INCREASED SENSITIVITY OF OLD RATS TO BACTERIAL ENDOTOXINS

Adriaan Brouwer

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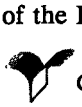
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STELLINGEN

behorende bij het proefschrift

'Increased sensitivity of old rats to bacterial endotoxins'.

1

Biologische veroudering is een zinvol en onvermijdelijk proces, waarvan slechts de snelheid van voortschrijden en de onnodige pathologische bijverschijnselen, die ontstaan door suboptimale leefgewoonten en omstandigheden, kunnen worden beïnvloed.

2

De opname van retinol door cellen is niet afhankelijk van receptoren voor retinolbindingseiwitten.

3

De pathofysiologische effecten van alcoholgebruik zijn in grote mate afhankelijk van de voedingstoestand en van de aanwezigheid van toxische cofactoren.

4

De procesgang bij het verlenen van een doctorstitel dient in overeenstemming te worden gebracht met het gegeven dat het doctoraat inmiddels is ge(d)evalueerd tot een vakdiploma dat slechts behoeft te getuigen van het vermogen om door anderen bedacht onderzoek naar behoren te helpen uitvoeren en beschrijven.

5

Het dienstbaar stellen van medisch-biologisch onderzoek aan concrete maatschappelijke belangen leidt tot voorwaarden en procedures die veelal strijdig zijn met een zinnige beoefening van de wetenschap.

6

Het streven naar handhaving van de maximumsnelheid van motorvoertuigen dient te worden ondersteund door regelgeving die de betrouwbaarheid van de snelheidsmeters van deze voertuigen garandeert.

A. Brouwer, 10 september 1992.

INCREASED SENSITIVITY OF OLD RATS TO BACTERIAL ENDOTOXINS

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 10 september 1992
te klokke 16.15 uur

door

Adrianus Brouwer

geboren te Nieuwer-Amstel in 1949

Promotiecommissie:

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	Prof.dr. P. Brakman

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CHAPTER 1

INTRODUCTION

Background

The studies presented in this thesis deal with the effects of aging on pathophysiological consequences of intravenous administration of bacterial lipopolysaccharides (LPS), also called endotoxins. One objective of the experiments was to gain insight into the mechanisms responsible for the increased sensitivity of older individuals to sepsis and to other severe challenges of homeostasis, such as major surgery, trauma and burns, which lead to a similar type of response (1).

The studies were also intended to contribute to further development of theoretical concepts of biological aging (2). The subject of the thesis, the increased morbidity and mortality of the older organism under challenge, is a central component of most commonly accepted definitions of biological aging. The most characteristic general feature of biological aging may be the reduced capacity of an organism to maintain homeostasis in response to external stimuli, in association with an increased mortality rate (2-6). The increased susceptibility of old rats and mice to the acute effects of gram negative bacteremia and endotoxins can be considered as a representative model to study these issues. An analysis of the regulatory changes in the response of old rats to endotoxins may, therefore, provide clues as to which homeostatic mechanisms might be particularly affected by aging.

Endotoxins are lipopolysaccharide (LPS) components from the outer cell wall of gram-negative bacteria (7). Endotoxemia, i.e. the appearance of endotoxins in the blood circulation in the course of gram-negative sepsis, has profound effects on mammals. Most pathophysiological consequences of gram-negative sepsis are attributable to endotoxins (1,7,8). Administration of endotoxin results in a variety of dose-dependent effects including fever, hypothermia, production of cytokines and acute phase proteins, intravascular coagulation, metabolic alterations, tissue damage, and, already at relatively low concentrations, circulatory shock and death (1). These phenomena have been extensively characterized in experimental animals, such as rats (1), mice (9,10), rabbits (11), minipigs (12) and dogs (13). These animal models have been shown to be of great value for

our understanding of the pathophysiology at the cellular and molecular level occurring during septicemia, septic shock and multi-organ failure in man. Such studies have revealed that the major effects of endotoxin, including fever, hemodynamic and metabolic disturbances and tissue damage, are not a direct effect of endotoxin, but are mediated and regulated by numerous mediators produced by macrophages and other cells (14). Several types of biological response modifiers, including cytokines and eicosanoids, are involved, many of which are produced by Kupffer and endothelial cells of the liver (15,16), as well as by circulating white blood cells and by macrophages and endothelial cells in other tissues (14), and by many other cell systems.

The response requires careful regulation, since it is also potentially harmful for the organism itself, because of possibly severe alterations in body temperature, tissue perfusion and metabolism that may cause damage to cells and tissues. The sequence of events involved in this response is extremely complex, and little is known about the regulatory processes that keep it in control, even though many of the components of the response have been identified. Recent experiments have shown that the regulation of the response to endotoxins in human volunteers is very similar to that in experimental animals, with the same types of cytokines appearing in the circulation (17-20). The same cytokines, which include tumor necrosis factor (TNF), interleukins (IL) 1 and 6, interferon γ, and plasminogen activator inhibitor, were found in plasma of septic shock patients (21-23). Other recent studies have shown that TNF-α, is likely to be a primary mediator of endotoxin effects (24,25). Administration of recombinant TNF-α in man results in a spectrum of changes similar to those observed in septicemia (25). These changes include fever, rapid reactions in white blood cell counts, activation of neutrofils and of coagulation, rapid activation and subsequent inhibition of fibrinolysis, and, the appearance of secundary mediators, such as interleukin-6, prostacyclin, epinephrine, norepinephrine, adrenocorticotropic hormone (ACTH), cortisol, and, glucagon (25). In addition, basal metabolic rate was increased and the metabolism of glucose and free fatty acids was activated (25).

The sensitivity to endotoxins is strongly species-dependent. Humans are among the most sensitive mammalian species (7), and clinical reports indicate that the elderly are more susceptible to and less likely to survive septic shock than young adults (1,26). In elderly, there is also a higher morbidity and mortality rate after injury and blunt trauma (27-29) and age is an independent risk factor for secondary complications and mortality after myocardial infarction (30), perforated ulcer (31), major surgery (32) and acute drug poisoning (33).

Clinically, septic shock is managed by intensive care treatment, including controlled plasma volume expansion and red cell substitution, supported by antibiotics. In cases of shock, hemodynamic parameters can be improved by epinephrine, dubotamine, dopamine and norepinephrine (34-40). Also, the use of inhibitors and antagonists of eicosanoids

and of other monokines, such as platelet activating factor and IL-1, are being tested in experimental models of septic shock (41-43). In addition, anti-coagulant therapy (antithrombin III substitution) has been reported to decrease mortality in patients with disseminated intravascular coagulation due to septic shock (44,45). One of the new developments that are currently being developed and tested include treatment with antibodies against bacterial lipopolysaccharide (46,47). No effect on survival of septic shock patients was found in the first reported clinical trial with human antibodies (48), but a significant reduction of mortality in patients with sepsis gram-negative bacteremia was found in a double-blind placebo controled clinical trial with human monoclonal anti-lipopolysaccharide antibodies (49).

The studies presented here form an extension of earlier work from our laboratory that was focused on the effects of aging on the biochemistry, morphology and function of liver cells, particularly non-parenchymal or sinusoidal cells (50-56). Briefly summarized, these rat studies indicated various age-related biochemical and (ultra)structural alterations in all liver cell types, including Kupffer and endothelial cells. However, most of these changes were rather subtle, while the possible consequences of these changes were unclear. These studies were in keeping with the limited knowledge on age-related changes in macrophage functions obtained in other studies (for a review see 57). In vitro studies displayed age-related changes in various enzymatic activities, in antigen processing and in activation by LPS (see Chapters 2 and 9). The functional and pathophysiological consequences of such changes for the elderly organism were unclear, and so was the significance of age-related changes in macrophages in relation to those in other cells and processes. In recent years, the role of macrophages in aging has been described in more detail (see Chapters 3 and 8-10).

Since Kupffer cells have important functions in the clearance and detoxification of endotoxins (58-61), and also contribute to the host response to endotoxin (15), we have focused subsequent studies on the effects of aging on the pathophysiological and cellular aspects of endotoxemia in rats. Although rodents have been widely used in aging research, relatively little was known about the reactions of aging animals under stress (1). It was thought that characterization of these processes in the aging rat model might reveal essential age-dependent changes in their regulation that could be relevant for the treatment and management of sepsis in the elderly. Currently, there is little understanding of the factors that determine the greater susceptibility of the elderly to septic shock.

Outline of the thesis

This thesis includes an analysis of the overall pathophysiological response and survival of rats of different strains, gender and age after administration of E. coli-derived endotoxin (Chapter 2). The further aims of the study were to delineate the crucial factors

and regulatory processes responsible for the increased susceptibility of old rats to endotoxin-induced shock. Therefore, more detailed experiments on the time sequence of appearance of tissue damage (Chapters 3 and 4), on fibrin deposition and fibrinolysis (Chapter 5) and on glucose dyshomeostasis (Chapter 6) were conducted in aging female BN/BiRij rats. Specific attention was given to the possible role of changes in the endocytic capacity of hepatic macrophages (Kupffer cells) (Chapter 7) and to the mediators produced by these cells in response to LPS (Chapter 8). Also, the induction of early response proteins by LPS was studied in Kupffer cells from young and old rats (Chapter 9). An integrated analysis of the results and a further definition of the factors that are crucial for the deregulation of the response of old rats to LPS is attempted in Chapter 10.

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CHAPTER 2

CHANGES IN ENDOTOXIN SENSITIVITY IN AGEING. ABSORBTION, ELIMINATION AND MORTALITY

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CHANGES IN ENDOTOXIN SENSTIVITY IN AGEING. ABSORBTION, ELIMINATION AND MORTALITY

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SUMMARY

In this paper we describe the influence of ageing on responses to intravenously-injected endotoxin in two rat strains. Old age had no apparent effect on the absorption of ^{51}Cr-labelled endotoxin from either jejunum or colon. Notwithstanding, aged animals appeared much more sensitive than their young counterparts to the lethal effects of intravenously injected endotoxin. Old animals exhibited virtually 100% mortality over the dose range 1—4 mg/100 g body weight while only sporadic deaths were seen in young animals. One consistent feature of dying animals was a profound and progressive hypothermia. At post mortem examination, the major findings were in the liver (leukocyte infiltrates and hepatocellular necrosis) and kidneys (acute tubular necrosis). Ageing was associated with slower removal of endotoxin from the circulation but not to an extent that could reasonably account for the enhanced sensitivity to endotoxin toxicity.

Key words: Endotoxin; Absorption; Elimination; Mortality

INTRODUCTION

Perhaps the first suggestion that bacterial products might influence (at least some) age-related phenomena appears in Metchnikoff's book *The Prolongation of Life* [1]. It has been suggested that gut-derived endotoxins (highly active components of the cell wall of gram negative bacteria) might induce some of the immunological changes typically associated with old age [2] and may be of some importance

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in maintaining the mucosal-associated lymphoid tissue, which appears not to be affected adversely during ageing [3].

Older mice appear to be more sensitive to the toxic effects of endotoxins than are their younger counterparts [4—6]. There is now overwhelming evidence that cells of the mononuclear-phagocyte system [7] provide the only major route for the elimination of endotoxins from the body [8—13]. A moderate age-related decrease in the clearance functions of the MPS using a variety of test colloids has been described [14,15] and we have previously reported such a decrease in aged rats of the BN/BiRij strain using endotoxin as the test substance [16,17]. It is therefore possible that reduced endotoxin uptake by cells of the MPS to some extent could explain the increased sensitivity to endotoxins seen in senescent animals.

In this study, we report further findings on endotoxin elimination and endotoxin-induced mortality together with findings on endotoxin absorption and the accompaniments of the endotoxin-induced deaths.

MATERIALS AND METHODS

Animals

Specific pathogen free-derived BN/BiRij and WAG/Rij rats maintained under 'clean, conventional' conditions were used [18]. Animals were free of common bacterial and viral pathogens as determined by microbiological, serological and histopathological monitoring [19]. The lifespan characteristics of these strains are given in Table I. Animals were maintained at an ambient temperature of 19—21 °C unless otherwise stated and were allowed free access to food (diet AM II, Hope Farms, Woerden, The Netherlands) and water. Animals were used in groups of five unless otherwise stated and were not starved prior to endotoxin administration.

In the earliest experiment employing an endotoxin dose of 4 mg/100 g body weight, animals were watched continuously for approximately 12 h but in subse-

TABLE I

SURVIVAL CHARACTERISTICS OF THE BN/BiRij AND WAG/Rij RAT STRAINS MAINTAINED IN THE AGEING COLONY OF THE INSTITUTE FOR EXPERIMENTAL GERONTOLOGY TNO

Strain	*Sex*	*No. of rats*	*Age (months) at survival points*			
			90%	*50%*	*10%*	*Max.*
BN/BiRij	M	286	20.8	30.5	35.5	39.9
BN/BiRij	F	885	22.4	31.8	37.6	41.3
WAG/Rij	M	276	20.1	27.2	33.5	36.5
WAG/Rij	F	984	24.5	33.2	39.2	44.5

quent experiments, observations were continued until the animal was killed in extremis or at the end of the experimental period. Any untoward event or change in behaviour was recorded. Animals in extremis were quickly anaesthetised, the abdomen opened and as much blood as possible was obtained from the inferior vena cava. Plasma samples were used for endotoxin measurements (when appropriate) and serum was stored for assay of transaminase activities.

After blood sampling, liver, lung and kidneys were removed and fixed by immersion in phosphate-buffered formalin. Kidneys were transected before immersion. Samples of fixed organs were embedded in paraffin, sectioned at 4 μm and stained with haematoxylin-phloxine-saffron (HPS). Two to three sections of liver (each obtained from a different lobe) and a single longitudinal section of the kidney were examined without knowledge of treatment group ('blind') by the same pathologist (SKD) and graded histologically by light microscopy. Numerical values assigned were as follows: 0 = absent, 1 = minimal, 2 = mild, 3 = moderate and 4 = severe. Categories of hepatic injury included: [1] sinus granulocytosis (SG) characterised by an accumulation of neutrophils within sinusoids, [2] portal tract haemorrhage (PTH), [3] isolated parenchymal cell necrosis (IHN) and [4] focal areas of parenchymal cell necrosis (FAN) defined as a group of more than ten adjacent necrotic parenchymal cells. Acute tubular necrosis was the only category of renal injury. The histology scores from each category and cumulative histology score for individual animals were analysed using the Kruskall—Wallace test followed by Dunn's multiple comparisons according to Newman-Keuls [20].

Measurement of body temperature

Colonic temperature was measured without restraint in conscious animals by inserting a lubricated, plastic-coated thermistor probe some 3 cm beyond the anus. This procedure caused no apparent distress and was well tolerated by the animals.

Endotoxin preparation and assay

A phenol-water extract of *E. coli* O26:B6 (Sigma Chemical Company, St. Louis, MO, U.S.A.; lot number 93f-4041) was used throughout. When required, this preparation was labelled with ^{51}Cr after the method of Braude and co-workers [21]. Briefly, 1 mCi of $Na^{51}CrO_3$ (Radiochemical Centre, Amersham, England; spec. act. 250—500 mCi/mg) was added to 27.5 mg of endotoxin in 17.5 ml water and incubated at 37 °C for 24 h. The radiolabelled endotoxin was then dialysed three times against distilled water and several times against 0.9% NaCl until no radioactivity could be further dialysed. In the final preparation, 95% of the radioactivity was still precipitable with absolute ethanol. The labelling procedure did not affect the ability of the endotoxin to activate Limulus Amoebocyte Lysate. Both the ^{51}Cr-labelled and

non-labelled endotoxins exhibited an activity in the Limulus test of approximately 2.5×10^6 endotoxin units (EU)/mg.

Endotoxin was also measured in plasma samples using the Limulus Amoebocyte Lysate (LAL) test. A reagent set obtained from Kabi Vitrum (Stockholm, Sweden) was used. All procedures were performed in a Class II microbiological safety cabinet using sterile and endotoxin-free materials. Plasma was diluted in endotoxin-free distilled water and heated at 75 °C for 10 min to remove inhibition. Samples were then assayed over a range of dilutions against standards from 0 to 1.15 EU/ml. After samples were at 37 °C, an equal volume of freshly reconstituted LAL was added and the incubation proceeded at this temperature for ten mins. Two volumes of the chromogenic reagent S2423 in buffer were then added and the incubation continued for exactly 3 min, then stopped with two volumes of 50% acetic acid. The absorbance was read at 405 nm in a Titertek Multiskan® MC (Flow Laboratories, Ayrshire, Scotland).

Endotoxin absorption

The method of Ouwendijk was used [22]. Two groups of nine female BN/BiRij rats aged 6 and 24 months, respectively, were used. Animals were placed under halothane anaesthesia and a midline laparotomy performed. To assess endotoxin absorption from the small intestine, a loop of about 15 cm length was chosen immediately distal to the duodenum. Two loose ligatures were placed approximately 15 cm apart, taking care not to damage the vasculature. A small incision was made just proximal to the first ligature and a polypropylene cannula (1 mm external diameter) was passed through so that its tip lay in the gut lumen between the two ligatures. The two ligatures were then tightened and the cannula secured by means of a third ligature just proximal to its site of entry. Two millilitres of ^{51}Cr-labelled endotoxin (0.5 mg/ml) were instilled into the in situ loop followed immediately by 0.25 ml of air. The cannula was sealed by heating and the abdomen closed in layers with clips to the skin. Animals were allowed to recover and killed 2.5 h after the instillation of labelled endotoxin.

Blood was obtained from the inferior vena cava and portal veins by aspiration into a syringe. The isolated loop was removed intact and the length measured after attaching a standard weight to one end. The remaining gut, together with the liver, spleen, kidneys, adrenals, heart and lungs, was removed and placed in plastic vials with the other organs and blood. Radioactivity was counted over 10 min in a gamma counter. The radioactivity in the blood and organs was expressed as a percentage of the total radioactivity recovered from them plus that in the isolated loop. Absorption was calculated as μg/cm gut per 2.5 h.

To assess absorption from the colon, the terminal 8 cm of bowel was chosen. The procedure described above was followed except that the colon was gently irrigated with 0.1544 M NaCl before the distal ligature was tightened and only 1 ml of endotoxin solution was introduced into the colonic lumen.

Endotoxin injection and blood sampling

Injections into the right femoral or jugular veins were performed through a 27 G needle under light halothane anaesthesia between 09.00 and 12.00 h. Endotoxin doses of either 1 mg, 2 mg or 4 mg per 100 g body weight were used except on one occasion when 10 mg/100 g body weight was administered to a group of three 6-month-old female BN/BiRij rats. Animals were then returned to the original cage to recover.

Blood samples of 100 μl were taken into heparinized glass capillaries from a small nick close to the tip of the tail. The capillaries were then sealed, spun and plasma drawn off. ^{51}Cr-labelled endotoxin in the plasma was determined by radioactivity counting in 20 μl aliquots over 5 min in a gamma scintillation counter. Plasma samples were also used in the test. Samples were taken at 10-min intervals for the first hour and then at 30—60-min intervals. Endotoxin disappearance curves were plotted for each animal and the plasma half-life was calculated over the period, 2—10 h following injection. The regression line, which characterised the elimination phase, comprised 4—10 data points. All regression lines were acceptable at the 5% level of probability. All data between groups were compared by means of a two-tailed Mann-Whitney *U*-test.

In one experiment, unlabelled endotoxin (1 mg/100 g body weight) was administered into the portal venous system. Ten 24-month-old female BN/BiRij rats were randomly allocated to one of two groups. Each animal was brought under halothane anaesthesia and a midline laparotomy performed. A loop of ileum was delivered through this incision and kept moist with gauze swabs soaked in 0.9% NaCl. A small mesenteric vein was cannulated with a 27 G needle and the injection made over two mins. The needle was then withdrawn and the puncture covered lightly with a cotton swab until any oozing of blood from the puncture had stopped. The loop of gut was then returned to the abdominal cavity and the abdomen closed in layers with clips to the skin. Rats from one group were killed at intervals (for purposes of histology — reported elsewhere) and rats of the other group were observed and killed when moribund.

RESULTS

Endotoxin absorption

After administration of endotoxin into the isolated intestinal loop, it was detected (with the LAL test) in the portal blood of all the animals but not in the peripheral blood. This endotoxin could have arisen from the isolated loop or from elsewhere along the length of the gut. Absorption of ^{51}Cr-labelled endotoxin from the isolated loops of jejunum and colon, as determined by the appearance of radioactivity in the blood and organs, did not differ significantly with age for absorption from either the jejunum or from the colon (Fig. 1).

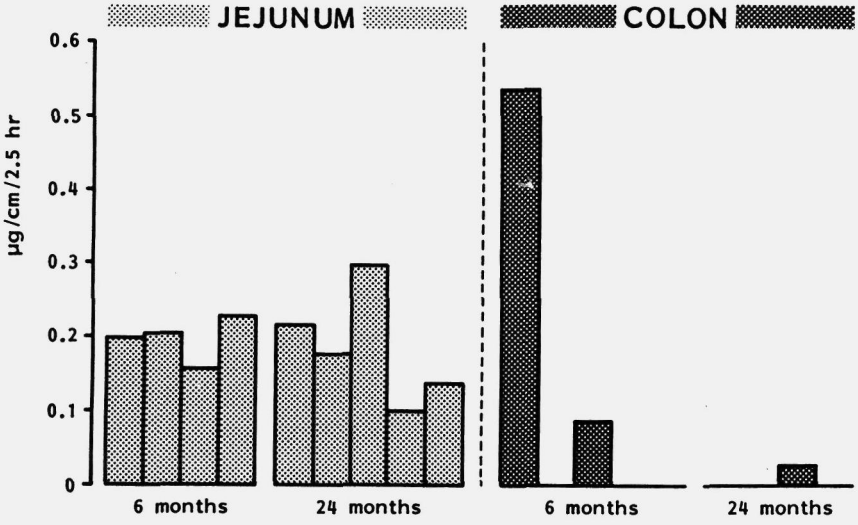

Fig. 1. Absorption of ^{51}Cr-labelled endotoxin from jejunal loops (containing 1 mg of endotoxin) and colonic loops (containing 0.5 mg of endotoxin) in situ.

Clinical aspects

All animals (regardless of age) reacted similarly soon after the systemic injection of endotoxin. Over the dose range 1—4 mg/100 g body weight, all animals showed reduced activity and attempted to reduce surface area/volume ratio by adopting a hunched posture. Epiphora, pilo-erection and shivering were universal features. After approximately 90 min, the blood appeared viscous and hypercoagulable. This state persisted for several hours in animals destined to survive (mainly young animals) and was then followed by a progressive return to normal appearance and behaviour. In those animals destined not to survive (most of the aged rats and the three young female BN/BiRij rats given 10 mg/100 g body weight of endotoxin), by about 4 h they started to uncurl and become increasingly lethargic. The precise timing of these events was dose dependent, occurring earlier with higher doses. At this point they felt cool to the touch and as time progressed they lapsed into coma and generalised convulsions were sometimes seen in the old rats. The animals were then judged to be moribund and were killed by exsanguination under anaesthesia.

Endotoxin-induced mortality

Table II shows the group size, age, sex and strain of rats used to assess endotoxin-induced deaths. All animals aged 24 months or more died following an injection of 1 mg per 100 g body weight of endotoxin or more. The time course and dose-response relationships are illustrated for 24-month-old female BN/BiRij rats in Fig. 2 and for 24-month old male BN/BiRij and 24-month-old female WAG/Rij rats in Fig. 3.

TABLE II

ENDOTOXIN-INDUCED FATALITY IN RATS OF DIFFERING AGE, SEX AND STRAIN ADMINISTERED DIFFERING DOSES OF *E. COLI* 026:B6 ENDOTOXIN INTRAVENOUSLY

Strain	*Sex*	*Age (months)*	*Group size*	*Endotoxin dose (mg/100 g body wt.)*	*No. of deaths*	*Time of first death (h)*	*Time of last death (h)*
BN/BiRij	M	24	5	1	5	6.3	9.0
BN/BiRij	M	6	5	1	0	—	—
WAG/Rij	F	24	5	1	5	10.5	29.5
WAG/Rij	F	6	5	1	0	—	—
BN/BiRij	F	6	3	10	3	9.0	12.0
BN/BiRij	F	36	5	4	5	5.3	7.0
BN/BiRij	F	24	4	4	4	7.7	10.09
BN/BiRij	F	6	5	4	0	—	—
BN/BiRij	F	3	5	4	1	22.4	—
BN/BiRij	F	36	3	2	3	5.6	7.9
BN/BiRij	F	24	5	2	5	11.5	14.0
BN/BiRij	F	6	5	2	0	—	—
BN/BiRij	F	3	5	2	0	—	—
BN/BiRij	F	24	5	1	5	9.0	29.0
BN/BiRij	F	6	5	1	0	—	—

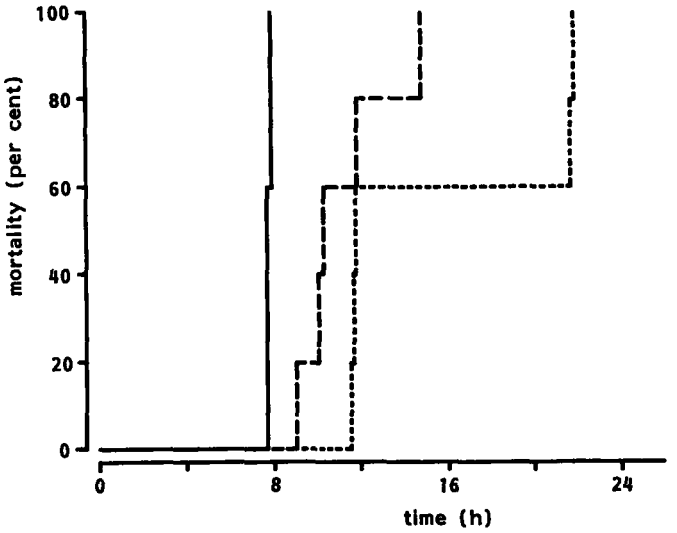

Fig. 2. Mortality curves for groups of 24-month-old BN/BiRij female rats injected intravenously with endotoxin; 4 mg/100 g body weight (continuous line), 2 mg/100 g body weight (broken line) and 1 mg/100 g body weight (dotted line).

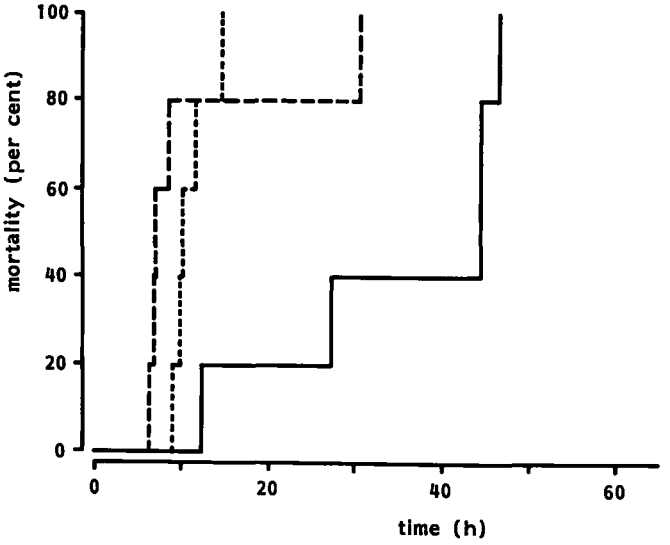

Fig. 3. Mortality curves for groups of 24-month-old male BN/BiRij rats (dotted line), 24-month-old BN/BiRij female rats (broken line) and 24-month-old WAG/Rij rats (continuous line) injected intravenously with 1 mg/100 g body weight of endotoxin.

