1980).

Sharp, J.G. and D.A. Crouse. A radioresistant thymic stem cell population in the adult mouse. Radiat. Res. 59, 51 (1974).

Sharp, J.G. and D.B. Thomas. Thymic regeneration in lethally X-irradiated mice. Radiat. Res. 64, 293 (1975).

Sharp, J.G. and E.B. Watkins. Cellular and immunological consequences of thymic irradiation. In: Immunopharmacologic Effects of Radiation Therapy (J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds.). p. 137. Raven Press, New York, 1981.

Sharrow, S.O., B.J. Mathieson and A. Singer. Cell surface appearance of unexpected MHC determinants on thymocytes from radiation bone marrow chimeras. J. Immunol. 126, 1327 (1981).

Sharrow, S.O., A. Singer, U. Hämmerling and B.J. Mathieson. Phenotypic characterization of early events of thymus repopulation in radiation bone marrow chimeras. Transplantation 35, 355 (1983).

Shen, F.W., M.J. Chorney and E.A. Boyse. Further polymorphism of the TLa locus defined by monoclonal Tl antibodies. Immunogenet. 15, 573 (1982).

Shortman, K. and H. Jackson. The differentiation of T lymphocytes. I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. Cell. Immunol. 12, 230 (1974).

Sidman, C.L., L. Forni, G. Köhler, J. Langhorne and K. Fisher Lindahl. A monoclonal antibody against a new differentiation antigen of thymocytes. Eur. J. Immunol. 13, 481 (1983).

Singer, A, K.S. Hatchcock and R.J. Hodes. Self-recognition in allogeneic radiation bone marrow chimeras. A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T helper cells. J. Exp. Med. 153, 1286 (1981).

Singer, A. K.S. Hatchcock and R.J. Hodes. Self-recognition in allogeneic thymic chimeras. Self recognition by T helper cells from thymus-engrafted nude mice is restricted to the thymic H-2 haplotype. J. Exp. Med. 155, 339 (1982).

Sprent, J. Effects of blocking helper T cell induction in vivo with anti-Ia-antibodies. Possible role of I-A/E hybrid molecules as

restriction elements. J. Exp. Med. 152, 996 (1980)

Springer, T., G. Galfre, D.S. Sechter and C. Milstein. Mac-1: A macrophage antigen identified by monoclonal antibody. Eur. J. Immunol. 9, 301 (1979).

Stout, R.D., M. Yutoku, A. Grossberg, D. Pressman and L.A. Herzenberg. A. surface membrane determinant shared by subpopulations of thymocytes and B. lymphocytes J. Immunol. 115, 508 (1975).

Stutman, O. Intrathymic and extrathymic T cell maturation. Immunol. Rev. 42, 138 (1978).

Takada, A., Y. Takada, C.C. Huang and J.L. Ambrus. Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129, 445 (1969).

Takada, A., Y. Takada, U. Kim and J.A. Ambrus. Bone marrow, spleen, and thymus regeneration patterns in mice after whole-body irradiation. Radiat. Res. 45, 522 (1971).

Trainin, N., M. Pecht and Z.T. Handzel. Thymic hormones: inducers and regulators of the T cell system. Immunol. Today 4, 16 (1983).

Trowbridge, J.S. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200, J. Exp. Med. 148, 313 (1978).

Trowell, O.A. Radiosensitivity of the cortical and medullary lymphocytes in the thymus. Int. J. Radiat. Biol. 4, 163 (1961).

Vliet, E. van, M. Melis and W. van Ewijk. Monoclonal antibodies directed stromal cell types of the mouse thymus. Eur. J. immunol. 14, 524 (1984a).

Vliet, E. van, M. Melis and W. van Ewijk. Immunohistology of thymic nurse cells. Cellular Immunol. 87, 101 (1984b).

Vliet, E. van, E.J. Jenkinson, R. Kinston, J.J.T. Owen and W. van Ewijk. Stromal cell types in the developing thymus of the normal and nude mouse embryo. Eur. J. Immunol. 15, 675 (1985).

Vliet, E. van, M. Melis, J.M. Floidart and W. van Ewijk. Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody. J. Histochem. Cytochem., in press (1986).

Wagner, H., C. Hardt, H. Stockinger, K. Pfizenmaier, R. Bartlett and M. Rollinghoff. Impact of thymus on the generation of immunocompetence and diversity of antigen-specific MHC-restricted cytotoxic T lymphocyte precursors. Immunol. Rev. 58, 95 (1981).

Weissman, I.L. Thymus cell maturation. J. Exp. Med. 137, 504 (1973).

Wekerle, H. and U.P. Ketelsen. Thymic nurse cells - Ia-bearing epithelium involved in T lymphocyte differentiation. Nature 283, 402 (1980).

Wijngaert, F.P. van de , M.D. Kendall, H.J. Schuurman, L.H.P.M. Rademakers and L. Kater. Heterogeneity of epithelial cells in the human thymus. An ultrastructural study. Cell Tissue Res. 237, 227 (1983).

Zinkernagel, R.M. H-2 restriction of virus-specific cytotoxicity across the H-2 barrier: separate effector T-cell specificities are associated with self H-2 and with the tolerated allogeneic H-2 in chimeras. J. Exp. Med. 144, 933 (1976).

Zinkernagel, R.M. Thymus and lymphohemopoietic cells: their role in T cell maturation, in selection of T cells H-2 restriction-specificity and in H-2 linked Ir gene control. Immunol. Rev. 42, 225 (1978).

Zinkernagel, R.M. and P.C. Doherty. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J. Exp. Med. 141, 1427 (1975).

Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein and J. Klein. On the thymus in the differentiation of H-2 selfrecognition by T cells: evidence for dual recognition? J. Exp. Med. 147, 882 (1978a).

Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, J.W. Streilein and J. Klein. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. J. Exp. Med. 147, 897 (1978b).

Zinkernagel, R.M., A. Althage, A. Waterfield, B. Kindred, R.M. Welsh, G.N. Callahan and P. Pinet. Restriction specificities, alloreactivity and allotolerance expressed by T cells from nude mice reconstituted with H-2 compatible or incompatible thymus grafts. J. Exp. Med. 151, 376 (1980).

CHAPTER II

INTRODUCTION TO THE EXPERIMENTAL WORK

As demonstrated in Chapter I, ionizing radiation can be divided in two main types of irradiation, i.e. electromagnetic radiation such as gamma- and X-rays, and particulate radiation such as alpha particles, beta particles and neutrons. X-rays and neutrons are indirectly ionizing radiation types. They are electrically neutral and have a fair penetrating power. Fast fission neutrons and X-rays produce recoil protons and fast electrons respectively, which are directly ionizing. However, the ionization density along the track of recoil protons is much higher than that of fast electrons. This difference in ionization density accounts for the observed differences in biological effectiveness after irradiation with either radiation type. These differences can be described by the RBE for fast fission neutrons, i.e. the ratio of the absorbed dose of 300 kVp X-rays to the absorbed dose of fission neutrons to produce the same biological effect.

Neutron RBE determinations are important for radiation protection, fast neutron radiotherapy and treatment after accidental exposure to fast neutrons. Using the fast fission neutron exposure facility in Petten, a number of RBE values have been obtained (see Section I.1). A marked difference in neutron sensitivity could be demonstrated between epithelial and bone marrow-derived tissues. As demonstrated in Section I.2, the thymus is composed of a lymphoid, bone marrow-derived component and a stromal, epithelial-derived component. Furthermore, it was shown
that the thymic stroma is involved in the differentiation and maturation of the bone marrow-derived T cell precursors. With respect to the radiosensitivity of both thymic components, there is only limited data available on the stromal sensitivity to irradiation whereas the lymphoid sensitivity to irradiation is characterized to some extent (see Section I.3).

However, since many thymocyte subpopulations can be identified in the thymus, different radiosensitivities could exist for these subpopu-

lations. In addition, the precise nature and progeny of the radioresistant intrathymic precursor cells is still poorly understood.

With respect to low LET irradiation, i.e. X-rays versus high LET irradiation, i.e. fast fission neutrons, only limited data are available on the effects after neutron irradiation. Furthermore, based on the above described generalization concerning the neutron sensitivity of epithelia and bone marrow-derived cells, it is conceivable that a similar marked difference in neutron sensitivity might be expected between cell populations in a lympho-epithelial organ as the thymus. In order to characterize these events in more detail, we decided to expose mice to whole-body irradiation with either fast fission neutrons or X-rays and we studied the effects of these irradiations on the lymphoid and stromal compartment in the thymus.

In Chapter III, we describe the cellular response of the thymus of CBA mice after whole-body irradiation with sublethal doses of fission neutrons of 1 MeV mean energy or 300 kVp X-rays. The CBA strain used is an inbred strain kept in Petten since 1962, originally derived from the CBA/Br strain. Furthermore, this chapter deals with the long-term effects of irradiation on the thymus.

In Chapter IV, monoclonal antibodies directed to cell surface differentiation antigens of T cells were used to study the distribution of T cell subpopulations in the thymus of CBA/H mice.

Chapter V deals with the degeneration of the thymus after sublethal fission neutron irradiation and the following bone marrow-independent repopulation of the thymus, brought by intrathymic radioresistant precursor cells. These events were characterized with flow cytofluorometry and immunohistology using monoclonal antibodies directed to the cell surface antigens Thy-1, T-200, Lyt-1, Lyt-2, MT-4, and MEL-14.

The following chapter (Chapter VI) describes the effects of sublethal fission neutron irradiation on the stromal cells of the thymus. These effects were characterized with immunohistology, using monoclonal

antibodies directed to I-A and H-2K (MHC) antigens as well as monoclonal antibodies defining various stromal cell types in the cortex and medulla of the thymus. In addition, this chapter studies the question whether the radiation-induced changes in the thymic microenvironment are related to the lymphoid repopulation (Chapter 5) in the thymus.

In Chapter VII, the long-term immunohistology of lymphoid and stromal compartments of the thymus, long-term after a single exposure to sublethal doses of whole-body irradiation, is investigated. This chapter describes a number of thymuses with an aberrant thymus lobe observed as a long-term effect after neutron irradiation.

Chapter VIII deals with the determination of the radiosensitivity and neutron RBE values of thymocyte subpopulations of mice exposed to graded doses of neutrons and X-rays, defined by monoclonal antibodies directed to T cell differentiation antigens using flow cytofluorometry. In addition, the radiosensitivity of the intrathymic radioresistant precursor cells was estimated.

In Chapter IX, experiments are described to investigate the radiosensitivity of the thymic stroma. To this purpose, thymuses of neonatal mice, exposed to graded doses of neutrons or X-rays, were excised and transplanted in MHC compatible nude mice. The immunohistology of the graft was investigated with antibodies directed to thymic stromal cells and T cell differentiation antigens and the graft size was measured. In order to investigate the reconstitution capacity of the thymic transplant after irradiation, the peripheral T cell pool was quantified using flow cytofluorometry with monoclonal antibodies directed to T cell antigens.

CHAPTER III

SHORT AND LONG-TERM EFFECTS OF WHOLE-BODY IRRADIATION WITH FISSION NEUTRONS OR X-RAYS ON THE THYMUS IN CBA MICE

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SUMMARY

Young adult (6 weeks old) female CBA mice were exposed to whole-body irradiation with either 2.5 Gy fast fission neutrons of 1 MeV mean energy or 6.0 Gy 300 kVp X-rays at center-line dose rates of 0.1 and 0.3 Gy/min, respectively. The weight of spleen and animal and the weight, cellularity and histological structure of the thymus were studied at different times after irradiation. Thymic recovery after whole-body irradiation showed a biphasic pattern with minima at 5 and 21 days after irradiation and peaks of regeneration at days 14 and 42 after X-irradiation or at days 14 and 70 after neutron irradiation. After the second phase of recovery, a marked decrease in relative thymus weight and cellularity was observed, which lasted up to at least 250 days after irradiation. Splenic recovery showed a monophasic pattern with an overshoot on day 21 after irradiation. After neutron-irradiation a late decrease in relative spleen and animal weight was observed.

The observed late effects on thymus and spleen weight and thymus cellularity are discussed in terms of a persistent defect in the bone marrow.

INTRODUCTION

The effects of X-irradiation on the thymus in mice have been extensively studied (1-6). After whole-body irradiation, the cellular regeneration of the thymus follows a biphasic pattern; an initial decrease in thymus weight and cellularity until day 5 is followed by a first regeneration period until day 14 and subsequently a second decrease from day 16 until day 22 is followed by a final recovery at day 40 after irradiation.

The first regeneration period has been attributed to proliferation of the fraction of prothymocytes which survived in the thymus after irradiation (2, 7). The secondary involution of the irradiated thymus has been explained by exhaustion (1) and limited proliferative capacity (8) of those prothymocytes and by an impaired production of precursors in the bone marrow due to radiation damage (2, 4). The final recovery of the thymus on day 40 after irradiation is due to its replenishment from bone marrow precursors (9, 10).

In contrast to the relatively short-term effects there is only little known about the long-term effects of radiation of the thymus. After whole-body irradiation with gamma-rays Coggle (10) found no detectable effects after the recovery on day 40 up to 15 months after irradiation. In the present study we describe the short and long-term effects of whole-body irradiation with 2.5 Gy fast fission neutrons of 1 MeV mean energy or 6.0 Gy 300 kVp X-rays on the weight, cellularity and histology of the thymus in CBA mice.

MATERIALS AND METHODS

Animals

Because of their homogeneity and a maximum of thymus weight about the 6th week of age (fig. 1), female mice of a CBA inbred subline were irradiated or sham-irradiated at the age of 42 ± 3 days. The procedures of animal care have been described elsewhere (11).

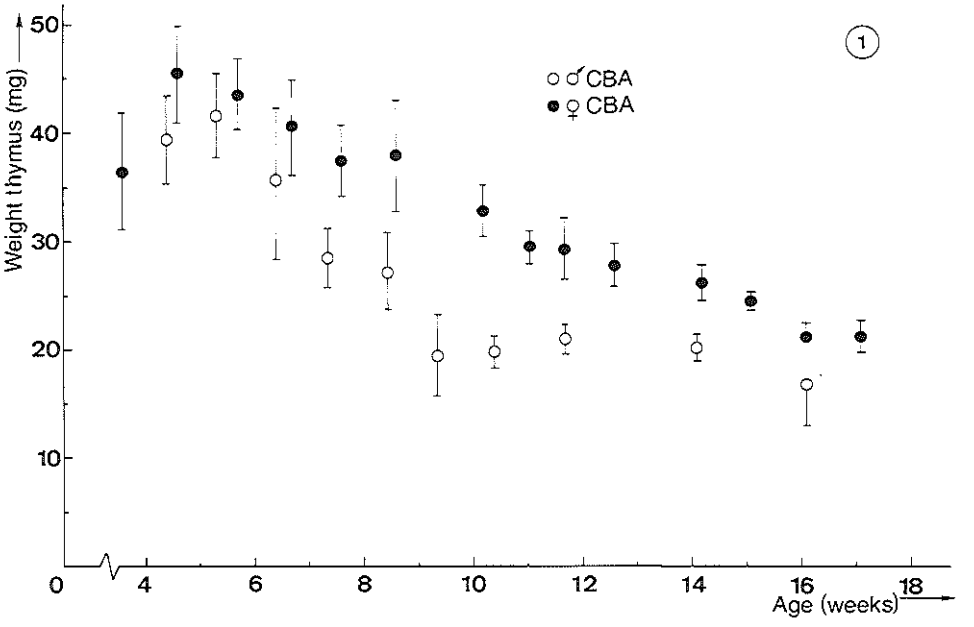


Fig. 1. Thymus weight as a function of age in male (○) and female (●) CBA mice.

Irradiation procedures

The animals were irradiated with fast fission neutrons from a ^{235}U-converter in the Low Flux Reactor at Petten. The design of the exposure facility, the tissue dosimetry and the neutron spectrometry have been described elsewhere (12).

The animals were exposed bilaterally at a fast neutron rate of 0.1 Gy/min. The absorbed doses are given as neutron center-line doses; they do not include the 9% gamma-ray contribution. The variation in neutron center-line dose rate over the 40 mouse positions in the exposure facility is within ± 2% of the mean value. The neutron spectrum has a mean energy of 1.0 MeV and the mean track average LET of the recoil protons produced in tissue is equal to 57 keV/µm in water.

X-irradiation was performed with a Philips Müller X-ray tube, operating at 300 kVp constant potential at 5 mA and with a measured HVL of 2.1 mm Cu. Simultaneously, twenty animals were irradiated while confined in polycarbonate tubes which were mounted in a rotating disk of perspex. The dose rate was equal to 0.3 Gy/min. in the center-line of the animals, the distance from focus to center-line being 69 cm.

Experimental procedures

The animals were irradiated with either 2.5 Gy fission neutrons or 6.0 Gy X-rays. Simultaneously sham-irradiated controls were put in similar plastic tubes as used for the irradiation. The doses used in this investigation were chosen because previous experiments on intestinal and gastric epithelium and hematopoietic tissue (13) indicated that from these doses about an equivalent radiation damage could be expected. The doses were expected to be in the sub-lethal range, the LD 50/30 day for 6 weeks old female CBA mice being 2.99 Gy for fission neutrons and 6.93 Gy for X-rays (Huiskamp et al., unpublished results).

At selected times after irradiation 7-8 mice from the sham-irradiated and the irradiated groups were killed by ether intoxication. The weight of the animal and spleen and the weight, cellularity and histological structure of the thymus were studied. The weight of thymus and spleen was measured with a VDF torsion balance "United" type 100 according to standard weighing procedures. Precautions were taken to avoid the influence of desiccation during the preparation and weighing procedure, otherwise evaporation may influence the recorded weight of the small post-irradiation thymuses. The thymuses of 5 animals were pooled, minced with scissors and gently pressed through a nylon gauge filter (pore size, 200 µm). All cells were kept in phosphate buffered saline supplemented with 5% newborn calf serum. The total number of nucleated cells was counted in a hemocytometer.

The thymuses of the remaining 2-3 mice were fixed in methanol-formaldehyde acetic acid anhydrous (85 : 10 : 5, v/v/v), embedded in paraffin and sectioned at 7 µm. The sections were stained by hematoxylin-eosine.

RESULTS

Among the unirradiated controls, the mortality was zero. After neutron- irradiation the lethality was 3.0% and in the X-irradiated group the lethality was zero.
The changes in relative animal weight due to irradiation with fission neutrons or X-rays were more pronounced after neutron irradiation. Especially, beyond 200 days after neutron irradiation, one can observe a decrease in relative animal weight (fig. 2).
After irradiation with 2.5 Gy fission neutrons a marked decrease in thymus weight and cellularity was observed until day 5 (fig. 3). During the subsequent 9 days thymus weight and cellularity increased to a maximum on the 14th day after irradiation. After this phase of recovery

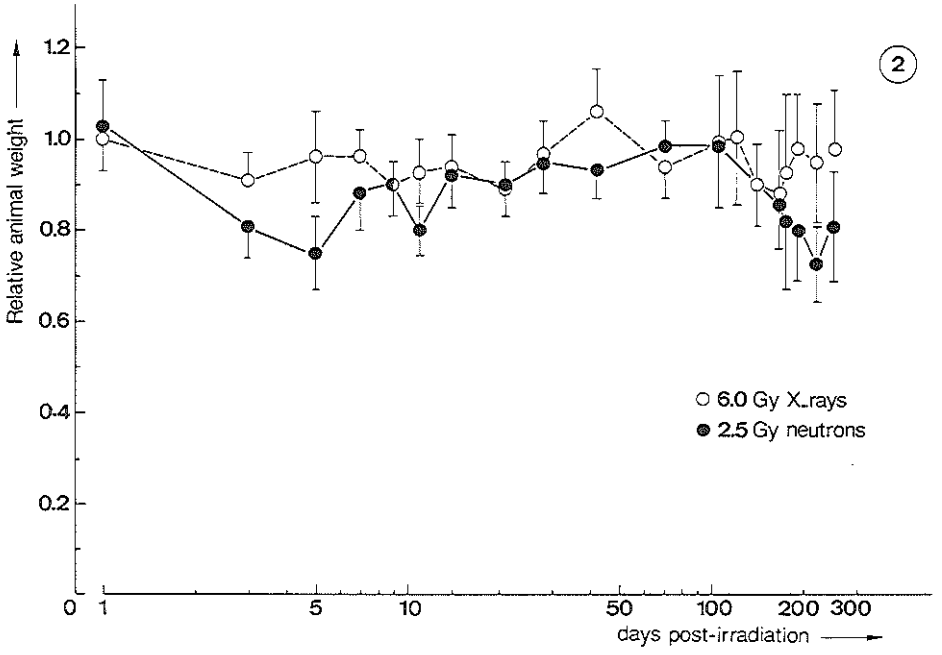


Fig. 2. Animal weight relative to that of age-matched sham-irradiated controls (mean ± SD) at various times after whole-body irradiation with 2.5 Gy neutron (●) or 6.0 Gy X-rays (○).

there was a second decrease followed by a second phase of recovery, starting after the 28th day and lasting until about the 50th-70th day after irradiation. After this second phase of recovery thymus weight and cellularity decreased again. This final decrease lasted up to at least 250 days after irradiation.

If the animals were exposed to 6 Gy X-rays, the same biphasic pattern of regeneration was observed (fig. 4). The marked decrease after the second phase of recovery was also observed.

Fig. 5A and 5B respectively show changes in thymus weight and cellularity after 2.5 Gy neutron or 6 Gy X-irradiation relative to their control values. During the first 5 days after irradiation, the effects on the thymus weight and cellularity due to neutron or X-irradiation were more or less the same. The first phase of recovery after this initial decrease was less pronounced after neutron irradiation. The increase during the second phase of recovery was evident after X-irradiation and nearly reached the control level on the 42nd day after irradiation. After neutron irradiation this increase was less pronounced and reached its maximum around the 70th-100th day after irradiation.

Beyond this second phase of recovery there was a marked decrease in thymus weight and cellularity after neutron as well as X-irradiation.

At the first day after neutron irradiation the thymus had, in comparison with unirradiated animals, a very thin cortex which was very hypocellular and contained a lot of debris (fig. 6A,B). The medulla of the thymus was only slightly affected by irradiation. On the 5th day after irradiation the cortex was still thin but a sparse distribution of small lymphocytes was present. At this stage there were many cells in mitosis. At the 14th day after irradiation, at the peak of the first phase of recovery, the histological appearance of the thymus was about normal with a large cortex containing many darkly stained lymphocytes (fig. 6C). The same observations were made after neutron irradiation.

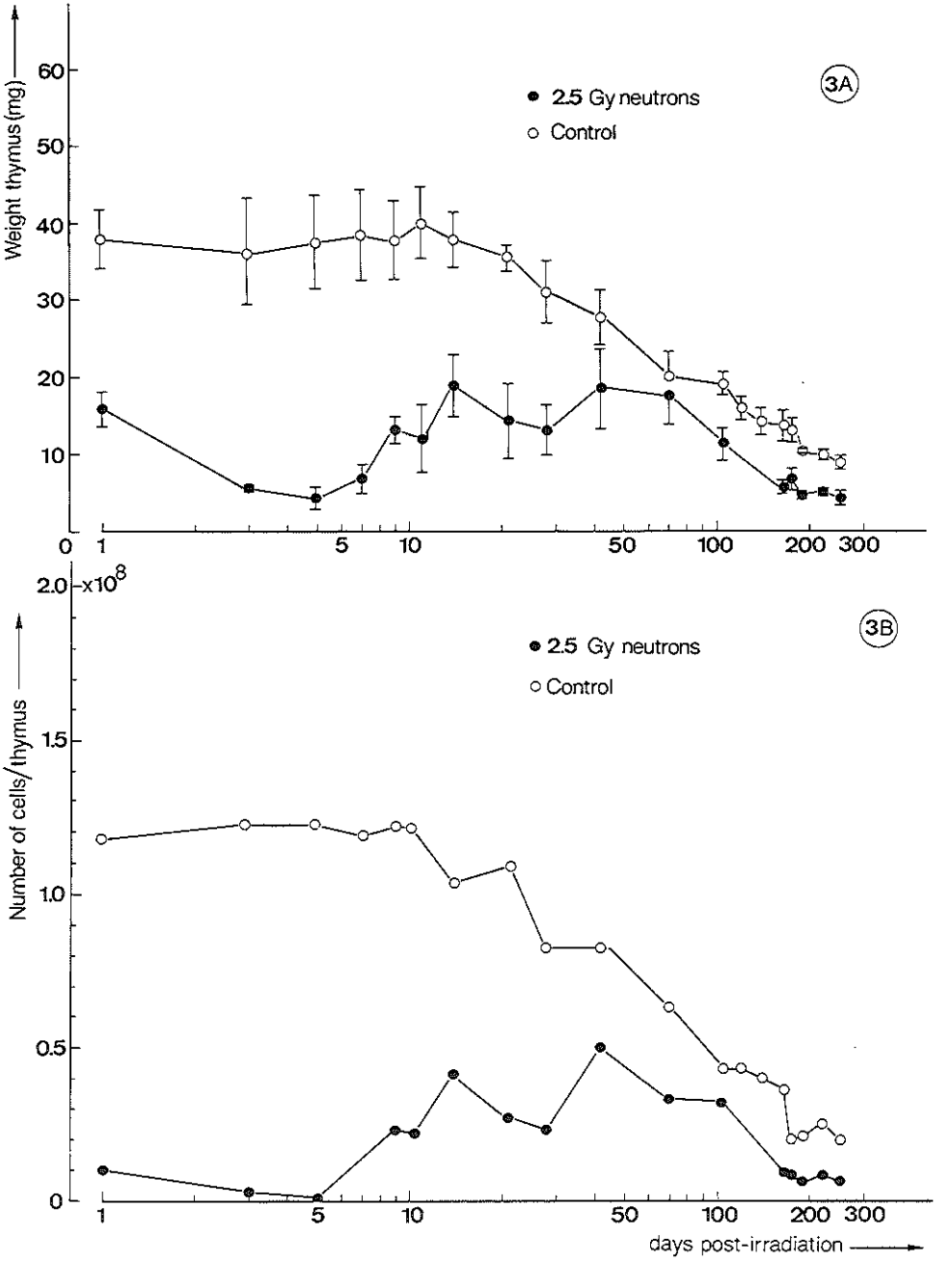


Fig. 3. (A): Thymus weight (mean ± SD of 5 animals) and (B): Thymus cellularity (total number of nucleated cells of 5 pooled thymuses/5) at various times after 2.5 Gy whole-body neutron (●) or sham-irradiation (0).

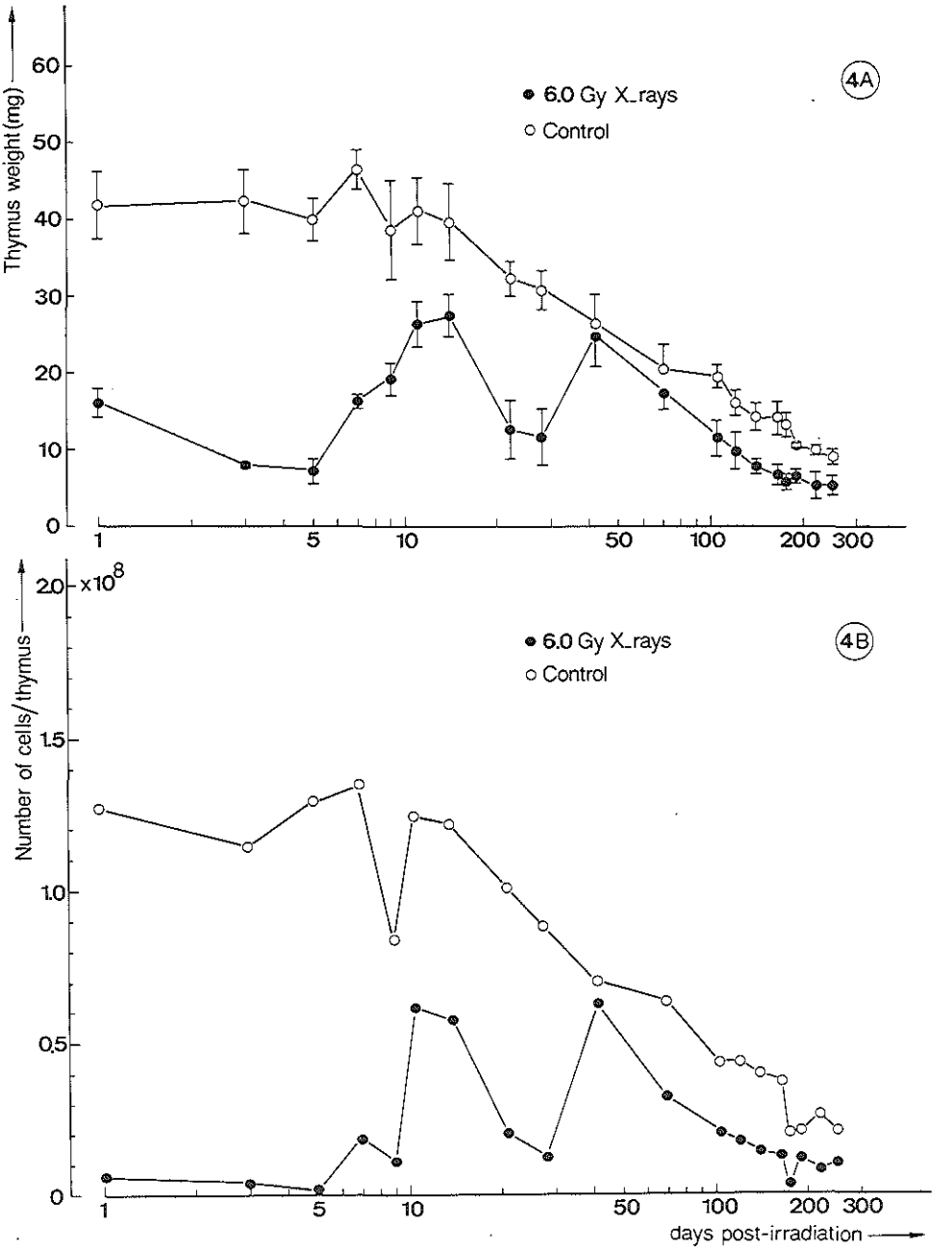


Fig. 4. (A): Thymus weight (mean ± SD of 5 animals) and (B): Thymus cellularity (total number of nucleated cells of 5 pooled thymuses/5) at various times after 6.0 Gy whole-body X- (●) or sham-irradiation (○).

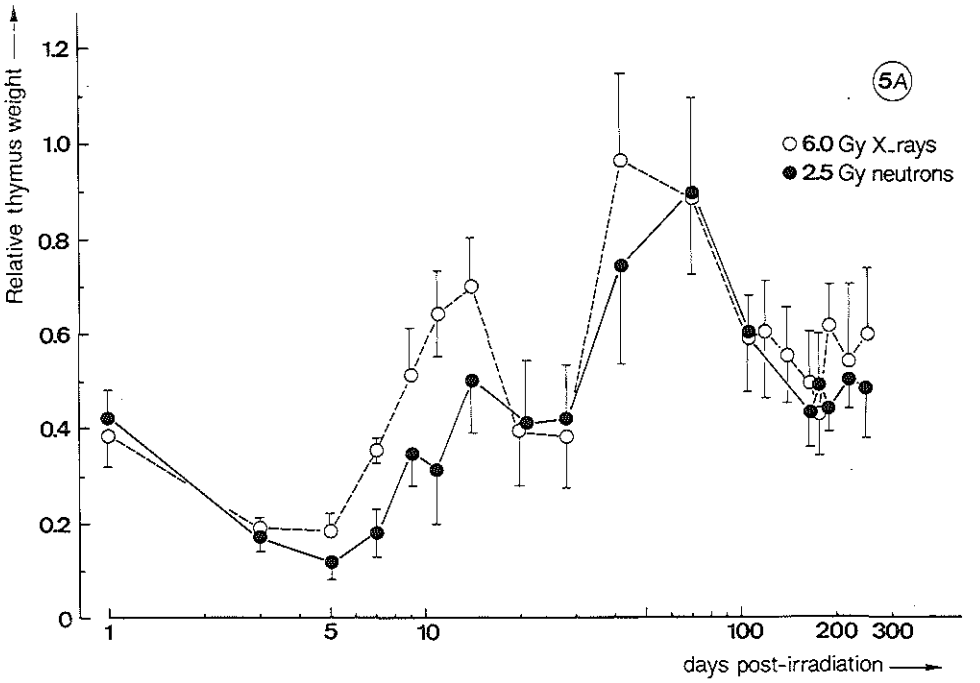
During the second phase of involution, the thymus cortex became thin again but contained more darkly stained small lymphocytes as during the first phase of involution. At day 42 after X-irradiation and at day 70 after neutron irradiation, the histological appearance of the thymus of irradiated animals was almost identical to that of unirradiated animals and remained identical up to at least 250 days after irradiation with neutrons or X-rays.

The loss in spleen weight that followed whole-body irradiation was less pronounced than the loss of thymus weight (fig. 7). After X-irradiation the spleen showed a monophasic regeneration pattern with a peak on day 21 after irradiation. After this peak the weight of the irradiated spleen remained somewhat below the control level. After neutron irradiation the overshoot at day 21 after irradiation was less pronounced than after X-irradiation. After this peak there was a drop below control level. At day 70 after irradiation the spleen weight reached nearly the control level and hereafter decreased to about 70% of the control level.

DISCUSSION

The biphasic pattern of thymus recovery in mice following whole-body X- irradiation has been known for some time (1-5, 14). After an initial decrease of thymus weight and cellularity due to interphase death of lymphocytes and induced cessation of mitosis (2), regeneration of the thymus in the irradiated animals takes place to a considerable extent and is attributed to proliferation of surviving pro-thymocytes in the thymus (7, 9, 15) and/or an influx of radioresistant transient precursor cells into the thymus (6). The second decrease in thymus weight and cellularity is ascribed to an exhaustion (1) and limited proliferative capacity of the surviving prothymocytes in the thymus (8) and to an impaired production of thymus restricted precursors in the bone marrow due to radiation damage (2, 4, 16). The second regenerative phase has been attributed to the replenishment of the pro-thymocyte pool of thymus precursors in the recovering bone marrow (2, 9, 10).

The results of the present investigation confirm the cyclic nature of this recovery patterns after 6.0 Gy X-irradiation as well as after


5A
6.0 Gy X-rays
2.5 Gy neutrons
Relative thymus weight
1.2
1.0
0.8
0.6
0.4
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0
1
5
10
100
200
300
days post-irradiation

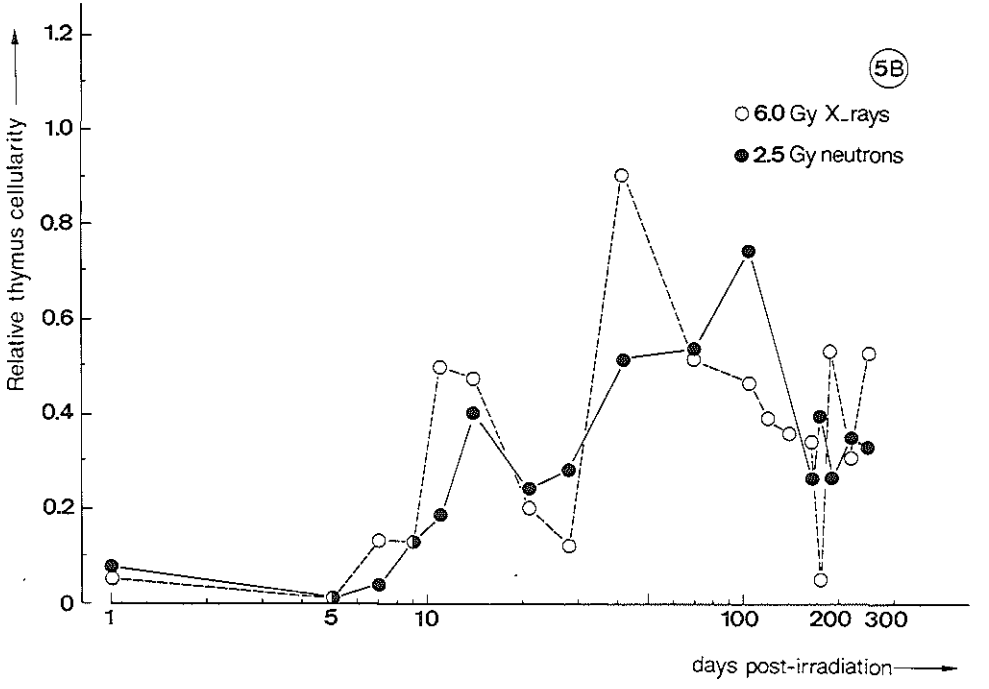

5B
6.0 Gy X-rays
2.5 Gy neutrons
Relative thymus cellularity
1.2
1.0
0.8
0.6
0.4
0.2
0
1
5
10
100
200
300
days post-irradiation

Fig. 5A. Thymus weight relative to that of age-matched sham-irradiated controls (mean ± SD) at various times after whole-body irradiation with 2.5 Gy neutrons (●) or 6.0 Gy X-rays (○).

Fig. 5B. Thymus cellularity relative to that of age-matched sham-irradiated controls (mean ± SD) at various times after whole-body irradiation with 2.5 Gy neutrons (●) or 6.0 Gy X-rays (○).

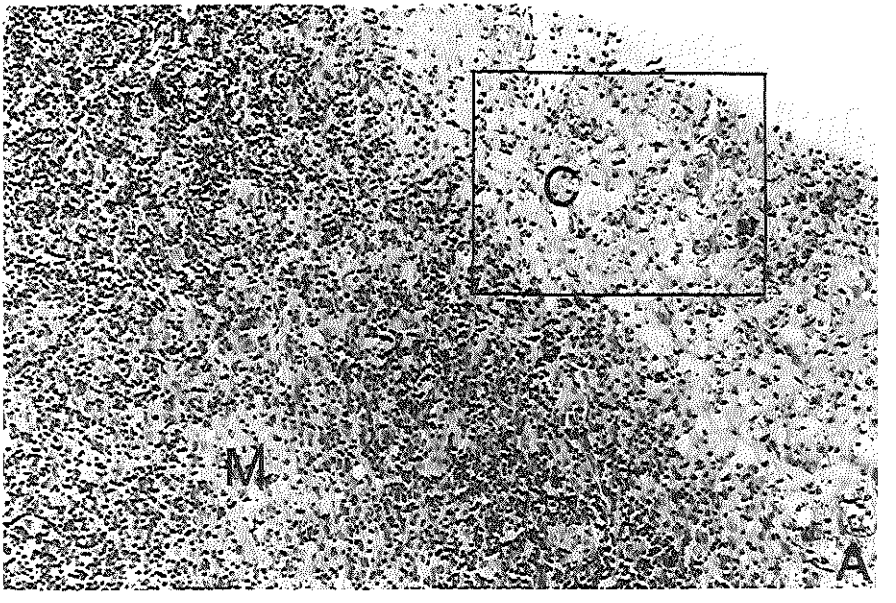
Fig. 6. Thymus after 2.5 Gy whole-body neutron irradiation

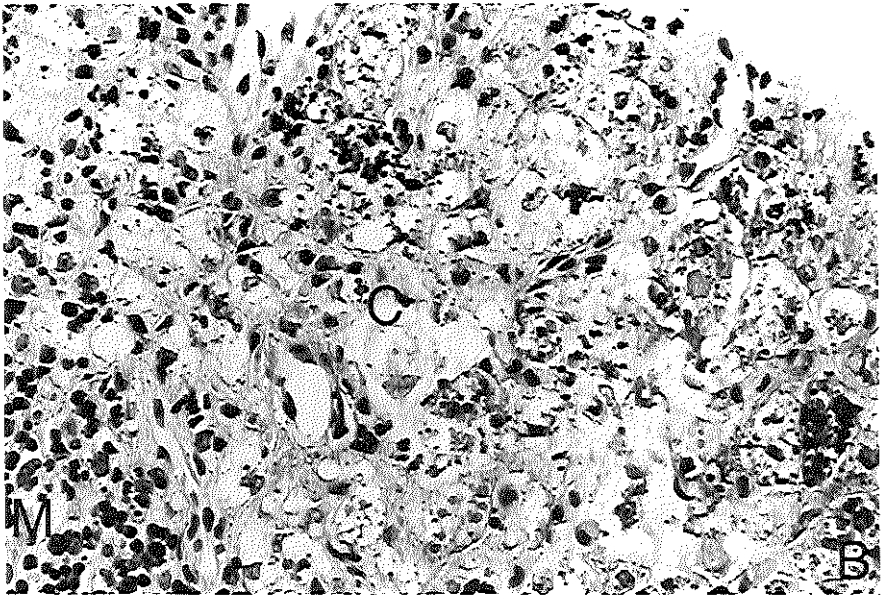
A. thymus 1 day postirradiation showing marked hypocellularity, particularly in the cortex; medulla only slightly affected (130 x).

B. detail of the thymus 1 day postirradiation with a cortex containing a lot of debris (330 x).

C. thymus 14 days postirradiation has an essentially normal appearance (130 x).

C = cortex, M = medulla.

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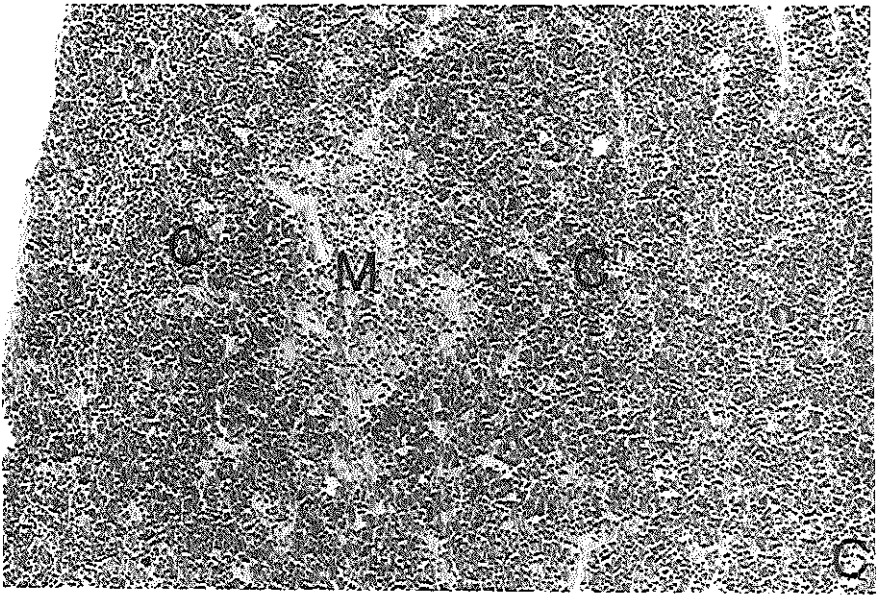
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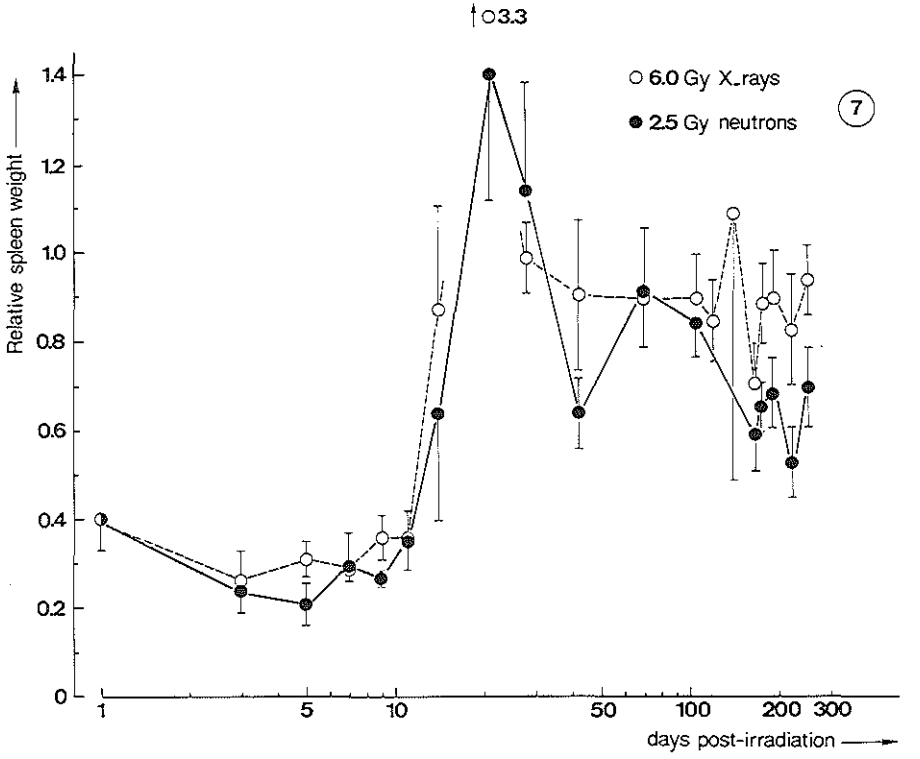

Fig. 7. Spleen weight relative to that of age-matched sham-irradiated controls (mean ± SD) at various times after whole-body irradiation with 2.5 Gy neutron (●) or 6.0 Gy X-rays (○).

2.5 Gy neutron irradiation (Fig. 3,4,5). The regeneration of the thymus after the dose of neutron irradiation we used is slower and less pronounced than after X-irradiation. In contrast to Coggle (10) who reported that no late effects were detectable in the cellularity of the thymus up to 15 months after whole-body irradiations with 2,4 and 6 Gy gamma-rays given to SAS/4 mice at 4 weeks of age, we observed after the second phase of regeneration a decrease in relative thymus weight and cellularity and this decrease persists up to at least 250 days after irradiation.

The decrease, late post-irradiation was not only observed in our own CBA inbred strain, but also in CBA/H mice (Huiskamp et al, unpubl. results). The decrease might be due to "accidental" thymic involution from infections, malnutrition or traumatic experiences (17). However, this explanation seems unlikely since the histological appearance of the thymus late post-irradiation is identical to that of the unirradiated thymus.

