THE RESPONSE OF THE RETICULOENDOTHELIAL SYSTEM IN MICE TO TISSUE TRANSPLANTATION AND IMMUNOSUPPRESSIVE AGENTS.

by

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CONTENTS

SUMMARY

The phagocytic activity and morphology of Reticuloendothelial organs of Balb/c mice were studied following tissue transplantation and immunosuppressive treatment. Mice bearing medium-sized (4 cm^2) and massive (8 cm^2) skin isografts and allografts showed significantly increased blood clearance rates of opsonized *Salmonella typhimurium C5*. Increased clearance of colloidal carbon was also observed in mice bearing 1 cm^2 grafts, but mice with 4 cm^2 and 8 cm^2 grafts had normal phagocytic indices. The sequelae of severe surgical trauma may indirectly limit carbon clearance in mice with large grafts by depleting serum opsonins. Splenic enlargement occurred in isografted and allografted mice. The spleens of isografted mice showed increased erythropoiesis but little alteration in the lymphatic nodules and surrounding structures. Allografted mice showed increased granulopoiesis and erythropoiesis and cellular depletion from the marginal zones during the first phase of splenomegaly; a second peak of splenomegaly which occurred after allograft rejection was characterized by normal haematopoiesis but a marked enlargement of lymphatic nodules and germinal centres. Hepatomegaly paralleled the splenic enlargement in allografted mice and was due to enlarged hepatocytes which contained increased concentrations of RNA. The development of hepatomegaly following skin transplantation may represent a response which provides increased amounts of (a) purines, to sustain the intense cellular proliferation in lymphoid organs, and (b) plasma proteins, notably α - and β - globulins and fibrinogen which are elevated following surgery or trauma.

The use of large numbers of mice bearing different sized grafts permitted

a detailed study of the relationship between skin allograft size and survival time. In each of three H - 2 incompatible donor-recipient combinations tested, survival times of massive allografts were 2 to 3 days greater than those of small allografts, a difference that was highly statistically significant. The prolonged survival of massive allografts may be due to immunodepression following severe surgical trauma.

Intravenous administration of F_1 hybrid spleen cells to Balb/c mice resulted in hepatosplenomegaly and a marked increase in phagocytosis. In this *host-versus-graft* (HVG) situation, the order of increase in the phagocytic index for carbon was comparable with that reported for the *graft-versus-host* (GVH) reaction. Although the HVG reaction was characterized by changes in Reticuloendothelial organs also found in the GVH situation, *viz.*, phagocytic stimulation and hepatosplenomegaly, significant differences existed in the onset and nature of these changes. They occurred earlier in the HVG reaction and persisted for shorter periods; the relative number of liver macrophages did not increase and the spleen showed marked enlargement of lymphatic nodules with prominent germinal centre formation.

Cortisone in a dose of 10 mg/kg profoundly impaired carbon clearance by reducing the number of Kupffer cells capable of phagocytosing carbon. Hepatocytes in mice treated with 10 mg/kg of cortisone showed a 3-fold increase in lipid, while doses of 50 mg/kg and higher caused severe fatty change and hepatomegaly. When given daily over a 10 day period, 5 to 50 mg/kg of azathioprine did not affect phagocytosis, and phagocytic impairment occurred only when a near lethal dose (75 mg/kg) was used. Mild hepatotoxic changes followed

the administration of 25 mg/kg of azathioprine, while higher doses caused severe alterations in liver structure. Spleens from mice given azathioprine at 10 mg/kg and higher showed decreased red pulp haematopoiesis and histological abnormalities of lymphatic nodules.

One and two doses of rabbit anti-mouse antilymphocyte serum (ALS) depressed carbon clearance, while multiple doses stimulated phagocytosis and induced marked hepatosplenomegaly. The liver enlargement was due to hepatocyte hypertrophy, although increased numbers of Kupffer cells were also found in some cases. The enlarged spleens showed profound lymphoid cell depletion and intense haematopoiesis. The changes in phagocytic activity and morphology of Reticuloendothelial organs in adult Balb/c mice treated with ALS closely parallel those found in neonatally thymectomized mice. These results support the concept that ALS can confer a state of "immunological thymectomy" when administered to adult mice.

SIGNED STATEMENT

This thesis contains no material which has been accepted or submitted for the award of any other degree or diploma in any University. Furthermore, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text of this thesis.

The work described herein has been the subject of the following publications:

GOTJAMANOS, T. (1970). The effect of skin allograft size on survival time following transplantation between mice differing at the H-2 locus.
Aust. J. Exper. Biol. Med. Sci. 48, 1-15.

GOTJAMANOS, T. (1970). A comparison of the changes produced in Reticuloendothelial organs of mice during host-versus-graft and graft-versus-host reactions.
Aust. J. Exper. Biol. Med. Sci., In Press.

GOTJAMANOS, T. (1970). Alterations in Reticuloendothelial organ structure and function following cortisone administration to mice.
RES, J. Reticuloendothelial Soc. 8, 421-433.

GOTJAMANOS, T. (1970). The effect of azathioprine on phagocytic activity and morphology of Reticuloendothelial organs in mice.
Pathology, In Press.

GOTJAMANOS, T. and GILL, P.G. (1970). Changes in phagocytic activity and morphology of Reticuloendothelial organs in mice induced by antilymphocyte serum.
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Signed:

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CHAPTER 1

INTRODUCTION

The term 'Reticuloendothelial System' (RES) was introduced by ASCHOFF (1924) and interpreted in the narrow sense to include reticulum cells found in the pulp cords of the spleen, lymph nodes and other lymphatic tissues, endothelial cells lining the (a) lymph sinuses of lymph nodes, (b) blood sinuses of the spleen and liver, and (c) capillaries of the bone marrow, adrenal cortex and anterior lobe of the pituitary gland. A broader interpretation of this system included the fixed macrophages (histiocytes) found in connective tissues and the blood monocytes.

Since Aschoff's original presentation, several authors have discussed their own interpretations of the RES and in some cases have made certain modifications to the original concept (JAFFE, 1927; ROULET, 1930; ROSSLE, 1939; DOAN, 1940; AMANO, 1948; FRESEN, 1953; ROHR, 1953; AKAZAKI, WATANUKI and TESHIMA, 1965). Although the functions performed by cells of the RES are many and varied, a property common to each is the capacity to phagocytose foreign material. The macrophages of the liver and spleen are responsible for removing about 90% of blood-borne foreign matter, and it is this fact that has enabled the development of methods for quantitatively assessing phagocytic activity of the RES (HALPERN, BENACERRAF and BIOZZI, 1953; BIOZZI, BENACERRAF and HALPERN, 1953).

Interest in the investigations to be described in the succeeding Chapters of this thesis arose from the demonstration by BRENT and MEDAWAR (1962) and MEDAWAR (1963) that prolongation of skin allografts could be achieved

if the RES was "blockaded" by the intravenous administration of trypan blue. It was of considerable interest to us to study the mechanism by which impairment of phagocytosis by fixed macrophages could modify the immunological response against foreign grafts. Further interest lay in determining whether particles other than trypan blue, e.g. S^{35} - labelled barium sulphate, could be employed as blocking agents and thereby potentiate the action of immunosuppressive agents in bringing about allograft prolongation.

A review of the literature, however, revealed the existence of very little basic information on the effect of tissue transplantation and immunosuppressive drugs on the RES. The work reported in this thesis has, therefore, been directed towards providing such information. Experiments were designed to study the phagocytic activity and morphology of the liver and spleen following skin transplantation and during treatment with commonly used immunosuppressive agents. The results of experiments dealing with the effect of primary and secondary skin allografts on the RES are reported in Chapter 4.

The use of large numbers of mice bearing different sized grafts provided an excellent opportunity to study the relationship between skin allograft size and survival time when such grafts are exchanged across strong histocompatibility barriers. The relevant findings are described in Chapter 3. In this section, attempts are made to reconcile controversies contained in the literature relating to the effect of graft size on survival time, and also to outline some essential criteria for assessing graft viability, in order to eliminate discrepancies in future work involving skin graft survival.

During the course of experiments on the effect of skin grafts on the RES, it became apparent that certain responses observed in allografted mice, e.g., splenomegaly, might not be a direct consequence of the rejection phenomenon, but rather effects secondary to the surgical trauma unavoidably inflicted during grafting. For this reason, it was decided to carry out a study on the RES during a host-versus-graft reaction in which most variables that might indirectly affect RES organs could either be eliminated or at least minimized. The experimental model employed was to inject spleen cells from F_1 donors into the systemic circulation of mice from one parental strain (i.e., the reverse situation to that which leads to the development of a graft-versus-host reaction). The injected F_1 cells are incapable of reacting against the recipient's tissues because the latter do not possess transplantation antigens that are foreign to the donor cells. On the other hand, the recipient animal is able to mount an immunological reaction against the donor cells because they possess transplantation antigens that have been derived from the other parental strain, and so a true host-versus-graft reaction ensues. The results of this and related cell grafting experiments are reported in Chapter 5 and provide a basis for comparison with the results obtained with fixed tissue grafts.

Azathioprine was the drug chosen initially for studying the effect of immunosuppression on the RES. A review of the literature on this subject revealed that most of the available data related to corticosteroids, and furthermore, opinion varied widely regarding the effect of cortisone on phagocytosis, ranging from inhibition, no effect, to stimulation. It was evident that these discrepancies might be partly related to differences in the species and even in the strains of

animals employed. Consequently, the effect of cortisone on the RES of Balb/c mice (the mouse strain used in most of the experiments reported in this thesis) was examined prior to performing similar studies with azathioprine, in order to provide a basis for comparison of the latter's action. While these experiments were in progress, GRAY et al. (1966) and MONACO, WOOD, GRAY and RUSSELL (1966) published results of their investigations on the immunosuppressive properties of heterologous antilymphocyte serum (ALS). Subsequent work carried out in our laboratories revealed profound morphological changes in the liver and spleen when ALS was administered to mice. It was decided to extend the investigation into the influence of immunosuppressive agents on the RES to include a detailed analysis of the effects induced by ALS. The results of all these studies are described in Chapter 6.

Terminology: The terms used in the succeeding Chapters of this thesis to describe the nature of transplanted tissue are shown in Table 1.1. This table has been reproduced from "The Biology of Tissue Transplantation" by Paul S. RUSSELL and Anthony P. MONACO (1964).

TABLE 1.1 TERMINOLOGY OF TISSUE TRANSPLANTATION.

OLD TERMINOLOGY	NEW TERMINOLOGY	NEW ADJECTIVE	DEFINITION
Autograft	*Autograft*	*Autologous*	Graft in which donor is also recipient.
Isograft	*Isograft*	*Isogeneic*	Graft between individuals identical in histocompatibility antigens.
Homograft	*Allograft*	*Allogeneic*	Graft between genetically dissimilar members of same species.
Heterograft	*Xenograft*	*Xenogeneic*	Graft between species.

In descriptions of the histological structure of the mouse spleen, the terminology employed is that suggested by Thelma B. DUNN (1954) in her monograph on "Normal and Pathological Anatomy of the Reticular Tissue in Laboratory Mice, with a Classification and Discussion of Neoplasms", *viz.*, the term *lymphatic nodule* (rather than *lymphatic follicle* or *lymphoid follicle)* is used to describe the anatomical units consisting of primary and secondary lymphoid centres. The nomenclature of the structures surrounding the lymphatic nodules and separating the white pulp from the red pulp, *viz.*, the *marginal sinus* and *marginal zone*, is that recommended by Theodore SNOOK (1964).

CHAPTER 2

MATERIALS AND METHODS

PAGE

PAGE

Origin, housing, breeding and maintenance of inbred mice

The inbred mice used in the experiments described in this thesis were derived from breeding pairs that had been obtained from the Microbiology Department in The University of Adelaide during 1966. These mice had originally come from The Walter and Eliza Hall Institute in 1965 which had, in turn, previously procured breeding pairs from the Jackson Laboratories beginning in 1947.

The mouse strains employed were Balb/c, CBA, C57 Black/6, C3H, AKR, and F_1 (Balb/c X C57 Black/6). Approximately 130 breeding pairs and between 400 and 600 stock were housed at any one time in an air-conditioned room at a constant temperature of 20°C. Breeding pairs were kept in aluminium cages measuring 14cm (width) x 11cm (depth) x 28 cm (length), weanling mice (approx. 20) in cages 28cm x 11cm x 33cm, and adult mice (approx. 25) in stock holding cages 30cm x 15cm x 46cm. This arrangement conformed closely with the housing guidelines recommended for experimental animals in the Public Health Service Publication No. 1024 (Revised 1965), prepared by the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, Washington, D.C.

All cages contained a layer of fine wood shavings and were cleaned out every 4 days. Breeding mice were supplied, in addition, with fine paper strips for building nests. Water and food were supplied *ad libitum*, the food consisting of standard mouse cubes with millet supplement 3 or 4 times weekly. The drinking water was fortified with a few drops of 'Pentavite' vitamin concentrate (Nicholas Pty. Ltd., Melbourne, Victoria) and water bottles were changed every week.

Animals undergoing immunosuppressive treatment with cortisone, azathioprine, or anti-sera were housed in a room separate from the breeding and stock animals. Spontaneous pathological conditions were encountered from time to time in some untreated animals, although such cases were quite rare and the affected animals were not used in any experiments.

Mouse combinations and sizes of grafts used in skin transplantation experiments.

Twelve to 16 week old female mice from strains Balb/c (H-2^d), C57 Black/6 (H-2^b), AKR (H-2^k), CBA (H-2^k) and C3H (H-2^k) weighing between 20 and 24 gm were used. Skin allografts were exchanged across strong H-2 barriers in the following combinations: C57 Black/6 to Balb/c; CBA to Balb/c; AKR to C57 Black/6. Four sizes of rectangular grafts were employed: small, 1cm^2 (1cm breadth x 1cm length); medium, 4 or 4.5cm^2 (2cm x 2cm or 1.5cm x 3cm); large, 6cm^2 (2cm x 3cm); massive, 8cm^2 (2cm x 4cm). Similar sized autografts and isografts in Balb/c and isografts in CBA and C57 Black/6 mice served as controls to ascertain the technical success of the grafting procedure. All grafts were placed on the dorsal surfaces of the animals, and in the case of the massive grafts these extended over the whole dorsal aspect and over part of each lateral surface (Fig. 3.1, top). Medium-sized allografts (4cm^2) were also transplanted from C3H to CBA mice which are identical at the H-2 locus but differ at the weaker H-3 and H-4 loci.

Technique of skin grafting and post-operative animal care.

The technique of skin grafting was basically that described by BILLINGHAM and MEDAWAR (1951), with certain modifications. Mice were anaesthetized by an intraperitoneal injection of 0.20 - 0.25 ml pentobarbitone

sodium ("Sagatal", May and Baker Ltd., Victoria). The stock solution containing 60mg/ml had been diluted 1 : 10 with 0.9% saline. A single dose of barbiturate was generally sufficient to keep each mouse anaesthetized throughout the grafting operation which extended for about 50 minutes in the case of massive grafts and about 25 minutes for small grafts. The fore- and hind-legs of each mouse were secured with rubber bands and stretched out on a cork board. Hair was first clipped and then shaved from the dorsal surface of each recipient animal. The whole area was finally swabbed with 70% alcohol.

Preparation of donor skin included shaving all hair to the level of the stratum corneum and removal of both the panniculus carnosus and panniculus adiposus by gentle scraping with a blunt scalpel blade. The use of pigmented donor skin was avoided because areas of pigmentation in both allografts and isografts were usually found not to heal readily following transplantation. In most cases donor skin was grafted within 3 hours following donor sacrifice and was kept in a moist atmosphere in a sterile Petri dish maintained at $4^{o}C$ during preparation of the graft bed of the recipient animal. Each graft was placed onto a bed of panniculus adiposus containing an intact blood supply and was secured under slight tension with both interrupted (at each corner) and continuous 5/0 silk sutures. Female mice were used in all grafting experiments because it was found that their prominent panniculus adiposus layer allowed a much better preparation of a highly vascular graft bed than that obtained when male animals were used. No dressings or plaster jackets were used to cover the grafts. This allowed the grafts to be visually inspected and the animals to be weighed accurately each day following surgery. On completion of the grafting procedure, the

animals were placed on a histological hot-plate maintained at about 37°C to counteract the hypothermia induced by the barbiturate anaesthetic.

During the preliminary stages of the investigation when the technique of skin grafting was being mastered, the procedure recommended by BILLINGHAM and MEDAWAR (1951) of applying tulle gras and plaster jackets around the grafts was followed. According to these authors, "the purpose of the outer dressings is to protect the graft physically, to hold it down with a slightly perpendicular pressure, and to prevent side slip". A total of 32 animals were grafted and covered with protective dressings. These animals were observed to be considerably restricted in their movement and feeding; wood shavings and faeces tended to adhere to the edges of their dressings. It was decided, therefore, to dispense entirely with protective coverings and to secure the grafts by sutures alone. Furthermore, it was felt that by placing the grafts on the dorsal surfaces and keeping each grafted animal in a separate cage, physical trauma would be largely eliminated. This procedure proved highly successful and was adopted in all subsequent grafting experiments.

Preparation and administration of dissociated spleen cell grafts.

Donor mice were sacrificed by cervical dislocation and their spleens removed aseptically through an abdominal approach. The spleens were collected in a fine mesh nylon strainer (Astex, Woolworth's Ltd., London) and kept moist by being bathed in liquid Medium 199 for tissue culture (Commonwealth Serum Laboratories (C.S.L.), Melbourne). This medium had been adjusted to pH 7.0 with sodium bicarbonate solution (C.S.L. "Sodium bicarbonate solution, 2.8% w/v, for tissue culture"). Each spleen was cut transversely with sharp scissors

2 - 3mm from one end and its contents gently squeezed out from the cut end by application of light pressure from a flat-ended glass rod. Dissociation of the splenic tissue was accomplished by passing it through the fine nylon mesh and into Medium 199 (adjusted with $NaHCO_3$ to pH 7.0) contained in a sterile Petri dish maintained at about $4^{o}C$. The cell suspension was uniformly dispersed with a Pasteur pipette and then transferred to a 10ml graduated glass centrifuge tube also maintained at about $4^{o}C$. The suspension was allowed to stand for 5 minutes to allow large clumps of cells and strands of connective tissue to settle out. The cell suspension was then centrifuged at about 800 r.p.m. for 5 minutes, the resultant supernatant was discarded and the cells resuspended in fresh Medium 199. Cell counts were made in a haemocytometer, and the volume of suspension adjusted to give a concentration of the order of 3×10^8 nucleated cells per ml. Viable counts using 1% trypan blue were routinely performed and these showed between 80 and 90% viability. Each mouse was injected intravenously via the lateral tail vein with 7×10^7 nucleated cells in a volume not exceeding 0.3ml using a 1 ml tuberculin syringe and a 25 standard wire gauge needle.

Technique of spenectomizing mice.

Mice were anaesthetized with 'Sagatal' (see p. 10), their fore- and hind-legs secured with rubber bands and then stretched out on a cork board. The skin overlying the spleen laterally was shaved and then swabbed with 70% alcohol. Access to the spleen was obtained through skin and peritoneal incisions and following its exposure, the major splenic vascular bundles were tied with 5/0 silk and then severed distally, allowing the spleen to be dissected free. The peritoneal and skin incisions were sutured separately, and the mice allowed to

recover from the anaesthetic by being placed on a warm histological plate. Rapid healing of the exposure site occurred within 2-3 days and no post-operative mortality was encountered.

Immunosuppressive agents.

1. Cortisone

Cortisone acetate dispersed in sodium carboxy-methyl-cellulose (Cortisyl, Roussel Laboratories Ltd., London) was appropriately diluted in 0.9% saline and administered subcutaneously to mice in doses ranging from 2 to 200 mg./kg. of body weight. The volume of solution injected did not exceed 0.5 ml.

2. Azathioprine

Azathioprine (6 - (1-methyl-4-nitro-imidazol-5-yl) - thiopurine) in solid form ('Imuran', Burroughs Wellcome and Co. (U.S.A.) Inc.) was added to 0.9% saline and the resulting solution/suspension was injected into the peritoneal cavity of mice in doses varying between 5 and 100 mg./kg. of body weight. The volume injected varied between 0.25 and 0.5 ml.

3. Antilymphocyte serum and antilymphocyte globulin

Antisera against mouse thymus or lymph node cells were raised in young adult sandy lop-eared or New Zealand white rabbits of both sexes according to the method of LEVEY and MEDAWAR (1966). In the results to be presented in Chapter 6, no distinction has been made between antilymphocyte and anti-thymocyte sera (hereafter called ALS). The particular ALS preparations used in these experiments were selected on the basis of their comparable immunosuppressive potency and absence of severe toxic effects.

ALS was administered to mice in either an absorbed or unabsorbed form, absorption being carried out to remove the haemagglutinins and haemolysins against mouse erythrocytes. The absorption procedure was as follows: Balb/c erythrocytes were obtained by puncturing the retro-orbital venous plexus and washed three times with cold 0.9% saline. Absorption was carried out at 37°C for 30 minutes, followed by 2 hours at 4°C, using a ratio of 3 volumes of serum to 1 volume of packed erythrocytes. The absorption procedure was repeated 3 times for each batch of serum. Antilymphocyte globulin (ALG) was prepared from pooled, non-toxic batches of ALS by elution of the IgG fraction from DEAE-cellulose with 0.0175 M phosphate buffer (pH 7.4) and concentrated by vacuum dialysis.

Use of colchicine for the study of mitotic activity.

For the study of mitotic activity of liver cells and epidermal cells in skin grafts, colchicine (B.D.H. Laboratory Chemicals Division, Poole, England) was dissolved in 0.9% saline and was administered subcutaneously to mice in a dose of 0.2 mg./100 g. of body weight. The volume of solution injected did not exceed 0.3 ml. The animals were sacrificed 4 hours after the injection of colchicine and their livers or skin grafts prepared for routine histological examination.

Techniques used to study phagocytic activity of the Reticuloendothelial System.

1. Rate of blood clearance of P^{32} - labelled *Salmonella typhimurium C5* by the liver and splenic macrophages.

a. Radioactive labelling of bacteria with P^{32}.

Salmonella typhimurium C5 organisms were grown in 50ml of minimal medium supplemented with Difco casamino acids (Difco laboratories, Detroit) as described by BENACERRAF, SEBESTYEN and SCHLOSSMAN, 1959). This 50ml of medium was contained in a 500ml flask which was held securely in a basin by a tight packing of cotton wool. One milli-curie of P^{32} as orthophosphate was added to the flask containing the inoculated medium which was then shaken at 37°C for about 18 hours.

At the end of the 18-hour incubation period, the P^{32} -labelled bacteria were collected by centrifugation at 5,000 r.p.m. The organisms were washed three times in a volume of 50ml of 0.9% saline in a polythene centrifuge tube, and resuspended in 13mls of casamino acid medium to give a concentration of the order of 10^9 organisms per ml. The suspension was then filtered through glass wool and the final concentration determined by taking optical density readings on a Shimadzu Q.R. 50 spectrophotometer at a wavelength of 675 millimicrons. Previous standardization had revealed that an optical density reading of 0.16 for a 1:30 dilution of bacterial suspension at 675 millimicrons represented a concentration of 10^9 viable organisms per ml. After spectrophotometric determination, the volume of bacterial suspension was adjusted to give the required concentration of 10^9 organisms per ml. The suspension was stored in a McCartney

bottle at 4°C and was used in clearance studies either on the day of preparation or on the following day.

b. Opsonization of bacteria.

Opsonization of the bacteria used in the blood clearance studies was carried out to minimize the effect of naturally occurring opsonins present in the serum of mice under investigation and thus increase the sensitivity of the test system (ROWLEY, D. and KOTLARSKI, Ieva, personal communication). Equal volumes of the standardized suspension of *S. typhimurium C5* and pig serum were mixed and placed in an incubator at 37° for 30 minutes before use. The pig serum had been obtained by pooling the blood from at least 3 healthy pigs slaughtered at the Adelaide abattoirs.

c. Intravenous administration of P^{32}-labelled *S. typhimurium C5* and collection of blood samples.

Approximately 2 x 10^8 opsonized, P^{32}-labelled *S. typhimurium C5* organisms were injected intravenously into each animal via the lateral tail vein in a volume of 0.2 ml using a 25 standard wire gauge needle attached to a 1ml tuberculin syringe. Blood samples (0.02ml) were taken at ½, 1, 2, 3, 4, 5 and 7 minutes following intravenous injection by puncturing the retro-orbital venous plexus with a fine glass capillary pipette previously calibrated to 0.02ml as described by BIOZZI, BENACERRAF and HALPERN (1953). After collection, each blood sample was immediately pipetted onto a circular disc of polythene-backed absorbent paper (Benchkote, W. and R. Balston Ltd., England) which had been pre-cut to fit the holders of the radioactive counting equipment.

d. Radioactive assay of blood samples.

The early work on bacterial clearance studies was carried out in the Microbiology Department and the radio-activity contained in each sample was assayed according to the method described by JENKIN and ROWLEY (1961). Briefly, the apparatus consisted of a thin mica end-window Geiger counter installed in a Nuclear Chicago automatic sample changer C 110 A (Nuclear Chicago, Chicago) with an automatic printing timer C 111, coupled to the model 183 scaling unit to record the results. This apparatus had been modified by fitting a helium-filled thin window Geiger counter tube so as to increase the sensitivity of the machine three-fold.

Later work was carried out in the Department of Surgery and the apparatus used to assay radioactivity was a Philips PW 4141 gas-flow radiation counter (Philips Electrical Pty. Ltd., Adelaide). This instrument was employed as a proportional counter to record beta radiation and was operated by using a 9 to 1 mixture of argon and methane gas. Radioactive blood samples contained on polythene-backed paper discs were placed on sample pans in a Philips PW 4001 automatic sample changer coupled to the PW 4141 instrument and the results were recorded on a Victor print-out machine.

e. Calculation of the phagocytic index.

The rate of clearance of bacteria from the circulation was expressed as the phagocytic index K, as described by BIOZZI, BENACERRAF and HALPERN (1953). The equation

$$K = \frac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_1}$$

was used to calculate the phagocytic index K, where C_1 and C_2 represent the radioactivity recovered in 0.02ml blood samples at times T_1 and T_2 after intravenous injection (BENACERRAF, SEBESTYEN and SCHLOSSMAN, 1959).

2. Rate of blood clearance of colloidal carbon by the liver and splenic macrophages.

The technique used was basically that described by BIOZZI, BENACERRAF and HALPERN (1953), with certain modifications.

(a) Preparation of carbon suspension

Pelikan carbon preparation C11/1431a (Gunther Wagner, Hanover) was diluted with a 2% solution of bacteriological gelatine in distilled water to give a carbon concentration of 16 mg /ml. The diluted suspension and the syringes used for injection were maintained at 37°C to prevent solidification of the suspension during experimentation.

(b) Intravenous administration of carbon suspension and collection of blood samples.

A dose of 16mg/100gm body weight of the carbon suspension was injected into the lateral tail vein of each mouse using a 0.25ml tuberculin syringe (B-D Yale, Becton Dickinson and Co., Rutherford, New Jersey). The volume injected did not exceed 0.25 ml. Blood samples (0.02ml) were taken at 1, 2, 3, 5, 8, 12 and 15 minutes following carbon injection as described previously. Each blood sample was immediately lysed in a small tube containing 3ml of an 0.1% solution of sodium carbonate and the lysate uniformly dispersed with a Pasteur pipette.

(c) Spectrophotometric estimation of the carbon content of blood samples

Optical density readings of the 3ml lysates were taken at 675 millimicrons on a Beckman DB-G grating spectrophotometer (Beckman Instruments Inc., Fullerton, California) using 0.02ml of normal blood lysed in 3ml of 0.1% Na_2CO_3 as the standard. The concentration of carbon in each 3ml lysate was calculated by reference to a standard graph which had been prepared by determining the optical densities of 3 ml samples of lysed blood containing known amounts of carbon.

(d) Calculation of the phagocytic index

The phagocytic index (K) was calculated from the equation given earlier, viz.

$$K = \frac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_1}$$

where C_1 and C_2 represent the carbon concentrations at times T_1 and T_2 after intravenous injection.

(e) Use of the corrected phagocytic index to compensate for changes in liver and spleen weights.

BIOZZI, BENACERRAF and HALPERN (1953) concluded from their extensive studies on the phagocytic activity of the RES in rats that such activity varied with the cube of the relative weights of the organs principally involved, viz., the liver and spleen. They derived the following equation in order to compensate for differences in liver

and spleen weights between animals of the same species and also between those of dissimilar species:

$$\alpha = \sqrt[3]{K} \times \frac{W}{wl + ws}$$

where α = corrected phagocytic index; K = phagocytic index; W = weight of animal (g); wl = weight of the liver (g); ws = weight of the spleen (g).

BIOZZI and co-workers were unable to explain the basis of this relationship between phagocytic activity and the cube of the weight of the liver and spleen, but suggested that the relative blood flow through these organs was an important factor.

In the succeeding Chapters of this thesis, the corrected phagocytic indices for all animals studied by the carbon clearance method are presented.

3. Identification and estimation of the relative numbers of phagocytic cells in the liver and spleen by their uptake of carbon.

The relative numbers of phagocytic cells in the liver and spleen were estimated by studying histologically the distribution of intravenously injected carbon in these organs. Animals were sacrificed when carbon uptake by the liver and splenic macrophages was complete. Complete clearance was considered to have occurred when optical density determinations of lysed blood samples failed to reveal the presence of carbon in the circulation.

Six micron-thick sections of the liver and spleen were prepared, examined and photographed under a standard magnification (X 100). It was found that the study of the relative number of carbon-containing macrophages was

facilitated if liver sections were left unstained and if spleen sections were lightly stained with 1% neutral red.

Weighing of animals and organs and expression of organ weights.

The body weights of individual mice bearing skin grafts were recorded daily or on alternate days using an Ohaus triple beam balance (Ohaus Scale Corporation, New Jersey, U.S.A.). The combined body weights of mice in groups which received either spleen cell grafts or immunosuppressive agents were recorded daily, and the percentage change in weight for the group during the experimental period was calculated. The weights of each animal's liver, spleen and 4 lymph nodes (2 axillary and 2 inguinal) were determined on a Shimadzu single beam balance (Shimadzu Seisakusho Ltd., Kyoto, Japan).

The procedure for removing and weighing organs was as follows: immediately after sacrifice of the animal by cervical dislocation, the abdominal cavity was opened, and the liver, spleen and lymph nodes removed and weighed in that order. Before weighing, each organ was blotted and freed from any extraneous adherent tissue; in the case of the liver, the gall bladder was pierced and its contents discharged. After being weighed, the liver, spleen and lymph nodes from each animal were placed immediately into a McCartney bottle containing fixative.

Organ weights were expressed as a percentage of each animal's total body weight. In cases where loss of body weight occurred as a result of the experimental procedure, expression of organ weights as percentages of total body weight was not based on each animal's body weight recorded at the end of the treatment period. Rather, the "corrected body weight" for each animal was

calculated by adjusting its final body weight according to the percentage weight loss experienced by the group as a whole, and this corrected weight was used to express organ weight percentages.

Preparation of tissues for light microscopy.

Pieces of skin and liver, whole spleens and lymph nodes were fixed in either buffered formol-saline or Bouin's fluid (see Appendix 1). In the case of the spleen, a fine cut through the capsule was made at either end to facilitate rapid penetration of the fixative. When Bouin's fluid was used the fixation period was 12-24 hours, following which the tissues were thoroughly washed in 70% alcohol for 1 hour. The fixation period with formol-saline varied between 1 day and 3 weeks. Dehydration was carried out in increasing strengths of alcohol as follows: 50 or 70% alcohol for $\frac{3}{4}$ hr; 80%, $\frac{3}{4}$ hr; 95, $\frac{1}{2}$ hr; 3 half-hourly changes in absolute alcohol. The tissues were cleared in methyl benzoate overnight and on the following day were rinsed in two changes of chloroform to remove the methyl benzoate. They were then passed through 3 half-hour changes of paraffin wax (Bioloid, Paraffin Embedding Compound; M. P. 53-55^{o}C, Will Scientific, Inc., U.S.A.) and embedded under vacuum.

Sections were cut at 6 microns on a Spencer 820 microtome, mounted on glass slides, stained with Ehrlich's haematoxylin and counterstained with eosin (see Appendix 1). In addition, sections of livers from animals treated with cortisone were stained with Sudan III stain to identify the presence of fat (CULLING, 1963).

Photomicrography.

Photomicrographs of histological sections were taken on Ilford Pan F and Adox KB14 film with the aid of a Leitz Microsix L light meter coupled to an Olympus PM6 camera attached to a Leitz Weitzlar microscope. Prints were made on Ilfobrom IB photographic paper (Ilford Ltd., Sussex, England).

Preparation of tissues for electron microscopy.

Specimens of liver tissue were prepared for electron microscopy by fixation in 6% glutaraldehyde for one hour (SABATINI, MILLER and BARNETT, 1964) and post-fixed for a further hour in 1% osmium tetroxide. Both fixatives had been adjusted to pH 7.4 using MILLONIG'S (1962) buffer and were maintained at approximately $2^{o}C$.

The procedure of obtaining the specimens was as follows. Within seconds of sacrificing each animal and exposing the liver by a mid-line ventral incision, small, wedge-shaped pieces of liver were cut *in situ* with a pair of sharp scissors and transferred to a flat glass surface containing a few drops of glutaraldehyde. Smaller pieces measuring approximately 1mm x 1mm were cut with the two halves of a sharp razor blade, using a cross-cut motion, and were then placed in fresh fixative.

Following fixation, tissues were dehydrated in increasing strengths of alcohol according to the following schedule: 15 min. in 50%; 15 min. in 70%; 15 min. in 90%; 15 min. in 95%; 30 min. in Absolute I; 30 min. in Absolute II. Dehydration was carried out at $0^{o}C$ in 50-95% alcohol and at room temperature in absolute alcohol. The specimens were then embedded in Araldite (See

Appendix 2) according to the method of LUFT (1961).

Thin sections (straw-coloured or pale gold when magnifications up to X4000 were required, and silver to grey for higher magnifications) were cut on a Si-ro-flex ultramicrotome using a diamond knife, floated on distilled water or a 10 per cent acetone solution, expanded with the aid of 1 : 2 Dichloro-ethane vapour, and picked up on Parlodion- Carbon shadowed copper grids (300 mesh).

The sections were stained with a saturated alcoholic solution of uranyl acetate for 20 minutes and then with lead citrate according to the procedure of REYNOLDS (1963). The stained sections were examined under a Philips EM 100 electron microscope at 60 kV. Electron micrographs were taken using Ilford Fine Grain Safety Positive Film and enlargements made on $6\frac{1}{2}$" x $8\frac{1}{2}$" Ilfobrom IB photographic paper.

Biochemical estimations of liver composition.

The liver was removed aseptically from each mouse, blotted, freed from any extraneous adherent tissue and weighed. It was then placed in a smooth-walled glass test-tube containing 5ml of distilled water, the volume made up to 10 ml, and homogenization performed by means of a mechanically rotated teflon pestle. Aliquots of the homogenate were taken for individual estimations of water, protein, DNA, RNA, glycogen and fat content.

a. Water.

Two ml of homogenate were pipetted onto a pre-weighed watch-glass and placed in an oven at 80°C. The watch-glass was weighed after 12-18 hours and the precedure repeated until a constant weight was obtained. From the

calculated dry weight of 2ml of homogenate, the total dry weight of the liver and hence its percentage water content could be determined.

b. Protein.

The procedure adopted for estimating the protein content was that described by CLELAND and SLATER (1953) using the Biuret reagent.

c. Deoxyribonucleic and Ribonucleic Acids.

DNA and RNA were determined according to the methods described by SCHNEIDER (1945 ; 1957) using diphenylamine reagent for DNA and orcinol for RNA.

d. Glycogen.

The method of CARROLL, LONGLEY and ROE (1956) using anthrone reagent was followed for glycogen determination.

e. Fat.

Two ml of the homogenate were pipetted into a Quickfit test tube equipped with ground glass stopper and shaken thoroughly for 1-2 minutes with approximately 8ml of ether. The ether containing dissolved fat was allowed to separate above the homogenate, drawn into a Pasteur pipette and discarded. This extraction procedure was carried out 5 times on each 2ml sample of homogenate. The extracted homogenate was pipetted onto a watch-glass of known weight, placed in an oven at $80^{o}C$ and weighed until constant weight was attained. The fat-free dry weight of the liver was calculated and the fat content determined by subtracting this weight from the previously determined total dry weight of the liver.

Statistical methods.

The body weight of each animal and the weights of its liver, spleen and 4 lymph nodes and the phagocytic index (K) were coded on data detail sheets for subsequent transfer to standard 80-column punch cards. Standard descriptive statistical analyses were carried out on the observed variables and the corrected phagocytic index (α) computed from the recorded values. The statistical parameters, which were estimated by conventional methods, included the mean, standard deviation, variance, standard error of the mean and the range of variation. These were calculated for each animal group using a CDC 6400 computer and standard programmes used in growth research carried out in the Department of Dental Science, University of Adelaide.

"Student's" t-test was used to determine the significance of differences between the mean values obtained for experimental and control groups. The variances of the two samples were assumed equal, and computations were performed with the aid of a Facit electronic calculator using the following formula given by BAILEY (1959):

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means for the respective test and control groups; s_1 and s_2 are the standard deviations of the means for the test and control groups containing n_1 and n_2 animals respectively. The probability that a difference between two means differed from zero was determined by consulting "Student's" t-test tables included in Documenta Geigy, using the disclosed value of t with

$(n_1 + n_2 - 2)$ degrees of freedom.

Survival times of skin allografts were expressed as the mean $\pm$ standard deviation of the mean, and the standard analysis of variance techniques were employed to test for differences between classes.

Due to the limited space imposed by the large amount of data included in some of the Tables in Chapters 4, 5 and 6, the figure zero has not always been placed before the decimal point in cases where the value obtained was less than one; *e.g.*, a mean phagocytic index $\pm$ S.E.M. of 0.028 $\pm$ 0.002 is shown as .028 $\pm$ .002.

CHAPTER 3

THE RELATIONSHIP BETWEEN SKIN ALLOGRAFT SIZE AND SURVIVAL TIME FOLLOWING TRANSPLANTATION BETWEEN MICE DIFFERING AT THE H-2 LOCUS

PAGE

INTRODUCTION

A subject that has immense theoretical and practical importance in the field of transplantation immunity is the fate of large quantities of foreign tissue transplanted between genetically dissimilar individuals. Since MEDAWAR's (1944, 1945) observations that the mean survival time (m.s.t.) of skin allografts in rabbits varied inversely with the dose of tissue grafted, many reports either supporting or refuting this phenomenon have been published and opinion is equally divided. With two exceptions (VEITH, MURRAY and MILLER, 1966; ZANELLA et al. 1968), all of these subsequent studies have used rats as the experimental animals.

MEDAWAR's (1944, 1945) finding that small allografts survived longer than large ones has been supported by LEHRFELD and TAYLOR (1953) and by ZOTIKOV, BUDIK and PUZA (1960), while CALNAN and BLACK (1962), MATTER et al. (1963) and VEITH, MURRAY and MILLER, (1966) found no significant difference between m.s.t.'s of small and large skin allografts. Zotikov's group (1960) reported the additional finding that 15 out of 31 massive skin allografts, covering more than one-third of the total body surface of their rats, showed prolonged survival, although they did not give m.s.t.'s. In four further studies (CALNAN and KULATILAKE, 1962; CONVERSE, SIEGEL and BALLANTYNE, 1963; BALLANTYNE and STETSON, 1964; ROTHER, ROTHER and BALLANTYNE, (1967) in which rats were allografted with skin comparable in area to that used by ZOTIKOV et al. (1960), increases from 4 to 12 days in m.s.t. of massive grafts were reported. The numbers of animals used in some of these investigations were small and the genetic disparity between donors and recipients not clearly defined.

The use of highly inbred mouse strains makes it possible to perform uniform skin grafting experiments on large numbers of genetically defined animals and thereby carry out detailed statistical analyses of survival times. ZANELLA et al. (1968) used inbred mouse strains differing at the H-2 histocompatibility locus to evaluate the effect of skin graft size on survival time. In an investigation involving approx. 120 mice these workers reported that massive allografts extending over parts of the dorsal, lateral and ventral surfaces of each animal survived for periods up to 27 days, large grafts (4.5 $cm.^2$) for periods up to 19 days, while much smaller grafts were rejected at about 9 days. The sole criterion for rejection of massive and large grafts used by these investigators was to examine each graft visually and to classify it as rejected when there were "less than 10% surviving epithelial cells".

In this Chapter, results of experiments involving transplantation of different sized skin allografts from either C57 Black or CBA donors to nearly 400 Balb/c recipients are presented. These grafts were performed on mice being studied for changes in RES function following skin transplantation (to be described in Chapter 4). Control animals from other studies designed to test the effectiveness of immunosuppressive agents were also included. A further 30 allografts exchanged between AKR and C57 Black mice have also been carried out since publication of the report by ZANELLA et al. (1968) to try and confirm their results obtained with this strain combination.

The Present Investigation

In the present study the terms "time of graft rejection" and "graft survival time" have been interpreted to mean the point at which the entire graft

epidermis is rendered non-viable by the immunological response of the host. Before commencing the main investigation, the macroscopic and microscopic appearances of 27 allografts and 27 isografts carried out in parallel were correlated, and the macroscopic features indicative of graft non-viability adopted as criteria for graft rejection. Six mice were grafted on any one day, 3 with large (6 $cm.^2$) C57 Black allografts, and 3 with similar sized isografts. Each of the 9 groups of 6 animals was sacrificed at either 2, 4, 6, 8, 10, 12, 15, 18 or 25 days after grafting and careful note made of the macroscopic appearance of each allo- and isograft. Following sacrifice each graft was gently removed and divided mid-longitudinally and mid-transversely with a sharp razor, thus providing four different surfaces for microscopic examination.

The macroscopic features of allo- and isografts of similar ages were correlated with their microscopic appearances. In this way the macroscopic features of epithelium showing complete destruction on microscopic examination were adopted as the criteria for allograft rejection. In all subsequent experiments, an allograft was judged as being rejected when its entire surface exhibited each of the following features: a colour change from light or dark pink to a dark yellow-brown; a wrinkled appearance which raised its level above that of the surrounding host skin; and pitted areas which exposed the underlying exuding dermis. These criteria applied to the area of the graft excluding the borders secured by sutures. In both iso- and allografts, within 4 to 5 days after grafting, these sutured areas always showed a narrow (1-2 mm.) band of dead tissue as a result of pressure necrosis.

During the course of the skin grafting - RES experiments, additional viability studies on transplanted skin were carried out. One of the fundamental properties of cells in the basal and prickle cell layers (stratum malpighii) of skin epidermis is to continually proliferate and thereby replace cells lost from the more superficial layers. The stratum malpighii of normal mouse epidermis is usually only 2 to 3 cells in thickness and mitotic activity occurs largely, if not entirely, in the basal layer (MONTAGNA, 1956). It was decided, therefore, to use the continued ability of basal cells to divide *in situ* as a specific microscopic criterion for indicating a viable and adequately functioning skin graft. The plant alkaloid colchicine has the property of arresting dividing cells at the metaphase stage of mitosis. Colchicine is a reliable marker in cell proliferation studies (BERTALANFFY, 1964) and has been used in the present investigation to render dividing cells more easily recognizable in histological sections (see Fig. 3.4., top left).

For the study of proliferation of cells in the basal layers of grafted skin, 9 groups of Balb/c mice (3 animals per group), bearing 8 $cm.^2$ CBA allografts at 4, 5, 6, 7, 8, 9, 10, 12 and 14 days were injected subcutaneously with a single dose of colchicine and sacrificed 4 hr. later. Balb/c mice bearing massive isografts at 4, 6, 8, 10, 12 and 15 days were similarly treated and served as controls. A further 5 pairs of CBA mice bearing successful 4 $cm.^2$ C3H allografts (as judged by macroscopic criteria) at 20, 24, 30, 40 and 45 days were also injected with colchicine and sacrificed after 4 hr. Injections of colchicine were carried out between 6 a.m. and 7 a.m. and animal sacrifice performed between 10 a.m. and 11 a.m., since greatest mitotic activity in

mouse epidermis occurs at this period (COOPER and FRANKLIN, 1940). Following sacrifice, each skin graft was prepared for histological examination in the manner described previously.

RESULTS

Post operative recovery of grafted animals.

The technique of transplanting skin grafts varying in area from 1-8 square centimetres proved highly successful, Post-operative mortality was less than 5% and was due to an overdose of barbiturate anaesthetic. The mice were subdued for 1-2 days following surgery and showed a small loss in body weight, this being slightly greater in allografted animals than in isografted controls. Maximum body weight loss during this period was 6% and occurred in animals bearing massive allografts. By 2-3 days, however, most of the animals had regained their normal agility and pre-operative body weights and by 7-10 days all showed weight gains comparable to non-grafted controls (see Appendix 3). These observations contrast strongly with those previously reported for dogs (VEITH, MURRAY and MILLER, 1966) where high mortality occurred following placement of massive allografts. They also differ from results obtained with rats where considerable body weight loss was observed in allografted animals 1-8 days following skin grafting and which continued during the subsequent 2 weeks (CALNAN and BLACK, 1962).

Behaviour of autografts and isografts.

(a) Macroscopic. Three to 4 days following transplantation, autografts and isografts of all sizes had acquired a deep pink colour indicative

of a hypervascular response in the graft bed. This hypervascularity subsided at about 6 days and rapid healing followed thereafter. Hair growth was evident at 15-16 days (Fig. 3.1., middle) and by 24 days the original hair length and density had been almost restored to normal (Fig. 3.1., bottom). Gradual contraction of massive isografts occurred during the healing and hair growth phases and thereafter ceased (Fig. 3.2.).

(b) Microscopic. Histological examination of massive isografts transplanted to Balb/c mice that had been given a dose of colchicine on either the 4th. or 5th. day following grafting revealed a hyperplastic response of their epithelium with many mitoses evident in the basal and prickle cell layers. This hyperplastic behaviour of isografted epithelium persisted until about 8 days. Thereafter, the frequency of mitotic figures in the deeper layers decreased, and the epithelium gradually assumed its normal thickness. Mitotic activity of basal cells , orderly maturation of all epidermal layers and elaboration of new stratum corneum continued normally during the period 10-15 days. Infiltrating mononuclear cells at the interface of donor and host tissue and in parts of the dermis which first became evident 2 days after grafting were present throughout the entire healing phase, but had largely disappeared during the period 20-25 days.

Behaviour of allografts exchanged across identical H-2 but different H-3 and H-4 loci.