There is a clear relationship between (a) the injected dose and the time at which the first animal died in each group and (b) the injected dose and the time elapsing between the death of the first animal and the death of the last animal in each group. Most animals died over a very short time-period and before 8 h had elapsed from the time of endotoxin administration. With the lowest dose (1 mg/100 g body weight), deaths did not occur before 11 h and the time course over which they occurred was considerably prolonged. Rats aged 36 months died considerably earlier (between 5 and 8 h after administration of 2 mg or 4 mg per 100 g body weight). Only sporadic deaths were observed in the 3- and 6-month-old animals (2 deaths in total) in the dose range 1—4 mg per 100 g body weight but all three animals died within 12 h of giving 10 mg per 100 g body weight.

This dramatically increased sensitivity to the lethal effects of endotoxin occurred independently of sex and strain in that it was also observed in 24-month-old male BN/BiRij rats and 24-month-old female WAG/Rij rats. One interesting observation is that the deaths in the WAG/Rij rats occurred over a much longer time period (up to 50 h after intravenous injection of 1 mg per 100 g body weight of endotoxin). BN/BiRij rats of an intermediate age (18 months) given the same dose of endotoxin showed an intermediate response in that only two of the five animals died within 48 h, though the three survivors were in poor condition and would probably have died if the experiment had continued.

Changes in body temperature

One of the most prominent associations of endotoxin-induced deaths is a profound and progressive hypothermia. The basal body temperature (Table IV) obtained before the injection of endotoxin did not vary significantly within or between groups. Following the injection, all animals appeared to attempt to generate heat by shivering. Non-shivering thermogenesis was not assessed. Furthermore, animals appeared to attempt to conserve heat by adopting a hunched posture, thus reducing the surface area/volume ratio. Over the first 90 min following endotoxin injection, all animals experienced a modest fall in body temperature followed by a return towards normal. This return towards normal continued in survivors, which usually managed to generate modest increases in body temperature compared to basal. In non-survivors, body temperature again started to fall, a trend which continued until death. Examples of these responses are shown in Figs. 4—6. The time course of the progression of the hypothermia follows precisely the clinical progression observed in the two rat strains with the time course considerably prolonged in the WAG/Rij rats. Intermediate responses were observed in the 18-month-old rats (Fig. 5).

TABLE III

TERMINAL HALF-LIFE OF INTRAVENOUSLY INJECTED *E. COLI* 026:B6 ENDOTOXIN IN FEMALE BN/BiRij RATS OF DIFFERING AGE DETECTED BY RADIOACTIVITY COUNTING (RA) AND BY THE LIMULUS AMOEBOCYTE LYSATE TEST (LAL)

Dose (mg/100 g body wt.)	*Age (months)*	*Detection method*	*Half-life (h)*
1	6	RA	5.16 ± 0.30
	24	RA	8.41 ± 1.09**
2	3	RA	5.70 ± 0.44
	6	RA	4.93 ± 0.33
	24	RA	7.28 ± 0.33**
	36	RA	8.13 ± 1.23*
	3	LAL	2.00 ± 0.14
	6	LAL	2.28 ± 0.13
	24	LAL	3.28 ± 0.28**
	36	LAL	3.29 ± 0.64
4	3	RA	7.02 ± 0.93
	6	RA	6.38 ± 0.38
	24	RA	7.65 ± 0.53*
	36[a]	RA	6.05 ± 0.33

Statistical analysis by the Mann-Whitney *U*-test. $P < 0.05$. Results expressed as mean ± S.E. of the mean. Five animals per group (except [a] $n = 4$).

*Significantly different from 6-month-old animals.

**Significantly different from 3-month-old animals.

TABLE IV

BASAL BODY TEMPERATURE IN RATS OF DIFFERING AGE, SEX AND STRAIN

Strain	*Sex*	*Age (months)*	*Body temperature (°C)*
WAG/Rij	F	6	37.44 ± 0.29
BN/BiRij	M	6	37.06 ± 0.23
WAG/Rij	F	24	37.50 ± 0.31
BN/BiRij	M	24	36.62 ± 0.55

Results expressed as mean ± S.E. of the mean.

In one experiment, we attempted to reduce endotoxin-induced deaths by preventing the fall in body temperature. To this end, a group of five female BN/BiRij rats aged 24 months were housed in a heated cabinet in which the ambient temperature could be controlled. Figure 7 shows the body temperature of all five animals until death or the end of the experiment at 48 h following endotoxin administration. The three surviving animals were removed from the cabinet for a short time at 30 h and

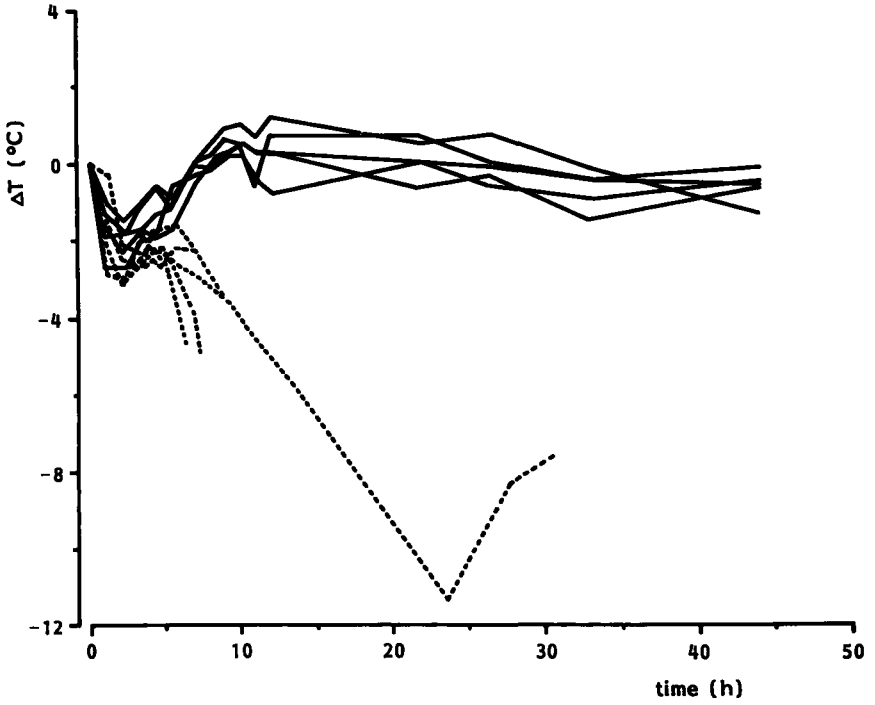

Fig. 4. Changes in body temperature compared with basal body temperature in five male BN/BiRij rats aged 6 months (unbroken lines) and five aged 24 months (dotted lines) following the injection of 1 mg/100 g body weight of endotoxin.

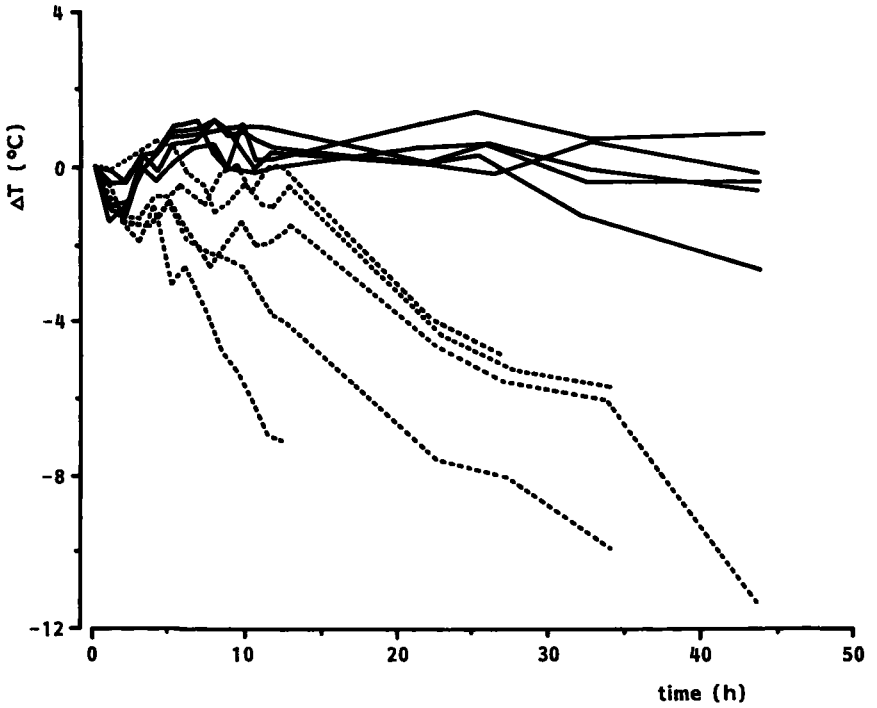

Fig. 5. Changes in body temperature compared with basal body temperature in five female WAG/Rij rats aged 6 months (unbroken lines) and five aged 24 months (dotted lines) injected intravenously with 1 mg/100 g body weight of endotoxin.

were kept at an ambient temperature of 21 °C (Fig. 7, arrow) and body temperature started to fall but returned to normal when the animals were returned to the cabinet. Survival beyond 24 h for rats of this strain given this dose of endotoxin had not been previously observed by us.

Endotoxin elimination

The values for the disappearance half life of endotoxin from the blood obtained by both radioactivity counting and the LAL test are shown in Table III. For any given group, the values obtained with the LAL test were considerably lower than with radioactivity counting, but the relative differences between age groups were similar by either method. The values obtained by radioactivity counting varied only modestly between groups and significant differences were obtained between values in 24-month-old-animals and the two younger age groups for all three doses of endotoxin. In 36-month-old rats, a significant increase in half-life time of disappearance

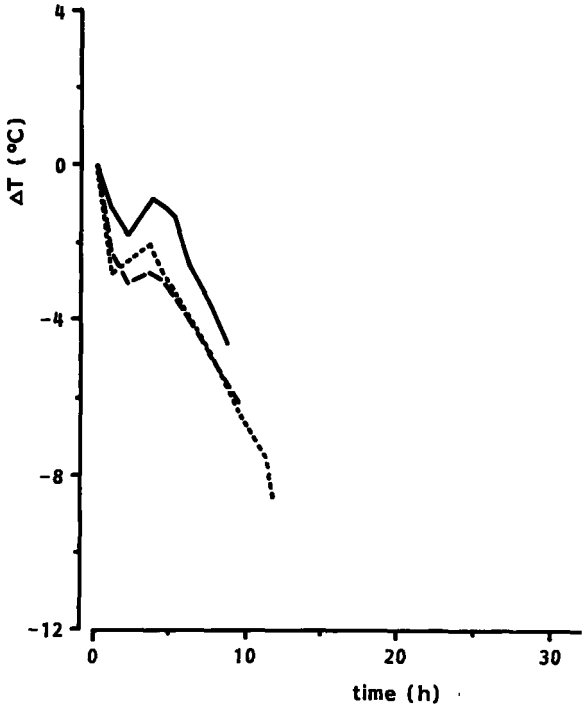

Fig. 6. Changes in body temperature compared with basal body temperature in three BN/BiRij female rats aged 6 months following the intravenous injection of 10 mg/100 g body weight of endotoxin.

was found only with the 2 mg/100 g body weight dose and then, only with radioactivity counting.

Histological findings

Morphological evidence of liver injury was more pronounced in endotoxin - treated aged rats compared with their younger counterparts (Table V). There was a greater inflammatory cell infiltration of polymorphonuclear leukocytes in sinusoids (sinus granulocytosis) in aged rats. Large numbers of polymorphonuclear leukocytes were also observed in close proximity to multifocal areas of hepatocellular injury and necrosis present in aged rats. The cumulative scores of liver damage were significantly greater in female 24-month-old rats of both strains compared with their 6-month-old counterparts (Table V). Acute tubular necrosis, predominantly involving the proximal renal tubular epithelium, was the only category of renal injury that occurred and this lesion was only observed in the aged rats (data not shown).

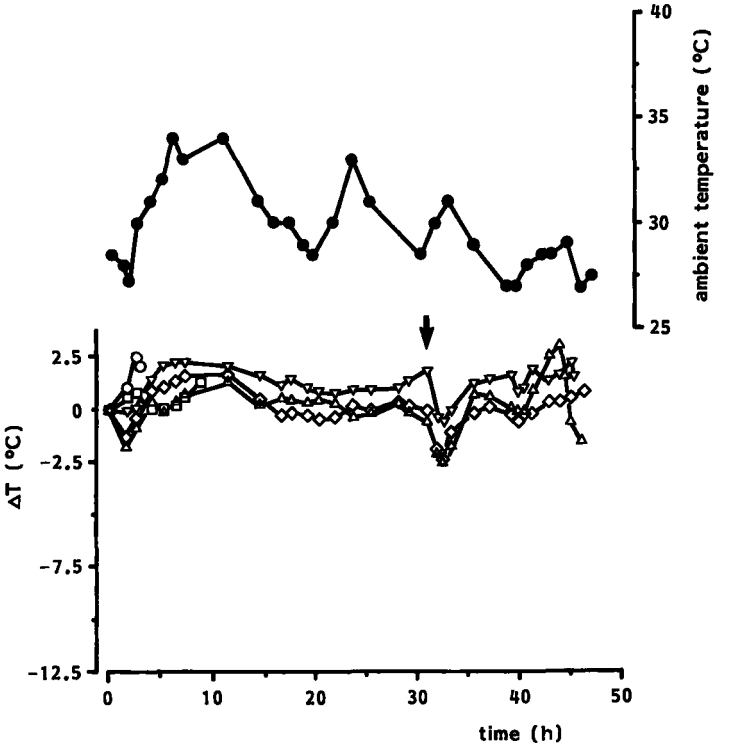

Fig. 7. Changes in body temperature compared with basal body temperature in five female BN/BiRij rats maintained in a thermostatically-controlled cabinet following the intravenous injection of 1 mg/100 g body weight of endotoxin. (Arrow indicates removal from the cabinet for 1 h.)

TABLE V

MEAN HISTOLOGY SCORES FOR LIVERS OF BN/BiRij AND WAG/Rij RATS INJECTED WITH *E. COLI* 026:B6 ENDOTOXIN

Strain	*Sex*	*Age (months)*	*SG*	*PTH*	*IHN*	*FAN*	*Cumulative score*
BN/BiRij	F	6	1.6	0.4	0.4	0.8	3.2 ± 1.2
BN/BiRij	F	18	0.9	0.6	0.8	1.6	3.9 ± 1.7
BN/BiRij	F	24	2.4	0.4	2.6	0.2	5.6 ± 0.8*
BN/BiRij[a]	F	24	1.0	0.8	0.6	1.8	4.4 ± 2.6
BN/BiRij[b]	F	6	2.7	0.7	2.0	0.6	6.0 ± 1.6**
BN/BiRij	M	6	0.3	0.2	0	0.6	1.1 ± 1.1
BN/BiRij	M	24	1.2	0	0.2	0.2	1.6 ± 0.8
WAG/Rij	F	6	0.6	0	0	0.4	1.0 ± 0.8
WAG/Rij	F	24	1.4	0.6	1.2	3.2	6.4 ± 1.0*

[a]These animals were maintained at thermoneutrality in a heated cabinet during the experiment.
[b]These animals were injected with 10 mg/100 g body weight of endotoxin (n = 3). All other animals were injected with 1 mg/100 g body weight (n = 5 per group).
*Differ significantly from their 6-month-old counterparts.
**Differ significantly from their 1 mg per 100 g body weight counterparts.

DISCUSSION

The most distinct finding obtained from the current experiments is the marked difference in sensitivity to the lethal effects of endotoxin between young and old rats of the BN/BiRij and WAG/Rij strains. All animals aged 24 months or more died following the intravenous injection of 1, 2 or 4 mg of endotoxin per 100 g body weight. Within this dose range, old animals receiving the highest dose died at earlier time points. Both male and female animals of the BN/BiRij strain were compared and no difference in response was observed. Only females of the WAG/Rij strain were used. One interesting finding with this strain is that, although all the older animals died, the time course of the events was considerably prolonged over that characteristic of the BN/BiRij strain.

Such an age-related increase in sensitivity to the lethal effects of endotoxin has already been reported for mice. Habicht [4] reported that approximately 65% of 24-month-old BALB/c and C57BL/6J mice were killed by the intravenous injection of 50 μg (approx. 125 μg/100 g body weight) of a phenol extract of *E. coli* O111:B4. No deaths were observed in 3—4-month-old animals while animals of intermediate age showed an intermediate response. Similar results were obtained by Hoffman-Goetz and Keir [5]. Neither of these studies gave details about the precise time course over which the deaths occurred, nor did they give any details of the clinical accompaniments.

We observed that both young and old animals responded similarly during the initial period following endotoxin administration. The behavioural changes were those to minimise heat loss. In addition, shivering was observed but non-shivering thermogenesis was not assessed in this study. As time passed, a return to normal activity was observed in those animals that eventually survived whereas those that eventually died no longer minimised heat loss by behavioural means and exhibited a profound and progressive hypothermia. Prevention of hypothermia had a markedly beneficial effect assessed over 48 h, though it did not prevent deaths in all animals.

In mice, the ability to regulate body temperature has been linked to survival [23]. Aged C57BL/6 mice show greater variation in body temperature over time at an ambient temperature of 22 °C than do younger animals of the same strain. Old animals able to maintain their basal body temperature survived longer, though a progressive fall in body temperature always preceded death. Aged C57BL/6J mice are less able to withstand thermal stresses [24]. Under thermoneutral conditions, old animals (19—20 months) had a significantly lower body temperature than their young (6—7 months) counterparts and were unable to maintain their body temperature at an ambient temperature of 15 °C. Animals of an intermediate age (13—14 months) were able to maintain their body temperature but became hypothermic when a humidity stress was superimposed. It would appear that the abnormality that underlies altered thermoregulation with age commences well before old age since it can be uncovered with the superimposition of a humidity stress. In small animals

(such as rats and mice), heat production is a considerably more important mechanism in thermostasis than the regulation of heat loss [25]. The reverse is true for large animals (such as man), though behavioural responses seem to have equal importance for both large and small animals.

Kiang-Ulrich and Horvath [26] examined the responses of male F344 rats of different ages to a prolonged cold stress of 5 °C. Younger animals (3—12 months old) increased food intake by over 100% and maintained their body weight. Animals aged 21 months increased food intake even more but were unable to maintain body weight. Animals aged 25 months experienced severe reductions in body weight as well as a high mortality (54% at three weeks). Kiang-Ulrich and Horvath [26] suggested that the two older age groups were unable to generate 'metabolic heat' (non-shivering thermogenesis) and that this could explain the high mortality; 'The age-related difference in energy economy and response to cold could be due to different responsiveness in brown adipose tissue at different stages of life'. In rodents, brown adipose tissue is the major effector of non-shivering thermogenesis and heat production may be increased over 50-fold on exposure to cold [27]. Brown adipose tissue mass and activity have been assessed in rats of the BN/BiRij strain during ageing [28]. Animals aged 3, 6, 24 and 36 months were studied and the thermogenic response to noradrenaline was shown to be significantly reduced in the older animals. Brown adipose tissue mass was unaffected by age but tissue protein content, specific mitochondrial cytochrome oxidase activity and thermogenic activity (assessed from mitochondrial purine nucleotide binding) all declined markedly with age.

There is overwhelming evidence that the MPS is the major route through which circulating endotoxins are taken up and eliminated. There is also evidence that the density of Kupffer cells in the hepatic sinusoids at least partly explains the considerable inter-species variation in sensitivity to endotoxins. A number of studies in both man and laboratory rodents using a variety of test colloids have shown that the activity of the MPS declines with age [14,15]. Indeed, these observations were in large part responsible for our undertaking the studies reported here. We have demonstrated a clear age-related decline in the removal from the blood of endotoxin administered as an intravenous bolus. However, it must be emphasised that the very modest prolongation of plasma half-life is unlikely to explain the dramatically increased sensitivity of old animals to endotoxin. Furthermore, the relationship between MPS activity and sensitivity to endotoxins is not simple and attempts to modulate endotoxin sensitivity by enhancing or suppressing MPS activity give rather confusing results (see Ref. 29). Some MPS stimulants (e.g., muramyl dipeptides) sensitise to endotoxins while others (e.g. di-ethyl stilboestrol) have no effect. The reverse is also true; methyl palmitate suppresses MPS activity and reduces sensitivity to endotoxins, while another MPS suppressant, Trypan blue, increases sensitivity.

The MPS may influence sensitivity to endotoxins by mechanisms other than uptake and elimination. MPS products may actually mediate some endotoxin

effects. Beutler and Cerammi [30] have suggested that tumour necrosis factor may be the mediator of endotoxin toxicity. Leukotrienes can also mediate many of the features of endotoxin shock [31]. In rodents and lagomorphs, hepatobiliary elimination with partial or complete cleavage of amino acids is the major route of elimination for amino acid-substituted leukotrienes. This pathway is inhibited by endotoxins and thus, their toxic effects are potentiated. There is also evidence that leukotrienes may mediate endotoxin-induced liver injury [32, 33], the major manifestations of which are hepatocellular necrosis and inflammatory cell infiltrates [34—36]. Despite extensive investigation, the precise mechanisms responsible for endotoxin-induced hepatic injury remain to be elucidated.

In the current study, morphological evidence of liver injury was more pronounced in endotoxin-treated aged rats compared to their younger counterparts. A similar phenomenon has been reported for other organ systems as well. A recent ultrastructural study of the lung compared endotoxin-induced pulmonary injury in young and old endotoxin-treated rats [6]. In that study, pulmonary endothelial cell injury and polymorphonuclear leukocyte microsequestration was more pronounced in the old than in the young rats. Platelet aggregation was observed only in endotoxin-treated old rats. In the current study, a greater inflammatory cell infiltrate of polymorphonuclear leukocytes in sinusoids and in close proximity to areas of hepatocellular necrosis occurred in old rats. In addition, only the old animals had evidence of renal tubular necrosis, presumably reflecting compromised renal perfusion.

In conclusion, the results of the current studies indicate that aged rats of the BN/BiRij and WAG/Rij strains are considerably more sensitive to the toxic effects of endotoxins than their younger counterparts. With the doses used, all the old animals died while only sporadic deaths were observed among the young animals. The most obvious accompaniment of this increased sensitivity is the failure to maintain body temperature. There is also a profound disturbance in carbohydrate metabolism (to be reported elsewhere). The explanation for this enhanced sensitivity is most likely multifactorial and a manifestation of age-effects in numerous systems which are uncovered by the stress of endotoxaemia.

ACKNOWLEDGEMENTS

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CHAPTER 3

PLATELET PARTICIPATION IN THE INCREASED SEVERITY OF ENDOTOXIN-INDUCED PULMONARY INJURY IN AGED RATS

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PLATELET PARTICIPATION IN THE INCREASED SEVERITY OF ENDOTOXIN-INDUCED PULMONARY INJURY IN AGED RATS

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SUMMARY

Recent studies have demonstrated that aged rats are more susceptible to the lethal effects of endotoxin as compared with young rats. The morphogenesis of early endotoxin-induced pulmonary injury in young (6 months) and aged (24 months) rats was examined by combined light and transmission electron microscopy to elucidate cell populations that may be responsible for these effects. Pulmonary endothelial cell injury was of greater severity and occurred at earlier time periods in aged rats as compared with young rats. Platelet sequestration and aggregation were observed only in aged rats in this study, and occurred in conjunction with the initial degenerative changes in the endothelium. Morphological evidence of granulocyte degranulation and fragmentation was also observed only in aged rats. These results suggest that pulmonary endothelial cells of aged rats are more susceptible to endotoxin-induced injury and that platelets may play an important role in the enhancement of initial endothelial damage. Furthermore, the extent of injury to the endothelial cell population may play an important role in accounting for differences in endotoxin-induced mortality between young and aged rats.

KEY WORDS—Endotoxin, age-related response, pulmonary endothelial cell, platelets

INTRODUCTION

Lipopolysaccharide components of Gram-negative bacteria (endotoxins: ET) are extremely biologically-active substances which have a wide spectrum of target organs, the lung being prominent among these.[1,2] There is a broad range of sensitivity to the effects of ET between various animal species, but the pathophysiological responses to intravenous infusion of the substance appear qualitatively similar.[1] The clinical relevance of the effects of ET has generated numerous studies over the years to elucidate the pathogenesis and pathophysiology of ET-induced injury and mortality. Despite the extensive attention that this subject has received, the precise mechanism(s) responsible for these effects remains unclear.

Recent clinical and experimental data indicate that ET may be involved in a number of age-related changes and that the biological responses of elderly individuals to ET-induced effects may differ markedly from young adults.[3,4] Previous studies have demonstrated increased susceptibility and mortality in aged rats following the systemic injection of ET.[5,6] The pathophysiological effects of ET were similar in the young and aged of different strains of rats, which indicates that a common mechanism(s) is most likely responsible for the observed age-related effects in this species.[5] The sequential development of ET-induced pulmonary injury in young (6 months) and aged (24 months) rats was examined by combined light and transmission electron microscopy in order to characterize the participation of various cell

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populations in this process, and to delineate those populations that may be responsible for observed differences in susceptibility and mortality.

MATERIALS AND METHODS

Young (6 months) and aged (24 months) female BN/Rij rats were fed pelleted food (Diet AM II, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The rats were free of common bacterial and viral pathogens as determined by microbiological, serological, and histopathological monitoring.[7] The rats received 1 mg of endotoxin (ET) (phenol–water extract of *Escherichia coli* 026: B6 purchased from Sigma Chemical Company, St. Louis, MO) per 100 g body weight administered as a single bolus via the jugular vein. Young and aged ET-treated rats ($n=2$ per group per time period) were randomly selected and killed by exsanguination after anaesthesia at 15 min, 30 min, 1 h, 3 h, 5 h, and 7 h following injection. Young and aged controls ($n=2$ per group per time period) were killed in a similar manner at 15 min and 7 h after saline injection.

The tracheas were exposed and cannulated. The lungs were fixed *in situ* by tracheal perfusion at a constant pressure of 20 cm of water and at 4°C with modified Karnovsky's fixative in 0·15 M cacaodylate buffer, pH 7·4.[8] Two 1-mm thick transverse sections of the left lung were cut for embedding in Epon by routine methods after 24 h of fixation. The first section was taken immediately caudal to the hilus; the second section was taken midway between the hilus and the most caudal aspect of the lung. Each lung specimen was then cut into six to seven equivalent size pieces, embedded in Epon by routine methods, sectioned at 0·5 μm, stained with methylene blue-Azure II, and examined by light microscopy. The identification of the segments of the microvasculature was based on topographical ocation and ultrastructure of a given blood vessel.[9–11] 0·5-μm thick sections, of which at least 75 per cent of the specimen contained alveolar parenchyma, were examined at ×400 magnification by light microscopy. The number of cells having morphological features of granulocytes (multilobulated nucleus, cell diameter approximately 12 μm) per high-power field was enumerated. A minimum of 30 alveolar fields from each animal were examined. Ultrathin sections from each block were supported on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 410 transmission electron microscope (Philips, Eindhoven, The Netherlands).

RESULTS

The morphological alterations are tabulated according to temporal sequence (Table I). No pulmonary morphological alteration was observed in young or aged rats at 15 min after saline injection. Similar numbers of granulocytes were observed in saline-treated young and aged rats (data not shown), and the data were pooled for graphical representation (Fig.1). The mean number of granulocytes per high-power field were higher in ET-treated aged rats as compared with both ET-treated young animals and saline-treated age-matched controls at all times examined (Fig. 1). There was an accumulation of granulocytes, predominantly neutrophils and small numbers of eosinophils, in close proximity to capillary endothelium as early as 15 min in both young and aged ET-treated rats (Fig. 2). Morphological alterations in the endothelium were initially observed in aged rats at the initial sampling time after ET administration. These changes included the attenuation, vesiculation, and separation and loss of capillary endothelial cell cytoplasmic processes from the underlying basal lamina (Fig. 3). Similar changes of greater frequency and severity were observed in the endothelium lining capillaries and small muscular arteries of aged rats at 30 min after ET administration. Alterations in the endothelium of young rats were rare, initially observed at 30 min after ET, and consisted of attenuation and vesiculation of endothelial cell cytoplasmic processes.