The observed late decrease in thymus weight and cellularity suggests a persistent defect in the thymus due to irradiation with either 6.0 Gy X-rays or 2.5 Gy neutrons. The reason for this phenomenon is still unknown, but according to Sharp & Watkins (18) long-term effects may only be expected to occur when the radiation dose was sufficient to damage the thymic micro-environment. However, in bone marrow reconstituted mice following 10 Gy gamma-rays, it was possible to detect donor derived functionally active T lymphocytes (16, 19), and after thymus-only irradiation of infant mice with doses up to 15 Gy gamma-rays, thymus weight was transiently depressed, but was essentially normal on day 180 postirradiation (18). Thus it seems unlikely that the doses employed in this investigation were high enough to damage the thymic micro-environment to a considerable extent. With increasing age intrinsic changes in the thymus occur. The absolute number of thymosin α positive cells and the thymic activity to promote T-cell differentiation decrease with age-related thymic involution (20, 21). Furthermore, the level and/or proliferative capacity of the thymus restricted progenitor cells in the bone marrow, which seem to be under the control of thymic humoral factors (24), are decreased in aging mice (22, 23). However, in a recent study, Sado and co-workers (25, 26), investigating the immunological competence of aging mice exposed to 1.5 - 4.5 Gy X-rays or 3.1 - 6.1 Gy gamma-rays during their young adulthood, were not able to demonstrate clear evidence of accelerated aging of the immune system due to earlier radiation exposure. Coggle (10), finding no late effects on thymus weight and cellularity after irradiation, suggested that the failure of finding such effects reflects the efficient long-term recovery of the bone marrow pool. Irradiation causing a long-lasting depression in pluripotent stem cells (CFU-S) has been described by a number of authors (27-29). However, a lower number of CFU-S in the bone marrow, cannot be taken as conclusive evidence of an injury to the

bone marrow because an increased number of total marrow cells or, alternatively, a faster turnover of the progenitor cells might compensate (30).

After 5 Gy gamma-irradiation the repopulation ability of the bone marrow was not only impaired by the loss of stem cells but also by the induction of genetic damage in stem cells (31).This residual damage, i.e. non-lethal stem cell damage, has been observed for at least 7 months following irradiation (32).

In addition, the differentiation of thymus precursors into prothymocytes seems to be associated with a decrease in both radiosensitivity and cortisone resistance (9). So, in our opinion, the observed late effects on the thymus after irradiation might be a result of a loss of pluripotent stem cells in combination with a residual damage in the surviving stem cells in the bone marrow.
The most notable change in lymphohematopoietic tissues was the overshoot seen in the spleen weight 21 days postirradiation (fig. 8). This phenomenon has been observed many times and may represent not only extra medullary hematopoiesis compensating completely for a deficiency in the bone marrow (31) and residual "trapping" of damaged lymphohematopoietic elements (33).

A 2.5 Gy neutron dose appears to have more effect on the spleen weight, especially from day 100 postirradiation on. In CBA/H mice we observed a slight decrease in spleen weight after 6.0 Gy X-rays and a larger decrease after 2.5 Gy neutrons (Huiskamp et al, unpublished results) suggesting a late defect in the extra-medullary hematopoiesis in combination with the earlier mentioned damage in the bone marrow.

The decrease of thymus, spleen and animal weight and of the cellularity of the thymus are in general larger after neutron than after X-irradiation. We assume that this is due to an incomplete comparability of the doses of the two types of irradiation. For instance, the differences in relative animal weight (fig. 2) in response to neutron or X-irradiations could be attributed to a larger value of the Relative Biological Effectiveness (RBE) of fission neutrons, i.e. the ratio of the absorbed dose of X-rays to the absorbed dose of neutrons required to produce the same biological effect. A absorbed dose of 2.5 Gy fission neutrons reduces the population of intestinal-crypt stem cells in

CBA mice to a fraction of 0.05 and the neutron RBE for this effect is 3.2 (34). Therefore, the heavier loss in animal weight early after neutron- irradiation can be explained by the fact that 2.5 Gy neutrons cause more damage of the intestinal stem cell population than 6.0 Gy X-rays.

A similar explanation might pertain to the loss in animal weight between 100 and 200 days after neutron-irradiation. However, the proliferating cell population involved in the latter effect has not been identified. Because of the differences in effect of the neutron and X-ray doses used, it is interesting to compare the thymic recovery after graded doses with the two types of ionizing radiation used in this investigation.

REFERENCES

1. Blomgren, H. and L. Révész, The effect of bone marrow protection on the cellular composition of irradiated mouse thymus. Exp. Cell Res. 53: 261 (1968).

2. Takada, A., Y. Takada, C.C; Huang and J.L. Ambrus, Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129: 445 (1969).

3. Takada, A., Y. Takada, U. Kim and J.A. Ambrus, Bone marrow spleen and thymus regeneration patterns in mice after whole-body irradiation. Radiat. Res. 45: 522 (1971).

4. Blomgren, H., The role of bone marrow of X-irradiated mice in the thymic recovery. Cell Tissue Kinet. 4: 443 (1971).

5. Sharp, J.G., and D.B. Thomas, Thymic regeneration in lethally X-irradiated mice. Radiat. Res. 64: 293 (1975).

6. Sharp, J.G., and D.B. Thomas, Origin of the radio resistant precursor cells responsible for the initial phase of thymic regeneration after X-irradiation. J. Reticuloendothel. Soc. 22: 169 (1977).

7. Kadish, J.L. and R.S. Basch, Thymic regeneration after lethal

irradiation: evidence for an intra-thymic radio resistant T cell precursor. J. Immunol. 114: 452 (1975).

8. Dukor, P., J.F.A.P. Miller, W. House and V. Allman, Regeneration of thymus grafts. I Histological and cytological aspects. Transplantation 3: 639 (1965).

9. Hiesche, K.D. and L. Révész, Effect of cortisone and X-irradiation on cellular depletion and regeneration between thymus lymphocyte precursors in the bone marrow and in the thymus. Path. Res. Pract. 164: 157 (1979).

10. Coggle, J.E., The absence of late effects of radiation on the cellularity of the mouse thymus. Int. J. Radiat. Biol. 40: 229 (1981).

11. Davids, J.A.G., Bone marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone marrow transplantation. Int. J. Radiat. Biol. 17: 173 (1970).

12. Davids, J.A.G., A.P.J. Mos and A. de Oude, Fast neutron facility for biological exposures in an Argonaut reactor: design, tissue dosimetry and neutron spectrometry. Phys. Med. Biol. 14: 573 (1969).

13. Davids, J.A.G., Acute effects of 1 MeV fast neutrons on the hematopoietic tissues, intestinal epithelium and gastric epithelium in mice. In Advances in Radiation Research (J.F. Duplan & A. Chapiro, Ed.) Vol. II. p. 565. Gordon and Breach Science Publishers New York, 1973.

14. Watkins, E.B., J.G. Sharp, D.A. Crouse and W.K. Metcalf, Lympho-hematopoietic consequences of infant and adult thymic irradiation. In Experimental Hematology Today 1980 (S.J. Baum, G.D. Ledney and D.W. van Bekkum, Ed.), p. 81. Karger, Basel, 1980.

15. Hiesche, K.D. and L. Révész, Cellular repopulation in irradiated mouse thymus and bone marrow. Beitr. Path. Biol. 151: 304 (1974).

16. Boersma, W., I. Betel, R. Daculsi and G. van der Westen, Post-irradiation thymocyte regeneration after bone marrow transplantation I regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet. 14: 179 (1981).

17. Fisher, E.R., Pathology of the thymus and its relation to human disease. In Thymus in Immunobiology (R.A. Good and A.E. Gabrielsen, Ed.), p. 676, Hoeber Medical Division, Harper & Row, Publishers, New York, 1964.

18. Sharp, J.G. and E.B. Watkins, Cellular and immunological consequences of thymic irradiation. In Immunopharmacologic Effects of Radiation Therapy. (J.B. Dubois, B. Serrou and C. Rosenfeld, Ed.), p. 137, Raven Press, New York, 1981.

19. Gorczynski, R.M. and S. MacRae, Differentiation of functionally active mouse T lymphocytes from functionally inactive bone marrow precursors. I kinetics of recovery of T cell function in lethally irradiated bone marrow reconstituted thymectomized and non-thymectonized mice. Immunol. 33: 697 (1977).

20. Hirokawa, K. and T. Makinodan, Thymic involution: effect on T cell differentiation. J. Immunol. 114: 1659 (1975).

21. Hirokawa, K., J.E. McClure and A.L. Goldstein, Age-related changes in localization of thymosin in the human thymus. Thymus 4: 19 (1982).

22. Tyan, M.L., Impaired thymic regeneration in lethally irradiated mice given bone marrow from aged donors. Proc. Soc. exp. Biol. 152: 33 (1976).

23. Tyan, M.L., Age-related decrease in mouse T-cell progenitors. J. Immunol. 118: 846 (1977).

24. Cohen, J.J. and S.S. Fairchild, Thymic control of proliferation of T cell precursors in bone marrow. Proc. Natl. Acad. Sci. USA 76: 6587 (1979).

25. Sado, T., S. Kobayashi, H. Kamisaku, H. Kurokawa and Y. Kataoka, Immundological competence of aging mice exposed to X-rays or gamma-rays during young adulthood. In Late Biological Effects of Ionizing Radiation vol. II p. 115, IAEA, Vienna (1978).

26. Sado, T., Late effects of radiation on immune system: a review. In Radiation Research, proceedings of the sixth international congress of radiation research (S. Okada, M. Imamura, M. Terashima and H. Yamaguchi, Ed.) p. 688, Tokyo (1979).

27. Covelli, V. and P. Metalli, A late effect of radiation on the hemopoietic stem cells of the mouse. Int. J. Radiat. Biol. 23: 83 (1973).

28. Schofield, R. and T.M. Dexter, CFU-S repopulation after low-dose whole-body radiation. Radiat. Res. 89: 607 (1982).

29. Ainsworth, E.J., R.J.M. Fry, D. Grahn, F.S. Williamson, P.C. Brennan, S.S. Stearner, A.V. Carrano, J.H. Rust, Late effects of neutron or gamma irradiation in mice. In Biological effects of neutron irradiation p. 359 IAEA, Vienna (1974).

30. Coggle, J.E. and C. Proukakis, Late effects of radiation on the hemopoietic stem cells in the mouse. Int. Congr. Radiat. Res. (Evian) Abstr., 163, p. 45 (1970).

31. v. Wangenheim, K.H., M.P. Siegers and L.E. Feinendegen, Repopulation ability and proliferation stimulus in the hematopoietic system of mice following gamma-irradiation. Exp. Hematol. 8: 694 (1980).

32. Hübner, G.E., K.H. v. Wangenheim and L.E. Feinendegen, An assay for the measurement of residual damage of murine hematopoietic stem cells. Exp. Hematol. 9: 111 (1981).

33. Rubin, P. and G.W. Casarett, Clinical Radiation Pathology VII. p. 778. Saunders, Philadelphia, 1968.

34. Davids, J.A.G., Application of a fast-neutron facility in an Argonaut reactor for radiobiological research. In Radiobiological Applications of Neutron Irradiation p. 27. IAEA, Vienna (1972).

CHAPTER IV

THE IMMUNOHISTOLOGY OF T CELL SUBPOPULATIONS IN THE THYMUS OF CBA/H MICE

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SUMMARY

The T cell composition of the thymus of CBA/H mice was analyzed with immunohistology, using monoclonal antibodies directed to the cell-surface antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14. The results of this investigation show that the large majority of cortical cells in the thymus is bright Thy-1$^+$, bright T-200$^+$, bright Lyt-2$^+$, MT-4$^+$ and variabel in Lyt-1 expression. In contrast, medullary cells are dull Thy-1$^+$, bright T-200$^+$, bright Lyt-1$^+$ and either MT-4$^+$ or Lyt-2$^+$. However, in the subcapsular area of the cortex, small subpopulations of lymphoblasts can be identified that are Thy-1$^+$, T-200$^-$ or Thy-1$^+$, T-200$^+$ and negative for the other tested antigens. In addition, scattered cortical cells and a small number of medullary cells are MEL-14$^+$, a receptor involved in the homing of lymphocytes in the peripheral lymphoid organs. We discuss the distribution of the various thymocyte subpopulations in relation with recently published data on T cell differentiation.

INTRODUCTION

It is well established that the thymus plays a crucial role in the generation of immunologically competent T lymphocytes (1). Bone marrow-derived precursor cells migrate to the thymus (2, 3), proliferate in the thymus and give rise to a number of functional T-cell subpopulations such as helper T cells, suppressor T cells and cytotoxic T cells (1). During the differentiation antigens such as Thy-1, TL, and Lyt,

are acquired on their cell surface (1, 4). By means of the Lyt antigens, functional subpopulations can be identified. Thus, T-amplifier and T-helper cells express the Lyt-1 antigen, whereas T-cytotoxic and T- suppressor cells express the Lyt-2 antigen (4). However, more recent studies have indicated that Lyt-1 is detectable on all T cells, albeit at higher density on the Lyt-2$^-$ subpopulation (5, 6). In addition, recently developed monoclonal antibodies, designated GK1.5 (7, 8) and MT-4 (9), appear to be specific for T-amplifier and T-helper cells.

In the present investigation, we have used immunohistology to characterize the T cell composition of the thymus in CBA/H mice with monoclonal antibodies directed to the cell-surface differentiation antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2, and MEL-14. The results of this investigation show that the thymus contains several T cell subpopulations which are localized in different thymic compartments.

MATERIALS AND METHODS

Animals

Male and female CBA/H mice, age 5 to 7 weeks, were used for the present study. The procedures for animal care have been described elsewhere (10).

Antisera

Monoclonal antibodies directed to cell-surface determinants of mouse-lymphoid cells were obtained from the tissue-culture supernatant of hybrid-cell lines prepared and characterized by Ledbetter and Herzenberg (11). Clone 59-AD-2.2 secreted anti-Thy-1 antibodies, clone 53-7.3.13 secreted anti-Lyt-1 antibodies, clone 53-6.72 secreted anti-Lyt-2 antibodies and clone 30-G-12 secreted anti -T-200 antibodies. MEL-14 monoclonal antibodies specific for a lymphocyte- surface molecule that appears to function as the homing receptor for perperipheral lymph node high endothelial venules (12), were the kind gift of Dr. E.L. Butcher, Stanford University, Stanford, USA. Clone H129.19 secreted anti-MT-4 antibodies, generated and characterized by Pierres

et al. (9), was a kind gift of Dr. M. Pierres, INSERM-CNRS, Marseille, France.

The monoclonal antibodies were detected in an indirect immuno assay using a polyvalent rabbit-anti-rat immunoglobulin serum, conjugated with horse radish peroxidase (RaRa Ig-HRP, Dakopatts, Denmark).

Tissue preparation for immunohistology

4.5 µm frozen sections of freshly isolate thymuses, were prepared and stained using the indirect immunoperoxidase method as described elsewhere (13). Briefly, acetone fixed frozen sections were overlayered with monoclonal antibodies for 45 min, rinsed, and overlayered with RaRa Ig-HRP, supplemented with 1% normal mouse serum, for another 45 min. Antibody binding was visualized by incubation of the frozen sections with diaminobenzidene, according to Graham & Karnovsky (14). To enhance the contrast of the precipitate, the sections were incubated with a solution containing 1% $CuSO_4$ and 0.9% NaCl. The sections were then postfixed in 1% glutaraldehyde. For photography, the contrast of the image was further enhanced with a 490 nm IL interference filter (Schott, W., Germany).

RESULTS

For the description of the immunohistology of the thymus, we divide the thymus in four major regions: 1. a subcapsular cortical area with large lymphocytes and frequent mitosis, 2. the cortex with small thymocytes and epithelial cells with long reticular processes, 3. the cortico-medullary area with large blood vessels and 4. the medulla, characterized by thymocytes intermediate in size and spindle-shaped epithelial cells. The results of this investigation were obtained from comparative observations using carefully chosen serial adjacent frozen sections.

Frozen sections of the thymus stained with monoclonal anti-Thy-1 antibodies, a pan T cell antibody, show that the Thy-1 antigens are

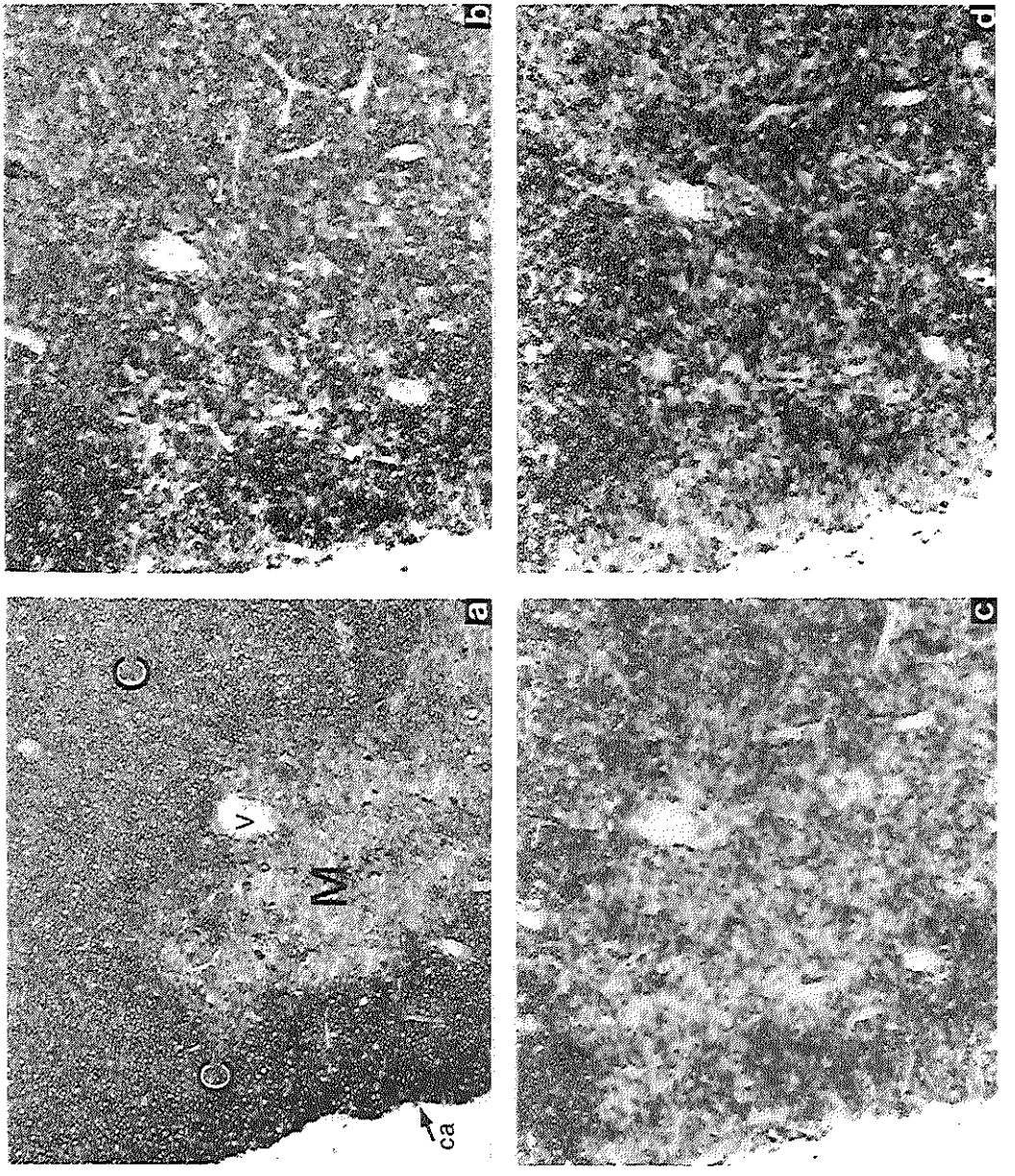
expressed on virtually all thymocytes. The Thy-1 staining is most intense in the subcapsular area and gradually decreases towards the medulla (Fig. 1a). In general, the medullary compartment can be distinguished from the cortex on basis of a weak Thy-1 staining. However, this demarcation is not always very clear.

Virtually all thymocytes express T-200 antigens, i.e. the common leukocyte antigen. Cortical thymocytes are bright T-200$^+$, whereas cells in the cortico-medullary area and in the medulla show a higher density of this antigen. In the subcapsular area and in the medulla some negative to dull T-200$^+$ cells can be observed (Fig. 1b).

Serial sections stained with anti-MT-4 antibodies, an antibody which detects T helper cells, show that the large majority of cortical cells is MT-4$^+$. In the subcapsular area, however, the cells show variation in the level of MT-4 expression; a number of cells is even MT-4$^-$. In the medulla, the MT-4 expression is very heterogenous. Besides MT-4$^-$ cells, the majority of cells is dull MT-4$^+$ whereas individual bright MT-4$^+$ cells can also be observed (Fig. 1c).

Serial sections stained with anti-Lyt-1 antibodies reveal that almost all thymocytes are Lyt-1$^+$, but the density of this surface marker is also very heterogeneous. In the subcapsular area, the thymocytes are negative to dull Lyt-1$^+$ whereas the majority of cortical cells is dull Lyt-1$^+$. However, in the cortex and especially in the cortico-medullary area, foci of bright Lyt-1$^+$ cells are observed. Individual bright Lyt-1$^+$ cells are also observed in the subcapsular area. In the medulla, the majority of cells express high levels of Lyt-1 antigens, but there are also areas were the Lyt-1 expression resembles that of the cortical dull Lyt-1$^+$ thymocytes (Fig. 1d).

Serial anti-Lyt-2 stained sections show that most cells in the cortex are bright Lyt-2$^+$ with scattered foci of negative to dull Lyt-2$^+$ cells, especially in the subcapsular area. The majority of medullary cells is Lyt-2$^-$ with scattered dull to bright Lyt-2$^+$ cells (Fig. 1e). Close comparison of adjacent serial frozen sections incubated with MT-4 and Lyt-2 reveals that medullary cells are either MT-4$^+$, Lyt-2$^-$ or MT-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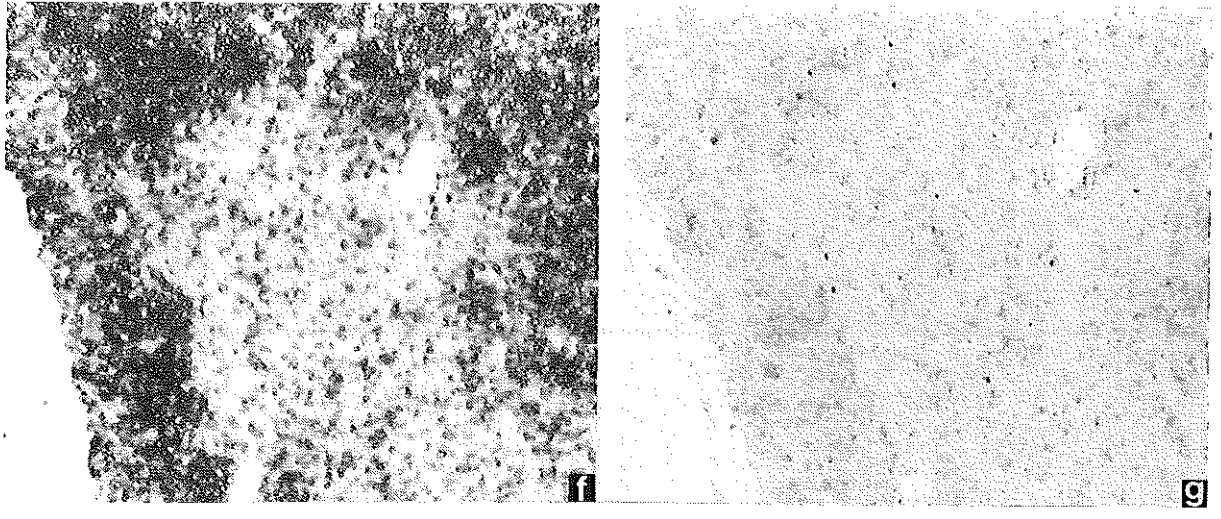

Fig. 1. Immunoperoxidase staining of serial frozen sections of the thymus of CBA/H mice incubated with monoclonal anti-Thy-1 (1a), anti-T-200 (1b), anti-MT-4 (1c), anti-Lyt-1 (1d), anti-Lyt-2 (1e), and anti-MEL-14 (1f). C = cortex, M = medulla, ca = thymic capsule, v = venule (x 140).

4^-, Lyt-2^+.

Frozen sections stained with MEL-14 antibodies reveal that this antigen is expressed in a dull way on cortical cells and, in this mouse strain also a small number of medullary cells. However, in the cortex one can observe individual bright MEL-14^+ cells (Fig. 1f).

DISCUSSION

In the present investigation we have used immunohistology to characterize the T cell distribution in the thymus of CBA/H mice with monoclonal antibodies directed to the cell-surface differentiation antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14. Our results show that the thymus contains several T-cell subpopulations, localized in different compartments. Virtually all thymocytes express Thy-1, T-200 and Lyt-1 antigens. The Thy-1 staining is most intense in the cortex and gradually decreases towards the medulla while the T-200 expression increases. However, a clear separation between the bright Thy-1^+ cortical cells and the dull Thy-1^+ medullary cells was not always possible since medullary cells sometimes express cortical-like Thy-1 staining levels. Relatively high levels of Thy-1 staining on dull Thy-1^+ cell populations, using indirect staining techniques, have also been observed with other anti-Thy-1 reagents (15) and indicate that anti-Thy-1 reagents have to be used with caution to discriminate between the cortical and medullary compartment of the thymus. In contrast, a clear subdivision of the cortical and medullary compartment was observed after staining of frozen sections with anti-MT-4 or anti- Lyt-2 antibodies. The majority of the cortical cells is MT-4^+ and bright Lyt-2^+ with varying levels of Lyt-1 expression, whereas medullary cells are in general bright Lyt-1^+ and either MT-4^+, Lyt-2^- or MT-4^-, Lyt-2^+. However, the MT-4^+ cells outnumber the Lyt-2^+ cells.

These results are in agreement with an earlier immunohistological analysis of the thymus, as described by van Ewijk et al. (13) and two colour flow-cytometric analysis of the Lyt phenotypes of mouse thymocytes (16, 17). Our immunohistological observations on the MT-4, Lyt-2 phenotype of cortical and medullary thymocytes are in concordance with recently published flow cytometric data by Scollay & Shortman (17, 18)

with Lyt-2 and GK1.5 (anti-L3T4), a monoclonal antibody described by Dialynas et al. (7, 8) analogeous to H129.19 (anti-MT-4). These studies showed that cortical thymocytes are L3T4$^+$, Lyt-2$^+$ and that medullary thymocytes are either L3T4$^+$, Lyt-2$^-$ or L3T4$^-$, Lyt-2$^+$. In this sense, medullary thymocytes are comparable with peripheral T cells.

Besides these major thymocyte subpopulations, we observed three minor subpopulations. First, individual bright Lyt-1$^+$ cells are scattered throughout the cortex, including the subcapsular area. These cells show, on basis of their Lyt-1 expression, a more "mature" phenotype. Similar cells have also be identified in the thymus of the human by high levels of T1 and T3 (19). Bright Lyt-1$^+$ cortical cells may represent a separate developmental lineage beside the major Lyt-1$^+$, Lyt-2$^+$ lineage in the cortex. A split into these lineages has been traced back to the level of subcapsular lymphoblasts (20). In addition, during ontogeny Lyt-1$^+$ "only" cells appear before the Lyt-1+, Lyt-2$^+$ cell (21, 23). Furthermore, Lyt-1$^+$ "only" cells can generate Lyt-1$^+$, Lyt-2$^+$ cells in the fetal thymus (24). Thus the bright Lyt-1$^+$ "only" cells that can be found in the embryonic as well as in the adult thymus could represent a precursor population for the medullary thymocyte population or alternatively for peripheral T cells. This precursor population could be correlated with the population of MEL-14$^+$ cells that are scattered throughout the cortex. It has recently been shown that these cells have, on the basis of peanut agglutinin and class I major histocompatibility complex antigens expression, a mature phenotype and represent the major source of thymus emigrants (25). However, on the basis of the present study we cannot conclude if the bright Lyt-1$^+$ cortical cells also express MEL-14 and only combined immunocytochemical and auto-radiographic analysis can provide evidence for this hypothesis.

Second, a small subpopulation bright Thy-1$^+$, T-200$^+$ cells can be observed in the subcapsular area that does not express functional markers such as MT-4 and Lyt-2 antigens. These cells are very dull Lyt-1$^+$ or Lyt-1$^-$. A third small subpopulation in the subcapsular area express exclusively Thy-1 antigens but no other T cell markers. Recently Scollay & Shortman (17) also identify such a negative subpopulation

by flow cytometry. They further showed that a substantial number of these cells is low Thy-1$^+$ or even Thy-1$^-$. These latter small subpopulations are particulary enriched in the thymus of cortisone- treated mice (13) and in the thymus of sublethal irradiated mice (Chapter IV). Furthermore, phenotypical similar cell types can also be identified during the ontogeny of the thymus (21, 26) and in the adult thymus (18) and represent therefore most likely precursor cells involved in the early events of T cell differentiation in the thymus of mice.

REFERENCES

1. Cantor, H., and I.L. Weissman, Development and function of subpopulations of thymocytes and T lymphocytes. Prog. Allergy 20:1 (1976).

2. Ford, C.E., H.S. Micklem, E.P. Evans, J.D. Gray, and D.A. Ogden, The inflow of bone marrow cells to the thymus. Studies with part body irradiated mice injected with chromosome marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci. 129:283 (1986).

3. Kadish, J.L., and R.S. Basch, Hemopoietic thymocyte precursors. III. A population of thymocytes with the capacity to return "home" to the thymus. Cell. Immunol. 30:12 (1977).

4. Cantor, H., and E. Boyse. Regulation of the immune response by T-cell subclasses. Contemp. Top. Immunobiol. 7:47 (1977).

5. Mathieson, B.J., S.O. Sharrow, P.S. Campbell, and R. Asofsky, An Lyt differentiated thymocyte subpopulation detected by flow microfluorometry. Nature 277:478 (1979).

6. Nakayama, E., H. Shiku, E. Stockert, H.F. Oettgen, and L.J. Old, Cytotoxic T cells: Lyt phenotye and blocking of killing activity by Lyt antisera. Proc. Natl. Acad. Sci. USA 76:1977 (1981).

7. Dialynus, D.P., D.B. Wilde, P. Marrack, A. Pierres, K.A. Wall, W.

Mavran, G. Otten, M.R. Loken, M. Pierres, J. Kappler, and F.W. Fitch, Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T-cell clones appears to correlate primaily with class II MHC antigen reactivity. Immunol. Rev. 74:30 (1983).

8. Dialynus, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quintas, M.R. Loken, M. Pierres, and F.W. Fitch, Characterization of the murine T-cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human LEU- 3/T4 molecule. J. Immunol. 131:2445 (1983).

9. Pierres, A., P. Naquet, A. van Agthoven, F. Bekkhoucha, F. Denizot, Z. Mischal, A. Schmitt-Verhulst, and M. Pierres, A rat anit- mouse T4 monoclonal antibody (Hl29.19) inhibits the proliferation of Ia-reactive T-cell clones and delineates two phenotypically distinct ($T4^+$, Lyt-2,3^-, and $T4^-$, Lyt-2,3^+) subsets among anti-Ia cytolytic T-cell clones. J. Immunol. 132:2775 (1984).

10. Davids, J.A.G., Bone-marrow syndrome in CBA mice exposed to fast neutrons of 1 MeV mean energy. Effect of syngeneic bone-marrow transplantation. Int. J. Radiat. Biol. 17:173 (1970).

11. Ledbetter, J.A., and L.A. Herzenberg, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63 (1979).

12. Gallatin, W.M., I.L. Wiessman, and E.C. Butcher, A cell surface molecule involved in organ-specific homing of lymphocytes. Nature 304:30 (1983).

13. van Ewijk, W., P.L. van Soest, and G.J. van der Engh, Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. J. Immunol. 127:2594 (1981).

14. Graham, T.C., and M.J. Karnovsky, The early stage of absorption of injected horse radish peroxidase in the proximal tubules of the mouse kidney: ulttastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291 1966).

15. Scollay, R, Inconsistencies detected by flow cytometry following immunofluorescence stained with anti-Thy-1 antibodies. J. Immunol. Meth. 52:15 (1982).

16. Ledbetter, J.A., R.V. Rouse, H. Spedding-micklem, and L.A. Herzenberg, T-cell subsets defined by expression of Lyt-1, 2, 3 and Thy-1 antigens. Two-colour immunofluorescence and cytotoxic analysis with monoclonal antibodies modifies current views. J. Exp. Med. 152:280 (1980).

17. Scollay, R., and K. Shortman, Thymocyte subpopulations: an experimental review, including flow-cytometric cross correlations between major murine thymocyte markers. Thymus 5:245 (1983).

18. Scollay, R., and K. Shortman, Identification of sacly stages of T Lymphocyte development in the thymus cortex and medulla. J. Immunol. 134: 3632 (1985).

19. Bhan, A.K., E.L. Reinherz, S. Poppema, R.T. McCluskey, and S.F. Schlossman, Localization of T cell and major histocompatibility-complex antigens in the human thymus. J. Exp. Med. 152:771 (1980).

20. Scollay, R., and I.L. Weissman, T-cell maturation: thymocyte and thymus-migrant subpopulations defined with monoclonal antibodies to the antigens Lyt-1, Lyt-2, and ThB. J. Immunol. 124:2841 (1980).

21. Mathieson, B.J., S.O. Sharrow, Y. Rosenberg, and U. Hämmerling, Lyt-$1^{+}23^{-}$ cells appear in the thymus before Lyt-123^{+} cells. Nature 289:179 (1981).

22. van Ewijk, W., E.J. Jenkinson, and J.J.T. Owen, Detection of Thy-1, T-200, Lyt-1, and Lyt-2 bearing cells in the developing lymphoid organs of the mouse embryo in vivo and in vitro. Eur. J. Immunol. 12:262 1982).

23. Scollay, R., K.W. Pike, and T. Mandel, Ontogeny of Lyt-1 and Lyt-123 lymphocytes in the mouse thymus. Adv. Exp. Med. Biol. 149:365 (1982).

24. Ceredig, R., R.P. Sekaly, and H. Robson-McDonald. Differentiation in vitro of Lyt-2^+ thymocytes from embryonic Lyt- 2^- precursors. Nature 303:248.

25. Reichert, R.A., W.M. Gallatin, E.C. Butcher, and I.L. Weissman, A homing receptor-bearing cortical thymocyte subset: implications for thymus-cell migration and the nature of cortisone-resistant thymocytes. Cell 38:89 (1984).

26. Ceredig, R., H. Robson-McDonald, and E.J. Jenkinson. Flow-cytometric analysis of mouse-thymus development in vivo and in vitro. Eur. J. Immunol. 13:185 (1983).

CHAPTER V

REPOPULATION OF THE MOUSE THYMUS AFTER SUBLETHAL FISSION NEUTRON IRRADIATION

I SEQUENTIAL APPEARANCE OF THYMOCYTE SUBPOPULATIONS

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SUMMARY

The T cell composition of the thymus of sublethal fission neutron irradiated CBA/H mice was analyzed with cytofluorometry and immunohistology, using monoclonal antibodies directed to the cell surface antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14.

The results of this investigation show that whole-body irradiation with 2.5 Gy fission neutrons results in a severe reduction and degeneration of the cortex whereas the medulla is affected to a less extent. Irradiation selects, within 24 hours, for a population of dull Thy-1$^+$, bright T-200$^+$, bright Lyt-1$^+$ cells localized in the medulla. Phenotype analysis of the regeneration of the thymus, which starts at about 5 days after irradiation, reveals the sequential appearance of: (1) "null" cells, i.e. lymphoblasts negative for all tests antigens, mainly in the subcapsular area but also in the medulla; (2) Thy-1$^+$ "only" and T-200$^+$ "only" cells in the subcapsular area; (3) Thy-1$^+$, T-200$^+$ cells and (4) Thy-1$^+$, T-200$^+$, MT-4$^+$, Lyt$^+$ cells in the cortex. In addition, an increased MEL-14 expression is observed in correlation with the expression of Thy-1 and T-200 determinants during the regeneration of the thymus. From day 10 on upto at least 150 days after irradiation, no differences can be observed in the thymus of irradiated and age-matched

sham-irradiated control mice, as measured by the expression and distribution of Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 antigens.

The observed sequence in phenotype shift in the regeneration of the thymus after irradiation is discussed in view of recently published data on the differentiation of the T cell system.

INTRODUCTION

The thymus is essential for the development and maintenance of cell-mediated immunity (1). It is considered to be a primary lymphoid organ that generates immunologically competent lymphocytes (2). Thymocytes originate from a subpopulation bone marrow-derived precursor cells (3) that migrate to the thymus (4-6). During differentiation in the thymus these cells aquire specific differentiation antigens on their cell surface such as Thy-1, TL, and the Lyt antigens (2,7). Immunohistological observations, with monoclonal antibodies directed to cell surface differentiation antigens, revealed during ontogeny of the thymus (8,9) and in the thymus of cortisone-treated mice (10) a specific phenotypical sequence order that seems to be a general rule in T cell differentiation.

Whole-body irradiation has severe effects on the thymus and leads to a strong depopulation. Thymus recovery, hereafter, follows a biphasic pattern (11-13). After an initial depopulation of the thymus, regeneration starts about 5 days after irradiation from a population of intrathymic radioresistant precursor cells (14,15) and leads to an almost complete recovery of the thymus when sublethal doses are employed. The following second depopulation of the thymus, starting about day 14 after irradiation, is ascribed to an exhaustion and limited proliferative capacity of these radioresistant precursors (16,17) and to an impaired production of the thymus restricted precursors in the damaged bone marrow (6,11). The second regenerative phase in thymus recovery has been attributed to the replenishment of the prothymocyte pool in the recovering bone marrow (11,18).

The existence of radioresistant intrathymic precursor cells and the following bone marrow independent repopulation of the thymus

brought by these cells after irradiation, provides a model to get insight in T cell differentiation. In the present investigation, we have exposed mice to a sublethal dose of fission neutrons, a type of irradiation with a high ionisation density. In contrast to X-rays, fission neutrons cause damage virtually without intracellular repair (19). This means that the regeneration of the lymphoid system after neutron irradiation, will take place virtually without the influence of repair mechanisms of sublethal damage in the investigated cell system. We analyzed the T cell composition of the thymus following irradiation with cytofluorometry and immunohistology, using monoclonal antibodies directed to the cell surface antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14.

The results of this investigation indicate that after an initial depopulation, which mainly affects the cortex of the thymus, regeneration starts with the appearance of large "null" cells followed by a sequential appearance of the other analysed antigens in a time-course of 5-9 days after irradiation. Hereafter, no differences could be observed between irradiated and age-matched unirradiated control mice. We relate these findings to recently published data on thymocyte differentiation in the mouse.

MATERIALS AND METHODS

Animals

Male and female CBA/H mice were irradiated or sham-irradiated at the age of 5-7 weeks. The procedures of animal care have been described elsewhere (20).

Irradiation procedure

Whole-body neutron irradiation was performed with fast fission neutrons from a ^{235}U-converter in the Low Flux Reactor at Petten as described elsewhere (13). Briefly, the animals were exposed bilaterally at a fast neutron dose rate of 0.10 Gy/min. The absorbed doses are given as neutron centerline doses and do not include the 9% gamma-ray contribution. The neutron spectrum had a mean energy of 1.0 MeV. The mice were irradiated with 2.5 Gy neutrons.

Antisera

Monoclonal antibodies directed to cell surface determinants of mouse lymphoid cells were obtained from the tissue culture supernatant of hybrid cell lines. Clone 59-AD-2.2 secreted anti-Thy-1 antibodies, clone 53-7.3.13 secreted anti-Lyt-1 antibodies, clone 53-6.72 secreted anti-Lyt-2 antibodies and clone 30-G-12 secreted anti-T-200 antibodies. All clones were originally prepared and characterized by Dr. J.A. Ledbetter (21). MEL-14 monoclonal antibodies specific for a lymphocyte surface molecule that mediates the recognition of lymph node high endothelial venules (22), were the kind gift of Dr. E.L. Butcher, Stanford University, Stanford, USA. Clone H129.19 secreted anti-MT-4 (L3T4) antibodies, generated and characterized by Pierres (23) was the kind gift of Dr. M. Pierres, INSERM-CNRS, Marseille, France.

Conjugates

Peroxidase-conjugated rabbit-anti-rat serum (RaRa Ig-HRP) was obtained from Dakopatts, Denmark. The conjugate was diluted in a 1:20 dilution and supplemented with 1% normal mouse serum (NMS). Fluores cein-conjugated rabbit-anti-rat serum (RaRa Ig-FITC) was obtained from Nordic Immunological Laboratories. This conjugate was used in a 1:15 dilution, also supplemented with 1% NMS.

were calculated by plotting the fluorescence profiles of the cell suspensions. The profiles of the negative control cell suspension were "smoothed", a "cut-off" channel was determined, and the percentage of cells above this "cut-off" channel i.e. the aspecific fluorescence, was calculated. In the experimental curves the percentage of cells above this "cut-off" channel was calculated and corrected for the aspecific fluorescence. In case, there was no overlap of control and experimental curves, no correction for aspecific fluorescence was performed.

Tissue preparation for immunohistology

Frozen sections of thymuses, isolated at various days after irradiation, were prepared and stained using the indirect immunoperoxidase method as described elsewhere (10).

RESULTS

In the first part of this section, we describe the immunohistology of the thymus in neutron-irradiated CBA/H mice, defined by monoclonal antibodies against the cell surface antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14, at various days after irradiation. These results were obtained from carefully choosen serial frozen sections. For the description of the immunohistology of the thymus in unirradiated control mice, we refer to previous papers (10,25). In the second part, we present quantitative data, such as cytofluorometry of the thymocyte subpopulations after irradiation, as analyzed with monoclonal antisera against Thy-1, T-200, Lyt-1 and Lyt-2.

1. Immunohistology of T cell subpopulations in the thymus of neutron-irradiated mice

Irradiation with 2.5 Gy fission neutrons causes, within 24 hours, extensive necrosis and phagocytosis in the cortex resulting in a dramatic reduction in the volume of the cortex of the thymus. In contrast, the medulla is only slightly affected. Until 3 days after irradiation, the cortex is very hypocellular and virtually negative for all tested antigens. In the medulla, this dose of irradiation selects for a population of dull Thy-1$^+$, bright T-200$^+$, bright Lyt-1$^+$ cells. Some of these cells show intermediate levels of MT-4 or Lyt-2 expression (data not shown).

4-5 Days after irradiation, the number of cells in the cortex starts to increase. These cells are located in the subcapsular area. Most of these cells are negative for all tested antigens ("null" cells) and generally larger in size than normal thymocytes (Fig. 1). Some of these cells express only Thy-1 or T-200 antigens (Thy-1$^+$ "only" and T-200$^+$ "only" cells). Deeper in the cortex, the majority of cells is dull Thy-1$^+$ with scattered bright Thy-1$^+$ cells. A number of these cortical cells are T-200$^+$ but virtually negative for the other tested antigens (Fig. 2). The expression of Thy-1 in the medulla at this time is very heterogeneous, varying from dull to bright. A small subpopulation of Thy-1$^-$ cells can be observed in this area. These cells are also negative for the other tested antigens. The majority of medullary cells is T-

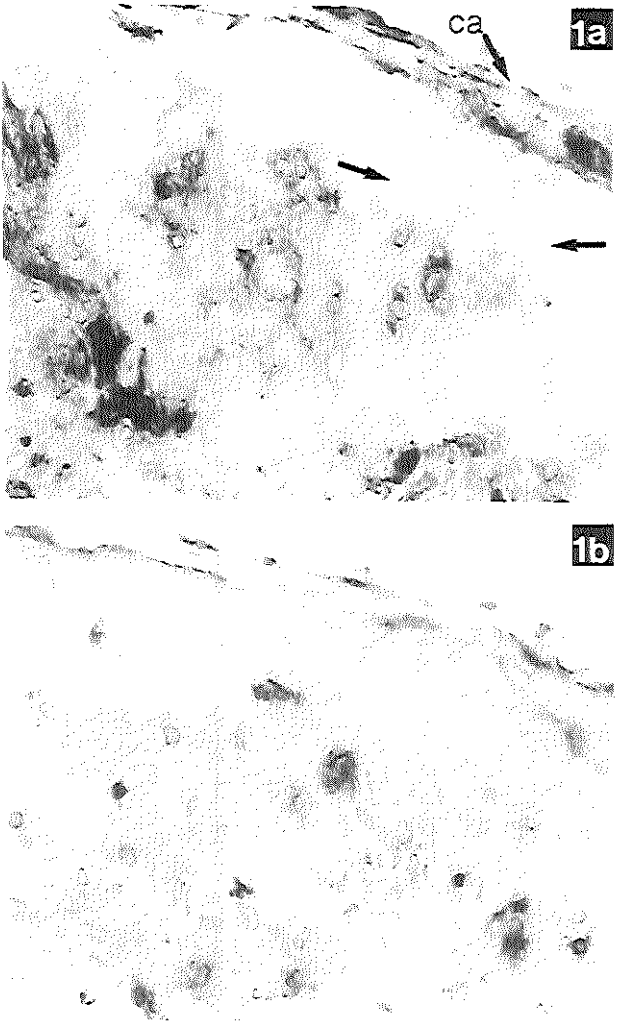


Fig. 1. High power magnification of the subcapsular area of the thymus 4-5 days after 2.5 Gy neutron irradiation. 1a Represents a frozen section incubated with monoclonal anti-Thy-1 antibodies. 1b Represents a serial section incubated with monoclonal anti-Lyt-1 antibodies. Arrows indicate large Thy-1$^-$ cells which are also Lyt-1 negative. CA = thymic capsule (x 595).