(a) Macroscopic. Allografts exchanged between C3H and CBA mice behaved similarly to CBA isografts. Healing was rapid and hair growth appeared at 17-18 days. In contrast to the isografts, however, hair density in

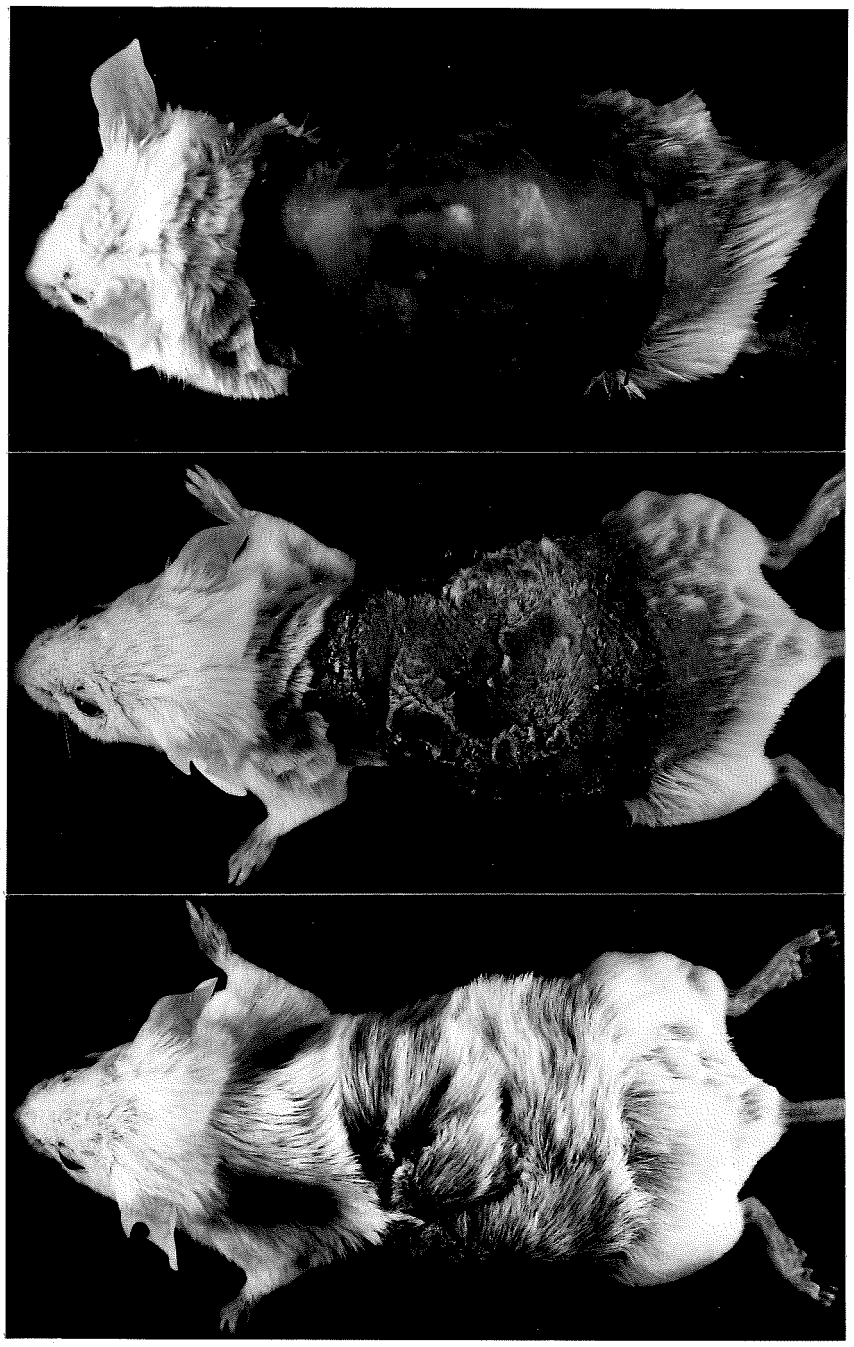

Fig. 3.1. Behaviour of a massive isograft transplanted to a Balb/c mouse. Top: 2 days after grafting. Middle: 15 days after grafting showing first evidence of continued hair growth. Bottom: 24 days after grafting; hair length and density have been almost restored to normal.

these allografts did not return to normal, not even in 2 CBA animals exhibiting otherwise successful C3H grafts at 233 and 240 days respectively.

(b) Microscopic. Twenty- to 45-day-old C3H to CBA allografts which appeared macroscopically healthy were found on histological examination to show healthy epithelium and normal mitotic activity of basal cells. A low-grade mononuclear infiltration was observed at the graft-host interface in most of these grafts examined. The 2 mice bearing successful allografts at 233 and 240 days showed a normal histological appearance of the grafted epithelium and dermis with no evidence of cellular infiltration in either the dermis or graft-host interface.

Fate of allografts exchanged across different H-2 loci.

(a) Macroscopic. Balb/c mice bearing either C57 Black or CBA allografts, and C57 Black recipients grafted with AKR skin showed evidence of a hypervascular response in the graft bed 3 to 4 days after grafting. However, by 6 to 7 days most allografts were no longer pink; their colour had become a grey or pale yellow-brown and in the following 2-3 days this had altered to a definite brown. During the transformation from pink to brown, the graft surface had become very dry and soon after numerous pitted areas became apparent, exposing the underlying moist dermis. At this stage, the grafts had lost their flat contour and became wrinkled, raising the level of their surfaces above that of the surrounding host skin. Considerable shrinkage of grafted skin occurred, particularly in the case of massive and large allografts, thereby reducing their surface area to about 60% or less of the original (Fig. 3.2.). These changes were evident to a varying degree at 7 days in all allografts examined, irrespective

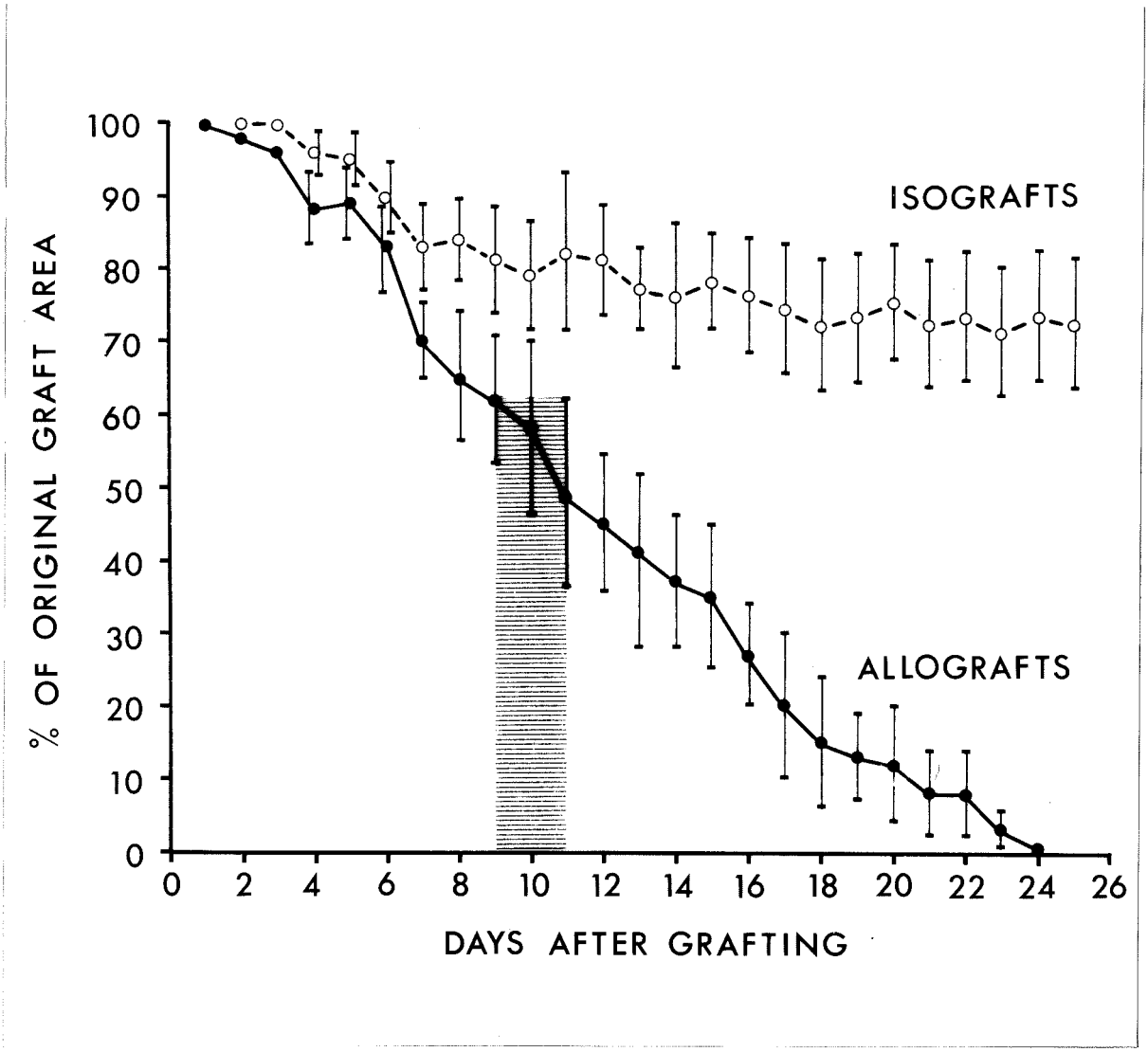

Fig. 3.2. Graph illustrating contraction of massive Balb/c isografts and CBA allografts transplanted to Balb/c mice. The vertical limits represent two standard deviations of each mean. The shaded area shows the period during which the allografts were classed as totally rejected.

of size, and, with massive and large grafts, became increasingly prominent during the next few days and involved the whole graft. According to the macroscopic criteria listed previously, m.s.t.'s of the different sized allografts are given in Table 3.1. Shedding of the rejected graft tissue in the form of a dry, shrunken scab occurred between 10 and 13 days in the case of small allografts, 17-20 days for medium and large grafts, 19-21 days for massive allografts in the combination AKR to C57 Black, and 22-25 days for massive CBA to Balb/c grafts (Fig. 3.3).

(b) Microscopic. Histological examination of massive CBA grafts transplanted to Balb/c mice that had been given a dose of colchicine revealed a thickened epidermis and a greater than normal number of mitoses in basal and prickle cell layers at 4 to 5 days after grafting (Fig. 3.4., top right). This hyperplastic response of allografted epithelium has been described previously (LAMBERT and FRANK, 1966). In 7- to 8-day-old allografts, however, very few dividing cells could be found in the deep epithelial layers while none were evident at 9 days (Fig. 3.4., bottom left). Microscopic examination of 9 to 11 day massive allografts which had been classed as rejected according to the macroscropic criteria outlined previously, revealed complete necrosis and dissolution of basal and spinosum layers, areas of oedema and haemorrhage beneath the necrotic epithelium, and an intense mononuclear and polymorphonuclear leucocyte infiltration throughout the graft dermis (Fig. 3.4., bottom right). These microscopic observations confirm the validity of criteria employed for macroscopic assessment of graft non-viability and support the accuracy of survival times given in Table 3.1. Following death of the graft, ingrowth of

TABLE 3.1. SURVIVAL TIMES OF DIFFERENT-SIZED SKIN ALLOGRAFTS TRANSPLANTED BETWEEN INBRED MICE DIFFERING AT THE H-2 LOCUS.

Donor Strain	Recipient Strain	Graft Size	Number of animals grafted	Survival time (days) Mean ± S.D.	Range (days)
C57 BL/6	Balb/c	$1cm^2$	64	7.1 ± 0.6	(6 - 8)
C57 BL/6	Balb/c	$4cm^2$	54	8.0 ± 0.8	(6 - 9)
C57 BL/6	Balb/c	$6cm^2$	58	8.3 ± 1.0	(7 - 10)
C57 BL/6	Balb/c	$8cm^2$	75	8.8 ± 0.8	(7 - 10)
CBA	Balb/c	$1cm^2$	39	7.4 ± 0.6	(6 - 8)
CBA	Balb/c	$4cm^2$	96	9.2 ± 0.6	(8 - 10)
CBA	Balb/c	$8cm^2$	12	10.0 ± 0.9	(8 - 11)
AKR	C57 BL/6	$1cm^2$	8	7.0 ± 0.5	(6 - 8)
AKR	C57 BL/6	$4cm^2$	10	8.9 ± 1.1	(7 - 10)
AKR	C57 BL/6	$8cm^2$	12	10.4 ± 0.9	(9 - 12)

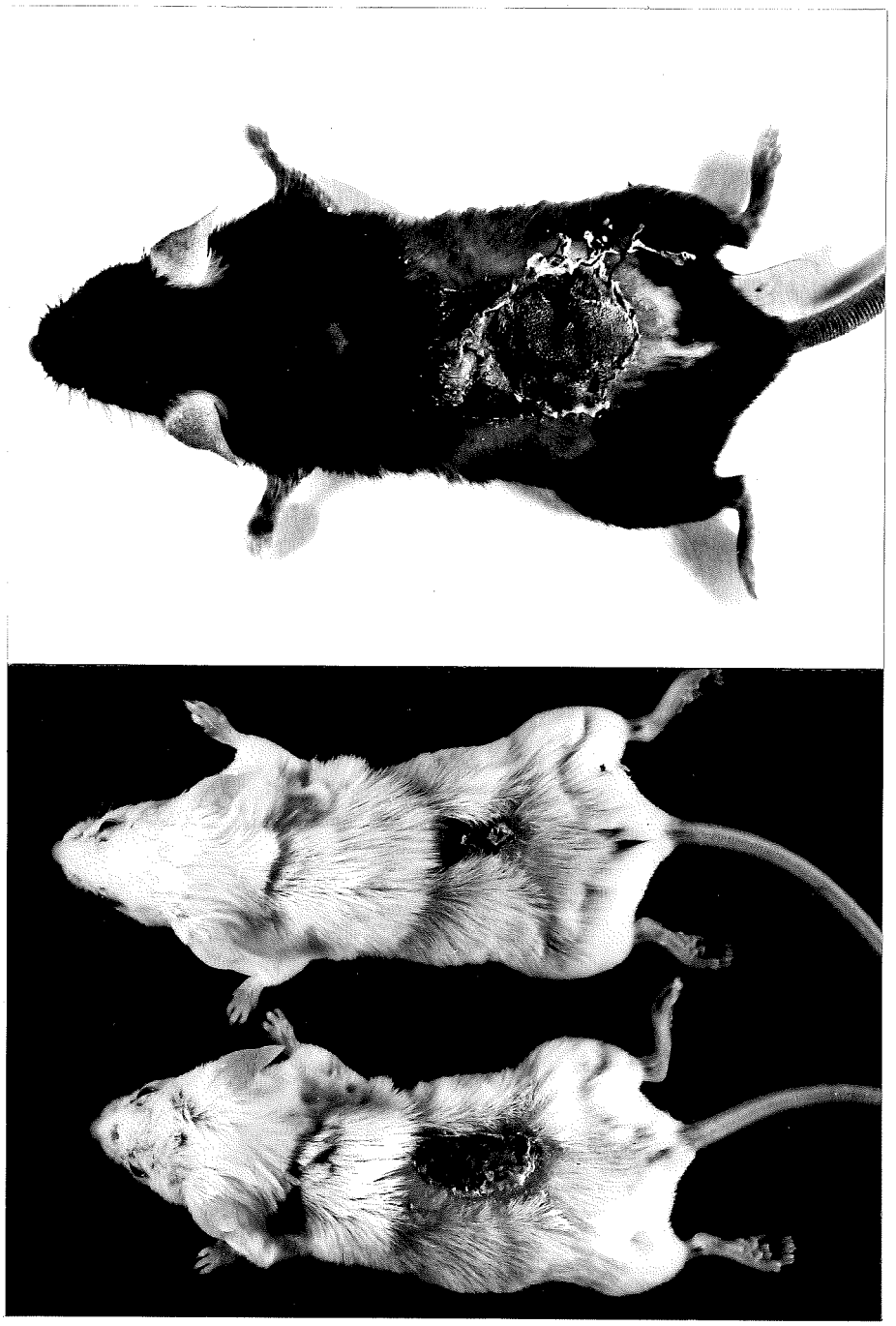

Fig. 3.3. The fate of massive allografts transplanted across strong H-2 barriers. These grafts were originally the same size as the isograft shown in Fig. 3.1. Top: C57 Black/6 mouse bearing remnants of AKR graft at 15 days; rejection of this graft had occurred at 11 days. Bottom: Balb/c mice with remnants of CBA grafts at 22 days; both of these grafts had been rejected at 10 days.

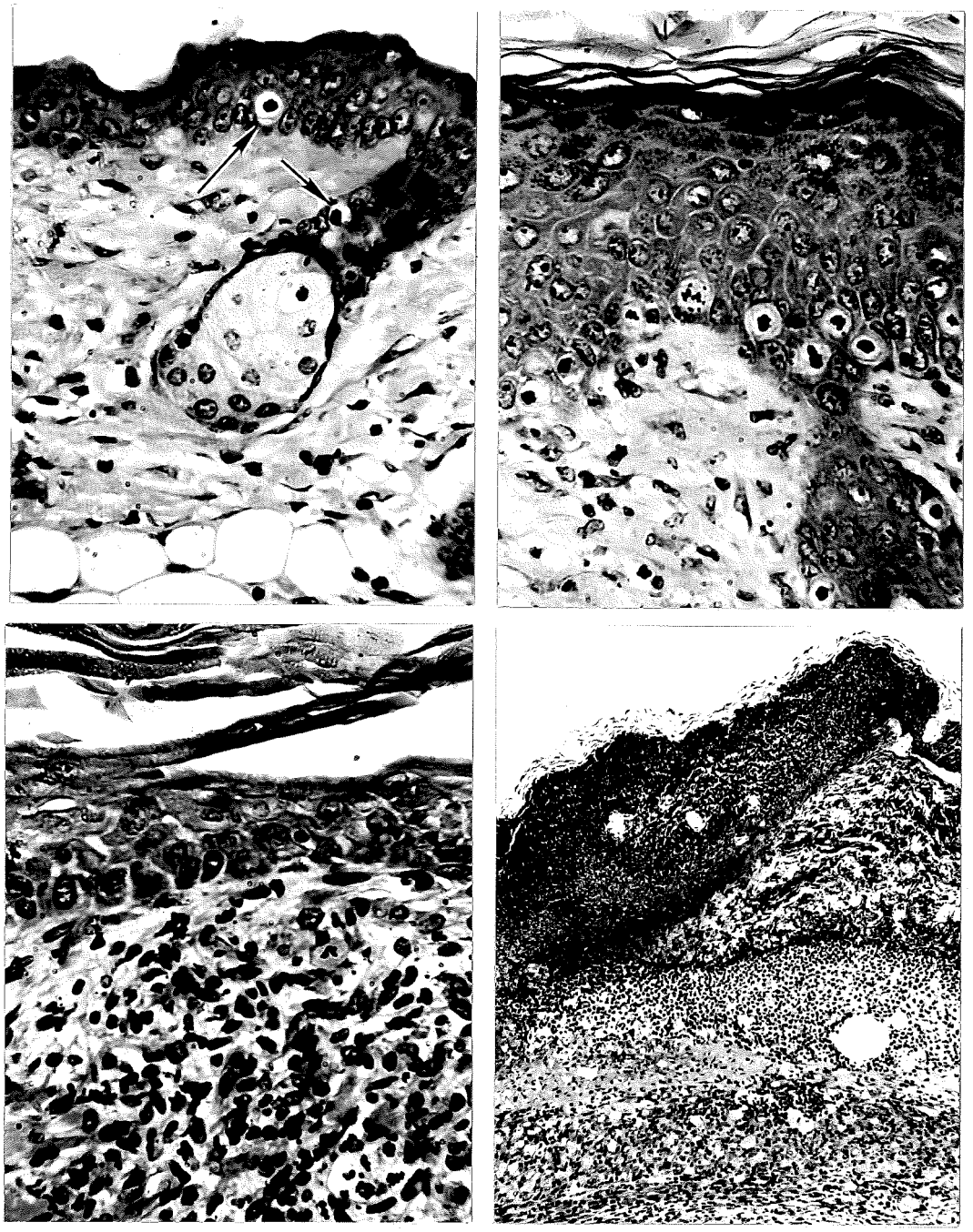

Fig. 3.4. Top left: normal mouse skin showing two basal cells (arrowed) arrested in metaphase stage of mitosis by the action of colchicine; x 240. Top right: massive CBA skin allograft 5 days after grafting; epidermis is hyperplastic with many mitoses in basal layer; x 240. Bottom left: massive CBA allograft 9 days after grafting; the hyperplastic phase has subsided, no dividing basal cells can be seen and all epidermal cells show degenerative changes; the dermis is heavily infiltrated with inflammatory cells; x 240. Bottom right; massive CBA allograft 11 days after grafting showing complete destruction of epidermis and presence of large numbers of inflammatory cells throughout the entire dermis; granulation tissue is forming below rejected graft; x 60. All sections stained with haematoxylin and eosin.

host epithelium undermined the rejected graft and eventually caused its exfoliation from the surface of the host.

Statistical analysis of graft survival times.

The experiments can be viewed as a ten class single classification and differences between classes can be tested by the standard Analysis of Variance techniques (Table 3.2). The nine degrees of freedom for between classes can be divided into three sets of degrees of freedom measuring differences between classes, within donor-recipient types, and two degrees of freedom measuring differences between donor-recipient types. Each set of degree of freedom measuring class differences can be further subdivided into (a) one degree of freedom measuring the significance of the linear regression on graft area, and (b) one or two degrees of freedom measuring departures from linearity.

In all three donor-recipient combinations tested the linear regression on graft area was highly significant (Table 3.2); *i.e.*, graft survival time showed a marked linear dependence on graft size. This is illustrated in the estimated regression lines (Fig. 3.5.) where it can also be seen that the combination CBA to Balb/c showed evidence of a small but significant curvature.

DISCUSSION

The results of the present study have shown that massive (8 $cm.^2$) skin allografts transplanted between inbred mice differing at the H-2 locus remain viable for longer periods than small (1 $cm.^2$) allografts. Although the difference in m.s.t.'s between massive and small allografts was found to be highly significant statistically, it is doubtful whether a 2 to 3 day increase in

TABLE 3.2. ANALYSIS OF VARIANCE OF GRAFT SURVIVAL TIMES

Component of variation	Degrees of Freedom		Sums of Squares	Mean Square	F Ratio
Class I : C57 Black to Balb/c					
Linear Regression on Area	1	99.08		99.08	182.67***
Departures from linearity	2	2.02		1.01	1.86 N.S.
Regression Total	3		101.10		
Class II : CBA to Balb/c					
Linear Regression on Area	1	93.11		93.11	171.66***
Departures from linearity	1	12.26		12.26	22.60***
Regression Total	2		105.37		
Class III : AKR to C57 Black					
Linear Regression on Area	1	54.97		54.97	101.34***
Departures from linearity	1	1.22		1.22	2.24 N.S.
Regression Total	2		56.19		
Differences between Donor-Recipient classes	2		55.78	27.89	51.42***
Between Subclass Total	9		318.44		
Within Subclasses (Error)	418		226.73	0.54	
Total	427		545.17		

*** Significant at 0.001 level. N.S. Not significant.

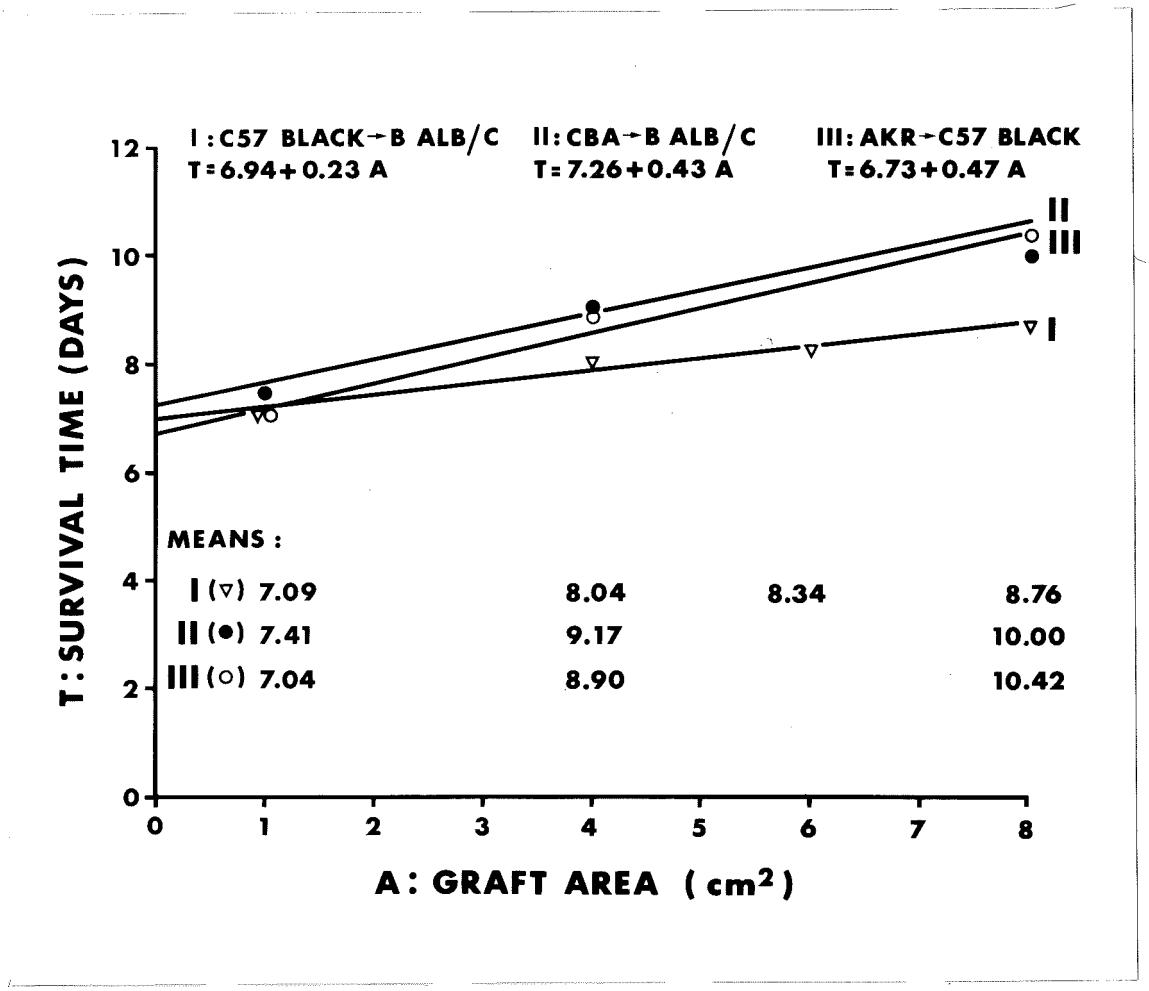

Fig. 3.5. Estimated regression lines of graft survival time on graft area in three different donor-recipient mouse combinations.

survival time for an 8-fold increase in graft size is of great significance from a practical standpoint. Animals bearing massive allografts experienced the greatest loss in body weight during the post-operative period, possibly reflecting the large degree of surgical trauma inflicted by the massive transplant procedure. Animal and human studies (RAPAPORT et al. 1964; CHAMBLER and BATCHELOR, 1969) have suggested that depression of immunological capacity following severe thermal injury may be responsible for the prolonged survival of skin allografts observed in such cases. A similar depression of the immune response associated with a large degree of surgical trauma may occur following placement of massive skin allografts and thereby contribute to their prolonged survival. Furthermore, the enormous quantity of foreign tissue involved with massive grafts would require a very great cellular response by the recipient animal to destroy and remove the grafted tissue, and this must take longer to occur than in the case of a small allograft. It is probable, therefore, that increased trauma to the recipient of a massive allograft leading to temporary immunodepression, together with the necessity of mounting a greater cellular response, may explain the slight increase in survival time of massive allografts over small ones.

Although the sizes of grafts employed in this study are closely comparable to those used by ZANELLA et al. (1968), the results do not substantiate the claim made by these workers that massive allografts survive for periods 2.0 to 2.6 times greater than small grafts. In the present study in which nearly 100 massive allografts were performed, none were observed to remain viable beyond 12 days. The area covered by each of these massive grafts would closely

approximate 25% of the animal's total body surface area. It is interesting to calculate from the estimated regression curves (Fig. 3.5.) that for a 16 cm.^{2} allograft, i.e., one covering approx. 50% of the animal's total body surface area, the m.s.t.'s of such grafts in donor-recipient combinations I, II and III would be 10.6, 14.1 and 14.3 days, respectively. ZANELLA et al. (1968) reported m.s.t.'s of massive allografts (covering approx. 33% of total body area) and large allografts (approx. 13% of total body area) ranging from 22 to 25 and 16 to 18 days, respectively, but did not provide evidence to demonstrate continued viability of these grafts. Their survival times correspond closely to periods at which the last remnants of scab graft were shed from our animals bearing massive and large allografts.

The presence of non-viable tissue anywhere in the body excites a cellular response which leads eventually to its removal. In the case of a skin allograft, physical removal of the rejected tissue is accomplished through an undermining of the necrotic material by growth of host epithelium across the graft bed. Shedding of graft remnants cannot occur until epithelial restoration is complete, and the time taken for epithelial bridging will be in direct proportion to the area involved. For this reason, remnants of rejected massive and large allografts will obviously remain attached to their beds for longer periods than remnants of very small allografts. Therefore, if one takes as the end point of rejection the time at which the last pieces of necrotic graft tissue are shed from the recipient, then graft survival time thus calculated will obviously be longer than the actual period during which the grafted tissue remains viable following transplantation.

Being entirely subjective, the macroscopic assessment of skin graft survival inevitably carries the likelihood of considerable inter-examiner variation unless specific criteria are closely followed. It is probable that the discrepancies in the previously reported results and conclusions relating to skin graft area and survival time in rats stem largely from the widely differing criteria for graft rejection employed by different investigators, and their failure to accurately define criteria for graft viability. Some groups (LEHRFELD and TAYLOR, 1953; CONVERSE, SIEGEL and BALLANTYNE, 1963; BALLANTYNE and STETSON, 1964; ROTHER, ROTHER and BALLANTYNE, 1967) have used stereomicroscopic examination of graft vessels, and have taken the end point for graft survival as being the time when haemostasis, followed by vascular disruption, occurred in the allografted blood vessels. Others (CALNAN and BLACK, 1962; CALNAN and KULATILAKE, 1962) have assessed skin graft survival using the macroscopic criteria of colour, consistence and attachment, and have classified a graft as rejected when it has changed from being pink, soft and clean to one that is yellow or brown, hard and moist. Another factor that may be partly responsible for the discrepant results is the uncertain genetic relationship between the experimental animals used in some of these studies. For example, the donor and recipient rats used by ZOTIKOV et al. (1960) were all albino and of unnamed genetic disparity. Their results must, therefore, be looked upon with reservation, particularly in the light of the work by MATTER et al. (1963). The latter group obtained greatly prolonged survival times of skin "homografts" exchanged between rats of the Holtzman strain which had been bred in a closed colony (but not inbred) for over 16 years and had obviously

reached a state of histocompatibility. Their experiments involving exchange of grafts across strong histocompatibility barriers between the Red Oklahoma King and Holtzman strains, however, demonstrated that there was no difference between m.s.t.'s of large and small skin allografts.

The criterion used by ZANELLA et al. (1968) to assess viability of their large and massive grafts was that of estimating visually the percentage of "surviving epithelial cells" and classifying a graft as rejected when there were less than 10% "surviving" cells. Although this method of assessment has been used by several investigators, it is, nevertheless, anatomically and physiologically unsound since the only layer of skin epidermis visible to the naked eye, the stratum corneum, is normally comprised of non-viable keratin. The viable cell layers of the epidermis, the strata spinosum and basale, are situated in the deeper regions and any accurate assessment of "surviving epithelial cells" in a skin graft can be made only by microscopic examination and demonstration of continued normal cellular activity in the deep epidermal layers.

In the present investigation, the validity of the macroscopic criteria used to classify a graft as non-viable has been confirmed by microscopic examination. Tissue viability implies continuation of normal physiological and biochemical function and it becomes necessary, therefore, to establish the existence of such normal function in grafted tissues before classifying them as viable. A full-thickness skin graft is composed of dermis and epidermis, and while the former contains mainly collagenous fibres, the latter is composed of several cell types, although they are all derived from the basal layer. The

dermal and epidermal tissues exhibit widely differing degrees of metabolic activity and obviously one specific biochemical criterion of "normal function" will be inappropriate for the whole graft. However, the maintenance of the integrity of the superficial epidermal layers is dependent upon normal proliferation of cells in the basal cell layer. Normal mitosis of these cells in turn depends on specific dermal-epidermal interactions (BRIGGAMAN and WHEELER, 1968). For these reasons, it is felt that continued proliferative activity of basal cells offers a simple, reliable and reproducible criterion for indicating that the epidermis of a skin graft is viable and the normal dermal-epidermal relationship is intact. In the present study, macroscopically healthy 15- to 45-day-old Balb/c isografts and allografts exchanged between C3H and CBA mice were found on microscopic examination to exhibit normal mitotic activity in their basal epidermal layers. On the other hand, epidermal cell proliferation in massive allografts transplanted from CBA to Balb/c mice was not observed to occur beyond 8 days following transplantation and total necrosis of graft epidermis occurred 2-3 days later.

Control auto- and isografts were carried out in parallel with the allografts throughout the entire course of the present investigation. These control grafts routinely showed evidence of hair growth by 16 days following transplantation and the original hair length and density had almost returned to normal by about 24 days. This observation aided the other macroscopic criteria of colour and surface characteristics used to assess graft viability. Hair growth also occurred at 17-18 days in allografts exchanged between C3H and CBA mice, and similar observations have been made in antilymphocyte serum-treated

Balb/c mice bearing successful CBA grafts for periods longer than 17 days (GILL, P. G. , personal communication). In the study by ZANELLA et al. (1968) and also in others where prolongation of skin allografts beyond 17 days has been reported (CONVERSE , SIEGEL and BALLANTYNE, 1963; BALLANTYNE and STETSON, 1964; ROTHER , ROTHER and BALLANTYNE, 1967), no mention is made of hair growth from these grafts. Furthermore, only one of these groups of workers (ROTHER , ROTHER and BALLANTYNE, 1967) carried out control isografts.

In order to avoid further confusion in future studies on transplantation phenomena in which skin graft survival is an important parameter, it is imperative that different investigators agree upon a set of uniform criteria for the assessment of skin graft viability. From the knowledge and experience gained by carrying out the present work, some suggestions are now offered. Undoubtedly, the list is incomplete, but is intended to serve merely as a baseline. It is desirable that donor skin be shaved completely before being grafted in order to facilitate naked-eye examination for colour and texture, and to ascertain whether hair growth is continuing. Auto-, or preferably isografts, must be carried out in parallel with the allografts and the appearance of the latter should be compared with that of the controls. Before a skin allograft is classed as being truly prolonged, it is essential that it exhibits a pink colour, has a smooth surface which is lying at the same level as the surrounding host skin and retains at least 60 to 70% of its original area; if greater than 16-17 days duration, it must show evidence of continued hair growth. Microscopically, the normal histology of the epidermis and dermis must be preserved and the cells of the basal layer

must show normal mitotic activity. The use of colchicine offers a simple, reliable and reproducible method for readily identifying dividing cells. The presence of an inflammatory cell accumulation at the graft-host interface, and even within parts of the dermis, need not necessitate labelling a graft as rejected. Our observations on animals showing prolonged survival of skin allografts, due either to transplantation across weak H-3 and H-4 barriers or to treatment with anti-lymphocyte serum, have been that many of these allografts exhibit an inflammatory cell infiltration at their graft-host interfaces, although their epidermal layers are quite intact.

As previously mentioned, the criteria outlined here will no doubt need to be augmented by other discriminating investigators. However, it is hoped that adoption of uniform criteria will eliminate further discrepancies arising from inter-examiner variation, and thereby allow rapid dissemination and ready acceptance of new findings in the field of transplantation immunity.

SUMMARY

Survival times of 428 skin allografts varying in area from 1 to 8 $cm.^2$ and exchanged between 4 inbred mouse strains differing at the H-2 locus are reported in this Chapter. As a preliminary to the main investigation, the macroscopic and microscopic appearance of allografts and isografts carried out in parallel were correlated and the macroscopic features indicative of graft non-viability adopted as criteria for classifying a graft as rejected.

In each of three different donor-recipient combinations tested, massive (8 $cm.^2$) allografts remained viable for 2 to 3 days longer than small

(1 cm.^{2}) allografts. The differences between survival times of massive and small grafts were highly statistically significant. Animals bearing massive allografts experienced the greatest loss in body weight during the post-operative period. It is suggested that temporary immunodepression following severe surgical trauma may be partly responsible for the prolonged survival of massive allografts.

Previous studies on the relation between skin allograft size and survival time have led to conflicting results and conclusions. These discrepancies probably stem from the widely differing criteria for graft rejection used by different investigators. In order to eliminate inter-investigator variation in future studies involving skin graft survival, it is necessary for different workers to adopt uniform criteria for assessing graft viability. Some suggestions have been offered in this Chapter, with special emphasis placed on the importance of demonstrating continued mitotic activity of basal epidermal cells before classifying a skin graft as viable and showing prolonged survival.

CHAPTER 4

THE EFFECT OF SKIN TRANSPLANTATION ON THE PHAGOCYTIC ACTIVITY AND MORPHOLOGY OF RETICULOENDOTHELIAL ORGANS IN MICE

PAGE

INTRODUCTION

The experiments to be reported in this Chapter were begun in 1966, up to which time only one report dealing with the effect of skin allografts on the phagocytic properties of the RES had been published by FISHER and FISHER (1964). The experiments reported by MARSHALL (1968) and MARSHALL and KNIGHT (1969a) on the effect of skin allografts on the RES of mice were carried out concurrently with those of the present study.

FISHER and FISHER (1964) used rats in their investigation and observed an increased rate of phagocytosis of intravenously administered carbon during the period of skin allograft rejection (7 to 14 days after grafting). Xenografts were also found to produce phagocytic stimulation, but this occurred only at 14 days and not during the rejection phase (7 to 10 days). No reference was made to any alterations in mass or histological structure of the RE organs in the test animals. Other investigators had previously reported morphological changes in the lymph nodes of rabbits and mice following allo-transplantation of skin (SCOTHORNE and McGREGOR, 1955; ANDRE et al., 1962; MICKLEM and BROWN, 1967), cartilage (CRAIGMYLE, 1958) and bone (BURWELL and GOWLAND, 1960). In each of these situations, the lymph nodes enlarged and histological examination revealed increased numbers of medium and large lymphoid cells in the cortex (mainly) and in the medulla; large numbers of young plasma cells also occupied the medullary cords. Although SCOTHORNE and McGREGOR (1955) were unable to detect any histological changes in the spleens of rabbits which had skin allografts applied to one ear, ANDRE et al. (1962) employing a similar technique found enlargement of lymphatic nodules and germ-

inal centres in the spleens of their allografted rabbits. Similar hyperplastic changes in the splenic white pulp of mice bearing skin allografts were reported by CONGDON (1962). In marked contrast, SHANFIELD et al. (1962) found profound lymphoid depletion in the spleens of dogs which had received renal allografts and which were subsequently maintained without immunosuppressive drugs. The lymphatic nodules were either absent or very small and did not show germinal centre development; plasma cell numbers in both the white and red pulp were increased. These discrepancies in the nature of the cellular responses reported by different investigators are probably due to 3 main factors: variations in the splenic histology exhibited by different animal species, differences in the type and size of allograft employed, and the different time interval after grafting at which the spleen was examined.

The Present Investigation.

The aim of the present investigation was to study mice bearing different sized skin allografts at varying times after transplantation and examine for any alterations in the phagocytic activity of the RES or morphology of the liver and spleen. Initially, phagocytic activity was estimated by measuring the rate of blood clearance of P^{32} - labelled *Salmonella typhimurium C5*. Groups of female Balb/c mice (3 per group) bearing skin allografts were examined at periods ranging from 2 to 30 days after transplantation. Isografted mice were similarly studied. Normal, ungrafted Balb/c mice served as controls. Phagocytic indices were calculated for individual mice, but the particular RE organs from animals in each group were weighed together and expressed as a

percentage of the mean body weight for that group. While this data permitted patterns of organ weight change to be followed, it precluded carrying out statistical analyses to test the significance of differences in organ weights between different groups. For this reason, only the mean phagocytic index and the standard error of the mean for each animal group studied by the bacterial clearance method have been included in the results.

A problem encountered occasionally was agglutination of bacteria, which would sometimes occur after opsonization and thereby give rise to spurious K values for the untreated control and grafted groups. For this reason, not all results of experiments in which P^{32}-labelled bacteria were used to assess phagocytic activity of grafted mice have been presented in this Chapter. Only those in which agglutination did not occur have been included.

During the course of preliminary experiments, the observation was made that the livers and spleens of mice bearing skin allografts enlarged considerably. To determine whether increased numbers of macrophages contributed to the increased organ mass, and to provide data on phagocytic activity of grafted mice which could be compared directly with that previously reported by FISHER and FISHER (1964), a new series of experiments was undertaken using colloidal carbon as the RES test particle. The general plan of these experiments was similar to that employed in the bacterial clearance studies, but with three main modifications. Four animals instead of three were included in each allografted and isografted group, and the weight of each animal's liver, spleen, and 4 lymph nodes was determined *individually* and expressed as a percentage of that animal's body weight. Knowledge of individual liver and spleen

weights and the phagocytic index permitted calculation of the corrected phagocytic index for each animal. Morphological changes in the liver and spleen were followed in H and E stained sections, while the relative numbers of macrophages which had phagocytosed carbon were assessed in unstained liver sections and neutral red-stained sections of spleen. In addition, electron microscopic examination and biochemical estimations were performed on the enlarged livers of allografted mice.

A review of the literature on drug-induced hepatomegaly revealed several references to liver enlargement in rats and mice treated with phenobarbital (CONNEY et al., 1960; REMMER, 1962; HERDSON, GARVIN and JENNINGS, 1964a; KUNZ et al., 1966; PLATT and COCKRILL, 1967). In all of these studies, hepatomegaly developed only when phenobarbital in a dose of 70 to 100 mg/kg was given daily for 4 consecutive days or longer. It seemed unlikely, therefore, that the liver enlargement found in allografted mice was due to the single 60 mg/kg dose of sodium pentobarbitone administered to induce anaesthesia. Nevertheless, to test this possibility, Balb/c mice which received a single 60 mg/kg dose of sodium pentobarbitone, but not subjected to any other experimental procedure, were examined at varying time periods after injection for any alteration in liver weight or phagocytic activity of the RES.

RESULTS

BACTERIAL CLEARANCE STUDIES

Phagocytic indices of Balb/c mice bearing primary 4cm^2 skin isografts and C57 Black allografts.

Table 4.1 shows K values obtained for allografted and isografted mice at varying times after transplantation. When compared with the untreated controls,

allografted mice exhibited significant phagocytic stimulation ($p < 0.01$) before, during and after the period of rejection. However, with the exception of the 16 day group, isografted mice showed the same degree of stimulation.

Phagocytic indices of Balb/c mice bearing secondary 4 cm^2 C57 Black skin allografts.

Balb/c mice which received a second 4 cm^2 C57 Black allograft 3-5 weeks after they had rejected a primary 4 cm^2 C57 Black allograft showed phagocytic indices which did not differ significantly from those obtained for untreated control mice (Table 4.2.).

Phagocytic indices of Balb/c mice bearing primary 8 cm^2 skin isografts and C57 Black allografts.

Phagocytic indices of mice with massive isografts and allografts are given in Table 4.3. Significant stimulation of phagocytosis ($p < 0.05$) was evident in the 6, 9 and 11 day isografted and allografted groups.

CARBON CLEARANCE STUDIES

Phagocytic indices of normal mice.

Mean phagocytic indices for groups of normal, 12-16 week-old, female Balb/c mice ranged from 0.026 to 0.028 with a standard deviation of 0.002 to 0.004 (Tables 4.4 to 4.10 inclusive). These figures compare with a mean phagocytic index $\pm$ S.D. of 0.021 ± 0.011 obtained by BENACERRAF et al., (1957) for outbred Swiss white mice. Preliminary clearance experiments performed on different mouse strains prior to commencing the present investigation revealed that mean K values varied between 0.015 and 0.028, and that this variation was probably related to differences in the relative size of the liver and

TABLE 4.1. RATE OF BLOOD CLEARANCE OF P^{32}-LABELLED *SALMONELLA TYPHIMURIUM C5* BY BALB/C MICE[a] BEARING PRIMARY 4CM^2 SKIN ISOGRAFTS AND C57 BLACK ALLOGRAFTS[b].

Group	Phagocytic Index (K) (Mean ± S.E.M.)
Untreated control	0.12 ± 0.01
2 day allografts	0.14 ± 0.01
5 day allografts	0.14 ± 0.01
6 day allografts	0.17 ± 0.01**
6 day isografts	0.16 ± 0.01**
8 day allografts	0.16 ± 0.01**
10 day allografts	0.15 ± 0.01**
10 day isografts	0.15 ± 0.01**
14 day allografts	0.14 ± 0.01
14 day isografts	0.17 ± 0.02*
16 day allografts	0.16 ± 0.01**
16 day isografts	0.14 ± 0.02
22 day allografts	0.16 ± 0.01
26 day allografts	0.12 ± 0.01
30 day allografts	0.13 ± 0.02

a There were 6 mice in the untreated control group and 3 mice in each grafted group.

b Allograft rejection occurred between 6 and 9 days.

* p 0.01 - 0.05; ** p 0.001 - 0.01 (test group versus untreated control group).

TABLE 4.2. RATE OF BLOOD CLEARANCE OF P^{32}-LABELLED *SALMONELLA TYPHIMURIUM C5* BY BALB/C MICE[a] BEARING SECONDARY $4CM^2$ C57 BLACK SKIN ALLOGRAFTS[b].

Group (Time after secondary grafting)	Phagocytic Index (Mean ± S.E.M.)
Untreated control	0.14 ± 0.01
2 days	0.16 ± 0.01
3 "	0.16 ± 0.01
4 "	0.16 ± 0.01
5 "	0.17 ± 0.02
6 "	0.15 ± 0.01
7 "	0.15 ± 0.01
10 "	0.17 ± 0.01
12 "	0.16 ± 0.01
15 "	0.14 ± 0.01
22 "	0.15 ± 0.01

a There were 6 mice in the untreated group and 3 mice in each grafted group.

b Rejection occurred between 6 and 8 days.

TABLE 4.3 RATE OF BLOOD CLEARANCE OF P^{32}-LABELLED *SALMONELLA TYPHIMURIUM C5* BY BALB/C MICE[a] BEARING PRIMARY $8CM^2$ SKIN ISOGRAFTS AND C57 BLACK ALLOGRAFTS[b].

Group	Phagocytic Index (Mean ± S.E.M.)
Untreated control	0.13 ± 0.01
2 day allografts	0.14 ± 0.02
2 day isografts	0.12 ± 0.01
4 day allografts	0.14 ± 0.01
4 day isografts	0.13 ± 0.01
6 day allografts	0.16 ± 0.01*
6 day isografts	0.16 ± 0.01*
9 day allografts	0.16 ± 0.02*
9 day isografts	0.16 ± 0.01*
11 day allografts	0.16 ± 0.01*
11 day isografts	0.15 ± 0.01*
16 day allografts	0.14 ± 0.01
16 day isografts	0.12 ± 0.01
22 day allografts	0.13 ± 0.01
22 day isografts	0.13 ± 0.01
29 day allografts	0.13 ± 0.01
29 day isografts	0.11 ± 0.01

a There were 6 mice in the untreated control group and 3 mice in each grafted group.

b Allograft rejection occurred between 7 and 10 days.