Alterations in platelet morphology, including loss of discoid contour and secretory granules, occurred only in aged rats. Platelet sequestration was frequently observed in capillaries and small muscular arteries of aged rats by light microscopy at 15 and 30 min (Fig. 4). At 30 min, platelet degranulation in association with endothelial cell injury was observed by electron microscopy in aged rats (Fig. 5). Platelet sequestration and aggregation were not present in young rats at any time after ET administration.

Neutrophils of aged rats, some of which were degranulated, were present in the interstitium at 30 min after ET injection. At 1 h after treatment, most neutrophils of ET-treated aged rats lacked secretory granules, were occasionally fragmented, and were

Table I—Temporal sequence of early ET-induced pulmonary morphological alterations in young and aged rats

Time	Young rats	Aged rats
15 min	PMN recruitment	Mild endothelial cell injury, platelet sequestration, PMN recruitment
30 min	Rare endothelial injury	Widespread endothelial injury, platelet aggregation, occasional PMN degranulation, interstitial oedema
1 h	Similar to 30 min	Widespread PMN degranulation and occasional fragmentation
3–7 h	Similar to 30 min	Alveolar haemorrhage and oedema

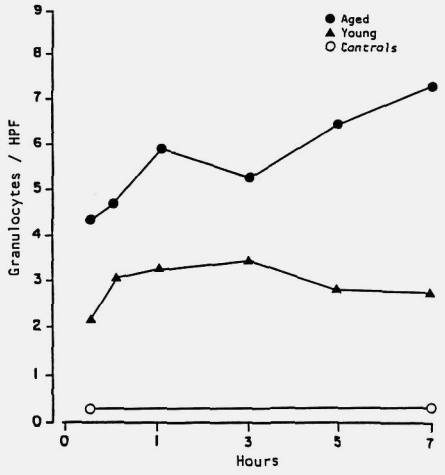

Fig. 1—Mean number of granulocytes per high-power field of endotoxin (ET)-treated young rats (▲), ET-treated aged rats (●), and pooled age-matched saline-treated controls (○)

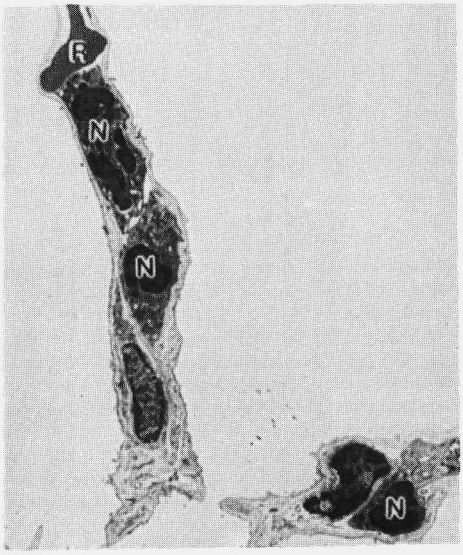

Fig. 2—Transmission electron micrograph (TEM) of alveolar septa of aged rat at 15 min after ET administration. Three neutrophils (N), containing numerous cytoplasmic granules, are present in adjacent capillary lumens. R = Erythrocyte. (Lead citrate and uranyl acetate, × 3000)

accompanied by interstitial oedema (Fig. 6). Sparse fibrogranular material and mild haemorrhage were initially observed by light microscopy in the alveolar spaces of aged rats at 3 h after treatment, and were increased in severity and frequency at 5–7 h. This granular material was determined by electron microscopy to be predominantly fibrin aggregates. Alveolar oedema or haemorrhage were not observed in young rats by either light or electron microscopy at any time after treatment.

DISCUSSION

The results of this study demonstrate that ET-induced pulmonary endothelial cell injury occurred at earlier time periods and were of greater frequency and severity in aged rats as compared

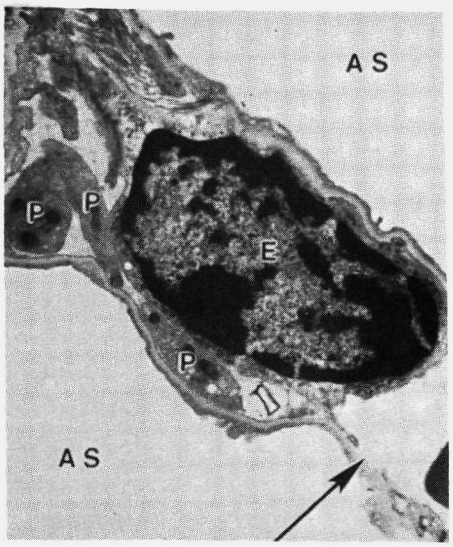

Fig. 3—TEM of alveolar septum of aged rat at 15 min after ET administration. There is focal loss of the endothelial (E) cell cytoplasmic process (arrow) from the subjacent external lamina. Numerous platelets (P) are present in the capillary lumen. AS = Alveolar space. (Lead citrate and uranyl acetate, × 9800)

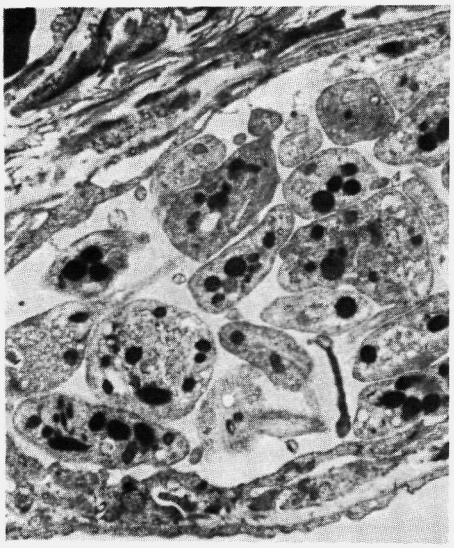

Fig. 4—TEM of small calibre pulmonary vessel of an aged rat at 30 min after ET administration. Numerous platelets occupy the vascular lumen. Several platelets have lost their secretory granules and discoid contour. (Lead citrate and uranyl acetate, × 10 900)

with young rats. Numerous possibilities and/or their combinations could account for the greater severity of ET-induced pulmonary injury in aged rats: (1) increased platelet–endothelial interactions in aged versus young rats; (2) increased granulocyte recruitment and degranulation in aged rats; and (3) increased susceptibility of endothelium of aged rats to the direct effects of endotoxin.

An intriguing possibility that arises from the current study is that the platelet is the cell population initially responsible for the marked differences in the severity of endothelial cell injury observed between young and aged rats, and that platelet–endothelial cell interactions play an important role in the development of ET-induced pulmonary injury in aged rats. Other studies that demonstrated platelet-induced endothelial cell injury lend further support for this premise.[12,13] Platelet sequestration and aggregation in close proximity to pulmonary endothelium were observed only in aged rats and occurred in conjunction with the initial endothelial changes.

Platelets may also play an important role in ET-induced pulmonary injury in elderly people. A recent study demonstrated an increase in platelet sensitivity to aggregation agents in platelets obtained from elderly persons.[14] Other investigators working with experimental animals reported that platelets from adult 'aged' rats (11 months) were more reactive upon exposure to an aggregating agent, collagen, when compared with young rats (1 month), as determined by a lower threshold to the aggregating agent, greater amplitude of the aggregation curve, and higher thromboxane B_2 formation.[15] These investigators also demonstrated a compensatory mechanism of higher vascular prostacyclin production in aged rats to counteract the greater platelet aggregation. These physiological and biochemical results may help to explain the proposed mechanism responsible for the observed morphological alterations in platelets in the current study, i.e., ET-induced injury to the endothelium impairs normal prostacyclin production and the endothelium is unable to counteract the greater platelet aggregation occurring in aged rats. Similar physiological and biochemical studies have not been performed in the strain of rat used in this study.

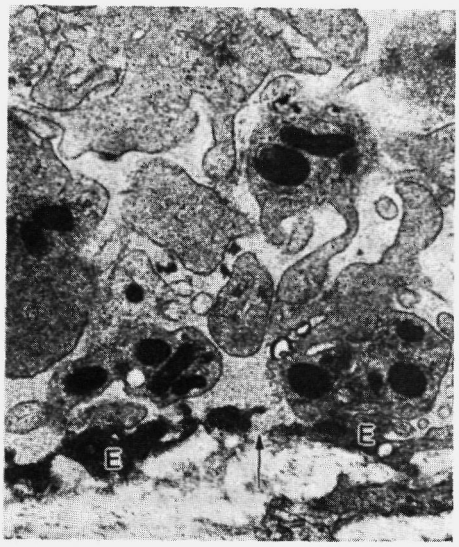

Fig. 5—TEM of a small peribronchiolar artery of aged rat at 30 min after ET administration. A platelet aggregate containing numerous markedly swollen platelets lacking secretory granules is in close association with focal loss of the endothelial (E) cell cytoplasmic process (arrow). (Lead citrate and uranyl acetate, × 19 700)

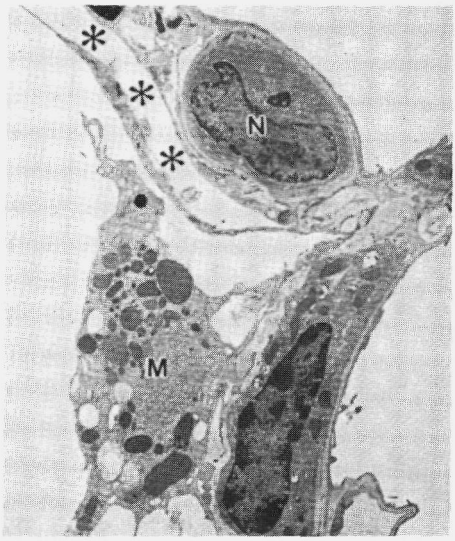

Fig. 6—TEM of aged rat at 1 h after ET. There is increased electron lucency of the interstitium indicative of oedema (asterisks) accompanied by a neutrophil (N) lacking secretory granules in the capillary lumen. A macrophage (M) is present in the alveolar space. (Lead citrate and uranyl acetate, × 4500)

Transient platelet sequestration in pulmonary capillaries has also been observed in ET-treated rabbits[16] and dogs.[17] Moderate to marked platelet degranulation was observed in aged rats in this study, whereas slight variation in the platelet contour without degranulation was reported in the rabbit.[16] Alterations in platelet morphology were not determined in the study in dogs. These studies in rabbits and dogs were also not performed in aged animals.

Initial alterations in platelet and endothelial cell morphology occurred at the first sampling time (15 min) in the current study only in aged rats. Additional studies that examine time periods prior to 15 min will be required to determine the sequential development of changes. The morphological changes in platelets of aged rats observed in the current study suggest that platelets must, at the very least, contribute to the development of ET-induced pulmonary injury in aged rats. Furthermore, other investigators have demonstrated that platelet aggregation and degranulation induce a transient increase in hydrostatic pressure,[18,19] which could enhance the development of pulmonary oedema and promote mortality. Additional studies will be required to determine whether changes in physiological parameters are also different between young and aged rats following ET administration. Another area for future studies would be to determine whether direct ET-induced effects are different between platelets from various age groups.

The extensive neutrophil degranulation observed in this study indicates that neutrophils contribute to the increased severity of endothelial cell injury in aged rats. The pronounced and persistent recruitment of neutrophils into the lungs of aged rats following ET is not surprising when viewed in the context of the pronounced and diffuse pulmonary endothelial cell injury. It is well recognized that neutrophils contain many enzymes capable of inducing cellular injury, and endothelial cell alterations have been reported following sequestration of neutrophils within pulmonary capillaries.[20,21] The widespread neutrophil degranulation that occurred in aged rats in this study most likely does not reside in a mechanism(s) related to increased sensitivity, similar to that proposed for platelets. This

preliminary conclusion is based on studies in mice and humans which report that aging results in significant reductions in numerous parameters of neutrophil functions following stimulation, including respiratory burst, exocytosis, and enzyme release.[22,23] Another investigator demonstrated that neutrophils of aged mice responded to perturbations induced following ET injection in a manner qualitatively similar to that occurring in young mice.[24] Similar studies in the strain of rat used in this study have not been performed. The neutrophil was the predominant cell recruited into the lung in the current study, whereas in the ovine experimental model of ET-induced lung injury, neutrophils and lymphocytes occur in equivalent numbers.[20] Thus, the lymphocyte may play a more substantial role in the ovine experimental model as compared with the rat model.

Another possibility to account for the increased severity of pulmonary injury in aged rats is that the endothelium of aged rats is more susceptible to the direct effects of ET. Although controversy still exists, current dogma suggests that endotoxin per se can cause direct damage to the endothelium, but the severity is less than that observed in the presence of granulocytes, complement, and other mediators.[25] Recent studies have examined the dichotomy of response of human and bovine endothelial cells to ET-induced toxicity and determined that the difference in response was mediated via the ability of the endothelium to oxidize the low density lipoprotein–endotoxin lipopolysaccharide complex.[26] It has not been determined whether a similar pathway exists in rats, or that alterations occur in this pathway with aging.

In conclusion, ET-induced pulmonary endothelial cell injury is more pronounced in aged rats as compared with young rats. The exact mechanism(s) responsible for the marked differences between young and aged animals in the severity and extent of ET-induced pulmonary endothelial cell injury remains to be elucidated. Subsequent experiments will be required to determine whether the widespread endothelial cell damage observed in this study predominantly originates from direct ET-induced endothelial cell cytotoxicity or is indirectly related to platelets, granulocytes, or other biological effects following ET administration.

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CHAPTER 4

COMPARATIVE ENDOTOXIN-INDUCED HEPATIC INJURY IN YOUNG AND AGED RATS

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SUMMARY

Recent studies have demonstrated that aged rats are more susceptible to the lethal effects of endotoxin (ET) than young rats. The early (15 min to 7 h) hepatic ultrastructural and biochemical changes induced by ET in young (6 months) and aged (24 months) rats were evaluated to elucidate cell populations and/or the mechanisms that may be responsible for the previously observed differential effects. Aged rats given ET had significantly increased numbers of neutrophils in hepatic sinusoids at 30 min and thereafter as compared with ET-treated young rats. Morphologic evidence of coagulation within hepatic sinusoids, including aggregates of fibrin enmeshed among polymorphonuclear leukocytes and platelet aggregates, was frequently observed in ET-treated aged rats but not in ET-treated young rats. In contrast, Kupffer cells of ET-treated young rats frequently contained phagocytized neutrophils and platelets, whereas this phenomenon was rarely observed in Kupffer cells of ET-treated aged rats. Hepatocellular morphologic injury was more pronounced and occurred at earlier time periods in ET-treated aged rats, and was accompanied by significant increase in hepatic transaminases. ET-treated aged rats had an earlier onset and greater severity of endothelial cell injury than did ET-treated young rats. The results of this study indicate a greater aggregation of blood elements in the hepatic sinusoids of aged rats following the intravenous administration of ET, which suggests that a greater diminution in microcirculation was induced in aged rats by ET. Additionally, the increased phagocytosis of inflammatory cells by Kupffer cells of young rats may be a mechanism which affords protection against endotoxin-induced lethality.

KEY WORDS—Endotoxin, liver, aged rats, ultrastructure.

INTRODUCTION

Endotoxins (ETs) are extremely biologically active substances derived predominantly from the lipopolysaccharide wall components of Gram-negative bacteria. Hepatic injury is a prominent feature observed in man and experimental animals following endotoxaemia.[1] Although there is a broad range of sensitivity to ET-induced lethality between various experimental animal species, the administration of ET results in similar pathophysiologic responses.[2] Numerous investigators have examined potential mechanism(s) responsible for ET-induced hepatic injury. Despite the extensive attention that this subject has received, the precise mechanism(s) responsible for the hepatic injury has yet to be elucidated, but popular hypotheses include ischaemia due to sinusoidal thrombosis and decreased sinusoidal blood flow, [3,4] release of lysosomal enzymes and cytokines from polymorphonuclear leukocytes and Kupffer cells,[5–7] and a direct cytotoxic effect on hepatic parenchymal cells.[8]

Clinical investigations indicate that the biological responses of elderly individuals to ET-induced effects may differ markedly from those of young adults.[9,10] Recent biochemical and morphological studies in experimental animals have demonstrated an increased severity of hepatic injury and mortality in aged rats following the intravenous injection of ET.[11,12] The age-related pathophysiologic effects induced by the administration of ET were similar in different strains of rats, which suggests that common mechanism(s) are most likely responsible for the increased susceptibility in older animals of this species.[12] ET pharmacokinetics have also been examined in the young and aged of this strain of

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rat.[13] In both young and aged rats, most of the injected ET was rapidly cleared from the circulation, which resulted in similar plasma ET levels during the first few hours after injection. During the elimination phase, a slight, but statistically significant, increase in the terminal half-life of endotoxin occurred in aged rats. It was concluded from the study that the increased susceptibility of aged rats to endotoxin did not reside in changes in the handling and distribution of endotoxin, but rather was due to differences in the sensitivity of target tissues to the primary and secondary consequences of a comparable degree of endotoxaemia. In this study, the sequential development of ET-induced hepatic injury in young (6 months) and aged (24 months) rats was examined by both light and transmission electron microscopy in order to characterize the participation of various cell populations in this process, and thereby delineate those populations and/or mechanism(s) that may be responsible for the observed differences in age-related susceptibility and mortality.

MATERIALS AND METHODS

Animals

Young (6 months) and aged (24 months) female BN/BiRij rats were fed pelleted food (Diet AM II, Hope Farms, Woerden, The Netherlands) and water *ad libitum*, maintained on a 12 h light cycle, and housed under 'clean conventional conditions'.[14] Life span characteristics of this rat strain include a 50 per cent survival of 32 months and a maximal life-span of approximately 41 months. Spontaneous pathology in this rat strain has been described previously.[15] The rats were free of common bacterial and viral pathogens as determined by microbiologic, serologic, and histopathologic monitoring.[16]

Experimental design

Rats under light ether anaesthesia received either saline (sham) or 1 mg endotoxin (phenol–water extract of *E. coli* 026 : B6 purchased from Sigma Chemical Company, St. Louis, MO) per 100 g body weight administered as a single bolus via the right jugular vein. The effects of anaesthesia lasted approximately 3–5 min. Animals that received endotoxin ($n=2$ per age and treatment group per time period) and to be used in electron microscopic studies were selected randomly and killed at 15 min, 30 min, 1 h, 3 h, 5 h, and 7 h. Age-matched controls to be used in electron microscopic studies were killed in an identical manner at 15 min and 7 h after saline injection.

Animals were anaesthetized to a surgical plane by vaporizing halothane with air to a concentration of 2 per cent. The abdominal viscera was exposed by an incision through the linea alba to the diaphragm, through the abdominal musculature parallel to the diaphragm to the dorsal surface, and the musculature was laid back. Blood was obtained via the vena cava; serum was collected. A cannula was inserted into the carotid artery and the animal was perfused with chilled (4°C) saline using a 2115 multiperpex peristaltic pump (LKB, Bromma, Sweden) at a perfusion rate of 8–10 ml/min until the vascular system was adequately perfused (approximately 2 min). The median lobe of the liver was ligated and excised for determinations not addressed in this paper. The perfusate was then changed to chilled (4°C) modified Karnovsky's fixative[17] in 0·15 M cacodylate buffer, pH 7·2, at a flow rate of 6–8 ml/min until adequate fixation (approximately 6 min). One to two millimetre-thick transverse specimens of the liver were post-fixed in 1 per cent osmium and embedded in EPON.

Plastic-embedded liver sections 1·5 μm-thick were cut with glass knives and stained with toluidine blue. These sections were examined at × 400 magnification by light microscopy. The number of cells having morphological features of polymorphonuclear leukocytes (multilobulated nucleus, cell diameter approximately 12 μm) per high-power field (HPF) was enumerated. The number of cells having morphological features indicative of phagocytized degenerating PMNs (multilobulated pyknotic nucleus with a pale-staining cytoplasm surrounded by sinusoidal cell cytoplasm) per high-power field (HPF) was also enumerated. A minimum of 25 HPFs from each animal were examined. Semi-quantitative data were analysed by Kruskall–Wallace followed by Dunn's multiple comparisons according to Newman–Keuls.[18] Areas of interest were mounted on size 0 epoxy blocks, cut into large trapezoid specimens with a TM60 trim machine (Reichert, Vienna, Austria), sectioned at 0·5 μm with glass knives, stained with either basic fuchsin–methylene blue–azure II or toluidine blue, and examined by light microscopy. The specimens were further cut into small trapezoids for thin-sectioning. Thin sections were supported on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 410 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Table I—Temporal sequence of early ET-induced hepatic morphologic alterations in young and aged rats

Time	Young rats	Aged rats
15 min	Kupffer cell phagocytosis of platelets; increased Kupffer cell phagosomes	Sinusoidal platelet aggregation; increased Kupffer cell phagosomes
30 min	Kupffer cell phagocytosis of PMNs; sinusoidal fibrin aggregates	Sinusoidal PMN influx; focal endothelial cell injury; sinusoidal and space of Disse fibrin aggregates
1 h	Similar to 30 min	Hepatocyte cytoplasmic/nuclear invagination and mitochondrial swelling; prominent autophagosomes in fat-storing cells
3 h	Similar, but less frequent and severe, changes to those in aged rat hepatocytes; rare sinusoidal thrombosis	Hepatocyte glycogen depletion, increased vesicular bodies, dilated endoplasmic reticulum; widespread sinusoidal thrombosis
5–7 h	Similar to previous periods	Progression in frequency and severity of hepatocyte injury and sinusoidal thrombosis

A minimum of three specimens were examined ultrastructurally at each sampling time from every individual perfused ET-treated rat or vehicle-treated control.

Other animals ($n=3$–5 per age and treatment group per time period) were treated with endotoxin or saline in an identical manner to the animals used for electron microscopic evaluation, and killed at similar (excluding 15 and 30 min) and additional (2 and 4 h) time points. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using a Reflotron automatic analyser (Boehringer, Mannheim, F.R.G.). Serum transaminase values were statistically analysed by Student's t-test followed by Bonferoni correction.[19]

RESULTS

No morphologic alterations were observed in vehicle-treated (sham) young and aged rats at 15 min after injection. The predominant differences observed in hepatic cell populations of young and aged vehicle-treated rats were the increased size and the number of lipid droplets in fat-storing cells of aged rats.

Endotoxin-induced morphologic alterations are tabulated according to temporal sequence (Table 1). At the initial time period examined, 15 min following endotoxin (ET) administration, morphologic alterations were observed in the Kupffer cells of both young and aged rats. The predominant change consisted of an increase in the number and size of phagocytic vacuoles accompanied by the disappearance of primary lysosomes. In young rats, Kupffer cell phagocytosis of platelets, many of which still contained secretory granules, was frequently observed at 15 min after ET (Fig. 1a), whereas phagocytosis of platelets by Kupffer cells rarely occurred in aged rats (Fig. 1b). Morphological features of platelet aggregation, including degranulation and disruption of platelet membranes, were frequently observed in aged rats, but not in young rats at 15 min after ET. There were larger numbers of polymorphonuclear leukocytes (PMNs) in the liver of both ET-treated young and aged rats at all time periods as compared with their age-matched vehicle-treated cohorts (Fig. 2a). A significant increase in the number of PMNs was observed in the liver of ET-treated aged rats at 30 min and thereafter as compared with ET-treated young rats ($P<0{\cdot}05$) (Fig. 2a). In marked contrast, greater numbers of PMNs were observed in Kupffer cell phagosomes of ET-treated young rats as compared with ET-treated aged rats ($P<0{\cdot}05$) (Fig. 2b). Kupffer cells of ET-treated young rats were prominent and had large phagocytic vacuoles containing degenerating PMNs, which was readily evident by both light and transmission electron microscopy (Figs 3a and 3b). Phagocytosis of PMNs by Kupffer

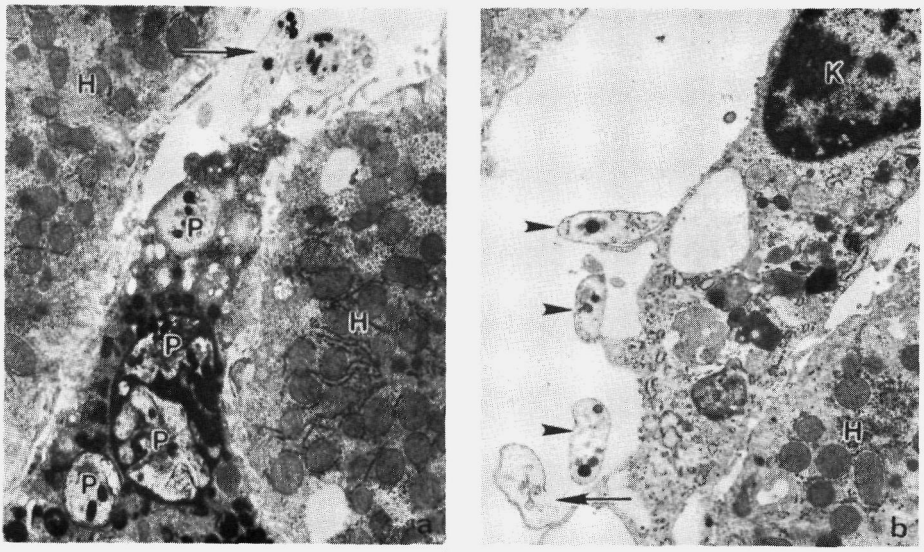

Fig. 1—(a) Transmission electron micrograph (TEM) of the liver of a young rat 15 min after endotoxin (ET). Numerous platelets (P) containing several secretory granules are present within phagolysosomes of the Kupffer cell. A couple of platelets are present in the sinusoidal lumen (arrow). H = Hepatocyte. (b) TEM of an aged (24 months) rat 15 min after ET. There is a marked decrease in the number of electron-dense lysosomes within the Kupffer (K) cell. Numerous platelets within the sinusoidal lumen have decreased numbers of secretory granules (arrowheads). There is focal disruption of the platelet plasma membrane accompanied by loss of secretory granules (arrow). H = Hepatocyte. Lead citrate and uranyl acetate; × 6480

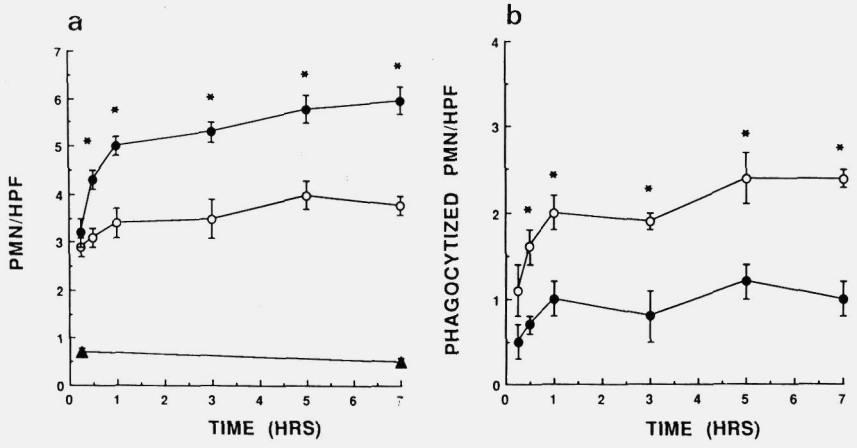

Fig. 2—(a) PMNs per high-power field (HPF) of ET-treated young rats (○) and ET-treated aged rats (●). PMNs in vehicle-treated (▲) young and aged rats were similar. $*P < 0{\cdot}05$, ET-treated aged rats as compared with ET-treated young rats. (b) Phagocytized PMNs per HPF of ET-treated young rats (○) and ET-treated aged rats (●). $*P < 0{\cdot}05$, ET-treated aged rats as compared with ET-treated young rats. Data represent the means and standard deviation of the means

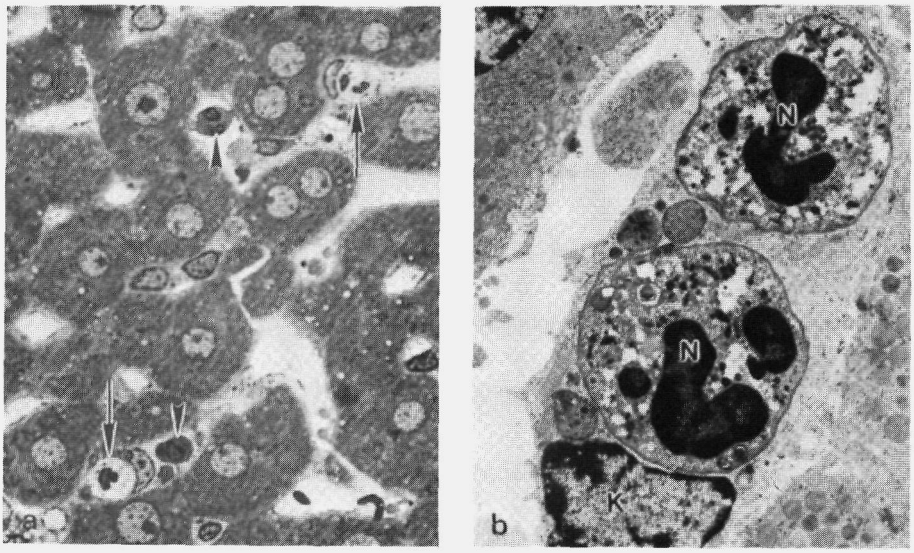

Fig. 3—(a) Light photomicrograph of the liver of a young rat at 30 min after ET. There are two degenerating neutrophils (arrows) with translucent cytoplasm which have been phagocytized by Kupffer cells. Note the large elipsoidal nucleus of the Kupffer cell adjacent to the degenerating neutrophils. Other neutrophils (arrowhead) are also present within the sinusoidal lumen. Methylene blue–azure II; × 600. (b) TEM of the liver of a young rat 30 min after ET in which two degenerating neutrophils (N) are contained within phagolysosomes of the Kupffer (K) cell. Lead citrate and uranyl acetate; × 5744

cells of ET-treated aged rats was rarely observed (Fig. 2b). Kupffer cell phagocytosis of leukocytes did not occur in age-matched vehicle-treated controls. Focal swelling of endothelial cell cytoplasmic processes not associated with morphologic evidence of intravascular coagulation was occasionally observed only in aged rats at 30–60 min after ET (Fig. 4). Fibrin aggregates were frequently observed at this time in the sinusoidal lumen of both young and aged rats, and occasionally in the space of Disse of aged rats (Fig. 5). Injury of hepatocytes was observed only in aged rats at 60 min after ET and consisted of cytoplasmic invagination of the nucleus and occasional mitochondrial swelling accompanied by increased electron lucency (Fig. 5). Large autophagosomal structures, containing abundant fibrillar membranous and lipid material, were frequently observed in fat-storing cells of ET-treated aged rats at this time period (Fig. 6). Similar autophagosomal structures were rarely observed in saline-treated aged rats. The previously described changes in endothelial or fat-storing cells of aged rats at 30–60 min after ET were not observed in ET-treated young rats.