2b

2d

2a
M
O

2c

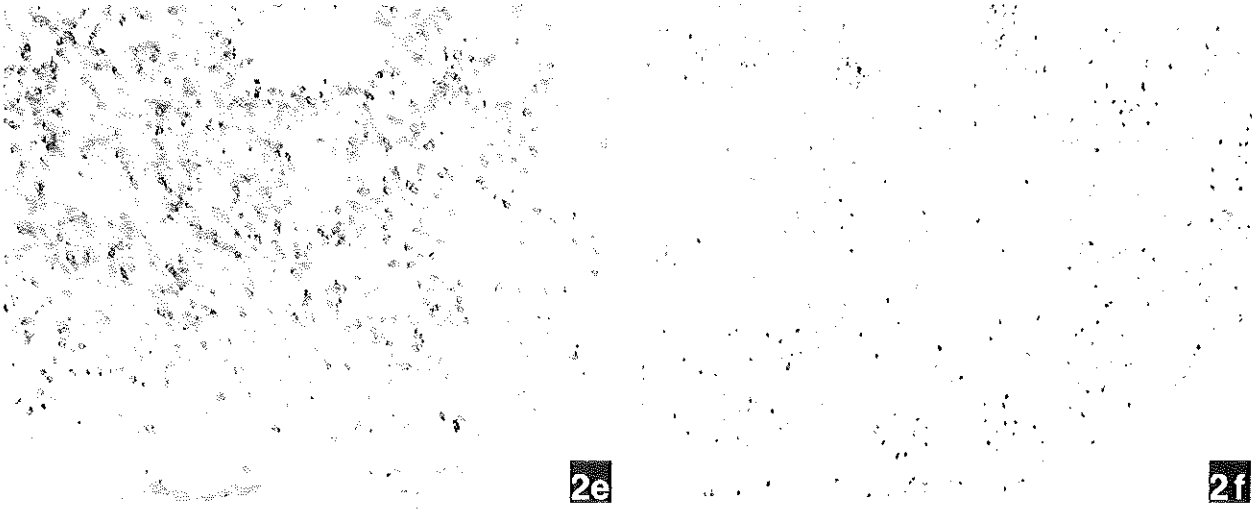
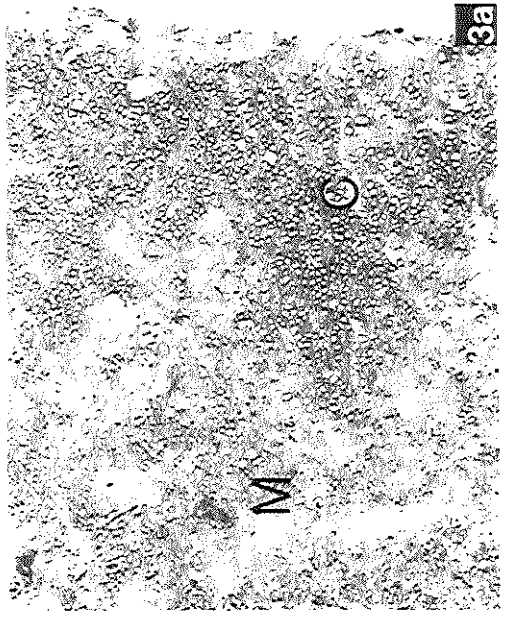


Fig. 2. Immunoperoxidase staining of serial frozen sections of the thymus 4-5 days after 2.5 Gy neutron irradiation incubated with monoclonal anti-Thy-1 (2a), anti-T-200 (2b), anti-MT-4 (2c), anti-Lyt-1 (2d), anti-Lyt-2 (2e), and anti-MEL-14 (2f). C = cortex, M = Medulla (x 140).


3b


3d


3a
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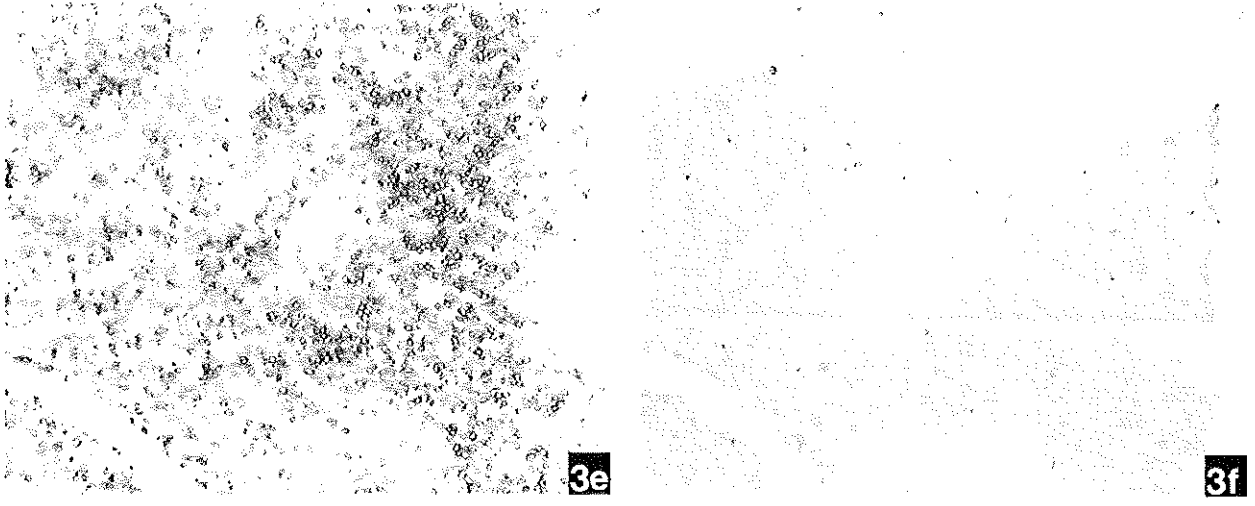


Fig. 3. Immunoperoxidase staining of serial frozen sections of the thymus 6-7 days after 2.5 Gy neutron irradiation incubated with monoclonal anti-Thy-1 (3a), anti-T-200 (3b), anti-MT-4 (3c), anti-Lyt-1 (3d), anti-Lyt-2 (3e), and anti-MEL-14 (3f). C = cortex, M = Medulla (x 140).

200$^+$ and show varying levels of Lyt-1 expression. Both MT-4$^+$ cells as well as Lyt-2$^+$ cells are scattered throughout the medulla (Fig. 2).

At 6-7 days after irradiation, virtually all thymocytes are Thy-1$^+$, except for a small number of subcapsular cells. These particular cells are negative for the other tested antigens. The cells in the cortex are in general large (about 7-8 µm) in size. The Thy-1 staining is most intense in the cortex and gradually decreases towards the medulla (Fig. 3). Comparison of serial sections shows that the majority of bright Thy-1$^+$ cells are also dull T-200$^+$, however T-200$^-$, as well as bright T-200$^+$ cells can be observed. Observations of individual cells further show that most Thy-1$^+$, T-200$^+$ cells are also MEL-14$^+$. The regeneration of the thymus at this stage is not uniform since adjacent areas in the cortex are often in different regenerative stages: in areas where cells express high Thy-1 and intermediate T-200 levels, the expression of MT-4, Lyt-1, Lyt-2 is developed to some extent, whereas in areas where Thy-1$^+$ cells express very low levels of T-200 antigens, the expression of the other tested antigens, except MEL-14, is poorly developed. The expression of Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 in the medulla, 6-7 days after irradiation, is starting to resemble that of non-irradiated control mice (Fig. 3). However, at this time-point, the medulla contains also a number of Thy-1$^-$ cells. These cells are also negative for all other tested antigens (Fig. 4) and are present in small cell clusters.

At 8-9 days after irradiation, the regeneration of the thymus as measured by the expression of Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 antigens, is nearly completed and from day 10 on upto at least 150 days after irradiation with fission neutrons, no differences can be observed in the thymus of irradiated and age-matched sham-irradiated control mice.

In summary, irradiation with 2.5 Gy fast fission neutrons selects within 24 hours for a population of dull Thy-1$^+$, bright T-200$^+$, bright Lyt-1$^+$ cells with scattered MT-4 and Lyt-2 expression. These cells are located in the medulla. Phenotype analysis of the regenerating thymus reveals the sequential appearance of (1) "null" cells; (2) Thy-1 "only" cells; T-200 "only" cells in the subcapsular area and (3) Thy-1$^+$, T-200$^+$ cells; (4) Thy-1$^+$, T-200$^+$, MT-4$^+$, Lyt$^+$ cells in the cortex in a

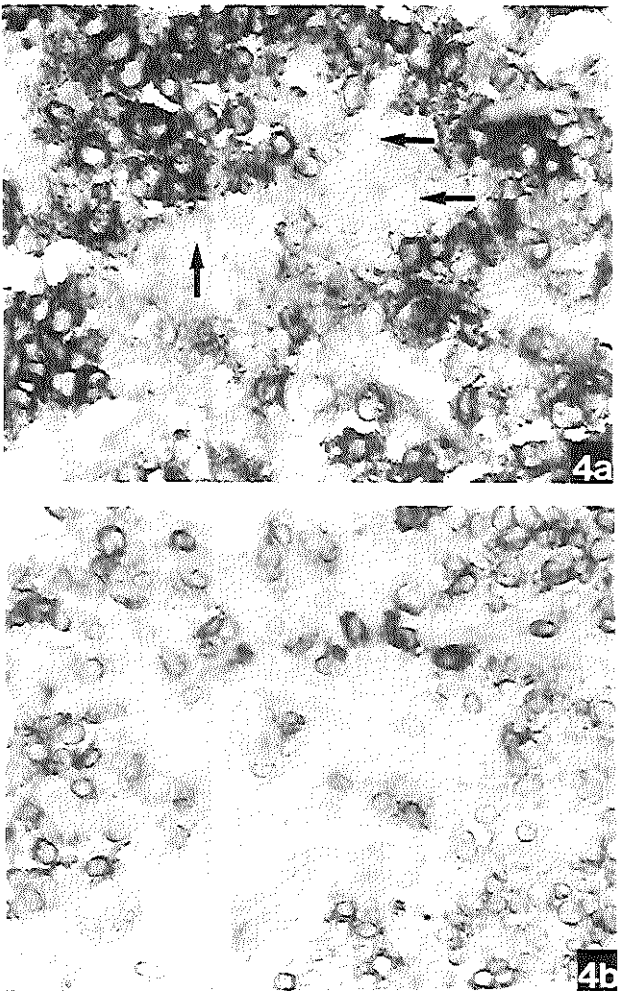


Fig. 4. High power magnification of the medulla of the thymus 6-7 days after 2.5 Gy neutron irradiation. 4a Represents a frozen section incubated with anti-Thy-1 antibodies. 4b Represents a frozen section incubated with anti-Lyt-1 antibodies. Arrows indicate Thy-1$^-$ cells which are also Lyt-1 negative (x 595).

time period from 5-9 days after irradiation. "Null" cells were also observed in the medulla of the regenerating thymus. Furthermore, an increased MEL-14 expression seems to be correlated with the Thy-1$^+$, T-200$^+$ stage in the regenerative process.

Since these histological data cannot easily be quantified, we show in the following section quantitative data of the thymus after irradiation.

2. Quantitative aspects of the thmymus of control and neutron-irradiated mice

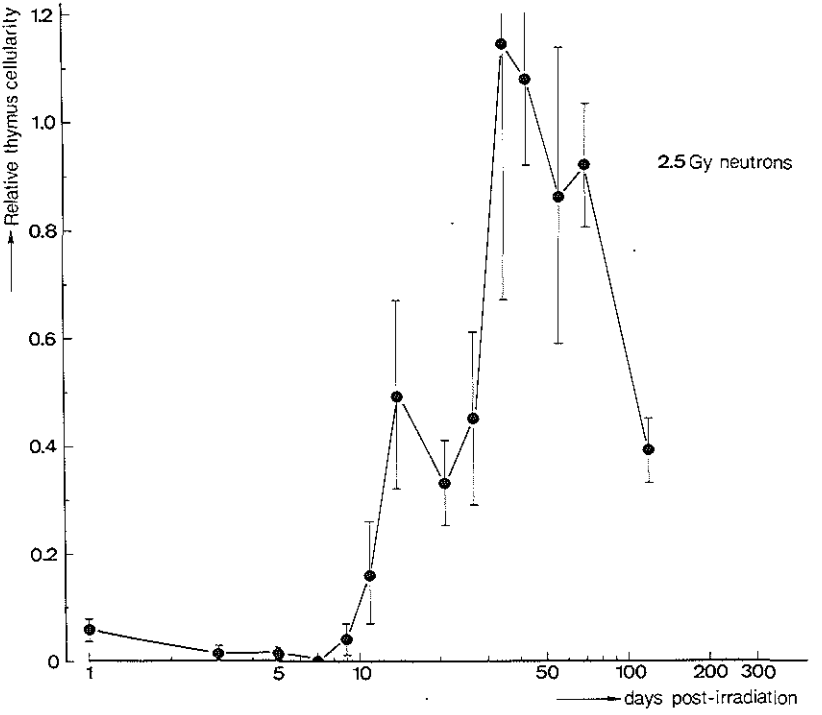


Fig. 5. Thymus cellularity relative to that of age-matched sham-irradiated controls (mean ±SD) at various days after 2.5 Gy whole-body irradiation with fission neutrons.

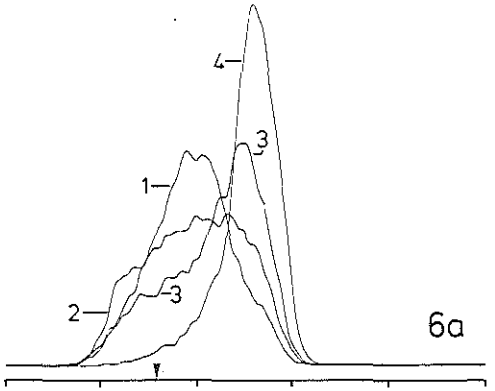
2A. Cellular quantification of the thymus after irradiation

Irradiation with 2.5 Gy fission neutrons causes a marked decrease in the relative thymic cellularity to less than 1% of the control values at day 6 after irradiation. Hereafter, a biphasic regeneration pattern can be observed followed by a marked decrease which lasts upto at least 150 days after irradiation (Fig. 5).

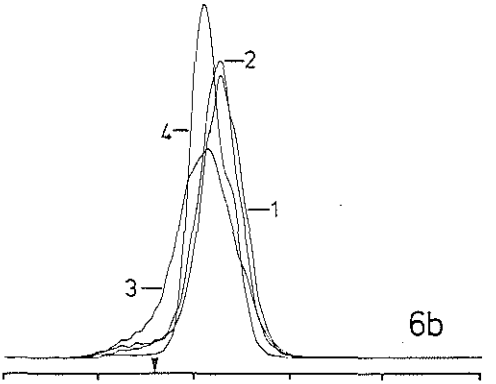
2B. Flow cytofluorometric analysis of thymocytes of control and neutron irradiated mice

Flow cytofluorometric analysis of the thymus was started on day 3 after irradiation since at day 1 and 2, necrosis and phagocytosis in the irradiated thymus caused autofluorescence, which severely influenced the analysis. The monoclonal antibodies used for flow cytofluorometry were Thy-1, T-200, Lyt-1 and Lyt-2. MT-4 and MEL-14 were not available for this purpose.

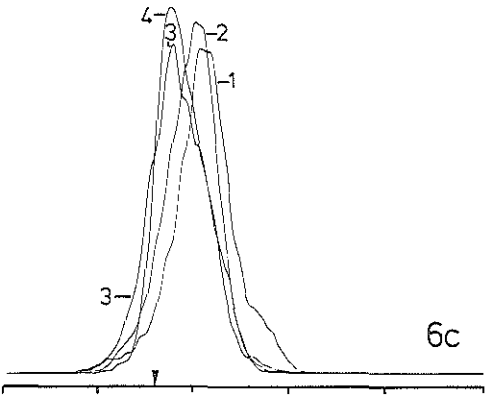
The fluorescence profile of thymocytes from control mice, stained with the anti-Thy-1 monoclonal 59-AD-22 is shown in Fig. 6a. Virtually all thymocytes are Thy-1^+ (see also Table I), but show a wide range of fluorescence intensities. The fluorescence profile of Thy-1^+ cells show in general a slight shoulder at the left side, indicating a dull subpopulation, although the distinction between the low and bright Thy-1^+ subpopulations is not always very clear. Irradiation with fission neutrons results at day 3 after irradiation in a overall reduction of the percentage and absolute number Thy-1^+ cells (Table I, Fig. 7) and selects for the dull Thy-1^+ subpopulation (Fig. 6a). From day 5 on after irradiation, one can observe an increase of the percentage Thy-1^+ cells but the number of Thy-1^+ cells is still decreasing (Fig. 7). The fluorescence profile at day 6 after irradiation shows Thy-1^- and bright Thy-1^+ cells besides the surviving dull Thy-1^+ cell population. 7 Days after irradiation, the bright Thy-1^+ subpopulation has further increased while the Thy-1^- cell population is reduced (Figs. 6a, 7). From day 8 after irradiation on, the fluorescence profile of Thy-1^+ thymocytes resembles that of control mice.



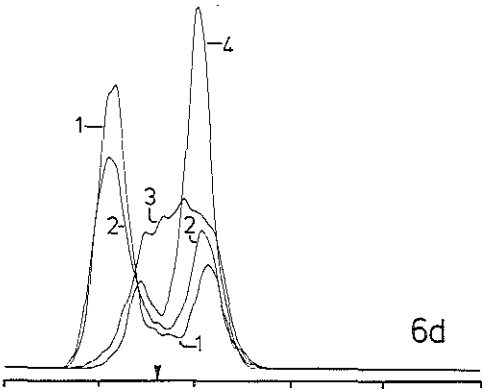
6a



6b



6c



6d

Fig. 6. Fluorescence histograms of thymocytes stained with monoclonal anti-Thy-1 (6a), anti-T-200 (6b), anti-Lyt-1 (6c), and anti-Lyt 2 (6d) antibodies. The fluorescence intensity is determined over 250 channels with a multichannel analyser and plotted on a logarithmic scale. Fluorescence intensity increases from the left to the right on the ordinate, on which a 250 channel scale is indicated. For each channel number the relative frequency of cells with the corresponding fluorescence intensity is expressed (abscissa).
In each histogram, 4 profiles are shown: Profile 1 represents the specific staining of thymocytes 3 days after 2.5 Gy neutron irradiation; Profile 2 represents the staining pattern of thymocytes 6 days after neutron irradiation; Profile 3 represents the staining pattern of thymocytes 7 days after irradiation whereas Profile 4 represents the staining pattern of control thymocytes.
The arrows of the ordinate indicate the cut-off channel used for the determination of the percentage positive cells in a thymocyte population.

TABLE I Fluorescence characteristics and percentage of labeled cells in the thymus of control and irradiatated mice at various days after 2.5 Gy neutron whole-body irradiation

Staining	Thy-1			Lyt-1			Lyt-2			T-200		
	P[b]	A[b]	%[c]	P	A	%	P	A	%	P	A	%
Day[a]												
0 (Control)	133	129	93	92	96	90	103	97	82	108	110	93
3	105	100	72	104	106	83	60	73	20	114	113	86
4	106	98	59	106	101	77	51	69	18	111	109	84
5	102	98	67	98	98	81	55	74	25	113	109	85
6	101	101	64	101	99	77	56	76	28	116	114	85
7	125	111	76	92	93	69	96	92	61	108	106	80
8	125	118	92	83	87	75	101	88	74	100	102	92
9	128	122	92	87	89	81	97	94	83	104	105	94

[a] Days after 2.5 Gy (250 rad) neutron irradiation. Control: age-matched sham-irradiated mice. Values are given from one of two identical experiments.

[b] See materials and methods

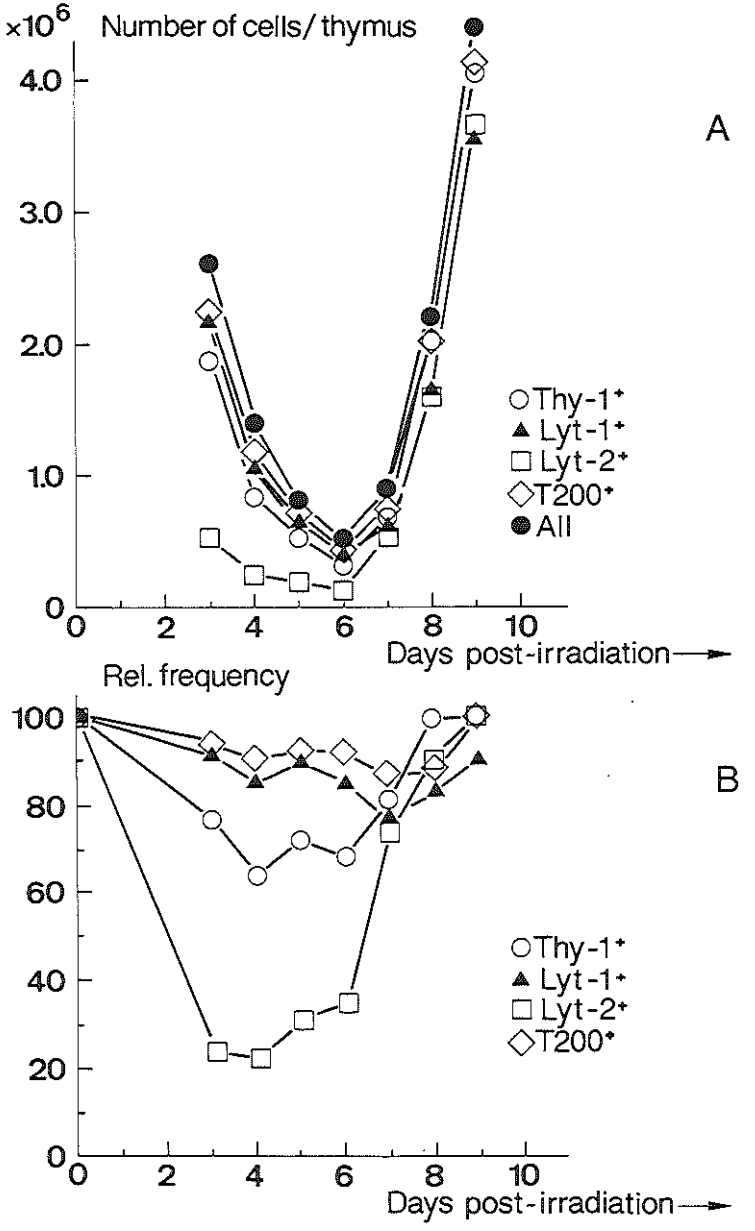
[c] % = percentage positively labeled cells in a thymocyte population, corrected for aspecific fluorescence*.

The fluorescence distribution of thymocytes of control mice stained with anti-T-200 antibodies shows that virtually all thymocytes are T-200$^+$ and a shoulder at the right side of the profile, i.e. the bright T-200$^+$ subpopulation (Fig. 6b, Table I). After neutron irradiation, the percentage T-200$^+$ cells remains at a somewhat lower level than in the sham-irradiated control mice, but the absolute number of T-200$^+$ cells decreases and there is a selection for the bright T-200$^+$ subpopulation (Figs. 6b, 7). From day 6 on after irradiation, dull T-200$^+$ and T-200$^-$ cells appear besides the surviving bright T-200$^+$ population (Fig. 6b). At about day 8 after irradiation, the fluorescence profile of T-200$^+$ thymocytes resembles that of control mice.

The fluorescence profile of Lyt-1$^+$ cells in the thymus of control mice shows that the large majority is Lyt-1$^+$ and that these cells have a wide range of fluorescence intensities. Furthermore, the profile has a small shoulder at the right side of the curve, indicating a subpopulation of bright Lyt-1$^+$ cells (Fig. 6c, Table I). 3 Days after irradiation, one can observe a slight decrease of the percentage Lyt-1$^+$ cells but the overall number of Lyt-1$^+$ cells is severely reduced (Fig. 7). In addition, irradiation selects for the bright Lyt-1$^+$ subpopulation (Fig. 6c). Between day 6 and 7 after irradiation, a further decrease of the percentage Lyt-1$^+$ cells occurs while the total number of cells has increased (Fig. 7), indicating the appearance of Lyt-1$^-$ and dull Lyt-1$^+$ cells (Fig. 6c). From day 8 on, the fluorescence profile is starting to resemble that of control mice.

The Lyt-2 fluorescence distribution of thymocytes of control mice shows a bimodal profile with a major bright Lyt-2$^+$ subpopulation and a minor (about 15-20%) population with no or dull Lyt-2 expression (Fig. 6d). Irradiation causes a marked decrease of the percentage Lyt-2$^+$ cells to about 20%. This low level is maintained until day 6 after irradiation, but the absolute number of cells further decreases (Fig. 7). From this timepoint on, a marked increase of the percentage Lyt-2$^+$ cells is observed, with varying levels of Lyt-2 expression (Figs. 6d, 7). About 9 days after irradiation, the Lyt-2 fluorescence distribution resembles that of sham-irradiated control mice.

Comparison of cytofluorometric data of irradiated and age-matched sham-irradiated mice at longer periods after irradiation, does not reveal any significant differences.


×10^6
Number of cells/ thymus
4.0
3.0
2.0
1.0
0
0
2
4
6
8
10
Days post-irradiation
A
○Thy-1+
▲ Lyt-1+
□ Lyt-2+
◇T200+
● All
Rel. frequency
100
80
60
40
20
0
0
2
4
6
8
10
Days post-irradiation
B
○Thy-1+
▲ Lyt-1+
□ Lyt-2+
◇T200+

Summarized, irradiatation selects for bright T-200$^+$, bright Lyt-1$^+$ cells (Table I). The large majority of these cells is dull Thy-1$^+$ and Lyt-2$^-$. However, the results further indicate the existence of a smal subpopulation of cells that express T-200 and Lyt-1 antigens but no Thy-1 antigens. Regeneration starts with the appearance of "null" cells, bright Thy-1$^+$ and T-200$^+$ cells, followed by the reappearance of dull Lyt-1$^+$ cells and an increase of the percentage Lyt-2$^+$ cells.

DISCUSSION

In the present investigation we have used immunohistology and flow cytofluorometry to characterize the T cell composition of the thymus of sublethal fission neutron-irradiated CBA/H mice, using monoclonal antibodies directed to the cell surface antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14. The neutron irradiation system enables us to study the regeneration of cell systems virtually without the influence of repair of sublethal damage.

Fig. 7.

(A) Absolute number of cells stained with anti-Thy-1, anti-T-200, anti- Lyt-1, or anti-Lyt-2 antibodies and the total number of cells in the thymus as a function of time after 2.5 Gy neutron irradiation.

(B) Frequency of positive cells stained with anti-Thy-1, anti-T-200, anti-Lyt-1, or anti-Lyt-2 antibodies relative to that of control thymocytes as a function of time after 2.5 Gy neutron irradiation.

The results of these experiments show that sublethal whole-body irradiation with 2.5 Gy fast fission neutrons has severe effects on the thymus and results in a dramatic reduction of the cortex, whereas histologically the medulla seems to be slightly affected.
However, the present investigation shows clearly that only 20-30% of the medullary cell pool survives the irradiation. In addition, cell survival studies with graded doses of irradiation reveal that the radioresistant medullary cell population present at day 2 after irradiation, cannot be detected anymore at day 5 after irradiation (Huiskamp et al., manuscript in prep.). This explains the observed discrepancy between the time of maximal cortical atrophy and the time of maximal thymic cell depletion. Though radioresistant medullary thymocytes have a similar phenotype, i.e. dull Thy-1$^+$, bright T-200$^+$, and bright Lyt-1$^+$, as shown for cortisone resistant thymocytes (10,26), they differ with respect to their MEL-14 expression. Many cortisone resistant thymocytes are bright MEL-14$^+$ and could be cortisone resistant cortical bright MEL-14$^+$ cells which are repositioned in the medulla as proposed by Reichert et al. (27). However, radioresistant thymocytes are MEL-14$^-$ and can therefore not be compared with cortisone resistant thymocytes. In addition, the present flow cytofluorometric data indicate the existence of a subpopulation T-200$^+$, Lyt-1$^+$ cells which are Thy-1$^-$. However, immunohistology does not reveal such a sub population. The reason for this discrepancy remains to further investigated.

We further demonstrated that the regeneration of the thymus follows a biphasic pattern and starts with the repopulation of the cortex with large "null" cells. These "null" cells probably represent the proposed intrathymic radioresistant precursor cells (14) that repopulate the thymus in situ following irradiation as shown by Sharrow et al. (15). Large Thy-1$^-$ lymphoblasts were also observed in the thymus of cortisone-treated mice (10). We propose that the "null" cells are comparable with the population of cells, expressing no differentation antigens, which can be identified at day 14 of gestation in the thymus during ontogeny (8,9). Our present observation that "null" cells appear in the subcapsular area as well as in foci in the medulla are in concordance with studies showing that foci of proliferating cells appear in the medulla of the repopulating embryonic avian thymus at the same

time as they appear in the subcapsular area of the cortex (28). These observations and recently published data by Ezine et al. (29) on thymic repopulation in lethally irradiated Thy-1 congeneic bone marrow reconstituted mice, support the current thinking that cortex and medulla have independent generation kinetics (30-33). However, in the present system we are unable to delineate the further phenotypical differentiation of the medullary "null" cells since their frequency is low. Moreover, during their further differentiation, these cells become indistinguishable from the surviving surrounding medullary thymocytes.

In addition, we found a subpopulation of large bright Thy-1$^+$ cells in the cortex that, by comparing adjacent sections incubated with the other tested antigens, have to be Thy-1$^+$ "only" cells. Besides, based on the percentage T-200$^+$ cells and the present immunohistological observations, there is also a T-200$^+$ "only" subpopulation. However, the 30-G-12 antibody does recognize macrophage associated T-200 antigens (34). Moreover, serial frozen sections stained with antibodies directed to Mac-1 show the presence of some macrophages (Huiskamp, unpubl. observations) but their very low incidence cannot account for all the observed T-200$^+$ "only" cells. This observation indicates that, in the immature thymocyte population in the cortex, different blast subpopulations can be distinguished. In as much these blast subpopulations are related to the split in Lyt-1$^+$ and Lyt-1$^+$, Lyt-2$^+$ lineages that has been traced back to the level of the lymphoblasts (35), remains to be established.

When the regeneration proceeds, virtually all thymocytes become Thy-1$^+$, T-200$^+$. They are generally large sized (7-8 µm) and the majority of these cells develop Lyt-1, Lyt-2 and MT-4 antigens. Morphological studies have shown that only thymocytes of 7-8 µm nuclear diameter or larger divide (36,37). Thus, these large cells still have to be considered as a precursor population and are probably comparable with the Lyt-2$^+$ blast population that has been observed in the thymus of normal control mice (26,31).

The regeneration of the thymus is not uniform. We observed in adjacent areas of the cortex often different regenerative stages: in areas, where Thy-1$^+$ cells express low levels of T-200 antigens, the expression of all other tested antigens is also poorly developed,

whereas in areas where cells express more or less control levels of Thy-1 and T-200 antigens, the expression of the other tested antigens has further developed. These differences in regeneration kinetics are probably related with the differences in expression of MHC determinants on the thymic epithelial cells in the forementioned areas. In this context, we stress that irradiation has namely a severe effect on the thymic stroma. This is for instance shown by the dendritic staining pattern of MHC determinants that can be seen in the thymus (10,38), which is lost until 7 days after 2.5 Gy neutron irradiation. Hereafter, the dendritic staining pattern is observed again in those parts of the cortex where the Thy-1$^+$, T-200$^+$ cells express intermediate levels of MT-4, Lyt-1 and Lyt-2 antigens (39). A close correlation between the expression of MHC determinants on thymic epithelial cells and the onset of proliferation of lymphoblasts has also been observed in the embryonic thymus (40). In addition, in vivo treatment of neonatal mice with monoclonal anti-I-A antibodies has been shown to interfere with the differentation of I-A-restricted T helper cells (41) and emphasizes the role of MHC determinants in the T cell maturation process.

A further interesting point is, that the Thy-1$^+$, T-200$^+$ cell stage in the thymus regeneration seems to be correlated with an enhanced MEL-14 expression. A similar correlation can also be observed with the Thy-1$^+$ lymphoblast stage during thymocyte differentiation in the ontogeny of the thymus (E. van Vliet, pers. commun.). These immature cells already express a marker normally found on mature recirculating peripheral T cells and on scattered thymocytes, mainly located in the cortex. These MEL-14$^+$ cells might be the cortical precursors of peripheral T cells (27). However, only combined immunocytochemical and autoradiographic analysis can provide evidence for this hypothesis.

Finally, about 8-9 days after irradiation, we observed that the regeneration of the thymus, as measured by the expression of Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 antigens, was nearly completed and that from day 10 upto at least 150 days after irradiation no differences could be observed between the thymus of irradiated and age-matched sham-irradiated control mice. Thus, the observed second decrease of the thymic relative cellularity, starting about 14 days after irradiation, and the following regeneration, as well as the ultimate decrease of the relative thymic cellularity after irradiation, have no reflections on

the T cell composition in the thymus and are probably related with the number of thymus restricted precursors available in the bone marrow after irradiation (13).

The different phases in the initial bone marrow-independent thymic regeneration, where sequentially the following phenotypes were ob- observed: 1. "null" cells; 2. Thy-1$^+$ "only" and T-200$^+$ "only" cells; 3. Thy-1$^+$, T-200$^+$ cells; 4. Thy-1$^+$, T-200$^+$, MT-4$^+$, Lyt$^+$ cells, were also observed in the thymus of 6.0 Gy X-irradiated mice although quantitative differences, due to the two types of irradiation, were also observed. This phenotypical sequence in the thymus regeneration after irradiation is similar to those that have been found in ontogeny (8,9) and in the thymus of cortisone-treated mice (10), and might therefore be a general rule in the T cell differentiation in the mouse.

REFERENCES

1. Miller, J.F.A.P., and D. Osoba, Current concepts of the immunological function of the thymus. Physiol. Rev. 47:437 (1967).

2. Cantor, H., and I.L. Weissman, Development and function of subpopulations of thymocytes and T lymphocytes. Prog. Allergy 20:1 (1976).

3. Jones-Villeneuve, E.V., J.J. Rusthoven, R.G. Miller, and R.A. Philips, Differentiation of Thy-1 bearing cells from progenitors in long-term bone marrow cultures. J. Immunol. 124:596 (1980).

4. Ford, C.E., H.S. Micklem, E.P. Evans, J.G. Gray, and D.A. Ogden, The inflow of bone marrow cells to the thymus. Studies with part body irradiated mice injected with chromosome marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci. 129:283 (1966).

5. Kadish, J.L., and R.S. Basch, Hemopoietic thymocyte precursors. III. A population of thymocytes with the capacity to return "home" to the thymus. Cell. Immunol. 30:12 (1977).

6. Boersma, W., I. Betel, R. Daculsi, and G. van der Westen, Post-irradiation thymocyte regeneration after bone marrow transplantation. I. Regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet. 14:179 (1981).

7. Cantor, H., and E. Boyse, Regulation of the immune response by T cell subclasses. Contemp. Top. Immunobiol. 7:47 (1977).

8. van Ewijk, W., E.J. Jenkinson, and J.J.T. Owen, Detection of Thy-1, T-200, Lyt-1, and Lyt-2 bearing cells in the developing lymphoid organs of the mouse embryo in vivo and in vitro. Eur. J. Immunol. 12:262 (1982).

9. Ceredig, R., H. Robson-McDonald, and E.J. Jenkinson, Flow-cytometric analysis of mouse thymus development in vivo and in vitro. Eur. J. Immunol. 13:185 (1983).

10. van Ewijk, W., P.L. van Soest, and G.J. van der Engh, Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. J. Immunol. 127:2594 (1981).

11. Takada, A., Y. Takada, U. Kim, and J.A. Ambrus. Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129:445 (1969).

12. Sharp, J.G., and D.B. Thomas, Thymic regeneration in lethally irradiated mice. Radiat. Res. 64:293 (1975).

13. Huiskamp, R., J.A.G. Davids, and O. Vos, Short- and long- term effects of whole-body irradiation with fission neutrons or X- rays on the thymus in CBA mice. Radiat. Res. 95:370 (1983).

14. Kadish, J.L., and R.S. Basch, Thymic regeneration after lethal irradiation: Evidence for an intrathymic radioresistant T cell precursor. J. Immunol. 114:452 (1975).

15. Sharrow, S.O., A. Singer, U. Hämmerling, and B.J. Mathieson, Phenotypic characterization of early events of thymus repopulation in radiation bone marrow chimeras. Transplantation. 35:355 (1983).

16. Dukor, P., J.F.A.P. Miller, W. House, and V. Allman, Regeneration of thymus grafts. I. Histological and cytological aspects. Transplantation. 3:639 (1965).

17. Blomgren, H., and L. Révész, The effect of bone marrow protection on the cellular composition of the irradiated mouse thymus. Exp. Cell Res. 53:261 (1968).

18. Coggle, J.E, The absence of late effects of radiation on the cellularity of the mouse thymus. Int. J. Radiat. Biol. 40:229 (1981).

19. Broerse, J.J., and G.W. Barendsen, Relative biological effectiveness of fast neutrons for effects on normal tissues. In: Current Topics in Radiation Research. Vol 8. M. Ebert and A. Howard eds. North-Holland, Amsterdam. p. 309 (1973).

20. Davids, J.A.G, Bone-marrow syndrome in CBA mice exposed to fast neutrons of 1 MeV mean energy. Effect of syngenic bone-marrow transplantation. Int. J. Radiat. Biol. 17:173 (1970).

21. Ledbetter, J.A., and L.A. Herzenberg, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63 (1979).

22. Gallatin, W.M., I.L. Weissman, and E.C. Butcher, A cell surface molecule involved in organ-specific homing of lymphocytes. Nature. 304:30 (1983).

23. Pierres, A., P. Naquet, A. van Agthoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A.M. Schmitt-Verhulst, and M. Pierres, A rat anti-mouse T4 monoclonal antibody (H129.19) inhibits the proliferation of Ia-reactive T cell clones and delineates two phenotypically distinct ($T4^+$, Lyt-2, 3^- and $T4^-$, Lyt-2, 3^+) subsets among anti-Ia cytolytic T cell clones. J. Immunol. 132:2775 (1984).

24. van Ewijk, W., P.L. van Soest, A. Verkerk, and J.F. Jongkind, Loss of antibody binding to prefixed cells: fixation parameters for immunocytochemistry. Histochem. J. 16:179 (1984).

25. van Ewijk, W, Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentation. Amer. J. Anat. 170:311 (1984).

26. Scollay, R., and K. Shortman, Thymocyte subpopulations: an experimental review, including flow cytometric cross correlations between the major murine thymocyte markers. Thymus. 5:245 (1983).

27. Reichert, R.A., W.M. Gallatin, E.L. Butcher, and I.L. Weissman, A homing receptor-bearing cortical thymocyte subset: implications for thymus cell migration and the nature of cortisone- resistant thymocytes. Cell. 38:89 (1984).

28. Jotereau, F.V., and N.M. Le Douarin, Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic perinatal life. J. Immunol. 129:1869 (1982).

29. Ezine, S., I.L. Weissman, and R.V. Rouse, Bone marrow give rise to distinct cell clones within the thymus. Nature 309:629 (1984).

30. Shortman, K., and H. Jackson, The differentiation of T lymphocytes, I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. Cell. Immunol. 12:230 (1974).

31. Goldschneider, I., K. Shortman, D. McPhee, D.S. Linthicum, G. Mitchell, F. Battye, and F.J. Bollum, Identification of subsets of proliferating low Thy-1 cells in thymus cortex and medulla. Cell. Immunol. 69:59 (1982).

32. Scollay, R, Intrathymic events in the differentiation of T lymphocytes: a continuing enigma. Immunol. Today. 4:282 (1983).

33. Mathieson, B.J., A reviewing of thymocyte differentiation. In: Leucocyte Typing. Human leucocyte differentiation antigens detected by monoclonal antibodies, specification-classification-nomenclature. A. Bernard, L. Boumsell, J. Daussel, C. Millstein and S.F. Schlossman eds. Springer Verlag, Berlin p 645 (1984).

34. Ralph, S.J., and M.V. Berridge, Expression of antigens of the "T-200" family of glycoproteins on hemopoietic stem cells: evidence that thymocyte cell lineage antigens are represented on "T-200". J. Immunol. 132:2510 (1984).

35. Scollay, R., and I.L. Weissman, T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to the antigens Lyt-1, Lyt-2 and Th.B. J. Immunol. 124:2841 (1980).

36. Metcalf, D., The thymus: its role in immune responses, leukaemia development and carcinogenesis. In: Recent results in cancer research, vol. 5. Springer Verlag, Berlin (1966).

37. Bryant, B.J., Renewal and fate in the mammalian thymus: mechanisms and influence of thymocytokinetics. Eur. J. Immunol. 2:38 (1972).

38. Rouse, R.V., W. van Ewijk, P.P. Jones, and I.L. Weissman, Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122:2508 (1979).

39. Huiskamp, R., E. van Vliet, and W. van Ewijk. Repopulation of the mouse thymus after sublethal fission neutron irradiation. II Sequential changes in the thymic microenvironment. J. Immunol., 134: 2170 (1985).

40. Jenkinson, E.J., J.J.T. Owen, and R. Aspinall, Lymphocyte differentiation and major histocompatibility complex antigens expression in the embryonic thymus. Nature. 284:177 (1980).

41. Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer, and J.J. Mond, Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia antibodies interferes with differentiation of I restricted T cells but not with K/D restricted T cells. J. Exp. Med. 157:1932 (1983).

CHAPTER VI

REPOPULATION OF THE MOUSE THYMUS AFTER SUBLETHAL FISSION NEUTRON IRRADIATION

II SEQUENTIAL CHANGES IN THE THYMIC MICROENVIRONMENT

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SUMMARY

The stromal cells of the thymus of sham-irradiated and sublethal fission neutron irradiated CBA/H mice were analyzed with immunohistology, using monoclonal antibodies directed to I-A and H-2K antigens as well as specific determinants for cortical and medullary stromal elements.

In the control thymi, I-A expression in the thymus shows a reticular staining pattern in the cortex and a confluent staining pattern in the medulla. In contrast, H-2K expression is mainly confluently located in the medulla.

Whole-body irradiation with 2.5 Gy fission neutrons reduces within 24 hours the cortex to a rim of vacuolized "nurse cell like" epithelial cells, largely depleted of lymphoid cells. The localization of I-A antigens changes in the cortex and I-A determinants are no longer associated with or localized on epithelial reticular cells. Medullary stromal cells, however, are more or less unaffected. A high rate of phagocytosis is observed during the first three days after irradiation.

About 5 days after irradiation, the thymus becomes highly vascu-

larized and lymphoid cells repopulate the cortex. The repopulation of the thymic cortex coincides with the appearance of a bright H-2K expression in the cortex which is associated with both stromal cells as well as lymphoid blasts. During the regeneration of the thymus, the thymic stromal architecture is restored prior to the expression of cell surface associated reticular MHC staining patterns. The observed sequential changes in the thymic microenvironment are related to the lymphoid repopulation of the thymus.

INTRODUCTION

There is ample evidence that the thymic non-lymphoid cells are involved in the process of differentiation and maturation of T cells. Direct cell-cell interactions between thymocytes and thymic stromal cells through receptors for major histocompatibility complex (MHC) antigens, have been implicated to be involved in the aquisition of MHC restricted self recognition and selection of the developing T cells (1-6). Immunohistological studies of the thymic microenvironment are in line with this hypothesis and show that MHC determinants are mainly expressed on thymic stromal cells. These studies show that the epithelial reticular cells and the bone marrow derived "interdigitating reticular cells (IDC) are the major stromal elements bearing MHC determinants (7-9). In addition, the initial expression of MHC determinants on the epithelial cells has been shown to correlate with the onset of proliferation of the large lymphoblasts in the embryonic thymus (10). Furthermore, treatment of mice with monoclonal I-A antibodies interfered with the generation of I-A specific T helper cells (11,12) and further emphasizes the role of MHC determinants in the selection process of T cells in the thymus.

Lympho-stromal interactions have been demonstrated by the recent, in vitro, isolation of lympho-stromal complexes, such as "thymic nurse cells" (TNC) (13,14) and thymocyte rosettes (Ros) (15), and may represent the in vitro correlate of an in vivo lympho-stromal interaction.

After irradiation, the thymus primarily regenerates from a population of radioresistant intrathymic precursor cells (16,17). In

previous paper we have shown that a distinct phenotypical sequence order, in expressing cell surface differentiation antigens, is observed in the regenerating thymocyte population (18). The implication, that the reappearance of lymphostromal complexes in the regenerating thymus is closely related to the initiation of the intrathymic T cell differentiation (14,15), had led us to characterize the thymic microenvironment in this regeneration process after irradiation.

In the present investigation, we describe the effects of sublethal neutron irradiation on the architecture of the thymic stroma using immunohistological methods. We employ monoclonal antibodies directed to MHC antigens and recently developed monoclonal antibodies defining various thymic stromal cell types (19). We relate the observed changes in the thymic microenvironment after irradiation with the T cell differentiation process in the regenerating thymus.

MATERIALS AND METHODS

Animals

Female and male CBA/H mice were irradiated or sham-irradiated at the age of 5–7 weeks. The procedures of animal care have been described elsewhere (20).

Irradiation procedure

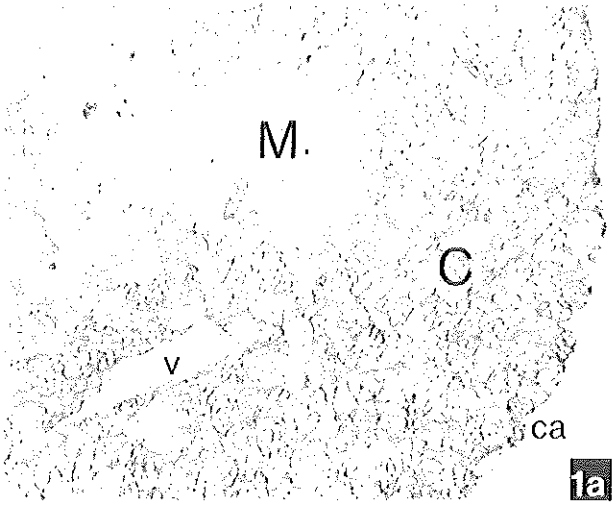
Whole-body neutron irradiations were performed as described elsewhere (21). Briefly, the animals were exposed bilaterially with 2.5 Gy fast fission neutrons at a center-line dose rate of 0.10 Gy/min. The neutron spectrum had a mean energy of 1.0 MeV. The absorbed doses do not include the 9% gamma-ray contribution.