* p 0.01 - 0.05 (test group vs untreated control group).

spleen in different strains. Balb/c mice were found to have the largest RE organs relative to body weight, and accordingly showed the highest phagocytic indices of all strains examined (see Appendix 4).

Liver and spleen weights and phagocytic indices of Balb/c mice given a single intra-peritoneal dose (60 mg/kg) of sodium pentobarbitone.

The results presented in Table 4.4 show that a single, 60 mg/kg dose of sodium pentobarbitone administered to Balb/c mice did not affect the weights of the liver or spleen, nor did it alter the phagocytic index.

Liver and spleen weights and phagocytic indices of Balb/c mice bearing primary 1 cm^2 skin isografts and CBA allografts.

A significant increase in the phagocytic index of mice with 1 cm^2 isografts and allografts first became evident 7 days after transplantation and thereafter persisted for a further 2 to 3 weeks (Tables 4.5 and 4.6). Isografted and allografted mice had enlarged spleens at 4 and 7 days, while hepatomegaly was found only in the 7, 22 and 29-day allografted groups.

Liver and spleen weights and phagocytic indices of Balb/c mice bearing primary 4 cm^2 isografts and CBA allografts.

Phagocytic indices of mice with 4 cm^2 isografts and allografts did not differ significantly from those obtained for untreated control mice (Tables 4.7 and 4.8). Isografted and allografted mice showed splenomegaly (up to a 2-fold enlargement) when examined at 6, 8 and 10 days after grafting (Tables 4.7 and 4.8; Fig. 4.1). However, whereas mice in the 22-day allografted group had spleens which were enlarged 3-fold, mice in the corresponding isografted group had normal-sized spleens.

TABLE 4.4. LIVER AND SPLEEN WEIGHTS AND PHAGOCYTIC INDICES[a] OF BALB/C MICE[b] GIVEN A SINGLE INTRA-PERITONEAL DOSE (60mg/kg) OF SODIUM PENTOBARBITONE (MEANS ± S.E.M.).

Group (Time after injection)	No. of animals studied	Liver weight as % of Total Body Weight (T.B.W.).	Spleen weight as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	4	4.89 ± .11	.51 ± .01	.028 ± .001	5.62 ± .11
4 days	6	5.27 ± .07	.49 ± .02	.027 ± .002	5.22 ± .14
6 "	6	4.77 ± .07	.49 ± .02	.027 ± .001	5.71 ± .13
8 "	10	5.19 ± .13	.54 ± .03	.028 ± .001	5.32 ± .11
9 "	15	5.22 ± .09	.51 ± .02	-	-
10 "	21	4.63 ± .05	.54 ± .01	.026 ± .001	5.72 ± .05

a The carbon clearance test was performed on 4 animals from each group with the exception of the 9 day group.

b Female mice aged 14-18 weeks and weighing between 19 and 23 g were used.

TABLE 4.5. LIVER AND SPLEEN WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING 1CM^2 SKIN ISOGRAFTS (MEANS ± S.E.M.).

Group (Time after grafting).	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.05 ± .06	.52 ± .03	.027 ± .002	5.40 ± .15
4 days	5.36 ± .16	.75 ± .02**	.030 ± .001	4.86 ± .10*
7 "	5.31 ± .12	.69 ± .02**	.044 ± .003**	5.29 ± .19
16 "	5.03 ± .11	.52 ± .01	.036 ± .002*	5.72 ± .16
22 "	5.14 ± .19	.54 ± .01	.033 ± .001*	5.66 ± .15

* p 0.01 - 0.05; ** p 0.001 - 0.01 (grafted group vs untreated control group).

TABLE 4.6. LIVER AND SPLEEN WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING $1CM^2$ PRIMARY CBA SKIN ALLOGRAFTS[a] (MEANS ± S.E.M.).

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.05 ± .06	.52 ± .03	.027 ± .002	5.40 ± .15
4 days	4.93 ± .33	.94 ± .07**	.027 ± .003	5.17 ± .44
7 "	5.83 ± .12**	.95 ± .04***	.042 ± .003**	5.10 ± .13
10 "	5.07 ± .07	.56 ± .01	.040 ± .002**	6.08 ± .15*
16 "	5.04 ± .07	.57 ± .04	.033 ± .001*	5.71 ± .10
22 "	5.61 ± .14**	.52 ± .02	.039 ± .002**	5.55 ± .11
29 "	5.51 ± .16*	.52 ± .02	.037 ± .001**	5.52 ± .09

a Rejection occurred between 6 and 8 days.

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (grafted group vs untreated control group).

TABLE 4.7. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING 4 CM^2 SKIN ISOGRAFTS (MEANS ± S.E.M.).

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.).	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	4.91 ± .08	0.50± .02	.15 ± .01	.028 ± .002	5.44± .19
6 days	5.36 ± .23	0.81± .05***	.18 ± .01**	.031 ± .002	5.07± .07
8 "	5.35 ± .10	1.05± .05***	.18 ± .01**	.030 ± .001	4.87± .12*
10 "	5.02 ± .10	0.82± .04***	.19 ± .01**	.029 ± .001	5.23± .13
22 "	5.18 ± .14	0.57± .03	.16 ± .01	.027 ± .002	5.01± .14

a Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (grafted group vs untreated control group).

TABLE 4.8. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING PRIMARY 4 CM^2 CBA SKIN ALLOGRAFTS[b] (MEANS ± S.E.M.)

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	4.91 ± .08	0.50 ± .02	.15 ± .01	.028 ± .002	5.34 ± .19
4 days	4.66 ± .09	0.71 ± .01***	.17 ± .01	.021 ± .002	5.10 ± .14
6 "	5.87 ± .17**	0.89 ± .04***	.24 ± .01***	.023 ± .001	4.20 ± .07***
8 "	7.43 ± .14***	1.19 ± .07***	.25 ± .01***	.023 ± .001	3.33 ± .09***
10 "	6.95 ± .29***	1.37 ± .05***	.24 ± .01***	.026 ± .002	3.59 ± .17***
12 "	5.94 ± .26**	1.44 ± .06***	.24 ± .01***	.023 ± .003	3.87 ± .25**
15 "	5.79 ± .22*	1.20 ± .06***	.21 ± .01***	.023 ± .003	4.03 ± .06***
18 "	5.38 ± .09	1.24 ± .07***	.22 ± .01***	.027 ± .001	4.55 ± .13**
22 "	6.56 ± .21***	1.57 ± .13***	.22 ± .02**	.037 ± .004	4.13 ± .28**
26 "	6.02 ± .08***	1.03 ± .20***	.22 ± .01***	.031 ± .003	4.47 ± .22*
36 "	5.34 ± .21	1.03 ± .20***	.21 ± .01***	.029 ± .001	4.86 ± .19

a Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

b Rejection occurred between 8 and 10 days.

* p 0.01-0.05; ** p 0.001-0.01; *** p < 0.001 (grafted group vs. untreated control group).

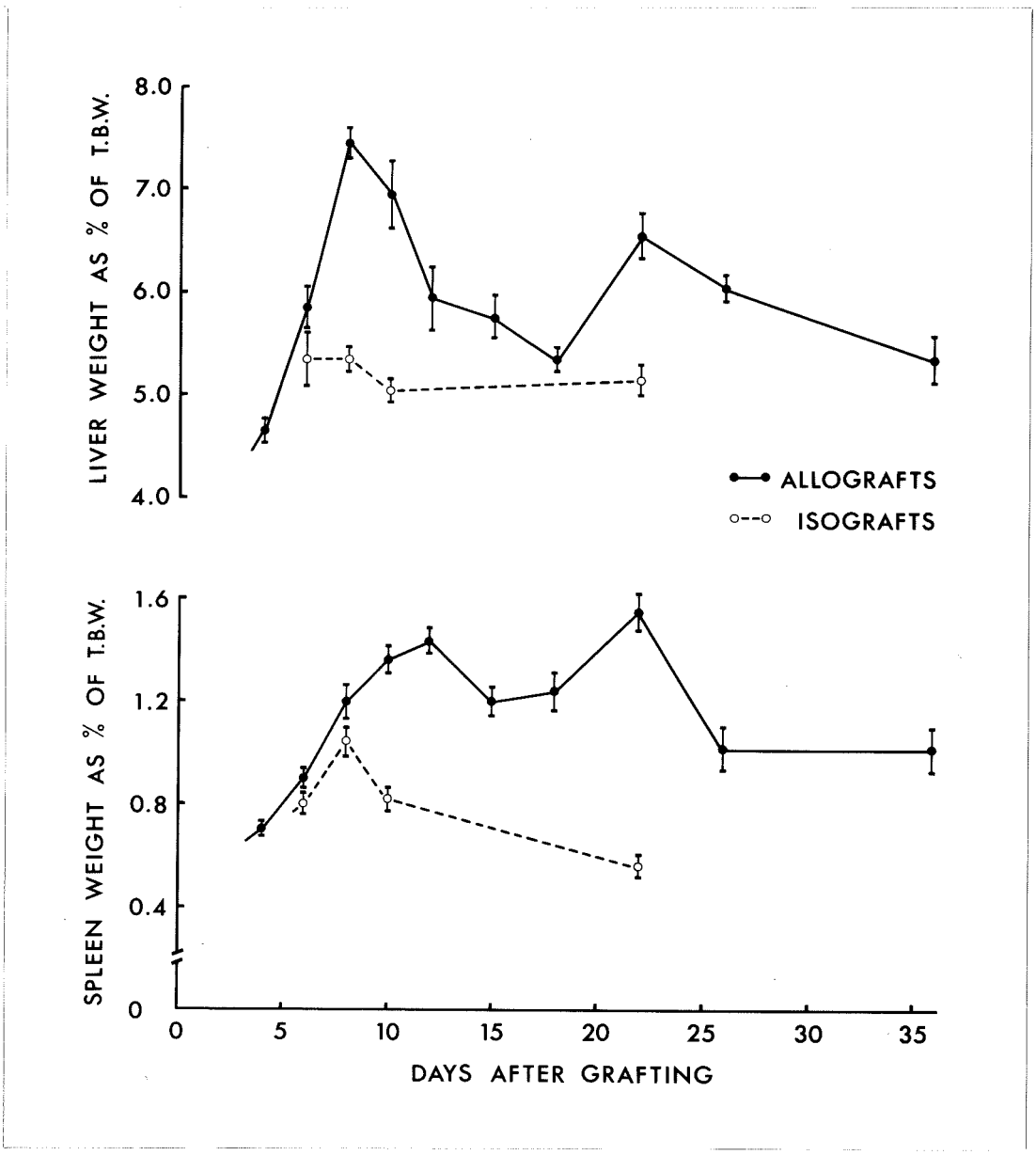

Fig. 4.1. Liver and spleen weights (expressed as a percentage of total body weight, T.B.W.), of groups of Balb/c mice at varying times after transplantation of primary 4 cm^2 skin isografts and CBA allografts. The vertical limits represent two standard errors of each mean.

Hepatomegaly became evident prior to allograft rejection, reached a maximum during the rejection phase and persisted for a variable time thereafter (Table 4.8; Fig. 4.1.). A second peak of liver enlargement which occurred at 22 days coincided with the second peak of splenomegaly (Fig. 4.1.). Due to the considerable degree of hepatosplenomegaly exhibited by the allografted mice, the corrected phagocytic indices for the 6 to 26-day groups showed a significant decrease (Table 4.8.). No alteration in liver weight was noted in any of the isografted mice (Table 4.7.).

Liver and spleen weights and phagocytic indices of Balb/c mice bearing secondary 4 cm^2 CBA allografts.

Phagocytic indices of Balb/c mice which received a second 4 cm^2 CBA graft 3 to 6 weeks after rejecting a primary 4 cm^2 CBA graft did not differ from the values obtained for untreated control mice (Table 4.9). Enlargement of the liver and spleen occurred between 3 and 4 days after application of the secondary graft and persisted until 18 days. As in the case of primary 4 cm^2 allografts, mice bearing secondary 4 cm^2 allografts showed a second peak of hepatosplenic enlargement at 22-23 days. Because the weights of the liver and spleen were increased while the K values remained unaltered, the corrected phagocytic indices for the groups examined between 4 and 23 days after grafting were significantly lower than that of the control group.

Liver and spleen weights and phagocytic indices of Balb/c mice bearing primary 8 cm^2 isografts and CBA allografts.

Mice bearing massive isografts and allografts had phagocytic indices which did not differ significantly from those recorded for untreated control mice

TABLE 4.9. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING SECONDARY 4 CM^2 CBA SKIN ALLOGRAFTS[b] (MEANS ± S.E.M.).

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.01 ±.09	0.58± .03	.17± .01	.026 ±.001	5.27± .09
3 days	5.43 ±.14	0.82± .03**	.21± .02	.026 ±.001	4.76± .11
4 "	6.27 ±.07***	0.89± .05**	.21± .01*	.029 ±.001	4.31± .05***
5 "	6.12 ±.25**	0.90± .07**	.21± .01*	.031 ±.002	4.54± .19*
6 "	6.50 ±.05***	1.09± .02***	.17± .02	.031 ±.001	4.21± .05***
7 "	5.70 ±.05***	1.21± .04***	.18± .03	.034 ±.002	4.68± .09**
8 "	5.70 ±.07***	1.37± .10***	.17± .02	.032 ±.001	4.52± .10**
10 "	6.21 ±.14***	1.34± .07***	.20± .02	.033 ±.002	4.25± .11***
12 "	5.86 ±.24*	0.96± .07**	.15± .03	.033 ±.002	4.70± .12**
15 "	5.43 ±.15	0.90± .03***	.15± .01	.028 ±.001	4.82± .10*
18 "	6.02 ±.32*	0.93± .11*	.16± .02	.033 ±.002	4.66± .18*
20 "	5.22 ±.09	0.64± .02	.18± .02	.027 ±.001	5.10± .07
22 "	6.46 ±.08***	1.20± .39	.18± .04	.034 ±.001	4.26± .20**
23 "	6.28 ±.15***	2.27± .15***	.18± .02	.034 ±.002	3.79± .08***

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Rejection occurred between 6 and 8 days.

* p 0.01-0.05; ** p 0.001-0.01; *** p < 0.001 (grafted group vs. untreated control group).

(Tables 4.10 and 4.11). When examined between 4 and 16 days after the massive transplant procedure, allografted and isografted mice were found to exhibit the same degrees of enlargement of their livers and spleens. The corrected phagocytic indices for all grafted groups exhibiting hepatosplenomegaly were significantly less than that of the untreated control group.

Morphologic changes in the enlarged livers of mice bearing skin grafts as seen under light microscopy.

Examination of unstained histological sections prepared from the enlarged livers of mice bearing 4 cm^2 allografts 8 to 10 days after transplantation failed to reveal any increase in the relative number of Kupffer cells capable of phagocytosing carbon. In H and E-stained sections, the hepatocytes were seen to be enlarged (Fig. 4.3; compare with normal-sized liver parenchymal cells shown in Fig. 4.2), suggesting that the hepatomegaly was the result of hypertrophy of hepatocytes rather than an increase in their number. Support for this conclusion was derived from an experiment in which groups of Balb/c mice (3 per group) bearing 4 cm^2 CBA allografts at 3, 4, 5, 6, 7 and 8 days after grafting were injected with colchicine and their livers examined for mitotic figures. No significant increase in mitotic rate could be detected in any section examined, indicating that increased cell proliferation did not contribute to the liver enlargement.

The enlarged livers exhibited by mice bearing massive allografts and isografts were also found to contain hypertrophied parenchymal cells.

TABLE 4.10. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING 8 CM^2 SKIN ISOGRAFTS (MEANS ± S.E.M.).

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.11 ± .11	0.55 ± .02	.14 ± .01	.028 ± .002	5.35 ± .09
4 days	6.24 ± .20**	0.95 ± .03***	.19 ± .02*	.030 ± .002	4.35 ± .16**
8 "	6.37 ± .39*	1.56 ± .04***	.16 ± .01	.032 ± .004	4.03 ± .28**
12 "	6.63 ± .28**	0.82 ± .10*	.19 ± .01**	.027 ± .003	4.05 ± .29**
16 "	5.82 ± .20*	0.83 ± .05**	.19 ± .01**	.029 ± .002	4.65 ± .14**
32 "	4.99 ± .10	0.53 ± .01	.13 ± .01	.024 ± .001	5.21 ± .03

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (grafted group vs untreated control group).

TABLE 4.11. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE BEARING PRIMARY 8 CM^2 CBA SKIN ALLOGRAFTS[b] (MEANS ± S.E.M.).

Group (Time after grafting)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.11± .11	0.55 ± .02	.14± .01	.028 ± .001	5.35± .09
4 days	5.94± .11**	1.10 ± .05***	.16± .01	.033 ± .002	4.99± .14
8 "	6.17± .20**	1.54 ± .05***	.19± .01***	.032 ± .003	4.33± .14***
10 "	6.33± .13***	1.25 ± .14***	.20± .01**	.023 ± .003	3.74± .11***
12 "	5.92± .09**	0.94 ± .05***	.17± .02	.024 ± .003	4.18± .15***
15 "	6.41± .14***	1.04 ± .07***	.18± .01*	.023 ± .001	3.63± .10***
22 "	5.89± .10**	0.69 ± .05*	.19± .01**	.023 ± .001	4.14± .17***
26 "	5.63± .08**	0.57 ± .02	.19± .01**	.023 ± .002	4.60± .13**
32 "	5.76± .28	0.58 ± .02	.16± .01	.024 ± .001	4.56± .23*
50 "	5.26± .06	0.57 ± .01	.16± .01	.025 ± .002	5.05± .16

a Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

b Rejection occurred between 8 and 11 days.

* p 0.01-0.05; ** p 0.001-0.01; *** p < 0.001 (grafted group vs. untreated control group).

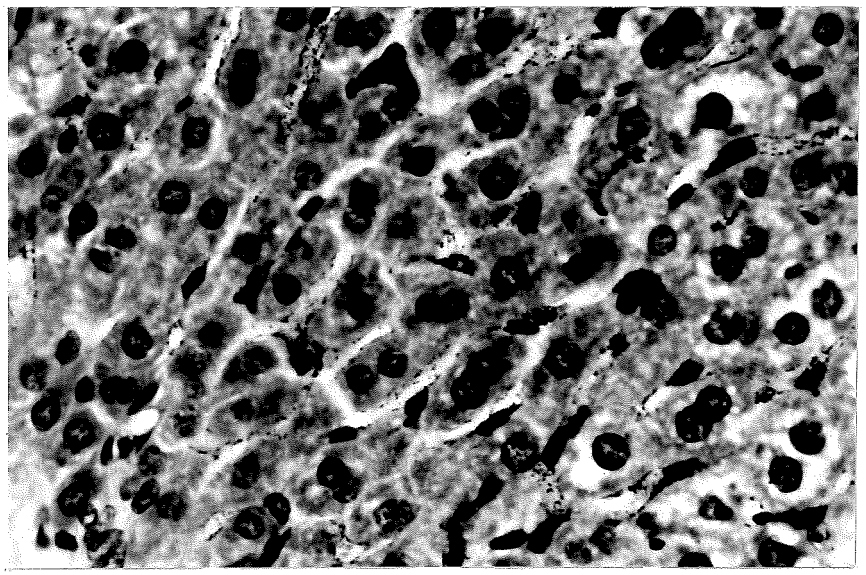

Fig. 4.2. Liver of a normal Balb/c mouse sacrificed 15 minutes after receiving a standard dose of colloidal carbon intravenously. Many Kupffer cells have phagocytosed carbon. Note the relative size of the hepatic parenchymal cells (This liver represented 5.07% of the animal's total body weight). H and E X400.

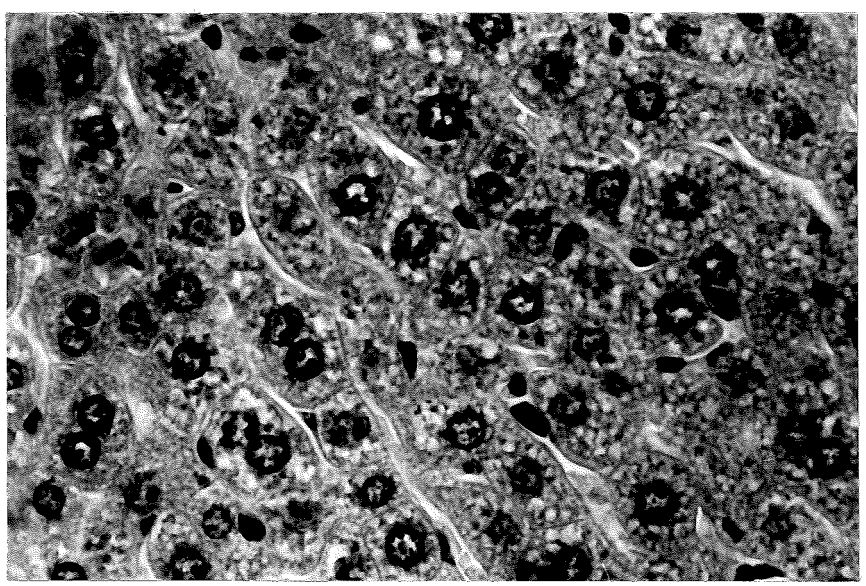

Fig. 4.3. Liver of a Balb/c mouse sacrificed 8 days after receiving a 4 cm^2 CBA skin allograft (This liver was significantly enlarged, representing 6.38% of the animal's total body weight). The enlargement of hepatocytes is due to an increase in the quantity of cytoplasm. Only the nuclei of Kupffer cells are evident along the sinusoids since this mouse was not injected intravenously with carbon. H and E X400.

Alterations in the fine structure of hepatocytes from enlarged livers of mice bearing skin allografts.

The most prominent alteration in the fine structure of hepatocytes in the enlarged livers of mice with 4 cm^2 allografts was an increase in the amount of rough-surfaced endoplasmic reticulum (RER) (Fig. 4.5; compare with normal appearances shown in Fig. 4.4). The RER was distributed in an inter-mitochondrial and peri-mitochondrial position, and many ribosomal strands were closely apposed to the mitochondrial membranes. The relative number and size of mitochondria were not greatly altered, although some hypertrophied hepatocytes did appear to contain increased numbers of mitochondria which were larger than normal.

Biochemical changes in livers of mice bearing skin allografts and isografts.

The percentage composition of water, protein, fat, glycogen and DNA in the livers of mice grafted with 4 cm^2 allografts and isografts did not differ significantly from the corresponding values obtained for untreated control mice. RNA concentration was between 19 and 26% higher in the allografted and isografted mice than that recorded for the controls. The differences in concentration of liver RNA between allografted and control mice and between isografted and control mice were highly significant ($p < 0.001$ in each case), but there was no significant difference between the values for allografted and isografted mice.

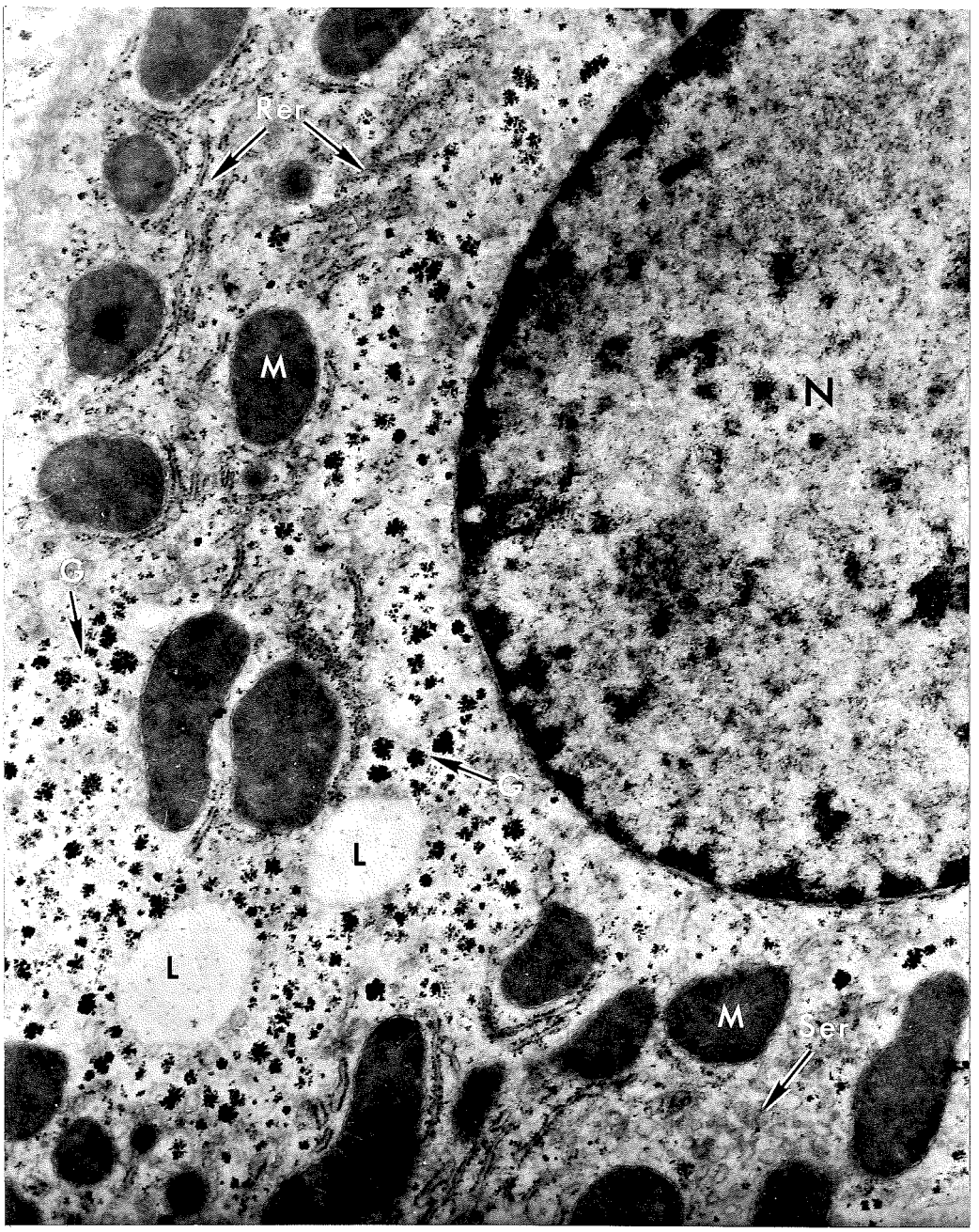

Fig. 4.4. Portion of a liver parenchymal cell from a normal, control Balb/c mouse. N = nucleus; M = mitochondria; L = lipid droplets; G = clusters of glycogen granules; Rer = rough-surfaced endoplasmic reticulum; Ser = smooth endoplasmic reticulum. X 16,500.

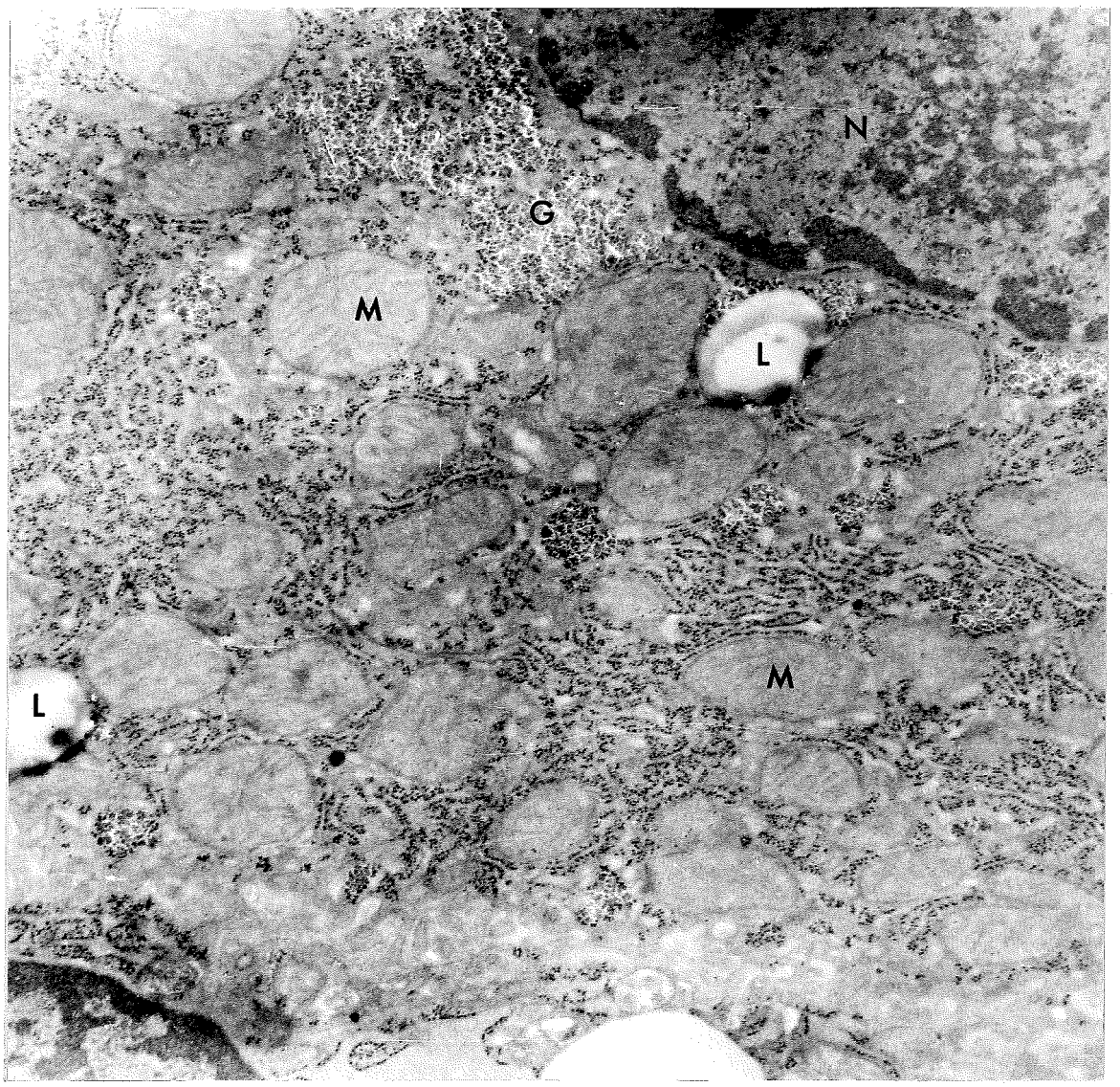

Fig. 4.5 Portion of a liver parenchymal cell from an enlarged liver of a Balb/c mouse sacrificed 8 days after receiving a 4 cm^2 CBA skin allograft. Increased amounts of rough-surfaced endoplasmic reticulum are packed between and around the mitochondria (M). N = nucleus; L = lipid droplets; G = glycogen. X 14,500.

Histological changes in the spleens of Balb/c mice following skin transplantation.

(a) Normal histology of control spleens from female Balb/c mice.

The white pulp in normal spleens of 12-18 week-old female Balb/c mice consists of well-defined lymphatic nodules, some of which contain small, relatively pale-staining germinal centres adjacent to their central arterioles (Fig. 4.6). In addition to blood cells within sinuses, fixed reticulum cells, macrophages, plasma cells and fibroblasts, the red pulp contains areas of extramedullary haematopoiesis, which according to DUNN (1954) occurs so regularly in the spleens of mice that it should be considered a normal condition. Granulopoietic activity is identified by the presence of young granulocytic forms adjacent to the capsule and fibrous trabeculae; megakaryocytes are usually found in close proximity (Fig. 4.7). In normal spleens of untreated, young adult Balb/c mice, the intensity of erythropoiesis far exceeds that of granulopoiesis. Erythropoiesis is characterized by collections of nucleated red cells with small, darkly-staining nuclei (Fig. 4.7). In H and E-stained sections of spleens, immature erythrocytes are readily identified by their distinct purple coloured nuclei which stain intensely, and are easily distinguished from small and medium-sized lymphocytes which exhibit a blue-purple nuclear colouration and stain less intensely. However, in black and white, light photomicrographs of these same sections, the morphological features of erythrocyte precursor cells resemble those of lymphocytes, and the two cell types are not always readily distinguishable.

As described for rats by SNOOK (1964) and NOSSAL et al. (1966), and for rabbits by WEISS (1964) and BURKE and SIMON (1970), the spleens of Balb/c

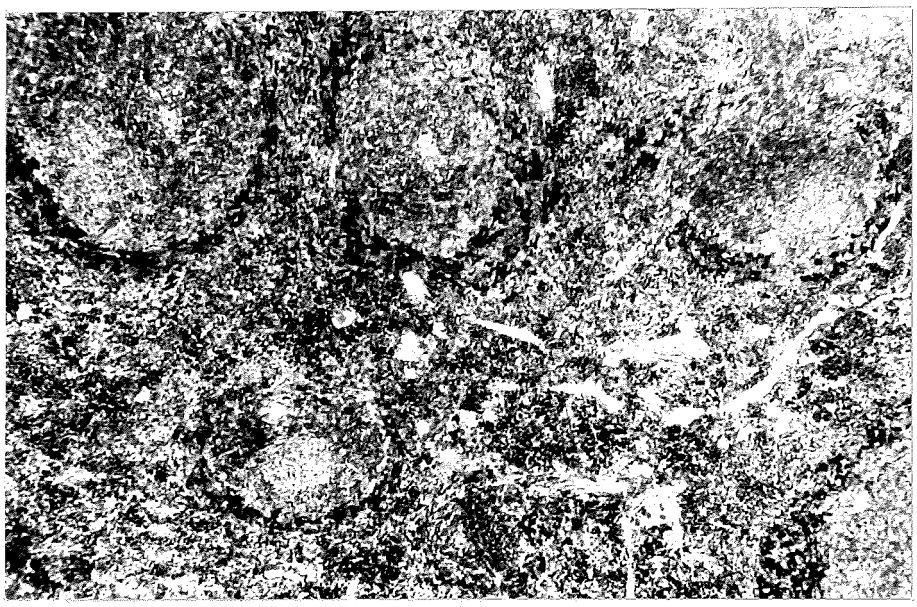

Fig. 4.6 Spleen of a normal Balb/c mouse showing the relative proportions of white and red pulp. The lymphatic nodules exhibit small, pale-staining germinal centres adjacent to their central arterioles. Carbon particles surround the lymphatic nodules. (This mouse was injected intravenously with a standard dose of colloidal carbon and sacrificed 15 minutes later). Haematopoietic activity in the red pulp is confined to the areas immediately surrounding the fibrous trabeculae. H and E X53.

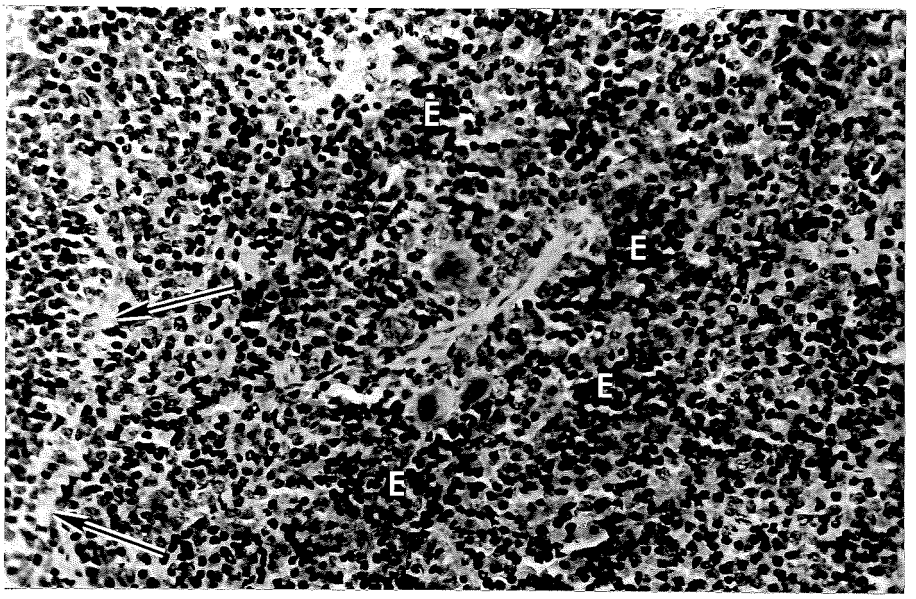

Fig. 4.7 (a). Extramedullary haematopoiesis in the red pulp of the spleen from a normal, untreated Balb/c mouse. Collections of nucleated erythrocyte precursor cells (E) surround part of a fibrous trabecula. A few pale-staining granulocytes and multinucleated megakaryocytes lie adjacent to the trabecula. The arrows indicate the marginal sinus of a neighbouring lymphatic nodule. H and E X175.

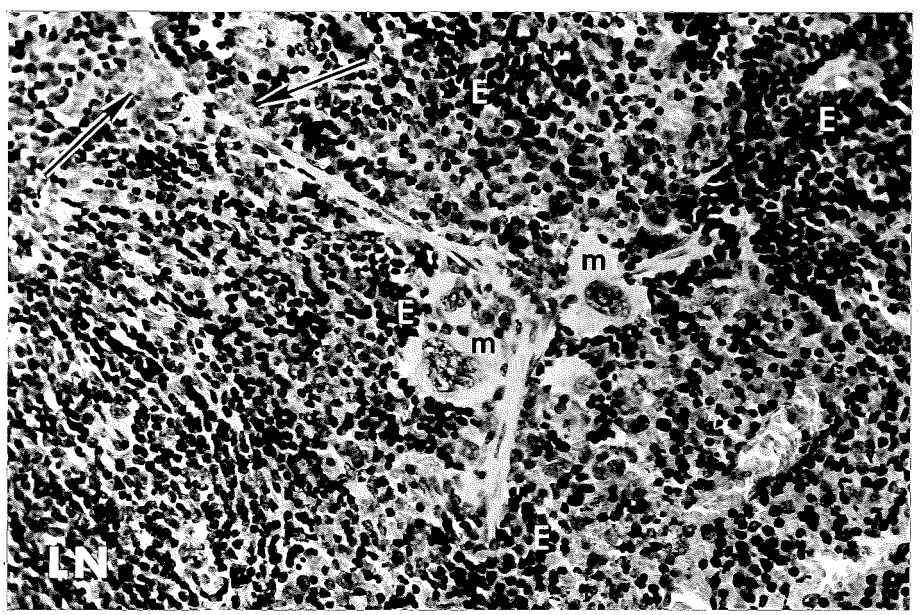

Fig. 4.7 (b). Another area of the red pulp of the spleen shown in Fig. 4.7 (a). Erythropoietic activity (E) is evident around the fibrous trabeculae. A few megakaryocytes (m) and pale-staining granulocytes (arrowed) lie alongside the trabeculae. LN = part of a lymphatic nodule. H and E X250.

mice display a narrow marginal sinus which separates the white pulp tissue of each lymphatic nodule from its surrounding marginal zone (Fig. 4.8, top). Initial localization of intravenously-injected carbon particles occurs mainly between the cells of the marginal zone (Fig. 4.8, bottom), although some carbon can occasionally be found in the interstices of the immediately adjacent red pulp (see Fig. 4.6).

(b) Histology of spleens from Balb/c mice bearing primary 4 cm^2 CBA allografts.

The sequence of splenic histological changes which occurred in response to the transplantation of medium-sized skin allografts was followed in H and E -stained sections prepared from mice sacrificed between 2 and 36 days after grafting. A slight enlargement of lymphatic nodules and a moderate increase in extramedullary haematopoiesis was evident at 2 days. By 6 days, the lymphatic nodules were reduced in size, while haematopoiesis (granulopoietic and erythropoietic) continued to increase. At 8 days (*i.e.*, at the time of, or just before graft rejection), some lymphatic nodules exhibited germinal centre formation; most of them showed reduced cellularity in their outermost portions as well as in the marginal zones (Fig. 4.9); very small amounts of carbon localized in these cell-depleted marginal zones (compare Fig. 4.9 with Fig. 4.8, bottom). Granulopoiesis was particularly prominent at this time (Fig. 4.10). During the period between 10 and 18 days after grafting, haematopoietic activity decreased, although it continued above normal levels; the lymphatic nodules showed slight enlargement and prominent germinal centre development. Very few plasma cells were found in the perinodular regions and throughout the red

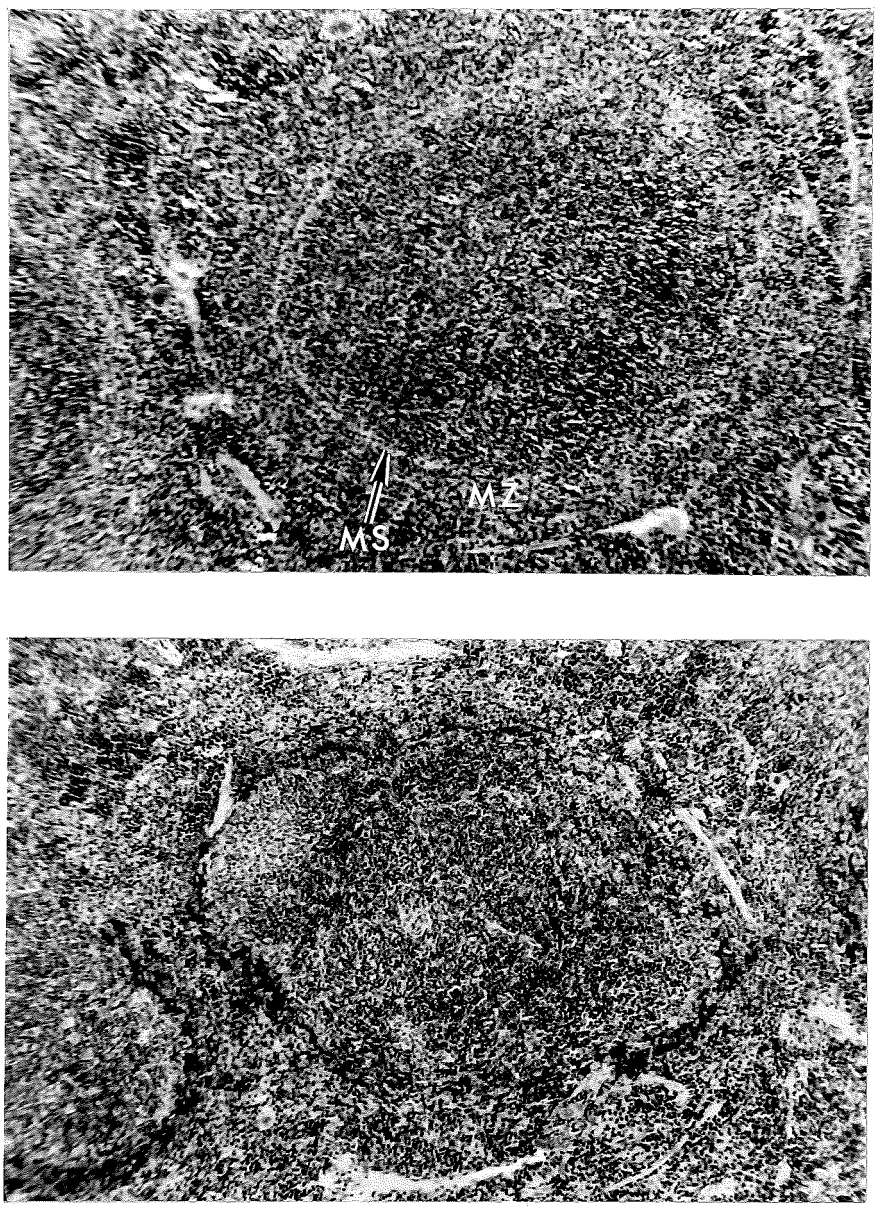

Fig. 4.8. Top: spleen of a normal, untreated Balb/c mouse. A marginal sinus (MS) separates the white pulp tissue of a lymphatic nodule from a surrounding marginal zone (MZ). The sinus cannot be traced completely around the nodule, nor is it evident in the part of the lymphatic nodule seen at the bottom left of the photograph. H and E X70. Bottom: spleen of a control Balb/c mouse sacrificed 15 minutes after receiving a standard intravenous dose of colloidal carbon. The carbon particles have localized in the marginal zones. H and E X70.

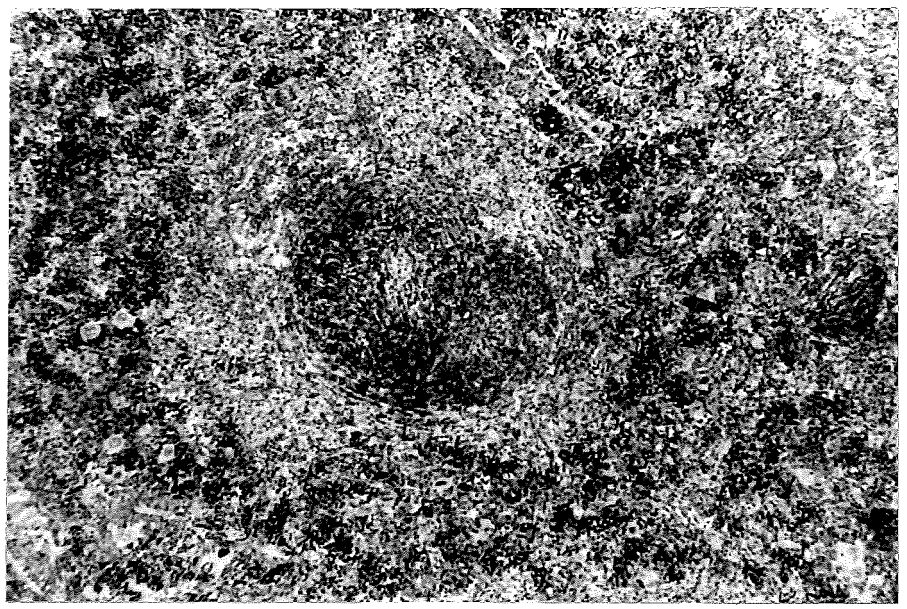

Fig. 4.9 Spleen of a Balb/c mouse sacrificed 8 days after receiving a primary 4 cm^2 CBA skin allograft. The lymphatic nodule seen in this field shows irregular cellular depletion in its outermost portions. The marginal zone also shows cellular depletion and contains very little trapped carbon. Intense haematopoiesis (granulocytic and haematopoietic) is evident in the red pulp . H and E X53.