There was an earlier and significant elevation of hepatic transaminases (ALT, AST) of ET-treated aged rats as compared with ET-treated young rats (Figs 7a and 7b). At 3 h, there was a marked decrease in glycogen granules accompanied by an increase in multivesicular bodies in the hepatocytes of ET-treated aged rats. Additional changes observed in the hepatocytes of aged rats included swelling and vesiculation of the rough endoplasmic reticulum, swelling of the perinuclear envelope, and mitochondria within autophagosomal vacuoles (Fig. 8). Similar, but less severe and less frequent, morphologic changes in hepatocytes occurred at 3 h in ET-treated young rats. Morphologic alterations indicative of sinusoidal thrombosis, including aggregates of platelets that had lost their discoid contour and secretory granules enmeshed among fibrin aggregates and PMNs, were frequently observed within the sinusoidal lumens of ET-treated aged rats (Fig. 9). Sinusoidal thrombosis

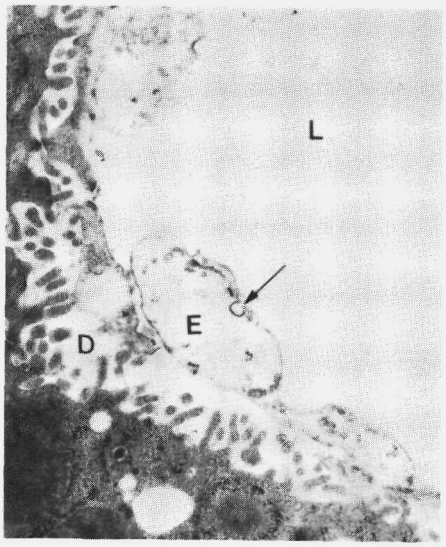

Fig. 4—TEM of the liver of an aged rat at 30 min after ET in which there is focal swelling of the endothelial (E) cell cytoplasmic processes. The endothelial cell has prominent surface calveoli (arrow). No morphologic evidence of coagulation within the sinusoidal lumen (L) or space of Disse (D) is present. Lead citrate and uranyl acetate; × 16 520

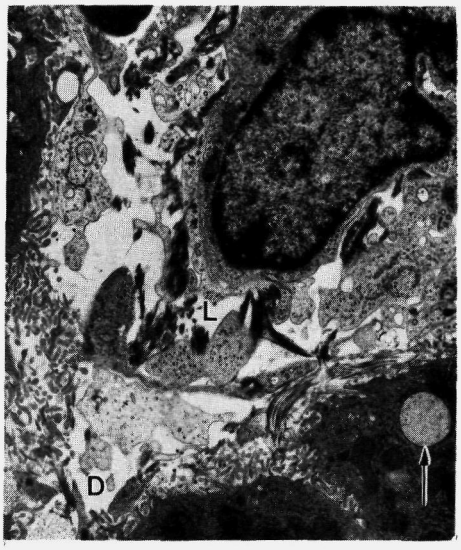

Fig. 5—TEM of the liver of an aged rat at 60 min after ET. Fibrin aggregates are present within the sinusoidal lumen (L) and space of Disse (D). There is swelling and increased electron lucency of a mitochondrion (arrow) within a hepatocyte. Lead citrate and uranyl acetate; × 8990

rarely occurred in young rats. No additional morphologic alterations were observed in young and aged rats at 5 and 7 h after ET. However, there was a progression in the frequency and severity of sinusoidal thrombosis and hepatocellular injury in aged rats.

DISCUSSION

The results of this study indicate a greater aggregation of blood elements in the sinusoids of ET-treated aged rats. Previous studies have demonstrated the importance of sinusoidal thrombosis in the development of ET-induced hepatic injury and the reduction or abrogation of hepatic damage by anti-coagulant therapies.[20,21] Numerous pathophysiologic mechanisms could partially account for the observed difference in occurrence of ET-induced intravascular coagulation between young and old rats, including differences in onset and severity of endothelial cell injury or platelet aggregability, alterations in hepatic blood flow and microcirculation, or differential activation of the intrinsic coagulation system.

Endothelial cell injury occurred at earlier time periods in aged rats in the present study. Additionally, endothelial cell injury not associated with adjacent intravascular coagulation was observed only in aged rats. This observation suggests that the endothelium of aged rats is more susceptible to the detrimental effects of ET as compared with young rats. This is consistent with other reports in which widespread endothelial cell damage was observed in the lungs of aged rats (but not those of young rats) of this strain following the administration of the same dose of ET.[22]

Increased platelet aggregability in aged rats is another likely contributing factor. Other investigators have documented that platelets from adult 'aged' rats (11 months) were more reactive upon exposure to an aggregating agent (collagen) when compared with young rats (1 month) as determined by a lower threshold to the aggregating agent, a greater amplitude of the aggregation curve, and greater thromboxane B_2 formation.[23] These investigators also documented a compensatory mechanism of higher vascular prostacyclin production in aged rats to counteract the greater platelet

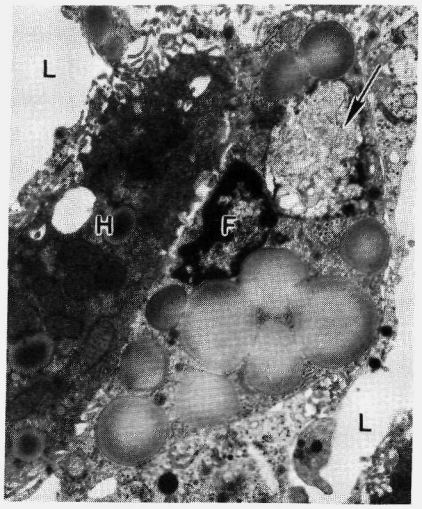

Fig. 6—TEM of the liver of an aged rat at 60 min after ET. A large autophagosomal structure (arrow) containing fibrillar, membranous and lipid material is present within the fat-storing (F) cell. H, hepatocyte; L, sinusoidal lumen (lead citrate and uranyl acetate, × 8200)

aggregability. However, the ET-induced injury to the endothelium observed in the present study would most likely impair normal prostacyclin production and the endothelium would be unable to counteract the greater platelet aggregability occurring in aged rats. Increased platelet sensitivity to aggregation agents has also been observed in platelets obtained from elderly persons.[24] Similar physiological and biochemical studies on platelet aggregability have not been performed in the strain of rat used in this study.

Differential alterations in hepatic blood flow and microcirculation are another mechanism which could partially account for the observed dissimilarity in the occurrence of ET-induced intravascular coagulation between young and aged rats. A 'pre-existing' age-related decline in hepatic blood flow is not likely, as, in contrast to man, it appears that hepatic blood flow in rats is minimally affected by ageing after reaching maturity.[25] Although ET will induce a decreased hepatic blood flow and alter the hepatic microcirculation in rodents,[26–28] age-related differences that may exist in the hepatic circulation following endotoxaemia have not been examined directly. If there were an age-related decline in the hepatic blood flow in response to ET, it would possibly result in dichotomous effects in terms of liver damage; i.e., milder effects in Kupffer cells due to decreased ET uptake and a greater effect on the endothelium by ET that was not phagocytized by Kupffer cells. This hypothesis is supported by recent findings performed in the rat strain used in this study which documented a significantly longer plasma half-life of endotoxin in aged rats as compared with young rats.[29] In the present study, the observed marked swelling of Kupffer cells in ET-treated young rats could also result in a decreased hepatic blood flow. However, pronounced sinusoidal thrombosis and other related morphologic evidence that could precipitate alterations in the hepatic microcirculation were not observed in ET-treated young rats.

Age-related differences in the activation of the intrinsic coagulation system in rats have not been examined in detail. However, studies that examined the effects of ageing on blood coagulation indicate that a greater thrombotic potential exists in elderly humans and aged rats.[30,31] A recent study in this strain of rat demonstrated a decreased fibrinolytic capacity and an increased plasminogen activator inhibitor in aged rats treated with endotoxin.[32]

The results of the present study demonstrated marked differences in Kupffer cell phagocytosis of platelets and neutrophils between the young and aged rats, which suggests that the increased phagocytosis of inflammatory cells by Kupffer cells of young rats may be a mechanism which affords protection against endotoxin-induced lethality. Decreased phagocytic removal of colloidal material, such as radiolabelled heat-aggregated colloidal albumin, has been documented in Kupffer cells of aged rats.[33] In contrast, other investigators initially suggested that the decreased phagocytic activity by Kupffer cells may be a protective mechanism against the detrimental effects of ET based on a study that demonstrated deficient Kupffer cell phagocytosis and lower levels of lysosomal enzymes in the C3H/HeJ mouse, a strain which is genetically resistant to doses of ET that are lethal in syngeneic C3HeB/FeJ mice.[34] However, additional studies demonstrated that the C3H/HeJ mouse fails to produce significant quantities of the cytokine, tumour necrosis factor-alpha (TNF-α), and that mice passively immunized with antiserum to TNF will abrogate endotoxin-induced lethality.[35] Studies are being performed to determine whether there is an age-related difference in the production and secretion of this cytokine in

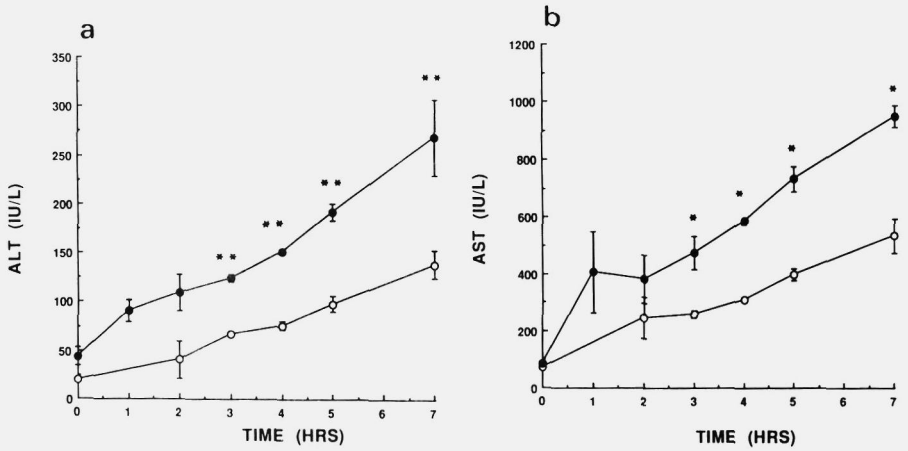

Fig. 7—(a) Alanine aminotransferase (ALT) values of young (○) and aged (●) female BN/BiRij rats following the administration of ET (arrow). $^{**}P < 0{\cdot}01$. (b) Aspartate aminotransferase (AST) values of young (○) and aged (●) female BN/BiRij rats following the administration of ET. $^{*}P < 0{\cdot}05$. Data represent the means and standard error of the means

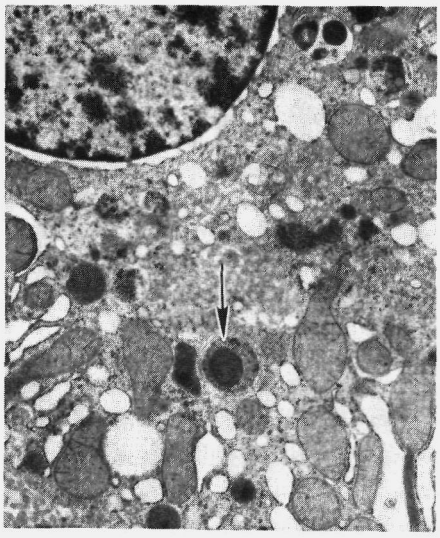

Fig. 8—TEM of the liver of an aged rat at 3 h after ET. There is swelling and vesiculation of the endoplasmic reticulum and perinuclear envelope of the hepatocyte. A mitochondrion is present within an autophagosome (arrow). Lead citrate and uranyl acetate; × 11 800

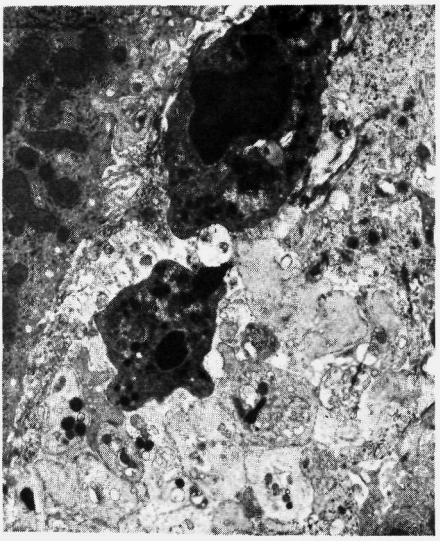

Fig. 9—TEM of the liver of an aged rat at 3 h after ET. There are morphologic changes indicative of sinusoidal thrombosis, including aggregates of platelets that have lost their discoid contour and secretory granules enmeshed among fibrin aggregates and neutrophils, within the sinusoidal lumen. Lead citrate and uranyl acetate; × 12 960

the strain of rat utilized in the present study. The relationship(s), if any, between ET-induced cytokine production and release, and Kupffer cell phagocytic activity has yet to be elucidated.

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CHAPTER 5

ON THE FIBRINOLYTIC SYSTEM IN AGED RATS, AND ITS REACTIVITY TO ENDOTOXIN AND CYTOKINES

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SUMMARY

Aged rats are more susceptible to endotoxin-induced effects, including microthrombosis and platelet aggregation, than are young rats. To investigate whether changes in the fibrinolytic system might be involved, we investigated the fibrinolytic activity in plasma euglobulin fractions and tissues (lung and heart) of young (6-months old) and aged (24-months old) rats under base-line conditions and after challenge with endotoxin. Aged rats had lower plasma levels of tissue-type plasminogen activator (t-PA) and of urokinase-type PA (u-PA) activity. PA inhibitor (PAI) activity was higher in the plasma of aged rats, as was t-PA activity in lung and heart.

Rats were treated with either a low dose (1 μg/kg) or a high dose (10 mg/kg) of endotoxin. Both treatments induced a transient phase of increased blood fibrinolytic activity, as evidenced by higher levels of tissue-type plasminogen activator (t-PA) activity and decreased levels of PA inhibitor (PAI) activity. Over time, the fibrinolytic activity decreased, probably due to increased levels of PA inhibitor.

Both the early increase in t-PA activity, and the subsequent increase in PAI activity, were more pronounced in the aged rats, as compared with the younger rats, after the high dose of endotoxin. The aged rats also responded to an injection of interleukin-1β or tumor necrosis factor-α with a larger increase of PAI activity than did the younger rats.

Together the data suggest that, compared to young rats, aged rats have a decreased base-line plasma fibrinolytic activity, while their fibrinolytic system is more responsive to challenge by endotoxin and cytokines.

INTRODUCTION

Endotoxin, a major component of the cell wall of gram-negative bacteria, profoundly affects the fibrinolytic system of both humans (1-5) and experimental animals (6-10). Intravenous injection of endotoxin results in a transient phase of increased fibrinolytic activity, followed by a more extended period of reduced activity. In addition to these fibrinolytic effects, endotoxin also activates coagulation (5). Through these two pathways, endotoxins may be involved in clinical consequences of gram-negative sepsis, such as fibrin deposition and microthrombosis (11).

Elderly individuals are more susceptible to endotoxin-induced effects than the young (12). An earlier study (13) documented an increased mortality, and a more pronounced disturbance of temperature regulation, glucose metabolism, and tissue blood supply in aged rats as compared with young rats following endotoxin injection. Morphological

evidence (14,15) of an increased incidence of microthrombosis and platelet aggregation was observed in the liver and lungs of aged rats following endotoxin administration. To see whether changes in the fibrinolytic system in aged rats might be involved in this increased sensitivity to endotoxin, we investigated in the present study fibrinolytic components in young (6-months old) and aged (24-months old) rats, and evaluated in these two groups the response of the fibrinolytic system to an i.v. injection of endotoxin, interleukin-1β, and tumor necrosis factor-α. The results suggest that aged rats have lower base-line levels of plasma fibrinolytic activity, but respond more strongly to challenge.

MATERIALS AND METHODS

Rats

Brown Norway (BN/BiRij) rats were obtained from the breeding colony of the Institute. The animals were fed standard food pellets and tap water *ad lib.* The rats were free of common bacterial and viral pathogens as determined by routine microbiological, serological and histopathological monitoring (16). In the experiments on the effects of endotoxin on the rat fibrinolytic system male rats were used. The aged rats used in our experiments were female, as no male rats of the appropriate age were available. Separate studies demonstrated, however, no differences between the fibrinolytic parameters of (young) male and female rats, and no differences between the two genders in the induction of PAI activity by endotoxin (data not shown).

Experimental design

Rats were anaesthetized with Nembutal (60 mg/kg i.p.), and i.v. injected with the compound under study, i.e. endotoxin (1 μg/kg or 10 mg/kg), interleukin-1β (1 μg/kg) or tumor necrosis factor-α (10 μg/kg). Controls were injected with sterile, endotoxin-free saline (1 ml/kg). At various times after the injection, as specified below, rats were bled from the aorta under Nembutal anaesthesia. (In the experiment involving serial blood sampling, halothane anaesthesia was used, and blood was obtained from a tail vein). Blood (9 parts) was immediately mixed with 1 part of pre-cooled trisodiumcitrate (0.13 M), centrifuged at 4°C to prepare platelet-poor plasma, and stored at -70°C. Tissues were freed of adherent fat and connective tissue, rinsed in saline, frozen in liquid nitrogen and stored at -70°C.

Assays

The dilute blood clot lysis time was determined as previously described (17,18). In brief, blood (0.2 ml) was diluted with 1.7 ml of 0.12 M sodium acetate buffer (pH = 7.4), clotted with thrombin (0.1 ml of 20 NIH Units/ml) and incubated at 37°C. The lysis

time was read in minutes.

The activity of t-PA was determined in the plasma euglobulin fraction (pH = 6.0; dilution 1:20) by the method of Verheijen et al. (19), in the presence or absence of quenching rabbit anti-rat t-PA Ig (20) or 100 μM amiloride (21; see ref 20 for details). The PA activities are expressed in units (U), one U giving the same rate of change in absorbtion, in the spectrophotometric assay, as one international unit (IU) of human t-PA, as defined by the International Standard.

Plasma plasminogen activator inhibitor (PAI) activity was determined spectrophotometrically by titrating (diluted) plasma with human t-PA, as described by Verheijen et al. (22). PAI activity is expressed in units (U), one U being equivalent to the amount that inhibits one IU of human t-PA.

Fibrinogen was determined by radial immunodiffusion against rabbit anti-rat fibrin monomer antiserum (23).

Plasminogen activity was determined spectrophotometrically, essentially as described by Mussoni et al. (24), using microtitre equipment.

α_2-antiplasmin activity was determined spectrophotometrically using microtitre equipment, as detailed elsewhere (25).

Fibrin autography of plasma (2 μl) was performed by the method of Granelli-Piperno and Reich (26). Gels were incubated for 48 hrs at 37°C.

Tissues were triturated in liquid nitrogen, homogenized in an acid acetate buffer and centrifuged, as described (27). Tissue concentrations of t-PA activity were determined spectrophotometrically in the presence and absence of anti-rat t-PA Ig (27). Tissue t-PA concentrations will be expressed as U per gram wet weight (U/g).

Materials

Escherichia coli endotoxin (serotype 026:B6), prepared by phenolic extraction, was obtained from Sigma (St. Louis, MO). Nembutal® was from Sanofi (Paris, France). Plasmin and the tripeptide substrate S-2251 were obtained from Kabi-*Vitrum* (Stockholm, Sweden), human urokinase and bovine thrombin from Leo Pharmaceuticals (Ballerup, Denmark). The International Standard for t-PA (lot 83/517) was obtained from the National Institute for Biological Standards and Control (South Mimms, UK). Recombinant human interleukin-1-β (rhIL-1β; 10^8 units/mg protein) was a gift from Dr. S. Gillis (Immunex, Seattle, WA).

Recombinant human tumor necrosis factor-α (rhTNFα; 0.25 x 10^8 units/mg protein) was kindly provided by Dr. J. Tavernier (Biogent, Gent, Belgium).

RESULTS

Effect of endotoxin on rat fibrinolytic components

To evaluate the effects of endotoxin injections on rat fibrinolysis, 6-months old rats were injected with one of two doses of endotoxin: 10 mg/kg, a sublethal dose in aged rats, or 1 μg/kg, a dose previously found to give a measurable increase in PAI activity in rat plasma (8). Both doses induced qualitatively similar effects, that were also similar to those seen in humans (1-5): a transient phase of increased blood fibrinolytic activity was followed by a prolonged period of reduced activity (Table 1). These effects were more pronounced after the high dose of endotoxin (Table 1).

Table 1. Effect of two dosages of endotoxin on rat fibrinolytic activity in six-months old male BN rats.

	Dilute blood clot lysis time (min)	PAI activity (U/ml)	t-PA activity (U/ml)
Controls (n = 9)	108 ± 16*	6.3 ± 1.7	0.34 ± 0.06
Endotoxin (10 mg/kg i.v.)			
30 min (n = 4)	38 ± 7	0	4.77 ± 1.23
60 min (n = 4)	48 ± 13	9.3 ± 8.4	0.77 ± 0.78
4 hrs (n = 5)	> 72 hrs	268 ± 86	0.21 ± 0.26
24 hrs (n = 3)**	6-24 hrs	24 ± 9	0.13 ± 0.06
Endotoxin (1 μg/kg i.v.)			
30 min (n = 3)	82 ± 8	3.8 ± 0.4	0.61 ± 0.23
60 min (n = 3)	96 ± 32	5.9 ± 1.3	0.20 ± 0.11
4 hrs (n = 3)	> 72 hrs	16.0 ± 1.3	0.12 ± 0.09
24 hrs (n = 3)	167 ± 35	7.2 ± 3.4	0.32 ± 0.20

* All data are mean ± standard deviation.

** Three animals died; two spontaneously during the night and one during re-anaesthesia at 24 hrs.

At 30 min and at 1 hr after endotoxin, the blood fibrinolytic activity was increased, as evidenced by a decrease in the dilute blood clot lysis time and an increase in euglobulin t-PA activities. PAI activity was decreased at 30 min (even to undetectable levels after the 10 mg/kg dose), but had increased to control values after 60 min.

At 4 hrs, PAI activity had increased to 2.5 times the base-line value in animals injected with the low dose, and to as much as 40 times the base-line value in animals injected with the high dose of endotoxin. At this time, dilute blood clots did not lyse, and euglobulin t-PA activity was strongly reduced.

At 24 hrs, PAI and t-PA activities had returned to control values in the group given 1 μg/kg group, but fibrinolytic activity was still reduced in the group injected with 10 mg

of endotoxin per kg (Table 1).

Plasminogen and fibrinogen showed in both groups small changes, which were significant at 4 and 24 hrs (Table 2), although these changes are presumably not of biological importance; α_2-antiplasmin levels did not change (Table 2).

Table 2. Effect of two dosages of endotoxin on rat plasma fibrinolytic components in six-months old male BN rats

	Plasminogen (%)	α_2-Antiplasmin (%)	Fibrinogen (%)
Controls (n = 9)	100 ± 6*	100 ± 5	100 ± 13
Endotoxin (10 mg/kg i.v.)			
30 min (n = 4)	97 ± 9**	102 ± 4	90 ± 21
60 min (n = 4)	89 ± 9	88 ± 12	103 ± 16
4 hrs (n = 5)	94 ± 4	84 ± 7	76 ± 11***
24 hrs (n = 3)	69 ± 10***	92 ± 19	135 ± 15***
Endotoxin (1 µg/kg i.v.)			
30 min (n = 3)	97 ± 2	103 ± 2	84 ± 12
60 min (n = 3)	102 ± 2	106 ± 3	97 ± 8
4 hrs (n = 3)	94 ± 4	102 ± 8	97 ± 6
24 hrs (n = 3)	75 ± 10***	108 ± 5	124 ± 31***

* All data are mean ± standard deviation.

** Percentage of mean value in the nine control animals.

*** Significantly different from controls by one-way ANOVA ($p < 0.01$).

Plasma fibrinolytic components in young and aged rats

A comparison of young (6-months old) and aged (24-months old) rats showed that plasma euglobulin fraction from aged rats contained less t-PA activity, and more PAI activity (Table 3). The u-PA activity was also lower in the aged rats. The decreased PA activity in aged animals was not an artefact consequent on the increase in PAI activity, as no correlation was found between t-PA (or u-PA) and PAI activities. This conclusion can not yet be supported by antigen assays, as no test sensitive enough to measure t-PA or u-PA antigen concentrations in rat plasma are at present available. Concentrations of plasminogen, α_2-antiplasmin and fibrinogen showed no differences between the two age groups. In contrast to the plasma t-PA activity, the tissue t-PA activity (which accounted for 95-98% of tissue total PA activity) was higher in lung and heart of the aged rats (Table 3).