Antisera

Syngeneic and xenogeneic monoclonal antibodies used in the present investigation are listed in Table I. For the detection of the binding of these antisera, the indirect immunoperoxidase method was

TABLE I Monoclonal antibodies used in the present study

Antibody	Reacts with / Target antigen	Reference
11-52-1.9	$I\text{-}A^k$	(22)
11-4.1	$H\text{-}2K^k$	(22)
M1/42.3.9.8	H-2K	(23)
ER-TR1	cortical and medullary stromal cells (I region of MHC)	(19)
ER-TR2	" " " "	(19)
ER-TR3	" " " "	(19)
ER-TR4	cortical epithelial cells	(19)
ER-TR5	medullary epithelial cells	(19)
ER-TR6	medullary "IDC and macrophages" and lymphoid cells	(19)
ER-TR7	reticular fibroblasts	(19)



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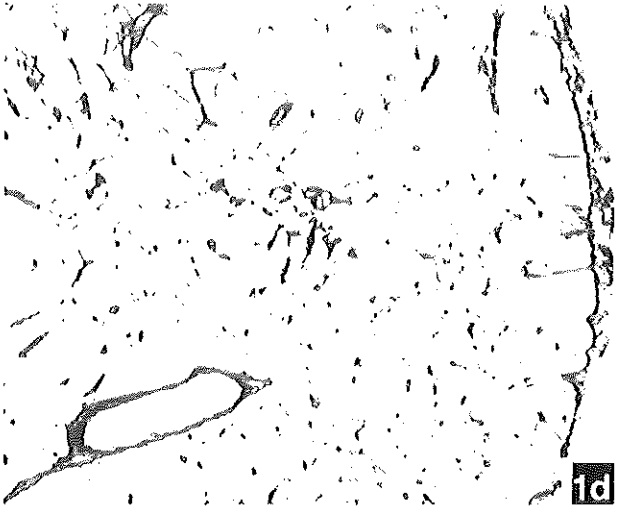

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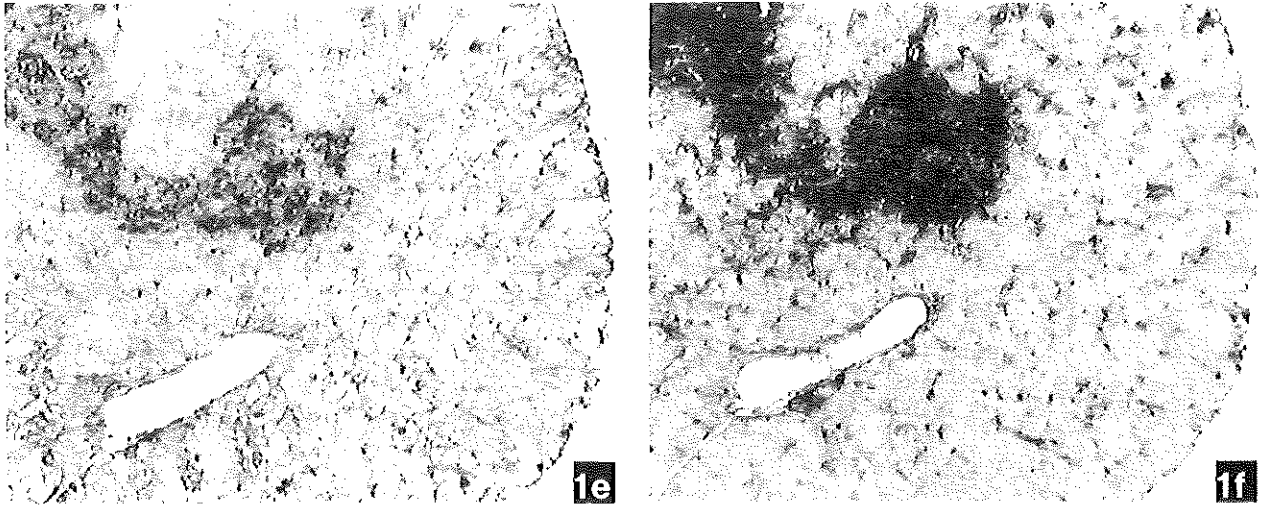


Fig. 1. Immunoperoxidase staining of serial frozen sections of a lobule of the sham-irradiated control thymus incubated with ER-TR4 (1a), ER- TR5 (1b), ER-TR6 (1c), ER-TR7 (1d), ER-TR1 (1e), and anti-H-2K monoclonal antibodies (M1/42.3.9.8)(1f). C = cortex, M = medulla, CA = thymic capsule, v = blood vessel (x 90).

used. Mouse monoclonal antibodies were detected by a polyvalent rabbit-anti-mouse peroxidase conjugated immunoglobulin (RAM-Ig-HRP, DAKO), which was extensively absorbed with mouse thymocytes before use. Rat monoclonal antibodies were detected by a polyvalent rabbit-anti-rat peroxidase conjugated immunoglobulin (RaRa-Ig-HRP, DAKO) supplemented with 1% normal mouse serum.

Tissue preparation for immunocytochemistry

Frozen sections of thymuses, isolated at various days after irradiation, were prepared and stained using the indirect immunoperoxidase method as described elsewhere (24). Briefly, frozen sections were overlayered with undiluted tissue culture supernatant containing monoclonal antibodies for 45 minutes, rinsed and overlayered with the appropriate conjugate for another 45 minutes. Antibody binding was visualized by incubation of the frozen sections with diaminobenzidene. After enhancing the contrast of the precipitate with a solution containing 1% $CuSO_4$ and 0.9% NaCl, the sections were postfixed in 1% glutaraldehyde.

RESULTS

In this section, we describe the immunohistology of the thymic stromal cells in (1) sham-irradiated control CBA/H mice and (2) irradiated CBA/H mice at various days after 2.5 Gy neutron irradiation, using monoclonal antibodies directed to MHC determinants, epithelial cells, interdigitating cells (IDC) and mesenchymal elements of the thymus.

1. Immunohistology of thymic stromal cells in sham-irradiated control mice

Frozen sections incubated with ER-TR4 antibodies, which define exclusively cortical epithelial-reticular cells, reveal a characteristic reticular staining pattern in the thymic cortex, whereas the medulla is virtually negative (Fig. 1a). In contrast, ER-TR5 and 6 antibodies react with the stromal cells in the medulla, but have different staining patterns (Figs. 1b, 1c). ER-TR5 antibodies define selectively medullary epithelial cells, whereas ER-TR6 reacts with other medullary

stromal cells, i.e. IDC and macrophages, and with medullary lymphoid cells. In addition, ER-TR6 reacts with blood vessel walls, the thymic capsule and occasionally with cortical non-lymphoid cells, i.e. macrophages (van Vliet unpubl. obs.).

Incubation of frozen sections with ER-TR7 antibodies reveal the staining of reticular cells in the medulla, the thymic capsule and the walls of blood vessels (Fig. 1d). In general, ER-TR7 defines the mesenchymal elements of the thymus.

Observations of serial sections incubated with ER-TR1, 2, and 3, which detect Ia antigens and anti-I-A antibodies (clone 11-52-1.9) reveal a fine reticular staining pattern in the cortex, whereas the medulla shows a more confluent staining pattern (Fig. 1e). Incubation with anti-H-2K antibodies reveal a confluent staining in the medulla and only a very weak staining in the cortex (clone 11-4.1). Clone M1/42.3.9.8, however, staines to some extent cortical stromal elements and lymphocytes (Fig. 1f). In general, anti-MHC antibodies detect epithelial reticular cells in the cortex, epithelial cells and IDC in the medulla and medullary lymphocytes.

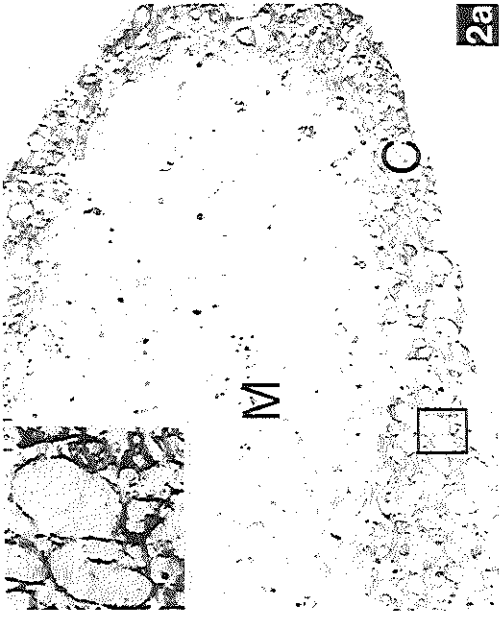
2. Immunohistology of thymic stromal cells in irradiated mice

Irradiation with 2.5 Gy fission neutrons has severe effects on the thymus. In a previous paper we have shown that, within 24 hours, the cortex is very hypocellular, whereas the medulla is only slightly affected (18).

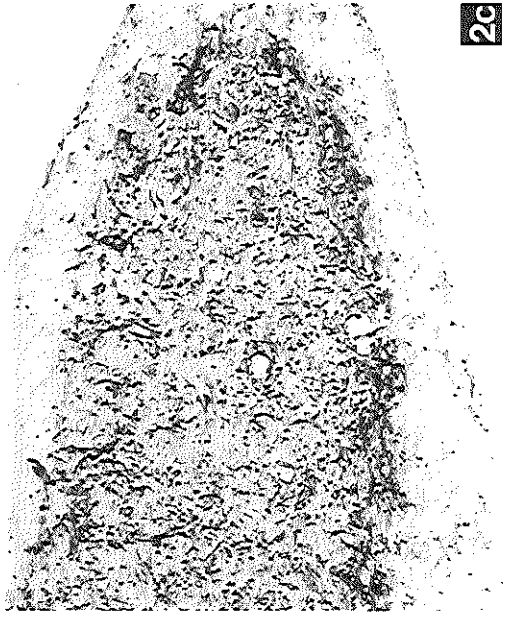
Incubation of frozen thymus sections with ER-TR4 shows the reduction of the cortex to a small rim of vacuolized epithelial cells (Fig. 2a). Within these rounded "nurse cell like" epithelial cells, the remnants of thymocytes are seen. The medullary stromal cell compartment is slightly affected by irradiation. The density of the medullary epithelial cells (ER-TR5) has increased to some extent in the cortico-medullary area (Fig. 2b) whereas the density of IDC and macrophages (ER-TR6) is markedly increased in both medulla as cortico-medullary area (Fig. 2c). The ER-TR7+ve mesenchymal elements of the thymus appear not to be

2b

2d



2a



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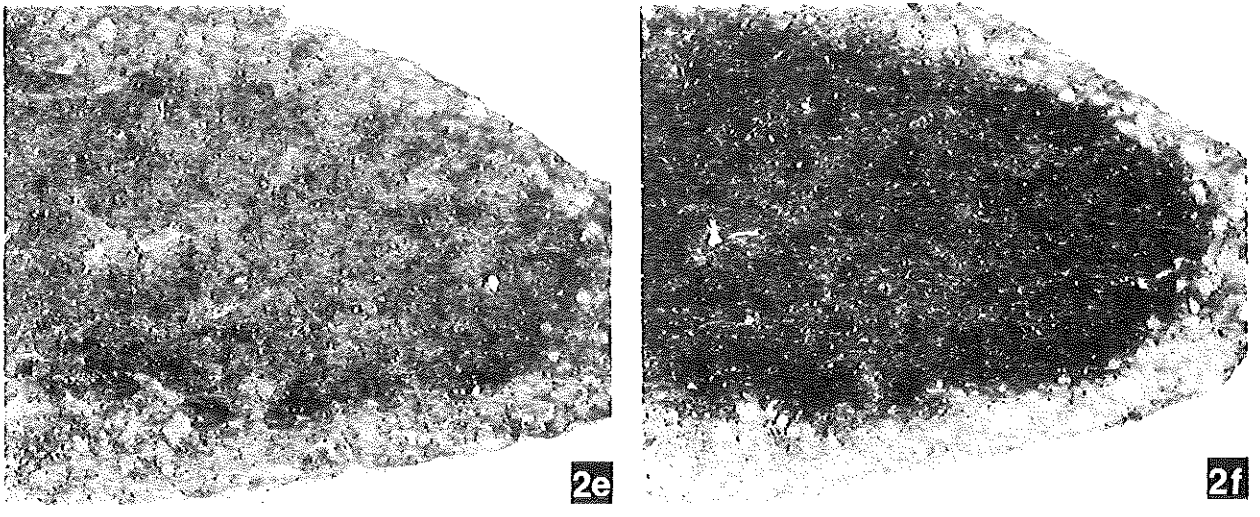
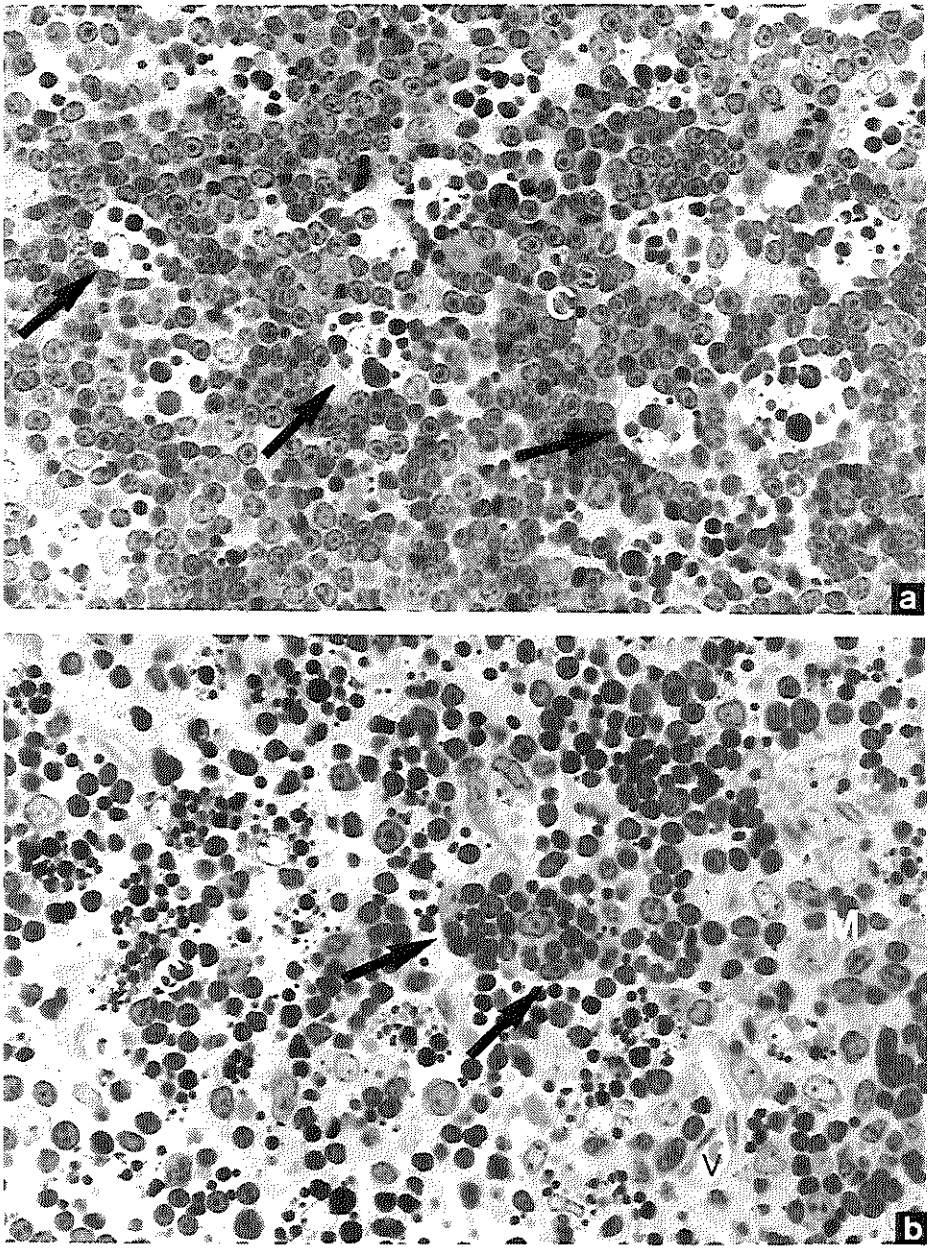


Fig. 2. Immunoperoxidase staining of serial frozen sections of the thymus 24 hours after 2.5 Gy fission neutron irradiation incubated with ER-TR4 (2a), ER-TR5 (2b), ER-TR6 (2c), ER-TR7 (2d), ER-TR1 (2e) and anti-H-2K monoclonal antibodies (M1/42.3.9.8)(2f). The inset is a higher magnification of the area indicated by the square. C = cortex, M = medulla (x 90; inset 360 x).


a
b
V

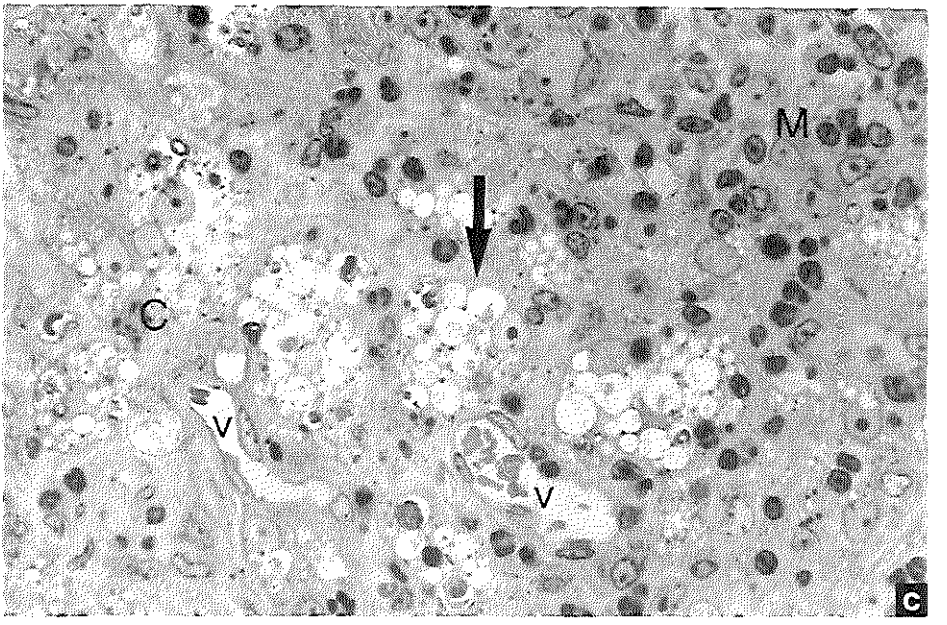


Fig. 2A Semithin sections (1 µm) of the thymus after sublethal whole-body irradiation, stained with toluidine blue:

a) 3 hours after irradiation showing scattered rounded vecuolized epithelial cells (arrows) containing necrotic thymocytes (x 595).

b) 7 hours after irradiation showing epithelial cells (arrows) enveloping a number of thymocytes (x 595).

c) 24 hours after irradiation revealing the epithelial matric (arrow) with remnants of thymocytes (x 595) C = cortex; M = medulla; V = blood vessel.

influenced at this time after irradiation (Fig. 2d). Incubation with ER-TR1, 2, 3 or 11-52-1.9 antibodies show that the I-A determinants in the cortex are not longer confined to the epithelial cells but shed in the cortex (Fig. 2e). In addition, the I-A expression has increased in the medulla, especially in the cortico-medullary area. However, H-2K expression appears to be unaffected in the medulla (Fig. 2f).

From day 2 until day 4 after irradiation, the size of the thymic cortex further diminishes and the vacuoles in the cortical epithelial cells disappear. The density of medullary stromal cells becomes comparable again with that of sham-irradiated control mice whereas the density of blood vessels throughout the thymus increases (data not shown).

From 5-6 days after irradiation, the size of the cortex starts to increase. The ER-TR4+ve cortical epithelial cells become less dense again, with in between lymphoid cells, and are sometimes more or less spherical (Fig. 3a). In contrast, no irradiation effects are detectable anymore in the medullary stromal cell compartment (Figs. 3b, c) but now the ER-TR6 antibodies also react to a considerable extent with the cortical lymphoid cells (Fig. 3c). Staining with ER-TR7 antibodies shows that the thymus, particularly the cortex becomes strongly vascularized (Fig. 3d). Frozen sections incubated with ER-TR1, 2, 3 or 11-52-1.9 antibodies show that the I-A staining is still not confined to the cell membranes of cortical epithelial reticular cells. However, some cortical epithelial cells already start to re-express I-A determinants in a membrane bound fashion (Fig. 3e). The expression of H-2K in the thymus at this timepoint after irradiation, is markedly changed. Staining with anti-H-2K antibodies reveals that the cortex is brightly $H\text{-}2K^{+}$ (Fig. 3f). Comparison of Fig. 3a and Fig. 3f shows that the bright cortical H-2K expression is associated with stromal cells as well as with the sparse distributed lymphoid cells and coincides with the repopulation of the cortex with lymphoid cells. However, the expression of MHC determinants in the medullary compartment of the thymus is comparable with that of sham-irradiated mice.

7-8 Days after irradiation, incubation with ER-TR4, 5 or 6 antibodies shows control-like staining patterns in the thymus, indicating

that the distribution of cortical epithelial cells (ER-TR4) and medullary stromal cells (ER-TR5 and 6) resembles that of control animals (Figs. 4a, b, c). In contrast, the number of blood vessels per surface area of the thymus, as measured by the ER-TR7 staining, remains at a high level (Fig. 4d). Incubation with ER-TR1, 2, 3 or 11-52-1.9 antibodies reveal a confluent staining pattern in the medulla and signs of a reticular staining pattern in the cortex (Fig. 4e). However, the regeneration of the thymus is not uniform since "shedding" and reticular staining patterns can be observed in adjacent areas of the cortex. The expression of H-2K determinants in the irradiated thymus starts to resemble that of control mice but the cortex is still stained to some extent (Fig. 4f).

From about 9 days upto at least 150 days after irradiation with fission neutrons, no differences can be observed in the distribution of stromal cell types and H-2 microenvironments in the thymus of irradiated and age-matched sham-irradiated control mice.

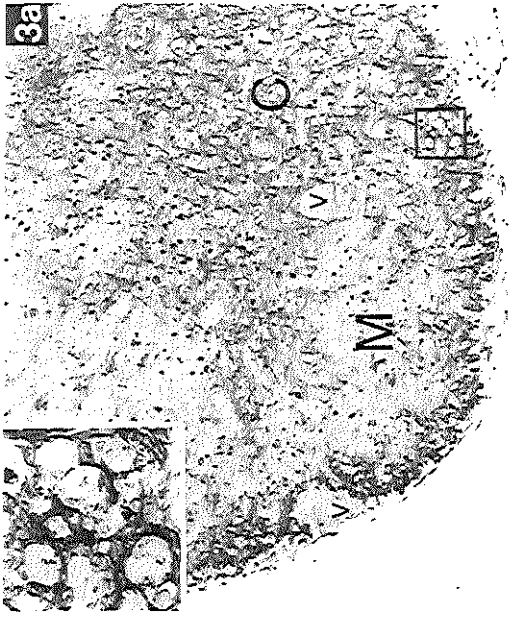
DISCUSSION

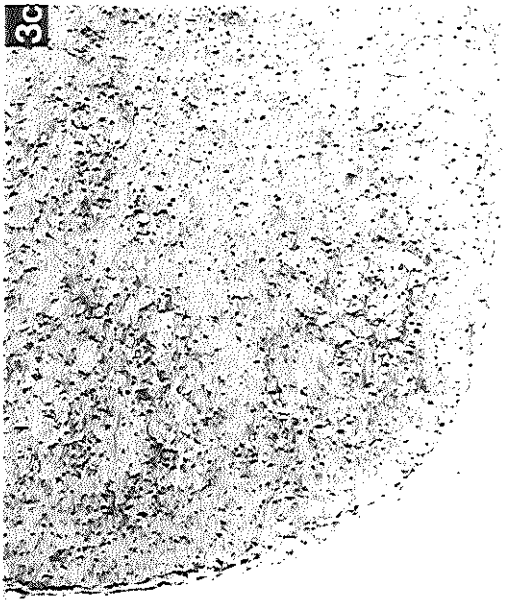
In the present investigation, we have used immunohistology to characterize the thymic stroma of irradiated and sham-irradiated control CBA/H mice with monoclonal antibodies directed to MHC antigens and determinants on stromal cell types. Our results show that: (A) a distinct stromal architecture and MHC microenvironments can be identified in the normal control thymus; (B) irradiation reduces the cortex to a rim of vacuolized ER-TR4+ve epithelium, largely depleted of lymphocytes; (C) the reticular I-A staining pattern disappears after irradiation and I-A determinants are "shed" in the cortex but ER-TR4, 5, and 6 do not dissappear; (D) increase cortical H-2K expression coincides with the lymphoid repopulation of the cortex; (E) restoration of the thymic stromal architecture after irradiation precedes reticular membrane associated MHC staining patterns.

In the control thymus, the three major types of non-lymphoid cells were detected. ER-TR4 detects cortical epithelial-reticular cells which are the major reticular elements in this area (9,25,26). This


3b


3d


3a
C
M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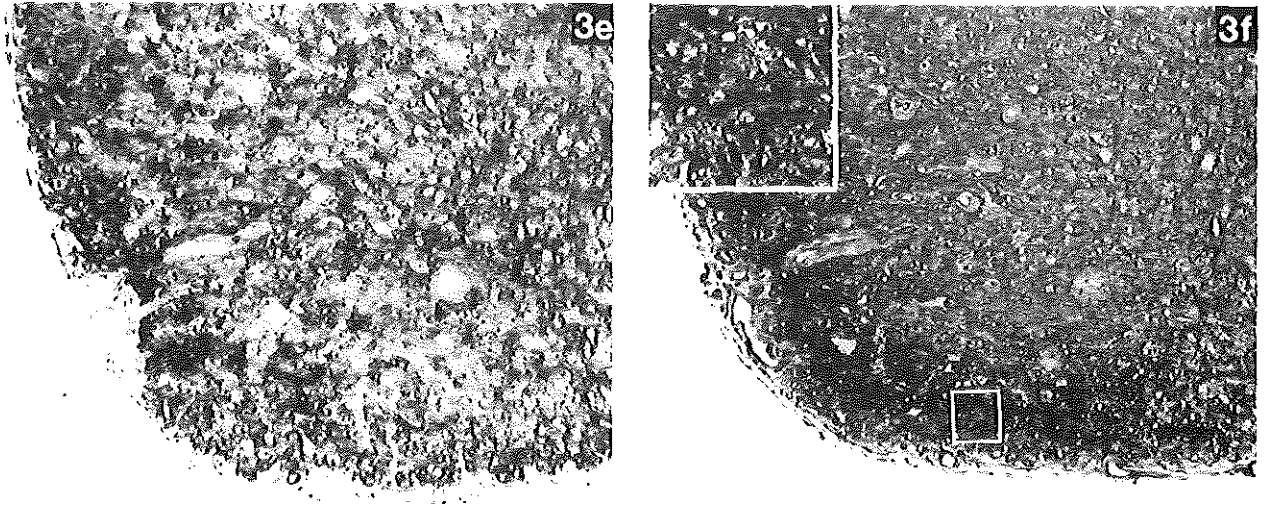
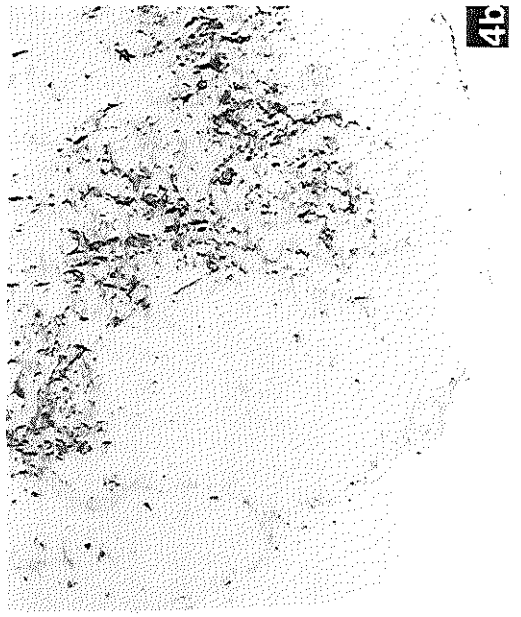
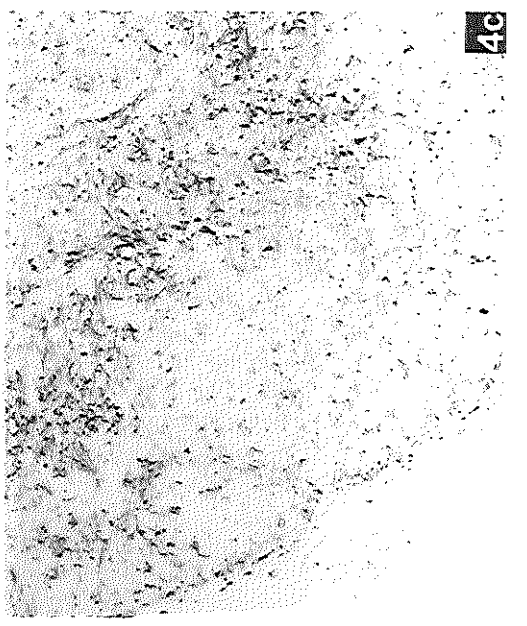


Fig. 3. Immunoperoxidase staining of serial frozen section of the thymus 6 days after 2.5 Gy neutron irradiation incubated with ER-TR4 (3a), ER-TR5 (3b), ER-TR6 (3c), ER-TR7 (3d), ER-TR1 (3e) and anti-H-2K monoclonal antibodies (M1/42.3.9.8)(3f). The inset is a higher magnification of the area indicated by the square. C = cortex, M = medulla, v = blood vessel (x 90; inset 360 x).


4b


4d


4a
M
C
ca


4c

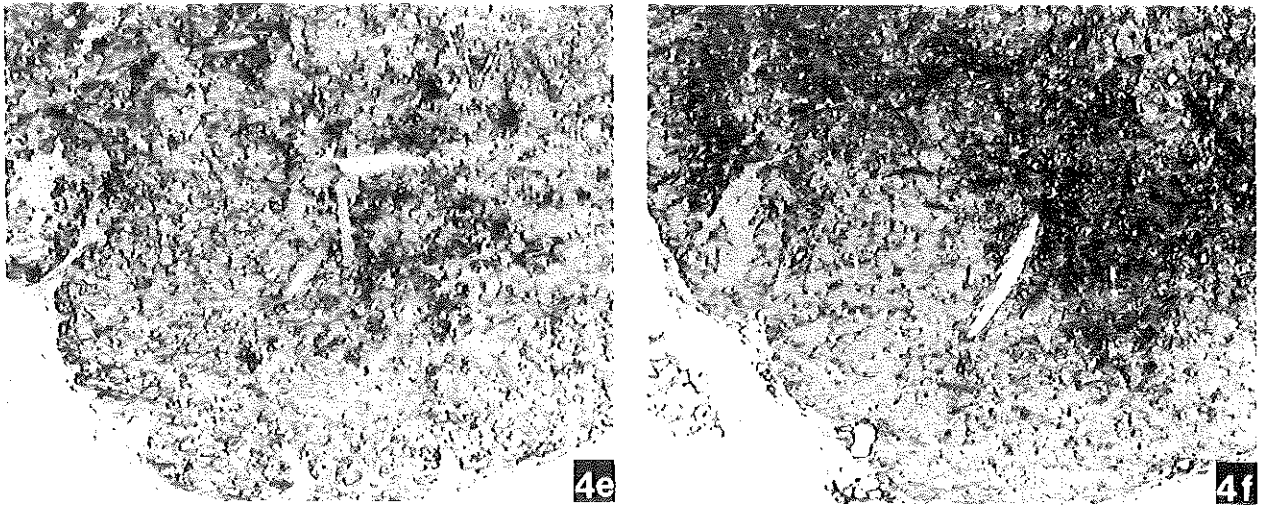


Fig. 4. Immunoperoxidase staining of serial frozen sections of the thymus 8 days after 2.5 Gy neutron irradiation incubated with ER-TR4 (4a), ER-TR5 (4b), ER-TR6 (4c), ER-TR7 (4d), ER-TR1 (4e) and anti-H-2K monoclonal antibodies (M1/42.3.9.8)(4f). C = cortex, M = medulla, CA = thymic capsule (x 90).

study and previous studies (7,8,19), show that this cell type expresses surface associated MHC antigens at a high level. In the medulla, epithelial cells are detected with ER-TR5 but two other types of stromal cells also are present in this area, namely IDC and macrophages detected by ER-TR6. These cells are bone marrow derived and also express MHC antigens at a high level (27). The present study, and previous investigations (7,8,19) show that the MHC staining in the medulla is of a confluent type. I-A determinants are not only confined to cellular membranes but also present in the cytoplasm and in the extracellular space between the cells. This suggests that MHC molecules are secreted by stromal cells in this compartment (28). However, double labeling with ER-TR5 or 6 and anti-I-A antibodies demonstrated partly overlapping populations demonstrating the heterogeneity of medullary stromal cells with respect to their I-A expression (van Vliet, unpubl. obs.) and needs to be further investigated.

Although stromal cells, in particular epithelial cells, are thought to be radioresistant (29,30), the present investigation shows that the thymic stromal architecture is severly affected by irradiation. Sublethal whole-body irradiation induces predominantly thymocyte necrosis in the cortex and cortico-medulary area. Within 24 hours, the cortex is reduced to a rim of vacuolized rounded epithelial cells in which the remnants of thymocytes can be observed. We have termed these cells "nurse cell like" since about 7 hours after irradiation, routine histology shows the presence of scattered epithelial cells that completely have enveloped a number of thymocytes (Huiskamp & Ploemacher, see fig. 2A). These cells match the properties of TNC in vitro, described by Wekerle & Ketelsen (13) and Kyewski & Kaplan (14). These TNC have been shown to be the in vitro representatives of ER- TR4+ve cortical epithelial-reticular cells in vivo (31). When the degeneration of the cortex proceeds, the epithelial matrix of these cells remains but the enveloped thymocytes become necrotic (Huiskamp & Ploemacher, see fig. 2A).

The I-A expression in the cortex after irradiation is not longer confined to the epithelial cells but seems to be shed in the cortex and may reflect radiation damage to the epithelial cells, resulting in a

direct loss of I-A determinants in or on the epithelial cells. Alternatively this "shedding" pattern might be related to the enhanced macrophage activity that has been observed in the cortex of the thymus after irradiation (9). However, the issue of I-A expression on macrophages is still controversial (27,32).

In the cortico-medullary area, irradiation also produces thymocyte necrosis to a considerable extent and this results in an increased density of the epithelial cells. In addition, the number of macrophages and IDC have increased during the first two days after irradiation. The latter cell type is brightly I-A positive and known to be involved in the phagocytotic process in the cortico-medullary area of the irradiated thymus (9,27). About 3 days after irradiation, the medullary stromal cells have an essentially "control-like" appearance, whereas the cortex further diminishes by losing the vacuoles in the epithelium.

Moreover, the loss of the thymic parenchyma is further illustrated by the increased vascularization of the thymus. This increase of the number of blood vessels, especially in the cortex, reflects the active tissue response during the regeneration process after irradiation.

When the thymus regenerates, large lymphoid cells can be observed in the cortex which express no differentiation antigens ("null" cells) (18) and may be identical to the proposed intrathymic radioresistant precursor cells (16) that repopulate the thymus in situ (17). The occurrance of these immature lymphoid cells coincides a bright H-2K expression that is associated with both stromal and the sparsely distributed lymphoid cells. The H-2K staining pattern however is not strictly defined to the cortical stromal cells, but resembles the I-A staining pattern. This could indicate that H-2K determinants are also shed in this thymus microenvironment. Furthermore, by comparing serial sections, we conclude that "null" cells are bright H-2K$^+$. Negative or low Thy-1$^+$, bright H-2K$^+$ thymocyte precursors have also been identified in the fetal thymus and in adult bone marrow (33-35), and favor the hypothesis that all thymocytes are descendants of low or negative Thy-1$^+$, bright H-2K$^+$ precursors and that separate precursor cell subsets exist

for cortical and medullary thymocytes (35,36).

During the regeneration of the thymus after irradiation, "null" cells sequentially acquire the differentiation antigens Thy-1, T-200, Lyt-1, Lyt-2 and MT-4 (18) and this coincides with the reduction of the H-2K expression in the cortex to control-like levels. In addition, the reappearance of the reticular I-A staining pattern in the cortex, is correlated with the aquisition of Lyt-1, Lyt-2 and MT-4 differentiation antigens (18) and this suggests a regulatory role of MHC antigens in T cell differentiation. In line with this assumption is the following evidence: 1. "Shedding" and reticular I-A staining patterns are observed in adjacent areas of the cortex 7 days after irradiation and in these areas different stages in thymocyte development can be observed (18). 2. A close correlation between the expression of MHC antigens on thymic epithelial cells and the onset of proliferation of lymphoblasts has been observed in the embryonic thymus (10). 3. Nude mice do not express I-A antigens on epithelial cells of the embryonic thymus (37). 4. In vivo administration of monoclonal anti-I-A antibodies to mice interfered with the generation of I-A specific T helper cells (11,12). Furthermore, the initiation of intra-thymic differentiation of functional T cells has been implied to be directly related to the reappearance of in vitro isolated TNC in the irradiated thymus, though in vivo the thymocytes involved, probably are not completely enveloped by the $I\text{-}A^+$ epithelial cells (14,31). Initial lympho-epithelial association after lymphoid repopulation may therefore be reflected by the reappearance of the I-A reticular staining pattern in the thymus after irradiation.

In conclusion, a close relationship exists between the stromal architecture of the thymic microenvironment after irradiation and the reappearance of specific differentiation markers on lymphoid cells that repopulate the thymus during the first phase of regeneration after sublethal irradiation.

REFERENCES

1. Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein, On the thymus in the differentiation of "H-2

self-recognition" by T cells: evidence for dual recognition? J. Exp. Med. 14:882 (1978).

2. Fink, P.J., and M.H. Bevan, H-2 antigens of the thymus determine lymphocyte specifity. J. Exp. Med. 148:766 (1978).

3. Wagner, H., C. Hardt, H. Stockinger, K. Pfizenmaier, R. Bartlett, and M. Röllinghoff, Impact of thymus on the generation of immunocompetence and diversity of antigen-specific MHC-restricted cytotoxic T lymphocyte precursors. Immunol. Rev. 58:95 (1981).

4. Kruisbeek, A.M., S.O. Sharrow, B.J. Mathieson, and A. Singer, The H-2 phenotype of the thymus dictates the self specifity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus engrafted nude mide. J. Immunol. 127:2168 (1981).

5. Sharrow, S.O., B.J. Mathieson and A. Singer, Cell surface appearance of unexpected MHC determinants on thymocytes from radiation bone marrow chimeras. J. Immunol. 126:1327 (1981).

6. Singer, A., K.S. Hatchcock, and R.J. Hodes, Self recognition in allogeneic thymic chimeras. Self recognition by T helper cells from thymus engrafted nude mice is restricted to the thymic H-2 haplotype. J. Exp. Med. 155:339 (1982).

7. Rouse, R.V., W. van Ewijk, P.P. Jones, and I.L. Weissman, Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122:2508 (1979).

8. van Ewijk, W., R.V. Rouse, and I.L. Weissman, Distribution of H-2 microenvironments in the mouse thymus. Immunoelectronmicroscopic identification of I-A and H-2K bearing cells. J. Histochem. Cytochem. 28:1089 (1980).

9. Duijvestein, A.M., Y.G. Köhler, and E.C.M. Hoefsmit, Interdigitating cells and macrophages in the acute involuting rat thy-

mus. An electronmicroscopic study on phagocytotic activity and population development. Cell Tiss. Res. 224:291 (1982).

10. Jenkinson, E.J., J.J.T. Owen, and R. Aspinall, Lymphocyte differentiation and major histocompatibility complex antigens expression in the embryonic thymus. Nature. 284:177 (1980).

11. Sprent, J., Effect of blocking helper T induction in vivo with anti-Ia antibodies. Possible role of IA-IE hybrid molecules as restriction elements. J. Exp. Med. 152:996 (1980).

12. Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer, and J.J. Mond, Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia antibodies interferes with differentiation of I restricted T cells but not with K/D restricted T cells. J. Exp. Med. 157:1932 (1983).

13. Wekerle, H., and U.P. Ketelsen, Thymic nurse cells. I-A bearing epithelium involved in T lymphocyte differentiation? Nature 283:402 (1980).

14. Kyewski, B.A., and H.S. Kaplan, Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. J. Immunol. 128:2287 (1982).

15. Kyewski, B.A., R.V. Rouse, and H.S. Kaplan, Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow derived stromal cells in the mouse thymus. Proc. Natl. Acad. Sci. USA 70:5646 (1982).

16. Kadish, J.L., and R.S. Basch, Thymic regeneration after lethal irradiation: Evidence for an intrathymic radioresistant T cell precursor. J. Immunol. 114:452 (1975).

17. Sharrow, S.O., A. Singer, U. Hämmerling, and B.J. Mathieson, Phenotypic characterization of early events of thymus repopulation in radiation bone marrow chimeras. Transplantation. 35:355

(1983).

18. Huiskamp, R., and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. I. Sequential appearance of thymocyte subpopulations. J. Immunol., 134:2161 (1985).

19. van Vliet, E., M. Melis, and W. van Ewijk, Monoclonal antibodies to stromal cell types of the mouse thymus. Eur. J. Immunol. 14:524 (1984).

20. Davids, J.A.G., Bone-marrow syndrome in CBA mice exposed to fast neutrons of 1 MeV mean energy. Effect of syngeneic bone-marrow transplantation. Int. J. Radiat. Biol. 17:173 (1970).

21. Huiskamp, R., J.A.G. Davids, and O. Vos, Short- and long- term effects of whole-body irradiation with fission neutrons or X- rays on the thymus in CBA mice. Radiat. Res. 95:370 (1983).

22. Oi, V.T., P.P. Jones, J.W. Goding, L.A. Herzenberg, and L.A. Herzenberg, Properties of monoclonal antibodies to mouse Ig allotypes. Curr. Top. Microbiol. Immunbiol. 81:115 (1975).

23. Springer, T.A., Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. In Monoclonal Antibodies. R.H. Kennet, T.J. McKearn, and K.B. Bechtol, Eds. Plenum Press, New York, p. 185 (1980).

24. van Ewijk, W., P.L. van Soest, and G.J. van den Engh, Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. J. Immunol. 127:2594 (1981).

25. Hoshino, T., Electronmicroscopic studies of epithelial reticular cells in the mouse thymus. Z. Zellforsch. 59:513 (1963).

26. Hwang, W.S., T.Y. Ho, and G.T. Simon, Ultrastructure of the rat thymus. Lab. Invest. 31:473 (1974).

27. Duijvestein, A.M., R. Schutte, Y.G. Köhler, G. Korn, and E.C.M. Hoefsmit, Characterization of the population of phagocytotic cells in thymic cell suspensions. A morphological and cytochemical study. Cell Tiss. Res. 231:313 (1983).

28. van Ewijk, W., Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentation. Amer. J. Anat. 170:311 (1984).

29. Sharp, J.G., and E.B. Watkins, Cellular and immunological consequences of thymic irradiation. In Immunopharmacologic Effects of Radiation Therapy. J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds. Raven Press, New York. p. 137 (1981).

30. Fink, P.J., I.L. Weissman, H.S. Kaplan, and B.A. Kyewski, The immunocompetence of murine stromal cell-associated thymocytes. J. Immunol. 132:2266 (1984).

31. van Vliet, E., M. Melis, and W. van Ewijk, Immunohistology of thymic nurse cells. Cell. Immunol., 87:101 (1984).

32. Beller, D.I., and E.R. Unanue, I-A antigens and antigen-presenting function of thymic macrophages. J. Immunol. 124:1433 (1980).

33. Ritter, M.A., Embryonic mouse thymus development: stem cell entry and differentiation. Immunology 34:69 (1980).

34. Kamarck, M.E., and P.D. Gottlieb, Expression of thymocyte surface alloantigens in the fetal mouse thymus in vivo and in organ culture. J. Immunol. 119:407 (1977).

35. Goldschneider, I., K. Shortman, D. McPhee, D.S. Linthicum, G. Mitchell, F. Battye, and F.J. Bollum, Identification of subsets of

proliferating low Thy-1 cells in thymus cortex and medulla. Cell. Immunol. 69:59 (1982).

36. Boersma, W.J.A., Characterization of mouse prothymocytes. Thesis, Erasmus University. Publication of the Institute for Ex- perimental Gerontology TNO, Rijswijk, The Netherlands (1982).

37. Jenkinson, E.J., W. van Ewijk, and J.J.T. Owen, Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice. J. Exp. Med. 153:280 (1981).

CHAPTER VII

UNILATERAL T CELL MATURATION ARREST IN THE THYMUS OF CBA/H MICE AS A LONG-TERM EFFECT AFTER NEUTRON IRRADIATION

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SUMMARY

Thymuses of CBA/H mice were investigated upto 570 days after whole-body irradiation with 2.5 Gy fast fission neutrons or 6.0 Gy X-rays. A number of these thymuses, observed 220-270 days after neutron irradiation, have two equal-sized lobes of which one has a abnormal T cell distribution. The present paper reports on the distribution of lymphoid and stromal cell types in these thymuses. To this purpose, we employed immunohistology using the indirect immunoperoxidase method. We incubated frozen sections of these aberrant thymuses with 1) monoclonal antibodies directed to cell surface differentiation antigens on lymphoid cells, such as Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14; 2) monoclonal antibodies directed to major histocompatibility complex (MHC) antigens, such as I-A and H-2K and 3) monoclonal antibodies directed to determinants an various thymic stromal cell types.

The results of this study show a T cell differentiation arrest in only one of the two thymic lobes. T cells in the aberrant lobe express Thy-1, T-200 and MEL-14 antigens but are MT-4$^-$ and Lyt-1$^-$. In some lobes, a weak Lyt-2 expression was observed. The observed T cell maturation arrest is mainly restricted to the cortex since in the medulla, in addition to cells with an aberrant cortical phenotype, normal T cell phenotypes are observed. This indicates that cortex and medulla have independent generation kinetics in T cell maturation.

The stromal cell composition in these abnormal lobes is not dif-

ferent from that in the normal lobe but the size of the medulla tends to be smaller. Furthermore, the I-A expression on the cortical epithelial cells does not reveal the characteristic reticular staining pattern that is observed in the normal lobe, since the I-A determinants are not strictly confined to the epithelial cells. In addition, cortical lymphoid and stromal cells in these lobes are slightly H-$2K^+$. These alterations in MHC expression in the cortex are discussed in relation to the observed T cell maturation arrest.