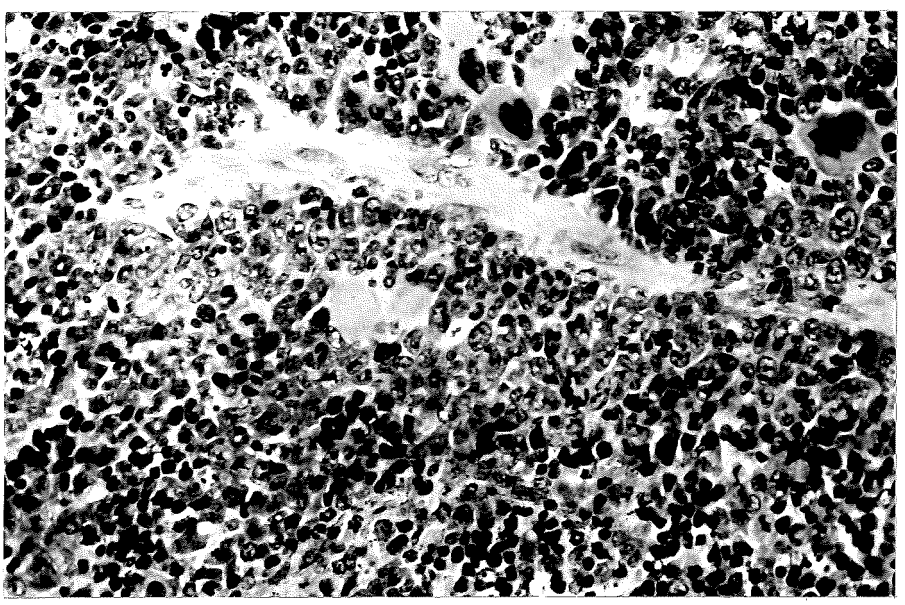

Fig. 4.10. Haematopoiesis in the red pulp of the spleen shown in Fig. 4.9. Numerous pale-staining granulocytes lie in close proximity to a fibrous trabecula. Megakaryocytes and immature erythrocytes with dark-staining nuclei are also present. H and E X250.

pulp during this entire period.

Reference was made earlier to the occurrence of a second peak of splenomegaly at 22 days (see Table 4.8 and Fig. 4.1). Histological examination of these spleens revealed normal haematopoietic activity, but a very marked enlargement of lymphatic nodules and active germinal centre development (Fig. 4.11). Enlarged nodules with germinal centres were still evident in spleens examined 36 days after allografting.

(c) Histology of spleens from Balb/c mice bearing secondary 4 cm^2 CBA allografts.

The pattern of histological changes observed in the spleens of mice bearing secondary allografts was similar to that found with primary allografts. Increased haematopoietic activity (erythropoietic and granulocytic) was evident at 3 days and reached a maximum between 6 and 10 days after grafting; during this period the lymphatic nodules showed considerable reduction in size. Between 12 and 15 days germinal centres formed and haematopoietic activity subsided. Spleens examined 18 to 20 days after grafting showed normal histology of the white and red pulp. The most striking microscopic feature of the greatly enlarged spleens from mice in the 23-day group (see Table 4.9) was the markedly enlarged lymphatic nodules and germinal centres which made up approximately 90% of the splenic mass.

(d) Histology of spleens from Balb/c mice bearing 4 cm^2 isografts.

The changes in the white and red pulp of spleens from mice with medium-sized isografts differed from those found in mice bearing similar-sized primary allografts. At the peak of splenic enlargement (8 days after isografting),

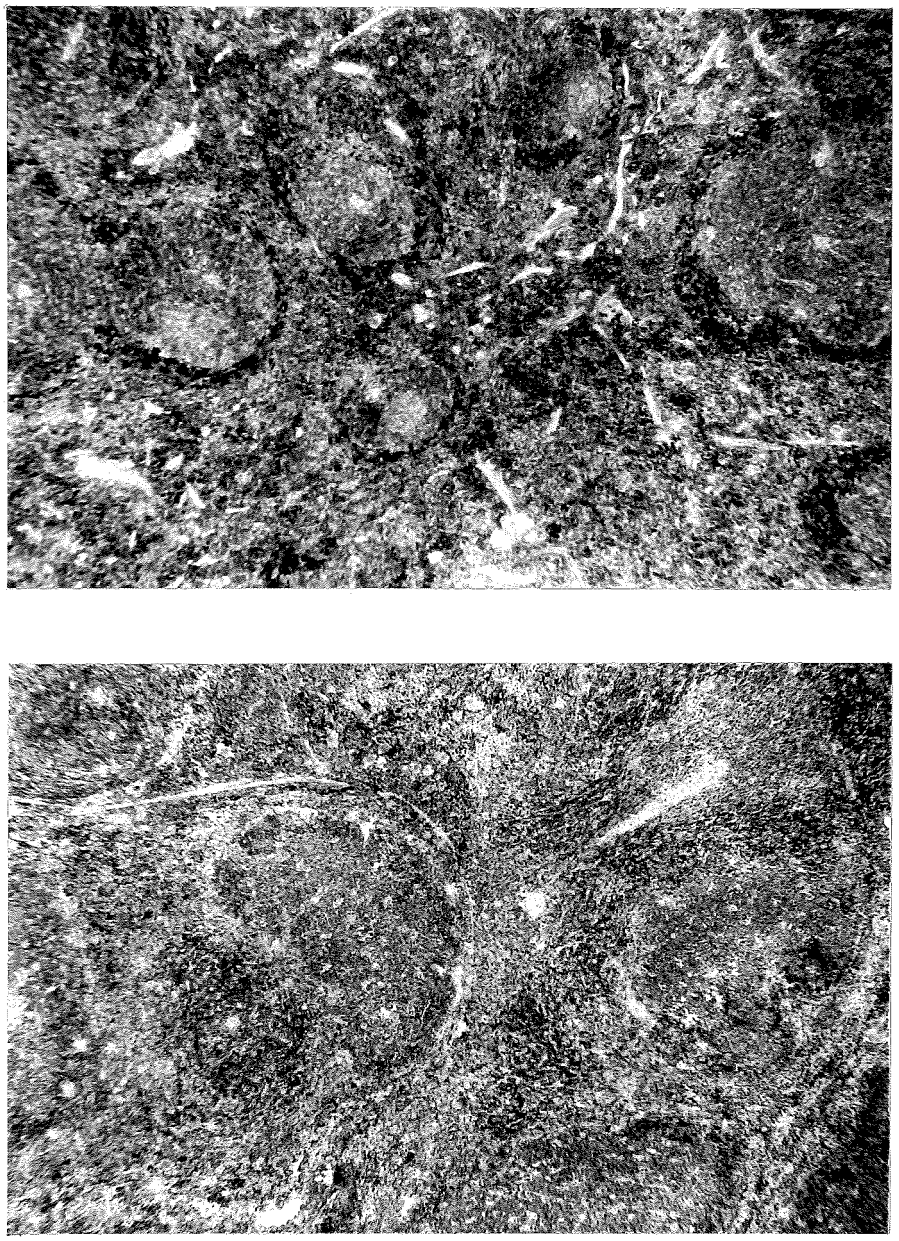

Fig. 4.11. Bottom: spleen of a Balb/c mouse sacrificed 22 days after receiving a primary 4 cm^2 CBA skin allograft. (This spleen was enlarged 3-fold). The lymphatic nodules are greatly enlarged and exhibit large, active germinal centres. H and E X35. For comparison, the normal spleen shown in Fig. 4.6 is included above. H and E X35.

the lymphatic nodules were not significantly altered in size, nor did they exhibit prominent germinal centre development; furthermore, their marginal zones showed only slight cellular depletion (Fig. 4.12; compare this with Fig. 4.9). The red pulp showed intense haematopoiesis (Fig. 4.12), but whereas granulopoiesis was a noticeable feature in the spleens of allografted mice, splenic haematopoiesis in isografted mice was predominantly erythropoietic (Fig. 4.13). The normal-sized spleens of mice bearing 22-day isografts had a normal histological appearance.

(e) Histology of spleens from Balb/c mice bearing primary 8 cm^2 CBA allografts.

The spleens of mice bearing massive allografts showed histological changes which were similar to those found in the case of medium-sized allografts, at least for the periods preceding and during rejection. Haematopoietic activity (granulopoietic and erythropoietic) was maximal at the time of rejection (8-11 days) (Fig. 4.14), and had declined considerably by 15 days. Up to this time, very little carbon localized in the marginal zones which displayed reduced cellularity (Fig. 4.14). The lymphatic nodules decreased in size prior to rejection and thereafter showed a moderate enlargement with germinal centre formation. The intense hyperplasia of the splenic white pulp observed at 22 days in mice with 4 cm^2 allografts was not found following transplantation of massive allografts. The spleens of mice bearing massive allografts at 22 and 26 days after grafting had a normal histological appearance.

(f) Histology of spleens from Balb/c mice bearing 8 cm^2 isografts.

The enlarged spleens from mice which received massive isografts showed a reduction in the size of the lymphatic nodules 4 to 8 days after grafting;

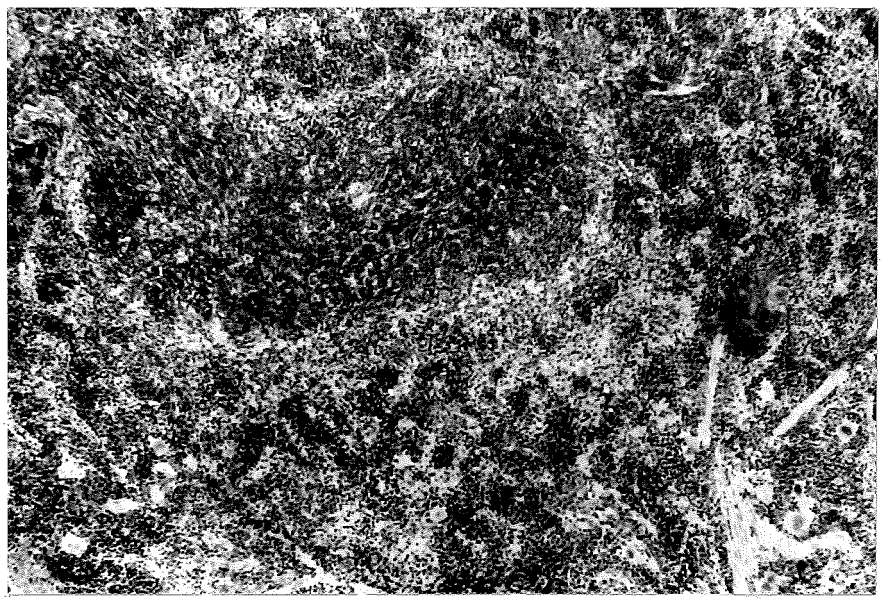

Fig. 4.12. Spleen of a Balb/c mouse sacrificed 8 days after receiving a 4 cm^2 skin isograft. The one lymphatic nodule visible in this field shows slight cellular depletion at its periphery; intense haematopoiesis is evident in the red pulp. H and E X53.

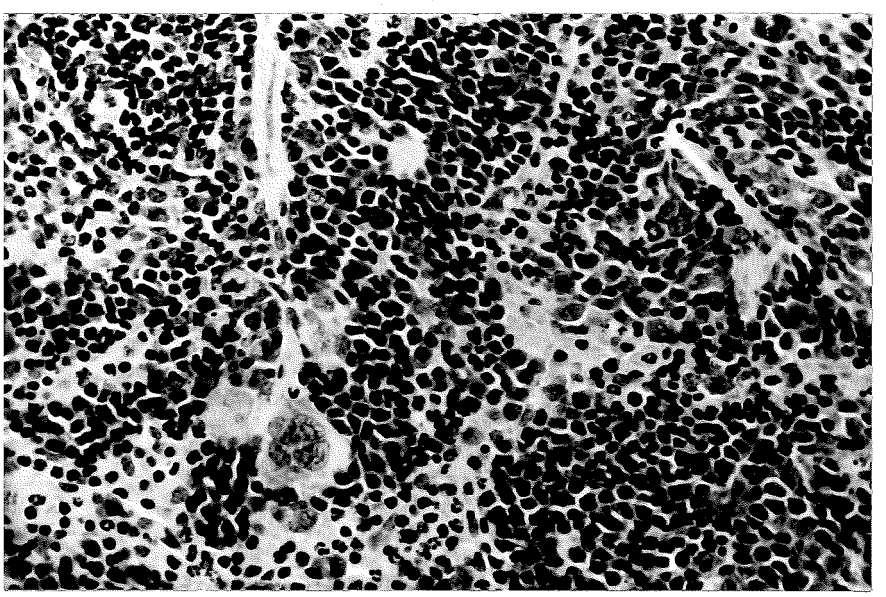

Fig. 4.13. Haematopoiesis in the red pulp of the spleen shown in Fig. 4.10. Most of the cells present are immature erythrocytes; a few granulocytes lie adjacent to fibrous trabeculae. H and E X250.

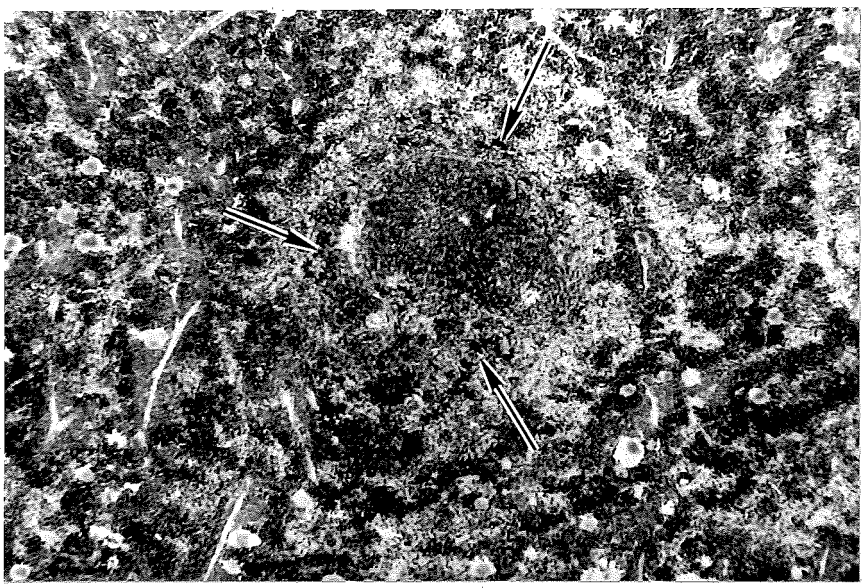

Fig. 4.14. Spleen of a Balb/c mouse sacrificed 8 days after receiving an 8 cm^2 CBA skin allograft. The lymphatic nodule visible in this field is smaller than normal and shows irregular cellular depletion at its periphery. Reduced cellularity of the marginal zone is also evident and very little carbon (arrowed) has localized in this region. The red pulp shows intense granulopoiesis and erythropoiesis as well as many megakaryocytes. H and E X53.

Fig. 4.15. Spleen of a Balb/c mouse sacrificed 8 days after receiving an 8 cm^2 skin isograft. The 2 lymphatic nodules seen in this field show only very slight depletion peripherally; small amounts of carbon (arrowed) have localized in the marginal zones. Increased haematopoietic activity is evident in the red pulp. H and E X53.

thereafter the nodules attained their normal size but did not develop germinal centres. The most marked histological change was the increase in haematopoiesis (erythropoietic and granulocytic) which reached maximum intensity at 8 days (Fig. 4.15), and although it had subsided considerably by 16 days, still continued above normal levels.

DISCUSSION

The increased rate of blood clearance of labelled bacteria exhibited by Balb/c mice bearing medium-sized (4 cm^2) and massive (8 cm^2) C57 Black skin allografts is in agreement with the results reported by MARSHALL (1968) and MARSHALL and KNIGHT (1969a) for the AKR $\rightarrow$ B_{10} D_2 strain combination. The present results differ notably from theirs in that mice with 4 cm^2 and 8 cm^2 isografts *also* showed significant phagocytic stimulation, and furthermore, no statistically significant differences existed between the phagocytic indices recorded for corresponding groups of isografted and allografted mice. (Similar results were obtained when colloidal carbon was used as the RES test particle, *vide infra*). These observations suggest that the phagocytic stimulation found in the present study was not directly related to allograft rejection, but rather, was a consequence of the surgical procedure of skin grafting. Several factors could be responsible for stimulating macrophage activity after skin grafting, irrespective of whether the tissue transplanted was isogeneic or allogeneic, *e.g.*, destruction of erythrocytes, leukocytes and other cells of donor or host origin at the local site and the release of cell breakdown products into the circulation; development of an infection would also stimulate the

phagocytic activity of fixed macrophages (BIOZZI et al., 1957). Although aseptic precautions were followed throughout the skin transplantation procedure, relatively large areas of recipient and donor tissue were unavoidably exposed to the atmosphere for short periods, and this could have allowed the entry of bacteria into the tissues of the graft bed. The hair-bearing skin adjacent to the operative site could have also served as a source of bacterial contamination. Nevertheless, any infection that may have become established must have been of low-grade intensity, since the phagocytic indices of grafted mice were only minimally raised (from 0.12 to 0.17 for medium-sized grafts, and from 0.13 to 0.16 for massive grafts), in contrast to the 2-fold increase in the K values reported by BIOZZI et al. (1957) for experimentally infected mice.

The discrepancy between the results of the present study and those of MARSHALL (1968) and MARSHALL and KNIGHT (1969a) would appear to arise largely from the different methods of statistical analysis employed in either case to test for differences between the phagocytic indices obtained for isografted and allografted mice. In the present investigation, "Student's" t-test was used to determine the significance of differences between the mean phagocytic indices obtained for corresponding groups of isografted and allografted mice. In all instances, no statistically significant differences existed between the values obtained for isografted and allografted mice. A direct comparison of the raw data with that of MARSHALL (1968) and MARSHALL and KNIGHT (1969a) is not possible since they did not report the standard deviations of the means, and furthermore, their raw data is no longer available (MARSHALL, V.R., personal communication). Although the statistical methodology is not given

in detail, it would appear that MARSHALL and KNIGHT (1969a) used the analysis of variance technique to test for differences between the *pooled* data from all isografted groups of mice and the pooled data derived from all the allografted groups. In view of the non-uniformity of the mean values obtained within the isografted and allografted groups, together with the fact that the curves for the two sets of data intersected, this method of statistical analysis is of doubtful validity.

In the present series of experiments in which colloidal carbon was used as the RES test particle, significant phagocytic stimulation was found in mice bearing small (1 cm^2) allografts and isografts, but not in mice with 4 cm^2 and 8 cm^2 grafts. This result, when considered along with the finding that 4 cm^2 and 8 cm^2 grafts resulted in stimulated activity of phagocytes towards opsonized bacteria, suggests that the large degree of surgical trauma inflicted on mice receiving medium-sized and massive grafts may have *indirectly* limited their capacity to clear carbon particles from the circulation at a rate *faster* than normal. Opsonins are essential for the efficient phagocytosis of carbon particles (JENKIN and ROWLEY, 1961; NORMANN and BENDITT, 1965; FILKINS and DILUZIO, 1966), and it follows that a reduced availability of these serum factors would limit the rate of carbon clearance. SABA and DILUZIO (1969) used a lipid emulsion as their RES test particle and found a significant impairment in the phagocytic activity of rats subjected to the surgical stress of a laparotomy. They suggested that the surgically-induced depression of RE function may have been mediated by a decrease in serum opsonins. Evidence that a transient but *significant* reduction in opsonic activity of rat serum does

in fact occur after surgery has recently been provided by SABA (1970). The surgical procedure employed on rats by SABA and DILUZIO (1969) was relatively minor, consisting of a midline abdominal incision approximately 2.5 cm long, with exposure of the peritoneal cavity. It is conceivable that the comparatively greater surgical trauma inflicted on mice receiving 4 cm^2 and 8 cm^2 skin grafts could lead to a depletion of serum opsonins, possibly mediated through their increased participation in the disposal of dead or damaged cells following surgery, and for this effect to continue over a much longer period than that found in rats subjected to a laparotomy. Another factor which might operate to limit carbon clearance in mice with large grafts is increased blood levels of cortisol mediated through an adreno-cortical response to surgically-induced stress. Relatively low levels of cortisone markedly inhibit phagocytosis of carbon by the Kupffer cells of mice (see Chapter 6, Section 1).

Liver enlargement was a prominent feature in mice bearing small, medium and massive allografts and also in mice with massive isografts. The increased liver mass was not related to the anaesthetic dose of phenobarbital, since hepatomegaly was not found in mice given a single dose of phenobarbital but not subjected to any surgical procedure. Furthermore, electron micrographs prepared from the enlarged livers of allografted mice did not show increased amounts of smooth endoplasmic reticulum which is the characteristic ultrastructural feature of phenobarbital-induced hepatocyte hypertrophy (REMMER and MERKER, 1963; REMMER, 1964; 1966; HERDSON, GARVIN and JENNINGS, 1964a; FOUTS and ROGERS, 1965). The hypertrophied liver parenchymal cells from allografted mice showed a large increase in their content

of rough-surfaced endoplasmic reticulum (RER) which was densely packed between and around the mitochondria. According to PICCARDO (1958) the intermitochondrial distribution of RER is the most common arrangement, occurring in about 85% of normal liver parenchymal cells, whereas only about 15% of normal hepatocytes have strands of RER in a perimitochondrial position. PICCARDO (1958) suggested that the close apposition of RER around the mitochondria allowed a very intense metabolic interrelationship between these two cell components. This suggestion derives support from the present finding that RNA formation was significantly increased in the enlarged livers of grafted mice. The question thus arises regarding the significance of the observed increases in liver mass and RNA formation.

RNA is involved in the synthesis of purines by the liver which acts as the main source of purine supply for bone marrow cells and possibly for the cells of most peripheral tissues (LAJTHA and VANE, 1958). It is possible that part of the hypertrophy of liver parenchymal cells in skin-grafted mice is a response which provides increased amounts of purines and thereby sustains the intense cellular proliferation in lymphoid tissues. Considerable enlargement of the spleen and lymph nodes occurred in mice bearing allografts of all sizes and also in mice with massive isografts. In many instances the liver enlarged at the same time as, or soon after the spleen showed an increase in mass, and thereafter remained enlarged for a corresponding period (see Tables 4.8 to 4.11 inclusive). This close parallel between liver and spleen enlargement was further emphasized by the fact that in mice which received either primary or secondary 4 cm^2 allografts, the second peak of hepatomegaly coincided with the

second peak of splenomegaly (see Tables 4.8 and 4.9, and Fig. 4.1). It seems reasonable to infer from these observations along with the electron microscopic and biochemical data, that the liver of a grafted mouse hypertrophies partly in order to meet the increased metabolic demands imposed by the intense cellular proliferation in lymphoid organs. CONGDON and KRETCHMAR (1963) who reported hepatocyte hypertrophy in bone marrow chimaeras, have also postulated that the increased liver mass found in their mice represents a "work hypertrophy" and provides the large amounts of purines required to sustain regeneration of haematopoietic and lymphoid tissues.

Factors unrelated to the maintenance of cellular proliferation in lymphoid tissues are no doubt also involved in bringing about hepatomegaly in grafted mice, since significant liver enlargement has been observed in *splenectomized* Balb/c mice examined 8 to 10 days after receiving 8 cm^2 CBA skin allografts (GOTJAMANOS, T. unpublished data). The liver produces all plasma proteins except the gamma globulins (MILLER and BALE, 1954) and it is probable that part of the first phase of parenchymal cell hypertrophy which develops in grafted mice a few days after surgery reflects an increased synthesis of plasma proteins. Loss of plasma proteins follows surgery or trauma (WUHRMANN and WUNDERLY, 1960). Such a loss would be considerable in mice receiving 4 cm^2 and 8 cm^2 skin grafts, and this would create a demand for increased protein production by the liver. Increased hepatic synthesis of proteins, notably α - and β- globulins, has been shown to follow surgical manipulation or acute injury in rats (GJESSING and CHANUTIN, 1947; NEUHAUS, BALEGNO and CHANDLER, 1961; 1966; LIU and NEUHAUS, 1968). Data

derived from human studies have also shown increased levels of α-globulins and fibrinogen following surgery or trauma (LUETSCHER, 1947; HOCH-LIGETI, IRVINE and SPRINKLE, 1953; BIRCH and JEPSON, 1956; PETERMANN, 1960).

The splenic enlargement which developed in mice a few days after skin grafting was due to increased haematopoiesis. In the case of isografts, the haematopoietic activity was mainly erythropoietic and most likely occurred in response to blood loss following surgery. Although the quantity of blood lost during the grafting procedure and post-operative period was probably less than 0.5 ml, this amount is appreciable in relation to the total blood volume of a mouse (between 3.0 and 3.5 ml per 20g of body weight). Since the red pulp of the mouse spleen is an important site of blood formation (DUNN, 1954), it would be expected to respond readily to a haematopoietic demand following surgery. The possibility of a viral aetiology for the increased erythropoiesis is extremely unlikely, since the histological changes in the spleens of grafted mice did not resemble those induced by viruses (METCALF, D., personal communication).

The spleens of allografted mice showed not only increased erythropoietic activity, but also intense granulopoiesis. While the increased production of granulocytes may reflect the need to replace cells lost through haemorrhage, alternative explanations are more likely, *e.g.*, it may represent a response which provides the large numbers of polymorphonuclear leukocytes required to dispose of dead tissue at the graft site. A common finding in histological sections of the CBA skin grafts transplanted to Balb/c mice was the presence of large numbers of polymorphs within the donor skin and in the adjacent host tissues.

TITUS and SHORTER (1962) have also observed large numbers of polymorphs in rejecting skin allografts in Swiss-Webster mice. Another explanation for the increased splenic granulopoiesis can be found by reference to the work of FORD (1966). His investigations indicated that cells of myeloid origin are more efficient than true lymphoid cells as progenitors of lymphoid tissue in mouse radiation chimaeras. Based on these findings, FORD (1966) suggested that in the normal, non-irradiated animal, established lymphoid tissue may be replaced naturally by new cells derived originally from a myeloid source. McGREGOR (1968) also concluded that immunologically competent lymphocytes are derived from myeloid cells. In view of the intense lymphoid cell response evoked by transplanting skin grafts between mice differing at the H-2 locus, it is conceivable that the splenic granulopoiesis exhibited by Balb/c mice bearing CBA skin grafts may be a mechanism whereby large numbers of lymphoid cells are readily made available, thereby ensuring a rapid and efficient immunological reaction against the foreign graft. The findings of McNEILL (1970) provide yet another possible explanation. His study showed that a variety of antigens injected into mice produced a significant increase in the number of spleen cells which form granulocytic and monocytic colonies *in vitro*. Since the H-2 histocompatibility antigens are the most potent at eliciting transplantation immunity against skin (AMOS, 1962), it is possible that the intense splenic granulopoiesis observed in Balb/c mice bearing CBA skin grafts is stimulated by the release of $H\text{-}2^k$ histocompatibility antigens carried by CBA cells.

The lymphatic nodules in the spleens of mice bearing 4 cm^2 allografts showed first evidence of active germinal centre formation at 8 days, *i.e.*, at

the time of, or just before graft rejection. Germinal centres became increasingly prominent 10 to 18 days after grafting and at 22 days maximum splenomegaly occurred as a result of a marked increase in the number and size of germinal centres. Since humoral antibody formation is associated with germinal centre development (COTTIER, HESS and STONER, 1967), the present observations regarding the time of appearance of these structures in the spleen following skin allografting are consistent with the finding that humoral antibodies become readily detectable in the serum at the onset of graft rejection (MICKLEM and LOUTIT, 1966). In addition, the observations support the idea advanced by MICKLEM and LOUTIT (1966) that the maximum antibody response occurs *after* a skin allograft is destroyed, possibly as a result of phagocytosed graft tissue which provides a more potent immunological stimulus than the intact graft. Reference to Table 4.8 and Fig. 4.1 shows 2 peaks of splenomegaly following transplantation of medium-sized allografts, the first occurring at 12 days and the second at 22 days, times at which germinal centre formation was prominent. Since humoral antibody determinations were not carried out in the present study, an association between germinal centre formation and humoral antibody formation can only be inferred; nevertheless, the observations warrant some comment and a brief comparison with the results of BLINKOFF (1966). In his study on the primary antibody response in mice to *Salmonella adelaide*, a bimodal spleen weight curve was obtained, with peaks on days 4 and 10 after immunization. Centrifugation of spleen homogenate supernatants in density gradients showed that the first peak detected in the spleen consisted entirely of γM antibody, while the second spleen peak was composed solely of γG antibody.

Extrapolation of this data to the skin allograft situation is not justified at present, but it does suggest a potentially fruitful line of further investigation. Furthermore, the failure of mice with massive allografts to show a second peak of splenomegaly and histological evidence of intense antibody formation also warrants further study. In Chapter 3 it was suggested that the prolonged survival of massive allografts in mice may be due to a depression of immunological capacity resulting from the sequelae of severe surgical trauma. If mice bearing massive allografts are in fact immunodepressed following extensive surgery, or alternatively, are antigenically "overloaded" as suggested by CONVERSE, SIEGEL and BALLANTYNE (1963), then their inability to mount a total humoral antibody response may reflect another aspect of their immunological impairment.

SUMMARY

Balb/c mice bearing skin isografts or CBA allografts of varying sizes were studied for changes in phagocytic activity and morphology of their reticuloendothelial organs. The rate of blood clearance of opsonized, P^{32} - labelled *Salmonella typhimurium C5* was significantly increased in mice with 4 cm^2 and 8 cm^2 isografts and allografts. When colloidal carbon was used to assess RES activity, significant phagocytic stimulation was found in mice bearing 1 cm^2 grafts, but *not* in mice with 4 cm^2 and 8 cm^2 grafts. It is likely that the surgical trauma involved in transplanting large grafts indirectly limits carbon clearance, possibly by bringing about a depletion of serum opsonins.

Hepatomegaly developed in mice bearing allografts of all sizes as well as in mice with massive isografts. The increased liver mass was due to

hypertrophied hepatocytes which were seen in electron micrographs to contain increased amounts of rough-surfaced endoplasmic reticulum. Biochemical estimations of the enlarged livers revealed increased concentrations of RNA. It is probable that the increase in liver mass and RNA formation represents a hypertrophic response which provides increased amounts of (a) purines, and thereby sustains the intense cellular proliferation in lymphoid tissues, and (b) plasma proteins, notably α- and β- globulins and fibrinogen which are known to be elevated following surgery or trauma.

Mice bearing 4 cm^2 and 8 cm^2 isografts and allografts showed splenomegaly which reached a maximum between 8 and 12 days after transplantation; in the case of 4 cm^2 allografts, a second peak of splenic enlargement occurred at 22 days. The spleens of isografted mice showed increased erythropoietic activity but little change in the lymphatic nodules. Allografted mice showed intense granulopoiesis as well as increased erythropoiesis during the first peak of splenomegaly; the second peak was characterized by normal haematopoietic activity and a marked enlargement of lymphatic nodules with prominent germinal centre development, indicating an intense humoral antibody response. Mice with massive allografts showed some evidence of germinal centre formation in their spleens at about the time of allograft rejection, but did not exhibit a second peak of splenomegaly nor a cellular response indicative of intense antibody formation such as that seen in mice with 4 cm^2 allografts. If the prolonged survival of massive allografts is due to a depression of immunological capacity resulting from the sequelae of severe surgical trauma, or is the result of antigen "over-loading", then the inability of mice with massive allografts to mount a total humoral antibody response may reflect another aspect of their immunological impairment.

CHAPTER 5

ALTERATIONS IN PHAGOCYTIC ACTIVITY AND MORPHOLOGY OF RETICULO-ENDOTHELIAL ORGANS IN MICE FOLLOWING INTRAVENOUS ADMINISTRATION OF DISSOCIATED SPLEEN CELL GRAFTS

INTRODUCTION

Studies on the alterations in RES function which follow the administration of dissociated cell grafts have been limited almost entirely to the graft-versus-host (GVH) reaction which results when immunologically competent spleen or lymph node cells from one parental strain are transplanted into F_1 hybrid animals. There is general agreement that the GVH reaction is characterized by marked changes in RE organs, notably phagocytic stimulation (HOWARD, 1961; COOPER and HOWARD, 1961; FOX and HOWARD, 1963; BIOZZI et al., 1965; DILUZIO, 1967, 1968), splenomegaly (SIMONSEN et al., 1958; GORER and BOYSE, 1959; HOWARD, 1961; HOWARD, MICHIE and SIMONSEN, 1961; SIMONSEN, 1962; FOX and HOWARD, 1963; FOX, 1966; DILUZIO, 1967; 1968), and hepatomegaly (SIMONSEN et al., 1958; HOWARD, 1961; SIMONSEN, 1962; DILUZIO, 1967, 1968).

The injection of F_1 hybrid lymphoid cells into animals of one parental strain would not result in a GVH reaction since the donor cells are genetically incapable of reacting against the host tissues. The injected cells, however, will be destroyed by the host-versus-graft (HVG) reaction. This experimental design has been employed in the present study to provide information on the RES responses evoked during such a reaction. The results provide a basis for comparison with the RES changes found to follow the transplantation of skin allografts which were reported in the preceding Chapter.

The Present Investigation.

The present investigation was carried out on female Balb/c mice aged 12-16 weeks and of closely comparable body weight. The experiments can be conveniently divided as follows:

1. Control: Balb/c spleen cells transplanted to Balb/c mice.

Twenty-seven Balb/c mice were divided into 9 groups (3 mice per group); each mouse was injected i.v. with 7×10^7 Balb/c spleen cells, and mice from each group were subjected to the blood clearance test with P^{32}-labelled *S. typhimurium C5* at 1, 2, 4, 6, 8, 10, 12, 15 or 20 days later. This experiment was repeated on 6 groups (4 mice per group) using colloidal carbon; clearance studies were performed between 2 and 30 days after i.v. cell injection.

2. F_1 (Balb/c x C57 Black) spleen cells transplanted to Balb/c mice.

Fifty-six Balb/c mice (14 groups, 4 mice per group) received 7×10^7 F_1 (Balb/c x C57 Black) spleen cells i.v.; mice from each group were subjected to the carbon clearance test at 4 hr., or 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18, 22 or 30 days later.

3. F_1 (Balb/c x C57 Black) spleen cells transplanted to *splenectomized* Balb/c mice.

Twenty-four Balb/c mice (6 groups, 4 mice per group) that had been splenectomized 2 weeks previously were injected i.v. with 7×10^7 F_1 (Balb/c x C57 Black) spleen cells; the carbon clearance test was carried out on each group between 3 and 22 days after cell injection.

4. CBA spleen cells transplanted to Balb/c mice.

This experiment was carried out to study the RES responses in a situation where the potential existed for HVG and GVH reactions to occur simultaneously, since the CBA cells can react against the $H\text{-}2^d$ antigens of Balb/c cells, and immunologically competent Balb/c cells can react against the $H\text{-}2^k$ antigens carried by the CBA cells.

Fifty-two Balb/c mice (13 groups, 4 mice per group), were injected i.v. with 7 x 10^7 CBA spleen cells; phagocytic activity of the RES was assessed using P^{32}-labelled *S. typhimurium C5* at periods varying between 1 and 30 days after cell injection. This experiment was repeated with carbon as the test particle on 44 Balb/c mice (11 groups, 4 mice per group) at times varying between 2 and 40 days after i.v. cell injection.

In all experiments outlined above, a close check was kept on the body weights of each group of mice during the period between cell injection and sacrifice. The combined body weights of mice in each group were recorded daily or on alternate days. In the bacterial clearance experiments, the livers, spleens and lymph nodes from all mice in each group were weighed together and expressed as a percentage of the mean body weight for that group. Although this data provided information on the overall pattern of organ weight changes, it precluded carrying out statistical analyses to test the significance of differences between groups. For this reason, only the mean phagocytic index and the standard error of the mean for each animal group have been included in the results on bacterial clearance. On the other hand, the liver, spleen and 4 lymph nodes from each mouse subjected to the carbon clearance test were weighed *individually* and expressed as percentages of that animal's total body weight. These results have all been included in the appropriate tables.

RESULTS

Mortality and body weight changes in Balb/c mice injected intravenously with isogeneic and allogeneic spleen cells.

Death following intravenous injection of spleen cells occurred in about 15% of mice treated and this necessitated injecting additional mice to maintain the requisite number in each group. Death occurred with equal frequency in mice injected with isogeneic and allogeneic cells. In all fatal cases, death resulted within 1-2 minutes after cell injection, and the observable signs were strongly suggestive of pulmonary embolism as being the cause. A significant feature was that nearly all fatalities occurred when spleen suspensions had been prepared from 24-30 week old donor mice. Death in recipient animals was rare when spleen suspensions from 10-18 week old donors were used.

During the period between cell injection and sacrifice, Balb/c mice given either Balb/c, F_1 hybrid or CBA spleen cells showed increases in body weight comparable to those recorded for normal controls (see Appendix 3).

Phagocytic activity of the RES and liver and spleen weights of Balb/c mice given Balb/c spleen cells.

Balb/c mice injected with 7×10^7 Balb/c spleen cells and subjected to blood clearance studies with labelled bacteria or colloidal carbon exhibited normal phagocytic indices (Tables 5.1 and 5.2). The liver and spleen weights of these mice were within normal limits with the exception of the 18 day group which showed a significant reduction (Table 5.2). The corrected phagocytic index for this group was, accordingly, significantly raised.

TABLE 5.1. RATE OF BLOOD CLEARANCE OF P^{32} -LABELLED *SALMONELLA TYPHIMURIUM C5* BY BALB/C MICE[a] GIVEN 7×10^7 BALB/C SPLEEN CELLS INTRAVENOUSLY.

Group (Time after cell injection)	Phagocytic Index (K) (Mean ± S.E.M.)
Untreated control	0.13 ± 0.01
1 day	0.15 ± 0.02
2 days	0.14 ± 0.01
4 "	0.13 ± 0.01
6 "	0.12 ± 0.01
8 "	0.11 ± 0.01
10 "	0.11 ± 0.01
12 "	0.14 ± 0.01
15 "	0.14 ± 0.01
20 "	0.12 ± 0.01

[a] There were 6 mice in the untreated control group and 3 mice in each spleen cell-injected group.

TABLE 5.2. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 7 X 10^7 BALB/C SPLEEN CELLS INTRAVENOUSLY (MEANS ± S.E.M.).

Group (Time after cell injection)	Liver weight as % of Total Body weight (T.B.W.).	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.06 ± .11	.56 ± .02	.14 ± .01	.028 ± .002	5.39 ± .04
2 days	5.16 ± .19	.55 ± .03	.15 ± .01	.028 ± .001	5.34 ± .09
5 "	5.12 ± .11	.51 ± .02	.16 ± .01	.026 ± .001	5.29 ± .07
8 "	4.86 ± .13	.50 ± .01	.15 ± .01	.027 ± .001	5.60 ± .11
12 "	4.97 ± .14	.56 ± .01	.14 ± .01	.027 ± .001	5.43 ± .06
18 "	4.65 ± .08**	.46 ± .02**	.15 ± .01	.027 ± .001	5.90 ± .10**
30 "	5.29 ± .15	.58 ± .03	.14 ± .01	.027 ± .001	5.15 ± .17

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

** p 0.001-0.01 (test group vs. untreated control group).

The response of the RES in Balb/c mice to the intravenous administration of F_1 (Balb/c x C57 Black) spleen cells.

(a) Changes in phagocytic activity.

The phagocytic index was significantly elevated 1 day after spleen cell injection and rose very rapidly thereafter to reach a 7-fold increase at 4 days (Table 5.3). The intense phagocytic stimulation exhibited by the 4-day group was emphasized by the fact that animals in this group took 7.3 ± 0.3 minutes to clear 95% of the total amount of carbon injected as compared with 49.0 ± 2.3 min. taken by untreated controls. Phagocytic indices remained significantly raised during the next 8 days and had returned to normal levels by 15 days. As a consequence of the great increase in K values between 3 and 8 days after cell injection, the corrected phagocytic indices showed a significant increase during the corresponding period (Table 5.3).

(b) Changes in liver mass and histology.

Mice examined 3-12 days after cell injection exhibited significant hepatomegaly which coincided with the period of intense phagocytic stimulation (Table 5.3). When unstained sections of livers from mice in the 4-day group were examined under low magnification (X100) and compared with those from control animals, no alteration in the relative number of carbon-containing Kupffer cells was evident (Fig. 5.1). Examination at higher magnification (X400) revealed a striking increase in the size and carbon content of many Kupffer cells and moderate enlargement of hepatocytes (Fig. 5.2.).

(c) Changes in spleen mass and histology.

Splenomegaly first became evident 1 day after F_1 cell injection and reached maximum proportions (2-3 fold enlargement) between 3 and 12 days (Table 5.3).

TABLE 5.3. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 7×10^7 F_1 (BALB/C X C57 BLACK) SPLEEN CELLS INTRAVENOUSLY (MEANS ± S.E.M.).

Group (Time after cell injection)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	5.06 ± .11	0.56 ±.02	.14 ± .01	.028 ±.002	5.39 ± .04
4 hours	5.16 ± .06	0.59 ±.01	.18 ± .01*	.024 ±.002	5.03 ± .14
1 day	5.25 ± .14	0.65 ±.03*	.20 ± .01**	.038 ±.002*	5.70 ± .21
2 days	5.38 ± .06	0.77 ±.03**	.22 ± .02**	.041 ±.002**	5.59 ± .13
3 "	5.91 ± .23*	1.26 ±.07***	.17 ± .02	.136 ±.003***	7.20 ± .26***
4 "	5.85 ± .13**	1.31 ±.11***	.20 ± .02*	.207 ±.024***	8.26 ± .41***
5 "	5.32 ± .09	1.46 ±.04***	.20 ± .01**	.135 ±.007***	7.55 ± .12***
6 "	5.48 ± .13*	1.65 ±.04***	.19 ± .01*	.132 ±.007***	7.14 ± .12***
8 "	5.76 ± .09**	1.40 ±.02***	.23 ± .01***	.106 ±.009***	6.60 ± .11***
10 "	5.80 ± .19*	1.42 ±.03***	.26 ± .01***	.054 ±.011	5.17 ± .53
12 "	5.84 ± .13**	1.41 ±.05***	.24 ± .01***	.048 ±.007*	5.00 ± .27
15 "	5.00 ± .07	0.61 ±.03	.18 ± .01*	.025 ±.002	5.23 ± .15
18 "	4.84 ± .13	0.59 ±.01	.16 ± .03	.027 ±.001	5.53 ± .16
22 "	4.93 ± .02	0.59 ±.02	.18 ± .01*	.026 ±.001	5.39 ± .03
30 "	5.15 ± .05	0.55 ±.01	.15 ± .01	.024 ±.001	5.09 ± .05

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

* p 0.01-0.05; ** p 0.001-0.01; *** p < 0.001 (test group vs. untreated control group).

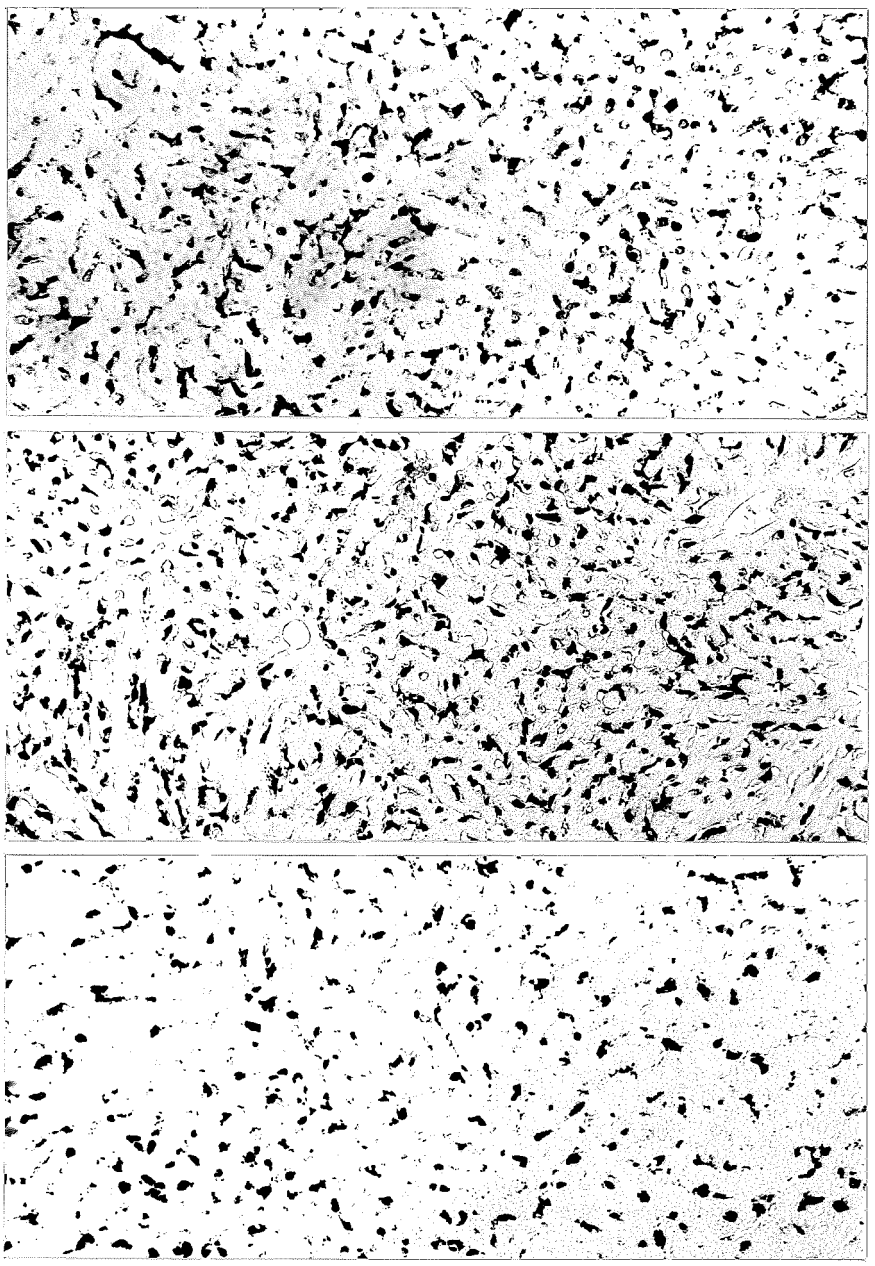

Fig. 5.1. Unstained liver sections from an untreated control Balb/c mouse (top), a Balb/c mouse sacrificed 4 days after receiving 7 x 10^7 F_1 (Balb/c x C57 Black) spleen cells intravenously (middle), and a *splenectomized* Balb/c mouse sacrificed 4 days after receiving 7 x 10^7 F_1 (Balb/c x C57 Black) spleen cells i.v. (bottom). Each mouse was subjected to the carbon clearance test and sacrificed when carbon could no longer be detected in the blood. No significant difference is apparent between the relative number of carbon-containing Kupffer cells in the untreated control and F_1 cell-injected mice. The *splenectomized* F_1 cell-injected mouse however, has fewer than normal carbon-containing cells in its liver. All sections X100.