Table 3. Fibrinolytic components in young (six-months old) and aged (twenty-four-months old) female BN rats

	Young (n = 6)	Aged (n = 6)
Euglobulins		
Total PA activity (U/ml)	2.49 ± 0.82*	1.40 ± 0.44**
t-PA activity (U/ml)	1.37 ± 0.26	0.67 ± 0.38**
u-PA activity (U/ml)	1.17 ± 0.40	0.69 ± 0.35
Plasma		
PAI activity (U/ml)	7.4 ± 0.7	9.6 ± 1.6**
Plasminogen (%)	100 ± 10	102 ± 14***
$Alpha_2$-antiplasmin (%)	100 ± 9	103 ± 21***
Fibrinogen (%)	100 ± 8	105 ± 9***
Tissues		
Lung t-PA activity (U/g)	1938 ± 356	2351 ± 335
Heart t-PA activity (U/g)	391 ± 40	545 ± 75**

* All data are mean ± standard deviation.

** Significant difference between young and aged rats (Students t-test; $p < 0.025$).

*** Percent of mean value of the group of young rats.

Effects of endotoxin in young and aged rats

In the next series of experiments, the effects of endotoxin in young (6-months old) and aged (24-months old) rats were compared. Due to the limited number of aged rats available, serial blood samples were used in this experiment. Before endotoxin, the PAI activity in the plasma of the aged rats (7.2 ± 2.5 U/ml) was again higher than the PAI activity of young rats (5.1 ± 2.5 U/ml), though in this case not significantly.

Injection of endotoxin at a dose of 1 μg/kg induced a similar pattern of change in PAI activity in both young and aged rats which was also similar to the response in the six-months old male rats described above (data not shown). However, after injection of endotoxin at the dose of 10 mg/kg, the increase in PAI activity was by two-way ANOVA significantly larger in the aged rats between 2 and 7 hours after injection (Fig. 1).

Due to the limited volume of plasma that was available from the serial blood samples, the plasma t-PA activity could not be assayed by the euglobulin procedure. Thus, fibrin autography was used instead to assess changes in plasma PA activity. As shown in Figure 2, both young and aged rats showed a similar, small, amount of free t-PA activity and of t-PA-PAI complex prior to endotoxin injection. Endotoxin induced a large increase in free t-PA activity at the earlier time points (15 and 30 min); the increase in free t-PA activity was larger, and persisted longer (up to 1 hr) in the aged

rats, compared with the young ones (Fig. 2). At later points in time an increased activity was seen at the high molecular weight position, presumably representing complexes between t-PA and PAI formed due to the increase in PAI activity. This increased activity at the high molecular weight position persisted for 24 hrs, at a comparable level in both age groups (Fig. 2).

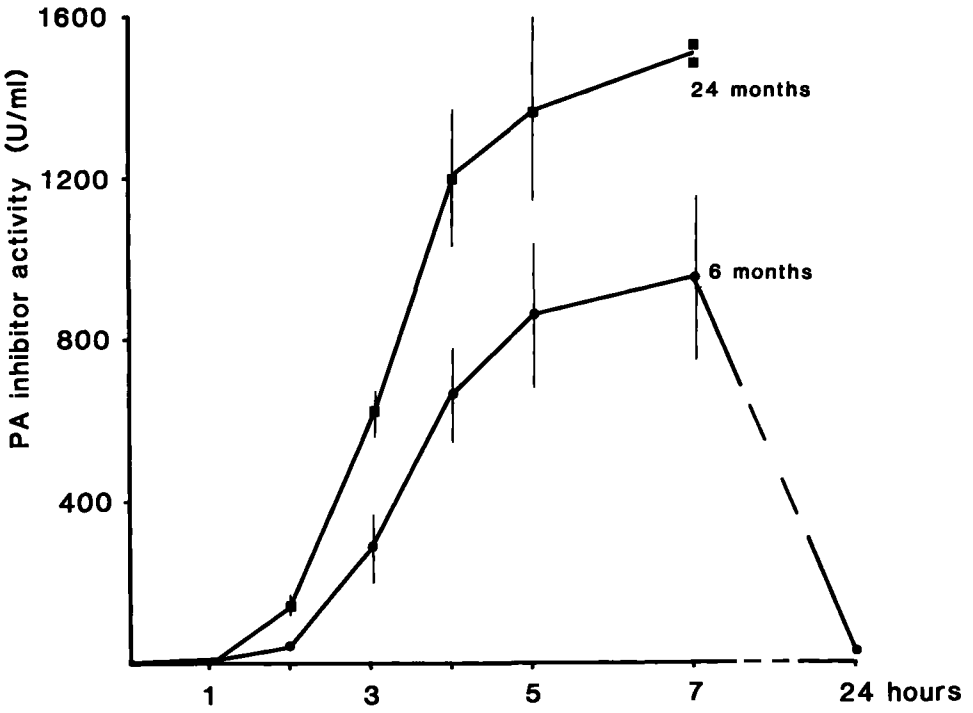

Figure 1. Time course of the effect of endotoxin (10 mg/kg; i.v.) on PA inhibitor activity in plasma from young (6-months old; ●) and aged (24-months old; ■) rats. Serial blood samples were obtained, using five young rats and three aged rats. Data shown are mean ± s.d. (one aged rat died during anaesthesia at seven hours).

Effects of cytokines in young and aged rats

As we had shown previously that the cytokines interleukin-1β (8) and tumour necrosis factor-α (28) also increased PAI activity in rats, aged and young rats were i.v. injected with rhIL-1β (1 μg/kg) (8) or with rhTNF-α (10 μg/kg) (28) (the dosages used were chosen on the basis of previously reported data; refs 8 and 28). The aged rats responded to both compounds with a larger increase in PAI activity than the young ones: at three hours after treatment with rhIL-1β or with rhTNF-α plasma PAI concentrations were approximately twice as high in the aged group as in the young group (Table 4). At 24

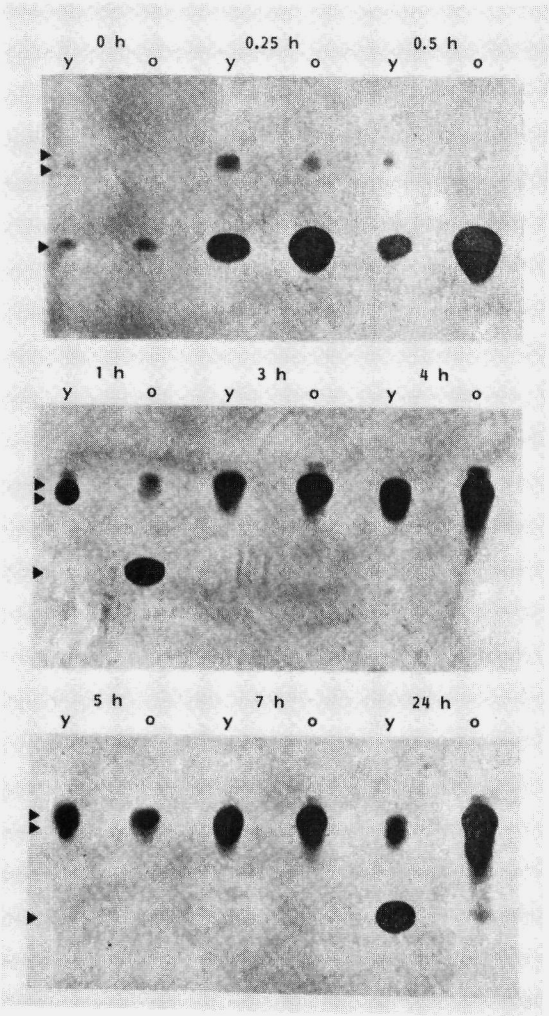

Figure 2. Time course of the effect of endotoxin (10 mg/kg; i.v.) on the PA activity pattern in plasma. Shown are the fibrin autographics of serial plasma samples from a young (6-months old) rat (y), and from an aged (24-months old) rat (o).
Note that up to one hour after endotoxin injection the lysis zone of free t-PA (arrowhead) is larger in the aged rat, compared with the young rat. At these time points (0.25, 0.5 and 1 hour) the t-PA-PAI complex lysis zone (double arrowhead) is smaller in the aged rat, possibly due to a more rapid titration of PAI-1 by t-PA in this animal. Similar patterns were found in the other rats studied.

hours after cytokine injection, plasma PAI concentrations were again normal in both groups (not shown). Tissue levels of t-PA activity, both in lung and heart, were slightly reduced (by some 10 %) after three hours, but had returned to normal levels after 24 hours (not shown). Plasma levels of t-PA were, in contrast, still reduced by about two-thirds at 24 hours (young rats 0.49 $\pm$ 0.11 U/ml, n=5; aged rats 0.15 and 0.21 U/ml, n=2). Four aged rats (two injected with rhIL-1β, two injected with rhTNF-α) died within 24 hours after cytokine injection, as opposed to one young rat.

Table 4. Plasma PA inhibitor activity in young and aged female BN rats after injection of rhIL-1β or rhTNF-α

	Plasma PA inhibitory activity (U/ml)	
	Young rats	Aged rats
Saline controls	7.4 ± 0.7 (6)*	9.6 ± 1.6 (6)**
At three hours after		
rhIL-1β (1 μg/kg i.v.)	90 ± 26 (3)	190 ± 30 (3)**
rhTNF-α (10 μg/kg i.v.)	34 ± 7 (3)	55; 80 (2)***

* All data are mean ± standard deviation (number of animals in parentheses).

** Significant difference between young and aged rats (Students t-test; $p < 0.025$).

*** One animal died at two hours after i.v. injection.

DISCUSSION

Intravenous injection of endotoxin into rats induced a rapid increase in fibrinolytic activity which lasted for about 1 hr, and was followed by a period of decreased activity caused by increased PAI activity (Table 1). Similar effects, e.g. a rapid transient increase in plasma fibrinolytic activity followed by a long-lasting decrease in activity, have been observed in man after endotoxin injection (3), and have recently been shown to be caused by increased levels of t-PA, followed by increased levels of PAI (4,5). In our rats, the rapid increase in PA activity was also due to an increase of t-PA, as shown by quenching experiments using anti-rat t-PA (Table 1), and by fibrin autography (Fig. 2). In view of the speed of the changes in t-PA levels, this increase in circulating t-PA was most likely the result of the release of preformed t-PA from stores in endothelial cells, rather than of increased synthesis (29). Endotoxin also induces release of von Willebrand factor, another plasma protein that is stored in endothelial cells, both in humans (4,5,30) and animals (31,32). The increase in PAI activity is due to an increased synthesis of PAI-1 as tissue concentrations of PAI-1 mRNA and activity increase in rats after endotoxin injection (33). The response to endotoxin of the fibrinolytic system is thus highly comparable between man and rat.

A comparison of plasma fibrinolytic components in young and aged rats showed that, under base-line conditions, the aged rats had lower levels of euglobulin t-PA activity and higher levels of plasma PAI activity (Table 3). The reaction to endotoxin was found to be more pronounced in the two-year old rats, both with regard to the increase in t-PA activity at the early time points after injection (Fig. 2), and with regard to the increase in PAI activity (Fig. 1). This enhanced reaction to endotoxin of the fibrinolytic system would agree with the greater responsiveness to endotoxin found in other systems in aged

rats (13-15).

The overall response to endotoxin of an organism involves a complex set of reactions including direct cellular effects of endotoxin, as well as indirect effects mediated by endotoxin-induced cytokines such as IL-1β, TNF-α and IL-6. The greater response, in aged rats, to endotoxin might therefore be explained by a greater response to those cytokines and/or by a greater induction of those cytokines by endotoxin. A greater response to the cytokines IL-1β and TNF-α was indeed observed. However, it is unlikely that the observed increased response to endotoxin is due to cytokine-related mechanisms. As will be shown elsewhere (manuscript in preparation), both suppression of (endotoxin-induced) IL-1 and TNF formation as well as inhibition of the action of these cytokines has no effect whatsoever on the induction of PAI by endotoxin. In parallel experiments we have also found that IL-6 did not induce PAI in (young and aged) animals (not shown). Endotoxin thus induces the PAI response independent of cytokines. As our data demonstrated an increased response to endotoxin, interleukin-1β as well as TNF-α, it is likely there exists a generalized increase in responsiveness of the fibrinolytic system in aged rats.

Little is known about the effect of aging on the regulation of the fibrinolytic system in man. Various studies (e.g. 35-40) have described age-dependent changes in the basal plasma concentrations of t-PA and PAI-1 in normal, healthy people. Whether an age-dependent increase in the reactivity of the fibrinolytic system, e.g. to endotoxemia, occurs in man as it occurs in rats remains to be determined. An answer to this question might be of interest, as an enhanced "response to insult" of the fibrinolytic system has been implicated as a factor contributing to the incidence of postoperative thrombosis (41).

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CHAPTER 6

EXPERIMENTAL ENDOTOXAEMIA IN AGING RATS: EFFECTS ON GLUCOSE HOMEOSTASIS

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SUMMARY

Old rats are much more susceptible to endotoxin-induced injury than young adult rats. Administration of endotoxin can lead to severe changes in the regulation of glucose metabolism. In this study, the metabolic response of young and old rats to endotoxin was compared. Female BN/Rij rats, 6 and 24 months of age, were given various doses of endotoxin and changes in the levels of glucose and some intermediary metabolites were determined in plasma and liver at several time intervals after injection. In non-fasted animals, both the hyperglycemic reaction of the first hour, and the hypoglycemia occurring later, were more severe in old rats, while only an increased tendency to develop hpoglycemia was observed in fasted old rats. These phenomena may be related to a decreased capacity of hepatic gluconeogenesis, partly caused by damage to liver cells. There were, however, only minor differences in the time-dependent changes in the concentrations of glucose metabolites after endotoxin treatment of young and old fasted rats.

INTRODUCTION

In earlier studies it was established that old rats are much more susceptible to endotoxin-induced injury than young adult rats. The increased severity of endotoxin effects in old rats was reflected in mortality rate, hypothermia, and hypercoagulability of blood (1), and, severe cell damage in tissues, notably lung (2) and liver (3). This was already apparent at 24 months, at which age no major signs of age-related pathology have yet developed (4).

Administration of endotoxin can lead to severe metabolic alterations which are associated with changes in body temperature (5,6) and glucose regulation (7-9). This response has been characterized in by dysregulation of plasma glucose (7-9), lactate (10) and lipid (11-13) levels, and induction of glycogenolysis (14,15). Increased tissue glucose utilisation, insufficiently compensated by increased gluconeogenesis and glucose output by the liver, plays a major role in the hypoglycemia induced by LPS (16-18).

In this study, the metabolic response of young and old rats to endotoxin was investigated to define possible alterations in homeostatic control mechanisms that may be responsible for, or are associated with, the increased sensitivity of old rats to endotoxin. Plasma glucose, lactate, insulin and glucagon levels, and hepatic contents of intermediate metabolites and glycogen were determined after administration of various dosages of endotoxin to young and old rats.

MATERIALS AND METHODS

Experimental design

Female BN/Rij rats, 6 and 24 months of age, were injected via the external jugular vein with endotoxin (phenol-water extract of *E.coli* O26:B6), at dosages between 100 ng and 7.5 mg per 100 g body weight. In the course of this study, two batches of endotoxin with slightly different biological activities were employed (I and II). Within each experiment the same batch was used. Animals were sacrificed at various time points after injection (between 15 min and 24 h) by exsanguination through the inferior vena cava. In some experiments, serial blood samples (± 120 μl) were taken to follow the time course of changes in the blood glucose concentrations and other parameters within each animal. These rats were killed at 24 hours after injection.

For the determination of glucose metabolites, livers were perfused with ice-cold saline at a flow of 5 $ml.min^{-1}$ for two min and frozen in liquid nitrogen.

Biochemical determinations

Plasma or serum samples were prepared for analysis of glucose, lactate, glucagon and insulin levels. Glucose levels were determined in heparinized whole blood by means of the Reflotron system (Boehringer Mannheim GmbH, FRG) or in plasma (see below). Plasma samples containing 4.7% trasylol were used for the determination of glucagon (NOVO-kit, a radio-immunoassay containing a specific antiserum against pancreas-glucagon). Serum insulin concentrations were determined with a radio-immunoassay obtained from Incstar corporation.

Freshly frozen liver tissue was used for the determination of glycogen by a modification of the method of Huijing (20). Liver samples were homogenized in water to a 25% w/v homogenate. Glycogen was digested in 1:10 diluted homogenate using α-amylase (0.04 $mg.ml^{-1}$) and α-amyloglycosidase (0.2 $mg.ml^{-1}$) in 0.1 M acetate buffer (pH 4.8) for 1 h. The amount of glucose liberated was quantified by the Gluc-DH^R method (Merck, Darmstadt, FRG), which employs β-D-glucose:NAD oxydoreductase and NAD^+ as an acceptor. The same assay was used to determine liver and plasma glucose. Lactate was determined in plasma using the enzymatic Monotest^R (Boehringer Mannheim GmbH, FRG). For the determination of intermediary metabolites, frozen liver was pulverized in liquid N_2 using mortar and pestle, deproteinized in 0.6 N perchloric acid. The neutralized supernatant (10,000 g, 10 min, 4 °C) was used for the determinations of glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (F-1,6-dP), pyruvate, and, phosphoenolpyruvate (PEP), as described in (21).

Histology

Liver tissue was fixed in 10 % buffered formalin and embedded in paraffin. Two samples

of liver, each obtained from a different lobe, were serially sectioned at 5 μm and routinely stained with hematoxylin-phloxine-saffron (HPS) (4). Periodic acid Schiff (PAS) was applied for staining of polysaccharides, including glycogen. The specificity of this staining for glycogen was checked by comparison with a serial section which was pretreated with diastase, which removes glycogen, but has no effect on other (muco)polysaccharides (4).

RESULTS

Effect of age on blood glucose levels in untreated rats

The basal non-fasting blood glucose concentration was not different between the 6-month-old animals (7.33 ± 0.31 (mean ± SEM, n = 5)) and the 24-month-old ones (6.93 ± 0.53 (n = 5)). When rats were fasted overnight, the blood glucose concentration at 10-11 a.m. was also not significantly different between the 6-month-old animals (8.4 ± 1.3 (n = 16)) and the 24-month-old ones (7.1 ± 0.4 (n = 8)).

Pilot experiments with an oral glucose tolerance test showed that the increase in plasma glucose levels after a glucose load was similar in 6- and 30-month-old rats, although the concomittant rise in plasma insulin was greater in the old rats (results not shown). In another experiment, the response to insulin (0.5 $U.kg^{-1}$; i.p.) was tested and the same degree of hypoglycemia (< 2 mM) was induced in 6- and 36-month-old BN rats. However, the return to normal values was delayed in old rats (personal communication, Dr. H. Van de Berg, TNO Toxicology and Nutrition Institute ITV, Zeist).

Alterations in blood glucose concentration after endotoxin administration to young and old rats

The effect of endotoxin on blood glucose levels was strongly dependent on time and dose and also on the age and nutritional condition of the rats. The general pattern of response consisted of several phases (Fig. 1). During the first 30 min after endotoxin administration a brief, small decrease in plasma glucose was noted. During the next 90 min, glucose levels rose up to severe hyperglycaemia. Beyond two-three hours after injection, all rats displayed a decline in glucose levels, resulting in mild to severe hypoglycemia at between 2 and 6 hours. Between about 8 and 24 hr, glucose levels might, or might not return to normal levels, dependent on the dose and age-group.

The dose dependency was different for young and old rats. When 6-month-old non-fasted rats were injected with 75 mg (batch 1) per kg, which is close to the approximate LD 50 in this age group (c.f. 1), only a brief and moderate hyperglycaemia of 12 mM was observed at 2 h. Old rats injected with 10 mg endotoxin (batch I) per kg, which

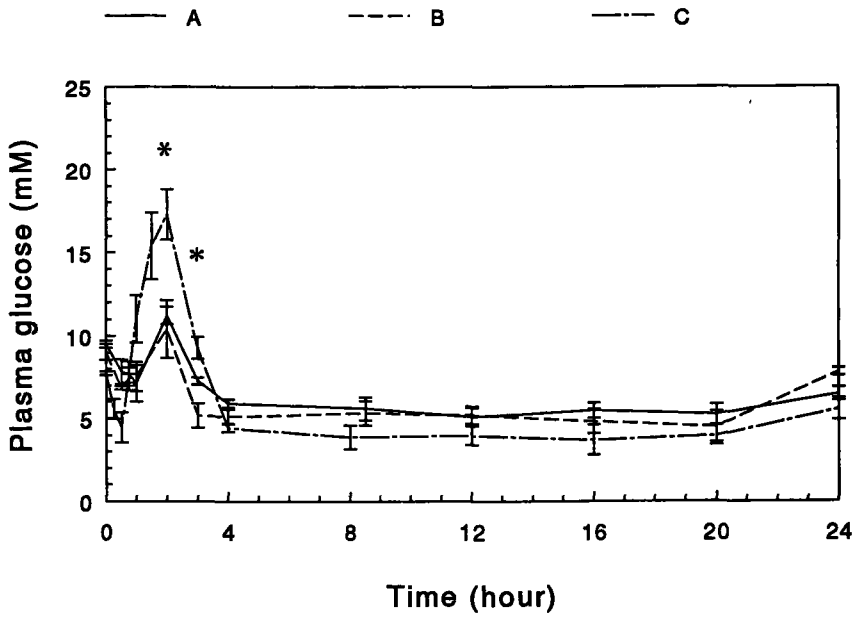

Figure 1. Blood glucose levels of 6- and 24-month-old BNBI/Rij rats at various time points after administration of various doses of endotoxin. Dose: 6-month-old: 75 mg LPS.kg^{-1} (n = 6) (A); 24-month-old: 10 mg LPS.kg^{-1} (n = 6) (B), and, 20 mg LPS.kg^{-1} (n = 8) (C). Values are mean ± SEM. * indicates a significant difference between the old rats treated with 20 mg LPS.kg^{-1} and the other treatment groups ($p < 0.05$; Student-t test).

approximates the LD 50 for this age-group, had a response similar to that of young ones receiving 75 mg per kg. A much stronger response was noted in old rats injected with 20 mg per kg during each phase of the response (Fig. 1).

In a second series, both young and old rats were injected with the same dose of 10 mg endotoxin (batch II) per kg (Fig. 2). The initial hyperglycemia was again higher, and beyond two hours after injection, all old rats showed a steep decline in glucose levels, resulting in moderate to severe hypoglycemia. Between 2 and 5 hours, only a few young animals did not maintain basal glucose levels, and most young animals had normal levels at 24 hours. All animals that did not survive, including an occasional young rat (1 out of 10), had a blood glucose concentration less than 2 mM at the time of death (Fig. 2). The few old rats that survived for 24 hr (3 out of 10) did not develop a hypoglycaemia as severe as the non-survivors but they still had a glucose concentration below normal at 24 h after injection.

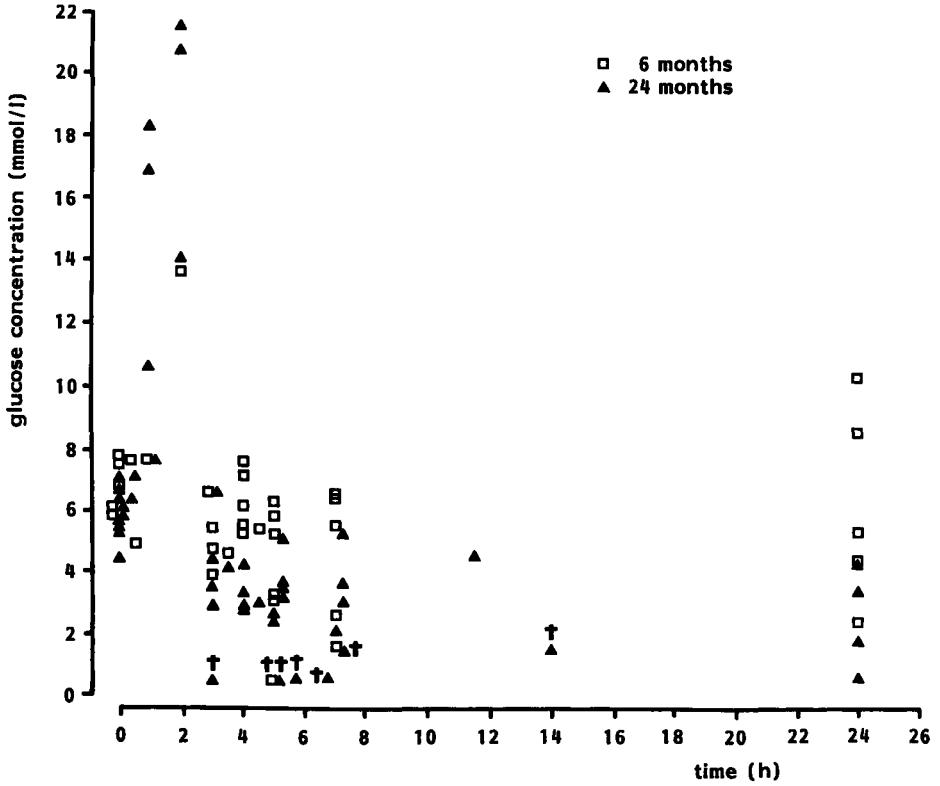

Figure 2. Blood glucose levels of 6- and 24-month-old BNBI/Rij rats at various time points after injection of 10 mg LPS.kg^{-1}. Each point represents a single determination. (†): died spontaneously.

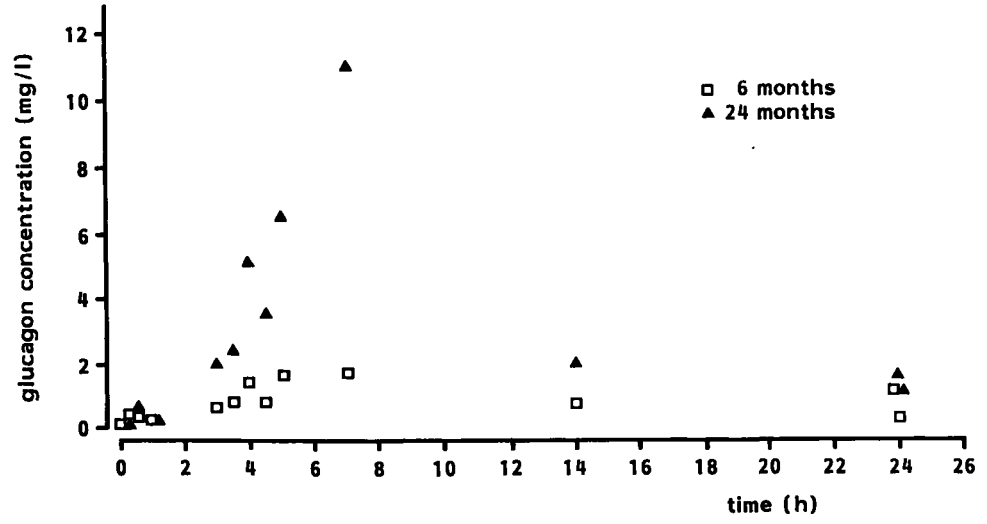

Figure 3. Plasma glucagon levels of 6- and 24-month-old BNBI/Rij rats after injection of 10 mg LPS.kg^{-1}. Each point represents one single animal. For the period between 3 and 14 hr after injection, a significant difference between the two age groups was noted ($p < 0.05$; Student-t test for paired observations.)

Effects of endotoxin on plasma glucagon and serum insulin levels

Dramatic differences between the two age groups were found in glucagon levels following endotoxin administration (10 mg.kg^{-1} in both age groups). Elevated glucagon levels were found at 3 h after injection and later in all rats, which coincided with the appearance of hypoglycemia. However, the increase was much more pronounced in old rats than in young adult ones between 3 and 14 hr after LPS administration (Fig. 3).

The insulin levels remained within normal limits (below 30 mU.ml^{-1}) in both young and old rats, with some fluctuations, but without a consistent pattern (Fig. 4).