INTRODUCTION

The thymus which is essential for the full development and maintenance of cell-mediated immunity (1,2), is highly susceptible to ionizing radiation. Sublethal whole-body irradiation results in a severe depopulation of the thymus. The subsequent thymic regeneration follows a biphasic pattern (3-5). The first phase of regeneration is a result of the proliferation of radioresistant intrathymic precursor cells (6,7), while the second phase depends on repopulation from progenitor cells generated in the recovering bone marrow (3,8).

Hereafter, a marked decrease can be observed in thymus weight and cellularity relative to that of age-matched control animals, which lasts upto at least 250 days after irradiation (5). This late effect after irradiation does not influence the histological appearance of the thymus and was regarded to be a result of pluripotent stem cell loss in combination with residual radiation damage in the surviving stem cells in the bone marrow (5). Furthermore, immunohistological analysis of the T cell distribution in the thymus of irradiated and age-matched sham-irradiated mice did not reveal any differences upto at least 150 days after irradiation (9).

However, while analysing the T cell distribution of the thymus of animals upto 500 days after irradiation, we noted a number of aberrant thymuses. These thymuses, which were first noted in mice 220 days after 2.5 Gy fast fission neutron irradiation, have two equal sized lobes, one of which did not express Lyt and MT-4 antigens.

In this study, we characterized in detail the T cell distribution patterns and thymic microenvironment composition of these thymuses with immunohistology using monoclonal antibodies directed to the cell sur-

face differentiation antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 as well as monoclonal antibodies directed to major histocompatibility comples (MHC) antigens and determinants on various thymic stromal cell types.

MATERIALS AND METHODS

Animals

Male and female CBA/H mice, age 5-7 weeks, were used for the present study. The procedures of animal care have been described elsewhere (10).

Irradiation procedure

Mice were irradiated sublethally with either 2.5 Gy fast fission neutrons from a ^{235}U converter in the Low Flux Reactor at Petten or with 6.0 Gy 300 kVP X-rays from a Philips Müller X-ray tube at centerline dose rates of 0.1 and 0.3 Gy/min respectively as described elsewhere (5).

Antisera

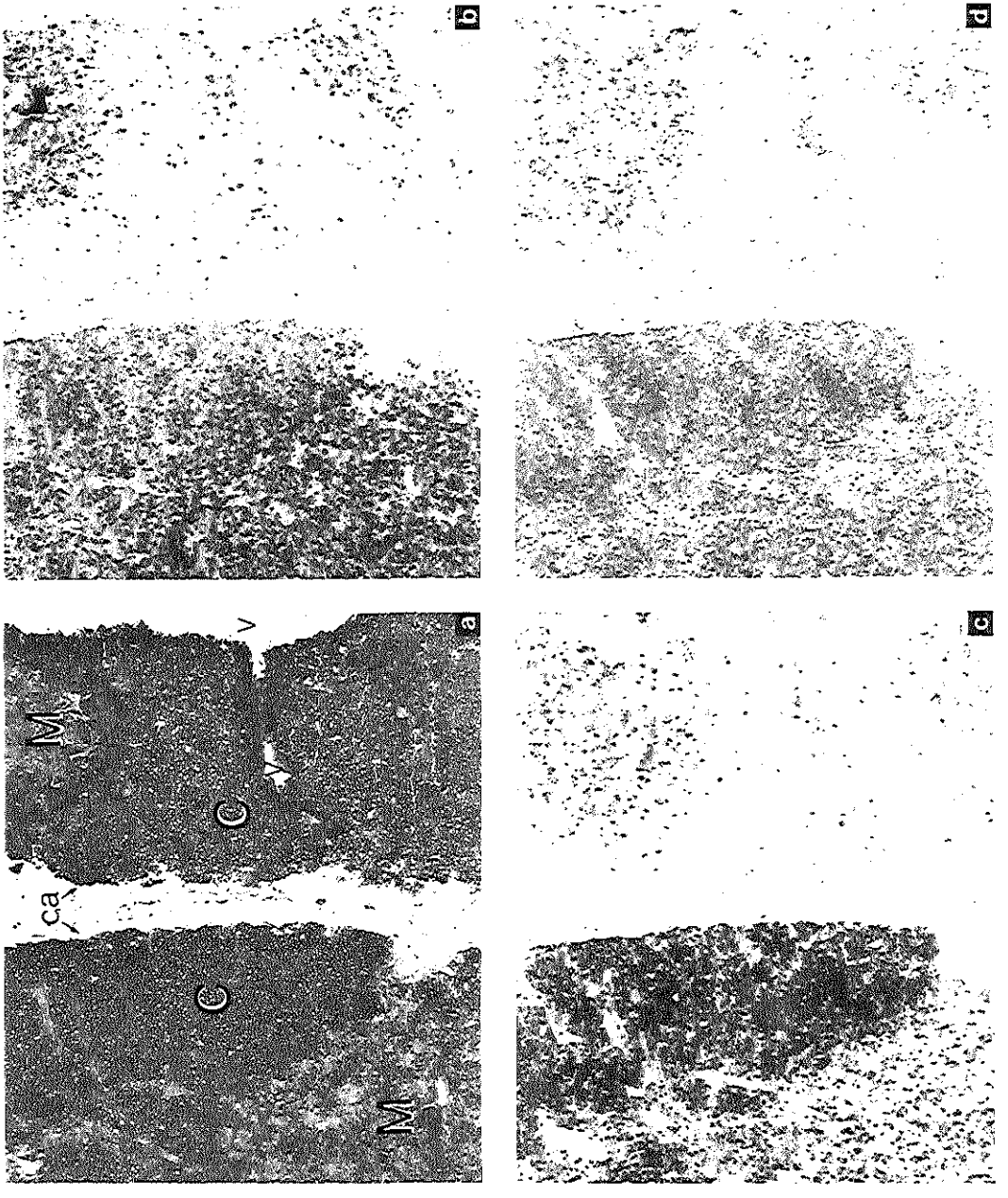
Syngeneic and xenogeneic monoclonal antibodies used in the present investigation are listed in Table I. The indirect immunoperoxidase method was used to detect the binding of these antisera. Mouse monoclonal antibodies were detected by a polyvalent rabbit anti mouse peroxidase conjugated immunoglobulin (RAM-Ig-HRP, Dakopatts, Denmark), extensively absorbed with mouse thymocytes prior use. Rat monoclonal antibodies were detected by a polyvalent rabbit-anti-rat peroxidase conjugated immunoglobulin (RaRa-Ig-HRP, Dakopatts, Denmark). Goat anti-rauscher GP70 antibodies were detected by a polyvalent rabbit-anti-goat peroxidase conjugated immunoglobulin (RaG-Ig-HRP, Nordic Immunological Laboratories). RaRa-Ig-HRP as well as RaG-Ig-HRP conjugates were supplemented with 1% normal mouse serum to prevent aspecific binding.

Tissue preparation for immunohistology

TABLE I Reagents used in the present investigation

Monoclonal antibody	Reacts with/ Target antigen	Reference
59-AD-22	Thy-1	(11)
30-G-12	T-200	(11)
H129.19	MT-4	(12)
53-7.3.13	Lyt-1	(11)
53-6.72	Lyt-2	(11)
MEL-14	Homing receptor for peripheral lymphnodes	(13)
ER-TR1	Cortical and medullary stromal cells (I region of MHC)	(14)
ER-TR2	" " "	(14)
ER-TR3	" " "	(14)
11-52-1.9	$I\text{-}A^k$	(15)
ER-TR4	Cortical epithelial cells	(14)
ER-TR5	Medullary epithelial cells	(14)
ER-TR6	Medullary "IDC and macrophages" and lymphoid cells	(14)
ER-TR7	Reticular fibroblasts	(14)
11-4.1	$H\text{-}2K^k$	(15)
M1/42.3.9.8	H-2K all haplotypes	(16)

Extensively purified goat anti-Rauscher MuLV GP70 was kindly provided by Dr. W. Varrato, National Cancer Institute, NIH, Bethesda, USA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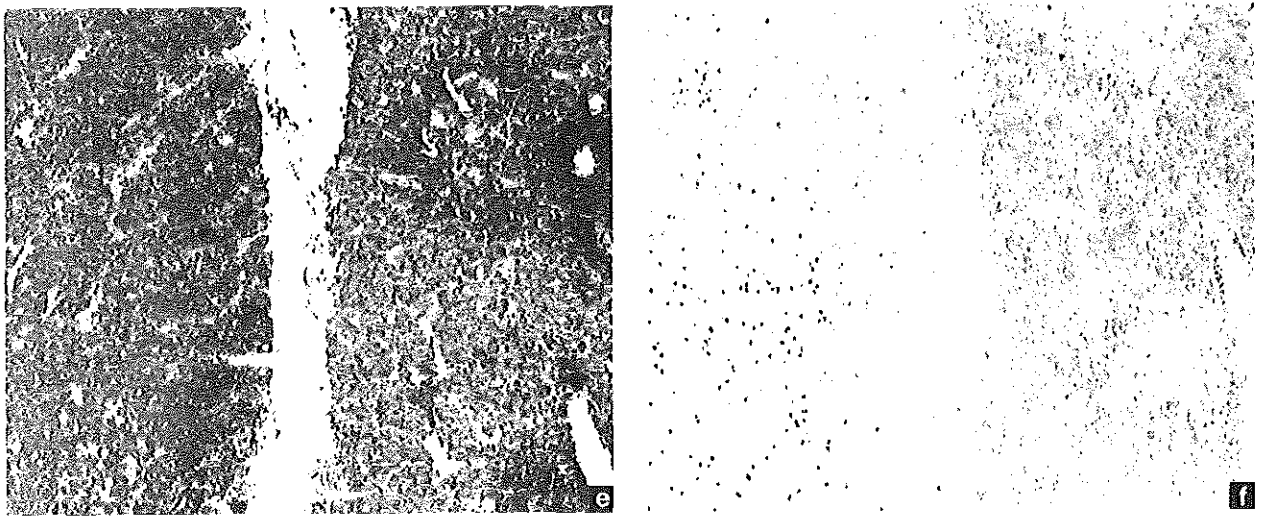


Fig. 1. Immunoperoxidase staining of serial frozen sections of an abnormal thymus, 270 days after 2.5 Gy fission neutron irradiation, incubated with monoclonal anti-Thy-1 (a), anti-Lyt-1 (b), anti-Lyt-2 (c), anti-MT-4 (d), anti-T-200 (e), and anti-MEL-14 (f). C = cortex, M = medulla, ca = thymic capsule, v = blood vessel. (x 90).

Frozen sections of thymuses, isolated at various timepoints, ranging from 200 to 500 days after irradiation, were prepared and stained using an indirect immunoperoxidase method as described elsewhere (17). Briefly, 4.5 µm frozen sections were overlayered with monoclonal antibodies for 45 minutes, rinsed and overlayered with the appropriate conjugate for another 45 minutes. Antibody binding was visualized by incubation of the frozen sections with diaminobenzidene. After enhancing the contrast of the precipetate with a solution containing 1% $CuSo_4$ and 0.9% NaCl, the sections were postfixed in 1% glutataraldehyde, dehydrated and coverslipped.

RESULTS

General remarks

For this investigation, we examined immunohistologically the T cell distribution in the thymus of a total of 119 CBA/H mice, devided in three experimental groups as listed in Table II. In 5 out of 52 mice of the neutron-irradiated groups, a thymus was observed with an aberrant T cell distribution in one of the equal sized lobes. This phenomenon was not observed in the 49 control and 18 X-irradiated animals. In section 3.2. we report on the tissue T cell distribution in these aberrant thymuses and section 3.3. concentrates on the thymic microenvironment in the aberrant lobes. In none of the described cases, standard post-mortem examination revealed pathological alterations.

Immunohistology of T cell subpopulations in thymuses with a normal (left) and aberrant (right) lobe

Incubation of frozen sections of the thymus with monoclonal anti-Thy-1 antibodies reveals hardly any differences between the two lobes (Fig. 1a). Cortical thymocytes are bright Thy-1^+ whereas medullary thymocytes are dull Thy-1^+. All cortical thymocytes in the aberrant right lobe, however, are larger (7-9 µm) in size and are comparable in size with Thy-1^+, T-200^+ blast cells in the supcapsular area of the normal (left) lobe.

TABLE II Experimental groups examined in this investigation

Radiation source	Dose (Gy)	Total number of mice	Time after (sham)-irradiation (days)	Number of abnormal thymuses
neutrons	2.5	32	220	4
"	2.5	20	270	1
X-rays	6.0	8	240	-
"	6.0	10	570	-
control	-	26	240	-
"	-	23	570	-
neutrons	2.5	37	17	-

Serial anti-Lyt-1 stained sections show a marked difference between the right and left lobe of the thymus (Fig. 1b). In the left normal lobe almost all thymocytes are Lyt-1^+, but the density of this marker is very heterogeneous. Cortical thymocytes are dull Lyt-1^+ with foci of bright Lyt-1^+ cells. In the medulla, the majority of cells is bright Lyt-1^+ but dull Lyt-1^+ cells are also observed. In contrast, virtually all cortical thymocytes in the right thymus lobe are Lyt-1^-. However, some individual bright Lyt-1^+ cells do occur scattered throughout the cortex. In contrast, in the medulla numerous dull and bright Lyt-1^+ cells can be observed.

Frozen sections incubated with monoclonal anti-Lyt-2 antibodies reveal also a marked difference between the two thymus lobes (Fig. 1c). In the left lobe, the majority of cortical cells are bright Lyt-2^+ whereas medullary cells are by enlarge Lyt-2^- with scattered dull to bright Lyt-2^+ cells. In the right lobe, however, virtually all cortical cells are Lyt-2^- but in the medulla scattered dull to bright Lyt-2^+ cells can still be observed.

Anti-MT-4 stained serial sections show a similar difference between the thymus lobes (Fig. 1d). In the left lobe, virtually all cortical thymocytes are MT-4^+ and a majority of the medullary cells ex-

press varying levels of MT-4 antigens. In the right lobe, however, the majority of cortical cells is MT-4$^-$ but some individual dull to bright MT-4$^+$ cells are scattered throughout the cortex. In the medulla, various dull to bright MT-4$^+$ cells are observed. Comparison of carefully chosen adjacent frozen sections incubated with either anti-Lyt-2 or anti-MT-4 antibodies shows that medullary cells in the normal lobe are either Lyt-2$^+$, MT-4$^-$ or Lyt-2$^-$, MT-4$^+$ whereas in the medulla of the aberrant lobe Lyt-2$^+$, MT-4$^-$ or Lyt-2$^-$, MT-4$^+$ and Lyt-2$^-$, MT-4$^-$ cells are observed.

When serial frozen sections are incubated with monoclonal anti-T-200 antibodies, only slight differences are observed between the two lobes (Fig. 1e). In both lobes all thymocytes express T-200 antigens but in the right lobe, the majority of cortical cells is dull T-200$^+$. In the medulla, the majority of cells express high levels of T-200 antigens.

Surprisingly, anti-MEL-14 stained sections reveal a dull staining level on cortical thymocytes in the left lobe but in the right aberrant lobe, MEL-14 expression on cortical thymocytes has markedly increased (Fig. 1f). To test the presence of viral antigens in these lobes, we incubated frozen sections with anti-GP70 antibodies. However, the thymocytes in the aberrant lobe are in all observed cases GP70$^-$ (data not shown).

Summarized, the phenotype of cortical thymocytes in the aberrant lobe is Thy-1$^+$, T-200$^+$, Lyt-1$^-$2$^-$, MT-4$^-$. In addition, the majority of these cells have an increased MEL-14 expression. In the medulla of the aberrant lobe, 3 subpopulations of cells can be observed: 1 Lyt-2$^+$, MT-4$^-$ cells; 2 Lyt-2$^-$, MT-4$^+$ cells and 3 Lyt-2$^-$, MT-4$^-$ cells. For the sake of brevity, we described only one of the five thymuses that express this phenomenon, in detail. The phenotype of cortical cells in the aberrant lobe of these other thymuses are listed in Table III and can be divided into two groups on the basis of their Lyt expression. In group 1, the cortical thymocytes do not express Lyt-1 or Lyt-2 antigens whereas in the second group, cortical thymocytes do express, to some extend, Lyt-2 but not Lyt-1 antigens. Similarities between the observed phenotypes are an almost normal expression of Thy-1 and T-200 antigens and an increased MEL-14 expression on the cortical cells. In all aberrant lobes, the cortical cells are larger in size than in the normal lobe. The medulla of the not described aberrant lobes do contain Lyt-2$^+$ cells but their frequency is lower than that in the normal lobe.

TABLE III Summary of the phenotypes observed in the cortex of the aberrant lobe of thymus observed 220-270 days after 2.5 Gy fission neutron irradiation

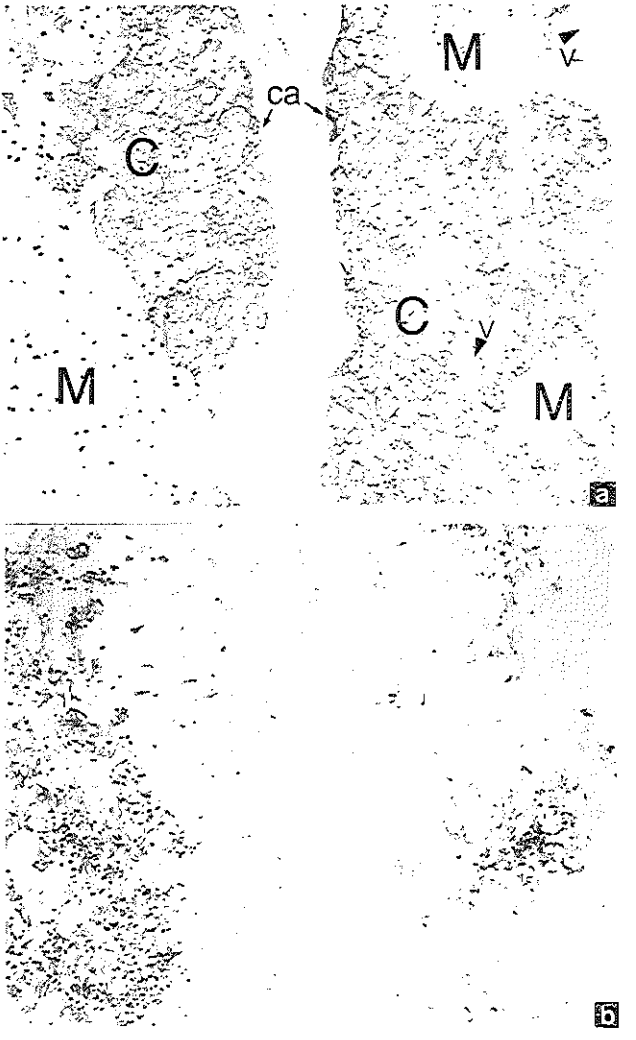
Mouse number	Specificity detected						
	Thy-1	T-200	Lyt-1	Lyt-2	MT-4	MEL-14	GP70
1	+[1]	+	-	-	ND	++	-
2	+	+	-	-	ND	++	-
3	+	+	-	-/+	ND	++	-
4	+	+	-	-/+	ND	++	-
5	+	+	-	-	-	++	-

[1] ++ = increased expression of antigen;
+ = normal expression of antigen; -/+ = weak expression of antigen;
- = antigen not detectable; ND = not determined

Immunohistology of thymic stromal cells in the aberrant thymus

Frozen sections of thymuses with abnormal T cell phenotypes in one of the lobes, were incubated with monoclonal antisera directed against various thymic stromal cell types and MHC antigens to analyse the thymic microenvironment of these thymuses.

Incubation of frozen sections with ER-TR4, an antiserum which detects epithelial-reticular cells of the thymic cortex, reveals in both lobes a normal reticular staining pattern (Fig. 2a). Frozen sections incubated with ER-TR5 (Fig. 2b), a monoclonal antibody which detects medullary epithelial cells, or frozen sections incubated with ER-TR6 (Fig. 2c), an antiserum which reacts with medullary interdigitating cells and macrophages, do not reveal any specific differences between the normal and aberrant thymus lobes.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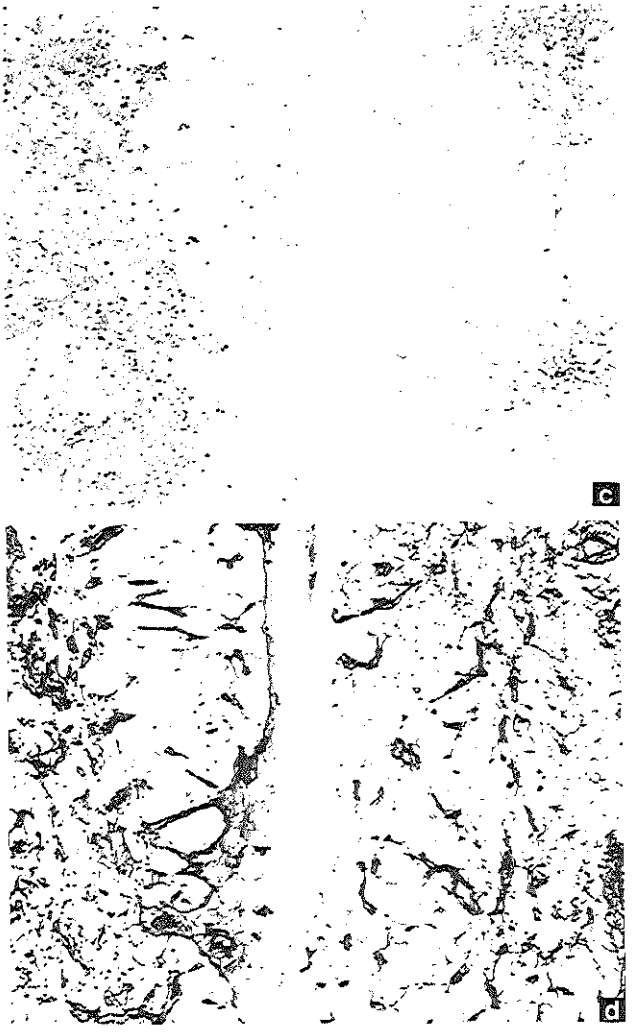
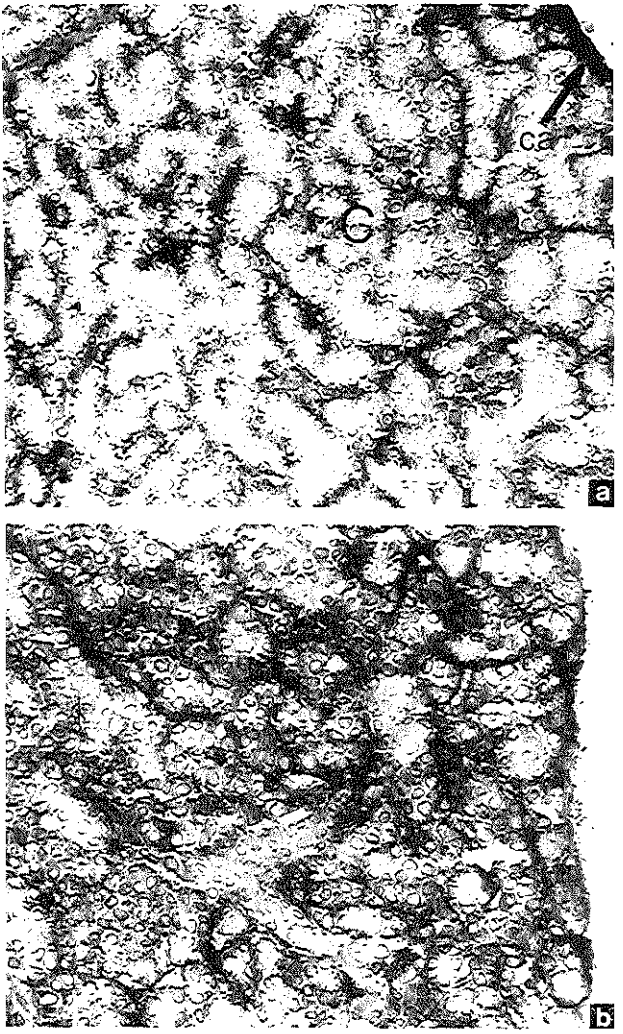


Fig. 2. Immunoperoxidase staining of serial frozen sections of an abnormal thymus, 270 days after 2.5 Gy fission neutron irradiation, incubated with ER-TR4 (a), ER-TR5 (b), ER-TR6 (c), ER-TR7 (d) monoclonal antibodies, Abbreviations as in Fig. 1. (x 140).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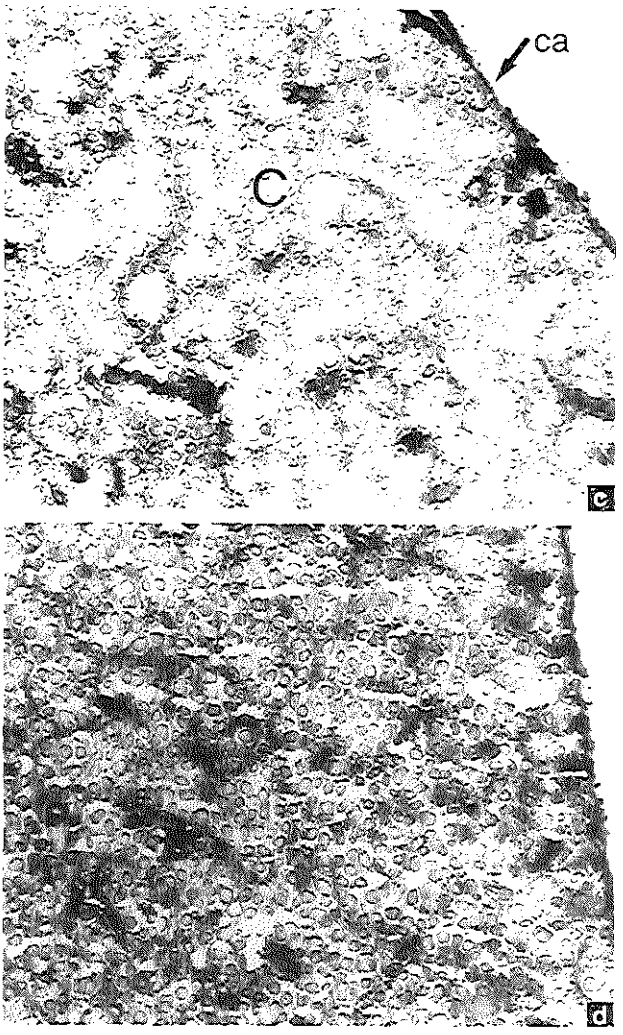

Fig. 3. Immunoperoxidase staining of serial frozen sections of the cortex of an abnormal thymus, 270 days after 2.5 Gy fission neutron irradiation, incubated with ER-TR3 (a,b) and anti-H-2K monoclonal antibodies (M1/42.3.9.8) (c,d). a and c represent sections of the normal lobe, b and d sections of the abnormal lobe. Abbreviations as in Fig. 1 (x 360).

However, based on comparisons of the ER-TR5 and ER-TR6 distribution patterns in normal and aberrant thymus lobes, we feel that the size of the medulla in the aberrant lobe has diminished.

Incubation of frozen sections with ER-TR7 (Fig. 2d), and antiseru which detects the reticular fibroblasts of the thymus, also shows no differences between the two lobes.

Observations of serial frozen sections incubated with either ER-TR1, 2, and 3, monoclonal antibodies which detect Ia antigens (Fig. 3a) or anti-I-A antibodies (clone 11-52-1.9) reveal a characteristic reticular staining pattern in the cortex of the normal lobe. In the lobe with the abnormal T cell phenotypes, the I-A expression is still reticular but compared to the normal lobe, the staining pattern of I-A determinants is not clearly confined to the cell surface of the epithelial reticular cells. In addition, many thymocytes in these lobes are dull $I\text{-}A^{+}$ (Fig. 3b). Incubation of frozen sections with anti-H-2K antibodies (clone M1/42.3.9.8) show in the cortical region of the normal lobe only a weak staining of stromal elements and lymphocytes (Fig. 3c). However, in the abnormal lobe, the H-2K staining level has increased and almost all cortical thymocytes are now dull $H\text{-}2K^{+}$ (Fig. 3d).

DISCUSSION

In the present paper, we describe aberrant thymuses of CBA/H mice, 220-270 days after whole-body irradiation with 2.5. Gy fast fission neutrons, using immunohistology with various monoclonal antibodies directed against T cells, stromal cell types and MHC antigens. Our results show that the described thymuses are abnormal in the sense that in one of the equal sized lobes, T cell differentiation in the cortex is arrested in a immature stage. Cortical T cells in this lobe express Thy-1 and T-200 antigens and have an increased MEL-14 expression. However, these cells are $Lyt\text{-}1^{-}$, $MT\text{-}4^{-}$ whereas in some cases a weak Lyt-2 expression is observed. The stromal cell distribution in the aberrant lobes is not different from that of the completely differentiated lobe. However, I-A and H-2K expression on stromal cells and lymphocytes in aberrant lobes has changed. Furthermore, the medulla in the aberrant lobe tends to be smaller and contains $Lyt\text{-}2^{-}$, $MT\text{-}4^{-}$ cells.

Interlobal independence in the thymus has also been noted during the development of radiation induced thymic leukemia in AKR mice (18). This immediately raises the question whether the present observations are related to a possible induced thymic leukomogenesis. The cell surface phenotypes of thymic leukemias have been shown to be very heterogeneous. Mathieson et al. (19) showed that BALB/c and AKR/J leukemic thymocytes expressed predominantly either Lyt-1 or Lyt-2 cell surface determinants. This is, however, no general rule since AKR thymomas can also be Lyt-1^+, Lyt-2^+ and can show other variations of Lyt expression (20). Furthermore, AKR leukemogenesis is accompanied by an increased Ia and H-2K expression on thymocytes (21). Leukemic thymocytes of HRS mice do have similar phenotypes as in AKR mice but do not show an increased Ia expression (22). Moreover, in a comprehensive study, Hogarth et al. (23) showed that radiation-induced C57Bl/6 and CE/J thymomas showed a variety of phenotypes with respect to their Lyt phenotypes. Besides Lyt-1^+, 2^+ and Lyt-1^-, 2^- phenotypes, the majority of the thymomas was Lyt-1^-, 2^+. The Ia expression on these thymomas was very heterogeneous since Ia^+ as well as Ia^- thymomas were observed. Scott et al. (24) recently reported a flow cytometric analysis of the phenotypes of virus as well as radiation-induced thymomas with the same monoclonal antibodies as used in the present investigation. They showed that many thymoma phenotypes are similar to those of thymocyte subpopulations that can be identified in the normal thymus. Furthermore, in 18 of 20 investigated thymomas, tumor cells showed an increased MEL-14 expression.

However, in a comprehensive study, Mole & Davids (25) detected no thymomas in CBA/H mice upto 800 days after graded doses up to 2.0 Gy neutron irradiation. The present investigation and other long-term studies also did not reveal any signs of thymomas (5,9). Based on these considerations, we feel that no leukemia has developed in the investigated thymuses. This opinion is further substantiated by the observation that these thymuses are $GP70^-$ whereas radiation induced thymic lymphomas have binding sites for the viral envelope glycoprotein GP70 of leukemogenic viruses (26).

As mentioned earlier, cortical thymocytes in the aberrant lobe express Thy-1, T-200 and MEL-14 antigens and are negative for MT-4 and Lyt-1. In some cases, a weak Lyt-2 expression was observed. In addition, cortical thymocytes in the aberrant lobe are all larger in size

than thymocytes in comparative areas in the normal lobe. Large-sized Thy-1$^+$, T-200$^+$ "only" cells have been identified during ontogeny (27) and during the regeneration of the thymus after irradiation (9) or after cortisone treatment (17). These cells are considered as an immature blast cell stage in thymocyte differentiation (9,28) and such cells show an increased level of MEL-14 expression (9). In addition, large bright MEL-14$^+$ blast cell have also been identified during the regeneration after cortisone treatment (29). We regard it therefore likely that the observed phenotypes in the abnormal thymus lobe have to be considered as cells belonging to an immature blast cell population. In the normal thymus such a blast cell population is located in the subcapsular area and acquires Lyt and MT-4 antigens during further differentiation (9,28). However in the aberrant lobe, this subset of thymic cells apparently does not further differentiate since deeper in the cortex, the same phenotypes are found as in the subcapsular area.

The thymic stroma in which these T cells have to proliferate, is known to support T cell differentiation. Thymic stromal cells probably mediate their effects on T cell differentiation by secretion of various hormones (30,31) as well as direct receptor mediated cell-cell contact of T cells with stromal cells (32). Furthermore, major histocompatibility complex (MHC) antigens, expressed on thymic stromal cells (33,34), have been implicated to be involved in dictating or selecting restriction specificities for self MHC of maturing T cells (35-39). In addition, during the regeneration of the thymus we observed that cortical epithelial cells, shortly after irradiation, loose their I-A determinants. Thereafter, the reappearance of a control-like reticular I-A staining pattern on the epithelial cells is closely correlated to the acquisition of Lyt and MT-4 antigens on the maturing surrounding thymocytes. This process is accompanied by a reduction of H-2K expression on epithelial and lymphoid cells in the cortex (40). A close correlation between the onset of proliferation of lymphoblasts and the expression of MHC determinants on epithelial cells has also been observed in the embryonic thymus (41). Furthermore, antibody blocking of cell surface-associated MHC determinants on stromal cells in the thymus has been shown to reduce the generation of MHC restricted T cells severely (42,43). These observations all suggest a regulatory role of MHC determinants in T cell differentiation. Our present observations on the

thymic stromal cell compartment in the aberrant lobe show that the stromal cell distribution in cortex and medulla is not different from that of the normal lobe. However, I-A determinants on the cortical epithelial cells in the aberrant lobe are not present in a strict membrane-bound fashion as observed in a normal lobe. Furthermore, cortical lymphoid and non-lymphoid cells in the aberrant lobe are slightly H-$2K^+$. This altered MHC expression in the cortex of the aberrant lobe might therefore be responsible for the observed T cell maturation arrest.

These long-term changes observed in the abnormal thymus lobe are not the result of a disturbed regeneration of the thymus shortly after irradiation since this phenomenon could not be observed 17 days after irradiation (Table II). Furthermore, we cannot definitely conclude whether this phenomenon is related to the radiation type since the X-ray group is rather small. The reason why these long-term alterations in T cell development and MHC expression occur at this time interval after the neutron irradiation, is as yet unclear and remains to be further investigated.

A further interesting point that arises from the present observations is that the medulla is only partly unaffected. In the medulla, numerous cells express Lyt-1 antigens. In addition, cells with varying levels of Lyt-2 and MT-4 expression are also observed. The present immunohistological observations and flow cytometric analysis of normal medullary thymocytes (44) show that medullary thymocytes are either Lyt-2^+, MT-4^- or Lyt-2^-, MT-4^+. In this sence, medullary thymocytes are comparable with peripheral T lymphocytes. Although Lyt-2^-, MT-4^- cells are also observed, the presence of these two subsets of thymocytes in the medulla of the aberrant lobe indicates that T cell differentiation in the medulla is only partly influenced by a cortical T cell maturation arrest. Furthermore, this indicates that the single lineage model in thymocyte differentiation, orginally proposed by Cantor and Boyse (45), suggesting that cortical Lyt-1^+,2^+ are the precursors of medullary Lyt-1^-,2^+ and Lyt-1^+,2^- cells, is not applicable in these aberrant thymic lobes since all cortical cells in these lobes are Lyt-1^-,2^-. In line with this finding are observations that cortex and medulla are independently seeded in the embryonic avian thymus (46). Moreover, in a congenic reconstitution model, Ezine et al. (47) also demonstrated two

independent thymus precursor lineages: a clone of cells which repopulated cortex and medulla and a clone of cells which repopulated only the medulla without cortical involvement.

Our present observations favour an independent generation of T cells in the medulla and are in line with the current view in thymocyte differentiation that cortex and medulla have independent generation kinetics (48,49). However, as already mentioned above, a substantial number of Lyt-2^-, MT-4^- cells is also observed in the medulla of the aberrant lobe. The presence of these cells with the phenotype of aberrant cortical cells suggests that the medulla is not strictly independently seeded but also seeded from cortical precursors as proposed by Weissman (50). If this hypothesis is true, the double negative but MEL-14^+ cortical blasts loose their MEL-14 expression while entering the medulla. Furthermore, the observed T cell differentiation arrest of cortical thymocytes might also result in a diminished seeding of cortical blasts into the medulla and explain the decreased size of the medulla in the aberrant lobe. In conclusion, our observations on aberrant thymus lobes after irradiation indicate the presence of a cortex independent medullary precursor lineage and a cortex to medulla lineage in T cell differentiation. The maturation of the latter lineage is arrested in an immature stage which is probably caused by the altered MHC expression in the cortex of these aberrant lobes. The present observations emphasize the role of the thymic microenvironment in T cell differentiation.

REFERENCES

1. Miller, J.F.A.P. and D. Osoba, Current concepts of the immunological function of the thymus. Physiol. Rev. 47: 437 (1967).

2. Cantor, H. and I.L. Weissman, Development and function of subpopulations of thymocytes and T lymphocytes. Prog. Allergy 20, 1 (1976).

3. Takanda, A., Y. Takada, C.C. Huang and J.L. Ambrus, Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129: 445-457 (1969).

4. Sharp, J.G. and D.B. Thomas, Thymic regeneration in lethally X-irradiated mice. Radiat. Res. 64: 293 (1975).

5. Huiskamp, R., J.A.G. Davids and O. Vos, Short- and long-term effect of whole-body irradiation with fission neutrons or X-rays on the s thymus in C3A mice. Radiat. Res. 95: 370 (1983).

6. Kadish, J.L. and R.S. Basch, Thymic regeneration after lethal irradiation: Evidence for an intra-thymic radioresistant T cell precursor. J. Immunol. 114: 452 (1975).

7. Sharrow, S.O., A. Singer, U. Hämmerling, and B.J. Mathieson, Phenotypic characterization of early events of thymus repopulation in radiation bone marrow chimeras. Transplantation 35: 355 (1983).

8. Sharp, J.G., and E.B. Watkins, Cellular and immunological consequences of thymic irradiation. In Immunological Effects of Radiation Therapy (J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds.), p. 137. Raven Press, New York, 1981.

9. Huiskamp, R. and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. I. Sequential appearance of thymocyte subpopulations. J. Immunol. 134: 2161 (1985).

10. Davids, J.A.G., Bone marrow syndrome in CBA mice exposed to fast neutrons of 1 MeV mean energy. Effect of syngeneic bone-marrow transplantation. Int. J. Radiat. Biol. 14: 573 (1969).

11. Ledbetter, J.A., and L.A. Herzenberg, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47: 61 (1979).

12. Pierres, A., P. Naquet, A. van Agthoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A.M. Schmitt-Verhulst, and M. Pierres, Rat anti-mouse T4 monoclonal antibody (dl29.19) inhibits the proliferation of Ia-reactive T cell clones and delineates two phenotypically distinct ($T4^+$, Lyt-2,3^- and $T4^-$, Lyt-2,3^+) subsets among anti-Ia cytolytic T

cell clones. J. Immunol. 132: 2775 (1984).

13. Gallatin, W.M., I.L. Weissman, and E.C. Butcher, A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304: 30 (1983).

14. van Vliet, E., M. Melis, and W. van Ewijk, Monoclonal antibodies to stromal cell types of the mouse thymus. Eur. J. Immunol. 14: 524 (1984).

15. Oi, V.T., P.P. Jones, J.W. Goding, L.A. Herzenberg, and L.A. Herzenberg, Properties of monoclonal antibodies to mouse Ig allotypes. Curr. Top. Microbiol. Immunobiol. 81: 115 (1975).

16. Springer, T.A., Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. In Monoclonal antibodies (R.H. Kennet, T.J. McKearn and K.B. Bechtol, Eds.), p. 185. Plenum Press, New York, 1980.

17. van Ewijk, W., P.L. van Soest, and G.J. van der Engh, Fluorescence analysis and anatomical distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. J. Immunol. 127: 2594 (1981).

18. Siegler, R., W. Harris, and M.A. Rich, Pathogenesis of radiation-induced thymic lymphoma in mice. J. Natl. Canc. Inst. 37: 105 (1966).

19. Mathieson, B.J., P.S. Campbell, M. Potter, and R. Asofsky, Expression Ly-1, Ly-2, Thy-1 and TL differentiation antigens on mouse T-cell tumors. J. Exp. Med. 147: 1267 (1978).

20. Zielinski, C.C., D.L. Waters, S.K. Datta, and S.D. Waksal, Analysis of intrathymic differentiation patterns during the course of AKR leukemogenesis. Cell. Immunol. 59: 355 (1981).

21. Zielinski, C.C., S.K. Datta, and S.D. Waksal, Increased expression

of I-A antigens on thymocytes from late preleukemic and leukemic AKR mice. Immunogenetics 14: 169 (1981).

22. Tempelis, L.D., Leukemic transformation in HRS mice occurs in an immature non-activated thymocyte subpopulation cell. Immunol. 83: 142 (1984).

23. Hogart, P.M., M.M. Henning, and I.F.C. McKenzie, Alloantigenic phenotype of radiation-induced thymomas in the mouse. J. Natl. Canc. Inst. 69: 619 (1982).

24. Scott, M.L., M.B. Feinberg, K.E. Fry, D.E. Percy, and M. Lieberman, Pattern of thymocyte differentation markers on virus and radiation induced lymphomas of C57BL/Ka mice. Int. J. Radiat. Oncol. Biol. Phys. 11: 71 (1985).

25. Mole, R.H. and J.A.G. Davids, Induction of myeloid leukemia and other tumours in mice by irradiation with fission neutrons. In Neutron Carcinogenesis (J.J. Broerse and G.B. Gerber, Eds.), p. 31. Commission of the European Communities, Luxembourg, EUR 8084, 1982.

26. McGrath, M.S. and I.L. Weissman, A receptor-mediated model of viral leukemogenesis: hypothesis and experiments. In Differentiation of Normal and Neoplastic Hemopoietic Cells (B. Clarkson, P.A. Marks, and J.E. Till, Eds.) p. 577, Cold Spring Harbor Laboratory, 1978.

27. van Ewijk, W., E.J. Jenkinson, and J.J.T. Owen, Detection of Thy-1, T-200, Lyt-1, and Lyt-2 bearing cells in the developing lymphoid organs of the mouse embryo in vivo and in vitro. Eur. J. Immunol. 12: 262 (1982).

28. van Ewijk, W., Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. Amer. J. Anat. 170: 311 (1984).

29. Reichert, R.A., W.M. Gallatin, E.C. Butcher, and I.L. Weissman, A homing receptor-bearing cortical thymocyte subset; implications for thymus cell migration and the natur of cortisone-resistant thymocytes. Cell 38: 89 (1984).

30. Kruisbeek, A.M., Thymic factors and T cell maturation in vitro: a comparison of the effects of thymic epithelial cultures with thymic extracts and thymus dependent serum factors. Thymus 1: 163 (1979).

31. Trainin, N., M. Pecht, and Z.T. Handzel, Thymic hormones: inducers and regulators of the T cell system. Immunol Today 4: 16 (1983).

32. Stutman, O., Intrathymic and extrathymic T cell maturation. Immunol. Rev. 42: 138 (1978).

33. Rouse, R.V., W. van Ewijk, P.P. Jones, and I.L. Weissman, Expression of MHC antigens by thymic dendritic cells, J. Immunol. 122: 2508 (1979).

34. van Ewijk, W., R.V. Rouse, and I.L. Weissman, Distribution of H-2 microenvironments in the mouse thymus. Immunoelectronmicroscopic identification of I-A and H-2K bearing cells. J. Histochem. Cytochem. 28: 1089 (1980).

35. Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein, On the thymus in the differentiation of H-2 self recognition by T cells: evidence of dual recognition? J. Exp. Med. 147: 882 (1978).

36. Wagner, H., C. Hardt, H. Stockinger, K. Pfizenmaier, R. Bartlett, and M. Röllinghoff, Impact of thymus on the generation of immunocompetence and diversity of antigen-specific MHC-restricted cytotoxic T lymphocyte precursors. Immunol. Rev. 58: 95 (1981).

37. Kruisbeek, A.M., S.O. Sharrow, B.J. Mathieson, and A. Singer, The H- 2 phenotype of the thymus dictates the self-specificity

expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. J. Immunol. 127: 2168 (1981).

38. Kruisbeek, A.M., S.O. Sharrow, and A. Singer, Differences in the MHC-restricted self-recognition repertoire of intra-thymic and extra-thymic cytotoxic T lymphocyte precursors. J. Immunol. 130: 1027 (1983).

39. Kast, W.M., L.P. de Waal, and C.J.M. Melief, Thymus dictates major histocompatibility complex (MHC) specificity and immune response gene phenotype of class II MHC-restricted T cells but not of class I MHC-restricted T cells. J. Exp. Med. 160: 1752 (1984).

40. Huiskamp, R., E. van Vliet, and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. II. Sequential changes in the thymic microenvironment. J. Immunol. 134: 2170 (1985).

41. Jenkinson, E.J., J.T.T. Owen, and R. Aspinall, Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus. Nature 284: 177 (1980).

42. Sprent, J., Effects of blocking helper T cell induction in vivo with anti-Ia antibodies. Possible role of I-A/E hybrid molecules as restriction elements. J. Exp. Med. 152: 996 (1980).

43. Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer, and J.J. Mond, Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-IA-antibodies interferes with differentiation of I-restricted T cells but not with K/D-restricted T cells. J. Exp. Med. 157: 1932 (1983).

44. Scollay, R. and K. Shortman, Thymocyte subpopulations: an experimental review, including flow cytometric cross-correlations between the major muring thymocyte markers. Thymus 5: 245 (1983).

45. Cantor, H. and E.A. Boyse, Regulation of cellular and humoral immune response by T-cell subclasses. Cold Spring Harbor Symp. Quant. Biol. 41: 23 (1976).

46. Jotereau, F.V. and N.M. Le Douarin, Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. J. Immunol. 129: 1869 (1982).

47. Ezine, S., I.L. Weissman, and R.V. Rouse, Bone marrow cells give rise to distinct cell clones within the thymus. Nature 309: 629 (1984).

48. Scollay, R., Intrathymic events in the differentiation of T lymphocytes: a continuing enigma. Immunol. Today 4: 282 (1983).