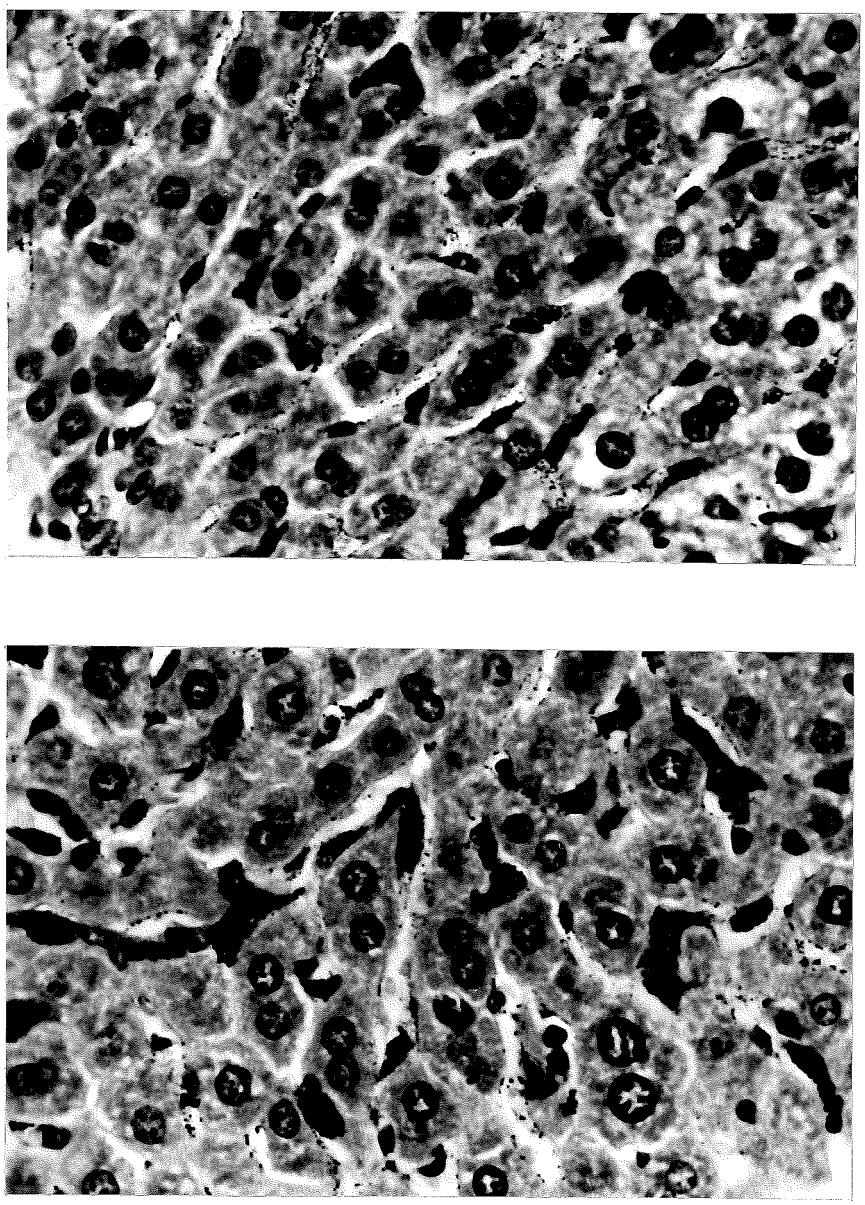

Fig. 5.2. Top: Liver from an untreated control Balb/c mouse given a standard dose of colloidal carbon and sacrificed 15 minutes later. Bottom: liver from Balb/c mouse 4 days after injection of 7×10^7 F_1 (Balb/c x C57 Black) spleen cells intravenously (this mouse also received a standard dose of carbon and was sacrificed 15 minutes later). The Kupffer cells from the F_1 cell-injected mouse are greatly enlarged and contain large quantities of carbon; several hepatocytes also show moderate nuclear and cytoplasmic enlargement. Both sections stained with H and E and photographed at X400.

The injected cells localized in the red pulp of the spleen some time between 1 and 2 days after being introduced into the circulation (Fig. 5.3). At this stage the lymphatic nodules showed slight enlargement and prominent cellular depletion at their peripheries. Rapid destruction of the injected cells occurred between 2 and 3 days, resulting in a relatively acellular red pulp containing mainly masses of pyknotic nuclei and other cell debris (Fig. 5.4). A notable feature during this reaction was the absence of active phagocytic cells from the red pulp. Between 4 and 12 days after cell injection, the enlarged spleens exhibited very large lymphatic nodules with germinal centres (Fig. 5.5). A rapid return to normal size and histological structure of the spleen (including normal localization in the marginal zones of intravenously injected carbon) occurred between 12 and 15 days (Fig. 5.6). Prior to this, only very small amounts of carbon were taken up by the spleen, indicating that removal of carbon from the circulation during the period of intense phagocytic stimulation was accomplished almost entirely by the liver macrophages.

A significant increase in the size of lymph nodes closely paralleled the splenic enlargement (Table 5.3).

The response of the RES in *splenectomized* Balb/c mice to the intravenous administration of F_1 (Balb/c x C57 Black) spleen cells.

(a) Phagocytic activity.

Splenectomized Balb/c mice subjected to clearance studies between 3 and 22 days after injection of F_1 cells had phagocytic indices which did not differ significantly from those obtained for non-injected splenectomized controls (Table 5.4). The mean K value for splenectomized control mice (0.034) was,

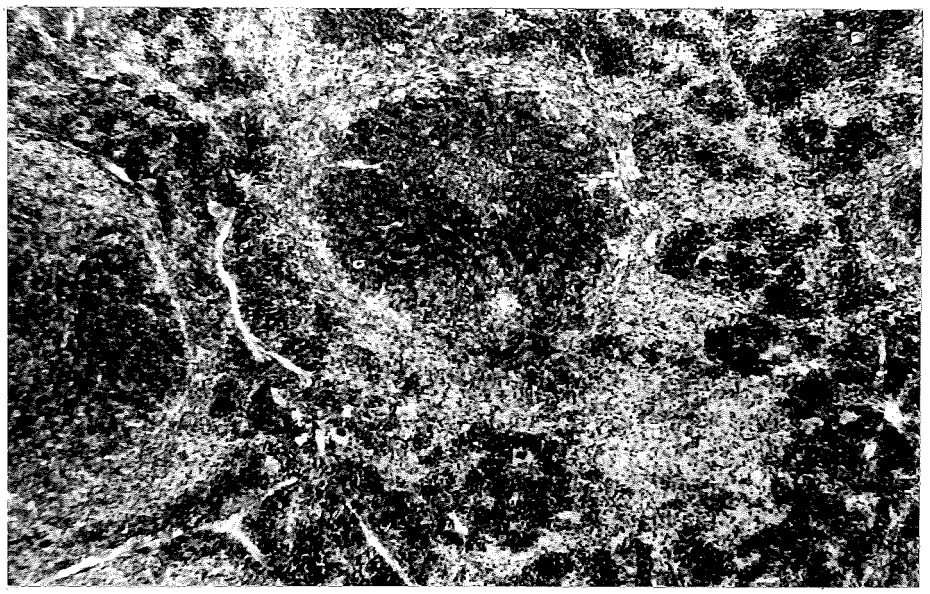

Fig. 5.3. Spleen of a Balb/c mouse sacrificed 2 days after being injected intravenously with 7 x 10^7 F_1 (Balb/c x C57 Black) spleen cells; (this mouse was injected with carbon 15 minutes before sacrifice). The lymphatic nodules show cellular depletion at their peripheries and there is very little carbon in this region. The injected F_1 cells have localized in the red pulp. H and E X53.

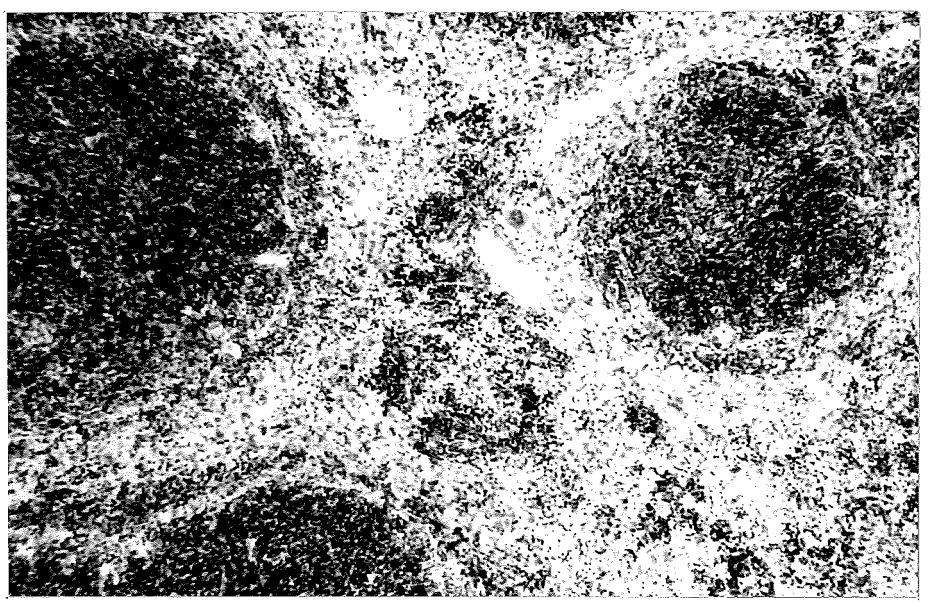

Fig. 5.4. Spleen of a Balb/c mouse 3 days after F_1 (Balb/c x C57 Black) cell injection. Most of the F_1 cells which localized in the red pulp (see Fig. 5.3) have been destroyed. H and E X53.

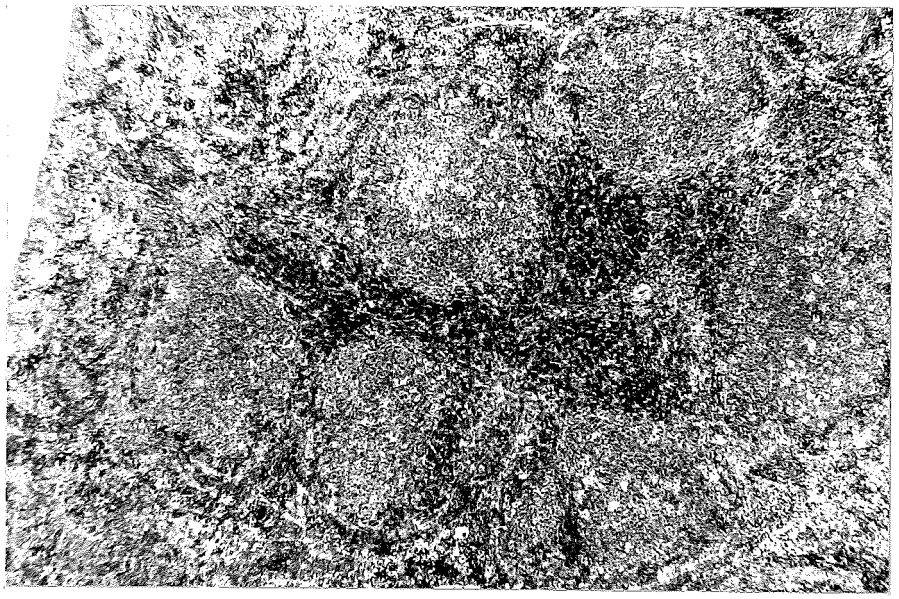

Fig. 5.5. Greatly enlarged spleen of a Balb/c mouse 12 days after F_1 (Balb/c x C57 Black) spleen cell injection. The one lymphatic nodule seen in this field shows 6 prominent germinal centres. There is no evidence of trapped carbon at the periphery of the nodule. H and E X35.

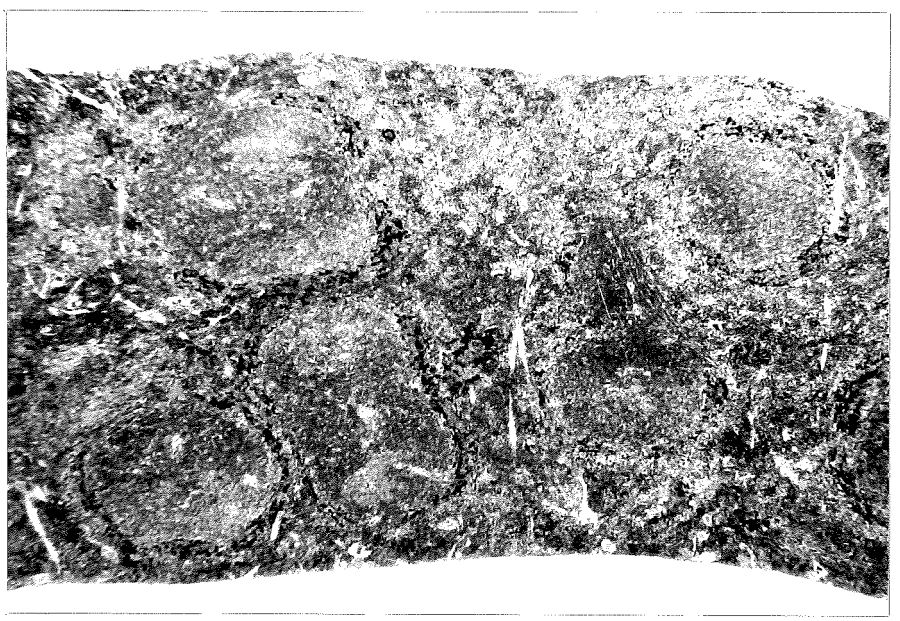

Fig. 5.6. Normal-sized spleen of a Balb/c mouse 15 days after F_1 (Balb/c x C57 Black) spleen cell injection. The lymphatic nodules are of normal size, but still show some evidence of germinal centre formation. Carbon localization in the marginal zones is normal. H and E X35.

TABLE 5.4. LIVER AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF *SPLENECTOMIZED* BALB/C MICE GIVEN 7×10^7 F_1 (BALB/C X C57 BLACK) SPLEEN CELLS INTRAVENOUSLY (MEANS ± S.E.M.).

Group (Time after cell injection)	Liver weight as % of Total Body Weight (T.B.W.)	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	4.75 ± .11	.17 ± .02	.025 ± .001	5.60 ± .16
Splenectomized, non-injected control	5.30 ± .03	.20 ± .01	.034 ± .002	6.08 ± .07
3 days	5.23 ± .15	.14 ± .03	.033 ± .002	6.13 ± .10
4 "	5.17 ± .13	.22 ± .02	.039 ± .004	6.57 ± .13*
6 "	5.31 ± .17	.20 ± .02	.034 ± .002	6.12 ± .32
10 "	5.28 ± .03	.19 ± .02	.037 ± .002	6.28 ± .14
15 "	5.01 ± .06	.18 ± .02	.035 ± .003	6.52 ± .21
22 "	5.16 ± .09	.21 ± .01	.031 ± .003	6.03 ± .17

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

* p 0.01-0.05 (test group vs. splenectomized, non-injected, control group).

however, significantly higher than that (0.025) obtained for normal, untreated mice ($0.001 < p < 0.01$).

(b) Liver and lymph nodes.

Liver and lymph node weights in all groups of F_1 cell-injected, splenectomized mice did not differ significantly from those recorded for non-injected splenectomized and normal controls (Table 5.4). Examination of histological sections of livers from mice in the 4 day group revealed fewer than normal carbon-containing cells (Fig. 5.1, bottom).

The response of the RES in Balb/c mice to the intravenous administration of CBA spleen cells.

(a) Changes in phagocytic activity.

Balb/c mice given CBA spleen cells i.v. showed a significant increase in the rate of phagocytosis of carbon 2-4 days after cell injection (Table 5.5). The K value remained minimally raised during the next 8 days and between 12 and 18 days showed a 2-5 fold increase, maximum phagocytic stimulation (K and α) occurring at 16 days after cell injection. The results of the experiment in which labelled bacteria were used as the RES test particle (Table 5.6) closely paralleled those obtained with carbon, although the extent of phagocytic stimulation towards bacteria was much smaller than for carbon.

(b) Changes in liver mass and histology.

Significant hepatomegaly was evident at 2 days and between 12 and 22 days after cell injection (Table 5.5), periods which coincided closely with maximum phagocytic stimulation. Microscopic examination of livers from the 2, 16 and 18 day groups revealed enlargement of both Kupffer and parenchymal

TABLE 5.5. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 7×10^7 CBA SPLEEN CELLS INTRAVENOUSLY (MEANS ± S.E.M.).

Group (Time after cell injection)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	4.89 ± .11	0.55 ±.01	.15 ± .01	.028 ±.001	5.62 ± .11
2 days	5.78 ± .08***	1.11 ±.02***	.15 ± .01	.052 ±.003***	5.40 ± .08
4 "	5.26 ± .10	0.98 ±.02***	.15 ± .01	.042 ±.001***	5.60 ± .09
6 "	5.36 ± .11	0.79 ±.02***	.15 ± .01	.033 ±.003	5.22 ± .22
8 "	5.24 ± .10	0.76 ±.02**	.15 ± .01	.035 ±.002*	5.43 ± .04
10 "	5.25 ± .09	0.74 ±.01***	.14 ± .01	.035 ±.002*	5.45 ± .11
12 "	5.41 ± .16*	1.24 ±.06***	.18 ± .02	.061 ±.005***	5.93 ± .18
16 "	5.64 ± .18*	1.63 ±.05***	.25 ± .02***	.142 ±.010***	7.18 ± .19***
18 "	5.93 ± .09***	1.59 ±.05***	.25 ± .01***	.113 ±.009***	6.41 ± .19*
22 "	5.44 ± .09**	0.60 ±.02	.17 ± .02	.026 ±.002	4.89 ± .13
29 "	5.21 ± .18	0.59 ±.01	.14 ± .01	.025 ±.002	5.05 ± .23
40 "	5.05 ± .10	0.53 ±.04	.17 ± .02	.026 ±.004	5.31 ± .29

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

* p 0.01-0.05; ** p 0.001-0.01; *** p < 0.001 (test group vs. untreated control group).

TABLE 5.6. RATE OF BLOOD CLEARANCE OF P^{32}-LABELLED *SALMONELLA TYPHIMURIUM C5* BY BALB/C MICE[a] GIVEN 7×10^7 CBA SPLEEN CELLS INTRAVENOUSLY.

Group (Time after cell injection)	Phagocytic Index (K) (Mean ± S.E.M.)
Untreated control	0.13 ± 0.01
1 day	0.14 ± 0.01
2 days	0.18 ± 0.02***
4 "	0.16 ± 0.01***
5 "	0.14 ± 0.01
7 "	0.12 ± 0.01
8 "	0.14 ± 0.01
10 "	0.13 ± 0.01
12 "	0.21 ± 0.01***
14 "	0.19 ± 0.02**
16 "	0.22 ± 0.02**
19 "	0.18 ± 0.02
23 "	0.18 ± 0.02
30 "	0.12 ± 0.01

[a] There were 8 mice in the untreated control group and 4 mice in each spleen cell-injected group.

** p 0.001-0.01; *** $p < 0.001$ (test group vs. untreated control group).

cells. No increase in the relative number of carbon-containing Kupffer cells was noted in livers from any of these 3 groups.

(c) Changes in spleen mass and histology.

Balb/c mice showed significant splenic enlargement 2 days after receiving CBA spleen cells i.v. (Table 5.5). Spleen mass declined slightly during the next 8 days, but then rose to a peak (2-3 fold increase) between 12 and 18 days after cell injection. The injected cells localized in the red pulp of the spleen some time between 2 and 4 days after being introduced into the circulation, and were slowly destroyed during the period between 4 and 10 days. Simultaneous with the destruction of the transplanted cells in the red pulp, the lymphatic nodules showed moderate enlargement with germinal centre formation, while very little carbon could be seen at their peripheries. A very great increase in the size of the lymphatic nodules, many of which contained several prominent germinal centres, occurred between 12 and 18 days (Fig. 5.7; compare this appearance with that shown in Fig. 5.8). Thereafter, the weight and microscopic structure of the spleen rapidly returned to normal. Significant lymph node enlargement was found only in the 16 and 18-day groups (Table 5.5), coinciding with the peak of splenomegaly.

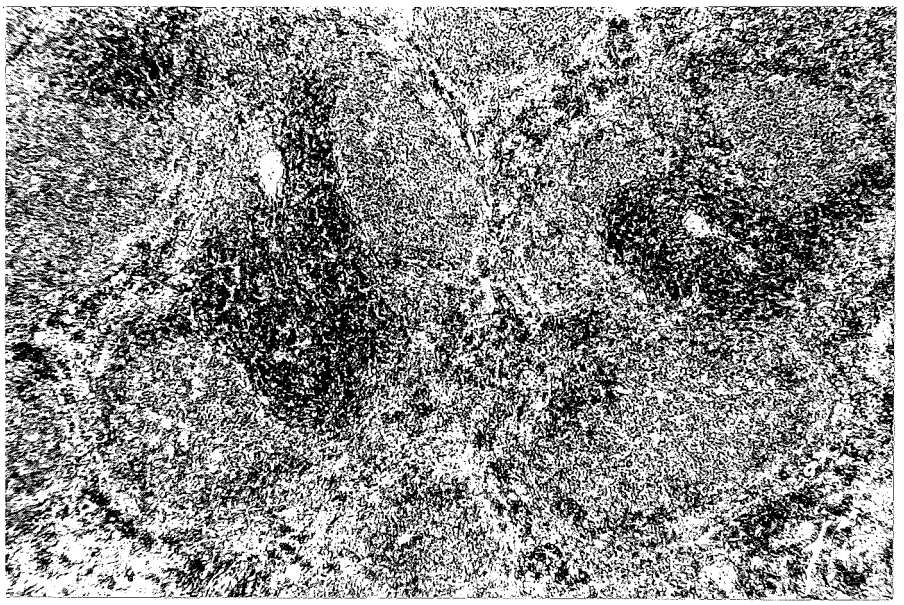

Fig. 5.7. Spleen of a Balb/c mouse 12 days after intravenous administration of 7 x 10^7 CBA spleen cells (this spleen was enlarged 3-fold). The 2 lymphatic nodules visible in this field show prominent germinal centre development. No carbon has localized around the nodules. H and E X35. (cf. this appearance with that produced during a graft-versus-host reaction shown in Fig. 5.8).

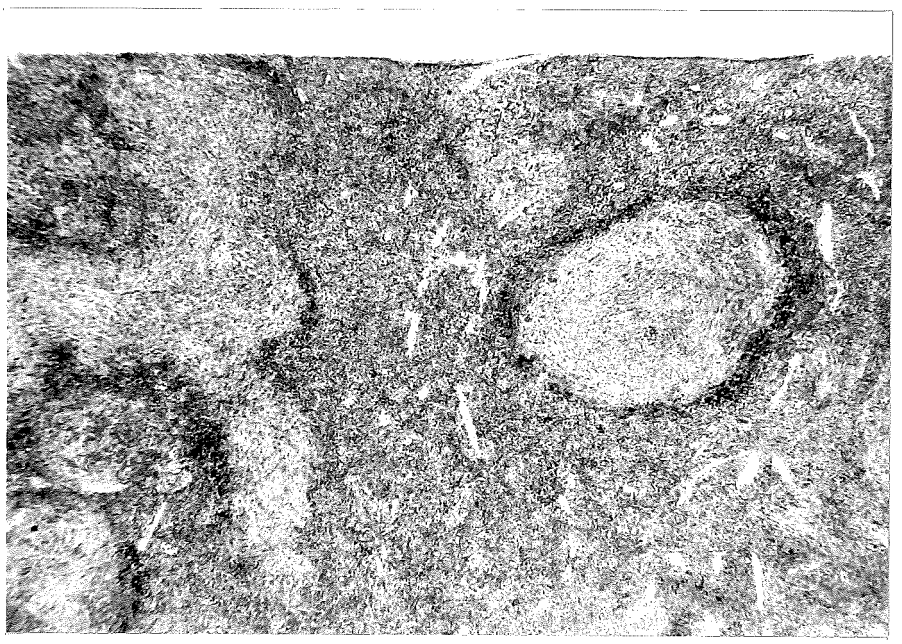

Fig. 5.8. Spleen of an F_1 (Balb/c x C57 Black) mouse 12 days after intravenous administration of 7 x 10^7 C57 Black spleen cells (this spleen was enlarged 2-fold). The lymphoid cells in the nodules have been replaced by pale-staining cells which also occupy the greater part of the red pulp. The latter contains very few foci of extramedullary haematopoiesis. Carbon localization in the marginal zone regions has not been greatly affected. H and E X35.

DISCUSSION

A host-versus-graft (HVG) reaction initiated by the transplantation of F_1 (Balb/c x C57 Black) spleen cells into Balb/c mice has been found in the present study to be accompanied by a very marked increase in the phagocytic activity of the RES. The order of increase in the phagocytic index as measured by colloidal carbon uptake was closely comparable with that previously reported for the graft-versus-host (GVH) reaction (HOWARD, 1961; COOPER and HOWARD, 1961; FOX and HOWARD, 1963; BIOZZI et al., 1965). The hyperphagocytic state which developed during the HVG reaction was characterized by considerable enlargement of Kupffer cells, many of which took up very large amounts of carbon from the circulation. This is in contrast to the GVH reaction, where the increased phagocytic activity has been found to be associated with an increased number of liver phagocytes (HOWARD, 1961). No evidence for a similar effect occurring in the HVG reaction was found. This result is not surprising in view of the fact that in the GVH reaction, the increased number of liver macrophages results from a transformation of the transplanted lymphoid cells into phagocytes which subsequently lodge in the liver sinusoids (HOWARD et al., 1965; HOWARD, BOAK and CHRISTIE, 1966). Since F_1 hybrid lymphoid cells injected into parental strain animals are rapidly destroyed by the HVG reaction, their transformation into phagocytes would not be expected to occur.

Although considerable enlargement of the spleen occurred during the HVG reaction, the splenic RE cells did not contribute to the increase in phagocytic activity. On the contrary, the amount of carbon which localized in the red pulp was much less than normal. It is likely that the hyperplastic

changes observed in the splenic white pulp in Balb/c mice given F_1 (Balb/c x C57 Black) spleen cells are indicative of a humoral response directed against the H-2^b antigens derived from the C57 Black parent. Enlargement of lymphatic nodules and germinal centre formation commenced soon after the F_1 cells settled in the spleen and continued for several days thereafter. The importance of germinal centres as the initial production sites of cells involved in antibody production has been emphasized by several workers (MARSHALL and WHITE, 1950; ORTEGA and MELLORS, 1957; CONGDON and MAKINODAN, 1961; CONGDON and GOODMAN, 1962; CONGDON, 1962; LANGEVOORT, 1963; HANNA, 1964; 1965). Germinal centre cells are believed to actively proliferate following antigenic stimulation and differentiate into members of the antibody-forming plasma cell series (CONGDON and GOODMAN, 1962; NOSSAL, 1962; HANNA, 1965).

While a significant elevation in the phagocytic index was evident 1 day after F_1 cell administration and before most of the transplanted cells had settled in the spleen, maximum phagocytic stimulation occurred after the F_1 cells had been destroyed. This suggests that the hyperphagocytic activity of liver macrophages was initiated through a release of cell breakdown products into the circulation, a suggestion supported by the finding that administration of F_1 cells to splenectomized mice failed to cause phagocytic stimulation. It seems likely that in the splenectomized animal, most of the F_1 cells would be removed from the circulation through phagocytosis by Kupffer cells. This would temporarily impair the ability of many liver macrophages to carry out additional phagocytosis of intravenously-administered carbon, and would be consistent

with the observation that the livers of splenectomized Balb/c mice injected with F_1 cells showed *fewer* carbon-containing cells than their non-splenectomized counterparts.

It is evident from the results obtained in the present study that the HVG reaction is characterized by the same main features found in the GVH reaction, viz., an increased rate of phagocytosis, splenomegaly and hepatomegaly. A more detailed analysis of each of these features is necessary in order to distinguish between the two reactions, particularly since splenomegaly and enhancement of phagocytic activity have been adopted as criteria for detecting the presence of a GVH reaction (SIMONSEN et al., 1958; HOWARD, 1961; 1964; FOX and HOWARD, 1963). The significant differences between the two reactions are summarized in Table 5.7. The data for the GVH reaction has been taken from HOWARD (1961) since the design of the present study on the HVG reaction (*i.e.*, injection of F_1 hybrid cells into one parental strain) is the reverse of the cell model used by HOWARD (1961). One notable difference not included in Table 5.7 is the influence of splenectomy. The intense phagocytic stimulation normally found in the HVG reaction can be abolished if F_1 cells are injected into mice that have been splenectomized prior to receiving the cells. In contrast, the intensity of phagocytic stimulation in the GVH reaction is only slightly lessened in F_1 hybrid mice that have been splenectomized before being injected with parental spleen cells (BIOZZI et al., 1964). However, BIOZZI and co-workers found that if splenectomy was performed 2-8 days *after* parental cell injection, pronounced phagocytic stimulation did not occur. They suggested that the stimulation of phagocytosis in the GVH reaction represented

TABLE 5.7. A COMPARISON OF THE CHANGES PRODUCED IN RETICULOENDOTHELIAL ORGANS OF MICE DURING HOST-VERSUS-GRAFT AND GRAFT-VERSUS-HOST[a] REACTIONS.

PHAGOCYTIC ACTIVITY	HVG	GVH
First evidence of stimulation	1 day after cell injection	7 days
Period of intense stimulation (4-fold or greater increase in K).	3-6 days	7-15(?) days
Maximum stimulation	4 days (7-fold increase in K).	15 days (10-fold increase in K).
LIVER		
First evidence of enlargement	3 days after cell injection	8 days
Period of enlargement	3-12 days	8-16 days
Maximum enlargement	3 days	13 days
Alteration in Kupffer cells	Enlarged; *no* increase in relative number.	Enlarged; relative number *increased*
SPLEEN		
First evidence of enlargement	1 day after cell injection	5 days
Period of enlargement	1-12 days	5-20 days
Maximum enlargement	6 days (3-fold enlargement).	9 days (3-fold enlargement).
White pulp reaction	Lymphatic nodules enlarged: prominent germinal centre development.	Lymphatic nodules destroyed.

[a] Data for GVH reaction taken from HOWARD (1961).

a host reaction to the presence of cell-breakdown products, and splenectomy eliminated a major focus of the reaction.

Reference to Table 5.7 is helpful in evaluating the significance of the RES responses found in the experiment involving the transplantation of CBA spleen cells to Balb/c mice (Tables 5.5 and 5.6). In this situation, the potential exists for an HVG and a GVH reaction to occur simultaneously. The fact that maximum increase in phagocytic activity (5-fold rise in K for carbon) and a 3-fold enlargement of the spleen occurred 16 days after cell injection could be interpreted as indicating the presence of a GVH reaction. However, a detailed examination of the situation reveals three important features which argue strongly against a GVH reaction, and indeed, support the existence of an HVG reaction: (1) all Balb/c mice given CBA spleen cells remained healthy and did not experience weight loss which usually accompanies the GVH reaction (GORER and BOYSE, 1959; SIMONSEN, 1962); (2) the enlarged spleens did not show atrophy of lymphatic nodules nor the presence of necrotic foci which develop during a GVH reaction (GORER and BOYSE, 1959; HOWARD, 1961); on the contrary, the spleens showed hyperplasia of the lymphatic nodules, identical with the changes found in Balb/c mice given F_1 spleen cells, and most likely reflected a humoral response directed against the H-2^k antigens carried by CBA cells; (3) an increase in the relative number of liver phagocytes which occurs in the GVH reaction (HOWARD et al., 1965; HOWARD, BOAK and CHRISTIE, 1966) could not be demonstrated when livers of Balb/c mice injected with CBA cells were examined histologically at the peak of the phagocytic response. It is concluded therefore, that the transplantation of CBA spleen cells

into Balb/c mice leads to the development of an HVG reaction, although it is delayed in onset and is of longer duration than that which occurs when F_1 hybrid spleen cells are transplanted into Balb/c recipients. Furthermore, the HVG reaction produced in the CBA → Balb/c combination is of sufficient intensity to prevent the transplanted cells from mounting a GVH reaction against the recipient animal. It remains to be determined whether the existence of different H-2 antigens in other donor-recipient strain combinations produce a similar result. HOWARD (1964) found that where non-H-2 antigens are involved, as in the CBA → F_1 (C57 Black x C3H) combination, a GVH reaction developed and suppressed the potential HVG reaction.

SUMMARY

A host-versus-graft (HVG) reaction initiated by the intravenous injection of F_1 (Balb/c x C57 Black) spleen cells into Balb/c mice is characterized by hepatosplenomegaly and a marked increase in the phagocytic activity of the RES. Although the HVG reaction is accompanied by changes in RE organs which are also found in the GVH situation, *viz.*, phagocytic stimulation, splenomegaly and hepatomegaly, significant differences exist in the onset, duration, and nature of the functional and morphological changes produced. They occur much earlier in the HVG reaction and persist for shorter periods. No increase in the relative number of liver macrophages occurs in the HVG reaction, and splenic enlargement is due to white pulp hyperplasia with prominent germinal centre development. In a situation where the potential exists for HVG and GVH reactions to occur simultaneously, such as that created by injecting CBA spleen cells into Balb/c mice, the HVG reaction develops and is of sufficient intensity to prevent the transplanted cells from mounting a GVH reaction against the recipient animal.

CHAPTER 6

THE EFFECT OF CORTISONE, AZATHIOPRINE AND ANTILYMPHOCYTE SERUM ON THE PHAGOCYTIC ACTIVITY AND MORPHOLOGY OF RETICULO-ENDOTHELIAL ORGANS IN MICE

SECTION 1: CORTISONE

INTRODUCTION

Since GORDON and KATSH (1949) published their detailed account of adrenal cortical influence on the structure and phagocytic function of macrophages, many investigators have studied the effect of corticosteroids on phagocytosis. Experiments performed on humans, rabbits, guinea pigs, rats and mice treated with cortisone or related compounds have focussed attention on the phagocytic properties of macrophages from the liver, spleen, lung and peritoneum, and also of polymorphonuclear leukocytes. Conclusions relating to the influence of cortisone on phagocytosis have ranged from inhibition, no effect, to stimulation.

Blood clearance studies carried out on rats (CLAWSON and NERENBERG, 1953; BENACERRAF et al., 1954; RAWLS et al., 1954), mice (GELL and HINDE, 1953) and rabbits (MARTIN and KERBY, 1952; HAUGEN, BASSØE and FLOOD, 1969) failed to demonstrate any impairment of phagocytosis following cortisone treatment, even when high doses (100mg/kg) were given daily for 7 days (CLAWSON and NERENBERG, 1953). On the other hand, NICOL and BILBEY (1958, 1960) showed that cortisone strongly depressed carbon clearance when administered to mice in daily doses of 17-25 mg/kg for 6 days. Depressed clearance of colloidal chromium phosphate was also found by HELLER (1955) to occur in mice and rats receiving between 12.5 and 140mg/kg of cortisone, while HORNES and RYGAARD (1960) were able to demonstrate significant inhibition of blood clearance of Au^{198} in mice which had received a single 10mg/kg dose of cortisone. However, in none of these studies where impairment of phagocytosis occurred was any suggestion advanced to explain the phagocytic inhibition induced by cortisone.

The Present Investigation

In an attempt to resolve the controversy outlined above, it was decided to carry out a detailed investigation on the effect of cortisone on the RES of an inbred strain of mice, with particular attention to any morphological changes in the main RE organs which could explain any alteration in phagocytosis induced by cortisone treatment.

Seven groups of female Balb/c mice (6 per group) were each given daily injections of one of the following dose levels of cortisone: 2, 5, 10, 25, 50, 100 or 200 mg/kg of body weight. This treatment was continued for 10 consecutive days, and one day after the last injection, 4 mice from each group were subjected to the carbon clearance test. The weights of each animal's liver, spleen and 4 lymph nodes were determined immediately after sacrifice and these organs were then prepared for histological examination.

Complete clearance studies were also carried out on control and cortisone-treated mice. A group of untreated controls and two groups which had received either 10 or 200mg/kg of cortisone daily for 10 days, were injected one day later with a standard dose of carbon and sacrificed when optical density determination of lysed blood samples indicated that carbon could no longer be detected in the circulation. The time taken for 95% of the injected dose of carbon to be cleared was taken as a basis of comparison between control and cortisone-treated mice. A further 4 groups of female Balb/c mice were treated daily for 10 days with 10, 50, 100 or 200mg/kg of cortisone, sacrificed 1 day after the last dose, and the amount of neutral fat contained in each animal's liver determined.

RESULTS

Mortality and body weight changes following cortisone administration to mice.

No deaths occurred in 48 mice given cortisone in daily doses varying between 2 and 100mg/kg for a period of 10 days, although all groups showed a loss in body weight which characteristically occurs in certain animal species receiving such treatment (Table 6.1; Fig. 6.1). Two out of 12 mice died on the 6th and 7th days respectively after receiving 200mg/kg; the remaining 10 animals survived the 10th dose and surprisingly, showed the least weight loss of all cortisone-treated groups.

Phagocytic activity of the RES in mice receiving cortisone.

Phagocytic indices for groups of mice receiving between 2 and 200mg/kg of cortisone are shown in Table 6.1. Considerable depression of phagocytosis followed administration of cortisone at a level of 10mg/kg, while treatment with higher doses inhibited phagocytosis even further. However, K values for the 200mg/kg group were not much lower than those obtained following treatment with only 10mg/kg of drug. The profound degree of phagocytic depression induced by cortisone was highlighted by the values for 95% carbon clearance times (Table 6.2). With the exception of the group which received 5mg/kg, all other groups showed a significant decrease in the corrected phagocytic indices (Table 6.1). This decrease was progressive with increasing dose levels of cortisone, the main factors contributing to this effect being loss in body weight, a decrease in the K value, and an increase in liver mass.

TABLE 6.1. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE FOLLOWING CORTISONE[b] ADMINISTRATION (MEANS ± S.E.M.).

Dose Group	% Alteration in Total Body Weight (T.B.W.) at end of injection regime.	Liver weight as % of T.B.W.[c]	Spleen weight as % of T.B.W.[c]	Lymph node weights as % of T.B.W.[c]	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	-	4.84± .05	.56 ± .01	.105 ± .003	.026 ± .001	5.50 ± .05
Saline control	+4.2	4.94± .13	.54 ± .02	.122 ± .003	.027 ± .002	5.52 ± .22
2mg/kg cortisone	-8.6	5.25± .14	.45 ± .01**	.185 ± .006***	.020 ± .001*	4.36 ± .18**
5mg/kg "	-11.7	4.66± .24	.38 ± .01***	.147 ± .009*	.022 ± .003	4.96 ± .23
10mg/kg "	-14.6	4.79± .13	.27 ± .02***	.040 ± .001***	.010 ± .001***	3.64 ± .09***
25mg/kg "	-22.1	4.54± .17	.19 ± .02***	.025 ± .003***	.009 ± .001***	3.38 ± .03***
50mg/kg "	-15.7	5.95± .09***	.15 ± .01***	-	.010 ± .001***	3.02 ± .07***
100mg/kg "	-16.4	5.86± .17**	.13 ± .01***	-	.007 ± .001***	3.06 ± .08***
200mg/kg "	-5.1	6.56± .35**	.12 ± .01***	-	.007 ± .001***	2.68 ± .15***

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Cortisone in the dose indicated was given subcutaneously each day for 10 days.

[c] Because of the body weight loss, organ weights have been expressed as a % of each animal's corrected body weight (see Materials and Methods Chapter).

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001; (test group vs. saline control group).

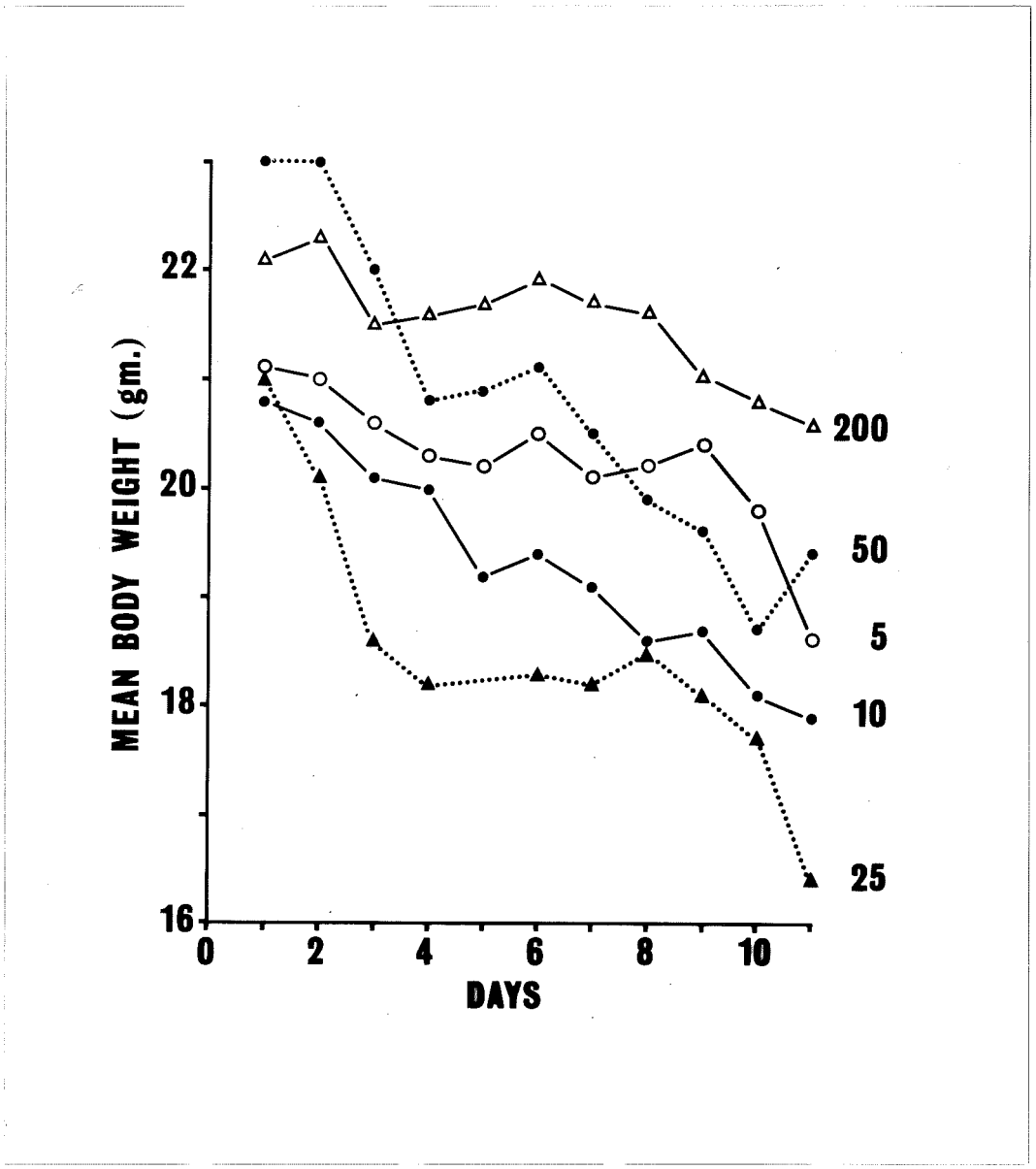

Fig. 6.1. Mean body weights of groups of Balb/c mice given 5, 10, 25, 50 or 200 mg/kg of cortisone daily for 10 days.

TABLE 6.2. 95 % CARBON CLEARANCE TIMES FOR CONTROL AND CORTISONE-TREATED[a] BALB/C MICE.

Group	95% Clearance time (minutes) (Mean ± S.E.M.)
Control	49 ± 2.3
Cortisone 10 mg/kg	135 ± 4.7
Cortisone 200 mg/kg	219 ± 4.7

[a] Mice were given cortisone subcutaneously each day for 10 consecutive days. Clearance test was carried out 1 day after the last dose of cortisone.

Changes in liver mass and morphology following cortisone treatment.

No alteration in liver mass occurred in mice which received between 2 and 25mg/kg of cortisone (Table 6.1). However considerable hepatomegaly was evident when doses of 50mg/kg and higher were administered; animals given these levels of drug had liver weights which were between 21 and 36 per cent greater than those of controls. Examination of H and E and Sudan III - stained histological sections of these enlarged livers revealed the characteristic picture of gross fatty change (Fig. 6.2). A few small areas of coagulative necrosis were seen in livers from the 100 and 200mg/kg groups, but the main histopathological change was the presence of excessive quantities of fat within hepatocytes. Abscess formation and inflammatory cell infiltration were not apparent in any section examined. Neutral fat determinations revealed up to an 8-fold increase in liver lipid in the high dose groups, while the 10mg/kg group showed a 3-fold increase (Table 6.3). A notable feature of livers from mice which received either 10 or 200mg/kg of cortisone, and which were subjected to complete clearance studies, was the marked reduction in the number of Kupffer cells capable of phagocytosing carbon (Fig. 6.3).

Changes in mass and morphology of the spleen and lymph nodes following cortisone treatment.

The profound influence of cortisone on the spleen was illustrated by the significant reduction in spleen mass in the group which received as little as 2mg/kg of drug (Table 6.1). Spleen size in groups receiving 10 and 25mg/kg were, respectively, only about 40 and 30% of normal, while higher doses resulted in further, but more gradual, reduction. Histologically, the lymphatic

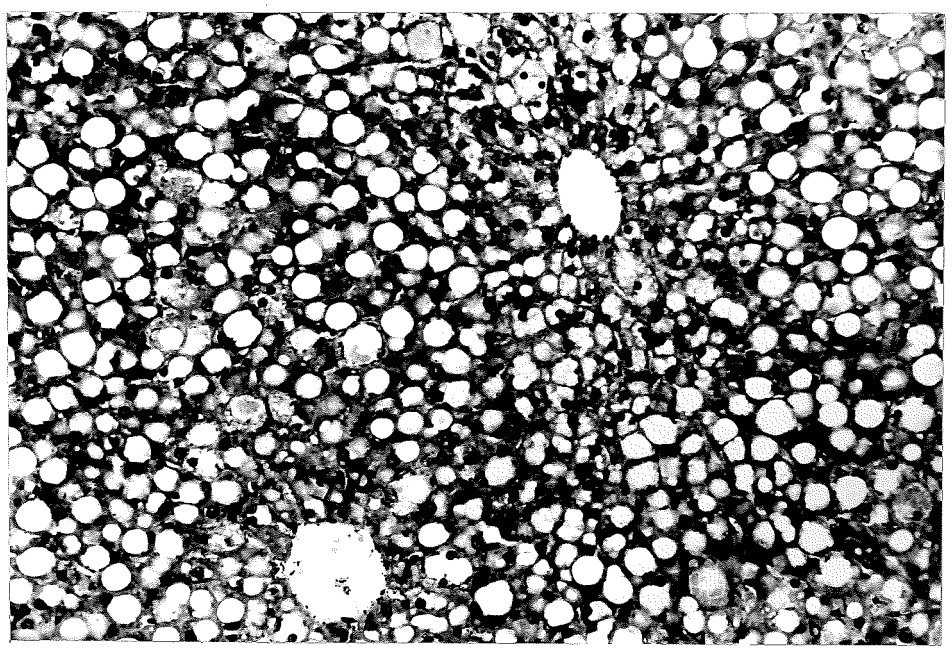

Fig. 6.2. Gross fatty change in a liver of a mouse given 50 mg/kg of cortisone daily for 10 days and killed one day later. Most hepatocytes are affected. H and E X100.

TABLE 6.3. FAT AND WATER CONTENT OF LIVERS FROM BALB/C MICE[a] TREATED WITH CORTISONE[b] (MEANS ± S.E.M.).

Group	Liver weight as % of total body weight	% Liver water	% Liver fat
Untreated control	4.86 ± .20	68.0 ± 0.4	1.8 ± 0.3
10 mg/kg cortisone	5.07 ± .21	64.2 ± 0.6 ***	5.0 ± 0.3 ***
50 mg/kg cortisone	6.14 ± .11 ***	67.7 ± 1.7	6.2 ± 0.8 ***
100 mg/kg cortisone	5.86 ± .38 *	61.4 ± 3.4	11.7 ± 1.2 ***
200 mg/kg cortisone	6.13 ± .29 **	59.2 ± 0.8 ***	14.0 ± 1.1 ***

[a] There were 4 female mice in each group.