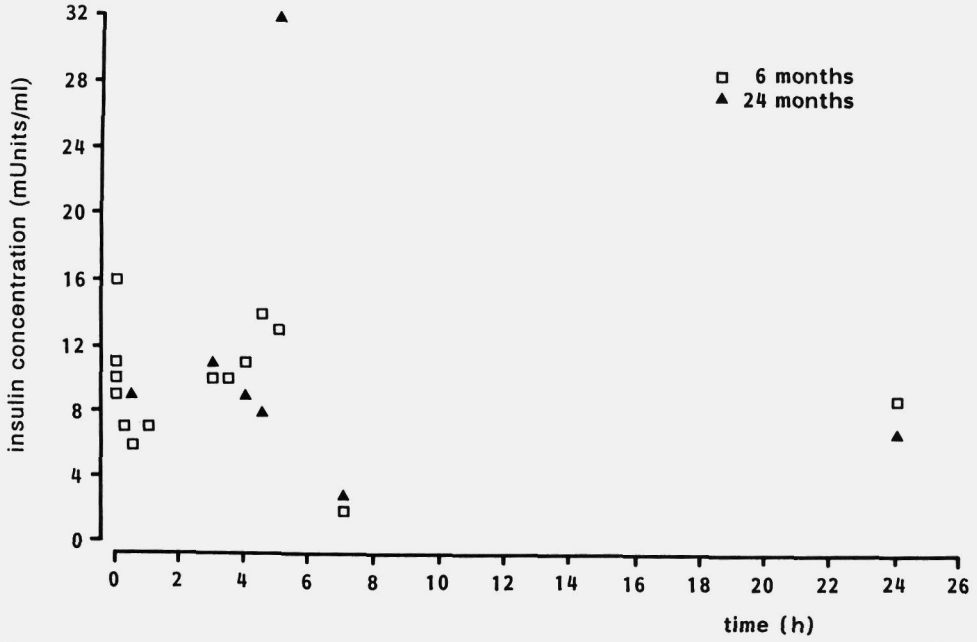

Figure 4. Serum insulin levels of 6- and 24-month-old BNBI/Rij rats after injection of 10 mg LPS.kg^{-1}. Each point represents one single animal.

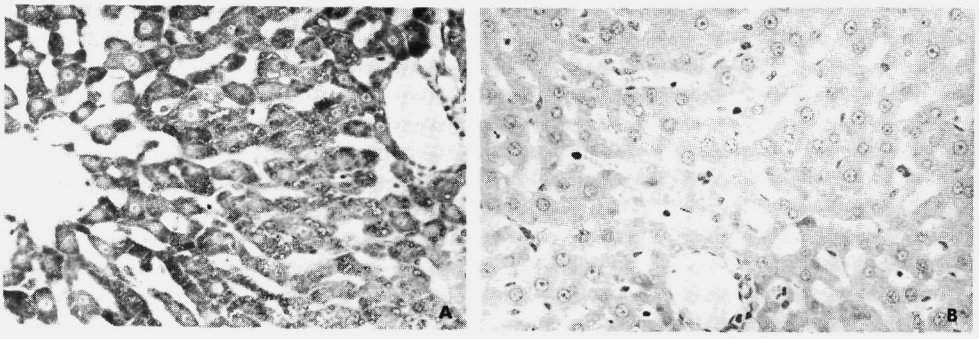

Figure 5 A-B. Determination of hepatic glycogen content by means of a PAS-positive staining in 24-month-old rats after administration of 10 mg LPS.kg^{-1}.Hepatocytes still contain abundant magenta-coloured material representing glycogen after 1 hour (a), while only some hepatocytes still contain some glycogen at 3 hours after administration of endotoxin (b) (X 500).

Liver glycogen depletion following endotoxin administration

Treatment of histological liver sections with diastase prior to PAS staining showed that almost all of the PAS-positive (muco)polysaccharide present in the liver of both age groups was represented by glycogen (not shown). Large quantities of glycogen were present in hepatic tissues of both young and old rats at the initial sampling times (not shown). A marked reduction in hepatic glycogen was observed in both young and old animals at 1 h after endotoxin administration (Fig. 5a). At 3 h (Fig. 5b), and all subsequent time points, liver glycogen stores were almost depleted. Biochemical determination of hepatic glycogen confirmed that glycogen levels were decreased to 20 mg per g liver at 1 h after injection and were below 3 mg per g at 3 h and later. The depletion in hepatic glycogen coincided with the onset of the decrease in plasma glucose levels observed at 3 h after endotoxin treatment in old rats.

Effects of fasting

The overnight fasting period had effectively removed glycogen from the liver of both young and old rats (Fig. 6a). There were no significant differences between fasted young and old rats in plasma or liver levels of glucose metabolites prior to LPS injection (Fig. 6b-e). In this experiment young and old rats each were injected with a dose of LPS that would produce a similar degree of endotoxic shock (approximate LD 50) in both age-groups. The 60 $mg.kg^{-1}$ dose in young rats produced the same type and degree of biological effects (temperature, lethargy, piloerection) as the 5 $mg.kg^{-1}$ dose in the old rats.

In fasted rats, the hyperglycemia was less pronounced than in non-fasted rats receiving the same dose, at least in old rats (Fig. 7). The hyperglycemic response was greater in young rats (up to 14 mM) than in old rats, which remained below 11 mM (Fig. 7). Plasma glucose levels returned to normal in young rats, while they decreased to below normal in the old. Plasma lactate levels (Fig. 8) initially rose and than decreased to below normal at 2 hr in young rats. In old rats, the initial rise was greater and lactate levels remained above normal.

There were some differences between untreated young and old fasted rats with regard to hepatic concentrations of some glucose metabolites, but these were of marginal statistical significance (Fig. 6). The levels of glucose (Fig. 6b) and hexosemonophosphates (Fig. 6c and 6d) in livers of both age groups all decreased during the hours after LPS administration. F-1,6-dP levels decreased transiently only in young rats, while they were increased at 5 h only in old ones (Fig. 6e). No major changes in hepatic pyruvate or phosphoenolpyruvate contents were found after endotoxin administration, but the pyruvate content tended to be somewhat higher in old rats.

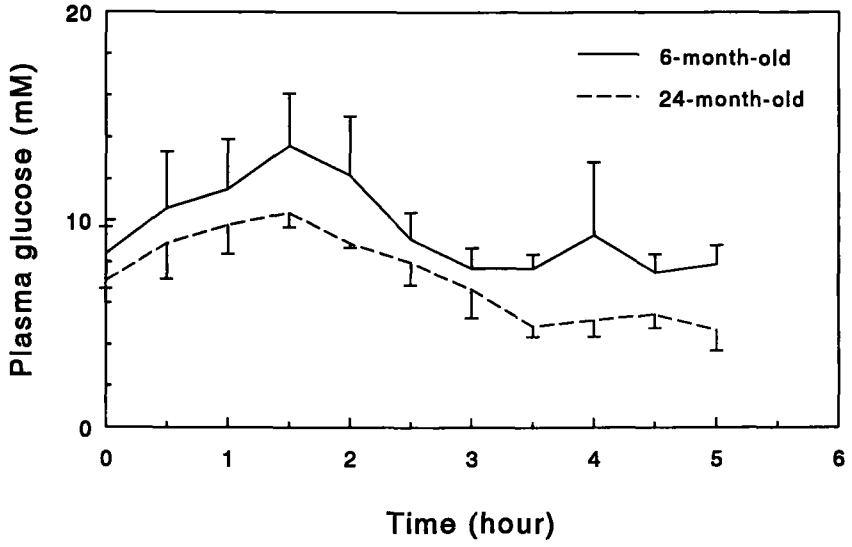

Figure 6. Plasma glucose concentration in 6- and 24-month-old rats after endotoxin administration (approx. LD50). Dose: 6-month-old: 60 mg LPS.kg^{-1};24-month-old: 5 mg LPS.kg^{-1}.Rats were fasted overnight. Values are mean ± S.D.. Statistical analysis (Student-t test for paired observations) indicated significant differences between the two age groups both from 0.5 to 2.5 hr ($p < 0.01$), and, from 2.5 to 5 hr ($p < 0.001$) after injection. Number of rats: 6-month-old rats: 0, 90, 180 and 300 min: n = 4. 24-month-old rats: 0 min: n = 5; 90, 180 and 300 min: n = 2.

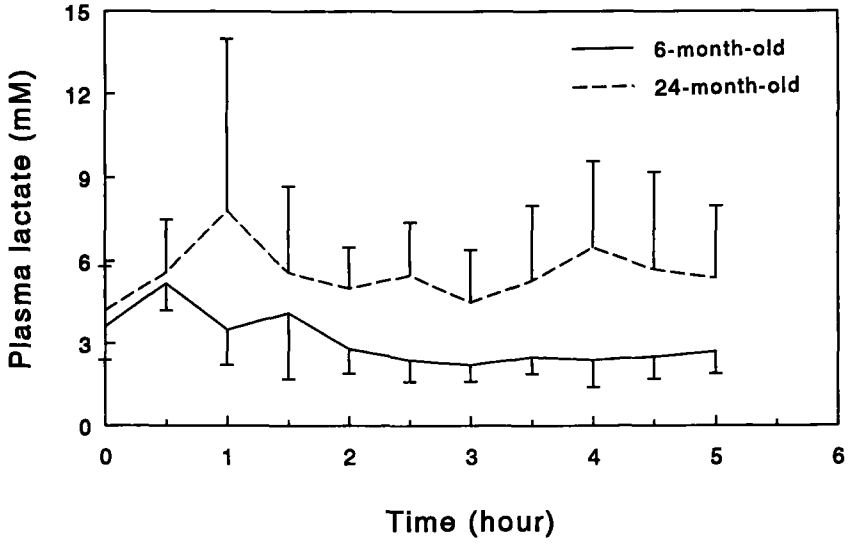

Figure 7. Plasma lactate concentration in 6- and 24-month-old rats after endotoxin administration (approx. LD50). Dose: 6-month-old: 60 mg LPS.kg^{-1};24-month-old: 5 mg LPS.kg^{-1}.Rats were fasted overnight. Values are mean ± S.D. (number of rats: see Figure 6). Statistical analysis (Student-t test for paired observations) indicated significant differences between the two age groups both from 0.5 to 2.5 hr ($p < 0.01$), and, from 2.5 to 5 hr ($p < 0.01$) after injection.

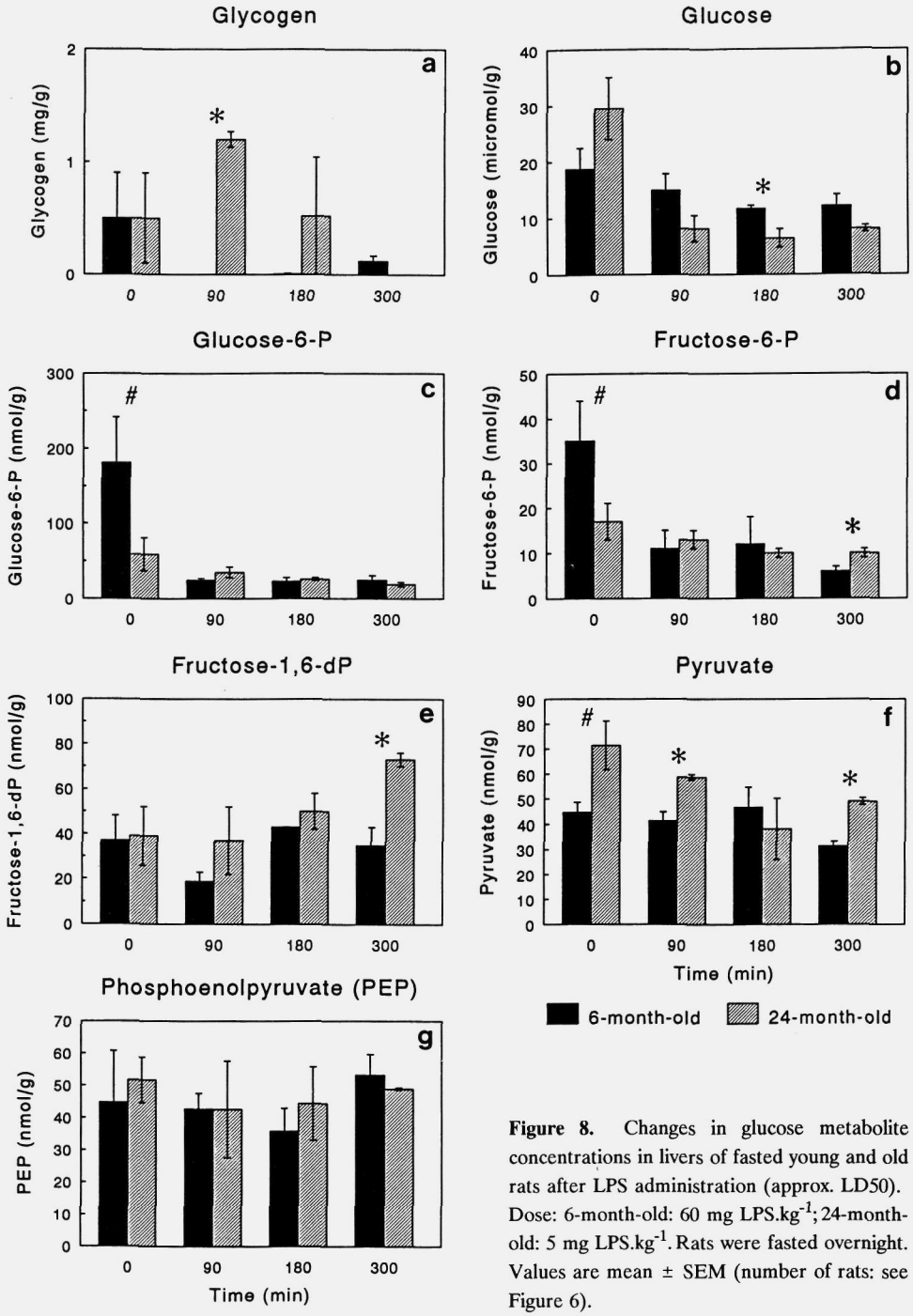

Figure 8. Changes in glucose metabolite concentrations in livers of fasted young and old rats after LPS administration (approx. LD50). Dose: 6-month-old: 60 mg LPS.kg^{-1}; 24-month-old: 5 mg LPS.kg^{-1}. Rats were fasted overnight. Values are mean ± SEM (number of rats: see Figure 6).

Statistical analysis was done by the Student-t test (#: $0.1 < p < 0.05$; *: $p < 0.05$).

DISCUSSION

Insulin and glucagon are the major regulators of glucose homeostasis in mammals (22,23), but most of the endotoxin-induced mediators, like eicosanoids (notably PGD_2 (14) and leukotrienes (24)), TNF (25), IL-1 (26), platelet activating factor (PAF) (27) and, α- and β-adrenergic hormones (28) also affect plasma glucose levels and hepatic and peripheral glucose metabolism.

The plasma glucose response to LPS consisted of 3 to 4 phases, dependent on dose and nutritional status. During the first 30 min after endotoxin administration (5-40 $mg.kg^{-1}$) a brief, small decrease in plasma glucose was noted, probably due to increased glucose utilisation by peripheral tissues, stimulated by insulin-like factors (16,29). During the next 90 min, glucose levels rose sharply, up to severe hyperglycaemia. Beyond two hours after injection, all rats displayed a decline in glucose levels, resulting in mild to severe hypoglycaemia at between 2 and 6 hours, at sufficiently high doses of LPS. Between about 8 and 24 hours, plasma glucose might, or might not, return to normal levels, dependent on the dose and age-group. These phases have also been described during sepsis and endotoxemia in humans (30,31) and experimental animals (18,19).

The dose dependency was different for young and old rats. Non-fasted old rats injected with 10 mg endotoxin per kg had a response similar to that of young ones receiving 75 mg per kg. The response of the old rats was much more pronounced than in young ones receiving the same dose, both during the hyper- and the hypoglycaemic phase. All animals that did not survive had a blood glucose concentration less than 2 mmol when moribond.

In non-fasted rats, the initially large quantities of glycogen present in the liver of both young and old rats were almost depleted at three hours after LPS administration. The higher hyperglycemia in old rats appeared to be dependent on glycogenolysis, since it was much less pronounced in fasted rats. Glucagon levels were elevated (and more so in old rats), but only after the hyperglycemic phase. The stronger early stimulation of hepatic glucose output in non-fasted rats could, alternatively, be related to activity of monokines, several of which are also known to induce hepatic glycogenolysis. Kupffer cell-derived mediators, particularly prostaglandins (mainly D_2), rather than TNF, are responsible for the induction of glycogenolysis by LPS in perfused rat liver (14). Increased production of these mediators may, however, not be the cause of the more severe hyperglycaemia in old rats, at least not as a result of intrinsic age-related changes in Kupffer cells. In vitro studies showed that Kupffer cells from the 24-month-old rats produce similar amounts of prostaglandins as those from young ones and no changes were observed in IL-1, IL-6 or TNF production and gene expression in response to endotoxin (Chapter 9). A more likely explanation is that the increased glycogenolysis induced by monokines is related to the activity of blood cells interacting with the endothelium, which is much

more damaged in the old rats (2,3).

The increased hypoglycaemia which occurred after the depletion of hepatic glycogen was accompanied by very high levels of plasma glucagon and an apparently diminished hepatic gluconeogenesis. The apparently insufficient glucose output by the liver during the hypoglycemic phase may largely be a secondary phenomenon, resulting from malperfusion with hypoxia and serious damage to liver parenchyma and endothelium (1,3). However, the increased tendency of old rats to develop hypoglycemia was also observed when both age groups were treated with a dose that induced similar degrees of shock. This increased hypoglycemia could be related to increased peripheral glucose consumption, but the results indicate that the maximal rate of gluconeogenesis is likely to be reduced in endotoxemic old rats, as was also suggested by others (18). Inhibited gluconeogenesis has been shown to augment the severity of endotoxin shock in rats (32).

Age-related changes of several components of the glucoregulatory system have been described, both in humans and in experimental animals, although the nature and magnitude of the reported changes vary between studies.

Much of the research on the effects of aging on glucose metabolism has been carried out in search of explanations for the high prevalence of non-insulin dependent (type II) diabetes mellitus (NIDDM) in the elderly (23). NIDDM is characterized by mild to severe fasting hyperglycemia and glucose intolerance (33). Both decreased uptake of glucose by muscle and liver, and decreased suppression of hepatic gluconeogenesis and glucose output in response to insulin contribute to the glucose intolerance observed, but to different degrees in different patients and stages of the disease (23).

Although aging does not generally cause glucose intolerance or elevated fasting glucose levels (34) some age-related changes that are similar to those observed in (early) NIDDM have been also observed in aged human volunteers (35,36).

In experimental animals, age-related changes in glucose metabolism have been described, but these were generally minor (37,38). There were no indications for major alterations in the normal glucose homeostasis in our 24-month-old BN rats. Basal hepatic concentrations of hexosemonophosphates were marginally lower, and those of glucose and pyruvate marginally higher in old rats. Other age-related changes in glucoregulation included only subtle changes in glucose tolerance test and in response to insulin, which were not strong enough to be interpreted as functional deteriorations.

The glucoregulatory response to endotoxin was strongly affected in the old rats. Similar results were obtained in a study on the effect of aging on hepatic carbohydrate metabolism in a rat model of septic shock induced by experimental peritonitis (18,19). The hyperglycemia noted at 5 hours after cecal incision was also stronger in aged rats, in spite of relatively low glycogen levels due to fasting. At 7 hours, old rats were severely hypoglycemic. Intermediate metabolites of glucose in liver were altered variably by endotoxin in our study, without major age-related differences. We did not determine

fructose-2,6-diphosphate, which was found to be increased in liver of endotoxin-teated rats by Miller et al. (39). F-2,6-dP, a potent allosteric activator of phosphofructokinase, which stimulates the phosphofructokinase/F-1,6-dP cycle in the direction of glycolysis, is likely to be the factor responsible for decreased gluconeogenesis in endotoxemia (39). We observed no changes in fructose-1,6-diphosphate (F-1,6-dP), except for an increase at 5 h in old rats, while Schumer (19) found an increase in hepatic F-1,6-dP in young, but not in old rats. Hexosemonophosphates were decreased, in both our young and old rats, while Schumer described an increase in old but not in young rats. Phosphofructokinase-1 activity was possibly suppressed in the old septic rats. The inhibition of glucose output by the liver at the level of glucose-6-phosphatase suggested by Schumer and Kuttner (18) is probably not present in our LPS treated old rats.

In conclusion, subtle intrinsic changes in the glucoregulatory system system were observed, but they do not appear to play a primary role in the increased sensitivity of old rats to LPS. The changes in glucose and lactate levels are probably consequences of shock, rather than causes. However, alterations in glucose response to LPS contribute to the increased severity of shock, both as a result of intrinsic changes in glucoregulation (gluconeogenesis) and as a secundary reaction to pathophysiological changes and secreted mediators.

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CHAPTER 7

AGE-RELATED CHANGES IN THE ENDOCYTIC CAPACITY OF RAT LIVER KUPFFER AND ENDOTHELIAL CELLS

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Age-Related Changes in the Endocytic Capacity of Rat Liver Kupffer and Endothelial Cells

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There are many indications that the functional capacity of the reticuloendothelial system (RES) declines with age. The aim of this study was to investigate the cellular basis of age-related changes in the clearance function of the RES. The experiments were focused mainly on Kupffer and endothelial cells of the liver which represent a major part of the RES and are primarily responsible for clearance of colloidal material from the circulation. The clearance capacity of the RES was tested clinically and experimentally by intravenous injection of colloids, such as radiolabeled heat-aggregated colloidal albumin.

Age-related changes in the endocytosis of ^{125}I-labeled colloidal albumin (CA) in rats were determined by clearance and organ distribution of different doses of intravenously injected CA, uptake of CA by Kupffer and endothelial liver cells *in vivo* as determined after isolation of the cells from injected rats and kinetic studies on CA uptake by Kupffer cells in culture.

The results show that, at a low dose, the clearance of CA is primarily determined by liver blood flow. At a higher saturating dose, plasma clearance and uptake by the liver are not significantly decreased with age. Endocytosis by endothelial cells, which accounts for about 60% of that of the whole liver, is also unchanged with age. In contrast, a significant decrease in endocytic capacity was observed for Kupffer cells *in vivo*. This age-related functional decline was also observed in Kupffer cells which were isolated from rats of different ages and maintained in culture. It is concluded that the overall capacity of the liver to clear the circulation of colloidal material is not necessarily affected by age. However, the Kupffer cell-specific decrease in endocytic capacity with advancing age may be important in the increased sensitivity to agents which are exclusively removed by Kupffer cells.

The reticuloendothelial system (RES) consists of macrophages and other phagocytic cells which contribute to natural resistance (1). The functions of the RES include clearance of potentially harmful substances from the plasma, catabolism of macromolecules, participation in immune responses and secretion of effector substances. Resistance to pathogenic microorganisms and to the lodging and proliferation of tumor cells appears to decrease significantly with aging in man and experimental animals. The RES, along with the immune system, has been implicated as being partly responsible for this decreased resistance [for a review, see (2)]. However, only a few studies of possible age-related deficiencies in specific functions of reticuloendothelial cells have been conducted (2).

Determination of the plasma clearance rate of intravenously injected colloidal test substances is generally used clinically and experimentally as an indicator of RES function (1). Several reports have described an age-related decline in the clearance capacity of the RES in man and experimental animals (2). In rats and mice, the magnitude of the decline and the age of onset varied considerably in different studies (2). As a consequence, age-related changes in the clearance capacity of the RES are still poorly described.

This study describes the age-related changes in the clearance of one particular RES test colloid, i.e., heat-aggregated colloidal albumin (CA), in rats. In addition, the uptake of CA by the liver and spleen was determined. Radioiodinated CA is an easily traced test substance that has a well-defined composition. It can be applied in humans and in studies with isolated cells (2, 3). Since previous studies from this laboratory showed that the main cell populations involved in clearance of CA from the blood are Kupffer and endothelial cells of the liver

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(3, 4), the endocytic capacity of these cell types *in vivo* was also assessed in rats of various ages. Cultured Kupffer cells were also used to study the kinetics of cellular endocytosis of CA in relation to aging.

MATERIALS AND METHODS

The experiments were performed with different age groups of female BN/BiRij rats from the Institute's aging colony. The 90, 50 and 10% survival data of female rats of this strain are 22, 30 and 37 months, respectively (5).

Bovine serum albumin was labeled with ^{125}I (6). Radioactively labeled bovine serum albumin was heat-aggregated at 75°C (7). The concentration of heat-aggregated CA in the final preparations was between 15 and 25 mg·ml^{-1}, with a specific activity of about 10^6 dpm per mg albumin. Each set of experiments was performed with the same batch of CA, thus excluding the possible influence of interbatch variation.

For determinations of clearance rate, organ distribution and uptake of CA by Kupffer and endothelial cells *in vivo*, mildly etherized rats were injected intravenously in the femoral vein with different doses of labeled CA in saline (see "Results"). The body temperature of the animals was kept at 37° to 38°C.

Plasma samples were obtained from heparinized blood collected from the tail vein at regular time intervals after injections of CA until sacrifice of the animals about 6 min later. The time interval between injection and sacrifice was always shorter than 6½ min to ensure that ingested CA was not broken down and excreted before the organs were removed. Minor variations in time intervals were accounted for in the calculations.

For determination of organ uptake of CA, the liver was blanched by perfusion with Gey's balanced salt solution for 2 min and then excised. The spleen was excised without prior perfusion.

In experiments on the uptake of CA *in vivo* by Kupffer and endothelial cells, the isolation procedure was started by perfusion of the liver through the portal vein, and was immediately followed by sacrifice of the animal by cutting the inferior vena cava, diaphragm and superior vena cava. Kupffer and endothelial cells were isolated by perfusion of the liver with Gey's balanced salt solution containing 0.2% pronase E at 8°C and gentle mechanical disruption of liver tissue at 0°C. The cells were purified by single layer density gradient centrifugation and centrifugal elutriation. The procedures resulted in purified populations of both Kupffer and endothelial cells (4). These isolation procedures were applied at low temperature to retain all ingested CA within the cells (4).

Radioactivity in plasma, tissues and cell preparations was determined in a gamma scintillation counter. In plasma samples, trichloroacetic acid-precipitable radioactivity was determined by addition of 5 m*M* KI and 10% trichloroacetic acid (final concentrations). More than 99% of total radioactivity was trichloroacetic acid-precipitable. From these determinations, the amount of CA in each sample was calculated on the basis of the radioactivity per mg CA in the original preparation. The plasma half-life was determined by linear regression analysis of a semilogarithmic plot of plasma radioactivity vs. time after injection.

Kupffer cells were obtained from untreated rats by treatment of the liver with pronase and collagenase at 37°C and purified by density centrifugation and centrifugal elutriation (8). Purified Kupffer cells were kept in maintenance culture (9). In brief, the cells were cultured at 37°C in Dulbecco's modification of Eagle's medium with L-glutamine containing 20 m*M* Hepes and 10 m*M* $NaHCO_3$ (pH 7.4) (DME medium), and supplemented with penicillin (100 units·ml^{-1}), streptomycin (100 μg·ml^{-1}) and 20% (v/v) fetal calf serum. The cells were used for experiments after 2 days of culture.

Kupffer cell cultures were incubated for 30 min at 37°C with labeled CA in DME medium with 10% fetal calf serum, and the uptake of CA by the cells was determined as described elsewhere (10). After thorough washing, the cells were dissolved in 0.5 *M* sodium hydroxide, and the extracts were counted in an auto-gamma scintillation spectrometer. Control incubations with CA were performed at 4°C, and the values obtained were subtracted from the radioactivity present in the cells after 30 min at 37°C, to correct for nonspecific binding. Cell-associated radioactivity almost exclusively represents endocytosed CA (10), and uptake of CA by cultured cells is linear for at least 30 min (6). The amount of cellular protein in the alkaline extracts was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard.