49. Mathieson, B.J., A re-viewing of thymocyte differentiation. In Leucocyte Typing: Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies (A. Bernard, L. Boumsell, J. Dausset, C. Milstein, and S.F. Schlossman, Eds.), p. 645. Springer Verlag, Berlin 1984.

50. Weissman, I.L., Thymus cell maturation. J. Exp. Med. 137: 504 (1973).

CHAPTER VIII

THE EFFECT OF GRADED DOSES OF FISSION NEUTRONS OR X-RAYS ON THE LYMPHOID COMPARTMENT OF THE THYMUS IN MICE

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SUMMARY

Young adult CBA/H mice were exposed to graded doses of whole-body irradiation with either fast fission neutrons or 300 kVp X-rays at centerline-dose rates of 0.1 and 0.3 Gy/min respectively.

Dose-response curves were determined at day 2 and 5 after irradiation for the total thymic cell survival and for the survival of thymocytes defined by monoclonal anti-Thy-1, -Lyt-1, -Lyt-2, and -T-200 antibodies as measured by flow cytofluorometric analysis.

Cell dose-response curves of thymocytes show, two days after irradiation, a two-component curve with a radiosensitive part and a part refractory to irradiation. The radiosensitive part of the dose survival curve of the Lyt-2^+ cells, i.e. mainly cortical cells, has a D_o value of about 0.26 Gy and 0.60 Gy for neutrons and X-rays respectively, whereas that of the other cell types has corresponding D_o values of about 0.30 Gy and 0.70 Gy. The radiorefractory part of the dose-response curves cannot be detected beyond 5 days after irradiation. At that time, the Lyt-2^+ cells are again most radiosensitive with a D_o value of 0.37 Gy and 0.99 Gy for neutrons and X-rays respectively. The other measured cell types have corresponding D_o values of about 0.47 Gy and 1.17 Gy. The fission neutron RBE values for the reduction

in the thymo cyte populations defined by either monoclonal anti-Thy-1, -Lyt-1, -Lyt-2, or -T-200 antibodies to 1.0 percent vary from 2.6 to 2.8.

Furthermore, the estimated D_o values of the Thy-1$^-$, T-200$^-$ intrathymic precursor cells which repopulate the thymus during the bone marrow independent phase of the biphasic thymus regeneration after whole-body irradiation, are 0.64 - 0.79 Gy for fission neutrons and 1.32 - 1.55 Gy for X-rays.

INTRODUCTION

The lymphoid compartment of the thymus is known to be very radiosensitive (1-4). After sublethal doses of irradiation, the thymus involutes dramatically and thereafter, the regeneration of the thymus follows a biphasic pattern (2, 5, 16). During the first phase of regeneration, the thymus regenerates from a population of radioresistant intrathymic precursor cells (3, 7). Phenotype analysis of the T cell distribution in the thymus during this first phase of recovery with flow cytofluorometry and immunohistology, using monoclonal antibodies directed to cell surface differentiation antigens, revealed a sequential appearance of distinct T cell subsets (8). The aforementioned study and a previous investigation (6) indicated, however, that in general the effects of irradiation on the thymus were greater after 2.5 Gy neutron than after 6.0 Gy X-irradiation.

In order to compare the biological effectiveness of two types of irradiation, the concept relative biological effectiveness (RBE) may be used. The RBE of fast fission neutrons as compared with X-rays, is the ratio of the absorbed dose of X-rays to the absorbed dose of neutrons required to produce the same biological effect. Neutron RBE determinations as a function of irradiation conditions, neutron-energy spectrum and biological endpoint are relevant for radiation protection, fast neutron therapy and treatment after accidental exposure with fast neutrons. In this context, it has been shown that, when neutron RBE data are compared, those for fast fission neutrons of 1 MeV mean energy are highest for each effect category investigated (9).

In this investigation, we exposed CBA/H mice to graded doses fast fission neutrons of 1 MeV mean energy or 300 kVp X-rays. We present dose-response curves and neutron RBE determinations for the total relative thymus cellularity at day 2 and 5 after irradiation. In addition, we present dose-response curves and neutron RBE determinations for the T cells in the thymus, defined by monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, Lyt-1, Lyt-2, or T-200 using flow cytofluorometry at day 2 and 5 after irradiation.

MATERIALS AND METHODS

Animals

Male and female CBA/H mice ($H\text{-}2^k$) were irradiated or sham-irradiated at the age of 5-7 weeks. The procedures of animal care have been described elsewhere (10).

Irradiation procedures

The animals were irradiated with fast fission neutrons of 1 MeV mean energy from a ^{235}U-converter in the Low Flux Reactor at Petten at a center-line dose rate of 0.10 Gy/min. The neutron doses do not include the 9% gamma-ray contribution. X-irradiation was performed with a Philips Müller X-ray tube, operating at 300 kVp constant potential at 5 mA with a measured HVL of 2.1 mm Cu. The center-line dose rate was equal to 0.30 Gy/min. For further details of the whole-body irradiation procedures, we refer to a previous paper (6).

Experimental procedures

Young adult female and male CBA/H mice were exposed to graded doses of whole-body irradiation. The doses used, varied from 0.50 to 2.50 Gy fission neutrons and from 1.25 to 6.25 Gy X-rays.
At day 2 and 5, mice were killed by anoxia. Depending on their size, 2-8 thymuses were pooled and processed for flow cytofluorometric analysis as described elsewhere (11, 12). Thymic cellularity was determined as described elsewhere (6).

Antisera

Monoclonal antibodies directed to cell surface determinants of mouse-lymphoid cells were obtained from the tissue culture supernatant of hybrid cell lines. Clone 59-AD-2.2 secreted anti-Thy-1 antibodies, clone 53-7.3.13 secreted anti-Lyt-1 antibodies, clone 53-6.72 secreted anti-Lyt-2 antibodies and clone 30-G-12 secreted anti-T-200 antibodies. All clones were originally prepared and characterized by Dr. J.A. Ledbetter (13).

Fluorescein-conjugated rabbit-anti-rat serum was obtained from Nordic Immunological Laboratories. This conjugate was used in a 1:15 dilution and supplemented with 1% normal mouse serum.

Flow cytofluorometric analysis of cell suspensions

Fluorescence of 4.10^4 cells/sample was analysed in a fluorescence activated cell sorter (FACS II, Becton and Dickinson FACS systems, Sunnyvale, California, USA). Integrated fluorescence signals were amplified using a logarithmic amplifier and analysed on a multichannel analyser (256 channels). The average fluorescence intensity channel number (A) was determined, over 256 channels at the multichannel analyser. Dead cells and debris were routinely excluded from analysis on the basis of low angle light scatter.

Percentages of positive cells were calculated by plotting the fluorescence profiles of the cell suspensions. The profiles of the negative control cell suspensions were smoothed, a cut-off channel was determined, and the percentage of cells above this cut-off channel i.e. the non-specific fluorescence, was calculated. In the experimental curves, the percentage of cells above this cut-off channel was calculated and corrected for the non-specific fluorescence. In case, there was no overlap of control and experimental curves, no correction was performed.

Regression analysis and statistical procedures

Dose-response curves and D_o values were determined by least squares regression analysis of the logarithm of the observed effect on

the radiation dose. Statistical comparisons of the D_o values of the various subpopulations were performed with a chi-square test.

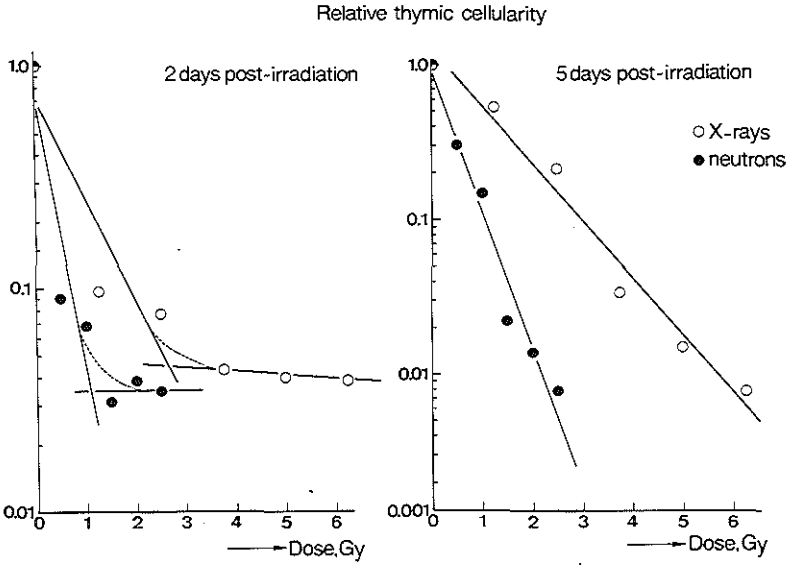

Fig. 1 Dose-response curves of the thymic cellularity relative to that of age-matched sham-irradiated control mice measured 2 and 5 days after irradiation with fission neutrons (●) or 300 kVp X-rays (o).

RESULTS

1. Thymic cell survival after irradiation

At day 2 and 5 after irradiation with graded doses of neutrons or X-rays, different dose-response curves on thymic cell survival are observed (Fig. 1). At day 2 after irradiation, thymic cell survival curves show a two component curve with a radiosensitive component and a component refractory to irradiation, i.e. dose-independent survival after irradiation (Fig. 1). After neutron as well as after X-irradiation more or less the same survival levels, i.e. between 3 and 4 percent, are reached at the highest doses. D_o values for the radiosensitive component were estimated by least squares analysis for the first three points, including the zero Gy point, after substraction of the contribution of the radiorefractory component. The limited data permit only a rough estimate of the D_o values of the radiosensitive component. The radiosensitive component has a D_o value equal to about 0.30 Gy for neutrons and about 0.74 Gy for X-rays. In contrast, thymic cell survival at day 5 after irradiation shows clear linear dose-response curves (Fig. 1) which are characterized by D_o values equal to 0.49 and 1.17 Gy for neutrons and X-rays respectively. RBE determination indicates an RBE value for fission neutrons equal to 2.54 ± 0.10 (s.e.m.) for a reduction of the relative thymic cellularity to 1.0 percent.

The radiobiological characteristics reported above, are for a functionally heterogeneous population of cells and therefore represent composite values. Therefore, we investigated the survival of T cells defined by monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, T-200, Lyt-1 and Lyt-2.

2. Survival of T cells in the thymus

Flow cytofluorometric analysis of the thymus, 2 days after irradiation with graded doses of X-rays or neutrons, shows that when the frequency of cells, expressing a specific marker, is measured, the Lyt-2^+ cell population is most radiosensitive (Table 1). The Thy-1^+ cell frequency is also affected and the average fluorescence intensity channel (A) indicates a selection for dull Thy-1^+ cells. In contrast, the

TABLE I Fluorescence characteristics and percentage of labeled cells in the thymus of CBA/H mice 2 days after graded doses of neutron or X-irradiation

		Staining							
		Thy-1		Lyt-1		Lyt-2		T-200	
Radiation exposure	a n	b A	c %	A	%	A	%	A	%
X-ray dose (Gy)									
1.25	2	112	81	110	85	94	48	126	90
2.50	3	102	69	113	87	92	36	129	89
3.75	4	101	69	112	86	89	32	129	89
5.00	6	100	67	112	85	93	41	128	88
6.25	8	100	66	110	84	93	40	129	91
neutron dose (Gy)									
0.50	2	111	75	106	81	86	38	103	71
1.00	3	103	76	108	86	82	34	107	80
1.50	4	99	74	105	83	70	16	104	76
2.00	6	99	60	104	72	74	14	104	66
2.50	8	98	70	107	84	76	19	103	73
Control	2	129	92	90	90	98	82	108	93

a. n = number of pooled thymuses

b. A = average fluorescence intensity channel*

c. % = percentage positively labeled cells in a thymocyte population, corrected for non-specific fluorescence*

* see materials and methods

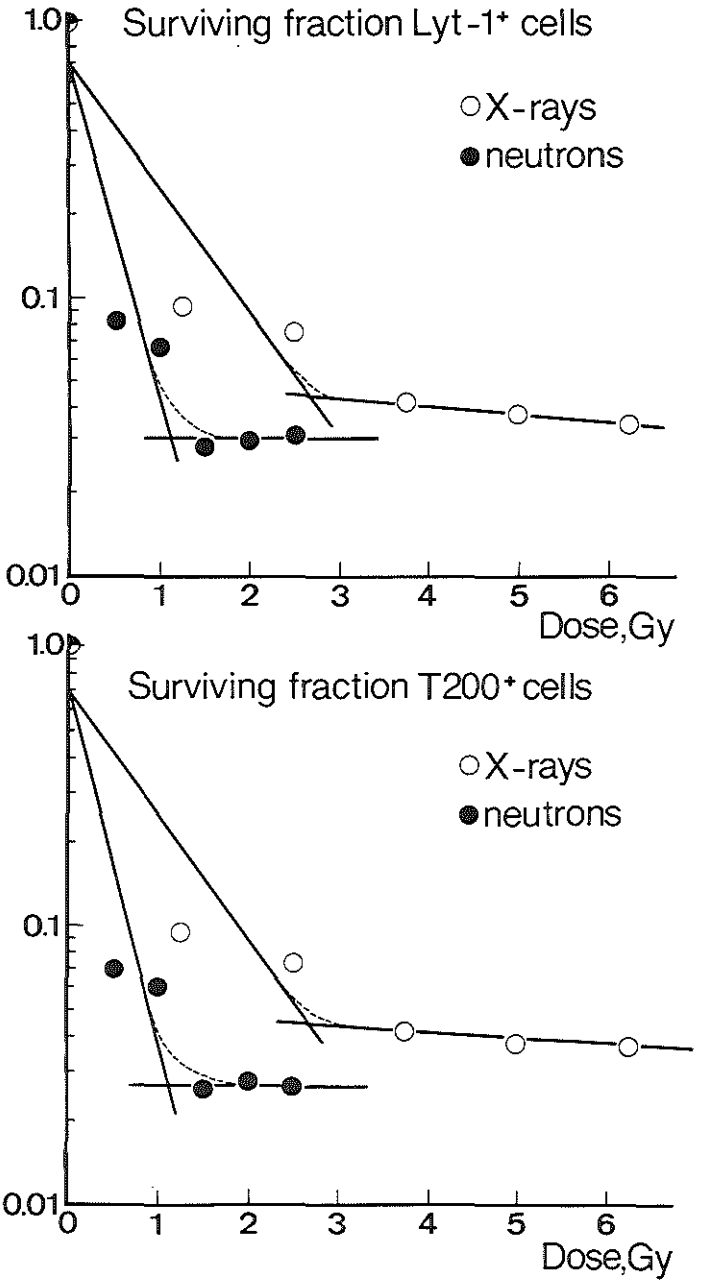

Fig. 2 Dose-response curves of the surviving fraction Lyt-1$^+$ and T-200$^+$ cells measured 2 days after irradiation with fission neutrons (•) or 300 kVp X-rays (o).

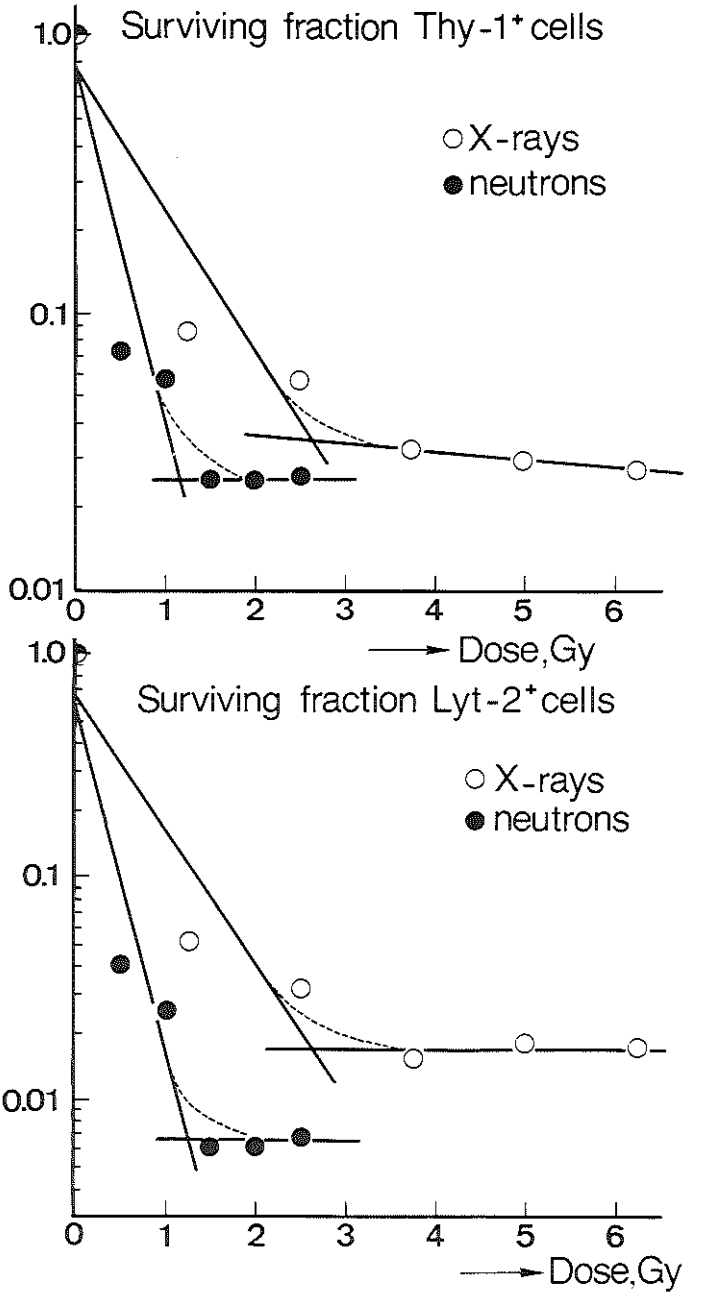

Fig. 3 Dose-response curves of the surviving fraction Thy-1$^+$ and Lyt-2$^+$ cells measured 2 days after irradiation with fission neutrons (•) or 300 kVp X-rays (o).

Lyt-1$^+$ frequency is not decreased after either neutron or X-irradiation. The T-200$^+$ cell frequency is not decreased after X-irradiation but is dose-independently decreased after neutron irradiation (Table I). For both markers, a selection for bright positive cells is observed, especially after X-irradiation (Table I).

Cell survival curves for Thy-1$^+$, Lyt-1$^+$, Lyt-2$^+$ and T-200$^+$ cells at day 2 after irradiation show also two component curves (Figs. 2,3). The radiosensitive component of the Thy-1$^+$, Lyt-1$^+$ and T-200$^+$ cell survival curves have similar D_o values, namely about 0.30 Gy for neutrons and 0.70 Gy for X-rays. The radiosensitive component of the Lyt-2$^+$ cell population has slightly lower D_o values, namely about 0.26 Gy for neutrons and 0.60 Gy for X-rays. The radiorefractory parts of the Lyt-1$^+$ cell survival curves after neutron and X-irradiation reach about the same survival levels (Fig. 2). This is also observed for the Thy-1$^+$ cell population (Fig. 3). However, the radiorefractory parts of the Lyt-2$^+$ cell survival curves lie at different survival levels 0.7 and 1.7 percent after neutrons and X-rays respectively (Fig. 3b). A difference in survival levels after neutron or X-irradiation is also observed for the radiorefractory parts of the T-200$^+$ cell survival curves (Fig. 2).

Flow cytofluorometric analysis at day 5 after irradiation, which marks the onset of thymic regeneration when the highest dose is employed (8), shows that the Thy-1$^+$ cell frequency and Lyt-2$^+$ cell frequency are clearly dose dependent (Table II). However, the T-200$^+$ cell frequency decreases only after neutron irradiation, whereas the Lyt-1$^+$ cell frequency varies after both types of radiation. Furthermore, the Thy-1$^+$ cells are of the dull positive type.

At day 5 after irradiation, survival curves for cells defined by Thy-1, Lyt-1, Lyt-2, or T-200 antigens, show a linear dose-response relationship (Figs. 4, 5). The D_o values and RBE determinations for Thy-1$^+$, Lyt-1$^+$, Lyt-2$^+$ and T-200$^+$ cells are listed in Table III. Comparison of the radiobiological characteristics of the Thy-1$^+$, Lyt-1$^+$ and T-200$^+$ cells shows no significant difference. In a previous investigation (8), we have shown that the surviving thymocytes after irradiation have in general the Thy-1$^+$, Lyt-1$^+$, T-200$^+$ phenotype. Therefore, for statistical purposes, we compared the D_o values of the other cell

TABLE II Fluorescence characteristics and percentage of labeled cells in the thymus of CBA/H mice 5 days after graded doses of neutron or X-irradiation

		Staining							
		Thy-1		Lyt-1		Lyt-2		T-200	
Radiation exposure	a n	b A	c %	A	%	A	%	A	%
X-ray dose (Gy)									
1.25	2	106	87	80	56	80	59	100	90
2.50	3	110	88	85	68	80	54	101	91
3.75	4	110	84	90	73	77	43	101	88
5.00	6	103	76	87	72	71	31	101	85
6.25	8	98	73	92	78	71	28	95	88
neutron dose (Gy)									
0.50	2	105	87	84	68	84	63	100	92
1.00	3	102	81	87	65	79	46	99	88
1.50	4	102	72	93	70	77	33	101	83
2.00	6	99	62	98	71	77	25	108	82
2.50	8	65	52	95	59	70	15	108	79
Control	2	130	93	91	90	97	82	107	93

a. n = number of pooled thymuses

b. A = average fluorescence intensity channel*

c. % = percentage positively labeled cells in a thymocyte population, corrected for non-specific fluorescence*

* see materials and methods

populations with the pooled D_o values obtained for the Thy-1$^+$, Lyt-1$^+$ and T-200$^+$ cells. Comparison of the D_o values of the Lyt-2$^+$ cell population with the pooled D_o values shows that the Lyt-2$^+$ cell population is significant more radiosensitive for both neutrons and X-rays with D_o values equal to 0.37 Gy and 0.99 Gy respectively.

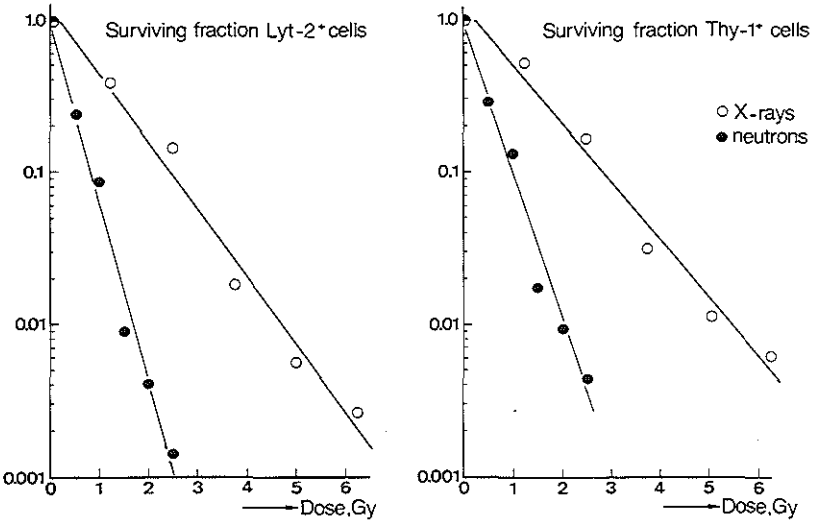

Fig. 4 Dose-response curves of the surviving fraction Lyt-2$^+$ and Thy-1$^+$ cells, 5 days after irradiation with fission neutrons (●) or 300 kVp X- rays (o).

The RBE values for the various cell populations are in general greater than that for the total thymic cellularity. This indicates the existence of other thymocyte subpopulations with lower RBE values which are not defined by the presently used monoclonal antibodies. It is known that besides the cells that express differentiation antigens,

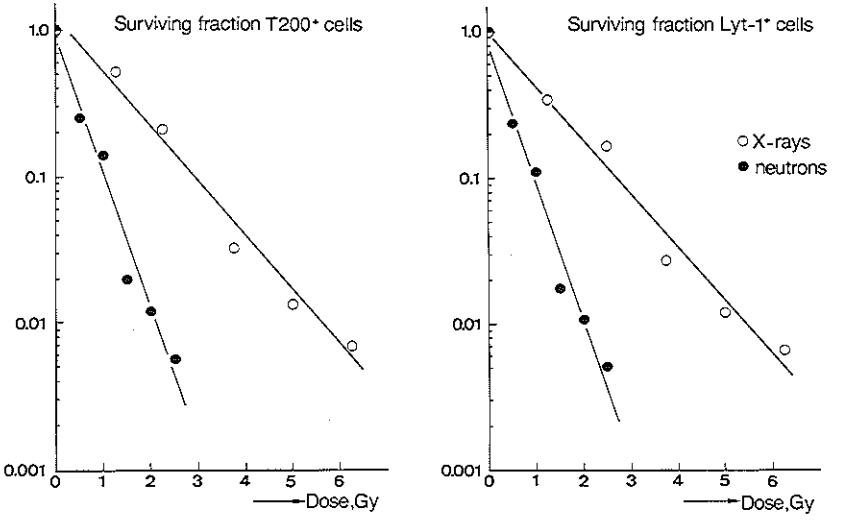

Fig. 5 Dose-response curves of the surviving fraction T-200$^+$ and Lyt-1$^+$ cells, 5 days after irradiation with fission neutrons (•) or 300 kVp X- rays (o).

TABLE III Radiobiological characteristics of thymocytes defined by Thy-1, Lyt-1, Lyt-2 or T-200 antigens, and Thy-1$^-$ and T-200$^-$ cells at day 5 after neutron or X-irradiation

	D_o ± sem[a] neutrons	D_o ± sem[a] X-rays	RBE ± SEM
Total cell population	0.49±0.05 Gy	1.17±0.10 Gy	2.56±0.12[b]
Thy-1$^+$ cells	0.44±0.03 Gy	1.14±0.09 Gy	2.75±0.13[b]
T-200$^+$ cells	0.48±0.04 Gy	1.17±0.10 Gy	2.64±0.12[b]
Lyt-1$^+$ cells	0.47±0.04 Gy	1.17±0.10 Gy	2.67±0.12[b]
Lyt-2$^+$ cells	0.37±0.02 Gy*	0.99±0.07 Gy*	2.81±0.11[b]
Thy-1$^-$ cells	0.79±0.10 Gy*	1.55±0.07 Gy*	2.22±0.19 (2.1)[c]
T-200$^-$ cells	0.64±0.07 Gy*	1.32±0.12 Gy	2.45±0.19 (2.2)[c]

a. D_o values determined by least squares regression analysis

b. RBE determined for surviving fraction 0.01

c. RBE determined for surviving fraction 0.1. Extrapolation for surviving fraction 0.01 in parentheses

* indicates a significant difference ($P<0.05$) from a pooled Thy-1$^+$, T-200$^+$, Lyt-1$^+$ D_o values which are equal to 0.46 ± 0.02 Gy and 1.16 ± 0.06 Gy respectively for neutrons and X-rays

thymic regeneration is characterized by the appearance of cells that initially do not express differentiation antigens and subsequently, express Thy-1 and T-200 antigens (8). Therefore it is likely that those cell populations with lower RBE values are composed of Thy-1$^-$ and T-200$^-$ cells. Dose response curves for the relative number of Thy-1$^-$ and T-200$^-$ cells reveal a linear dose response relationship (Fig. 6). The D_o and RBE values for these cell types are listed in Table III. The Do values are significantly higher than the pooled D_o values for Thy-1$^+$, Lyt-1$^+$, T-200$^+$ cells and the RBE values are less than those for the other cell populations. In general, the Thy-1$^-$ and T-200$^-$ cells are less sensitive to neutrons as well as X-rays than the more differentiated thymocytes.

DISCUSSION

In the present investigation, we exposed CBA/H mice to graded doses of fission neutrons or X-rays. At day 2 and 5 after irradiation dose response curves were determined for the total thymic cellularity and for the survival of thymocytes defined by monoclonal anti-Thy-1, -Lyt-1, -Lyt-2, or -T-200 antibodies. The results show: 1. the existence of a radioresistant population of cells at day 2 after irradiation which can no longer be detected at day 5 after irradiation; 2. Lyt-2$^+$ cells are most radiosensitive at day 2 as well as at day 5 after irradiation; 3. different D_o and RBE values are observed at day 2 and 5 after irradiation.

Analysis of thymic cell survival was started at day 2 after irradiation to prevent in the lower dose region, the influence of regeneration which starts about 5 days after irradiation when a 2.5 Gy neutron dose is used (8). Thymic cell survival curves at day 2 after irradiation reveal a two component curve with a radiosensitive part and a radiorefractory part. The estimated D_o values for the radiosensitive part, i.e. 0.30 Gy and 0.74 Gy for neutrons and X-rays respectively, are composite values since the population of cells measured is functionally heterogeneous. Therefore, we used monoclonal antibodies directed to cell surface differentiation antigens which thymocytes aquire during maturation in the thymus and define functional subpopulations

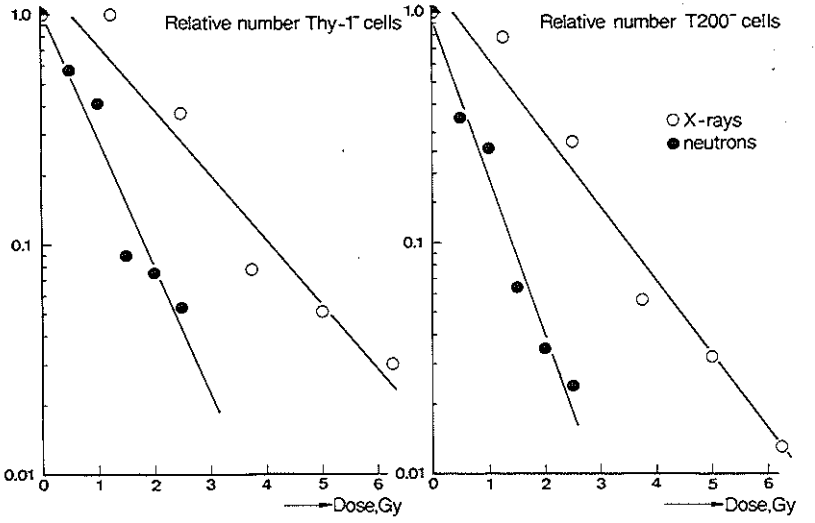

Fig. 6 Dose-response curves of number of Thy-1⁻ and T-200⁻ relative to age-matched sham-irradiated control mice, 5 days after irradiation with fission neutrons (•) or 300 kVp X-rays (o).

(14, 15) The results show clearly that for the markers investigated, the Lyt-2$^+$ cell population, comprising mainly cortical cells (11, 16), are most radiosensitive.

The radiosensitive components of the survival curves have estimated D_o values of about 0.26 Gy and 0.60 Gy for fission neutrons and X-rays respectively. The latter D_o value is in good agreement with those reported by Trowell (17) and Sato & Sakka (1). The radiosensitive components of the Thy-1$^+$, Lyt-1$^+$ and T-200$^+$ cell survival curves have similar D_o values of about 0.30 Gy for fission neutrons and about 0.70 Gy for X-rays.

The fluorescence characteristics of the radiorefractory part of the various cell survival curves indicate that the surviving cells at day 2 after irradiation are of medullary phenotype i.e. dull Thy-1$^+$, bright Lyt-1$^+$, bright T-200$^+$ cells which are mainly Lyt-2$^-$. This was also shown in a previous investigation using immunocytochemistry (8). These medullary cells represent the 3-4% cell survival, observed at day 2 after irradiation. In the control thymus, about 15% of the thymocytes belong to the medullary cell pool (18). Hence, only 20-30% of the medullary thymocytes survive neutron or X-irradiation at the highest dose levels. The T-200$^+$ and especially the Lyt-2$^+$ cell survival curves reveal in the radiorefractory part, different survival levels after neutron and X-irradiation. In contrast to the medullary phenotype, cortical cells are bright Thy-1$^+$, Lyt-1$^+$, T-200$^+$ and bright Lyt-2$^+$ (8, 11, 16). Thus, the D_o values for the Lyt-2$^+$ cells can be regarded as a measurement of the cortical cells whereas the D_o values for the Thy-1$^+$, Lyt-1$^+$, and T-200$^+$ cells can be regarded as a composite value of the cortical cells and the non-radiorefractory part of the medullary thymocyte pool.

The radiorefractory part of the medullary thymocyte pool cannot be detected in the thymus of CBA/H mice at day 5 after irradiation. Moreover, at the highest doses for both types of irradiation used, the relative thymic cellularity, observed at day 5 after irradiation, is markedly decreased in comparison with the level observed at day 2 after irradiation. This observation raises questions about the fate of this medullary subpopulation. These cells might have died between 2 and 5 days after irradiation. However, thymocytes are known to undergo interphase death within a few hours after the irradiation (19, 20). Furthermore, from day 2 after irradiation on, immunocytochemistry does not reveal any signs of thymocyte death (8). Alternatively, since thymus migrants have a medullary phenotype (21), the radiorefractory medullary thymocytes might have migrated out of the thymus to the peripheral lymphoid organs, which are devoid of T lymphocytes after irradiation (22, 23). The observed different survival levels of the radiorefractory Lyt-2$^+$ and T-200$^+$ cells at day 2 after neutron and X-irradiation might then be explained by different migration rates after neutron and X-irradiation. However, this hypothesis needs to be further investigated.

In contrast to the marked decrease of the relative thymic cellularity between day 2 and 5 after irradiation when the two highest doses are used, the relative thymic cellularity increases between 2 and 5 days after irradiation when the lowest two doses of both types of irradiation are used. This indicates proliferation of prothymocytes and/or proliferation of intrathymic precursors which survive the irradiation. However, we favor the latter possibility since bone marrow transplantation does not influence this first phase of the biphasic regeneration of the thymus after irradiation (3).

Five days after irradiation, the Lyt-2^+ cells are again most radiosensitive and have D_o values of 0.37 Gy and 0.99 Gy for fission neutrons and X-rays respectively. As mentioned earlier for the 2 days interval, the D_o values of cells defined by anti-Thy-1, -Lyt-1, or -T-200 can be regarded as a composite value of cortical and medullary cells. The fission neutron D_o values of these cells (D_o, 0.44-0.48 Gy) at day 5 after irradiation is comparable with that for hemopoietic stem cells of CBA mice (D_o, 0.45 Gy) (24). The X-ray D_o value of the cells defined by anti-Thy-1, -Lyt-1, or -T-200 are comparable with those reported for human thymocytes, cultured for 4 days after γ-irradiation (D_o, 1.35 Gy) (25) and for mouse thymocytes at day 4 after X-irradiation (D_o, 1.30 Gy) (4).

Comparison of the D_o values of the thymocytes defined by monoclonal anti-Thy-1, -T-200, -Lyt-1 or -Lyt-2 antibodies, at day 2 and 5 after irradiation with neutrons or X-rays, indicates that the precursor cells of the thymocytes present in the thymus at day 5 after irradiation, are less radiosensitive than the non-radiorefractory thymocytes present at day 2 after irradiation. In addition, comparison of the fission neutron RBE value for the reduction of the various cell types to 1.0 percent and that for the total thymic cellularity indicates the existence of other subpopulations with lower RBE values. In a previous investigation, we have shown that the intra-thymic bone marrow independent regeneration after 2.5 Gy neutron-irradiation is characterized by a sequential appearance of well-defined thymocyte subsets in a time period of 5-9 days after irradiation (8). This sequence starts at day 5 after irradiation with the appearance of "null" cells, i.e. large lymphoid cells that do not express cell surface differentiation antigens,

and probably represent the proposed intra-thymic radioresistant precursor cells (3). When the regeneration proceeds, Thy-1$^+$ "only" and T-200$^+$ "only" cells are observed, followed by Thy-1$^+$, T-200$^+$ and Thy-1$^+$, T-200$^+$, Lyt$^+$ cells (8). The present investigation shows clearly that thymocytes that do not express a specific T cell marker, are less sensitive to neutrons as well as X-rays, and have indeed a lower fission neutron RBE value than thymocytes expressing differentiation antigens.

Since we could not identify precursor cells by double negative fluorescence, we can only estimate the radiosensitivity of the intrathymic radioresistant precursor cells, i.e. "null" cells. However, as mentioned earlier, "null" cells acquire Thy-1 and T-200 antigens as a first step in the regenerative process. Therefore, a likely estimate of the D_o values of the "null" cells would be 1.32 - 1.55 Gy for X-rays and 0.64 - 0.79 Gy for fission neutrons (Table III). However, Sharp and Watkins (4) reported an X-ray D_o value for thymic precursor cells of 0.70 Gy by electronic cell counting, 10 days after irradiation. The reason for this discrepancy could be the higher doses of X-irradiation used by these authors (8.0 Gy v. 6.0 Gy in our experiments). Furthermore, the proliferative capacity of the intrathymic precursor cells might become limiting at higher doses (26). In addition, comparison of D_o values obtained by others is complicated by differences in methodology and mouse strains used. This is illustrated since X-ray D_o and neutron RBE values on thymic cell survival 5 days after irradiation, obtained from another inbred CBA strain (27,28) are higher than from CBA/H mice although the LD50/30 of both strains do not differ (Huiskamp & Davids, unpubl. observation). Based on the LD50/30 values for the two strains, an RBE of 2.3 can be determined for the bone marrow syndrone (6). This value agrees well with the estimated RBE value (2.1-2.2) for the intrathymic radioresistant precursor cells and points to the bone marrow as the ultimate origin of these cells.

In conclusion, cortical thymocytes are highly radiosensitive and have D_o values of about 0.26 and 0.60 Gy for fission neutrons and X-rays respectively. The medullary cell pool is composed of a radiosensitive part (70-80%) with D_o values of about 0.30 and 0.70 Gy for neutrons and X-rays respectively and a radiorefractory part (20-30%) which

cannot be detected 5 days after irradiation. Regeneration starts from cells with estimated D_o values of 0.64 - 0.79 Gy for fission neutrons and 1.32 - 1.55 Gy for X-rays.

REFERENCES

1. Sato, C. and M. Sakka, Cell population analysis of radiation damage and recovery of thymus and lymph nodes in mice. Radiat. Res. 38: 204 (1969).

2. Takada, A., Y. Takada, C.C. Huang, and J.L. Ambrus, Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129: 445 (1969).

3. Kadish, J.L. and R.S. Basch, Thymic regeneration after lethal irradiation: Evidence for an intra-thymic radioresistant T cell precursor. J. Immunol. 114: 452 (1975).

4. Sharp, J.G. and E.B. Watkins, Cellular and immunological consequences of thymic irradiation. In Immunopharmacologic Effects of Radiation Therapy (J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds.), p. 137. Raven Press, New York, 1981.

5. Sharp, J.G. and D.B. Thomas, Origin of the radioresistant precursor cells responsible for the initial phase of thymic regeneration after X-irradiation. J. Reticuloendothel. Soc. 22: 169 (1977).

6. Huiskamp, R., J.A.G. Davids, and O. Vos, Short- and long-term effects of whole-body irradiation with fission neutrons or X-rays on the thymus in CBA mice. Radiat. Res. 95: 370 (1983).

7. Sharrow, S.O., A. Singer, U. Hämmerling, and B.J. Mathieson, Phenotypic characterization of early events of thymus repopulation in radiation bone marrow chimeras. Transplantation 35: 355 (1983).

8. Huiskamp, R. and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. I. Sequential appearance of thymocyte subpopulations. J. Immunol. 134: 2161 (1985).

9. Broerse, J.J. and G.W. Barendsen, Relative biological effectiveness of fast neutrons for effects on normal tissues. Current Topics in Radiation Research Quarterly 8: 305 (1973).

10. Davids, J.A.G., Bone-marrow syndrome in CBA mice exposed to fast neutrons of 1.0 MeV mean energy. Effect of syngeneic bone-marrow transplantation. Int. J. Radiat. Biol. 17: 173 (1970).

11. van Ewijk, W., P.L. van Soest, and G.J. van der Engh, Fluorescence analysis and anatomical distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2, and T-200. J. Immunol. 127: 2594 (1981).

12. van Ewijk, W., P.L. van Soest, A. Verkerk, and J.F. Jongkind, Fixation parameters for immunocytochemistry. Histochem. J. 16: 179 (1984).

13. Ledbetter, J.A. and L.A. Herzenberg, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47: 61 (1979).

14. Cantor, H. and I.L. Weissman, Development and function of subpopulations thymocytes and T lymphocytes. Prog. Allergy 20: 1 (1976).

15. Cantor, H. and E.A. Boyse. Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev. 33: 105 (1977).

16. van Ewijk, W., Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. Am. J. Anat., 170: 311 (1984).

17. Trowell, O.A., Radiosensitivity of the cortical and medullary lymphocytes in the thymus. Int. J. Radiat. Biol. 4: 163 (1961).

18. Scollay, R. and K. Shortman, Thymocyte subpopulations: an experimental review, including flow cytometric cross correlations between the major murine thymocyte markers. Thymus 5: 245 (1983).

19. Yamaguchi, T., Relationship between survival period and dose of irradiation in rat thymocytes in vitro. Int. J. Radiat. Biol. 12: 235 (1967).

20. Ohyama, H., T. Yamada, and I. Watanabe, Cell volume reduction associated with interphase death in rat thymocytes. Radiat. Res. 85: 333 (1981).

21. Scollay, R., A. Wilson, and K. Shortman, Thymus cell migration: analysis of thymus emigrants with markers that distinguish medullary thymocytes from peripheral T cells. J. Immunol. 132: 1089 (1984).

22. Anderson, R.E. and N.L. Warner, Ionizing radiation and the immune response. Adv. Immunology 24: 215 (1976).

23. Anderson, R.E. and J.C. Standefer, Radiation injury in the immune system. In cytotoxic Insult to Tissue (C.S. Potten and J.H. Hendry, Eds), p. 67. Churchill Livingstone, Edinburgh, 1983.

24. Davids, J.A.G., Acute effects of 1 MeV fast neutrons on the haematopoietic tissues, intestinal epithelium and gastric epithelium in mice. In Advances in Radiation Research (J.F. Duplan and A. Chapiro, Eds.), Vol. II, p. 565. Gordon and Breach, New York, 1973.

25. Kwan, D.K. and A. Norman, Radiosensitivity of human lymphocyte and thymocytes. Radiat. Res. 69: 143 (1977).

26. Dukor, P., J.F.A.P. Miller, W. House, and V. Allman, Regeneration of thymus grafts. I. Histological and cytological aspects. Transplantation 3: 639 (1965).

27. Huiskamp, R., J.A.G. Davids, and O. Vos, Short- and long-term effects of whole-body irradiation with fission neutrons or X-rays on the thymus in CBA mice. Int. J. Radiat. Biol. 42: 78 (1983).

28. Huiskamp, R., J.A.G. Davids, O. Vos and W. van Ewijk, Cellular and immunohistological effects in the thymus of mice after whole-body irradiation with fission neutrons or X-rays. Abstract C1-15 in Proceedings of the seventh International Congress of Radiation Research. Martinus Nijhoff, The Hague, 1983.

CHAPTER IX

THE EFFECT OF GRADED DOSES OF FISSION NEUTRONS OR X-RAYS ON THE STROMAL COMPARTMENT OF THE THYMUS IN MICE.

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SUMMARY

The effect of irradiation on the supportive role of the thymic stroma in T cell differentiation was investigated in a transplantation model using athymic nude mice and transplanted irradiated thymuses. In this model, neonatal CBA/H mice were exposed to graded doses of whole-body irradiation with fast fission neutrons of 1 MeV mean energy or 300 kVp X-rays. The doses used varied from 2.75 up to 6.88 Gy fission neutrons and from 6.00 up to 15.00 Gy X-rays at centerline-dose rates of 0.10 and 0.30 Gy/min respectively. Subsequently, the thymus was excised and a thymus lobe was transplanted under the kidney capsule of H-2 compatible nude mice. One and two months after transplantation, the T cell composition of the thymic transplant was investigated using immunohistology with monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, Lyt-1, Lyt-2, MT-4 and T-200. Furthermore, the stromal cell composition of the thymic transplant was investigated with monoclonal antibodies directed to MHC antigens and with monoclonal antibodies defining different subsets of thymic stromal cells. In order to investigate the reconstitution capacity of the thymic transplant, the peripheral T cell number was measured using flow cytofluorometric analysis of nude spleen cells with the monoclonal antibodies anti-Thy- 1, -Lyt-2, and -MT-4.

The results of this investigation show that a neonatal thymus grafted in a nude mouse has a similar stromal and T cell composition as

a normal thymus in situ. In addition, grafting of such a thymus results in an significant increase of the peripheral T cell number. Irradiation of the graft prior to transplantation has no effects on the stromal and T cell composition but the graft size decreases. This reduction of size shows a linear dose-response curve after neutron irradiation. The X-ray curve is linear for doses in excess of 6.00 Gy. The RBE for fission neutrons for the reduction of the relative thymic graft size to 10 percent was equal to 2.1. Furthermore, the peripheral T cell number decreases with increasing doses of irradiation given to the graft prior to transplantation. The present data indicate that the regenerative potential of thymic stromal cells is radiosensitive and is characterized by D_0 values to 2.45 and 3.68 Gy for neutrons and X-rays respectively. In contrast, the ability of the thymic stromal cells to support T cell maturation is highly radioresistant.

INTRODUCTION

Stromal cells in the thymus are known to support the development of immunocompetent T cells. Thymic stromal cells probably mediate their effects on T cell differentiation by secreting various hormone-like factors (1,2) as well as by direct receptor mediated cell-cell contact of stromal cells with T cells (3). Furthermore, various in vivo experiments have indicated that major histocompatibility complex (MHC) antigens, mainly expressed on thymic stromal cells (4,5), are involved in dictating or selecting restriction specificities for self MHC of the maturing T cells (6-8). In addition, expression of MHC antigens on thymic epithelial cells have been implicated to play a regulatory role in the generation of T cells (9-12).