[b] Cortisone in the dose indicated was administered each day for 10 consecutive days and the mice sacrificed 1 day after the last injection.

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (test group versus untreated control group).

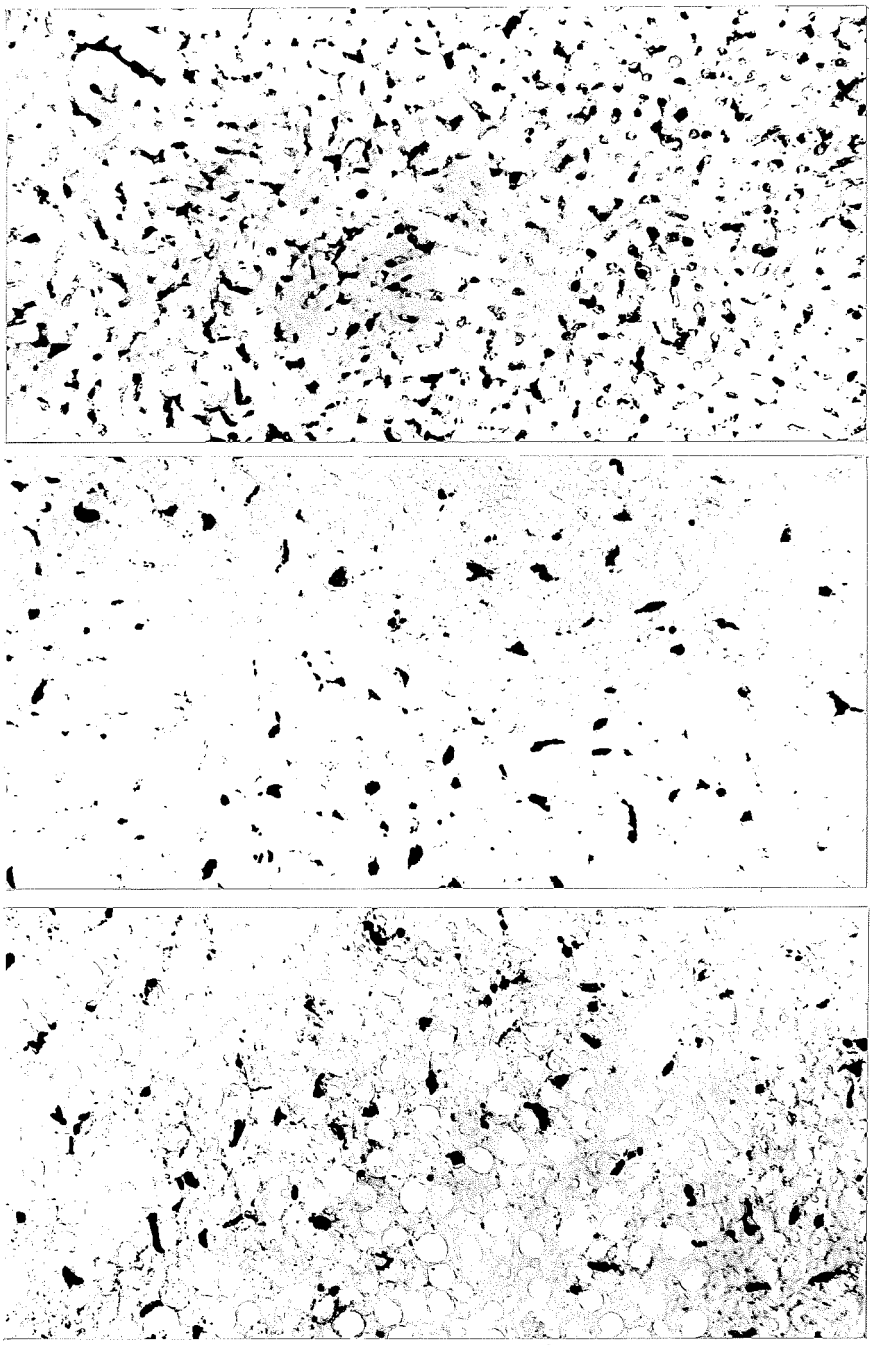

Fig. 6.3. Unstained liver sections from control and cortisone-treated mice showing distribution of carbon-containing Kupffer cells. Top: untreated control. Middle: liver of mouse treated daily with 10 mg/kg of cortisone for 10 days; a marked reduction in carbon-containing cells is apparent. Bottom: liver of mouse treated for 10 days with 200 mg/kg of cortisone; a marked reduction in carbon uptake is again evident, but this appearance does not differ greatly from that produced by only 10 mg/kg of cortisone. All magnifications X100.

nodules in spleens of animals given 5 - 100mg/kg showed reduction in size and number; the red pulp also exhibited decreased cellularity and a marked reduction in extramedullary haematopoiesis, particularly beneath the splenic capsule. In contrast to the situation found in the liver, the amount of carbon taken up by the spleens in mice treated with 10mg/kg of cortisone did not appear to differ significantly from that taken up by control spleens. However, a difference in the pattern of carbon distribution was evident in the spleens from these two groups. Whereas control spleens showed most of the carbon to be localized in the marginal zones and adjacent regions of the red pulp, spleens from cortisone-treated animals showed a much more even distribution of carbon throughout the entire red pulp (Fig. 6.4).

The lymph node response to cortisone administration presented a paradoxical situation in that the 2mg/kg group showed significantly increased lymph node weights when compared with controls, while at higher doses (10 and 25mg/kg), profound weight loss was observed (Table 6.1). Lymph nodes in mice which received between 50 and 200mg/kg of cortisone were so profoundly reduced in size that they could not be distinguished from their surrounding fascial tissues.

DISCUSSION

The results of the present investigation indicate that cortisone, when administered to mice at a dose of 10mg/kg and higher, has the ability to profoundly impair the ability of fixed macrophages to carry out phagocytosis. This finding supports the work of HELLER (1955) and HORNES and RYGAARD (1960) who also found phagocytic inhibition to follow administration of cortisone at comparable dose levels. The inability of cortisone-treated mice to clear carbon

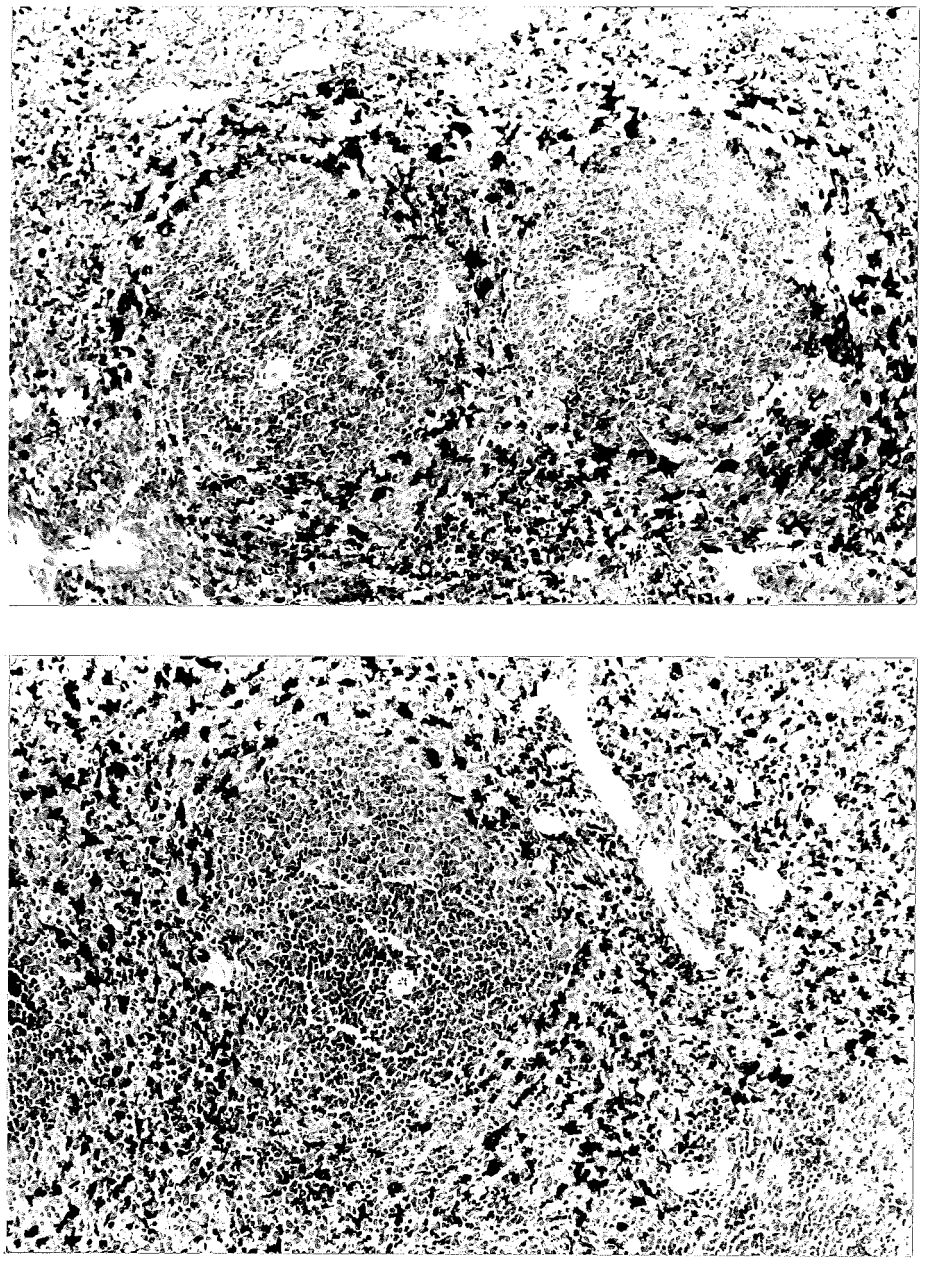

Fig. 6.4. Neutral red-stained spleen sections from control and cortisone-treated mice showing distribution of intravenously-injected carbon. Top: untreated control; most of the carbon is localized in the marginal zones and adjacent regions of the red pulp. Bottom: spleen of mouse given 10 mg/kg of cortisone for 10 consecutive days; the carbon particles are evenly distributed throughout the red pulp. X100.

particles from the circulation as rapidly and efficiently as control animals was found in the present study to be associated with a marked reduction in the number of Kupffer cells capable of phagocytosis. This conclusion was based on histological examination of the liver at a time when carbon could no longer be detected in the circulation. A reduced uptake of carbon by the liver following cortisone treatment was also observed by NICOL and BILBEY (1960). However, the fact that their animals were killed only one hour after carbon injection and not when all carbon had been cleared from the circulation precluded any firm conclusion as to whether a reduction in the number of active phagocytes had in fact occurred.

While a decreased uptake of carbon by the liver was found in the present study to follow cortisone treatment, there did not appear to be any significant decrease in the amount of carbon phagocytosed by splenic macrophages (at least for doses up to 10mg/kg), although a difference in the pattern of carbon distribution was noted between the spleens from cortisone-treated and control animals. This difference in carbon localization can probably be explained by reference to the work of SNOOK (1964) who showed that carbon and saccharated iron oxide particles, after being introduced into the bloodstream, first became trapped between cells situated just beyond the marginal sinus areas in the spleen; the trapped particles were subsequently found to have moved into the deeper regions of the red pulp where they were seen to lie within macrophages. It seems likely, therefore, that following cortisone treatment, because the phagocytic ability of liver macrophages is adversely affected, the spleen assumes a more important role in the removal of circulating particles; greater than normal amounts of blood-borne particles are taken up by this organ, localize initially

in the marginal zone area and soon move out into the central regions of the red pulp. In normal animals, on the other hand, most of the injected carbon will be taken up initially by the highly efficient liver macrophages, and splenic localization proceeds relatively slowly. This would explain the fact that at the termination of carbon clearance, most of the carbon was localized near the marginal zone regions in untreated control animals, whereas in cortisone-treated animals, carbon particles were uniformly distributed throughout the red pulp.

Apart from the reduction in numbers of Kupffer cells capable of phagocytosing carbon, the other most conspicuous alteration in liver morphology resulting from cortisone administration was the development of fatty change. Hepatocytes were mildly affected when a dose of 10mg/kg was used, while at 50mg/kg or higher, severe fatty change resulted. Liver abscess formation and inflammatory cell infiltration, such as observed by ASHBURN, WILLIAM and ARLANDER (1962) when cortisone was given to mice, were not found in the present study. Cortisone-induced fatty change associated with hepatomegaly has been previously described in rabbits (RICH, COCHRAN and McGOON, 1951; MORAN, 1962) and rats (HILL and DROKE, 1963), although the literature on this point is by no means in complete agreement. Increase in hepatic glycogen rather than lipid has been observed to occur following cortisone administration to mice (WILLIAMS, DAVIS and LOWE, 1956), rats (LOWE and WILLIAMS, 1953; WILLIAMS, DAVIS and LOWE, 1956), rabbits (LURIE et al., 1951), GERMUTH et al., 1951) and in hamsters (FRENKEL and HAVENHILL, 1963). On the other hand, ATERMAN and AHMAD (1953) concluded that negligible

changes occurred in livers of rats given 50mg/kg of cortisone daily for 10 days. These conflicting reports suggest that the susceptibility of liver parenchymal cells to cortisone-induced changes shows strain as well as species differences. These differences may equally apply to the changes produced in Kupffer cells and if so, may help to reconcile the previous discrepant results relating to the influence of cortisone on phagocytosis.

SUMMARY

Balb/c mice treated with cortisone at dose levels varying between 2 and 200mg/kg were studied for changes in the phagocytic activity and morphology of their reticuloendothelial organs. A level of 10mg/kg administered daily for 10 days profoundly impaired the clearance rate of intravenously injected carbon particles. The resulting phagocytic inhibition was due to a marked reduction in the number of Kupffer cells capable of phagocytosing carbon. The amount of carbon taken up by the spleen was not significantly affected by relatively low doses of cortisone, although the evidence obtained suggests that splenic uptake may have occurred more rapidly than normal as a consequence of the reduced phagocytic activity of liver macrophages. Liver parenchymal cells from mice treated with 10mg/kg of cortisone showed a 3-fold increase in fat content, while doses of 50mg/kg and higher brought about severe fatty change and hepatomegaly. Available evidence indicates that strain as well as species differences exist with respect to the susceptibility of hepatocytes to cortisone treatment. It is suggested that these differences might equally apply to the effects produced in Kupffer cells, and may thus reconcile previous conflicting reports regarding the influence of cortisone on phagocytosis.

SECTION 2 : AZATHIOPRINE

INTRODUCTION

In clinical practice, one of the most serious and often fatal complications resulting from immunosuppressive therapy with azathioprine and corticosteroids is infection (HILL, ROWLANDS and RIFKIND, 1964; WOODRUFF, 1969). It is not certain whether these drugs increase the risks of infection mainly be reducing the ability of host defence cells to phagocytose bacteria, or whether they severely impair the cells' capacity to destroy the organisms once they have been engulfed. Unlike cortisone, azathioprine has not been studied for its effect on phagocytic cells, nor have detailed reports appeared regarding its influence on the RES in general. Accordingly, the present study was designed to examine some effects of azathioprine treatment on RE organs, in particular, its effect on the phagocytic properties of liver and splenic macrophages and the morphology of lymphoid tissues, especially the spleen. At the same time it was considered worthwhile to examine closely the effect of azathioprine on liver morphology since there have been several reports of hepatotoxicity in humans treated with 3 to 5 mg/kg of the parent compound, 6-mercaptopurine (6-MP) (McILVANIE and MacCARTHY, 1959; CLARK, HSIA and HUNTSMAN, 1960; BURCHENALL and ELLISON, 1961; EINHORN and DAVIDSOHN, 1964; KRAWITT et al., 1967). Widespread hepatic necrosis has also been observed to follow administration of very high doses of 6-MP to rats (CLARKE et al., 1953; PHILIPS et al., 1954). Apart from the brief reference by CASEY (1968) to the occurrence of hepatocellular necrosis in mice given large doses of azathioprine, the literature does not appear to contain reports of studies dealing with the effect of azathioprine on the liver, despite the wide-

spread clinical use of this immunosuppressive agent. In reviewing the effectiveness and toxicity of the main immunosuppressive agents, BERENBAUM (1965) excluded azathioprine on grounds that it had not been adequately tested in animal species. The present study provides data on some of the effects produced by this drug in mice.

The Present Investigation.

Six groups of female Balb/c mice (6 per group), aged between 12 and 14 weeks were each given daily injections of one of the following dose levels of azathioprine: 5, 10, 25, 50, 75 and 100 mg/kg of body weight. This treatment was continued for 10 consecutive days, and one day after the last injection, 4 mice from each group were subjected to the carbon clearance test. The weights of each animal's liver, spleen and 4 lymph nodes were determined immediately after sacrifice and these organs were then prepared for histological examination. Specimens of the liver tissue taken from mice in the 50 and 75 mg/kg groups were also prepared for examination with the electron microscope.

RESULTS

Mortality and body weight changes following azathioprine administration to mice.

No deaths occurred when daily doses of 5 to 75 mg/kg were given for 10 consecutive days, although all groups showed body weight loss which ranged from 1.5% in the lowest dose group to 19.9% for the group given 50 mg/kg (Table 6.4; Fig. 6.5). All animals receiving 100 mg/kg daily died within 6 to 9 days of treatment.

TABLE 6.4. LIVER, SPLEEN AND LYMPH NODE [a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE FOLLOWING AZATHIOPRINE[b] ADMINISTRATION (MEANS ± S.E.M.).

Dose Group	% Alteration in Total Body Weight(T.B.W.) at end of injection regime.	Liver weight as % of T.B.W.[c]	Spleen weight as % of T.B.W.[c]	Lymph node weights as % of T.B.W.[c]	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	-	4.78 ± .05	.52± .01	.110 ± .004	.026± .001	5.59 ± .03
Saline control	+2.7	4.92 ± .16	.58± .03	.115 ± .003	.026± .001	5.42 ± .14
5mg/kg Azathioprine	-1.5	5.87 ± .20*	.59± .04	.145 ± .012*	.023± .002	4.26 ± .17**
10mg/kg "	-4.6	4.57 ± .12	.40± .02***	.097 ± .003	.024± .001	5.46 ± .09
25mg/kg "	-4.0	5.40 ± .13	.44± .01***	.180 ± .009***	.026± .001	4.84 ± .11*
50mg/kg "	-19.9	4.59 ± .15	.37± .03**	.097 ± .012	.028± .004	4.89 ± .32
75mg/kg "	-18.9	4.39 ± .16*	.37± .02***	.075 ± .006**	.013± .002**	4.04 ± .32**

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Azathioprine in the dose indicated was given intra-peritoneally each day for 10 days.

[c] Because of the body weight loss, organ weights have been expressed as a % of each animal's corrected body weight (see Materials and Methods Chapter).

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001; (test group vs. saline control group).

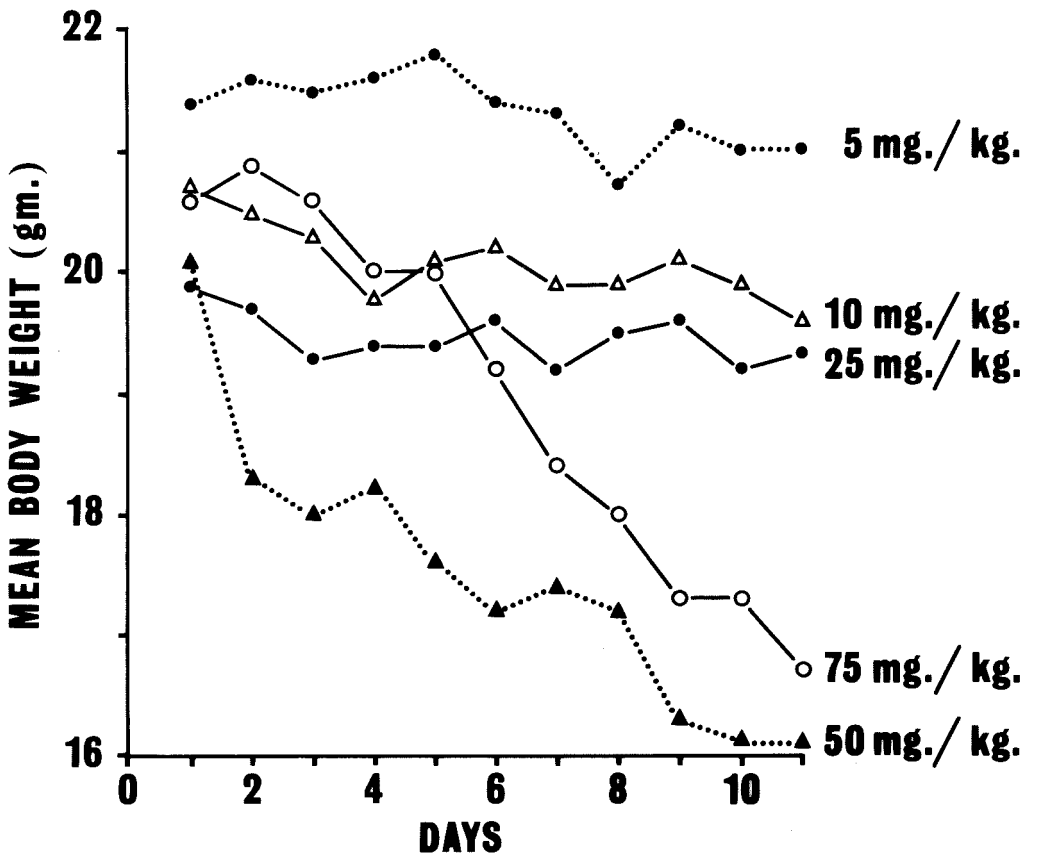

Fig. 6.5. Mean body weights of groups of Balb/c mice given 5, 10, 25, 50 or 75 mg/kg of azathioprine daily for 10 days.

Phagocytic activity of the RES in mice receiving azathioprine.

Phagocytic indices for mice receiving between 5 and 50 mg/kg of azathioprine did not differ from control values and significant depression of phagocytosis occurred only in the group which received 75 mg/kg of drug (Table 6.4).

Morphologic changes in livers from azathioprine-treated mice as seen under light and electron microscopy.

Although changes in liver mass followed treatment with certain dose levels of azathioprine, a trend was not apparent. The group given 5 mg/kg showed significant liver enlargement, while treatment with 75 mg/kg resulted in a significant reduction in liver mass (Table 6.4).

Extensive areas of necrosis and haemorrhage were found in livers of animals which had died following administration of 100 mg/kg of azathioprine (Fig. 6.6). Inflammatory cells were absent from within and around these necrotic areas (Fig. 6.7). In other regions many hepatocytes showed pyknosis, karyorrhexis or karyolysis of their nuclei, while their cytoplasm exhibited a vacuolated appearance. Similar changes were observed in the 50 and 75 mg/kg groups, although necrotic areas were fewer and less extensive. Hepatocytes in the centri-lobular areas showed prominent vacuolation of their cytoplasm while those adjacent to the portal blood vessels were unaffected (Fig. 6.8). The relative number, size and distribution of Kupffer cells and the total amount of carbon phagocytosed by individual cells was unaltered by low or high doses of azathioprine. In electron micrographs, many hepatocytes from livers of mice given 50 and 75 mg/kg of azathioprine were observed to contain numerous large

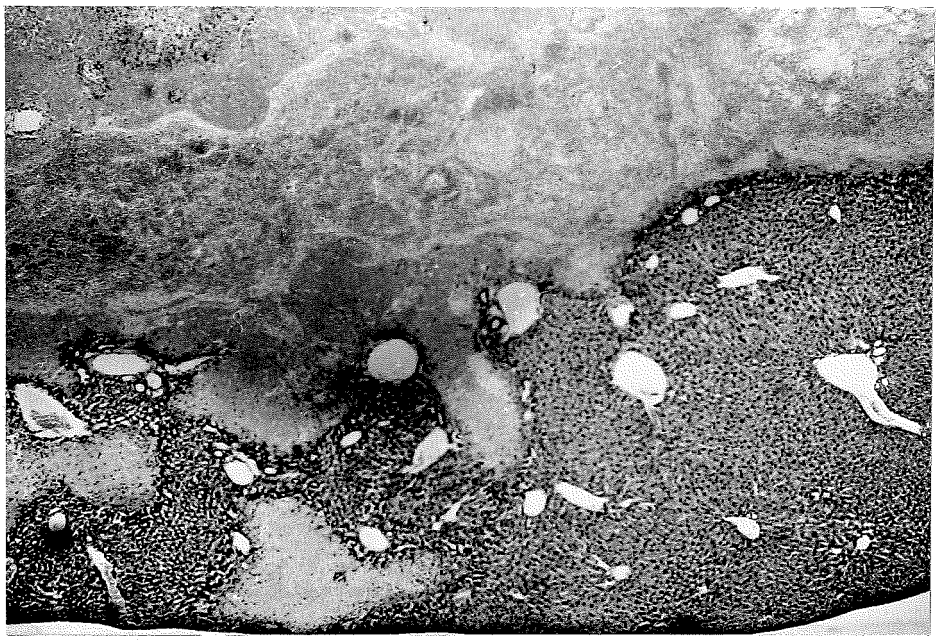

Fig. 6.6. Widespread necrosis and haemorrhage in the liver of a mouse given 100 mg/kg of azathioprine daily for 7 days and sacrificed 1 day later. H and E X 35.

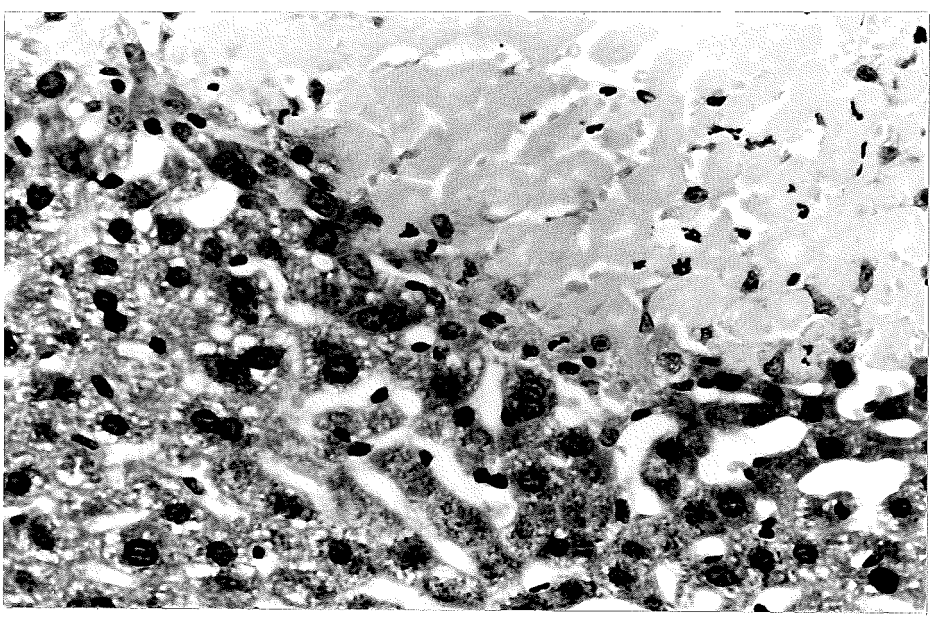

Fig. 6.7. Edge of necrotic area in the liver shown in Fig. 6.6. No inflammatory cells are visible. H and E X250.

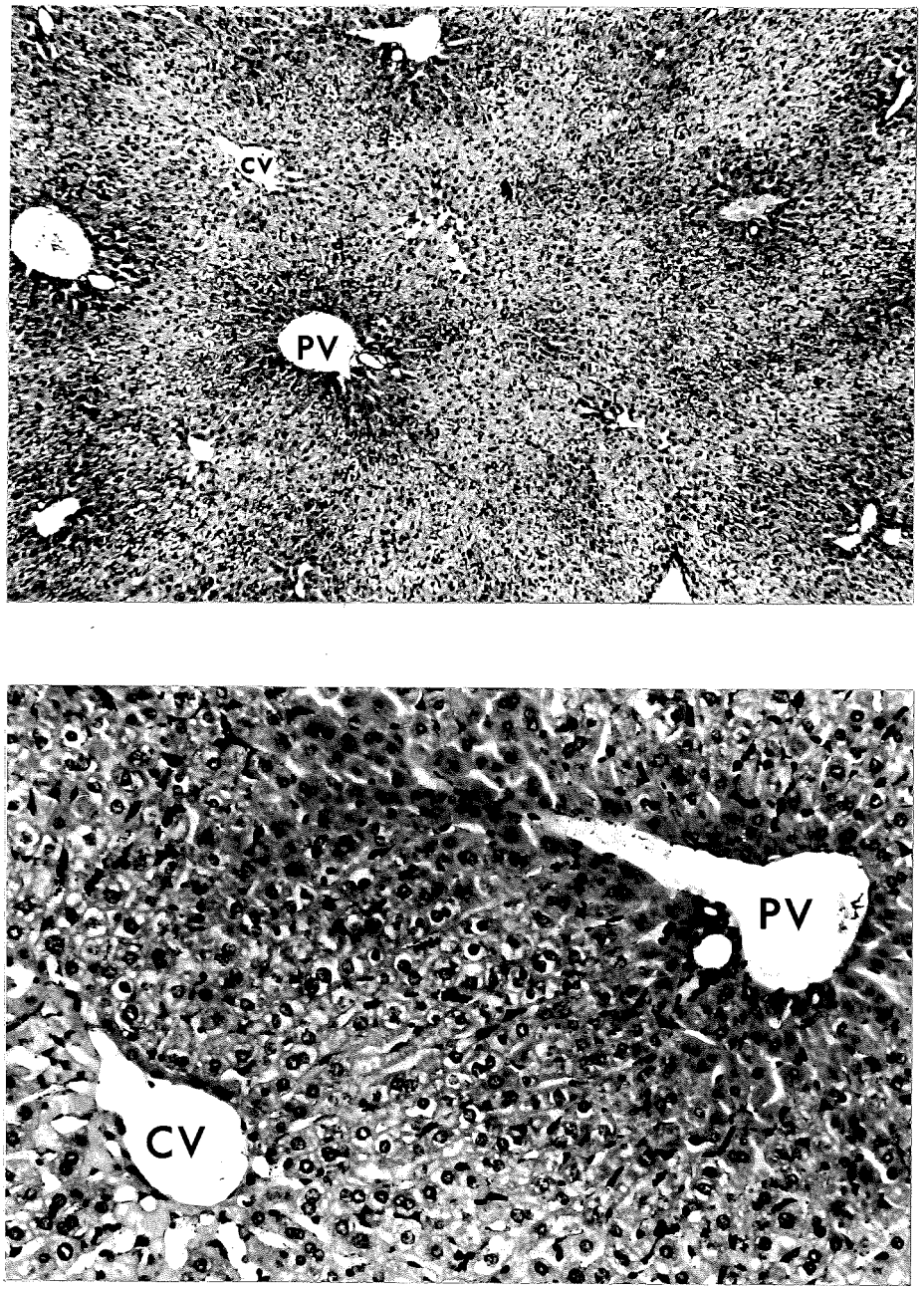

Fig. 6.8. Liver of a mouse killed one day after being given 50 mg/kg of azathioprine daily for 10 days. Hepatocytes around central veins (CV) show marked vacuolation of their cytoplasm while those in the immediate vicinity of the portal veins (PV) are unaffected. H and E. Top X60; Bottom X100.

lipid droplets in their cytoplasm, suggesting early fatty change (Fig. 6.9). In addition, some of these cells showed areas of complete cytoplasmic disorganization (Fig. 6.10). Although areas of necrosis and haemorrhage were not observed in livers of mice receiving 25 mg/kg of drug, nuclear and cytoplasmic alterations similar to those described for the higher dose groups were regularly observed. As far as could be determined by light microscopy, administration of 5 and 10 mg/kg of azathioprine produced no detectable alteration in liver cell morphology.

Alterations in lymphoid mass and morphology following azathioprine administration.

Spleen mass was significantly reduced in groups of mice given azathioprine at a dose of 10 mg/kg and higher (Table 6.4). Histological examination revealed alterations in both the red and white pulp. Extramedullary haematopoiesis was decreased, especially beneath the splenic capsule. Although lymphatic nodules were still clearly visible, their number and size were reduced, particularly following treatment with 50 and 75 mg/kg of drug. Furthermore, many of these nodules were atypical in that instead of having a normal pale-staining centre of reticulum cells surrounded by a darker rim of mature lymphoid cells, they were composed of adjacent "half-moon" areas each consisting of either pale or dark staining cells (Fig. 6.11). The ability of marginal zone cells to trap carbon particles from the blood was unaffected by 5 to 25 mg/kg of azathioprine, but higher doses resulted in a reduced uptake of carbon in this zone (Fig. 6.11).

Lymph nodes did not show a decrease in mass comparable with that found in the spleen. Mice receiving 5 and 25 mg/kg of drug had, in fact,

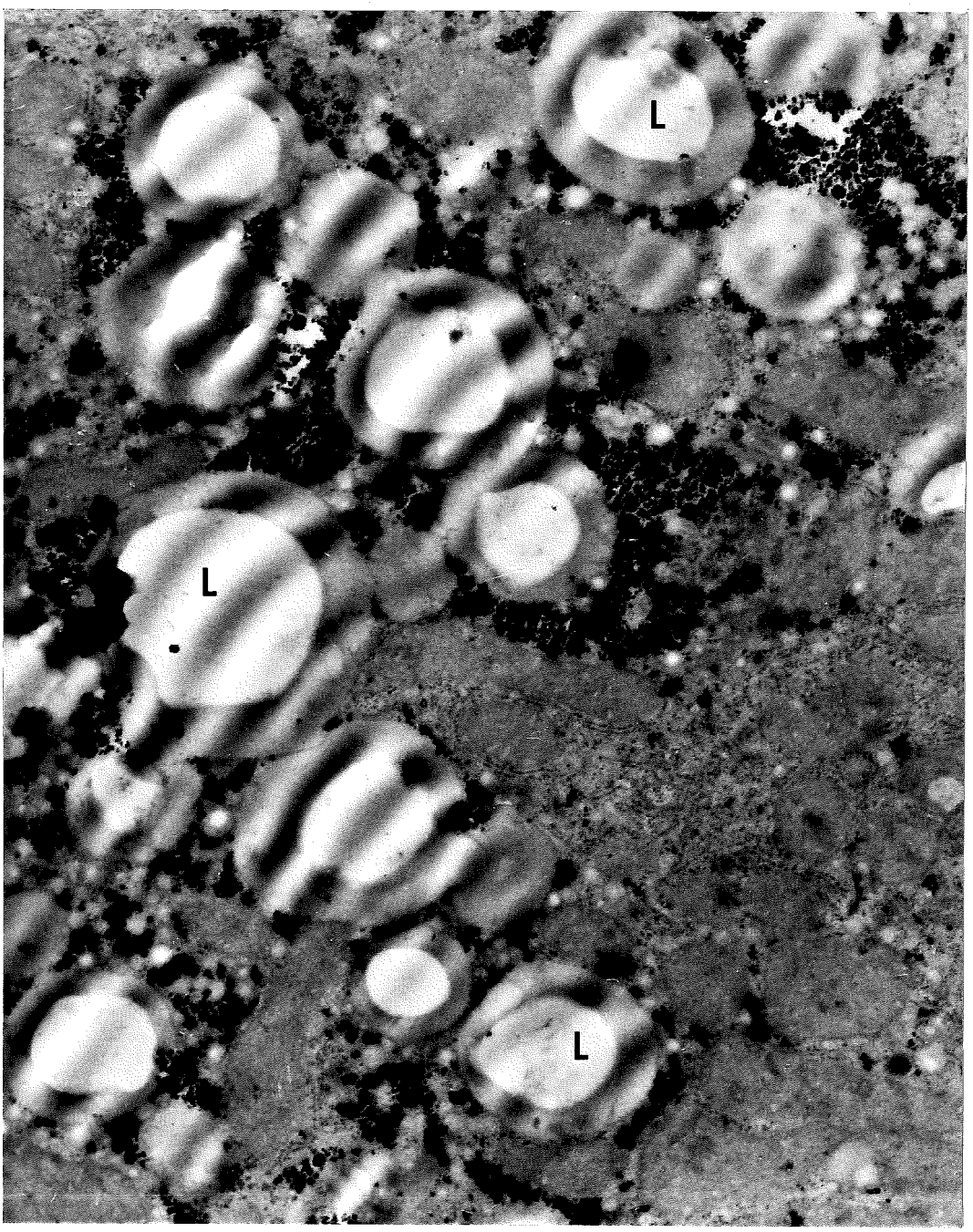

Fig. 6.9. Fatty change in an hepatocyte from a centrilobular region of the liver shown in Fig. 6.8. Numerous large lipid droplets (L) are present and are surrounded by clusters of glycogen granules. X 12,500.

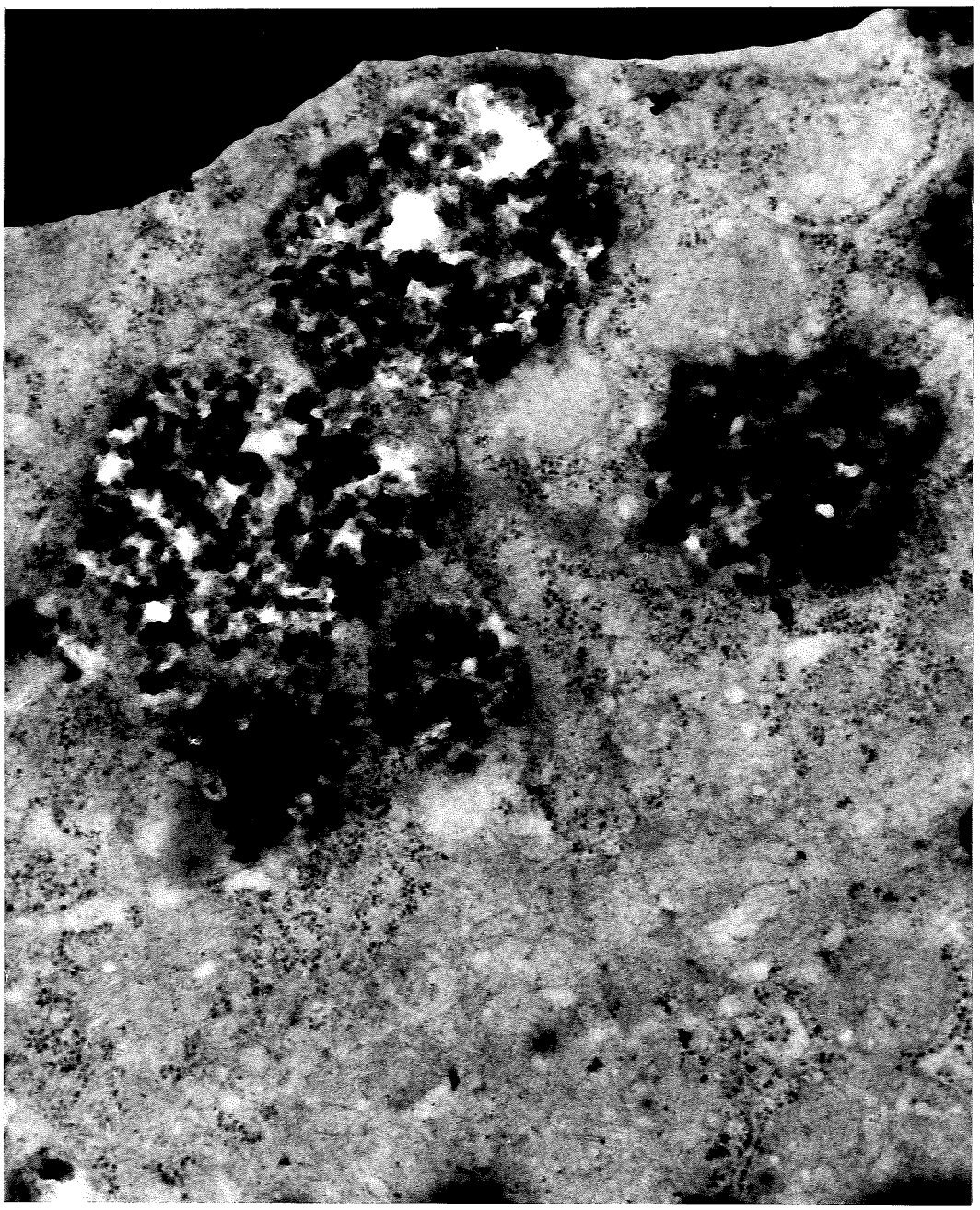

Fig. 6.10. Hepatocyte in a centrilobular region of the liver shown in Fig. 6.8., showing two areas of complete cytoplasmic disorganization. X 27,500.

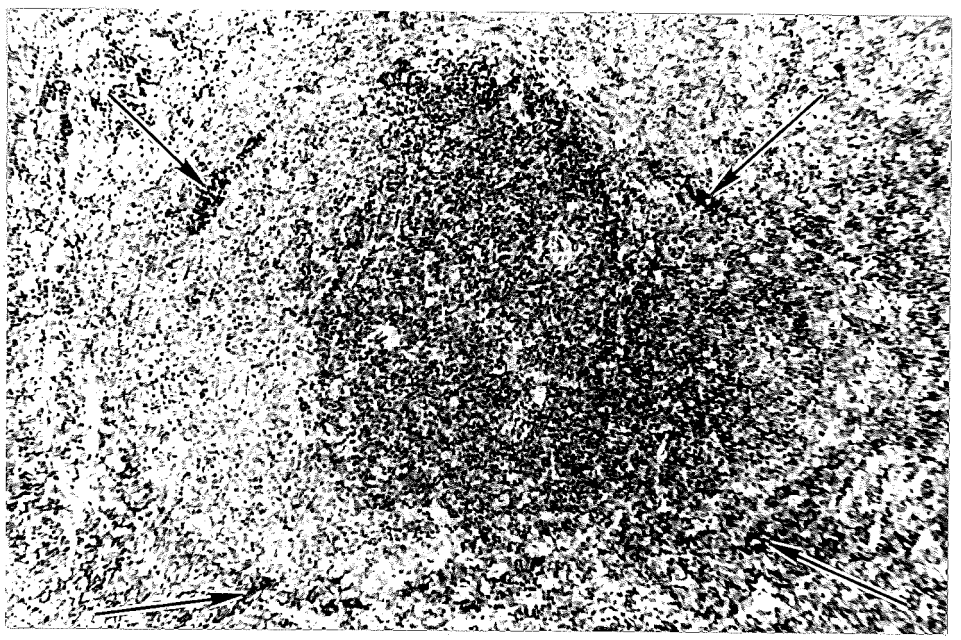

Fig. 6.11. Atypical lymphatic nodule in the spleen of a mouse given 50 mg/kg of azathioprine daily for 10 days and killed one day later. A few intravenously injected carbon particles (arrowed), which have localized in the marginal zone region, delineate the border of the lymphatic nodule; the latter is comprised of adjacent "half-moon" areas of pale and dark-staining cells. H and E X70.

enlarged lymph nodes and significant reduction in size was evident only in the 75 mg/kg group. The histological appearance of all lymph nodes was normal with the exception of those from the 75 mg/kg group which showed cellular depletion within the lymphatic nodules.

DISCUSSION

Impairment of phagocytic mechanisms was suggested by BERENBAUM (1967a) as probably contributing to the development of fatal infections in patients under immunosuppression, although acknowledgement was made of the fact that corticosteroids were the only immunosuppressive agents for which there was evidence of direct interference with phagocytosis. The present study has shown that azathioprine has little effect on phagocytosis of carbon by the liver and splenic macrophages of mice. Inhibition of phagocytosis became evident only when a near-lethal level of drug was administered. This finding is in accordance with the results of SCHWARTZ, EISNER and DAMASHEK (1959), and SCHWARTZ and ANDRE (1960) who observed normal clearance rates of heterologous erythrocytes and proteins from the circulation of 6-mercaptopurine-treated rabbits. It would seem therefore, that development of massive bacterial infection such as reported by HILL, ROWLANDS and RIFKIND (1964) in patients receiving combined azathioprine and prednisone therapy is probably due largely to two independent effects of these drugs. Corticosteroids inhibit phagocytosis by polymorphs (CREPEA, MAGNIN and SEASTONE, 1951) and fixed macrophages (HELLER, 1955; NICOL and BILBEY, 1958; 1960; HORNES and RYGAARD, 1960; GOTJAMANOS: see previous section on cortisone), and they also impair the bactericidal capacity of polymorphs (ALLISON and ADCOCK,

1962). The antimetabolite property of azathioprine indirectly causes a depletion of polymorphs by depressing their formation in haemopoietic tissue, and perhaps also through a direct effect on circulating cells. HILL, ROWLANDS and RIFKIND (1964) reported a virtual absence of polymorphs from infected organs of patients who had died during immunosuppressive treatment, and similar observations were made around the necrotic areas in the livers of azathioprine-treated mice studied in the present investigation.

Lymphoid tissue depletion is another factor that is undoubtedly responsible for conferring a state of weakened immunological response in recipients of immunosuppressive therapy. The ability of corticosteroids to cause lymphoid tissue involution has been well documented (SPAIN, MOLOMUT and HABER, 1950; DOUGHERTY, 1952; LURIE et al., 1951; MAGEE and PALMER, 1953; SANTISTEBAN and DOUGHERTY, 1954; BENACERRAF et al., 1954; FRENKEL and HAVENHILL, 1963). The data derived from the present study indicates that azathioprine also possesses the property of depleting lymphoid tissues, particularly the spleen, although this effect is not nearly as profound as that which follows cortisone treatment. For example, 10 mg/kg of cortisone given to mice daily for 10 consecutive days results in a reduction of spleen and lymph node mass by more than 50 per cent (see Table 6.1), whereas an identical regime of azathioprine treatment was found in the present study to deplete spleen mass by about 25 per cent and have a negligible effect on lymph node size. The reduction in spleen mass which followed azathioprine administration however, was not due entirely to depletion of lymphoid tissue, since extramedullary haematopoiesis was also inhibited. Differences in susceptibility of

lymphoid organs to azathioprine appear to exist among different species. Contrary to the present observations in mice, BILENKO (1967) reported complete disappearance of splenic lymphatic nodules in rabbits and dogs treated with 3 to 10 mg/kg of azathioprine. On the other hand, ELION et al., (1961) were unable to detect any histological abnormality in spleens from beagle pups given 1 to 4 mg/kg of azathioprine 5 times a week for 18 weeks.