RESULTS

An initial series of experiments was conducted to establish the possible age-related changes in plasma clearance of CA at a low dosage which is sometimes used for evaluation of the RES function in rodents. The results presented in Table 1 show that a dose of 20 μg CA per 100 gm of body weight is rapidly cleared with a half-life of about 1 min by 3-, 6- and 36-month-old rats. After 6 min, over 95% of the injected dose has been removed from the blood. Most of the injected CA (±90%) was taken up by the liver, with only minor contributions by spleen (±1%) and kidneys (<1%). The rate of uptake of CA by the liver can be expressed as the endocytic index by calculating the uptake of CA in relation to the average concentration of CA in the plasma during incubation. The endocytic index represents the amount of plasma to be cleared of CA to account for the amount of CA present in the liver. The results show that the endocytic index amounts to about 0.5 to 1 ml plasma per min per gm liver, which is similar to the total plasma flow through the liver (7). From this, it can be deduced that, at this dosage, the liver removes almost all CA from the plasma during the first passage. The rate of uptake of CA by the liver is limited primarily by liver blood flow. As a consequence, these experiments do not evaluate the clearance capacity of the RES in relation to age, but can reveal age-related differences in blood circulation. The results indicate that hepatic plasma flow is significantly greater at 6 and 36 months of age as compared with 3 months. The plasma flow per gram (wet) liver is higher in 6-month-old rats than in 3- and 36-month-old ones.

TABLE 3. *IN VIVO* ENDOCYTOSIS OF INTRAVENOUSLY INJECTED CA BY KUPFFER AND ENDOTHELIAL LIVER CELLS AS DETERMINED BY *ISOLATION OF LIVER CELLS AT LOW TEMPERATURE* (8°C)

Age (months)	n	Kupffer cells	Endothelial cells
3	5	32.4 ± 5.3[a]	53.5 ± 8.7
6	5	80.1 ± 10.5	43.3 ± 4.8
18	3	63.2 ± 5.3	59.6 ± 5.8
30	4	55.7 ± 4.1	69.2 ± 4.9
36	5	48.4 ± 6.7[a]	44.7 ± 7.7

Rats were injected with 2.5 mg CA per 100 gm of body weight and treated as in Table 2. The cellular uptake of CA was determined as described in "Materials and Methods." Values are given as nanograms per 10^6 cells per min (mean ± S.E.).

[a] Differs significantly from 6-month old rats ($p < 0.05$).

TABLE 4. ENDOCYTOSIS OF CA BY CULTURED KUPFFER CELLS FROM RATS OF VARIOUS AGES[a]

	Age (months)			
	3	12	30	36
Concentration of CA (μg/ml)				
15	1.06 ± 0.15	0.93 ± 0.13	0.65 ± 0.24	0.65 ± 0.09
29	1.59 ± 0.21	1.38 ± 0.20	1.10 ± 0.25	1.00 ± 0.17
78	2.55 ± 0.42	2.06 ± 0.29	1.60 ± 0.35	1.42 ± 0.19
114	4.27 ± 0.69	2.85 ± 0.42	2.13 ± 0.47	1.82 ± 0.18
219	5.27 ± 0.80	3.68 ± 0.46	3.01 ± 0.68	2.68 ± 0.25
463	8.54 ± 0.96	5.24 ± 0.62	4.35 ± 0.78	3.68 ± 0.40
908	11.24 ± 1.57	6.60 ± 0.69	5.84 ± 1.02	4.76 ± 0.56
2,150	15.33 ± 2.35	8.90 ± 0.67	7.42 ± 0.64	6.74 ± 0.83
3,930	16.58 ± 2.37	11.60 ± 1.54	8.18 ± 0.65	9.33 ± 0.91
Cellular protein (μg/10^6 cells)	92 ± 8	100 ± 3	118 ± 8	118 ± 6
n	5	4	3	4

Cultured Kupffer cells were incubated with CA for 30 min, and uptake was determined as described in "Materials and Methods."

[a] Values are expressed as micrograms of CA taken up per milligram of cellular protein per 30 min (mean ± S.E.).

TABLE 5. KINETICS OF ENDOCYTOSIS OF CA BY KUPFFER CELLS FROM FEMALE BNBI/RIJ RATS OF VARIOUS AGES[a]

Age (months)	n	Maximum rate of uptake (V_{max})	K_m
3	5	9.33 ± 1.36	0.124 ± 0.016
12	4	6.04 ± 0.71	0.089 ± 0.004
30	3	4.99 ± 0.81[b]	0.103 ± 0.025
36	4	4.28 ± 0.41[c,d]	0.091 ± 0.013

[a] Maintenance cultures of Kupffer cells were incubated with various concentration of CA as described in Table 4. The values were calculated, applying Michaelis-Menten kinetics, by linear regression from Lineweaver-Burke plots of the rate of uptake vs. the concentrations of CA. The maximum rate of uptake is expressed as micrograms of CA taken up per milligram of cellular protein per 30 min. The K_m is expressed as mg CA per milliliter. Values are the mean ± S.E.

[b] Difference with 3-month value: $0.05 < p < 0.10$ (Student's t test).

[c] Differs significantly ($p < 0.02$) from 3-month value.

[d] Difference with 12-month value: $0.05 < p < 0.10$.

maximum endocytic capacity, expressed as nanograms of CA per minute per 10^6 cells, was similar in both cell types. Since endothelial cells are about twice as abundant as are Kupffer cells in the liver, these results indicate that endothelial cells make a major contribution to CA clearance. This contribution exceeds that of Kupffer cells, even when the uptake capacity is maximally challenged. For endothelial cells, no significant age-related changes were observed in the uptake of CA. For Kupffer cells, there was a significantly higher uptake in 6-month-old animals as compared with both 3- and 36-month-old ones.

It is not possible to perform kinetic analysis of endocytosis of CA by liver cells *in vivo*, since liver blood flow is rate-limiting at low doses. Therefore, kinetic analyses were performed on purified Kupffer cells in maintenance culture. The cells were incubated with different concentration of CA (20 to 3,000 μg CA per ml). Uptake of CA by cultured Kupffer cells at the highest concentrations was 9 to 16 μg CA per mg protein per 30 min (Table 4). This is equivalent to 30 to 50 mg CA per 10^6 cells per min and is of the same order of magnitude as the rate of CA uptake by Kupffer cells *in vivo* (Table 3). The full dose-response relationships for all age groups are given in Table 4. Cells from 3-month-old rats take up more CA per milligram of cellular protein than do those from 30- and 36-month-old ones over the entire range of concentrations tested. The 12-month age group showed intermediate values which indicates that there is an age-related decline in CA endocytosis by Kupffer cells *in vitro* at all CA concentrations. Table 5 shows that there is a significant age-related decrease in the apparent V_{max}, the maximum rate of uptake of CA by Kupffer cells. The values for V_{max} are expressed per milligram of protein. Because the protein content of cells increases with age (Table 4), the percentage decrease in endocytosis with increasing age is less when V_{max} is expressed per 10^6 cells than per milligram of protein. K_m, which indicates the affinity of Kupffer cells for the substrate, is unaffected by age of the donor rat.

DISCUSSION

The results suggest that, in the rat, colloid clearance capacity, as tested with CA, does not decrease with age at low or high doses of CA. At the low dose, which is generally used for testing RES clearance capacity in man (12–14), the rate of clearance was primarily determined by the rate of blood flow through the liver. Liver blood flow showed only mild variations with age in this study. Since liver blood flow is easily affected by experimental conditions, liver weight per body weight ratio and many other parameters, it is not surprising that, in other studies, age-related changes in liver blood flow in rats during development and maturation were observed (15).

At a high, saturating dose of CA, CA clearance by the liver, which is the main organ responsible for clearance, is unchanged with age. Even when challenged to its maximum capacity, RES clearance function appears to be preserved even in old animals. Age-associated variations in the endocytic capacity of subpopulations of RES cells are not excluded and is suggested by the finding

Blood flow through the liver, which can be calculated from the plasma flow and hematocrit, shows a pattern of age-related changes comparable to that of plasma flow (Table 1).

To study the clearance capacity of the RES, the following experiments were performed with a saturating dose of CA which ensures that the RES endocytic capacity is the rate-limiting factor determining the clearance of CA. A dose of 2.5 mg CA per 100 gm of body weight was found to saturate, which is in accord with other studies (7).

TABLE 1. PLASMA CLEARANCE AND ORGAN DISTRIBUTION OF A LOW DOSE OF INTRAVENOUSLY INJECTED CA

	Age (months)		
	3	6	36
No. of rats (n)	4	4	3
Incubation time (min)	5.9 ± 0.1	6.0 ± 0.0	6.1 ± 0.3
Body weight (gm)	147 ± 5	162 ± 2	202 ± 6
Liver weight (gm)	4.65 ± 0.21	4.38 ± 0.13	5.95 ± 0.15
Spleen weight (mg)	401 ± 67	294 ± 3	464 ± 56
Hematocrit (%)	45.9 ± 2.8	50.5 ± 0.6	42.3 ± 2.5
Clearance half-life (min)	1.2 ± 0.1	1.06 ± 0.01	1.36 ± 0.22
Organ distribution			
Liver (% of dose)	92.2 ± 3.2	94.7 ± 1.3	86.8 ± 4.2
Kidney (% of dose)	0.70 ± 0.35	0.05 ± 0.01	0.18 ± 0.01
Spleen (% of dose)	1.18 ± 0.24	0.92 ± 0.12	0.52 ± 0.70
Plasma (% of dose)	3.73 ± 1.08	1.9 ± 0.4	5.3 ± 2.3
Liver uptake (μg CA/gm liver)	5.87 ± 0.13	7.46 ± 0.21	6.79 ± 0.23
Endocytic index liver			
(ml plasma/min)	2.77 ± 0.32	3.37 ± 0.35	3.28 ± 0.56
(ml plasma/min/gm liver)	0.595 ± 0.058	0.776 ± 0.089	0.552 ± 0.108
Liver blood flow (ml/min/gm liver)	1.30 ± 0.13	1.54 ± 0.19	1.33 ± 0.28

Rats were injected with 20 μg CA per 100 gm of body weight and sacrificed after about 6 min incubation time. Plasma clearance and organ distribution were determined as described in "Materials and Methods." Values are given as mean ± S.E.

The experiments were performed with 3-, 6-, 18-, 30- and 36-month-old rats. The amount of CA that was cleared during the 6-min incubation period was less than 25% of the injected dose. The low clearance rate of CA from plasma and the brief incubation period made it difficult to assess age-related changes from the clearance curves. The half-life of CA in plasma was 15 to 30 min without apparent changes in different age groups (not shown).

The uptake of CA by the liver and spleen is presented in Table 2. For the liver, uptake of CA was 8% of the injected dose, without significant age-related changes. The amount of CA endocytosed per gram liver wet weight varied between 57 and 72 μg and did not change with age.

Although the relative contribution of the spleen to clearance of saturating dose of CA was somewhat greater than with the low dose, it still represents only a minor component. The spleen contained between 0.5 and 1% of the dose. Since the spleen was not blanched by perfusion before excision, this amount includes a significant quantity of CA in blood (estimated to be about 0.3 to 0.4% of the injected dose, assuming 10% of the splenic weight to be occupied by blood). For these reasons, the moderate decrease in CA content per whole and per gram spleen observed in old rats may not reflect an age-related decrease in uptake capacity.

Endocytosis of CA by Kupffer and endothelial liver cells *in vivo* was determined by isolation and purification of these cell types from animals which were injected with a saturating dose of CA. The cells were isolated at low temperature to avoid degradation and excretion of label from cells during the isolation procedure. The uptake of CA by the two cell types is presented in Table 3. The

TABLE 2. ORGAN DISTRIBUTION OF A SATURATING DOSE OF IN VIVO INJECTED CA

	Age (months)				
	3	6	18	30	36
No. of rats (n)	5	5	3	4	5
Incubation time	6.0 ± 0.1	6.1 ± 0.1	6.6 ± 0.1	6.4 ± 0.9	6.3 ± 0.1
Body weight (gm)	159 ± 4	155 ± 5	201 ± 11	223 ± 10	219 ± 10
Liver weight (gm)	5.12 ± 0.13	5.02 ± 0.15	6.48 ± 0.34	7.20 ± 0.31	7.09 ± 0.32
Spleen weight (mg)	348 ± 19	304 ± 17	361 ± 29	484 ± 48	500 ± 47
Organ distribution (% of dose)					
Liver	8.54 ± 1.0	7.37 ± 0.89	8.42 ± 0.37	9.27 ± 0.89	8.88 ± 1.24
Spleen	0.92 ± 0.09	0.80 ± 0.05	0.67 ± 0.07	0.74 ± 0.10	0.56 ± 0.06
Plasma	81.4 ± 56.	85.0 ± 2.7	76.3 ± 3.2	80.9 ± 0.8	76.6 ± 1.8
Organ uptake (μg·CA/gm)					
Liver	66.2 ± 9.3	57.1 ± 6.9	65.0 ± 2.9	71.8 ± 6.9	68.8 ± 9.6
Spleen	104.6 ± 4.4	103.2 ± 8.2	93.6 ± 7.2	86.0 ± 3.4	61.5 ± 4.4

Rats were injected with 2.5 mg CA per 100 gm of body weight and sacrificed after about 6 min incubation time. Organ distribution was determined as described in "Materials and Methods." Values are given as mean ± S.E.

that the endocytic capacity of Kupffer cells decreases significantly between 6 and 36 months of age; no changes were observed for endothelial cells. Apparently, the decrease in Kupffer cell function is not sufficiently severe to cause a significant reduction in total liver uptake. This is not unexpected, since Kupffer cells are responsible for only about 30% of total liver clearance. Age-related decline in endocytosis of CA by Kupffer cells was also observed in maintenance cultures *in vitro*, although with a slightly different pattern. A progressive decrease was observed at between 3 and 36 months of age, resulting in a maximum reduction of 40%. The difference between the *in vivo* and *in vitro* results is mainly due to the 3-month-old rats, which show lower values *in vivo* than other age groups. The difference can be partly explained by the relatively low protein content of Kupffer cells from younger rats (see "Results"). *In vivo* clearance of colloids may fluctuate considerably during development of the rat [see Ref. (2)]. The 3-month-old rats should not be considered as adult animals. Any inference about the effect of aging on the uptake of CA should be drawn from comparisons between young adults (≧6 months old) and aged animals. The results of *in vivo* and the *in vitro* experiments suggest that the endocytic capacity of Kupffer cells decreases during aging.

The results on CA clearance by liver are in apparent contradiction with the previous suggestion that there is an age-related decline in the clearance capacity of the RES in man (12, 13, 16) and rodents [7, 15, 17–22; see also (2)]. However, the studies performed in man included injection with only low doses of (different) colloids and probably are indicative of age-related changes in liver blood flow rather than clearance capacity (2). The studies in rodents, surprisingly, did not include senescent animals. Several investigators attempted to increase the limited value of clearance parameters by calculating a (theoretical) maximum rate of uptake (V_{max}) by extrapolation of the results obtained after two consecutive increasing doses of a test colloid (12, 17). With this method, moderate age-related decreases in V_{max} were found in both man (12) and rats (17). However, this extrapolation is crude, since it is based only on theoretical assumptions concerning the relationship between clearance rate and injected dose.

The clearance studies in rodents showed contradictory results on the patterns of change with age observed in young and mature animals (7, 15, 17, 22). These discrepancies are probably results of the use of different species and strains of rodents, different types and concentrations of test colloids and other causes which cannot be distinguished from real age-related changes in RES clearance capacity. Therefore, we determined the clearance capacity by direct measurement of colloid uptake in the liver under saturating conditions and also determined uptake in specific liver cell populations. The results shows that the capacity of the whole liver to take up CA is not significantly affected even in very old age. However, a significant age-related decrease in endocytic capacity was observed for Kupffer cells. Since most RES test substances are, like CA, not exclusively cleared by Kupffer cells (3), this decrease can easily remain undetected. There are a number of potentially harmful agents, such as bacterial endotoxins, which are removed from the circulation only by Kupffer cells (3). The observed decrease in Kupffer cell endocytosis may, therefore, be an important factor in the increased sensitivity to infectious agents during aging.

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CHAPTER 8

STIMULATION OF KUPFFER CELLS FROM YOUNG AND OLD RATS BY ENDOTOXIN: 1. PRODUCTION OF EICOSANOIDS AND CYTOKINES

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SUMMARY

These studies are focused on the role of hepatic Kupffer cells in the increased sensitivity of old rats to bacterial endotoxins. Possible age-related changes in the production of the eicosanoids and the induction of gene expression and secretion of interleukin-1, tumor necrosis factor, and interleukin-6, by Kupffer cells were investigated in cells derived from both young and old animals.

The basal production of biological response modifiers was very low in cells of both young and old rats indicating that the resident macrophages of the liver were in an unactivated state in both age groups. Upon stimulation by LPS, the same types of monokines were produced as described for other types of macrophages, although the pattern was specific for Kupffer cells.

Eicosanoid production, predominantly PGD_2 and $PGF_{2\alpha}$, was observed mainly during the first hour after exposure to LPS. Endotoxin stimulated the synthesis of mRNA's of IL-1, IL-6 and TNFα in Kupffer cells in vitro, resulting in increased secretion of these cytokines into the medium. Kupffer cells from both young and aged animals appear to be exquisitely sensitive to endotoxin in respect of expression of mRNA for both IL-1α and IL-1β, and less sensitive in this regard with respect to IL-6 and TNFα gene expression. At relatively high LPS concentration IL-6 was secreted in particularly large amounts.

The effects of aging on any of these responses of Kupffer cells was minimal. No changes were observed at 24 months, and only a marginal increase in leukotriene and possibly $PGF_{2\alpha}$. There are no literature data available for direct comparison, but these results may be somewhat surprising in the light of reported age-related changes in other rat macrophages. It seems, however, unlikely that Kupffer cells, despite displaying some age-related changes in the uptake of and response to LPS, are a key factor in the increased susceptibility of old rats to LPS.

INTRODUCTION

Bacterial endotoxins are the ubiquitous lipopolysaccharide (LPS) components of bacterial cells walls which play a major role in the pathogenesis of septicaemia (1). The development of endotoxaemia is a medical emergency involving a state of cardiovascular collapse accompanied by severe inflammatory tissue damage which may be fatal and has been termed endotoxin shock (1). Mortality and tissue injury from experimental endotoxaemia have been shown to be highly age dependant, elderly subjects demonstrating greatly increased mortality and tissue damage when compared to younger age groups (2,3). Endotoxins are cleared from the circulation by cells of the mononuclear

phagocyte system and in this regard Kupffer cells play a central role: they comprise the largest population of fixed macrophages in the body, are responsible for the clearance and detoxification of gut-derived endotoxin under physiological conditions (4,5), and show an age-related decrease in the capacity to clear bacterial endotoxin from the circulation (6,7). However, this decreased clearance capacity is only marginal and can only partly explain the increased sensitivity of older rats to endotoxin (6,8). Other factors, including secondary reactions evoked by biological response modifiers such as the eicosanoids and the acute phase cytokines interleukin 1, interleukin 6 and tumor necrosis factor produced by activated macrophages (9) and other cells (1,10), might also play an important role in the increased sensitivity of older rats to endotoxins.

Therefore, possible age-related changes in the production of the eicosanoids and the cytokines interleukin-1, tumor necrosis factor, and interleukin-6 by endotoxin-treated Kupffer cells have been investigated in cells derived from both young and old animals.

MATERIALS AND METHODS

Isolation and culture of Kupffer cells from rat liver

Kupffer cells were isolated by pronase-collagenase treatment of the liver of untreated female BN/BiRij rats of various ages and purified by centrifugal elutriation, as described earlier (11). The cells were kept in maintenance culture for 20-24 hr in medium containing 20% fetal calf serum (FCS) (HyClone Laboratories Inc., Logan, Utah) (12). Medium components were used to ensure low basal endotoxin concentrations in the media (< 10 pg.ml^{-1}). This was checked by the quantitative chromogenic Limulus amoebocyte lysate (LAL) assay (KabiVitrum, Stockholm). Cultured Kupffer cells were incubated with various concentrations of E. coli O26B6 lipopolysaccharide (LPS) and the secretion of various monokines into the medium was determined.

Cytokine and eicosanoid assays

Interleukin 1 (IL-1), tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) were determined by sensitive and specific bioassays. IL-1 and IL-6 were determined by cell proliferation assays. For IL-1 the D10(N4)M subline of the murine T-cell cloned line D10.G4.1 was used, in the presence of saturating concentrations of human recombinant IL-2, as described by Hopkins and Humphreys (13). For IL-6, the method of Helle et al. (14), using B9 cells, was employed with minor modifications. TNFα was determined by cytotoxicity assay with L929 cells, essentially as described by Mathews and Neale (15).

Eicosanoids were determined using specific radioimmunoassay kits for prostaglandins D_2, E_2, $F_{2\alpha}$, and 6-keto-$PGF_{2\alpha}$, thromboxane B_2, and, a multi-specific assay for leukotrienes C_4, D_4 and E_4. All these kits were obtained from Amersham, UK.

Analysis of cytokine mRNA levels in Kupffer cells exposed to LPS

Cytoplasmic RNA was isolated from monolayer cultures of Kupffer cells essentially according to Gough (16). Vanadyl ribonuclease complexes were included in the cell lysis buffer at a concentration of 10 mM and the resultant RNA preparation was subject to 3 rounds of purification by extraction with phenol/chloroform/isoamyl alcohol.

RNA was quantitated by measurement of absorbance at 260 nm and aliquots containing equal amounts of RNA used to prepare the dot blots. RNA preparations were denatured at 65°C for 15 minutes in 16% formaldehyde/10 x SSC. Eight fold dilution series were prepared for each sample in 10 x SSC and 400 μl of each dilution spotted onto nitrocellulose (Hybond C extra - Amersham international) using a commercial dot-blotting manifold (Hybridot BRL Cat. no. 1050 MM). The filter was pre-wetted in 10 x SSC and after preparation was dried in air and baked for 2 hours at 80°C. Cytoplasmic RNA derived from isolated parenchymal liver cells was applied to each blot as a negative control.

The dot blots were probed with the following cDNAs. Full length rat IL-6 cDNA (RIL6-P1; 736 bp in a Bluescript vector) (17) was a generous gift from Dr. J. Gauldie (Dpt. of Pathology, McMaster University, Hamilton, Ontario, Canada). Full length mouse TNFα cDNA (pGEM-1/mTNF; 705 bp cDNA, including signal sequence) was a generous gift from Prof.Dr. W. Fiers (Dpt. Molecular Biology, Gent University, Gent, Belgium). Rat IL-1α and β cDNA's (Puc8il1alpharat (821 bp cDNA) and Pgemratil1b9 (276 bp cDNA) were obtained from the Glaxo Institute for Molecular Biology S.A. (Geneva, Switzerland). A rat β-actin probe consisting of a 1200 bp BglI fragment of the pRβA-1 cDNA clone isolated by P. Gunning (kindly supplied by C. Redfern, University of Newcastle upon Tyne, UK) served as control for RNA's which are not induced by LPS. Twenty five nanograms of each cDNA was labelled with ^{32}P-dCTP in a random primer labelling reaction using a commercial kit (Boehringer) and separated from unincorporated label by sepharose G50 column chromatography (NICK columns - Pharmacia). Probes were routinely labelled to a specific activity of $>10^9$ cpm.μg^{-1} for hybridizations (18).

Dot blots were pre-hybridised for 2 hours at 42°C in a mixture containing 50% formamide, 5 x Denhardts solution, 5 x SSC, 50 mM NaH_2PO_4 pH 6.5, 5 mM EDTA and 500 $\mu g.ml^{-1}$ denatured salmon sperm DNA. Hybridisation was carried out for 20 hours at 42°C in 50% formamide, 1 x Denhardts solution, 5 x SSC, 50 mM NaH_2PO_4 pH 6.5, 5 mM EDTA, and, 250 $\mu g.ml^{-1}$ denatured salmon sperm DNA. After hybridisation the filters were washed once in hybridisation buffer without salmon sperm DNA for 1 hour at 42°C, and then twice in 2 x SSC/0.1% SDS for 15 minutes at 42°C, finally 15 minutes in 0.2 x SSC/0.1% SDS at 65°C.

Hybridisations were visualised by autoradiography using pre-flashed film. All observations are supported by results from at least three independant experiments.

RESULTS

Production of eicosanoids by Kupffer cells from young and old rats

Incubation of isolated Kupffer cells in maintenance culture showed that these cells can be induced by endotoxin to produce considerable quantities of prostaglandins D_2 and $F_{2\alpha}$, and little or no thromboxane B_2, PGE_2, 6-keto-$PGF_{2\alpha}$ and leukotrienes (Table 1). Eicosanoids were produced mainly during the first hr of incubation, and little during the subsequent 23 hr. Kupffer cells isolated from 24-months-old rats produce similar quantities of these endotoxin-induced mediators both at 1 and at 24 h of incubation (Table 1). Only at 38 months of age, there was some increase in the excretion of $PGF_{2\alpha}$ and of leukotrienes.

Table 1. Endotoxin-induced eicosanoid production by Kupffer cells from rats of various ages.

Incubation time (hours)	Age (months)	Concentration of eicosanoids (ng/mg cell protein)		
		PGD_2	$PGF_{2\alpha}$	LT's (C_4, D_4 and E_4)
1	6	31 ± 11 (5)	4.6 ± 2.1 (5)	n.d. (4)
	24	28 ± 14 (5)	3.6 ± 1.3 (5)	n.d. (4)
	38	43 ± 17 (5)	6.2 ± 3.2 (4)	0.3 ± 0.3* (4)
24	6	41 ± 24 (5)	5.1 ± 3.6 (5)	n.d. (4)
	24	34 ± 16 (5)	7.5 ± 4.1 (5)	n.d. (4)
	38	45 ± 11 (5)	10.9 ± 2.2 (4)	0.5 ± 0.2* (4)

Cells were incubated with 100 μg E. Coli 026:B6 lipopolysaccharide ml. PGE_2 was always undectable, which implies that PGE_2 production did not exceed about 0.4 ng/mg protein.
Abbreviations: PG: prostaglandin; LT: leukotriene; n.d.: not detectable.
Values are given as means ± SEM (n).
* Differs significantly from the 6- and 24-month-old rats ($p < 0.05$, Student-t test).