To the lymphoid component of the thymus is severely affected after sublethal whole-body irradiation with fission neutrons or X-rays (13,14). However, the stromal compartment of the thymus has been shown to be fairly radioresistant. Davis and Cole (15) showed in sublethally irradiated bone marrow reconstituted mice that immune functions were restored even when the thymus was additionally exposed to 20 Gy X-rays. Furthermore, thymus grafts, exposed in vitro to 2060 R gamma-rays, showed evidence of lymphopoietic regeneration at 11 days after grafting (16). In newborn mice, no decline of several immunological functions

was observed after in situ exposure of the thymus to 2000 R X-rays (17). In a previous investigation (12), however, we have shown that within 24 hours after whole-body irradiation with 2.50 Gy neutrons or 6.00 Gy X-rays, especially the cortical microenvironmental architecture is severely disturbed. As shown by immunohistology, Ia determinants on cortical epithelial cells were no longer present in a membrane-bound fashion but appeared to be shed in the cortex. Moreover, the lymphoid repopulation of the thymus coincides with the appearance of an H-2K staining on the epithelial cells in the cortex which is not present in sham-irradiated control thymuses. Subsequently, the epithelial H-2K staining in the cortex disappeared and a reticular I-A staining in the cortex reappeared, similar to that observed in sham-irradiated control animals. This sequence indicates that the cortical epithelial cells are readily affected by sublethal doses of radiation with respect to their MHC expression.

These results led us to asses the radiosensitivity of the thymic stromal compartment in more detail. In the present investigation, we exposed neonatal CBA/H mice to graded doses fast fission neutrons of 1 MeV mean energy or 300 kVp X-rays upto 6.88 and 15.00 Gy respectively and transplanted the thymus in H-2 compatible athymic nude mice. Using immunocytochemistry, we investigated the size and the T cell composition of the thymic transplant, 1 and 2 months after transplantation. To this purpose, we used monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, Lyt-1, Lyt-2, MT-4, and T-200. Microenvironments in the transplanted thymuses were investigated using monoclonal antibodies directed to MHC antigens as well as monoclonal antibodies defining various thymic stromal cell types (ER-TR series, 22). In addition, the peripheral T cell number was investigated in the spleen of the nude mice with monoclonal antibodies directed to T cell surface differentiation antigens Thy-1, lyt-2 and MT-4 using flow cytofluorometry.

Our results indicate that the stromal and T cell distribution in the irradiated and sham-irradiated thymic grafts are similar to those observed in the normal thymus in situ. However, thymic graft size and peripheral T cell number are decreased when nude mice are reconstituted with irradiated thymic lobes. The present results allow an estimation of the radiosensivity of the thymic stromal compartment.

MATERIALS AND METHODS

Animals

Male and female neonatal mice of the inbred CBA/H substrain ($H\text{-}2^k$), maintained at Petten, were irradiated or sham-irradiated. Male C3H nude mice nu/nu ($H\text{-}2^k$), 6 weeks of age, were purchased in two deliveries, two weeks apart, from Bomholtgard, Ry, Denmark and maintained in a cross flow cabinet under semi-sterile conditions.

Irradiation procedures

Neonatal mice were irradiated with fast fission neutrons of 1 MeV mean energy from a ^{235}U-converter in the Low Flux Reactor at Petten, at a center-line dose rate of 0.10 Gy/min. The absorbed doses quoted do not include the 8% gamma-ray contribution. X-irradiation was performed with a Philips Müller X-ray tube, operating at 300 kVp constant potential at 5 mA with a measured HVL of 2.1 mm Cu. The center-line dose rate was equal to 0.30 Gy/min. For further details of the whole-body irradiation procedures, we refer to a previous paper (13).

Experimental procedures

Three neonatal CBA/H mice were exposed to each dose of whole-body irradiation. The doses used, varied from 2.75 up to 6.88 Gy fission neutrons and from 6.00 up to 15.00 Gy X-rays. Within 20 minutes after irradiation, the neonatal mice were decapitated and the thymus was excised. The two thymic lobes were separated and each lobe was transplanted under the kidney capsule of a C3H nude mouse, anesthetized beforehand with Avertin. At 1 and 2 months respectively after transplantation, the kidney with thymic transplant were excised and photographed. From enlarged photographs, the transplant volume was estimated using the formula $V = L(\text{ength}) \times [W(\text{idth})]^2/2$. Subsequently, the thymic transplants were processed for immunocytochemistry. In addition, the spleens of nude recipients were excised and processed for flow cytofluorometric analysis as described elsewhere (18, 19). Nude mice

grafted with a sham-irradiated thymic lobe were used as a positive control and sham-operated nude mice were used as a negative control.

Antisera

Syngeneic and xenogeneic monoclonal antibodies used in the present investigation are listed in Table I.

Peroxidase-conjugated rabbit-anti-rat immunoglobulin was obtained from Dakopatts, Denmark. The conjugate was diluted in a 1:20 dilution and supplemented with 1% normal mouse serum. Fluorescein-conjugated rabbit-anti-rat immunoglobulin was obtained from Nordic Immunological laboratories. This conjugate was used in a 1:15 dilution, also supplemented with 1% normal mouse serum.

TABLE I Reagents used in the present investigation

Monoclonal antibody	Reacts with/ Target antigen	Reference
59-AD-22	Thy-1	(20)
30-G-12	T-200	(20)
H129.19	MT-4	(21)
53-7.3.13	Lyt-1	(20)
53-6.72	Lyt-2	(20)
ER-TR1	Cortical and medullary stromal cells (I region of MHC)	(22)
ER-TR4	Cortical epithelial cells	(22)
ER-TR5	Medullary epithelial cells	(20)
ER-TR6	Medullary "IDC"	(20)
M1/42.3.9.8	H-2K, all haplotypes	(23)

Flow cytofluorometric analysis of spleen cell suspensions

Fluorescence of 4.10^4 cells/sample was analyzed in a fluorescence activated cell sorter (FACS II, Becton and Dickinson FACS systems, Sunnyvale, CA). Integrated fluorescence signals were amplified using a logarithmic amplifier and analyzed on a multichannel analyzer (256 channels). Dead cells and debris were routinely excluded from analysis on the basis of low angle light scatter gatting. Percentages of positive cells were calculated by plotting the fluorescence profiles of the cell suspensions. The profiles of negative control cell suspensions were "smoothed", a cut-off channel was determined, and the percentage of cells above this channel i.e. the aspecific fluorescence was calculated. In experimental profiles, the percentage of cells above this "cut-off" channel was calculated and corrected for the aspecific fluorescence. In case, there was no overlap of control and experimental profiles, no correction was performed.

Tissue preparation for immunocytochemistry

Frozen sections of thymus transplants, isolated 1 and 2 months after transplantation, were prepared and stained using the indirect immunoperoxidase method as described elsewhere [18].

Regression analysis and statistical procedures

Dose-response curves and D_o values were determined by least-squares regression analysis of the logarithm of the observed effect on the radiation dose. The mean percentage of positive cells of an experimental dose group for a specific staining was calculated from the pooled data obtained 1 and 2 months after transplantation. Statistical comparisons between experimental groups were performed with a Mann-Whitney U-test at the 0.05 level of significance.

RESULTS

In the first part of this section, we describe the tissue distribution of T cells and stromal cells in the thymic transplants in nude mice. The second part deals with the size of thymic transplants. The

third part concentrates on the peripheral T cell number measured in the spleen of these thymus grafted nude mice using flow cytofluorometry.

1. Immunohistology of thymic transplants

Monoclonal antibodies directed to the T cell differentiation antigens Thy-1, Lyt-1, Lyt-2, MT-4, and T-200, MHC antigens as well as antibodies defining various stromal cell types were used for immunohistological studies. The immunohistology of sham-irradiated control grafts, 1 and 2 months after transplantation, showed a normal T cell distribution and stromal architecture as compared with young adult the thymus in situ, as previously described (12, 22, 24).

Already one month after transplantation, all irradiated grafts were regenerated and completely developed, as judged by their immunohistological appearance. As an example of regeneration and development of irradiated thymic grafts, the immunohistology of an 15.00 Gy X-irradiated thymic graft, 2 months after transplantation is shown in Figures 1 and 2.

Frozen sections of thymic grafts incubated with anti-Thy-1 antibodies (Fig. 1a) reveal that the major population in the graft consists of bright Thy-1^+ cortical thymocytes. The Thy-1 staining is most intense in the subcapsular area and gradually decreases towards the medulla. In general, medullary cells are dull Thy-1^+.
Serial frozen sections incubated with anti-Lyt-1 antibodies (fig. 1b) reveal a variable expression of Lyt-1 in both cortex and medulla. The majority of cortical cells is dull Lyt-1^+ but scattered foci of bright Lyt-1^+ cells are also observed, especially in the cortico-medullary area. In the medulla, cells are in general bright Lyt-1^+ but also dull Lyt-1^+ cells and Lyt-1^- cells are observed.

Serial anti-Lyt-2 stained sections show that the majority of the cortical cells is bright Lyt-2^+. Scattered foci of negative to dull Lyt-2^+ are found in the subcapsular area. In the medulla, the majority of lymphoid cells is Lyt-2^- with scattered dull to bright Lyt-2^+ (fig. 1c).

Serial sections stained with anti-MT-4 antibodies demonstrate that the large marjority of cortical cells is MT-4^+. However in the subcap-

sular area, a number of cells is negative or dull MT-4$^+$. In the medulla, the MT-4 expression is very heterogeneous. The majority of medullary cells is dull MT-4$^+$ but MT-4$^-$ and bright MT-4$^+$ cells can also be observed (fig. 1d). Accurate comparison of adjacent sections incubated with Lyt-2 and MT-4 reveals that medullary cells are either Lyt-2$^+$, MT-4$^-$ or Lyt-2$^-$, MT-4$^+$.

Incubation of serial frozen sections with anti-T-200 antibodies shows that virtually all thymocytes are T-200$^+$ (fig. 1e). Cortical thymocytes are bright T-200$^+$ whereas cells in the cortico-medullary area and in the medulla have a higher density of this antigen. Some negative to dull T-200$^+$ cells can be observed in the subcapsular area and also in the medulla.

The stromal composition of thymic grafts is shown in fig. 2. Frozen sections incubated with ER-TR4 antibodies, which detect cortical epithelial cells, reveal a characteristic reticular staining pattern in the cortex (fig. 2a). ER-TR5 and ER-TR6 antibodies react with medullary stromal cells but show different staining patterns. ER-TR5 detects medullary epithelial cells (fig. 2b) and ER-TR6 reacts with other medullary stromal cells such as interdigitating cells and with medullary lymphoid cells (fig. 2c).

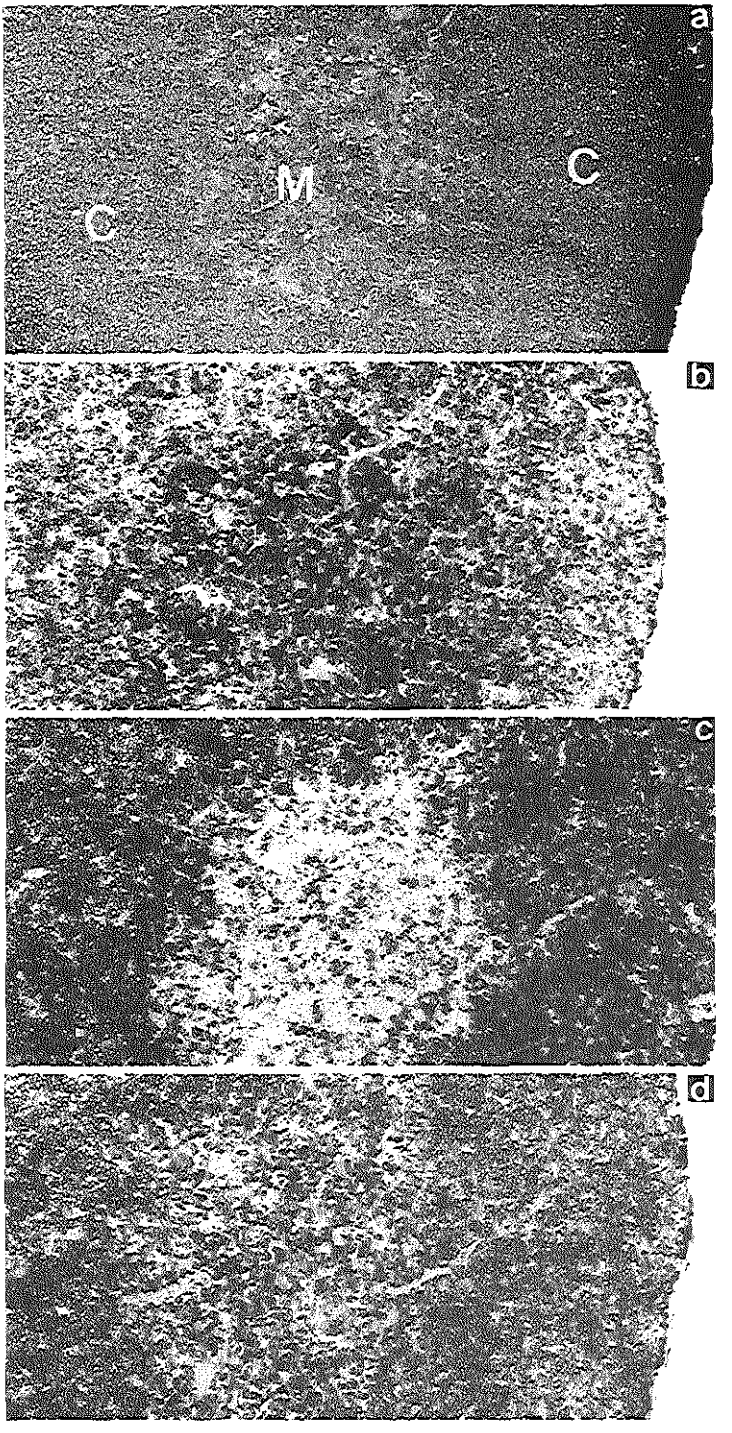
Incubation of serial frozen sections with ER-TR1, a monoclonal antibody which detects Ia antigens, show a reticular staining pattern in the cortex and a confluent staining in the medulla (fig. 2d).

Incubation with anti-H-2K antibodies (M1/42.3.9.8) reveal a confluent staining in the medulla whereas in the cortex to some extent, stromal and lymphoid cells are stained (fig. 2e).

A T cell and stromal cell distribution, identical to that observed in sham- irradiated grafts and in the thymus in situ was observed in all irradiated grafts.

2. The size of thymic graft

Volume determinations of identically pretreated thymic grafts, measured 1 or 2 months after transplantation, revealed no significant differences in size. Since immunohistology as well as size of these grafts, 1 and 2 months after transplantation, were identical, data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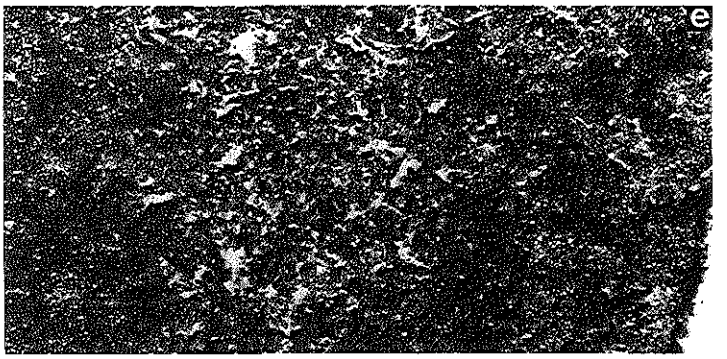
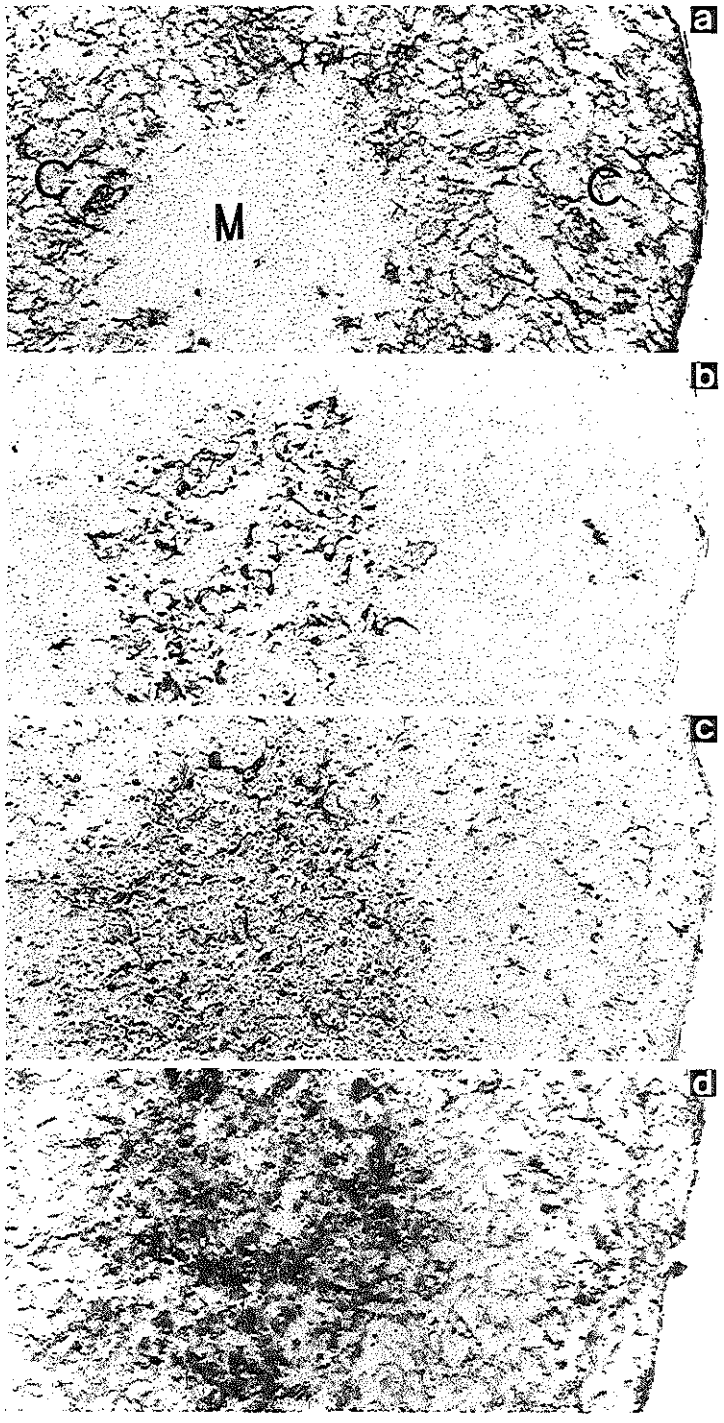


Fig. 1. Immunoperoxidase staining of serial frozen sections of a thymic graft irradiated with 15.00 Gy X-rays prior transplantation, 2 months after transplantation, incubated with monoclonal anti-Thy-1 (a), anti-Lyt-1 (b), anti-Lyt-2 (c), anti-MT-4 (d), and anti-T-200 (e). C = cortex, M = medulla. (x 140).

obtained from similar pretreated grafts will be discussed together. The transplanted sham-irradiated grafts grow very well and show an increase in graft size from 1.2 to 31.0 mm^3 in 4-8 weeks (fig. 3).

Comparison of the size of neutron or X-irradiated grafts with the size of sham-irradiated control grafts reveales evidently that the size of the irradiated grafts was deminished (Table II). Furthermore, this size reduction shows a clear dose-response relation after neutron irradiation (Fig. 3). The X-ray curve is linear for doses in excess of 6.00 Gy. For the determination of the relative biological effectiveness (RBE) of fast fission neutrons compared with X-rays, i.e. the ratio of


a
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M
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Fig. 2. Immunoperoxidase staining of serial frozen sections of a thymic graft irradiated with 15.00 Gy X-rays prior transplantation, 2 months after transplantation, incubated with ER-TR4 (a), ER-TR5 (b), ER-TR6 (c), ER-TR1 (d), and anti-H-2K monoclonal antibodies (M1/42.3.9.8) (e). For abbreviations see Fig. 1 (x 140).

the absorbed dose of neutrons to the absorbed dose of X- rays to produce the same biological effect, regression analysis was performed. An RBE value equal to 2.1 ± 0.1 (sem) was calculated for a reduction of the relative thymic graft size to 10% of the sham-irradiated graft size.

3. Flow cytofluorometric analysis of T cell subpopulations in the spleen of nude mice

Incubation of spleen cells of sham-operated nude mice with monoclonal anti-Thy-1 antibodies, an antibody which detects virtually all T

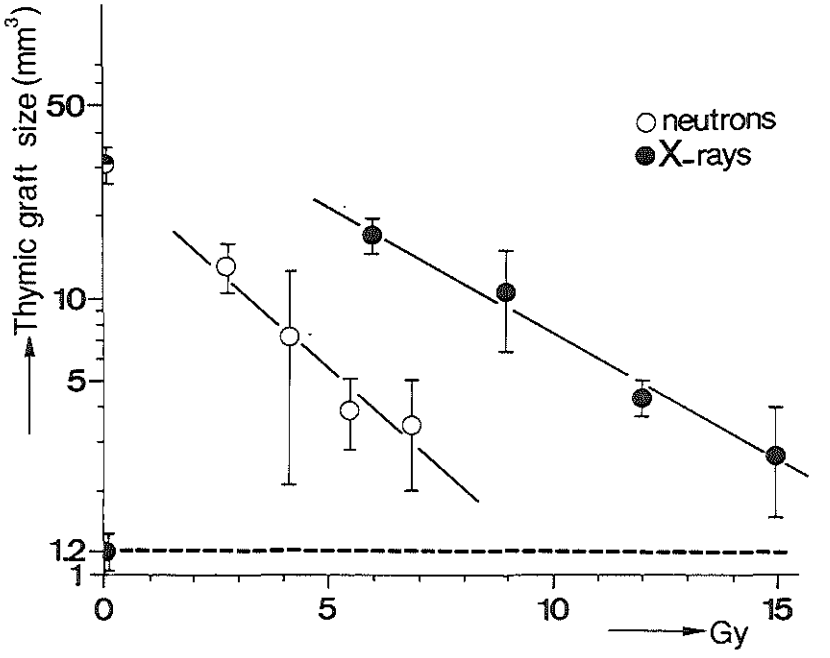

Fig. 3. Dose-response curves of the relative thymic graft size after irradiation with fission neutrons (o) or X-rays (•) prior to the transplantation. Dashed line indicates size of the transplanted thymic lobe, at time of transplantation.

cells, reveals that about 6 percent of the total nucleated spleen cel population is Thy-1$^+$ (Fig. 4). This number significantly increases upto 12 percent in mice grafted with a sham-irradiated thymus lobe. Irradiation of the graft prior to transplantation gives no conclusive dose-response relationship.

Incubation of splean cells of sham-operated nude mice with anti-Lyt-2 antibodies, an antibody which in the periphery detects cytotoxic and/or suppressor T cells, reveals that about 1.3 percent of the spleen cels shows Lyt-2$^+$ phenotype (Fig. 4). After grafting of a sham-irradiated thymus lobe, the percentage Lyt-2$^+$ cells significantly in-

TABLE II The size of thymic grafts after transplantation

Radiation exposure	n*	graftsize (mm^3) $\bar{x} \pm s$
X-ray dose (Gy)		
6.00	5	16.7 ± 2.0
9.00	5	10.5 ± 4.1
12.00	3	4.3 ± 0.6
15.00	5	2.7 ± 1.3
Neutron dose (Gy)		
2.75	5	13.1 ± 2.5
4.13	5	7.2 ± 5.1
5.50	3	3.9 ± 1.1
6.88	5	3.5 ± 1.5
Control	5	31.0 ± 4.6

*n = number of thymic graft recipients used for the volume determination of the transplants.

creases. Irradiation of the thymus grafts prior to transplantation with neutron or X-rays, upto 5.50 or 12.00 Gy respectively, results in a slight decrease of the percentage Lyt-2^+ spleen cells when compared with mice with sham-irradiated grafts. At the highest doses of both types of irradiation, the percentage Lyt-2^+ spleen cells is comparable with that in sham-operated nude mice (Fig. 4).

Incubation of spleen cells with anti-MT-4 antibodies which detect T helper cells, reveals that about 1.7 percent of the spleen celss in sham-operated nude mice are MT-4^+ (Fig. 4). Grafting of a nude mouse with a sham-irradiated thymus results in a significant increase of the percentage MT-4^+ spleen cells with a factor of about 4. After 2.75 Gy neutron irradiation or X-irradiation upto 12.00 Gy of grafts prior to

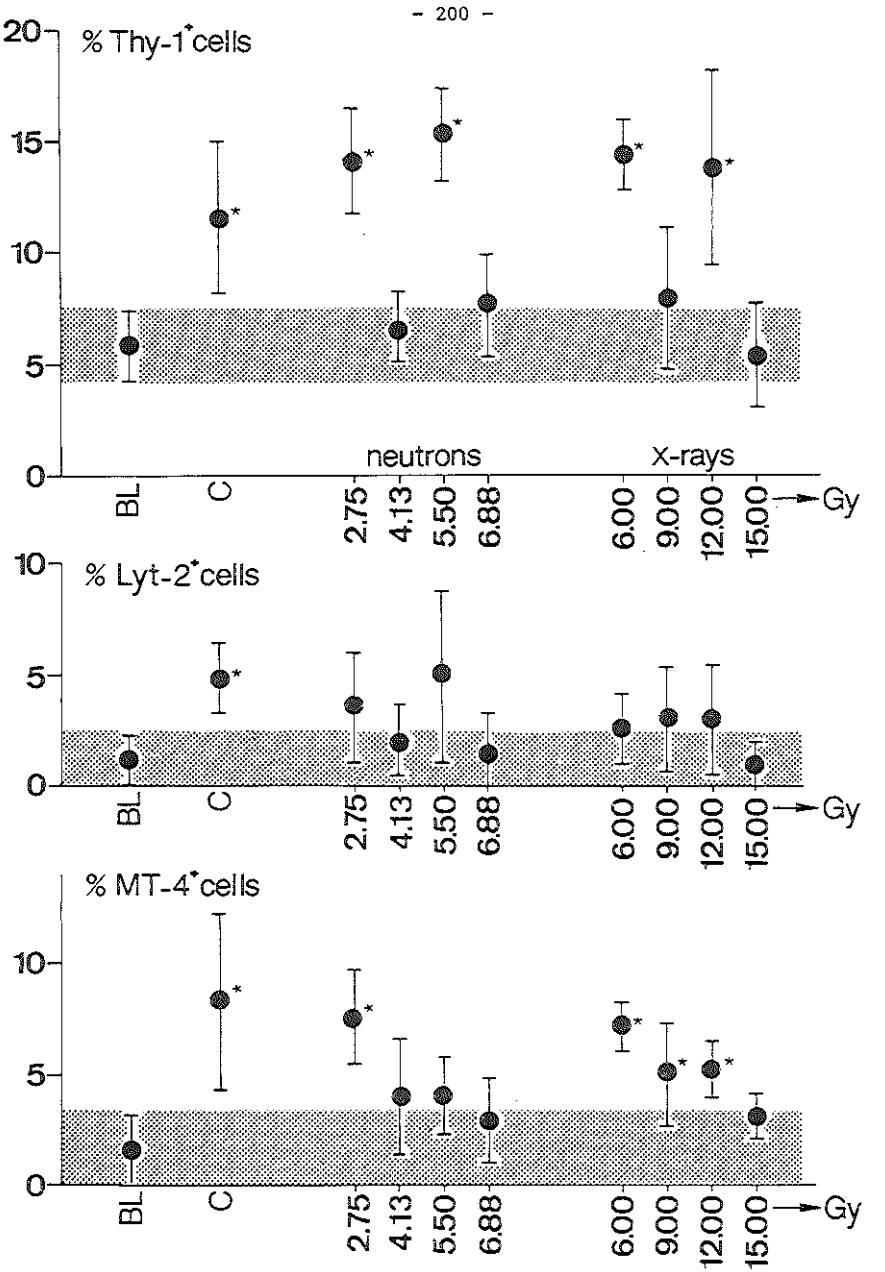

Fig. 4. Frequency of positive cells in spleen suspensions stained with anti-Thy-1, anti-Lyt-2, or anti-MT-4 in sham-operated nude mice (Bl), nude mice grafted with a sham-irradiated thymic lobe (C), and mice grafted with a neutron or X-irradiated thymic lobe. Asterics indicates significant difference from sham-operated group (BL).

transplantation, lower but still significant increases of the percentage MT-4$^+$ spleen cells can be observed when compared with sham-operated nude mice (Fig. 4). Further increasing of the dose of irradiation to the grafts results in a decrease of the percentage MT-4$^+$ cells to a comparable level as observed in sham-operated nude mice.

In summary, transplantation of sham-irradiated thymic lobes under the kidney capsule of nude mice significantly increases the Thy-1$^+$, Lyt-2$^+$ and MT-4$^+$ cell frequencies in the nude spleen. Irradiation with graded doses of fission neutrons or X-rays prior to transplantation results in a decrease of Lyt-2$^+$ and MF-4$^+$ cell frequencies to the levels observed in sham-operated nude mice.

DISCUSSION

In the present investigation, neonatal CBA/H mice were exposed to graded doses of fast fission neutrons or X-rays in order to investigate the effects of irradiation on the stromal compartment of the thymus. The thymic lobes were transplanted were the kidney capsule of nude mice. Using immunohistology, the T cell composition and the stroma of the thymic grafts were characterized with monoclonal antibodies. In addition, the T cell composition of the spleen of the nude recipients was investigated with flow cytofluorometry.

Our results clearly show that sham-irradiated thymic lobes completely regenerate and differentiate within one month after the transplantation under the kidney capsule of nude mice. The T cell and stromal cell distributions are indistinguishable from those described for the normal thymus in situ (12, 22, 24). Thus, thymocytes with a phenotype comparable to that of peripheral immunocompetent T cells, i.e. Lyt-2$^+$, MT-4$^-$ or Lyt-2$^-$, MT-4$^+$ (25), are observed in the medulla of the graft whereas the cortical thymocytes have predominantly the immature Lyt-2$^+$, MT-4$^+$ phenotype. Furthermore, the cortical epithelial cells have a reticular distribution pattern and are I-A$^+$ and almost H-2K$^-$. Medullary stromal cells show a confluent I-A staining pattern and are H-2K$^+$. This structural presence of MHC antigens on thymic stromal cells has been implicated to be a prerequisite for normal T cell development (9, 11, 12, 14). Frozen sections of thymus grafts

stained with anti-MEL-14 antibodies, an antibody considered to be an export marker for thymocytes (26), showed a normal distribution of MEL-14$^+$ cells indicating a normal export of mature thymocytes (Huiskamp, unpubl. observation).

Irradiation of the thymic lobes prior to transplantation with graded doses neutrons or X-rays upto 6.88 and 15.00 Gy respectively, does not influence the T-cell and stromal cell distribution within the graft as compared with those of the thymus in situ. This indicates that thymic stromal cells and their T cell maturation supporting function within the graft is highly radioresistant. In line with this assumption are observations reported by Davis and Cole (15) and Hirokawa and Sado (17). In an irradiation model of the thymus in situ, they showed that a complete restoration of immunological functions could be observed even after thymic irradiation with X-ray doses up to 2000 R.

Flow cytofluorometric analysis of T cell subpopulations in the spleen of sham-operated nude mice, reveals that about 6 percent of the spleen cells express the pan T cell marker Thy-1. However, only about 1.3 and 1.7 percent of the spleen cells are Lyt-2$^+$ and MT-4$^+$ respectively.
The present results confirm reports that athymic mice can contain cells that are phenotypically T cells (27, 28). However, about half the Thy-1$^+$ cells don't express the functional markers Lyt-2 or MT-4. This indicates the presence of T cells with an "abnormal" nonfunctional phenotype, i.e. Thy-1$^+$, Lyt-2$^-$, MT-4$^-$. Similar findings have been observed by Chen et al. (29). These authors further showed that the majority of the nude spleen T cells, even within the phenotypically "normal" subsets, appeared to be nonfunctional. However, functional T cells in the spleen of nude mice can be obtained by grafting neonatal thymuses in these mice (30, 31). The present data clearly show that transplantation of a sham- irradiated neonatal thymus lobe in a nude mouse results in a significant increase of the Thy-1$^+$, Lyt-2$^+$ and MT-4$^+$ cell frequencies.

Irradiation of thymus lobes prior to transplantation with sublethal doses of neutrons or X-rays, i.e. 2.75 and 6.00 Gy respectively, reveals frequencies of Thy-1$^+$ and MT-4$^+$ spleen cells similar to mice grafted with a sham-irradiated thymic lobe. The Lyt-2$^+$ cell

frequency, however, was slightly decreased. This indicates that sublethal doses of both types of irradiation influence to some extent the immunological reconstitution capacity of the thymic graft. With increasing doses of irradiation with neutrons or X-rays upto 6.88 and 15.00 Gy respectively, a decrease in Lyt-2^+, and MT-4^+ cell frequencies was observed, indicating a decreased reconstitution capacity of the graft. For Thy-1^+ spleen cells, however, the effect of irradiating the thymus with graded doses prior to transplantation reveales no dose effect relationship but varying responses. At the highest neutron and X-ray dose, peripheral T cell numbers are observed which do not differ significantly from those observed in sham-operated nude mice. These results are in agreement with other thymus grafting experiments showing impaired restoration of immune functions after grafting of an 1500 R X-irradiated neonatal thymus (17).

As shown in previous studies, thymus regeneration after in situ irradiation involves both stromal recovery and repopulation by thymocytes (12, 14). After grafting of an irradiated thymus in nude mice, unirradiated prothymocytes are readily available for repopulation of the graft. Hence, the thymic stromal cells seems to be the determining factor for the reconstitution of the immunological capacity and growth of the graft. Therefore, the observed effect of irradiation on the size of the thymus graft are reducible to effects on the thymic stroma. Thus, the RBE value equal to 2.1 for the reduction of thymic graft size to 10 percent can be regarded as an RBE value for the thymic stromal cell population. Furthermore, according to this hypothesis, growth of the graft after irradiation is directly related to the surviving stromal stem cell population. In order to estimate D_0 values for this cell population, dose-response curves were calculated (fig. 5). Stromal cell survival after neutron irradiation shows a clear linear dose-response relation, that is characterized by a D_0 value equal to 2.45 ± 0.46 (sem) Gy. The X-ray curve has a shoulder, due to accumulation of sublethal damage, and is linear for doses in excess of 6.00 Gy. The X-ray curve is characterized by a D_0 value equal to 3.68 ± 0.36 (sem) Gy. This latter D_0 is somewhat higher than the X-ray D_0 value (3.2 Gy) reported for dog thymus epithelium, estimated in vitro by Sharp & Watkins (33). Species and methodology differences can account for the difference.

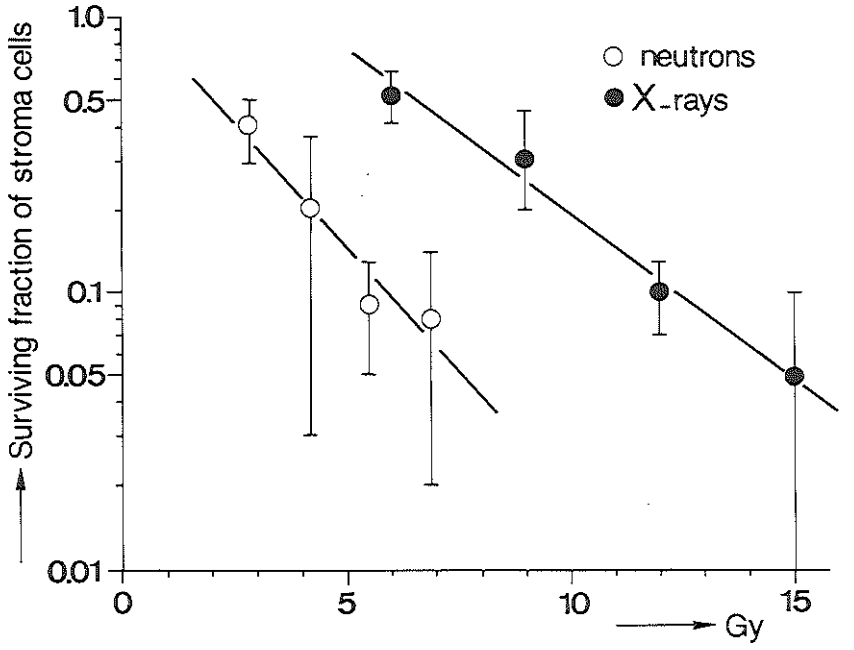

Fig. 5. Dose-response curves for the surviving fraction of stromal stem cells after fission neutrons (o) or X-rays (•). Lines calculated with linear regression analysis from the relative graft size, 4-8 weeks after transplantation, corrected for the initial graft size at transplantation.

The present results are in contrast with in vivo experiments in which only the thymus was X-irradiated. Doses up to 2000 R did not influence the immunological functions in these animals (15, 17). However, as shown in our experiments, the dose-response curve after X-rays for stromal cell survival has a large shoulder indicating repair of sublethal damage. The size of this shoulder might be smaller than that of a shoulder obtained with in situ irradiation since transplantation experiments involve additional necrosis (31). Therefore, differences in repair of sublethal damage can explain the fore mentioned difference.

As shown in a previous paper (13), whole-body irradiation with sublethal doses of neutrons or X-rays results in long-term effect on the thymus. This effect, i.e. decreases in the relative thymic weight and cellularity might besides bone marrow damage, also involve thymic stromal cell damage which due to the long turnover of the stromal cells, only emerges as a long-term effect after irradiation. Furthermore, these effects were in general larger after neutron irradiation (13) and this again may be explained by the large shoulder in the stromal cell survival curve after X-irradiation observed in the present study.

Finally, an alternative explanation for the reduced thymic graft size could be accelerated aging of the thymus graft (32). However, the immunohistology of thymic grafts showed no reduced Ia expression on thymic epithelial cells which is reported to be associated with aging of the thymus (33).

In conclusion, the results of the present investigation show that irradiation of the neonatal thymus with graded doses neutrons or X-rays prior to transplantation in nude mice, does not influence the T cell and stromal cell distribution in the graft. However, the graft size is dose-dependently decreased. Furthermore, the peripheral T cell number decreases when irradiation is given to the graft prior to transplantation. The present data indicate that the T cell maturation supporting ability of thymic stromal cells is highly radioresistant. However, the regenerative potential of the thymic stromal cells after the irradiation and transplantation is considered to be radiosensitive.

REFERENCES

1. Kruisbeek, A.M., Thymic factors and T cell maturation in vitro: a comparison of the effects of thymic epithelial cultures with thymic extracts and thymus dependent serum factors. Thymus 1, 163-185 (1979).

2. Trainin, N., M. Pech, and Z.T. Handzel, Thymic hormones: inducers and regulators of the T cell system. Immunol. Today 4, 16-21 (1983).

3. Stutman, O., Intrathymic and extrathymic T cell maturation. Immunol. Rev. 42, 138-184 (1978).

4. Rouse, R.V., W. van Ewijk, P.P. Jones, and I.L. Weissman, Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122, 2508-2515 (1979).

5. van Ewijk, W., R.V. Rouse, and I.L. Weissman, Distribution of H-2 micro-environments in the mouse thymus. Immunoelectronmicroscopic identification of I-A and H-2K bearing cells. J. Histochem. Cytochem. 28, 1089-1099 (1980).

6. Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein and J. Klein, On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence of dual recognition? J. Exp. Med. 14, 882-896 (1978).

7. Kruisbeek, A.M., S.O. Sharrow, B.J. Mathieson, and A. Singer, The H-2 phenotype of the thymus dictates the self specificity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. J. Immunol. 126, 2168-2176 (1981).

8. Kwast, M.W., L.P. de Waal, and C.J.M. Melief, Thymus dictates major histocompatibility complex (MHC) specificity and immune response gene phenotype of class II MHC-restricted T cells but not of class I MHC-restricted T cells. J. Exp.Med. 160, 1752-1766 (1984).

9. Jenkinson, E.J., W. van Ewijk, and J.J.T. Owen, Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice. J. Exp. Med. 153, 280-292 (1981).

10. Sprent, J., Effects of blocking helper T cell induction in vivo with anti-Ia antibodies. Possible role of I-A/E hybrid molecules as restriction elements. J. Exp. Med. 152, 996-1010 (1980).

11. Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer and J.J. Mond, Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia antibodies interferes with differentiation of I-restricted T cells but not K/D-restricted T cells. J. Exp. Med. 157, 1932-1946 (1983).

12. Huiskamp, R., E. van Vliet, and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. II. sequential changes in the thymic micro-environment. J. Immunol. 134, 2170-2178 (1985).

13. Huiskamp, R., J.A.G. Davids, and O. Vos, Short- and long-term effects of whole-body irradiation with fission neutrons or X-rays on the thymus in CBA mice. Radiat. Res. 95, 370-381 (1983).

14. Huiskamp, R. and W. van Ewijk, Repopulation of the mouse thymus after sublethal fission neutron irradiation. I. Sequential appearance of thymocyte subpopulations. J. Immunol. 134, 2161-2169 (1985).

15. Davis, W.F. and C.J. Cole, Retarded immunological recovery in sublethally X-irradiated mice by additional thymic exposure. Reversal with injected marrow cells. Proc. Soc. Exp. Biol. Med. 130, 1336-1344 (1969).

16. Dukor, P., J.F.A.P. Miller, W. House, and V. Allman, Regeneration of thymus grafts. I. Histological and cytological aspects. Transplantation 3, 639-668 (1965).

17. Hirokawa, K. and T. Sado, Radiation effects on regeneration and T-cell-inducing function of the thymus. Cell. Immunol. 84, 372-379 (1984).

18. van Ewijk, W., P.L. van Soest, and G.J. van den Engh, Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T-200, J. Immunol. 127, 2594-2604 (1981).

19. van Ewijk, W., P.L. van Soest, A. Verkerk, and J.F. Jongkind, Loss of antibody binding to prefixed cells: fixation parameters for immunocytochemistry. Histochem. J. 16, 179-193 (1984).

20. Ledbetter, J.A. and L.A. Herzenberg, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 61-90 (1979).

21. Pierres, A., P. Naquet, A. Agthoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A.M. Schmitt-Verhulst, and M. Pierres, Rat anti-mouse T4 monoclonal antibody (Hl29.9) inhibits the proliferation of Ia-reactive T cell clones and delineates two phenotypically distinct ($T4^+$, Lyt2, 3^- and $T4^-$, Lyt-2,3^+) subsets among anti-Ia cytolytic T cell clones, J. Immunol. 132, 2775-2782 (1984).

22. van Vliet, E., M. Melis, and W. van Ewijk, Monoclonal antibodies to stromal cell types of the mouse thymus. Eur. J. Immunol. 14, 524-529 (1984).

23. Springer, T.A., Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. In Monoclonal Antibodies (R.H. Kennet, T.J. McKearn, and K.B. Bechtol, eds.), pp 185-192. Plenum Press, New York, 1980.

24. van Ewijk, W., Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. Am. J. Anat. 170, 311-330 (1984).

25. Scollay, R. and K. Shortman, Thymocyte subpopulations: an experimental review, including flow cytometric cross-correlations between the major murine thymocyte makers. Thymus 5, 245-295 (1983).

26. Reichert, W.A., W.M. Gallatin, E.C. Butcher, and I.L. Weissman, A homing receptor bearing cortical thymocyte subject: implications for thymus cell migration and the nature of cortisone-resistant thymocytes. Cell. 38, 89-99 (1984).

27. MacDonald, H.R., R.K. Lees, B. Sordat, P. Zaech, J.L. Maryanski, and C. Bron, Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1, and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. J. Immunol. 126, 865-870 (1981).

28. Maryanski, J.L., H.R. MacDonald, B. Sordat, and J.C. Cerottini, Cell surface phenotype of cytolytic T lymphocyte precursors in aged nude mice. Eur. J. Immunol. 11, 968-972 (1981).

29. Chen, W.F., R. Scollay, K. Shortman, M. Skinner, and J. Marbrook, T-cell development in the absence of a thymus: the number, the phenotype, and the functional capacity of T lymphocytes in nude mice. Am. J. Anat. 170, 339-347 (1984).

30. Sharp, J.G. and E.B. Watkins, Cellular and immunological consequences of thymic irradiation. In: Immuno-pharmacologie Effects of Radiation Therapy (J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds.), pp. 137-179. Raven Press, New York, 1981.

31. Kindred, B., Nude mice in immunology. Prog. Allergy 26, 137-238 (1979).

32. Hirokawa, K., K. Sato and T. Makinodan, Influence of age of thymic grafts on the differentiation of T cells in nude mice. Clin. Immunol. Immunopathol. 24, 251-262 (1982).

33. Farr, A.G., and C.L. Sidman, Reduced expression of Ia antigens by thymic epithelial cells of aged mice. J. Immunol. 133, 98-103 (1984).

CHAPTER X

GENERAL DISCUSSION

As described in Chapter I of this thesis, the thymus is an extremely complex lympho-epithelial organ in which bone marrow-derived lymphoid precursor cells, i.e. prothymocytes, differentiate and mature in a stromal matrix. During their differentiation in this specialized microenvironment, thymocytes are selected on the basis of tolerance to self-MHC gene products and they acquire the capacity to recognize foreign antigens in the context of self-MHC antigens. Furthermore, during this differentiation, the thymocytes acquire a number of cell surface differentiation antigens. It is generally accepted that the thymic stromal cells are involved in the process of differentiation and maturation of T cells.

When animals are subjected to whole-body irradiation, severe effects develop in the thymus. As shown by many authors, irradiation with X-rays or gamma-rays, i.e. low LET radiation types, leads to a severe depopulation of the thymus and, subsequently, thymus recovery has been shown to follow a biphasic pattern (reviewed by Sharp & Crouse, 1980 and Watkins et al., 1980). The initial phase in this biphasic thymic recovery is brought about by **radioresistant intrathymic precursor cells** which are not derived immediately from bone marrow stem cells. Limited proliferative capacity and the resulting exhaustion of these **intrathymic precursor cells** as well as an impaired production of thymus precursor cells in the bone marrow are responsible for a second thymus involution. The final recovery of the thymus is due to its replenishment from **extrathymic** precursors in the regenerated bone marrow.

There are only limited data available concerning the **radiosensitivity** of lymphoid and stromal components of the thymus. In addition, the precise nature and progeny of the intrathymic radioresistant precursor cells are still poorly characterized. In contrast to many short term studies with X-rays, very limited information is available in the literature concerning their long-term effects and the effects of high LET radiation, such as fast fission neutrons, on the thymus. In

the foregoing chapters of this thesis, these aspects of the radiation effects on the thymus were investigated in detail.