In the present study marked hepatotoxic changes were observed in mice which received 50 mg/kg of azathioprine. Since this dose level is about 10 times greater than that used in man, extrapolation to the human situation may not apply. Available evidence suggests however, that in some cases the human liver may be susceptible to doses of immunosuppressive drugs far below those required to cause pathological changes in animals. CLARKE et al., (1953) found hepatic damage in about half of their rats given 100 mg/kg of 6-MP, whereas a high incidence of hepatotoxic changes has been reported in patients treated with less than 5 mg/kg of the same drug (McILVANIE and MacCARTHY, 1959; CLARK, HSIA and HUNTSMAN, 1960; BURCHENALL and ELLISON, 1961; EINHORN and DAVIDSOHN, 1964; KRAWITT et al., 1967). Similarly with cortisone, hepatomegaly associated with gross fatty change becomes evident in mice only when a dose of 50 mg/kg is given for 10 consecutive days (see Table 6.1). In contrast, STEINBERG, WEBB and RAFSKY (1952) observed rapid liver enlargement in a patient who had received approximately 5 mg/kg of cortisone for 6 days, while HILL (1961) described a fatal episode of fat embolism from a severe case of fatty liver in a young patient treated daily with prednisone (100mg) and hydrocortisone (40mg).

Combined azathioprine and prednisone therapy is used routinely in the management of transplant patients. Either drug is capable of causing hepatotoxic changes when given alone and it is possible that when the two drugs are administered simultaneously, toxic manifestations may occur more readily and at relatively low dose levels. Detailed histopathological studies of combined immunosuppressive treatment have not been reported, and it would obviously be of considerable value if such investigations were undertaken.

SUMMARY

Groups of Balb/c mice under immunosuppression with azathioprine at dose levels varying between 5 and 100 mg/kg of body weight were examined for any changes in phagocytic activity and morphology of their reticuloendothelial organs. When given daily over a 10 day period, 5 to 50 mg/kg of azathioprine had little effect on the ability of liver and splenic macrophages to phagocytose intravenously administered carbon particles, and phagocytic impairment occurred only when a near lethal level of drug (75 mg/kg) was used.

Mild hepatotoxic changes accompanied the administration of azathioprine at a level of 25 mg/kg, while doses of 50 to 100 mg/kg gave rise to severe alterations in liver structure, characterized by areas of frank necrosis, haemorrhage and degenerative changes in hepatocytes.

Significant reduction in spleen mass occurred when azathioprine was given at a dose of 10 mg/kg and higher. Alterations in both the splenic red and white pulp were apparent; these included decreased extramedullary haematopoiesis, a reduction in the number and size and abnormal development of lymphatic nodules. Lymph nodes did not show comparable changes except at the highest dose levels.

SECTION 3: ANTILYMPHOCYTE SERUM

INTRODUCTION

The profound immunosuppressive properties of heterologous anti-lymphocyte serum (ALS) have been adequately demonstrated and extensively studied in a variety of animal species. Its use has, however, been associated with serious complications, including increased susceptibility to viral infections (ABAZA et al., 1966; HIRSCH and MURPHY, 1968; ALLISON, 1970). A vast amount of experimental and clinical evidence strongly supports the concept that the RES constitutes an important defence against infection. An understanding of the way in which ALS modifies the phagocytic properties of reticuloendothelial cells would therefore be of considerable importance, particularly in view of the documented action of ALS in interfering with the inductive phase of certain immune responses (BERENBAUM, 1967b).

Previous studies on the effect of ALS on the phagocytic activity of macrophages have produced variable and conflicting results (BOAK et al., 1968; LOEWI et al., 1969; GROGAN, 1969; MARSHALL and KNIGHT, 1969b; SHEAGREN et al., 1969). In some of these investigations in which alterations in morphology of reticuloendothelial organs were also reported, satisfactory explanations for the observed changes were not advanced, nor was any attempt made to account for them in terms of the immunosuppressive properties of ALS. Accordingly, the aim of the present study was to provide detailed information regarding the effects of ALS on reticuloendothelial cells and organs, and to attempt to assess their significance in relation to the general mode of action of ALS in suppressing immune responses.

The Present Investigation

Male Balb/c mice aged 12-14 weeks and weighing between 22 and 27g were matched for age and weight and assigned to groups of 4. Each group received either 1, 2, 4 or 5 half-millilitre doses of ALS, ALG or normal rabbit serum (NRS). In the case of multiple injection regimes, each dose was given on alternate days. Phagocytic activity of the RES was estimated using colloidal carbon one day after single doses, and 2 days after the last multiple dose. Immediately after sacrifice, each animal's liver, spleen and 4 lymph nodes were removed, weighed, and then prepared for microscopic examination. Estimations of liver RNA, DNA, protein and water content were performed on a further 3 groups of male Balb/c mice (4 per group) which had received 5 doses of absorbed ALS, ALG or NRS.

For the experiments on RES activity, 4 separate batches of ALS were employed. These have been designated ALS I, ALS II, ALS IV and ALS 23A. Each of these antisera was capable of doubling the normal survival time of primary 2 cm^2 CBA skin allografts transplanted to Balb/c recipients (GILL, P.G., personal communication). The regime of antiserum administration used for this test of immunosuppressive potency consisted of 3 subcutaneous doses of ALS, each of 0.5ml; the first dose was given 2 days before grafting, and the other two on the 2nd and 5th days after grafting.

RESULTS

Mortality, body weight changes and haematologic findings following antiserum administration to mice.

Out of 64 mice given between 1 and 5 doses of ALS or ALG, only 2 died during the observation period. Mice given ALS that had been absorbed with erythrocytes had near normal haematocrits while those given unabsorbed ALS showed falls in haematocrit levels varying between 30 and 61%. (Table 6.5). Some groups given ALS subcutaneously experienced a loss of body weight, the unabsorbed sera causing the severest effect (Fig. 6.12; Tables 6.6 and 6.7). Intraperitoneal administration of ALS did not bring about any loss of body weight (Fig. 6.13; Table 6.8).

Effect of 1 or 2 subcutaneous doses of ALS, ALG and NRS on phagocytic activity of the RES.

Inhibition of phagocytosis followed the administration of 1 and 2 doses of unabsorbed ALS (Table 6.6). A similar effect accompanied 1 dose of NRS, while 2 doses of absorbed ALS also depressed phagocytosis, but to a lesser extent. Phagocytic activity was unaffected by one dose of ALG (Table 6.6).

Effect of 4 or 5 subcutaneous or intraperitoneal doses of ALS, ALG and NRS on phagocytic activity of the RES.

All groups of animals given 4 or 5 subcutaneous doses of NRS or ALS showed elevated K values (Table 6.7), the highest occurring in ALS-treated animals which showed the greatest increase in liver and spleen mass. Phagocytosis was unaffected by ALG treatment. Due to the considerable degree of hepatosplenomegaly exhibited by all serum- and globulin-treated animals, the corrected phagocytic indices (which express phagocytic activity per unit of tissue)

TABLE 6.5 HAEMATOCRITS OF MICE GIVEN UNABSORBED ANTILYMPHOCYTE SERUM[a] (ALS) AND ALS ABSORBED WITH ERYTHROCYTES.

Group	No. of doses	Haematocrits (Means ± S.E.M.)
Untreated control	-	44.0 ± 1.1
ALS I unabsorbed	4	31.0 ± 0.5
ALS I absorbed	4	39.5 ± 0.4
ALS IV unabsorbed	5	36.8 ± 0.9
ALS IV absorbed	5	43.8 ± 1.0
ALS 23A unabsorbed	2	16.7 ± 1.6
ALS 23A absorbed	2	41.9 ± 0.7

[a] Each 0.5 ml dose of ALS was given on alternate days and the animals were examined 2 days after the last dose.

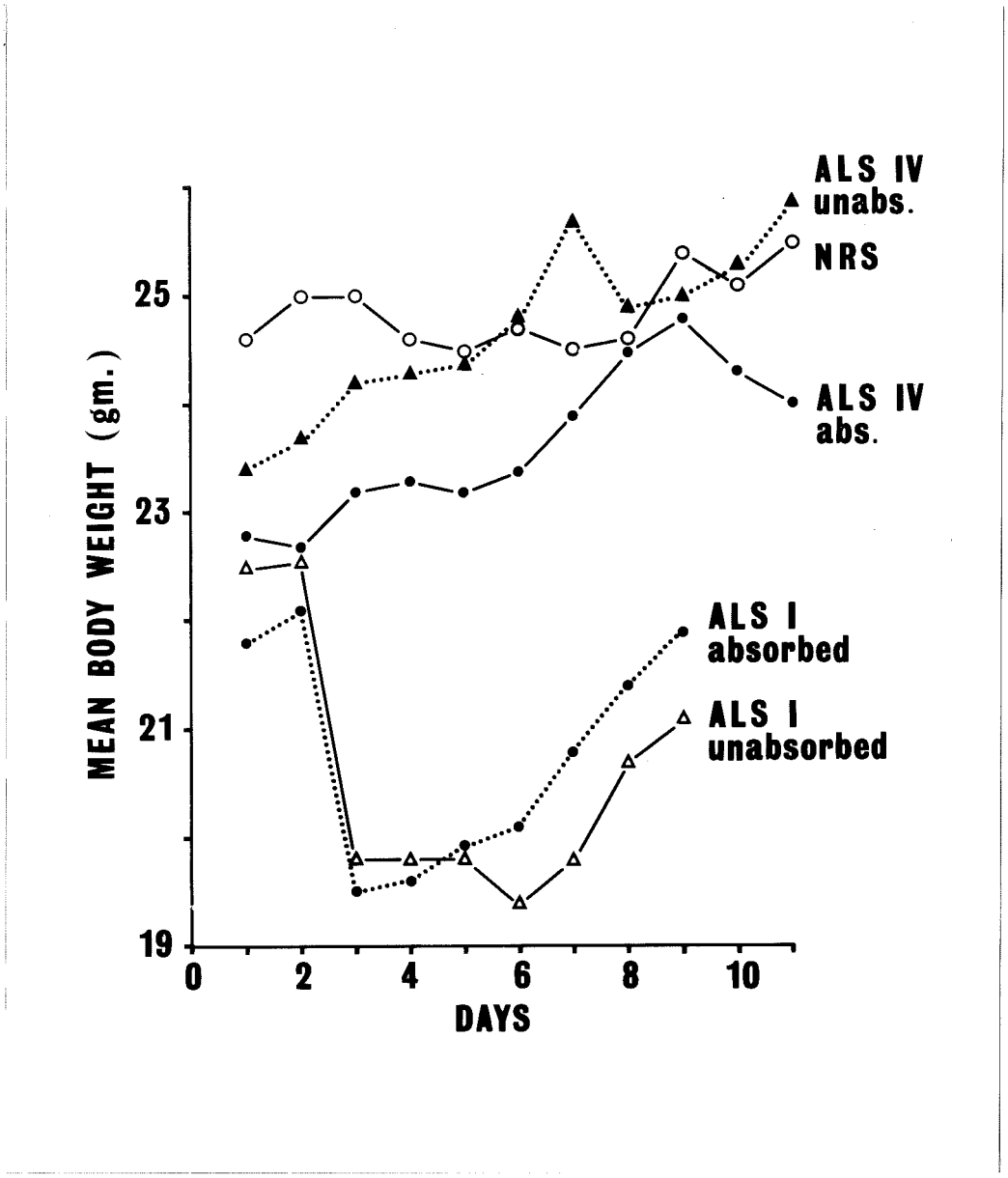

Fig. 6.12. Mean body weights of groups of mice given multiple subcutaneous injections of normal rabbit serum (NRS) or antilymphocyte serum (ALS). Four or 5 doses (each of 0.5 ml) of NRS or ALS were administered over an 8 to 10 day period, each dose being given on alternate days.

TABLE 6.6. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 1 OR 2 SUBCUTANEOUS DOSES[b] OF NORMAL RABBIT OR ANTILYMPHOCYTE SERA (MEANS ± S.E.M.)

Group	No. of doses	% Change in Total Body Weight (T.B.W.) at end of injection regime	Liver weight as % of T.B.W.[c]	Spleen weight as % of T.B.W.[c]	Lymph node weights as % of T.B.W.[c]	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	-	-	5.19 ±.07	0.50 ±.02	.120 ± .005	.028 ± .001	5.37± .14
NRS	1	+1.3	5.04 ±.06	0.42 ±.02**	.150 ± .007***	.015 ± .001***	4.51± .05**
ALS 23A (Unabs.)	1	-4.0	4.97 ±.04	0.79 ±.02***	.137 ± .026	.015 ± .003**	4.09± .34*
ALS 23A (Unabs.)	2	-11.6	4.72 ±.27	1.39 ±.16**	.117 ± .011	.012 ± .003**	3.23± .36**
ALS 23A (Absorbed)	2	-1.4	5.31 ±.21	0.73 ±.02***	.295 ± .012	.018 ± .002**	4.31± .21**
ALG	1	-1.8	5.54 ±.28	0.79 ±.06**	.241 ± .009***	.031 ± .002	4.91± .24

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Animals given single doses were sacrificed one day later; 2 doses of ALS were each given on alternate days and the animals were sacrificed 2 days after the second dose.

[c] Because of body weight loss, organ weights have been expressed as a % of each animal's corrected body weight (see Materials and Methods section).

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (test group vs untreated control group).

TABLE 6.7. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 4 OR 5 SUBCUTANEOUS DOSES[b] OF NORMAL RABBIT OR ANTILYMPHOCYTE SERA (MEANS ± S.E.M.)

Group	No. of doses	% Change in Total Body Weight (T.B.W.) at end of injection regime	Liver weight as % of T.B.W.	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	-	-	5.33 ± .08	0.56 ± .02	.137± .009	.027 ±.001	5.13± .04
Saline control	5	+6.4	5.50 ± .05	0.53 ± .03	.130± .004	.028 ±.001	5.07± .07
NRS	5	+3.7	6.45 ± .30*	1.27 ± .07***	.480± .006***	.037 ±.001**	4.33± .14**
ALS I (Unabs.)	4	-6.2	6.96 ± .17***	2.58 ± .09***	.200± .042	.041 ±.002**	3.61± .08***
ALS I (Abs.)	4	+0.4	8.29 ± .35***	2.30 ± .15***	.265± .023***	.050 ±.002***	3.49± .09***
ALS IV (Unabs.)	5	+10.0	6.10 ± .08***	1.07 ± .13**	.480± .070**	.034 ±.002	4.51± .12**
ALS IV (Abs.)	5	+5.3	6.66 ± .13***	1.30 ± .05***	.457± .046***	.038 ±.001**	4.22± .09***
ALG	4	+1.0	6.05 ± .15*	1.04 ± .08***	.380± .041**	.030 ±.004	4.38± .16***

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Doses of ALS and NRS were given on alternate days and the animals were sacrificed 2 days after the last injection.

* p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001 (test group vs. saline control group).

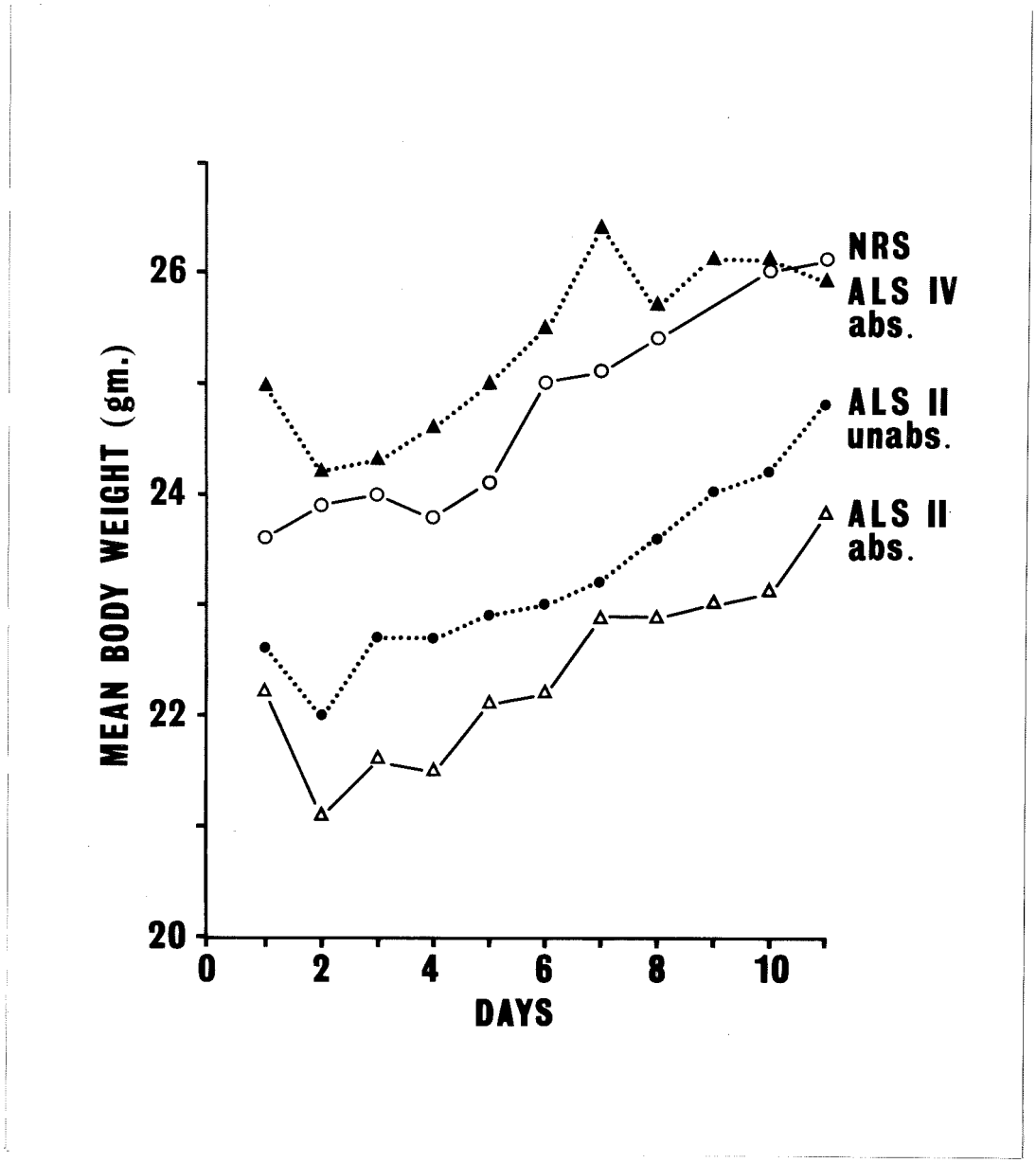

Fig. 6.13. Mean body weights of groups of mice given multiple intraperitoneal injections of normal rabbit serum (NRS) or antilymphocyte serum (ALS). Five doses (each of 0.5 ml) of NRS or ALS were administered over a 10 day period, each dose being given on alternate days.

TABLE 6.8. LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF BALB/C MICE GIVEN 5 INTRA-PERITONEAL DOSES[b] OF NORMAL RABBIT OR ANTILYMPHOCYTE SERA (MEANS ± S.E.M.)

Group	% Change in Total Body Weight (T.B.W.) at end of injection regime	Liver weight as % of T.B.W.	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
Untreated control	-	5.07 ± .06	0.54 ± .02	.112 ± .014	.026 ± .001	5.32 ± .10
Saline control	+4.8	5.29 ± .05	0.57 ± .02	.135 ± .009	.028 ± .001	5.20 ± .12
NRS	+10.6	5.99 ± .27*	0.95 ± .04***	.132 ± .010	.020 ± .003	3.87 ± .34**
ALS II (Unabs.)	+9.7	6.75 ± .13***	1.71 ± .13**	.245 ± .016***	.041 ± .002**	4.09 ± .09***
ALS II (Absorbed)	+7.2	6.44 ± .13***	0.86 ± .02***	.255 ± .025**	.029 ± .001	4.22 ± .08***
ALS IV (Absorbed)	+3.6	5.75 ± .09**	0.64 ± .08	.157 ± .018	.030 ± .002	4.89 ± .17

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed.

[b] Doses of ALS and NRS were given on alternate days and the animals were sacrificed 2 days after the last injection.

* p 0.01 - 0.05; ** p 0.001 - 0.01 *** p < 0.001 (test group vs saline control group).

showed a significant decrease.

Intraperitoneal administration of NRS or ALS did not affect phagocytosis as markedly as did the subcutaneous route, and only one ALS group showed a significantly elevated K value (Table 6.8).

Changes in liver mass following treatment with ALS, ALG and NRS.

Mice which received 1 or 2 subcutaneous doses of ALS, ALG or NRS showed no alteration in liver mass when examined 1-2 days later (Table 6.6). Four or 5 doses of ALS administered either subcutaneously or intraperitoneally brought about marked hepatomegaly (Tables 6.7 and 6.8); similar regimes of NRS and ALG also caused liver enlargement, although this response was not as marked as that obtained with some batches of ALS.

Morphologic changes in livers of mice given ALS and NRS as seen under light microscopy.

Liver parenchymal cells from ALS-treated mice showed considerable nuclear and cytoplasmic enlargement, while similar, but less marked, changes were observed in hepatocytes following NRS treatment (Fig. 6.14). Areas of extramedullary haematopoiesis were frequently present in livers from ALS- but not NRS-treated mice. An increase in the relative number of carbon-containing Kupffer cells was apparent when unstained liver sections from some mice given ALS were compared with those from NRS-treated and untreated controls (Fig. 6.14). However, this response did not occur with all batches of ALS.

Alterations in the fine structure of liver cells following administration of ALS and NRS to mice.

Electron microscopic examination of liver tissue from ALS-treated mice revealed normal size and cytoplasmic content of Kupffer cells. Many

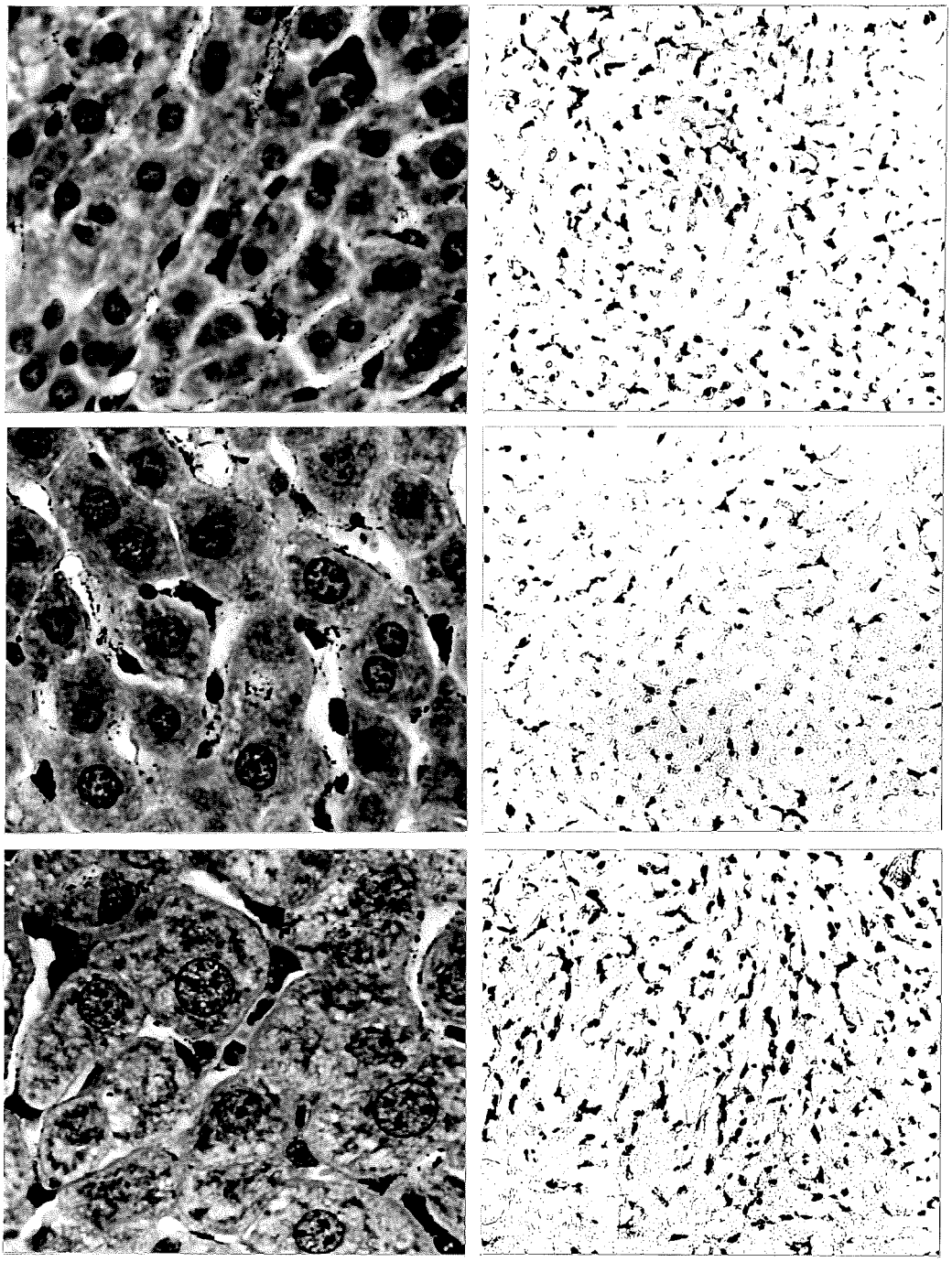

Fig. 6.14. Haematoxylin and eosin stained and unstained liver sections of an untreated control mouse (top) and mice given 5 doses of normal rabbit serum (middle) and antilymphocyte serum (bottom). Hepatocytes from NRS- and ALS-treated mice are considerably enlarged. (The control liver represented 5.07% of the animal's total body weight while livers from NRS- and ALS-treated mice were 6.45% and 8.49% respectively). When differences in the relative size of hepatocytes in the 3 mice are taken into account, the relative number of carbon-containing Kupffer cells in the ALS-treated liver is much greater than in the NRS and untreated control livers. All H and E sections X400; all unstained sections X100.

hepatocytes, on the other hand, were enlarged. Nuclear size was increased, and although the relative numbers and distribution of mitochondria and glycogen granules were normal, the amount of rough-surfaced endoplasmic reticulum (RER) was greatly increased (Fig. 6.15). Rows of RER were densely packed between the mitochondria and frequently could be traced almost around the entire mitochondrial membranes (Fig. 6.15, inset). Hepatocytes from NRS-treated animals also showed RER proliferation, but to a lesser extent than that found with ALS. Lipid droplets and glycogen granules were more abundant in livers from NRS-treated mice than in those from ALS-treated and untreated controls.

Biochemical changes in livers of mice given ALS, ALG and NRS

The enlarged livers from mice given 5 doses of NRS, ALS or ALG showed normal percentage composition of water, protein and DNA when analysed 2 days after the last dose. RNA concentration was significantly increased ($p < 0.001$) in all 3 groups over the untreated controls, the highest values occurring in those which received ALS and ALG.

Changes in spleen mass and morphology following subcutaneous administration of NRS, ALS and ALG.

(a) Effect of NRS.

Although spleen mass was significantly reduced 1 day after a single dose of NRS (Table 6.6), no histological abnormality could be detected. Spleens from animals given 5 doses of NRS showed a 2-fold enlargement (Table 6.7). Histologically, the lymphatic nodules were considerably enlarged, while haematopoiesis was unaffected (Fig. 6.17) (compare with normal spleen shown in Fig. 6.16).

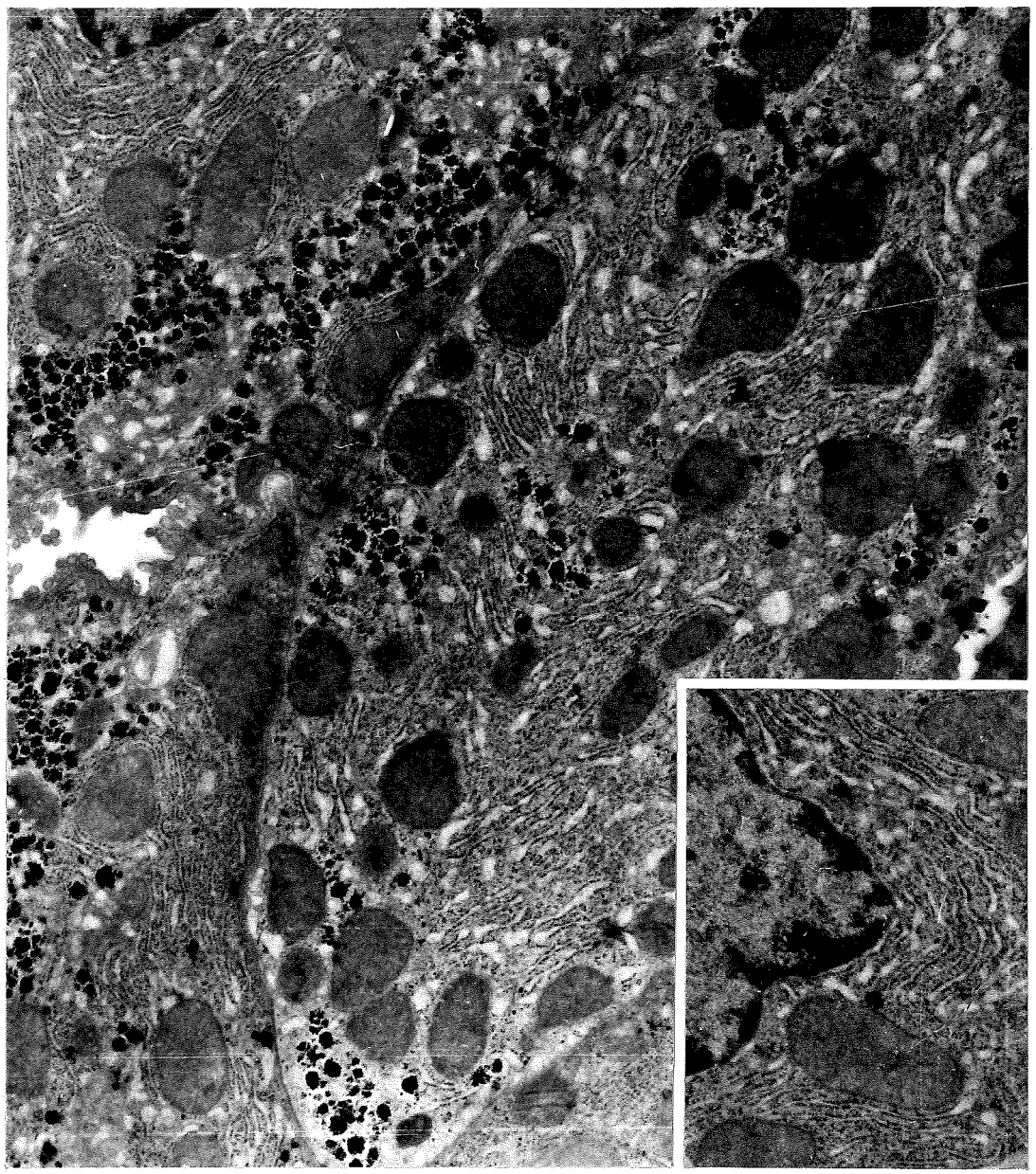

Fig. 6.15. Electron micrograph showing portions of 3 hepatocytes in an enlarged liver of a mouse given 5 doses of ALS. Proliferation of the rough-surfaced endoplasmic reticulum has occurred and in many areas the ribosomal strands are in intimate contact with the mitochondrial membranes (see inset). X10,500 and X16,500 (inset).

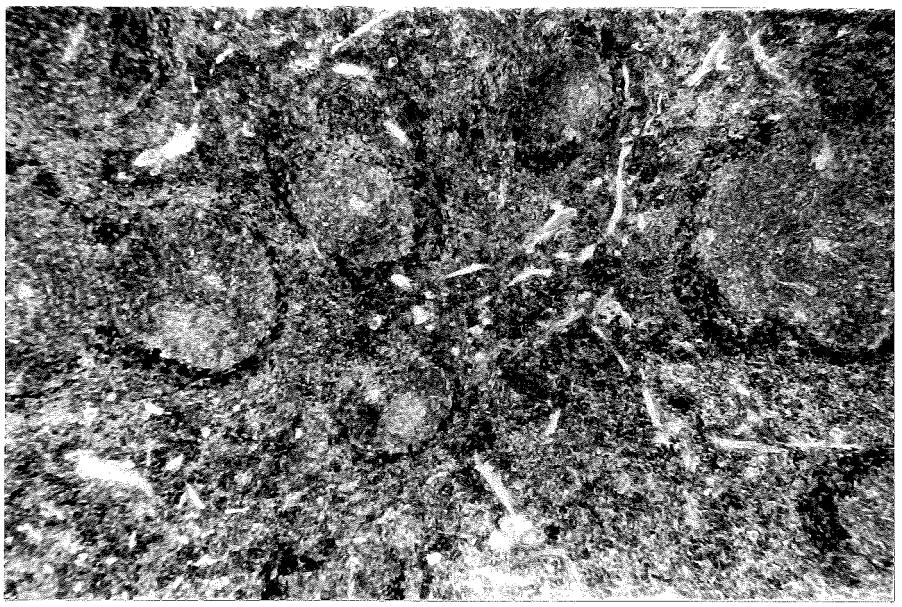

Fig. 6.16. Spleen of a control Balb/c mouse injected intravenously with colloidal carbon and sacrificed 15 minutes later. The carbon particles have localized in the marginal zones. H and E X 35.

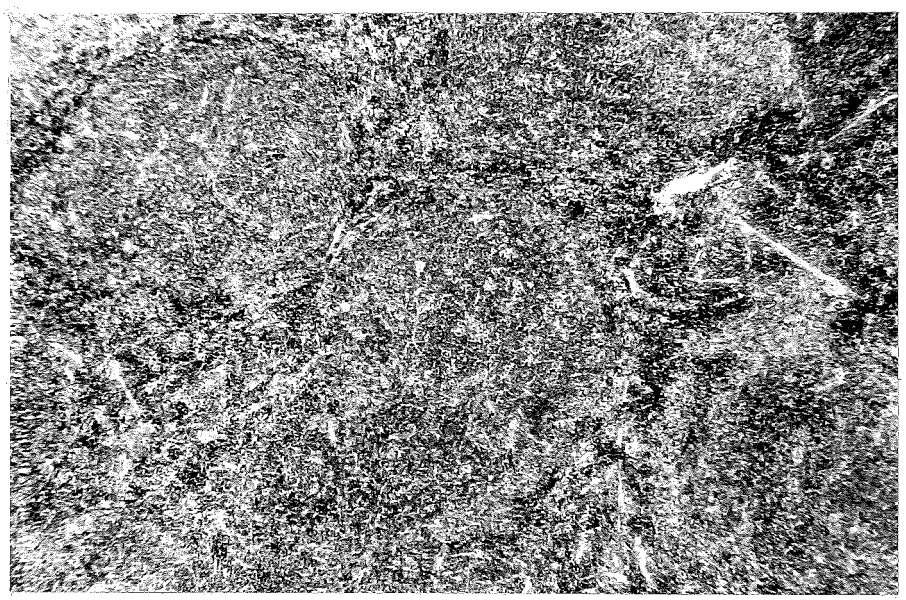

Fig. 6.17. Spleen of a mouse given 5 doses of normal rabbit serum over a 10-day period. The lymphatic nodules are greatly enlarged. Intravenously administered carbon particles have not localized in the perinodular regions. H and E X35.

(b) Effect of unabsorbed ALS

One dose of unabsorbed ALS significantly increased spleen size (Table 6.6). Microscopically, a reduction in the size of the lymphatic nodules was apparent and very little injected carbon could be seen localized in the marginal zones (Fig. 6.18). Large masses of sequestered erythrocytes were scattered throughout the red pulp and were obviously responsible for the increased spleen weight.

Spleens examined 2 days after a 2nd dose of unabsorbed ALS were enlarged 2-3 fold (Table 6.6). These showed a marked reduction in the size and also the relative number of lymphatic nodules, sequestered erythrocytes around the nodules and a diminished uptake of carbon (Fig. 6.19). The most striking histological feature, however, was the large number of foci of haematopoiesis (predominantly erythropoietic) which occupied the greater part of the spleen.

Up to a 4-fold increase in spleen mass resulted from the administration of 4 or 5 doses of unabsorbed ALS (Table 6.7). The few lymphatic nodules which were evident in histological sections were considerably reduced in size and exhibited marked cellular depletion around their central arterioles (Fig. 6.20). Large areas of haematopoiesis (erythropoietic and granulocytic) containing many megakaryocytes extended throughout the red pulp and occupied by far the greater part of the spleen (Figs. 6.20 and 6.21). Very small amounts of carbon localized in these spleens.

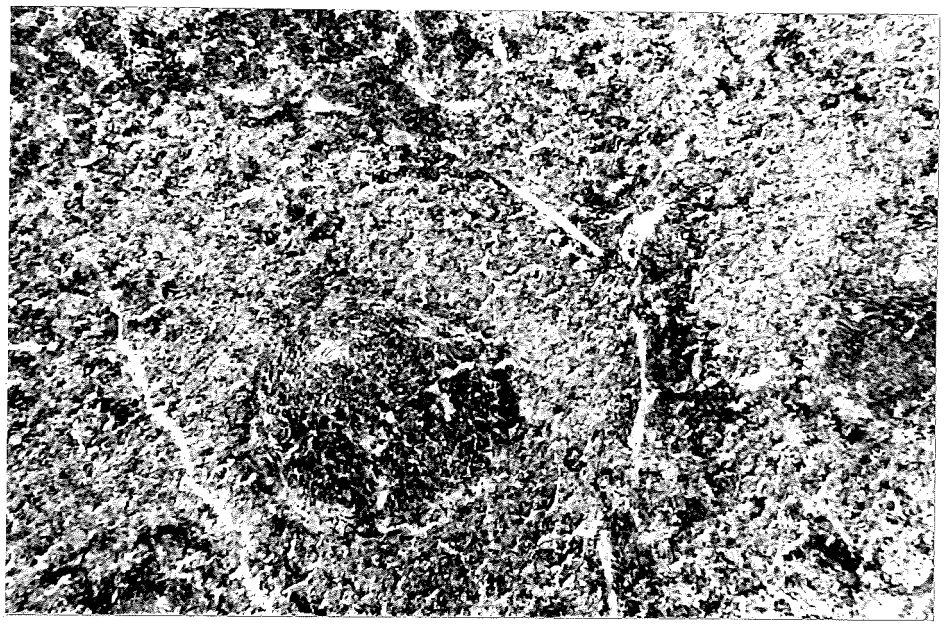

Fig. 6.18. Spleen of a mouse sacrificed 1 day after receiving a single dose of unabsorbed ALS. The lymphatic nodules are reduced in size and the red pulp contains large masses of sequestered erythrocytes. H and E X53.

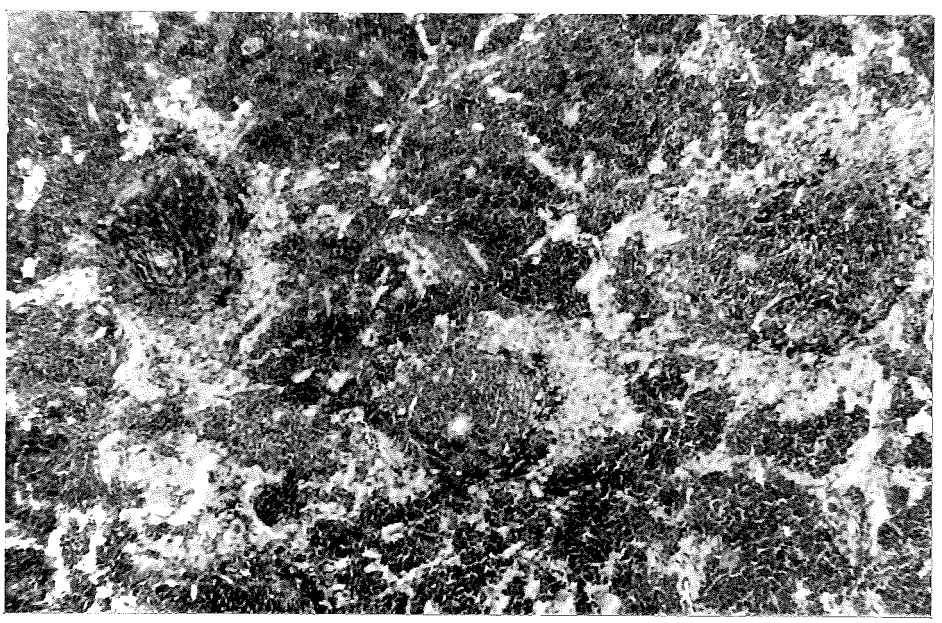

Fig. 6.19. Spleen of a mouse sacrificed 2 days after receiving a second dose of unabsorbed ALS. Some sequestered erythrocytes still surround the lymphatic nodules, but the most striking feature is the intense erythropoiesis in the red pulp. H and E X53.

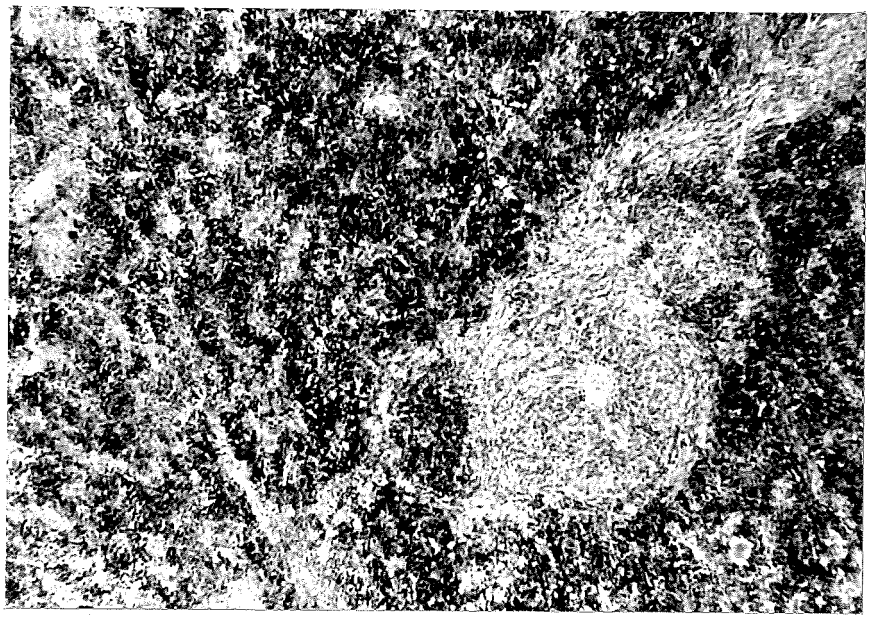

Fig. 6.20. Spleen of a mouse given 4 doses of unabsorbed ALS (each dose on alternate days) and killed 2 days after the last dose. The one lymphatic nodule visible in this field shows marked depletion of small lymphocytes around the central arteriole. The red pulp shows intense haematopoiesis (erythropoietic and granulocytic). H and E X53.

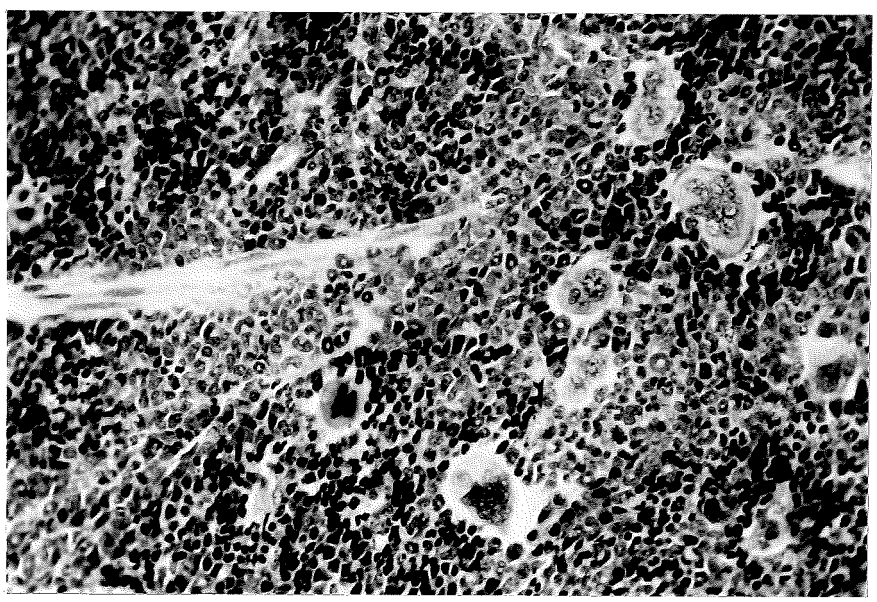

Fig. 6.21. Haematopoiesis in the red pulp of the spleen shown in Fig. 6.20. The relatively pale-staining granulocytes lie alongside a fibrous trabecula, while clusters of erythroid precursor cells with small, darkly staining nuclei surround multinucleated megakaryocytes. H and E X175.

(c) Effect of absorbed ALS

Significant splenomegaly followed the administration of 2 doses of ALS which had been absorbed with mouse erythrocytes prior to being injected (Table 6.6). No evidence of red cell sequestration was apparent histologically, the main feature being pronounced haematopoiesis (granulocytic and erythropoietic) and lymphoid depletion in the periarteriolar regions and marginal zones (Fig. 6.22). Identical changes in the spleens of Balb/c mice have been observed to follow 2 doses of ALS which had been absorbed with bone marrow cells as well as erythrocytes (GILL, P.G. and GOTJAMANOS, T., unpublished data).

The gross changes in the spleen which resulted from treatment with 4 or 5 doses of ALS absorbed with red cells were similar to those observed following multiple injections of unabsorbed ALS (Table 6.7). Loss of marginal zone cells followed the administration of absorbed ALS (Fig. 6.23) and the red pulp showed abundant granulopoietic activity and many erythroid precursor cells (Fig. 6.24).

(d) Effect of ALG

Significant splenic enlargement was evident 1 day after a single injection of ALG (Table 6.6). No decrease in the size of the lymphatic nodules was apparent, although several showed evidence of small lymphocyte depletion from the periarteriolar regions (Fig. 6.25). Carbon localization in the marginal zones was normal, and many new collections of granulocytes could be seen forming throughout the red pulp. Administration of 4 doses of ALG gave rise to a 2-fold enlargement of spleen size (Table 6.7). These spleens showed histological features identical with those observed following multiple injections of absorbed ALS (Fig. 6.26).

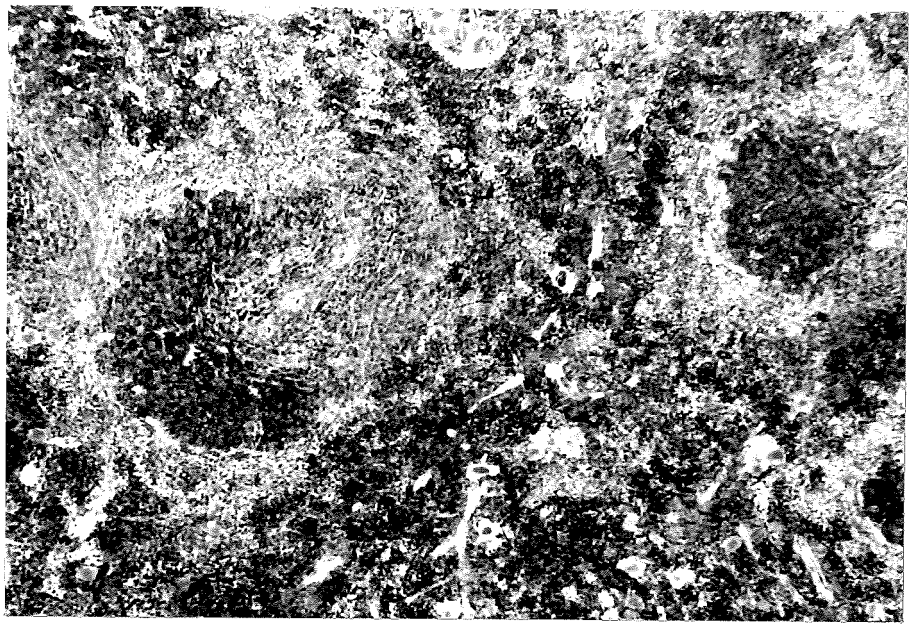

Fig. 6.22. Spleen of a mouse killed 2 days after receiving a second dose of ALS which had been absorbed with mouse erythrocytes before being injected. The lymphatic nodules show marked cellular depletion in the periarteriolar regions. The marginal zones also show reduced cell numbers. Numerous myeloid and erythroid precursor cells and megakaryocytes are evident in the red pulp. H and E X53.