The experiments described in Chapter III in which CBA mice were exposed to whole-body irradiation with either 2.5 Gy fast fission neutrons or 6.0 Gy X-rays, confirm the cyclic nature of the thymic recovery pattern after irradiation, first described by Takada et al. (1969). Furthermore, this pattern was also observed in a related mouse strain, i.e. CBA/H (Chapter V) which was used for the experiments described in the Chapters IV-IX. Irradiation causes a strong cellular depopulation of the thymus. Within 48 hours after irradiation, almost all cortical cells and 70-80% of the medullary cells are lost (Chapters III, V, VIII). Cortical thymocytes are very radiosensitive and have D_0 values of about 0.26 and 0.60 Gy for fission neutrons and X-rays, respectively (Chapter VIII). The latter D_0 value is in good agreement with those reported by Trowell (1961) and Sato and Sakka (1969). Furthermore, D_0 values of about 0.30 and 0.70 Gy for fission neutrons and X-rays, respectively, were estimated as a composite value for the cortical cells and the radiosensitive part of the medullary thymocyte pool. Apart from the radiosensitive thymocytes, a small fraction of about 3%, was observed that is refractory to radiation up to 2.5 Gy neutrons or 6.0 Gy X-rays (Chapter VIII). Based on the surface marker analysis of these cells, immunohistology of sham-irradiated and irradiated thymuses and recent studies of Müller-Hermelink et al. (1984), these cells have a medullary phenotype, i.e. dull Thy-1^+, bright Lyt-1^+, bright T-200^+ and mainly Lyt-2^-. Within 3 days hereafter, these cells are no longer present in the thymus (Chapter VIII). Similar results were reported by Müller-Hermelink and co-workers (1984). The fate of these cells is unclear. We favor the hypothesis that these cells leave the thymus and migrate to peripheral lymphoid organs. These radiorefractory cells could be similar to a fraction of peripheral T cells observed in chimeric mice that are host-derived and long-lived (Hirokawa et al., 1985).

We further demonstrated (Chapter VI) that the cortical stromal architecture is severely influenced by sublethal whole-body irradiation, whereas the medullary compartment is more or less unaffected.

Within 24 hours, the epithelial cells are rounded, vacuolized and have lost their membrane bound expression of I-A antigens. During regeneration of the thymus, these "nurse cell-like" epithelial cells loose their vacuoles. Subsequently, the cortex becomes repopulated by large lymphoid "null" cells, i.e. lymphoid cells expressing no differentiation antigens. These "null" cells are probably identical to the proposed **intrathymic radioresistant precursor cells** (Kadish & Basch, 1975) that repopulate the thymus after irradiation (Sharrow et al., 1983; Hirokawa et al., 1985). Based on our observation that these cells do not express differentiation antigens, an estimate of the **radiosensitivity** of these precursor cells was made (Chapter VIII). The D_0 values of the "null" cells were estimated to be 1.32-1.55 Gy for X-rays and 0.64-0.79 Gy for fission neutrons. However, the X-ray D_0 value reported in our studies, is markedly different from the one reported by Sharp and Watkins (1981) for intrathymic radioresistant precursor cells. Their assay, i.e. electronic cell counting, 10 days after a dose of 8.0 Gy X-irradiation, is different from the method used in Chapter VIII. The estimated RBE value (2.1 - 2.2) for the **intrathymic radioresistant precursor cells** points to the bone marrow as the ultimate origin of these cells (Chapter VIII).

The repopulation of the cortex with "null" cells coincides with the appearance of a bright H-2K staining on stromal as well as lymphoid cells. The "null" cells were identified as H-2K$^+$. Similar thymocyte precursors have been identified in the fetal thymus (Ritter, 1978; Kamarck & Gottlieb, 1977) and in the adult thymus (Goldschneider et al., 1982; Boersma, 1982). Furthermore, our results show that "null" cells appear in the subcapsular area as well as in the medulla (Chapter V). This observation is in concordance with recently published data on the repopulation of the avian thymus (Jotereau et al., 1982) and the murine thymus (Ezine et al., 1984) and points to mutually independent generation kinetics for cortical and medullary thymocytes. Recently, Scollay and Shortman (1985) proposed that the separate developmental streams of cortical and medullary thymocytes may be traced back to a single, **common**, precursor cell located in the subcapsular region. However, our observations on thymic lobes with an aberrant T cell differentiation, long-term after neutron irradiation (Chapter VII)

indicate also the presence of a cortex independent medullary precursor lineage besides a cortex to medulla lineage. In line with this observation is a recent immunohistochemical analysis performed by Ceredig and Schreyer (1984) on the course of bone marrow-derived repopulation of the thymus in chimeric mice with Thy-1 allelic differences. They showed that there are two sites of cellular entrance into the thymus, i.e. the subcapsular area and the medulla.

During the regeneration of the thymus after irradiation, "null" cells aquire the differentiation antigens Thy-1, T-200, Lyt-1, Lyt-2 and MT-4 (Chapter V). Sequentially, the following phenotypes were observed in the cortex: 1 Thy-1^+ "only" and T-200^+ "only" cells; 2 Thy-1^+, T-200^+ cells; 3 Thy-1^+, T-200^+, MT-4^+, Lyt$^+$ cells. Similar phenotypical sequences have been observed during ontogeny (van Ewijk et al., 1982; Ceredig et al., 1983) and in the thymus of cortisone-treated mice (van Ewijk et al., 1981, van Vliet et al., submitted for publication). In addition, cells with similar phenotypes have been identified in the normal adult thymus (Chapter IV; Scollay & Shortman, 1983, 1985). The cells that arise and differentiate from the "null" are less radiosensitive than similarly defined cells immediate after the irradiation (Chapter VIII). Five days after the irradiation, cortical cells have D_0 values of 0.37 and 0.99 Gy for neutrons and X-rays, respectively. The composite D_0 value for cortical and medullary cells is 0.44-0.48 Gy for fission neutrons and 1.14-1.17 Gy for X-rays. The latter D_0 value agrees rather well with the X-ray D_0 value reported by Sharp and Watkins (1981). The fission neutron RBE values for reduction of these cell populations to 1 percent vary from 2.6-2.8 (Chapter VIII).

The described sequence in thymocyte differentiation after irradiation coincides with the reduction of H-2K expression to control levels (Chapters V, VI). Furthermore, the reappearance of a reticular I-A staining pattern in the cortex is related with the acquisition of Lyt-1, Lyt-2 and MT-4 differentiation antigens (Chapter VI). A similar correlation between the regeneration of the thymic stroma and the expression of cell surface differentiation antigens was also observed in the thymus of cortisone-treated mice (van Vliet et al., submitted

for publication). Furthermore, the onset of T cell differentiation in the embryonic thymus correlates with the initial expression of MHC antigens on the thymic epithelial cells (Jenkinson et al., 1980). Another morphological indication for the relevance of the thymic stroma in T cell differentiation is the observed correlation of an abnormal MHC expression on cortical stromal cells in thymus lobes together with an aberrant T cell differentiation (Chapter VIII). Together these observations strongly argue that a normal stromal MHC expression is a prerequisite for normal T cell development.

Besides the morphological studies, a number of functional studies have directly proven that the thymic stroma can influence T cell differentiation. Sprent (1980) demonstrated that in vivo administration of monoclonal antibodies directed to I-A/E determinants interfered with the activation of Ia-restricted splenic T cells. Furthermore, neonatal mice given multiple injections with purified anti-I-A antibodies, lack thymic and splenic immunocompetent Ia restricted $L3T4^{+}$, $Lyt\text{-}2^{-}$ T helper cells and have a decreased Ia-antigen expression in the thymus, especially in the medulla (Kruisbeek et al., 1983, 1985). Recently, DeLuca (1986) further confirmed the role of class II MHC molecules in T cell differentiation. In an in vitro culture system of fetal thymic lobes, it was shown that addition of monoclonal anti-I-A antibodies to the culture resulted in a decreased lymphocyte yield and in a decreased responsiveness of these cells in a mixed lymphocyte culture assay. Furthermore, the treatment led to the disappearance of Ia-positive non-lymphoid cells. DeLuca also showed that in cocultures of Ia positive non-lymphoid cells enriched thymic lobes and fetal liver, bone marrow, or a normal thymic lobe, the thymus derived precursor cells but not precursor cells from bone marrow or fetal liver, are selected to proliferate only with thymic stroma bearing self-Ia antigens. Thus, both immunohistological as well as functional data indicate that the thymic microenvironment and especially the MHC molecules of the stromal cells regulate the differentiation of thymocytes and, after irradiation, a structuraly organized thymic stroma is required before the functional T cell differentiation process can start.

Although the thymic stromal cells are thought to be very radio-

resistant (Sharp & Watkins, 1981), the present studies (Chapters V, VI) clearly show that the thymic stromal architecture is severely influenced after irradiation with either fission neutrons or X-rays. However, using a transplantation model in which nude mice were grafted with an irradiated neonatal thymic lobe, we showed that the T cell distribution and the stromal cell composition of the graft, one month after transplantation, is not influenced by irradiation with doses up to 6.88 Gy neutrons or 15.00 Gy X-rays (Chapter IX). This observation indicates that the thymic stromal cells and the ability of these cells to support the T cell differentiation are highly radioresistant. In line with this conclusion are studies reported by Davis and Cole (1969) and Hirokawa and Sado (1984). They showed in an irradiation model of the thymus in situ, that thymic irradiation with doses up to 2000 R had no influence on the restoration of immunological functions in these mice. However, the present study (Chapter IX) shows that the graft growth decreases with increasing doses of irradiation given to the graft prior to the transplantation. In addition, when the highest neutron or X-ray dose was given to the graft, T cell numbers in the spleen of the engrafted nude mice were comparable with those observed in sham-operated mice. Thus, the immunological reconstitution of nude mice is impaired when high dose irradiated thymic lobes are grafted. We further conclude that injury of the thymic stromal cells is responsible for the decreased reconstitution capacity and decreased size of the graft after irradiation. Therefore, size, i.e. growth, of the graft is directly related to the surviving fraction of stromal stem cells in the graft after irradiation. The D_0 values for this cell population are equal to 2.45 Gy and 3.68 Gy for neutrons and X-rays, respectively. The latter D_0 value is somewhat higher than the X-ray D_0 value (3.2 Gy) reported by Sharp and Watkins (1981) for dog thymus epithelium but species and methodology differences may account for this.

The fission neutron RBE determination for the reduction of thymic size to 10 percent was equal to 2.1 and can be regarded as an RBE value for the thymic stromal cell population. This value is comparable with the fission neutron RBE value determined for the bone marrow (Davids, 1973) and the reported RBE value for the intrathymic precursor cells reported in Chapter VIII.

The decrease in immunological function of the graft observed in our experiments is in contrast with studies by Davis and Cole (1969) and Hirokawa and Sado (1984). The X-ray dose-response curve for stromal cell survival (Chapter VIII) has a large shoulder indicating accumulation of sublethal damage. Our experiments, however, also include necrosis induced by the transplantation of the irradiated graft. Therefore, the size of the shoulder in our experiments might be smaller than in experiments without transplantation. These differences in accumulation of sublethal damage might explain the differences between our results and those reported by Davis and Cole (1969) and Hirokawa and Sado (1984).

As shown in Chapters III and V, whole-body irradiation with 2.5 Gy fission neutrons or 6.00 Gy X-rays has also long-term effects on the thymus. The observed effect i.e. decreases in relative thymus weight and cellularity were in general larger after neutron irradiation. In Chapter III we attributed this effect to residual damage in the bone marrow causing an impaired production of prothymocytes. However, we have also shown that the proliferative capacity of the stromal cells is radiosensitive and already decreased at the above mentioned doses of irradiation (Chapter VIII). Thus, this long-term effect of radiation on the thymus might also be caused by stromal cell damage which only emerges as a long-term effect due to the long turnover of stromal cells. Repair of sublethal damage after X-irradiation could explain the differences between the magnitude of the effect after neutron and X-irradiation. A similar explanation might pertain why thymic lobes with an aberrant T cell distribution are observed only after neutron irradiation but the precise nature of this long-term effect following neutron irradiation remains to be further investigated.

In summary, this thesis describes in detail (short- and) long-term effects of high and low LET radiation types on the thymus. Besides determinations of the radiosensitivity of lymphoid and stromal cells in the thymus, morphological evidence emerges which indicates that the thymic stroma supports and regulates T cell differentiation after neutron and X-irradiation. Furthermore, this thesis provides

fission neutron RBE determinations pertaining to the cells in the thymus. These data may be used for fast neutron radiotherapy in the chest and neck regions, and protective treatment after accidental exposure to fast fission neutrons.

REFERENCES

Boersma, W.J.A. Characterization of mouse prothymocytes. Thesis, Erasmus University. Publication of the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands. 1982.

Ceredig, R. and M. Schreyer. Immunohistochemical localization of host and donor-derived cells in the regenerating thymus of radiation bone marrow chimeras. Thymus 6, 15 (1984).

Davids, J.A.G. Acute effects of 1 MeV fast neutrons on the haematopoietic tissues, intestinal epithelium and gastric epithelium in mice. In Advances in Radiation Research (J.F. Duplan and A. Chapiro, Eds.), Vol. II, p. 565. Gordon and Breach, New York, 1973.

Davis, W.F. and C.J. Cole. Retarded immunological recovery in sublethally X-irradiated mice by additional thymic exposure. Reversal with injected marrow cells. Proc. Soc. Exp. Biol. Med. 130, 1336 (1969).

DeLuca, D. Ia-positive nonlymphoid cells and T cell development in murine fetal thymus organ cultures: monoclonal anti-Ia antibodies inhibit the development of T cells. J. Immunol. 136, 430 (1986).

Ezine, S., I.L. Weissman and R.V. Rouse. Bone marrow cells give rise to distinct cell clones within the thymus. Nature 309, 629 (1984).

Goldschneider, I., K. Shortman, D. McPhee, D.S. Linthicum, G. Mitchell, F. Battye and F.J. Bollum. Identification of subsets of proliferating low Thy-1 cells in thymus cortex and medulla. Cellular Immunol. 69, 59 (1982).

Hirokawa, K. and T. Sado. Radiation effects on regeneration and T cell

inducing function of the thymus. Cellular Immunol. 84, 372 (1984).

Hirokawa, K. T. Sado, S. Kubo, H. Kamisaku, K. Hitomi and M. Utsuyama. Intrathymic T cell differentiation in radiation bone marrow chimeras and its role in T cell emigration to the spleen, an immunohistochemical study. J. Immunol. 134, 3615 (1985).

Jenkinson, E.J., J.T.T. Owen and R. Aspinall. Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus. Nature 284, 177 (1980).

Jotereau, F.V. and N.M. LeDouarin. Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. J. Immunol. 129, 1869 (1982).

Kadish, J.L. and R.S. Basch. Thymic regeneration after lethal irradiation: evidence for an intra-thymic radioresistant T cell precursor. J. Immunol. 114, 452 (1975).

Kamarck, M.E. and P.D. Gottlieb. Expression of thymocyte surface allo-antigens in the fetal mouse thymus in vivo and in organ culture. J. Immunol. 119, 407 (1977).

Kruisbeek, A.M., M.J. Fultz, S.O. Sharrow, A. Singer and J.J. Mond. Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia-antibodies interferes with differentiation of I-restricted T cells but with K/D-restricted T cells. J. Exp. Med. 157, 1932 (1983).

Kruisbeek, A.M., J.J. Mond, B.J. Fowlkes, S. Bridges and D.L. Longo. In vivo treatment of neonatal mice with anti-Ia antibodies: development of the $L3T4^{+}$ $Lyt\text{-}2^{-}$ T cell subset is dependent on thymic Ia bearing antigen-presenting cell function. J. Exp. Med. 161, 1029 (1985).

Müller-Hermelink, H.K., M. Gülden and R. Bathmann. Restitution of the thymus in lethally irradiated mice after transplantation of syngeneic or allogeneic bone marrow. Immunobiol. 167, 462 (1984).

Ritter, M.A. Embryonic mouse thymus development: stem cell entry and differentiation. Immunology 34, 69 (1978).

Sato, C. and M. Sakka. Cell population analysis of radiation damage and recovery of thymus and lymph node in mice. Radiat. Res. 38, 204 (1969).

Scollay, R. and K. Shortman. Thymocyte subpopulations: an experimental review, including flow cytometric cross correlations between the major murine thymocyte markers. Thymus 5, 245 (1983).

Scollay, R. and K. Shortman. Identification of early stages of T lymphocyte development in the thymus cortex and medulla. J. Immunol. 134, 3632 (1985).

Sharp, J.G. and D.A. Crouse. Role of the thymus in hematopoiesis. In Experimental Hematology Today 1980. (S.J. Baum, G.D. Ledney, and D.W. van Bekkum, Eds.) p. 63, Karger, Basel, 1980.

Sharp, J.G. and E.B. Watkins. Cellular and immunological consequences of thymic irradiation. In Immunopharmacologic Effects of Radiation Therapy (J.B. Dubois, B. Serrou, and C. Rosenfeld, Eds.) p. 137. Raven Press, New York, 1981.

Sharrow, S.O., A. Singer, U. Hämmerling and B.J. Mathieson. Phenotypic characterization of early events of thymic repopulation in the radiation bone marrow chimeas. Transplantation 35, 355 (1983).

Sprent, J. Effects of blocking helper T cell induction in vivo with anti-Ia antibodies. Possible role of IA/E hybrid molecules as restriction elements. J. Exp. Med. 152, 996 (1980).

Takada, A., Y. Takada, C.C. Huang and J.L. Ambrus. Biphasic pattern of thymus regeneration after whole-body irradiation. J. Exp. Med. 129, 445 (1969).

Trowell, O.A. Radiosensitivity of the cortical and medullary

lymphocytes in the thymus. Int. J. Radiat. Biol. 4, 163 (1961).

Watkins, E.B., J.G. Sharp, D.A. Crouse and W.K. Metcalf. Lymphohematopoietic consequences of infant and thymic irradiation. In Experimental Hematology Today 1980 (S.J. Baum, G.D. Ledney, and D.W. van Bekkum, Eds.) p. 81, Karger, Basel, 1980.

SUMMARY

As documented in Chapter I of this thesis, the thymus is essential for the development and maintenance of cell-mediated immunity. It is a lympho-epithelial organ that generates immunologically competent lymphocytes. The stromal cells of the thymus regulate the differentiation and maturation of the thymocytes. The thymus is very susceptible to radiation and after a sublethal dose of irradiation, a **biphasic** recovery pattern can be observed. The **first phase** of the regeneration starts from intrathymic radioresistant precursor cells, whereas the **second phase** is due to the replenishment of the prothymocyte pool from an extrathymic source, the recovering bone marrow. Sparse data are available concerning the radiosensititivy of stromal and lymphoid cells, the nature and progeny of the intrathymic radioresistant precursor cells and the long-term effects of irradiation on the thymus. Furthermore, very limited information is available with respect to the effects on the thymus of high LET radiation, i.e. fast fission neutrons. High LET irradiation causes large amounts of ionisations over a short distance. In this thesis, an attempt was made to obtain more information about these aspects of radiation and the thymus. The experimental work is briefly introduced in Chapter II.

In order to investigate the short- and long-term effects of radiation on the thymus, young adult CBA mice were exposed to whole-body irradiation with either 2.5 Gy fast fission neutrons of 1 MeV mean energy of 6.00 Gy 300 kVp X-rays (Chapter III). At various time-points after irradiation, the weight, cellularity and histological appearance of the thymus was studied. In addition, the weight of the spleen and the animal was recorded. The results of this investigation showed a biphasic regeneration pattern of the thymus, followed by a marked decrease in relative thymus weight and cellularity which lasted up to at least 250 days after both types of irradiation. **The nature of this late effect of radiation on the thymus is attributed to a possible loss of pluripotent stem cells and residual damage in the surviving stem cells in the bone marrow.** Recovery of the spleen followed a monophasic pattern with an overshoot due to extra-medullary hematopoiesis, at about 3 weeks after irradiation. After neutron irradiation, de-

creases in spleen and animal weight were observed late after irradiation. The loss of spleen weight, long-term after irradiation was also attributed to a persistent defect in the bone marrow.

In order to describe the T cell composition of the irradiated thymus (Chapter V), the immunohistology of T cell subpopulations in the thymus of normal CBA/H mice was analyzed first, using monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14 (Chapter IV). It was shown that the large majority of the cortical cells in the thymus is bright Thy-1^+, T-200^+, Lyt-2^+, MT-4^+ and has a heterogeneous Lyt-1 expression. MEL-14 is only expressed on scattered cortical cells. In contrast, medullary cells are dull Thy-1^+, bright T-200^+, bright Lyt-1^+ and either Lyt-2^+or MT-4^+. A small number of medullary cells was found to express MEL-14. Besides these major thymocyte subpopulations, three other minor subpopulations were observed. First, scattered bright Lyt-1^+ cells occur throughout the cortex. These cells may represent a precursor population for the medullary thymocyte population. Second, a small subpopulation of bright Thy-1^+, T-200^+ cells were observed in the subcapsular area which do not express functional T cell markers such as Lyt-2 and MT-4. In the subcapsular area a third small population expresses only Thy-1 antigens but no other T cell markers. The latter two cell populations are considered to be involved in the early events of T cell differentiation.

Chapter V describes the effect of sublethal fission neutron irradiation on the T cell composition of the thymus in CBA/H mice. The existence of radioresistant intrathymic precursor cells and the repopulation of the thymus by these cells after sublethal irradiation, provides a model to study sequential steps in the process of intrathymic T cell differentiation. To this purpose we used immunohistology and flow cytofluorometry with monoclonal antibodies directed to the cell surface differentiation antigens Thy-1, T-200, MT-4, Lyt-1, Lyt-2 and MEL-14. Whole-body irradiation with 2.50 Gy fast fission neutrons was shown to result in a severe reduction and degeneration of the cortex, whereas the medulla was less affected. Irradiation selects for a population of

dull Thy-1$^+$, bright T-200$^+$, bright Lyt-1$^+$ cells localized in the medulla comprising about 20-30% of the original medullary thymocyte pool. The regeneration of the thymus starts about 5 days after irradiation with the appearance of large lymphoblasts negative for all tested antigens. These cells were mainly located in the subcapsular area but also in the medulla. During further thymic regeneration, sequentially the following phenotypes were observed: 1. Thy-1$^+$ "only" and T-200$^+$ "only" cells in the subcapsular area; 2. Thy-1$^+$, T-200$^+$ cells; and 3. Thy-1$^+$, T-200$^+$, MT-4$^+$, Lyt$^+$ cells in the cortex. Furthermore, an increased MEL-14 expression was observed on Thy-1$^+$, T-200$^+$ cells. These cells could well be cortical precursors for peripheral T cells. The **sequence in thymus regeneration**, described above, is similar to those observed in ontogeny as well as in the adult thymus after cortisone treatment and might therefore reflect **a general rule** in T cell differentiation. In addition, the appearance of "null" cells in the cortex as well as in the medulla indicates **independent generation kinetics for thymocytes in the cortex and medulla.**

Chapter VI describes the effects of irradiation with fast fission neutrons on the stromal cells of the thymus in CBA/H mice in order to investigate whether the thymic stromal cells are involved in the regeneration process described in Chapter V. Therefore, we analyzed the irradiated thymus with immunohistology, using monoclonal antibodies directed to MHC antigens and monoclonal antibodies defining various stromal cell types. Within 24 hours after whole-body irradiation with 2.50 Gy neutrons the cortex was reduced to a rim of vacuolized "nurse cell"-like epithelial cells, depleted of lymphocytes. These cells have lost their membrane associated expression of I-A antigens. Medullary stromal cells were more or less unaffected. About 5 days after irradiation, the thymus became highly vascularized and the epithelial remnants were repopulated by large lymphoid "null" cells. The repopulation of the thymic cortex coincided with a bright H-2K expression in the cortex, associated with both stromal cells as well as lymphoid cells. Thus, the large lymphoid "null" cells are H-2K$^+$. This has also been shown for thymocyte precursor cells in the fetal liver or the bone marrow. Furthermore, the observed sequence in T cell differentiation

during the regeneration, described in Chapter V, coincides with the reduction of H-2K expression in the cortex to control levels, whereas the reappearance of a reticular I-A staining pattern in the cortex is related with the acquisition of MT-4 and Lyt antigens. Thus, **a structurally organized epithelium in the cortex is required before the functional T cell differentiation process can start after irradiation.**

In Chapter VII, thymuses of CBA/H mice were investigated up to 570 days after whole-body irradiation with 2.50 Gy fast fission neutrons or 6.00 Gy X-rays. Several thymuses, observed 220-270 days after neutron irradiation, revealed an aberrant T cell distribution. Further analysis using immunohistology with monoclonal antibodies directed to T cell differentiation antigens, monoclonal antibodies directed to MHC antigens and monoclonal antibodies directed to thymic stromal cell types was carried out to characterize the T cell distribution and stromal cell distribution in more detail. T cells in the aberrant lobe expressed Thy-1, T-200 and MEL-14 antigens but were negative for Lyt-1 and MT-4. Some lobes displayed a weak Lyt-2 expression. The observed T cell maturation arrest was mainly restricted to the cortex since in the medulla besides T cells with an aberrant phenotype also normal mature T cells were observed. The stromal cell distribution in these equal sized thymic lobes did not differ from each other but the medullary compartment in the lobe with an aberrant T cell distribution tended to be smaller. However, both I-A and H-2K staining patterns in the cortex of the aberrant lobe are different from those observed in the normal lobe. **These observed alterations in MHC expression in the aberrant lobe probably interfere with the normal T cell differentiation process, thus causing aberrant T cell phenotypes. In addition, the occurrence of normal functional T cell phenotypes in the medulla indicates a cortex-independent medullary precursor lineage.**

Chapter VIII describes the effect of graded doses of fission neutrons or X-rays on the lymphoid compartment on the thymus in order to investigate the radiosensitivity of thymocyte subpopulations for these radiation types. Dose-response curves were determined at Days 2 and 5 after irradiation for the thymic cellularity and for T cell subpopulations defined by anti-Thy-1, -T-200, -Lyt-1 and -Lyt-2 antibodies using

flow cytofluorometry. Two days after irradiation, a two component dose-response curve was observed with a radiosensitive and a radiorefractory part. The radiosensitive part of the dose survival curve of the Lyt-2^+ cells, i.e. cortical cells, has a D_0 value of about 0.26 and 0.60 Gy for neutrons and X-rays, respectively. The radiosensitive components of the Thy-1^+, T-200^+ and Lyt-1^+ cell survival curves are characterized by D_0 values of about 0.30 Gy for neutrons and about 0.70 Gy for X-rays. The radiorefractory part of the dose-response curves was no longer present at day 5 after irradiation, probably due to migration of these cells out of the thymus. The Lyt-2^+ cells, 5 days after irradiation, are again most radiosensitive and have D_0 values equal to 0.37 and 0.99 Gy for neutrons and X-rays, respectively. The other cell types have corresponding D_0 values of about 0.47 Gy and 1.17 Gy. Since "null" cells are probably identical to the intrathymic radioresistant precursor cells, we estimated the radiosensitivity of these cells from the Thy-1^- and T-200^- cells. The D_0 values for the Thy-1^-, T-200^- intrathymic precursor cells are 0.64-0.79 Gy for fission neutrons and 1.32-1.55 Gy for X-rays. Neutron RBE values for the reduction of Thy-1^+, T-200^+, Lyt-1^+ or Lyt-2^+ cell populations to 1% vary from 2.6 to 2.8. The RBE for the intrathymic radioresistant precursor cells is about 2.1-2.2 and therefore points to the bone marrow as the ultimate origin of these cells.

In Chapter IX, the effects of graded doses of fission neutrons or X-rays on the stromal compartment of the thymus is investigated. For this purpose, a transplantation model was used in which athymic nude mice received a neonatal thymic lobe under the kidney capsule. Prior to the transplantation, this lobe was irradiated with either neutrons or X-rays. One and two months after transplantation, the T cell composition and stromal cell distribution of the graft, as well as the graft size were investigated. Furthermore, the reconstitution capacity of the graft was measured using flow cytofluorometric analysis of nude spleen cells stained with anti-Thy-1, -Lyt-2 and -MT-4 monoclonal antibodies. Grafting of a thymus lobe results in a normal stromal and T cell distribution and in a significant increase of the splenic T cell number.

Irradiation of the graft prior to transplantation has no effects on the stromal and T cell distribution in the graft but the graft growth decreases in a dose-dependent way. The fission neutron RBE for the reduction of thymic graft size to 10 percent was equal to 2.1. The reduction of the graft size is directly related to the surviving stromal stem cell population. The D_0 values for this cell population are equal to 2.45 Gy for fission neutrons and 3.68 Gy for X-rays. The peripheral T cell number decreases to levels that are indistinguishable from those observed in sham-operated mice when the highest dose was given. These observations indicate that the ability of the thymic stromal cells to support T cell differentiation is highly radioresistant. However, the proliferative capacity of the stromal cells is radiosensitive.

SAMENVATTING

Zoals beschreven in Hoofdstuk I van dit proefschrift, kan ioniserende straling beschadigingen op moleculair niveau veroorzaken die tot biologische veranderingen (sterilisatie, celdood, mutatie, maligniteit, etc.) kunnen leiden. In dit proefschrift worden de effecten van ioniserende straling op de thymus beschreven. De thymus neemt een centrale plaats in bij het tot stand komen van het immunologisch afweersysteem. Het is een lymfo-epitheliaal orgaan dat immuno-competente T lymphocyten produceert. De stromale cellen van de thymus reguleren de differentiatie en rijping van de thymocyten. De thymus is erg gevoelig voor ioniserende straling en na een subletale dosis straling is het herstelpatroon bifasisch. De eerste fase van de regeneratie komt voort uit stralingsresistente precursorcellen aanwezig in de thymus. De tweede herstelfase wordt veroorzaakt door uit het beenmerg afkomstige thymusprecursorcellen. Er zijn tot dusver weinig gegevens bekend over de stralingsgevoeligheid van de stromale en lymfoïde cellen. Over de aard en de groei van de intrathymale stralingsresistente precursorcel en over lange termijn effecten van straling op de thymus is eveneens weinig bekend. Bovendien is er weinig informatie beschikbaar betreffende de effecten van straling met een hoge LET op de thymus. Straling met een hoge LET zoals bijvoorbeeld snelle splijtingsneutronen, veroorzaken veel ionisaties over korte afstand. In dit proefschrift werd getracht meer informatie te verkrijgen over de hierboven genoemde aspecten van straling en de thymus.

Om de korte en lange termijneffecten van straling op de thymus te onderzoeken, werden jong volwassen CBA muizen bloot gesteld aan een totale lichaamsbestraling van 2,50 Gy snelle splijtingsneutronen met een gemiddelde energie van 1 MeV of 6,00 Gy 300 kVp röntgenstraling (hoofdstuk III). Het gewicht, het aantal cellen en de histologie van de thymus werd op verschillende tijdstippen na de bestraling bekeken. Tevens werd het gewicht van de milt en van het dier bepaald. De resultaten van dit onderzoek geven aan dat de regeneratie van de thymus een bifasisch patroon volgt dat gevolgd wordt door een duidelijke afname van het relatieve thymus gewicht en het relatieve aantal thymocyten. Deze afname is tot minstens 250 dagen na de bestraling meetbaar. De

oorzaak van dit late effect na beide soorten straling wordt toegeschreven aan een verlies van pluripotente stamcellen en residuele schade in de overlevende stamcellen in het beenmerg. Het herstel van de milt na de bestraling volgt een monofasisch regeneratiepatroon met een "overshoot" tengevolge van compensatoire extramedullaire hematopoëse, ongeveer 3 weken na de bestraling. Na een neutronenbestraling wordt ook op langere termijn na de bestraling, een afname van de gewichten van de milt en het dier waargenomen. **Het verlies aan gewicht van de milt, op langere termijn na de bestraling, wordt ook toegeschreven aan schade in het beenmerg.**

Om de samenstelling van T cell subpopulaties in de **bestraalde** thymus te kunnen beschrijven (Hoofdstuk V), was het noodzakelijk de immunohistologie van T cel subpopulaties te bestuderen in de **onbestraalde** thymus van CBA/H muizen. In Hoofdstuk IV wordt de T cel samenstelling van de normale thymus geanalyseerd met behulp van een panel monoclonale antilichamen gericht tegen de T cel differentiatie antigenen Thy-1, T-200, MT-4, Lyt-1, Lyt-2 en MEL-14. De grote meerderheid van de **corticale** cellen blijkt sterk Thy-1^+, T-200^+, Lyt-2^+, MT-4^+ te zijn en heeft een variabele Lyt-1 expressie. Slechts enkele verspreide cellen zijn MEL-14^+. In de **medulla** zijn thymocyten zwak Thy-1^+, sterk T-200^+, sterk Lyt-1^+ en zijn ofwel Lyt-2^+ofwel MT-4^+. Een gering aantal medullaire cellen is MEL-14^+. Behalve deze drie hoofdgroepen thymocyten werden nog **drie kleine thymocytensubpopulaties** waargenomen. **Ten eerste,** sterk Lyt-1^+ cellen komen verspreid voor over de gehele cortex. Deze cellen kunnen een precursorpopulatie voor de medullaire thymocyten populatie zijn. **Ten tweede,** onder het kapsel van de thymus wordt een kleine subpopulatie sterk Thy-1^+, T-200^- cellen gevonden die de functionele markers Lyt-2 en MT-4 niet tot uiting brengt. **Een derde** kleine subpopulatie is slechts Thy-1^+ en wordt eveneens gevonden onder het kapsel van de thymus. **De laatstgenoemde twee subpopulaties zijn waarschijnlijk betrokken bij de vroege stadia van de T cel differentiatie.**

Hoofdstuk V beschrijft het effect van een subletale dosis neutronen straling op de T cel samenstelling van de thymus in CBA/H muizen. Het bestaan van de radioresistente precursorcel in de thymus en de

repopulatie van de thymus vanuit deze cellen, geeft een mogelijkheid om T cel differentiatie stapsgewijs te onderzoeken. Hiervoor werden immunohistologie en flow cytofluorometrie gebruikt in combinatie met monoclonale antilichamen gericht tegen de bovengenoemde T cel differentiatie-antigenen. Totale lichaamsbestraling met 2,50 Gy splijtingsneutronen veroorzaakt een sterke reduktie en degeneratie van de cortex. De medulla wordt veel minder aangetast. Bestraling selecteert voor een populatie zwak Thy-1$^+$, sterk T-200$^+$, sterk Lyt-1$^+$ cellen. Deze cellen worden waargenomen in de medulla maar vormen slechts 20-30% van de medullaire cellen die in de onbestraalde thymus gevonden worden. De regeneratie van de thymus begint ongeveer 5 dagen na de bestraling. Dit herstel gaat gepaard met het verschijnen van grote lymfoblasten die nog geen T cel differentiatie-antigenen tot expressie brengen. Deze cellen worden **zowel onder het kapsel** van de thymus alsmede **in de medulla** waargenomen. Gedurende de herstelperiode van de thymus worden stapsgewijs de volgende fenotypen waargenomen: 1. Thy-1$^+$, "only" en T-200$^+$ "only" cellen onder het kapsel van de thymus; 2. Thy-1$^+$, T-200$^+$ cellen en 3. Thy-1$^+$, T-200$^+$, MT-4$^+$, Lyt$^+$ cellen in de cortex. Bovendien wordt een verhoogde MEL-14 expressie waargenomen op Thy-1$^+$, T-200$^+$ cellen. Deze cellen kunnen mogelijk corticale precursorcellen zijn voor de T cellen in de perifere lymfoïde organen. De hierboven beschreven **fenotypische sequentie in T cel differentiatie** gedurende de regeneratie van de thymus na bestraling, is ook waargenomen tijdens de embryonale ontwikkeling van de thymus en in de thymus van met cortison behandelde muizen. Deze sequentie is daarom waarschijnlijk **representatief voor elke vorm van T cel differentiatie.** Dat "null" cellen zowel in de cortex als in de medulla voorkomen, lijkt een aanwijzing te zijn voor het voorkomen van **twee onafhankelijke differentiatielijnen van thymocyten.**

In Hoofdstuk VI worden de effecten van bestraling met neutronen op de stromale cellen van de thymus beschreven. Dit onderzoek werd uitgevoerd om na te gaan hoe de stromale cellen betrokken zijn bij het in Hoofdstuk V beschreven regeneratieproces. De bestraalde thymus is daarom immunohistologisch onderzocht met behulp van monoclonale antilichamen gericht tegen MHC antigenen en een panel monoclonale antilichamen gericht tegen stromale cellen. Totale lichaamsbestraling met 2,50 Gy

neutronen reduceert de cortex binnen 24 uur na de bestraling tot een band van "TNC-achtige" epitheelcellen. Deze cellen brengen geen membraangebonden MHC antigenen meer tot expressie. Medullaire stromale cellen worden onder deze omstandigheden nauwelijks aangetast.
Vijf dagen na de bestraling neemt de vascularisatie in de thymus sterk toe. Het stroma wordt gerepopuleerd door grote lymfoïde "null" cellen. Deze repopulatie gaat gepaard met een sterke expressie van klasse I MHC antigenen in de cortex die zowel met stromale als lymfoïde cellen geassocieerd is. De in Hoofdstuk V waargenomen stapsgewijze T cell differentiatie valt samen met een reductie van de expressie van klasse I MHC antigenen in de cortex tot controleniveau. Tegelijkertijd wordt opnieuw van een reticulair klasse II MHC kleuringspatroon in de cortex waargenomen en dit is gerelateerd met het verkrijgen van MT-4 en Lyt antigenen op het celoppervlak van de thymocyten. **Deze waarnemingen geven aan dat een structureel goed georganiseerd reticulum in de cortex een noodzakelijke voorwaarde is voor het functionele differentiatieproces van T cellen na bestraling.**

In Hoofdstuk VII worden lange termijn proeven beschreven waarin de thymus van CBA/H muizen onderzocht wordt die tot op 570 dagen eerder bestraald waren met 2,50 Gy neutronen of 6,00 Gy röntgenstraling. Tussen 220 en 270 dagen na een neutronen bestraling wordt een aantal thymussen waargenomen met een afwijkende T cel distributie. Met behulp van immunohistologie en monoclonale antilichamen gericht tegen MHC antigenen, T cel differentiatie-antigenen en monoclonale antilichamen gericht tegen stromale cellen, is de samenstelling van T cel subpopulaties en het stroma van deze thymussen nader gekarakteriseerd. T cellen in de afwijkende lob brengen Thy-1, T-200 en MEL-14 antigenen tot expressie maar zijn negatief voor de antigenen MT-4 en Lyt-1. Sommige thymussen hebben een afwijkende lob met een zwakke expressie van Lyt-2 maar niet van Lyt-1 en MT-4. De waargenomen storing in T cel differentiatie beperkt zich voornamelijk tot de cortex. In de medulla worden behalve T cellen met een afwijkend fenotype ook normale rijpe T cellen waargenomen. De stromale celverdeling in deze even grote thymuslobben verschilt niet van elkaar. Het medullaire compartiment van de lob met de afwijkende T cel verdeling is echter iets kleiner. De I-A en H-2K expressie in de afwijkende lob is anders dan in de normale lob. Deze

veranderingen in MHC expressie in de afwijkende lob interferen waarschijnlijk met de normale T cel differentiatie en veroorzaken afwijkende T cel fenotypen. De aan wezigheid van T cellen met een normaal fenotype in de medulla wijst er weer op dat naast de cortex-afhankelijke lijn, er een cortex-onafhankelijke medullaire precursorlijn bestaat.

Hoofdstuk VIII beschrijft de effecten van oplopende doses splijtingsneutronen of röntgenstraling op het lymfoïde gedeelte van de thymus. Het doel van deze experimenten is de bepaling van de stralingsgevoeligheid van thymocytensubpopulaties voor deze twee stralingssoorten. Dosis-effect curven zijn op dag 2 en dag 5 na bestraling bepaald voor de cellulariteit van de thymus en voor T cel subpopulaties gedefinieerd door monoclonale antilichamen gericht tegen de T cel differentiatie-antigenen Thy-1, T-200, Lyt-1 of Lyt-2. Twee dagen na de bestraling is een dosis-effect curve bepaald die uit een **stralingsgevoelig** en een **stralingsbestendig** gedeelte bestaat. Het **stralingsgevoelige** gedeelte van de Lyt-2^+ cellen, voornamelijk corticale cellen, heeft D_0waarden van ongeveer 0,26 Gy en 0,60 Gy voor respectievelijk neutronen- en röntgenstraling. De **stralingsgevoelige** gedeelten van de Thy-1^+, T-200^+ en Lyt-1^+ cel overlevingscurven worden gekarakteriseerd door D_0waarden van ongeveer 0,30 Gy voor neutronen en ongeveer 0,70 Gy voor röntgenstraling. De **stralingsbestendige** gedeelten van de dosis-effect curven zijn op dag 5 na bestraling niet meer aantoonbaar. Deze cellen zijn waarschijnlijk op dat tijdstip uit de thymus gemigreerd naar de perifere lymfoïde organen. Vijf dagen na de bestraling zijn de Lyt-2^+ cellen weer het meest stralingsgevoelig en hebben een D_0waarde gelijk aan 0,37 Gy en 0,99 Gy voor respectievelijk neutronen- en röntgenstraling. De andere celtypen gedefinieerd door T cel differentiatie markers hebben D_0 waarden gelijk aan 0,47 Gy en 1.17 Gy voor respectievelijk neutronen en röntgenstraling. "Null" cellen zijn waarschijnlijk identiek aan de radioresistente precursorcellen in de thymus en brengen geen differentiatie-antigenen tot expressie. Een schatting van de stralingsgevoeligheid van deze cellen kan gemaakt worden op grond van de dosis-effect curven voor de Thy-1^- en T-200^- cellen. De D_0waarden voor de radioresistente Thy-1^-, T-200^- precursor-

cellen in de thymus zijn 0,64-0,79 Gy voor neutronen en 1,32-1,55 Gy voor röntgenstraling. Neutronen RBE waarden voor een reduktie van de Thy-1$^+$, T-200$^+$, Lyt-1$^+$ of Lyt-2$^+$ cel populaties tot 1 procent variëren van 2,6 tot 2,8. **De RBE voor de radioresistente precursorcel in de thymus is ongeveer 2,1-2,2. Deze waarde geeft aan dat dit celtype oorspronkelijk uit het beenmerg afkomstig is.**

Hoofdstuk IX beschrijft de effecten van oplopende doses neutronen- en röntgenstraling op het stromale gedeelte van de thymus, met het doel de stralingsgevoeligheid van de stromale cellen te bepalen. Voor dit doel is van een transplantatiemodel gebruik gemaakt. In dit model zijn thymusloze "nude" muizen gebruikt waarin een bestraalde neonatale thymuslob is getransplanteerd onder het kapsel in de nier. Eén en twee maanden na de transplantatie is de samenstelling van de T cel subpopulaties, de stromale celverdeling en de grootte van het thymus transplantaat onderzocht. De capaciteit van het transplantaat om de perifere lymfoïde organen te voorzien van T cellen is in de milt van de "nude" muizen onderzocht met behulp van flow cytofluorometrie en de monoclonale antilichamen anti-Thy-1, -Lyt-2 en -MT-4.

Transplanteren van een onbestraalde thymus resulteert in een normale stromale cel en T cel verdeling in het transplantaat en een significante verhoging van het aantal T cellen in de milt van de recipiënt. Bestraling van het transplantaat vóór de transplantatie heeft geen effect op de immunohistologie van het transplantaat, echter de transplantaatgroei neemt met toenemende dosis af. De RBE voor splijtingsneutronen voor een reduktie van de transplantaatgrootte tot 10 procent is gelijk aan 2,1. De afname van de transplantaat grootte is direct gerelateerd aan het aantal overlevende stromale stamcellen. De D_0 waarde voor deze celpopulatie is gelijk aan 2,45 Gy voor splijtingsneutronen en gelijk aan 3,68 Gy voor röntgenstraling. Wanneer de hoogste dosis gebruikt wordt, neemt het aantal T cellen in de milt af tot het niveau dat in "nude" muizen zonder transplantaat wordt waargenomen. **Deze waarnemingen geven aan dat het vermogen van de stromale cellen om T cel differentiatie te stimuleren zeer radioresistent is. Het regeneratief vermogen van de stromale cellen is echter stralingsgevoelig.**

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 28 april 1955 te Apeldoorn geboren. Na het behalen van het diploma HBS-B aan het Christelijk Lyceum te Apeldoorn in 1972 werd in hetzelfde jaar de studie Biologie aangevangen aan de Rijksuniversiteit te Utrecht.

Het kandidaatsexamen B-1* werd afgelegd in september 1976. Het doctoraal examen bevatte de volgende disciplines: 1e hoofdrichting Biologische Toxicologie (onderzoek naar de neveneffecten van enkele houtconserveringsmiddelen en hun oplosmiddelen op juveniële ratten als model voor jonge vleermuizen onder begeleiding van Prof. Dr. H. van Genderen en Dr. P. Leeuwangh); 2e hoofdrichting Scheikundige Dierfysiologie (onderzoek naar het sterolmetabolisme, de titer en de fysiologische werking van asterosaponinen in de zeester Asterias rubens en onderzoek naar het werkingsmechanisme van de in vitro geïnduceerde "germinal vessicle break-down" in oöcyten van de zebravis Brachydanio rerio onder begeleiding van Prof. Dr. D.A. Zandee en Dr. P.A. Voogt) en het bijvak Vergelijkende Endocrinologie (kwantatief elektronenmicroscopisch onderzoek naar de effecten van oestradiol 17β op de lever van de mannelijke zebravis Brachydanio rerio onder de begeleiding van Prof. Dr. P.G.W.J. van Oordt en Dr. J. Peute). In juni 1979 werd het doctoraal examen afgelegd.

Vanaf januari 1980 was hij verbonden aan het Energieonderzoek Centrum Nederland (ECN) te Petten. Op dit instituut werd het in dit proefschrift beschreven onderzoek verricht in samenwerking met de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam onder leiding van Prof. Dr. O. Vos (EUR), Dr. W. van Ewijk (EUR) en Drs. J.A.G. Davids (ECN).

In de periode oktober 1984-mei 1985 was hij verbonden aan het Documentatie Centrum Toxicologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM). Sedert 1 juni 1985 is hij verbonden aan het Antoni van Leeuwenhoekhuis te Amsterdam. Hij werkt aan een door het Ministerie van Welzijn, Volksgezondheid en Cultuur gesubsidieerd project "Cancer treatment by targeted radio-isotopes".