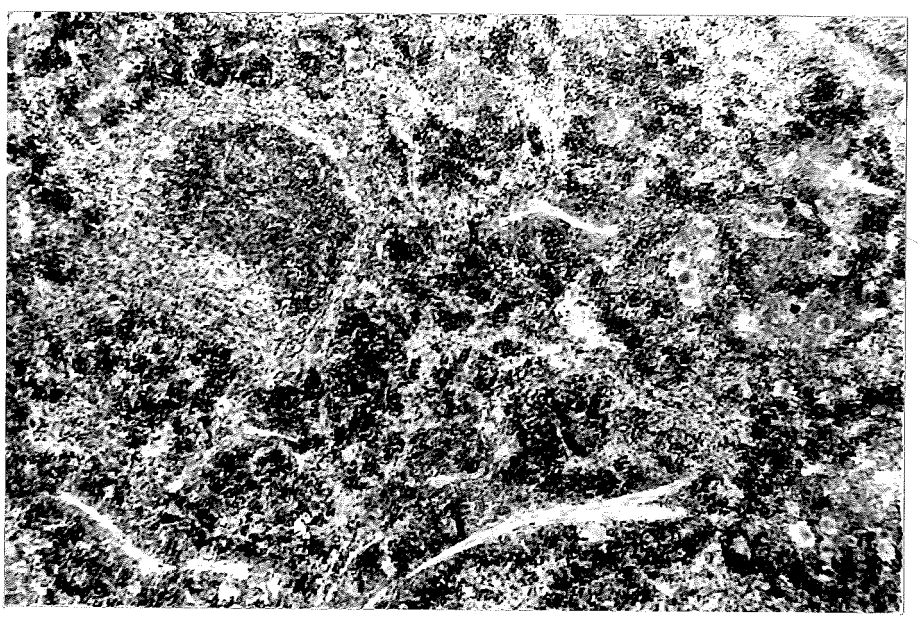

Fig. 6.23. Spleen of a mouse given 4 doses of absorbed ALS (each dose on alternate days) and killed 2 days after the last dose. The appearance is similar to that shown in Fig. 6.22, although extramedullary haematopoiesis is more intense. Carbon particles are not evident in the cell-depleted marginal zone. H and E X53.

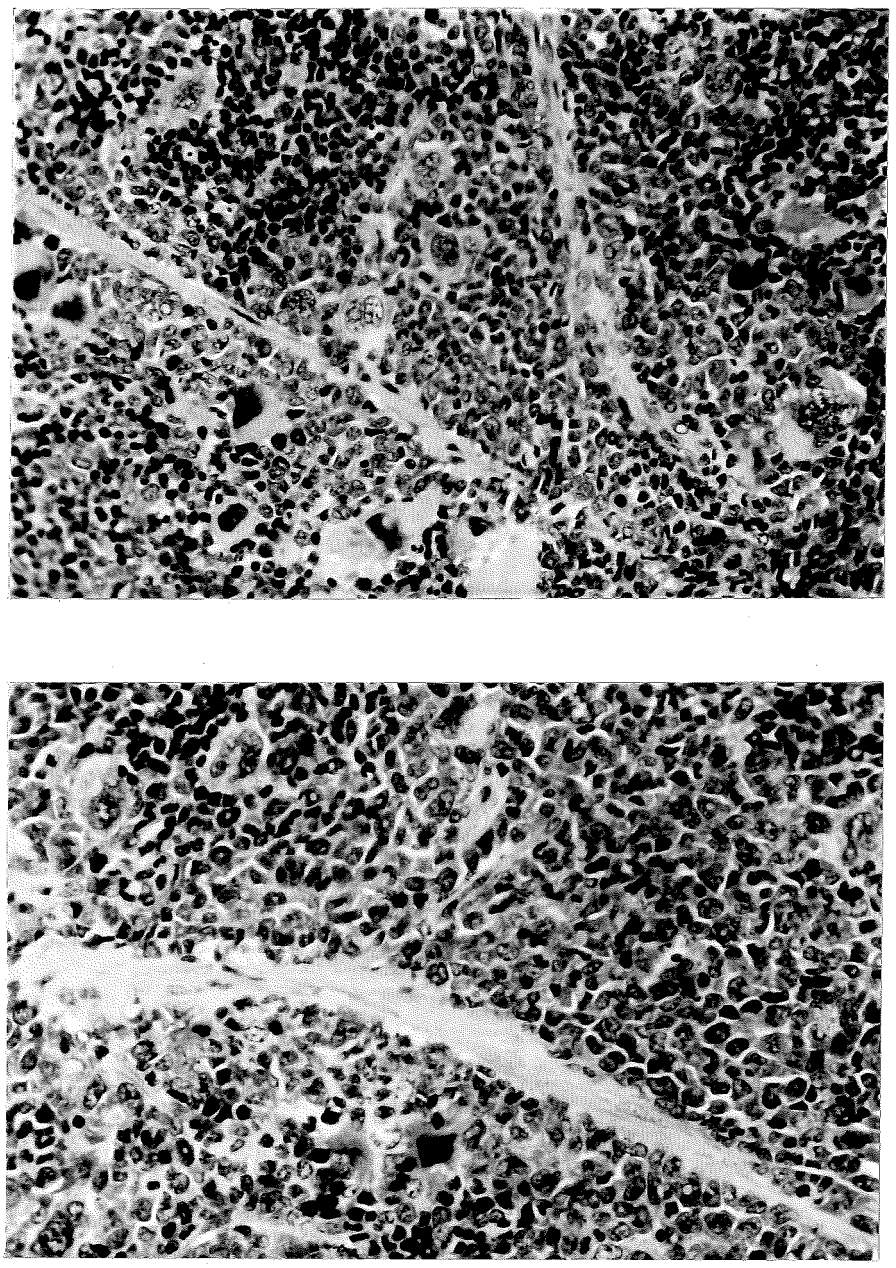

Fig. 6.24. Two regions of the red pulp of the spleen shown in Fig. 6.23. Intense granulopoiesis is evident around the fibrous trabeculae. Erythrocyte precursor cells with darkly staining nuclei are also present. Both sections stained with H and E. Top: X 175; bottom: X 250.

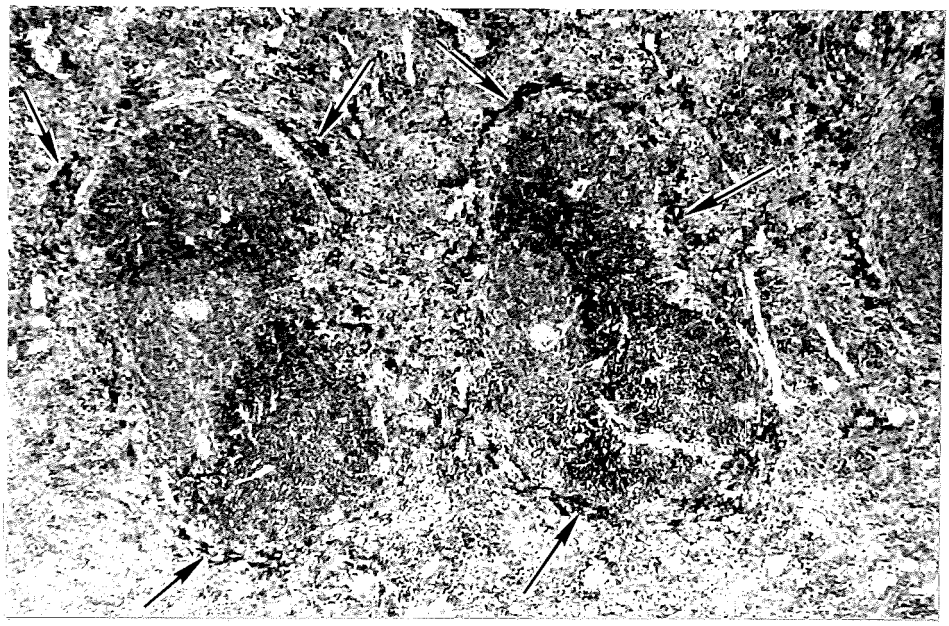

Fig. 6.25. Spleen of a mouse killed 1 day after receiving a single dose of antilymphocyte globulin (ALG). The two lymphatic nodules shown in this field exhibit depletion of small lymphocytes from the periarteriolar regions. Localization of intravenously-injected carbon particles (arrowed) in the marginal zones has not been affected. H and E X53.

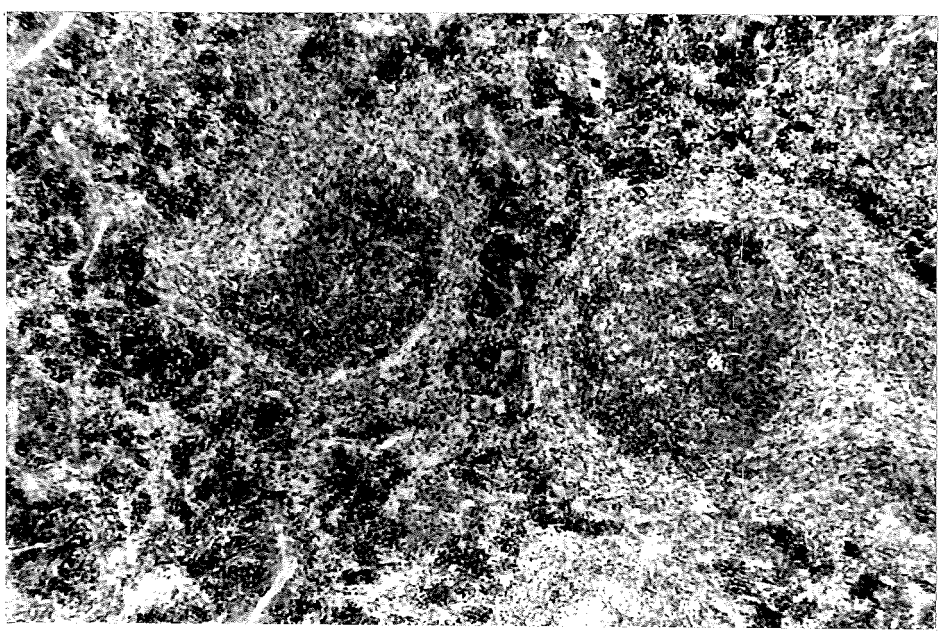

Fig. 6.26. Spleen of a mouse given 5 doses of ALG (each dose on alternate days) and killed 2 days after the last dose. The lymphatic nodules show cellular depletion in the periarteriolar regions. The marginal zones also exhibit reduced cellularity and do not contain any carbon particles. The red pulp is filled with myeloid and erythroid precursor cells. H and E X53.

Changes in lymph node mass and morphology following subcutaneous administration of NRS, ALS and ALG.

Enlargement of lymph nodes followed the administration of single subcutaneous doses of NRS and ALG (Table 6.6) and 2 to 5 doses of ALS given either subcutaneously or intraperitoneally (Tables 6.6, 6.7 and 6.8). Histological examination of NRS- and ALS-treated nodes revealed enlargement of the lymphatic nodules, some of which showed germinal centre formation with evidence of considerable mitosis and the presence of numerous tingible bodies. Granulopoiesis in the medulla was a prominent feature of lymph nodes following treatment with unabsorbed and absorbed ALS but not with NRS. Depletion of small lymphocytes from the paracortical areas was not evident following ALS treatment, although any actual depletion in these regions may have been obscured by the considerable degree of haematopoiesis occuring in the medulla. Lymph nodes from mice given 5 doses of ALG showed quite marked paracortical depletion of small lymphocytes. Widespread necrosis within lymph nodes was a rare finding and occurred in only one node following administration of 5 subcutaneous doses of absorbed ALS.

DISCUSSION

The results of the blood clearance studies reported in this Chapter have shown that ALS can either depress or stimulate the rate at which fixed macrophages remove carbon particles from the circulation, depending on the regime of antiserum administration. The depressed rate of carbon clearance which followed 1 and 2 doses of ALS is probably due largely to a limited availability of serum opsonins which are essential for the phagocytosis of carbon

particles (JENKIN and ROWLEY, 1961; NORMANN and BENDITT, 1965; FILKINS and DILUZIO, 1966). The necessity for phagocytic cells to dispose of large amounts of heterologous protein and damaged cells would result in a depletion of opsonins and thereby limit temporarily the capacity of macrophages to carry out additional phagocytosis of carbon particles. This explanation derives support from the observation that phagocytosis was also depressed following a single dose of NRS. Somewhat similar results with normal serum and ALS have been reported by SHEAGREN et al. (1969). In addition, GROGAN (1969) has provided indirect evidence showing that opsonic depletion contributes to the depressed rate of carbon clearance which results from a single dose of ALS. By administering normal, non-heated serum just prior to carbon injection, he was able to almost completely reverse the inhibitory effect. A further significant feature of the present study is that the liver showed no change in mass during the period of phagocytic depression induced by 1 or 2 doses of ALS. Although the spleen was significantly enlarged, the amount of carbon taken up in this case was less than that found in normal-sized spleens from NRS-treated and untreated control animals.

The phagocytic stimulation which resulted from 4 or 5 doses of ALS was accompanied by hepatomegaly which was very marked with some batches of ALS. An increased number of active Kupffer cells was evident in some cases only and cannot account for the consistent elevation in the phagocytic index exhibited by all groups of mice given multiple subcutaneous doses of ALS. The most likely explanation for this effect can be found by reference to the work of BENACERRAF et al., (1955) who showed that the rate of carbon uptake by

Kupffer cells was conditioned by the size and the amount of blood flowing through the liver. An increase in liver blood flow increased the rate of phagocytosis, an effect attributed largely to an increase in the number of carbon particles coming into contact with Kupffer cells per unit of time. Since an increase in blood flow through an hypertrophied liver would occur in response to the increased metabolic requirements of the enlarged hepatocytes, it is reasonable to conclude that the increased rate of phagocytosis observed in ALS-treated mice was due mainly to a raised liver blood flow. The fact that hepatomegaly and an increased rate of phagocytosis also occurred with multiple doses of NRS is consistent with this suggestion. An increased rate of phagocytosis following multiple injections of NRS and ALS has also been found to occur in rats by GROGAN (1969). While no alteration in liver mass was apparent in his study, it is possible that increased numbers of Kupffer cells contributed to the increased phagocytic activity, although histologic studies to verify this point were not performed.

Several factors may be responsible for the marked increase in liver mass brought about by administering ALS to mice. Hepatomegaly has been observed to develop during states of haemolytic anaemia induced by methylcellulose in dogs (HUEPPER, 1942), by zymosan in mice (GORSTEIN and BENACERRAF, 1960) and by phenyl-hydrazine in rats (JANDL et al., 1965). While this may explain the increased liver mass which followed treatment with unabsorbed ALS, it cannot account for the fact that absorbed ALS and ALG (which did not cause anaemia) also induced hepatomegaly. The evidence obtained from electron microscopic and biochemical studies carried out on the

enlarged livers points to an intense synthesis of RNA by hepatocytes. As previously referred to in Chapter 4, RNA is involved in the synthesis of purines by the liver which acts as the main source of purine supply for bone marrow cells, and possibly for the cells of most peripheral tissues (LAJTHA and VANE, 1958). It is possible that the hepatocyte hypertrophy induced by ALS is a response which provides increased amounts of purines to maintain the intense cellular proliferation occurring in lymphoid tissues. Considerable enlargement of the spleen and lymph nodes consistently followed ALS administration. Furthermore, hepatomegaly, splenomegaly and lymph node enlargement also resulted from NRS treatment. Although hepatosplenomegaly is a characteristic feature of the human granulocytic leukaemias (DAMASHEK and GUNZ, 1964), and ALS has the ability to potentiate virus oncogenesis in mice (ALLISON, 1970; LAW, 1970) including leukaemia induced by murine sarcoma virus (LAW, TING and ALLISON, 1968), the ALS-treated Balb/c mice examined in the present study did not exhibit the features of myeloid leukaemia described for mice by BARNES and SISMAN (1939).

The lymphoid depletion in the spleen and lymph nodes observed in the present study is consistent with previous findings in mice and rats receiving ALS (WOODRUFF and ANDERSON, 1964; RUSSE and CROWLE, 1965; GRAY et al., 1966; MONACO et al., 1966; PICHLMAYR et al., 1968; LANCE, 1968; TAUB and LANCE, 1968; BARTH et al., 1969). The selective depletion of small lymphocytes from the paracortical areas of lymph nodes and the periarteriolar regions of the spleen (thymus-dependent areas) is also in agreement with previous reports (PARROTT, 1967; TURK and WILLOUGHBY, 1967;

LANCE, 1968; TAUB and LANCE, 1968; BARTH et al., 1969). Cellular depletion within the marginal zones of the spleen previously reported by PICHLMAYR et al., (1968) and BARTH et al., (1969) was also noted in the present study. It is possible that the marginal zone which is the site of initial localization of antigen in the spleen (HUNTER, 1966; NOSSAL et al., 1966; WILLIAMS and NOSSAL, 1966), may also represent a thymus-dependent area.

The most striking histological alteration in the splenic red pulp observed in the present study was the enormous increase in haematopoiesis which followed treatment with ALS. A similar observation was made by TAUB and LANCE (1968), who suggested that this response was a consequence of anaemia related to haemolysis and bone marrow depression or the result of an immune reaction to the heterologous globulin. In the present study, however, the pronounced haematopoiesis in the spleen was not diminished by absorbing the ALS with mouse erythrocytes and furthermore, it occurred in animals which were not anaemic. In addition, serum immunoelectrophoresis performed on animals given multiple doses of ALS or NRS failed to reveal antibody against the rabbit globulin (GILL, P. G., personal communication). These findings indicate that the red pulp changes have a greater significance that simply a response to anaemia or heterologous protein. It is possible that the intense proliferation of myeloid cells observed in the present investigation occurred in response to the rapid elimination of peripheral lymphocytes by ALS. It has been suggested that ALS eliminates a sub-population of lymphocytes (MARTIN and MILLER, 1967; 1968; LEUCHARS, WALLIS and DAVIES, 1968; MOLLER and ZUKOSKI, 1968), and results in a proliferation of lymphoid cells of different

lineages (MARTIN and MILLER, 1967; 1968), a concept supported by DENMAN, DENMAN and EMBLING (1968) who demonstrated a pronounced alteration in the life span of peripheral blood lymphocytes in ALS-treated mice. The importance of myeloid cells as precursors of lymphoid cells in the mouse has been shown by FORD (1966), and it seems reasonable to propose that the pronounced red pulp myelopoiesis may largely represent the source of the new lymphocyte population in ALS-treated mice.

An increased rate of phagocytosis accompanied by an enlargement of reticuloendothelial organs, hepatomegaly associated in some cases with an increased number of Kupffer cells, and splenomegaly characterized by an enormous increase in extramedullary haematopoiesis and lymphoid cell depletion are changes that have been found by MILLER and HOWARD (1964) to occur in mice thymectomized at birth. The results of the present study thus tend to support the concept advanced by RUSSE and CROWLE (1965) that ALS has the ability to confer a state of "immunothymectomy" when administered to adult animals. The rapid onset of these changes following ALS treatment is probably due to the rapid and efficient elimination of thymus-derived cells in the periphery, as opposed to the gradual depletion following thymectomy which occurs as existing cells exhaust their life span. It would appear therefore, that the alterations in phagocytic function and morphology of reticuloendothelial organs in mice induced by ALS are largely a consequence of the principal mode of action of ALS.

SUMMARY

Rabbit anti-mouse antilymphocyte sera (ALS) and antilymphocyte globulin preparations were studied for their effects on the phagocytic activity and morphology of reticuloendothelial organs. One and 2 doses of ALS given to mice depressed the rate of carbon clearance from the circulation; multiple doses of ALS stimulated the rate of carbon clearance and induced marked hepatosplenomegaly. The increased liver mass in serum-treated mice was due mainly to hepatocyte hypertrophy, although some ALS-treated mice also exhibited an increase in the relative numbers of Kupffer cells. Splenic enlargement followed administration of unabsorbed ALS as well as ALS that had been absorbed with mouse erythrocytes. Histologically, these enlarged spleens showed marked lymphoid cell depletion and intense extramedullary haematopoiesis. The changes in phagocytic activity and morphology of reticuloendothelial organs observed in adult mice used in the present investigation closely parallel those which have been reported to occur in neonatally thymectomized mice. The results obtained thus tend to support the concept that ALS can confer a state of "immunothymectomy" when administered to adult animals.

CHAPTER 7

GENERAL DISCUSSION

The investigations described in Chapter 4 on the effect of skin transplantation on the RES of Balb/c mice were prompted by the findings of BRENT and MEDAWAR (1962) and MEDAWAR (1963) that the survival time of skin allografts could be prolonged if A-strain mice were given trypan blue at doses sufficient to bring about RES "blockade". The object of studying the phagocytic activity of the RES in mice bearing skin allografts was to determine what effect(s), if any, the allografts themselves had on the RES. The data obtained from blood clearance experiments on Balb/c mice do not permit a simple answer to this question. Any such answer must be qualified with information regarding the test particle used to assess phagocytic function, the area of skin transplanted, and the time after grafting at which RES activity was assessed. A close comparison between the changes observed in isografted and allografted mice reveals that the problem under investigation is complicated by the effects produced by the surgical technique of skin grafting. Significant alterations occurred in the weight and morphology of RE organs in grafted mice, although these effects were more pronounced in mice with allografts than in their isografted counterparts. Mice bearing 4 cm^2 and 8 cm^2 allografts and those with 8 cm^2 isografts developed hepatomegaly. Since the enlargement was due to hepatocyte hypertrophy, it seems likely that blood flow through the enlarged livers would have increased in order to support the increased metabolic requirements of the hypertrophied parenchymal cells. On the basis of the findings of BENACERRAF et al. (1955) regarding the influence of liver blood flow on the rate of phagocytosis

of carbon particles, it might be expected that the grafted mice with enlarged livers would have exhibited significantly *increased* rates of phagocytosis towards carbon particles. The fact that their phagocytic indices were *not* significantly raised strengthens the idea advanced in Chapter 4 that an inhibitory influence was operating on the RES of mice bearing medium-sized and massive grafts, and indirectly impaired their potential ability to rapidly remove carbon particles from the circulation. In view of the evidence available from the studies of SABA and DILUZIO (1969) and SABA (1970), it is likely that this inhibitory influence was a reduced availability of serum opsonins brought about by the sequelae of severe surgical trauma.

The results obtained from the carbon clearance experiments on mice bearing skin grafts differ from those reported for rats by FISHER and FISHER (1964). They found that adult male rats which received 5 cm x 7 cm allografts (corresponding to the medium-sized grafts used in the present study) showed significantly increased phagocytic indices just prior to rejection. At 7 days after transplantation, the mean K value for the unoperated control group was 0.011, for the autografted group it was 0.014, while the allografted group showed a mean K value of 0.026. Before attributing the discrepancy between their results and those of the present study to factors other than possible species differences, it is important to know first of all whether the allografted rats which exhibited phagocytic stimulation had enlarged livers. Unfortunately, this data was not provided. If considerable hepatomegaly does in fact develop in rats following allotransplantation of skin as it does in Balb/c mice, then the increased blood flow through the enlarged rat liver may be of such a magnitude to more than

compensate for any depletion of serum opsonins resulting from operative trauma, so that the *net* effect is an increase in the rate of phagocytosis of carbon by the liver macrophages.

Although allogeneic skin grafts produced only minimal alterations in the phagocytic activity of the RES in Balb/c mice, the transplantation of F_1 hybrid spleen cells markedly increased the rate at which the Kupffer cells of the recipient Balb/c mice phagocytosed carbon. The failure of splenectomized Balb/c mice injected with F_1 spleen cells to exhibit phagocytic stimulation indicates that the hyperphagocytic state found in normal Balb/c mice given F_1 cells is not an expression of a direct reaction against the transplanted cells. Most likely, it represents a response which is initiated *after* the F_1 cells have been destroyed in the spleen, and is probably mediated through the release of F_1 cell debris into the circulation. The general conclusion that can be drawn from the experiments on the transplantation of allogeneic skin and dissociated spleen cells (*i.e.*, situations which give rise to host-versus-graft reactions), is that the changes in phagocytic activity exhibited by the fixed macrophages of the liver and spleen have little or no relation to the immune reaction directed against the transplanted tissue.

Of much greater relevance to the immune reaction, however, is the development of germinal centres in the spleens of Balb/c mice following transplantation of CBA skin, and also following the intravenous administration of F_1 (Balb/c x C57 Black) and CBA spleen cells. In each of these situations, germinal centres first became prominent in the spleen a few days after allotransplantation, but it was not until several days *after* graft destruction that germinal centre

development reached maximum proportions. Although the existing knowledge on germinal centres is far from being complete, their development is generally regarded as signifying a particularly intense proliferative response to prolonged or repeated contact with antigen (COTTIER, HESS and STONER, 1967), and furthermore, this development is considered to be indicative of a secondary antibody response (GATTI, STUTMAN and GOOD, 1970; WHO Technical Report No. 448, 1970). Based on these premises, one interpretation of the histological changes previously described for the splenic white pulp of Balb/c mice bearing allogeneic grafts would be as follows. The transplantation of allogeneic skin or spleen cells to unsensitized Balb/c recipients evokes an early (weak?) primary humoral antibody response (very few plasma cells were seen in the spleens of allografted mice during the early stages of the reaction). As graft destruction proceeds, many of the allogeneic cell components and metabolic products are released into the tissues of the graft bed, are transported to the lymph nodes and spleen where they act as more potent immunogens than the intact transplanted cells. The marked development of germinal centres seen *after* the allogeneic tissue has been destroyed would therefore appear to represent the cellular manifestation of an intense secondary antibody response to a variety of iso-antigens carried by the transplanted cells.

Cellular depletion in the outermost portion of the lymphatic nodules (mantle layer or cuff of small lymphocytes) and within the adjacent marginal zone was noted in mice treated with ALS or ALG and in mice undergoing rejection of skin allografts. Studies in rats by WAKSMAN, ARNASON and JANKOVIC (1962) and in rabbits by GOOD and GABRIELSEN (1968) have shown that the mantle layers

of small lymphocytes are absent from the spleens and lymph nodes of neonatally thymectomized animals. In view of the close similarity between the effects produced in lymphoid organs by neonatal thymectomy and treatment with antilymphocyte or antithymocyte serum (Chapter 6), the reduced numbers of mantle layer lymphocytes in antiserum-treated mice can be accounted for on a similar basis. The loss of small lymphocytes from the mantle layers in mice undergoing rejection of skin allografts may be explained by mobilization and participation of these cells at the local site of graft rejection. The reduction in the numbers of marginal zone cells, on the other hand, cannot be accounted for as readily as in the case of mantle layer lymphocytes. The cells which make up the marginal zone are largely medium-sized lymphocytes (KRUMBHAAR, 1948; BAILLIF, 1953; MOORE, MUMAW and SCHOENBERG, 1964); blood cells and a few cells with phagocytic properties are also present (SNOOK, 1964; PETTERSEN, 1964). Although colloidal particles of carbon and saccharated iron oxide are not phagocytosed by marginal zone cells, initial localization of intravenously-injected colloidal particles occurs between these cells (SNOOK, 1964). It is not surprising, therefore, that very little carbon localized in the cell-depleted marginal zones of antiserum-treated and skin allografted mice (Chapters 4 and 6). Other studies have shown that the marginal zone is also an initial site of antigen localization (LA VIA, BARKER and WISSLER, 1956; HUNTER, 1966; NOSSAL et al., 1966; WILLIAMS and NOSSAL, 1966). The experiments of NOSSAL et al. (1966) with polymerized flagellin labelled with I^{125} demonstrated a progressive movement of label from the marginal zone, across the marginal sinus and into the white pulp. It was suggested that this movement of antigen most likely represented

either a migration of antigen-laden cells, or a transfer of antigen from cell to cell, the destination being the reticular cells in the "cap" region of germinal centres. A conclusion compatible with such a mechanism was reached by PETTERSEN, BORGEN and GRAUPNER (1967) who suggested that plasma cell precursors reside in the marginal zone and that soon after antigen administration, these precursor cells migrate into the lymphatic nodules where mitosis and subsequent differentiation into haemocytoblasts occurs in the presence of nodular macrophages. They further suggested that the haemocytoblasts then migrate across the marginal zone into the red pulp where differentiation into mature plasma cells occurs in the presence of red pulp macrophages. In Chapter 6 the suggestion was tentatively advanced that the marginal zone, like the periarteriolar regions of the splenic lymphatic nodules and paracortical areas of lymph nodes which also show cellular depletion following ALS and ALG treatment, may represent a thymus-dependent area. The observations of WAKSMAN, ARNASON and JANKOVIC (1962) and PETTERSEN and ROSE (1968) tend to argue against this possibility, since they found that neonatally thymectomized rats exhibited marginal zones which were normal in size and cell content. PETTERSEN, BORGEN and GRAUPNER (1967) did, however, suggest that the environment of the marginal zone may be a necessary prelude for the development of immunocompetence of cells derived from the thymus, bone marrow or other sources. It is noteworthy that in the investigations described in Chapters 4 and 6, depletion of cells from the marginal zones was prominent in mice undergoing rejection of skin allografts and in mice treated with ALG or absorbed ALS. Loss of cells from the marginal zone was accompanied by intense granulopoiesis in the red

pulp. In mice bearing skin isografts or treated with unabsorbed ALS, the increased haematopoiesis was predominantly erythropoietic, and significantly, the marginal zones showed very little cellular depletion. These observations suggest a possible association between loss of marginal zone cells and increased granulopoiesis in the red pulp. This suggestion is compatible with the conclusion of KRUMBHAAR (1948) who stated that the marginal zone in the rat spleen ("peri-follicular envelope") seemed to function as an auxiliary haematopoietic tissue. However, it is apparent that the current status of published data on the marginal zone does not permit any firm conclusions regarding the significance of the cellular depletion seen in this region in the spleens of mice treated with antisera or in mice undergoing rejection of skin allografts. In view of the importance of the marginal zone and its neighbouring structures in antigen localization and traffic of cells involved in antibody production, the problem warrants further detailed study.

Each of the immunosuppressive agents tested for their effects on the RE organs of Balb/c mice was found (at certain dose levels or injection regimes) to be capable of (1) reducing the phagocytic capacity of the liver and splenic macrophages, (2) depleting the splenic lymphoid tissue, and (3) inducing changes in the hepatic parenchymal cells (although cortisone and azathioprine caused hepatotoxic effects, it is unlikely that the RER proliferation seen in hepatocytes following ALS administration represents a pathological change, *vide infra)*. The ability of cortisone, azathioprine and ALS to exert such widespread effects in RE organs when administered singly is of considerable significance since combinations of these agents have been used to prolong allograft survival in

animals (HOEHN, WEIL and SIMMONS, 1967; JEEJEEBHOY, RABBAT and VELA-MARTINEZ, 1968; WEIL and SIMMONS, 1968; GROGAN and SHIVERS, 1969), and in humans (SHORTER, SPENCER and HALLENBACK, 1967; STARZL et al., 1967a, b and c; STARZL and PORTER, 1968; STARZL et al., 1968). Such combined immunosuppressive therapy has proved effective in suppressing allograft rejection and furthermore, has permitted the requisite doses of steroids and azathioprine to be reduced with a corresponding improvement in the prognosis for transplant patients (STARZL and PORTER, 1968; STARZL et al., 1968). However, the results of the investigations described in Chapter 6 should serve to emphasize some of the potential complications of immunosuppressive treatment. It is possible that hepatotoxic changes may occur when relatively low doses of corticosteroids, azathioprine and antilymphocyte globulin are administered simultaneously. Furthermore, the impairment of phagocytosis, the depletion of lymphoid tissue, and the suppression of cell-mediated and humoral antibody responses which would result from combined immunosuppressive therapy may severely impair the ability of some recipients to combat even minor infections. Obviously, the clinical testing of antilymphocyte globulin must continue. However, in order to circumvent potential complications and to devise optimal dose regimes, it is desirable that the effects of simultaneously administering ALG, corticosteroids and azathioprine be thoroughly evaluated in experimental animal studies before similar procedures become widespread in clinical practice.

The remarkable capacity of the liver to undergo changes in mass in response to a variety of experimental procedures was illustrated by the develop-

ment of hepatomegaly in Balb/c mice following the transplantation of small, medium and massive skin allografts and massive isografts, the intravenous administration of F_1 hybrid and CBA spleen cells, and the injection of cortisone acetate, normal rabbit serum, antilymphocyte serum and antilymphocyte globulin. While many forms of liver pathology are characterized by a marked increase in either liver lipid or water content, only in one of the conditions of hepatomegaly cited above (following cortisone administration) was the enlargement due to excessive accumulation of lipid within hepatocytes. No increase in water content occurred in the enlarged livers from mice given either cortisone, NRS or ALS or in mice bearing skin grafts. While there is no doubt that the hepatomegaly associated with fatty change represents a pathological effect of cortisone administration, it is unlikely that pathological processes are involved in bringing about liver enlargement in mice bearing skin grafts or treated with antisera. Before formulating any conclusions as to the significance of hepatomegaly observed in these two latter situations, it is important to consider other experimental situations which give rise to liver enlargement.

Previous studies have indicated that the increased liver mass exhibited by rodents is a sensitive indicator in toxicological studies. On the basis of results obtained from testing 200 organic compounds, SMYTH et al. (1952) concluded that liver weight (expressed in relation to total body weight) was about 10 times as efficient a criterion of injury than histopathological examination. In a subsequent report covering 364 studies with various compounds, ROWE et al. (1959) reported that an increase in liver weight occurred in 48% of these cases. Other studies have revealed liver enlargement in rats and mice given a variety

of drugs: coramine (COULSON and BRAZDA, 1947; BRAZDA and COULSON, 1948; WILSON and LEDUC, 1950), carbon tetrachloride (TSUBOI, STOWELL and LEE, 1951), thiourea (DOLJANSKI et al., 1956), ethyl-chlorophenoxy-isobutyrate (BEST and DUNCAN, 1964; HESS, STAUBLI and RIESS, 1965; AVOY, SWYRD and GOULD, 1965), 3-allyl-5-isobutyl-2-thiohydantoin (HERDSON, GARVIN and JENNINGS, 1964b), chlordane (HART and FOUTS, 1965), butylated hydroxynisol and butylated hydroxytoluene (GILBERT and GOLDBERG, 1965), ponceau Mx (HALL, LEE and FAIRWEATHER, 1966), flumedroxone acetate (HINES, 1967), phenobarbital (CONNEY et al., 1960; REMMER, 1962; HERDSON, GARVIN and JENNINGS, 1964a; KUNZ et al., 1966; PLATT and COCKRILL, 1967), and biphenyl methyl valeric acid (DE LA IGLESIA, SOSA-LUCERO and LUMB, 1970). Cytological examination to ascertain the nature of the hepatomegalic effect was carried out in only a few of these studies.

In those studies cited above where electron microscopic examination and biochemical estimations were performed on the enlarged livers, the results obtained did not resemble those reported in Chapters 4 and 6 for the enlarged livers from mice bearing skin grafts or treated with antisera. In each of the two latter situations, the large amounts of rough-surfaced endoplasmic reticulum contained within the hypertrophied hepatocytes had a normal ultrastructural appearance. The strands of RER did not exhibit any of the abnormalities such as disorganization of their parallel arrangement, vacuolization or detachment of ribosomes which have been observed in rat hepatocytes during carcinogenesis induced by the aminoazo dyes 3-Me-DAB (PORTER and BRUNI, 1959) or 2-Me-DAB (LAFONTAINE and ALLARD, 1964), dimethylnitrosamine (EMMELOT and

BENEDETTI, 1960), β-3-Thienylalanine (HRUBAN, SWIFT and WISSLER, 1963), a thiohydantoin compound (HERDSON, GARVIN and JENNINGS, 1964b), N-2-fluorenyldiacetamide (MIKATA and LUSE, 1964), ethionine (WOOD, 1965), or nitrosomorpholine (BANNASCH, 1968). This fact, together with the biochemical data which showed increased RNA concentration and normal composition of water, protein and DNA in the enlarged livers of grafted or serum-treated mice, strongly supports the following conclusion: *viz.*, that the liver enlargement which occurs in these situations does not in any way signify a pathological process, but rather, represents a physiological "work hypertrophy" or "hyperfunctional enlargement" brought about by a demand for increased production by hepatocytes of one or more substances which require(s) the utilization of RNA. In the case of administering antisera or normal rabbit serum to mice, the liver enlargement which resulted from such treatment was attributed to an increased production of purines by the liver in order to sustain the intense cellular proliferation within lymphoid tissues. This suggestion was advanced on the basis of the conclusion of LAJTHA and VANE (1958) relating to the role of the liver as the main source of purine supply for bone marrow cells and possibly for the cells of most peripheral tissues. Although electron microscopic examination and biochemical estimations were not performed on the enlarged livers of Balb/c mice injected with either F_1 hybrid or CBA spleen cells, it is likely that the ultrastructural and biochemical basis for the hepatocyte hypertrophy in these situations was similar to that for serum-treated mice, since liver enlargement was evident during the periods of considerable splenic enlargement (see Tables 5.3 and 5.5). In Chapter 4, increased production of purines as well as plasma

proteins by the liver were suggested as being responsible for the hepatomegaly in mice bearing skin grafts. All of these suggestions are readily amenable to experimental investigation, and it would be worthwhile to extend several of the experiments described in Chapters 4, 5 and 6 to determine the biochemical basis of the liver enlargement. Some of the most useful information which could be obtained from such experimentation is that pertaining to the dependence of lymphoid cells on the liver for purine supply. The conclusion of LAJTHA and VANE (1958) that the liver acts as the main source of purines was largely inferred from the demonstration that *de novo* synthesis of purines in bone marrow cells was greatly reduced by hepatectomy. The experimental situations described in Chapters 4, 5 and 6 (section 3) provide suitable experimental systems for the measurement of purine production by an enlarged liver in the presence of intense cellular activity within the lymphoid system.

Finally, it is important to consider the extent to which the changes in the livers and spleens of mice subjected to tissue transplantation or immunosuppressive treatment may apply to other animal species. The relative weights of the liver and spleen in 4 different species are shown in Appendix 5. While the relative weights of the human, rabbit and rat livers are closely comparable, the relative weight of the mouse liver is about twice that of each of the other three. This fact may have special significance in relation to the hepatotoxic effects produced in mice by certain dose levels of cortisone and azathioprine, the implication being that much lower doses of these drugs may be capable of producing hepatotoxic changes in the human, rabbit and rat. Reference has already been made to the high incidence of hepatotoxicity in patients treated with low levels of 6-mercaptopurine, and to the development of hepatomegaly asso-

ciated with fatty change in patients receiving corticosteroids. The data in Appendix 5 also shows that the relative weight of the mouse spleen is about twice that of the human and about 10 times that of the rabbit. This marked difference between the relative weights of the rabbit and mouse spleens probably explains in part why SCOTHORNE and McGREGOR (1955) found no alteration in the weight of the spleen in rabbits which had skin allografts applied to one ear, while up to a 3-fold splenic enlargement was found in Balb/c mice following skin allotransplantation (Chapter 4). Other factors contributing to this discrepancy are differences in the relative sizes of grafts employed and the site of their placement. In addition, significant histological differences exist among the spleens from different animal species. The rabbit spleen does not exhibit extramedullary haematopoiesis which is a normal feature of the mouse spleen. Significantly, the splenic enlargement which occurred in Balb/c mice a few days after skin grafting was due to increased haematopoiesis. According to WARD, JOHNSON and ABELL (1959), haematopoietic activity can be seen in the rat spleen. On the other hand, BAILLIF (1953) reported a total absence of splenic erythropoiesis in normal albino rats (strain unspecified). In a comparative study involving a small number of animals, the intensity of haematopoiesis in the spleens of female Balb/c mice has been found to be much greater than that in spleens of female chocolate brown rats (GOTJAMANOS, T., unpublished data). If this conclusion holds equally well for other strains of rats, then it may explain the difference between the effects produced by ALS on the Reticuloendothelial organs of Balb/c mice (Chapter 6) and those produced in Long-Evans rats (GROGAN, 1969). In the latter study, rats given 3 doses of ALS showed no alteration in spleen weight, whereas Balb/c mice which received 4 doses of ALS

showed up to a 4-fold splenic enlargement due to an enormous increase in haematopoiesis. The enlarged spleen was accompanied by a marked enlargement of the liver. It is significant that GROGAN (1969) did not find any alteration in either spleen or liver weight in rats treated with ALS. This observation supports the concept of a direct relationship between liver hypertrophy and intense cellular proliferation in the spleens of mice treated with ALS.

The few examples of species differences cited above should serve to illustrate how discrepancies in the results and conclusions can arise when similar experimental procedures are performed on different species of animals. Furthermore, they should emphasize the necessity of carefully considering the animal species to be used when attempting to reproduce the findings of other investigators or to extend their experiments. Animal experimentation will no doubt continue to play a dominant role in immunology and related disciplines. While extrapolation of data derived from animal studies to the human situation must be done with a full consideration of the species differences involved, nevertheless, such data will continue to prove invaluable in the diagnosis and treatment of immunological disorders, the immunological reactions accompanying the transplantation of tissues and organs, and the means by which rejection can be prevented by safe and effective immunosuppressive therapy. It is hoped that the investigations reported in this thesis and the discussion that has been stimulated by the experimental findings will form the basis for further studies in the fields of immunobiology, immunopathology and transplantation immunity. Even at this point of time, it seems appropriate to conclude with the words of Seneca:

> *"There is still much work to be done, and much will always remain, nor yet will those born a thousand generations hereafter, lack the opportunity of adding something further."*

APPENDIX I : HISTOLOGICAL METHODS

FIXATIVES

(a) Bouin's fluid

Saturated aqueous solution of picric acid	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid (added immediately before use)	5 ml

(b) Neutral Formol-saline

Formalin (40% formaldehyde)	10 ml
Sodium chloride	0.9 g
Distilled water	to 100 ml

Solution buffered to pH 7.0 by addition of 3.5 g of sodium dihydrogen phosphate (anhydrous) (NaH_2PO_4) and 6.5 g of disodium hydrogen phosphate (anhydrous) (Na_2HPO_4) per litre of the diluted solution.

STAINS

(a) Ehrlich's haematoxylin

Haematoxylin	6 g
Absolute alcohol	300 ml
Distilled water	300 ml
Glycerol	300 ml
Glacial acetic acid	30 ml
Potassium alum : added until deposit of crystals forms on bottom of container.	

Whole solution left in sunlight for 2 - 3 months before being used.

(b) Eosin

1% aqueous solution of water-soluble eosin.

APPENDIX 2

COMPOSITION OF ARALDITE[a] USED TO EMBED SPECIMENS FOR ELECTRON MICROSCOPY :

Araldite M (CY 212)	5.0 ml
Araldite Hardener (HY 964)	5.0 ml
Araldite Accelerator (DY 064)	0.2 ml
Di-butyl phthalate (plasticizer)	
or	0.5 ml
Dodecenyl succinic anhydride[b]	

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[a] Ciba Co. Ltd., Basel, Switzerland.

[b] Cargille Laboratories, Inc., New York.

APPENDIX 3. BODY WEIGHT[a] INCREMENT OF NORMAL BALB/C FEMALE MICE DURING PERIOD 12-20 WEEKS.

Group	Age (weeks) 12	13	14	15	16	17	18	19	20	% increase over 8 weeks	% increase per week
A	20.4	21.3	22.0	21.2	21.6	21.6	22.2	22.9	22.8	11.8	1.5
B	20.4	21.5	22.0	21.9	21.6	21.3	22.2	22.9	22.5	10.3	1.3
C	20.3	21.2	21.5	22.1	22.3	22.4	22.4	22.9	23.3	14.8	1.9
D	20.8	22.5	22.2	22.8	23.1	23.5	23.3	24.1	24.3	16.8	2.1

[a] Body weight of each group of mice is expressed in grams and represents the mean value of 4 mice.

APPENDIX 4: LIVER, SPLEEN AND LYMPH NODE[a] WEIGHTS AND PHAGOCYTIC INDICES OF DIFFERENT INBRED MOUSE STRAINS[b]. (MEANS ± S.E.M.).

Strain	Sex	Age (weeks)	Liver weight as % of Total Body Weight (T.B.W.)	Spleen weight as % of T.B.W.	Lymph node weights as % of T.B.W.	Phagocytic Index (K)	Corrected Phagocytic Index (α)
CBA	M	12	4.81± .12	.26 ± .01	.11± .01	.015± .001	4.91± .06
F_1 (Balb/c x C57 BL)	F	12	4.99± .16	.36 ± .01	.12± .01	.016± .001	4.77± .20
C57 BL/6	F	12	4.34± .10	.34 ± .01	.13± .01	.019± .001	5.74± .08
Balb/c	F	12	5.06± .11	.56 ± .02	.14± .01	.028± .002	5.39± .04
Balb/c	F	14	5.01± .09	.59 ± .03	.15± .01	.026± .001	5.27± .09
Balb/c	M	14	5.17± .13	.41 ± .02	.12± .01	.026± .001	5.30± .16
Balb/c	F	16	5.11± .11	.55 ± .02	.14± .01	.028± .002	5.35± .09

[a] Two axillary and 2 inguinal lymph nodes from each mouse were weighed. (Male and female mice from each of the strains included in the Table consistently exhibited only 2 axillary and 2 inguinal lymph nodes).

[b] Four mice from each strain were studied.

APPENDIX 5. RELATIVE WEIGHTS OF THE LIVER AND SPLEEN IN DIFFERENT ANIMAL SPECIES.

Species	Liver weight as a % of Total Body Weight (Means ± S.E.M.)	Spleen weight as a % of Total Body Weight (Means ± S.E.M.)
HUMAN *(post-mortem material)*	2.25 ± 0.15	0.25 ± 0.03
RABBIT *(outbred, sandy lop-eared)*	2.28 ± 0.28	0.05 ± 0.01
RAT *(inbred, chocolate brown)*	2.52 ± 0.08	0.19 ± 0.01
MOUSE *(inbred Balb/c[a])*	5.09 ± 0.11	0.53 ± 0.02

[a] The values for Balb/c mice have been derived by pooling the data for male and female Balb/c mice included in Appendix 4.

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Erratum

p. 85 Fig. 4.9, bracket should read :

(granulocytic and erythropoietic)