Interleukins 1 and 6 and TNFα secretion

At one hour after addition of LPS, there was still very little production of the interleukins or of TNFα (Fig. 1a,c,e). Secretion of IL-1 and TNFα was clearly stimulated by LPS at 24 hr, and the Kupffer cells especially produced large amounts of IL-6 in response to endotoxin (Fig. 1b,d,f). Maximal stimulation of the secretion of all three cytokines was already observed at 10 μg $LPS.ml^{-1}$. No significant changes were observed in the production of the three monokines by Kupffer cells from the various age-groups

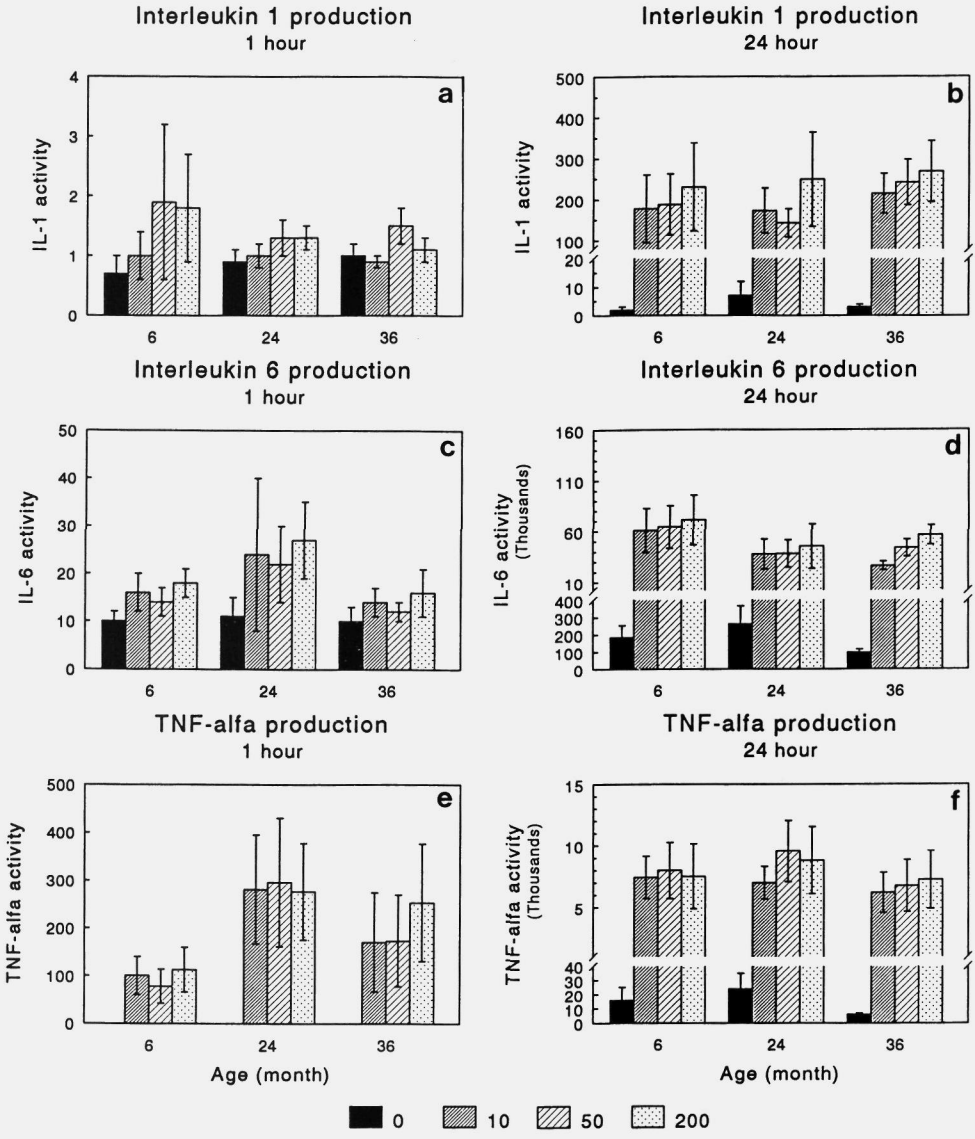


Figure 1 a-f. Interleukin 1, Interleukin 6, and tumor necrosis factor-α production by Kupffer cells from rats of various ages incubated with E.coli lipopolysaccharide.

Between 1 and 1.5 × 10^6 viable Kupffer cells were seeded per well. After 18-22 hr LPS was added at concentrations of 0, 10, 50 and 200 μg ET.ml^{-1}. Supernatants and cells were harvested separately after 1 hr (a, c, and, e) or 24 hr (b, d, and, f) incubation. Values are given as means ± SEM (n = 5).

a and b: IL-1 activities are expressed as units IL-1 (standard NIBSC) per 10^6 viable Kupffer cells.

c and d: IL-6 activities are expressed as units IL-6 per 10^6 viable Kupffer cells. One unit IL-6 represents approximately 1 pg recombinant human IL-6.

e and f: TNF-α activities are expressed as units TNF-α (standard NIBSC) per 10^6 viable Kupffer cells. TNF-α activity was below the detection limit at 1 hour incubation without LPS.

No significant differences ($p < 0.05$, Student-t test) between the age groups were present.

at any of the three LPS doses tested. Without added LPS, Kupffer cells of both young and old rats produced very small to undetectable amounts of all three monokines tested, which shows that the production of these mediators occurs only after stimulation of the cells.

Cytokine mRNA

Interleukin-1. Basal expression of IL-1α and IL-1β mRNA's was detected in unstimulated Kupffer cells. Some variability in the level of basal expression was seen (cf. Figs. 2, 3a and b). The dose-dependency of mRNA induction was studied after 2 hours incubation with endotoxin. Already at a dose of 25 ng.ml^{-1}, near maximal increases in expression were seen, when compared to a concentration of 100 μg.ml^{-1} (Fig. 2). Some increase in expression was observed at concentrations as low as 5 ng.ml^{-1} (not shown). Both young (6 months) and old (30 months) Kupffer cells showed similar responses.

The time dependency was studied at two doses. At a concentration of 100 μg.ml^{-1} some increase in expression was seen as early as 15 minutes after application of the stimulus, this response reaching a maximum at 30 to 60 minutes (Fig. 3) and being

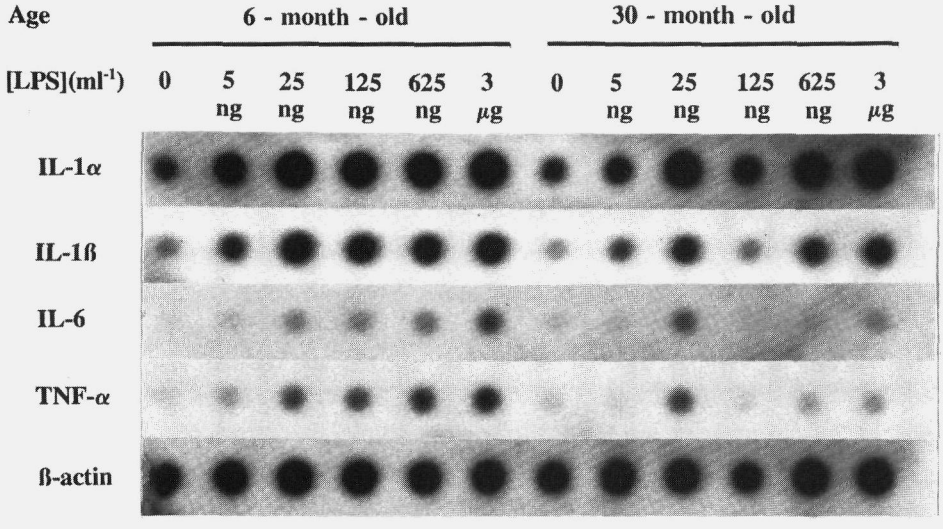
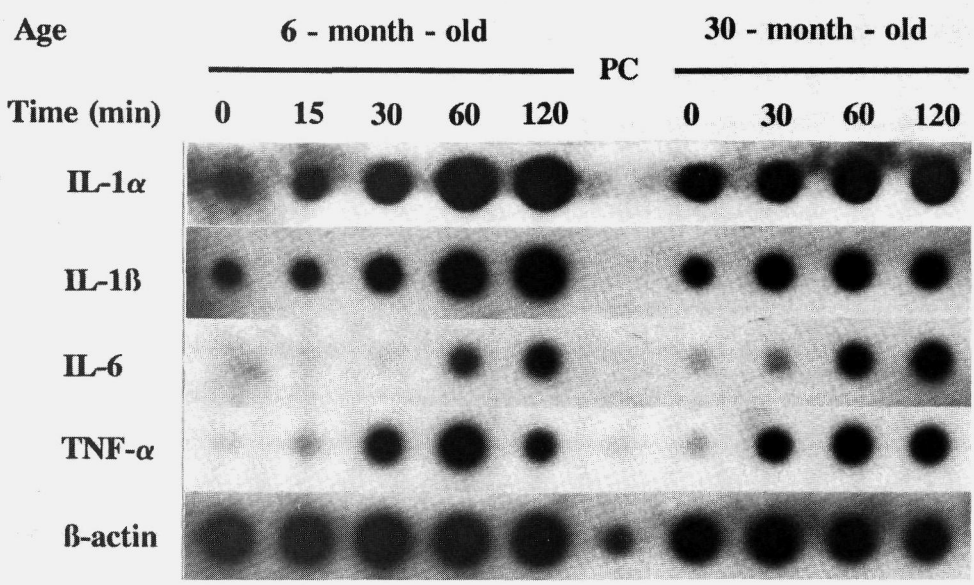


Figure 2. Effects of incubation with various concentrations of LPS on cytokine mRNA content of Kupffer cells isolated from young and old rats.
Cultured Kupffer cells from 6- and 30-months-old female BN/BiRij rats were incubated with the indicated concentrations of E. coli lipopolysaccharide for 2 hours. Total cytoplasmic RNA (about 1.5 μg for the first spot) was analyzed for the presence of specific mRNA species by dot blot hybridization (18).
Abbreviations: PC: parenchymal cells; cDNA probes: see Materials and methods section.

a



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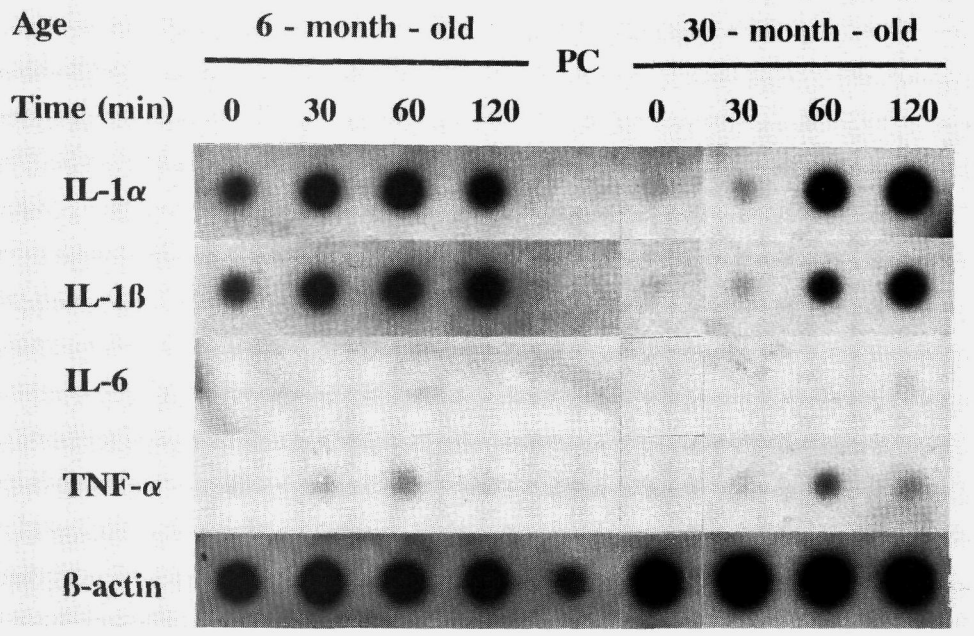

Figure 3 a-b.

Time course of induction of cytokine mRNAs by LPS in Kupffer cells isolated from young and old rats. Cultured Kupffer cells from 6- and 30-months-old female BN/BiRij rats were incubated with 100 μg LPS/ml (Fig. 3a) or 25 ng LPS/ml (Fig. 3b) for up to 8 hours. Methods and abbreviations as in Fig. 2.

sustained up to 8 hours in the continued presence of the stimulus (not shown). No differences were seen between cells from young and old rats. The only indication of a possible age effect was seen at a concentration of 25 $ng.ml^{-1}$: the old cells appeared to react more slowly to the stimulus, maximal expression being reached at 30 minutes in cells from young rats, and at 60 minutes in those from old ones (Fig. 3b).

TNFα and Interleukin-6. In contrast to the IL-1 species, expression of TNFα and IL-6 mRNA's was not detected in unstimulated Kupffer cells. After 2 hours incubation with endotoxin at a dose of 25 $ng.ml^{-1}$, expression was mostly somewhat higher than in control cells for IL-6, and more so for TNFα (Figs. 2 and 3b). A much stronger induction of both mRNA's was found with a concentration of 100 $\mu g.ml^{-1}$ (Figs. 2 and 3a). Both young (6 months) and old (30 months) Kupffer cells showed similar responses.

At a concentration of 100 $\mu g.ml^{-1}$ some increase in expression of IL-6 and TNFα mRNA's was seen at 30 minutes after application of the stimulus, this response reaching a peak at 30 to 60 minutes (Fig. 2a) and subsequently falling (though not returning to basal levels) by 4 to 8 hours in the continued presence of the stimulus (not shown). No consistent differences were seen between young and old cells.

DISCUSSION

Many of the features of endotoxin shock have been ascribed to cytotoxic mediators, like TNF, interleukins and eicosanoids. Macrophages, particularly Kupffer cells, are a likely source for most of these biological response modifiers (for reviews see: 9,19). The literature on cytokine production and secretion is difficult to interpret. For many years there was no consistency in nomenclature for cytokines and this confuses the issue up until about 1988 when international agreement on cytokine nomenclature was reached (20). Another confounding factor is that various sorts of activity, upon which biological assays were based, are shared between more than one cytokine (for a recent overview see 21).

The basal production of biological response modifiers was very low in cells of both young and old rats indicating that the resident macrophages of the liver were in an unactivated state in both age groups. Upon stimulation by LPS, the same types of mediators were produced as described for other types of macrophages, although the patterns were different.

Incubation of isolated cultured Kupffer cells with endotoxin in vitro showed that these cells can be induced to produce large quantities of prostaglandins D_2 and $F_{2\alpha}$ within two hours, and little or no thromboxane B_2, PGE_2, 6-keto-$PGF_{2\alpha}$ and leukotrienes. The production of PGD_2 and $PGF_{2\alpha}$ by rat Kupffer cells was described earlier by others (9,19,22,23). The absence of PGE_2, and particularly of 6-keto-$PGF_{2\alpha}$ (which is formed

after prostacyclin production) and thromboxane B_2, is still remarkable in view of other studies in which Kupffer cells produced considerable quantities of these cyclooxygenase products upon activation with phorbol ester, PAF, zymosan, and also with LPS (9). The pattern is also different from are own data on Ca-ionophore stimulated eicosanoid production by Kupffer cells, which included a large proportion of PGE_2, 6-keto-$PGF_{2\alpha}$ and leukotrienes (24). Apparently, LPS induces a specific selection of eicosanoids, while Ca-ionophore seems to trigger the conversion of arachidonic acid into the different eicosanoids in a non-specific manner. The complex regulation of eicosanoid synthesis depends greatly on the type of macrophage, its activation state, the stimulating agent, species, and it is strongly modulated by the presence of cytokines, hormones and eicosanoids themselves (9,25). Human Kupffer cells secreted more types of eicosanoids in response to endotoxin, particularly TxB_2 and PGE_2, than did rat Kupffer cells. However, this may be due to the fact that human Kupffer cells appeared already to be activated without incubation with LPS (26).

As reported by several others (see: 9,19) IL-1 and TNFα were also secreted in response to LPS. The cells especially produced large amounts of IL-6 in response to LPS, which was also described by Busam *et al.* (27) and Decker (9), as well by Feder *et al.* after in vivo LPS administration (28). The secretion of IL-1, IL-6 and TNFα by Kupffer cells started later, and was longer lasting, than that of eicosanoids. Endotoxin stimulated expression of IL-1, IL-6 and TNF-α mRNA's in Kupffer cells in vitro, an observation which has not previously been reported. The degree of induction, both for gene expression and secretion of the cytokines is striking, and emphasises the role of these cells as producers of important autocrine and endocrine biological response modifiers. Of particular interest is the dichotomy observed between the IL-1 response and the IL-6/TNFα response at the mRNA level. Kupffer cells from both young and aged animals appear to be exquisitely sensitive to endotoxin in respect of expression of mRNA for both IL-1α and IL-1β, and less sensitive in this regard with respect to IL-6 and TNFα gene expression.

The effects of aging on the induction of any of these mediators was minimal. Kupffer cells isolated from the 24-months-old rats produced similar quantities of the endotoxin induced eicosanoids as cells from young ones. Cells from older, 38-months-old, rats displayed some increase in $PGF_{2\alpha}$ and leukotriene production. Also, no changes were observed in the secretion of IL-1, IL-6 and TNFα by Kupffer cells from 6- and 30-months-old rats, only a possible tendency for a slower induction of mRNA.

There are few literature data on aging available for direct comparison, but these results on Kupffer cells may be compared to reported age-related changes in other types of macrophages. Several biochemical and functional characteristics of rodent macrophages have been reported to change, generally decrease, with aging, but unchanged activities were also found (for reviews see: 2,21,28 and Chapter 3).

Only few studies on age-related changes in the basal regulation of eicosanoid production are available. Plasma levels of prostanoids were altered in one study (30), and unaltered in another (31). The pattern of prostanoid synthesis in the kidney of older individuals appears different, with lower prostacyclin and higher thromboxane production (32).

The literature on the effects of aging on the synthesis and secretion of cytokines is limited. The message from animal studies is fairly clear: macrophages derived from aged mice and rats synthesise and/or secrete reduced quantities of IL-1 compared to those derived from young animals. Earlier studies used the relatively unspecific LAF assay, but those which utilised specific antisera (33) or cDNA probes (34) confirmed this age-associated defect. Studies in man do not show the same age related decline in IL-1 production by peripheral blood monocytes, using unspecific bioassays (c.f. 21 and Chapter 2). Two recent studies studies suggest a decline in the ability of aged peritoneal macrophages to synthesize and secrete TNF (33,35). No data on IL-6 and aging have been published. It is clear that despite a huge expansion of research into the general area of acute phase cytokines in the past ten years, our knowledge of the effect of aging on the signal molecules of the acute phase response in aged man is still deficient.

Taken together, our data and those of others show that overt functional deficits are not necessarily the rule or a predictible entity for macrophages of old individuals. However, dependent on genetic background and conditions of aging, some functional changes that contribute to the decreased plasticity of old age are likely to occur.

Our own data on early response protein synthesis provide confirmation at the cellular and molecular level that Kupffer cells responses to LPS are less vigorous in old than in young animals (chapter 9). This observation is more in keeping with our previous observations showing an age-related reduction in the clearance function of these cells regarding both LPS and colloidal albumin.

However, the in vivo experiments showed that old rats display an over-reaction, rather than a less vigorous one, after intravenous injection of LPS in many respects (c.f. chapter 3). Tissue responses such as pulmonary, hepatic and renal injury were more severe and activation of the fibrinolytic system was more pronounced. It seems, therefore, unlikely that Kupffer cells, despite displaying age-related changes in their response to LPS, are a key factor in the increased susceptibility of old rats to LPS. The stress of experimental endotoxemia appears to reveal age-related dysregulation in these important adaptive responses at multiple levels.

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CHAPTER 9

STIMULATION OF KUPFFER CELLS FROM YOUNG AND OLD RATS BY ENDOTOXIN: 2. INDUCTION OF EARLY RESPONSE PROTEINS

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SUMMARY

In order further to characterise the role of Kupffer cell responses in the age related increase in mortality following endotoxin administration, we examined the induction of Kupffer cell proteins by endotoxin in cells isolated from young (6 months) and old (30 months) female BN/Rij rats. The response to endotoxin was assessed by incorporation of ^{35}S-methionine into cellular proteins and visualised by 1 and 2 dimensional SDS/PAGE.

Kupffer cells isolated from female BN/Rij rats respond to endotoxin stimulation by the synthesis of a set of early response proteins similar to that described for murine peritoneal macrophages (1). One dimensional SDS/PAGE reveals induced proteins 3 hours after stimulation with apparent molecular weights of 32, 37, 52, 71 and 77 kD. Suppression of synthesis was consistently found at 38, 51 and 59 kD. This protein set is also induced in peritoneal macrophages derived from the same animal but tissue specific differences are seen, specifically a prominent induced band at 41 Kd which is seen in peritoneal macrophages but not Kupffer cells.

Kupffer cells from young and old animals differed markedly in quantitative aspects of their responses. Dose/response analysis showed that Kupffer cells isolated from young animals were approximately 2.5 times more sensitive to endotoxin than old cells in respect of specific protein induction during the third hour after application of the stimulus. Analysis of the time course of induction of these proteins demonstrated a slower response in the cells isolated from old rats. The rate of synthesis of induced proteins remained elevated for at least 12 hours in the continued presence of the stimulus.

Further characterisation of the Kupffer cell early response protein set by 2 dimensional isoelectric focusing (IEF)/SDS PAGE allowed identification of 3 proteins which appear to correspond to the 32, 50 and 77 kD bands and confirmed the reduced sensitivity of aged cells with respect to their induction by endotoxin.

INTRODUCTION

Activation of cells capable of transducing the initial signal of tissue damage or bacterial invasion is the crucial starting point for both local and systemic inflammatory responses. In this context activation of cells of the mononuclear phagocyte system by endotoxin - or bacterial lipopolysaccharide (LPS) - is of particular importance. Mononuclear phagocytes bear numerous surface receptors of different types and in recent years the mechanisms by which extracellular signals activate these cells to acquire functional

competencies such as cytotoxicity for various target cells, bacterial phagocytosis and killing, antigen presentation, the production of local inflammatory mediators (such as eicosanoids and oxygen free radicals) and systemic inflammatory signal molecules (such as tumor necrosis factor and interleukins-1 and -6) has been intensively studied (2).

Studies in murine peritoneal macrophages demonstrate that stimulation leading to the acquisition of functional competence for complex macrophage functions (such as tumour cell killing, antigen presentation and cytokine synthesis and secretion) is preceded and accompanied by a complex and highly regulated series of intracellular events. Hydrolysis of membrane inositol phosphates (3), changes in intracellular calcium concentration (4), protein phosphorylation (5) and the expression of a number of proto-oncogenes (6) is accompanied by the induction of a set of so-called "early response proteins" (1).

In these studies the rat macrophage early response protein set has been characterised in both Kupffer cells and peritoneal macrophages, and in order further to examine the role of Kupffer cells in the age associated increase in sensitivity to LPS, possible age related changes in the induction of these proteins have been investigated in cells from young and old animals.

MATERIALS AND METHODS

Materials

All laboratory glassware was autoclaved and baked prior to use. All tissue culture consumables (Falcon) were obtained from Becton Dickinson & Co (England). Collagenase H was obtained from Boehringer Mannheim GmbH (Mannheim, FRG) and Pronase E from Merck (Darmstadt, FRG). Methionine-free Minimum essential medium (Eagle) with Earles salt (MEM) and Geys balanced salt solution (GBSS) was obtained from Gibco (Grand Island, NY) and in vivo cell labelling grade 1 [^{35}S]-methionine (> 1000 Ci/mmol) from Amersham International (Amersham, UK). Fetal calf serum (FCS) was from HyClone Laboratories Inc. (Logan, Utah). All chemicals used for electrophoresis and isoelectric focusing were of the highest purity available unless otherwise stated. LKB ampholines were obtained from Pharmacia (Milton Keynes, UK) and 'Enhance' autoradiographic enhancer from New England Nuclear (Boston, Mass.).

Animals

Specific pathogen free female BN/Rij rats were maintained under 'clean conventional' conditions as previously described (7). The 90%, 50% and 10% survival ages of this rat strain are 21, 32 and 39 months, respectively. Rats were not fasted prior to cell isolation, which was performed under terminal ether anaesthesia.

Cell isolation and culture

Hepatic sinusoidal cells were isolated from 6 months and 30 months animals by pronase/collagenase perfusion and further purified by continuous density gradient centrifugation and centrifugal elutriation as previously described (8).

Kupffer cells were elutriated at 4°C at a flow rate of 70 to 85 ml/minute using a Beckman J6 elutriator rotor at 3250 rpm. Viability was routinely 89% (range 86-94%) as assessed by Trypan blue dye exclusion and purity was 79 - 95% as assessed by staining for endogenous peroxidase activity.

Cells were then suspended in MEM containing 20% fetal calf serum, penicillin (10^5 units/l) and streptomycin (100 mg/l), and plated out at a density of 3 x $10^5/cm^2$ in 35 mm wells or 24 well plates, as appropriate, cultured overnight and used for LPS stimulation experiments on the day after isolation. Medium components which ensured low basal endotoxin concentrations in the media (< 10 $pg.ml^{-1}$) were used.

Peritoneal macrophages were obtained by peritoneal lavage with 50 ml phosphate buffered saline (PBS)/0.34 M sucrose, they were washed 3 times in ice-cold PBS and suspended in MEM containing 20% fetal calf serum and antibiotics and plated out at a density of 6 x 10^5 cells/cm^2. After four hours culture at 37°C in 95% air/ 5% CO_2 the plates were washed vigorously 3 times with unsupplemented medium and the supplemented medium replaced. These cells were subsequently processed in an identical fashion to the Kupffer cells. Viability was greater than 86% and the resultant cell monolayer contained more than 90% macrophages.

Protein labelling with ^{35}S methionine

After an overnight period in culture the cells were washed 3 times in PBS and the medium replaced with serum-free, methionine-free MEM supplemented with 10 mM HEPES, 2 mM glutamine, antibiotics and varying amounts of LPS. Cells were cultured usually for 3 hours at 37°C in a 95% air/5% CO_2 atmosphere, 10 to 100 μCi of ^{35}S-methionine being added to each well during the final hour of incubation. After a further 1 hour incubation the cells were placed on ice, washed 5 times with ice cold PBS and prepared for electrophoresis as described below.

One dimensional SDS/PAGE

One-dimensional SDS/PAGE was carried out essentially as described elsewhere (9). Cell proteins were solubilised in 125 mM Tris HCl pH 6.8 containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and the protease inhibitors N-ethylmaleimide (NEM) (25 mM), thimerosal (0.1%), and, polymethylsulphonylfluoride (PMSF) (1 mM). Samples were frozen immediately in liquid nitrogen and stored at -70°C until used. Immediately prior to application to the gel the proteins were denatured for 2 minutes in a boiling water bath. Discontinuous 16 x 16 cm x 0.75 mm slab gels containing 12% acrylamide monomer

in the resolving layer were used throughout. Molecular weight markers were included in all gels and were pre-stained (Rainbow molecular weight markers, Amersham International, Amersham, UK)) with a molecular weight range of 14,300 - 200,000 kD.

Two-Dimensional IEF/SDS/PAGE

Two dimensional SDS/PAGE was carried out essentially as described elsewhere (10), with minor modifications (11). Briefly, cell monolayers were solubilised in 10 M urea containing 3% Nonidet NP40, 5% 2-mercaptoethanol, 3% ampholines pH 3.5-10, 2% ampholines pH 6-8, 25 mM NEM, 0.1% thimerosal, and, 1 mM PMSF. Samples were frozen in liquid nitrogen immediately after solubilisation and stored at -70°C until used. First dimension isoelectric focusing was in 12 cm x 3 mm, 6% acrylamide rod gels containing 8 M urea, 0.75% ampholines pH 3.5-10 and 0.25% ampholines pH 6-8. Samples were applied at the acidic end of the gel and the basic end protected by a 1 cm layer of ampholyte solution held in place with dialysis membrane. Applying the sample at the basic end of the gel resulted in protein precipitation in the first third of the rod and poor entry of the proteins into the second dimension gel. IEF was performed at 300 volts for 5000 Vhrs and 500 volts for the final hour. Second dimension SDS/PAGE was carried out in discontinuous 16 cm x 16 cm x 1.5 mm slab gels containing 12% acrylamide monomer in the resolving layer.

Autoradiography

Incorporation of ^{35}S-methionine into proteins separated by both techniques was visualised by autoradiography. Gels were fixed for 1 hour in 7% Acetic acid and soaked for 1 hour in ENhance, dried and exposed to Kodak Xomat R film Eastman Kodak, Rochester, USA) at -70°C for various times as appropriate. One-dimensional gels with 5 x 10^4 - 1 x 10^5 counts per minute (cpm) applied to each lane were exposed usually for three days and two dimensional gels with 5-10 x 10^5 cpm applied to the first dimension gel were exposed for two to three weeks. For quantitation of changes in band density on one dimensional SDS/PAGE gels, autoradiographs were scanned using a GDS 2000/61 digital gel documentation system together with SW 2000 image analysis software (UVP Inc., San Gabriel, USA).

RESULTS

Kupffer cells and peritoneal macrophages demonstrated essentially similar patterns of basal protein synthesis, with the exception that in peritoneal macrophages, a prominent 40 kD band was visible (Fig. 1). Stimulation for 3 hours with 50 $\mu g.ml^{-1}$ LPS induced a set of similar proteins, in both cell types. Induction was consistently seen at 33, 37, 50,

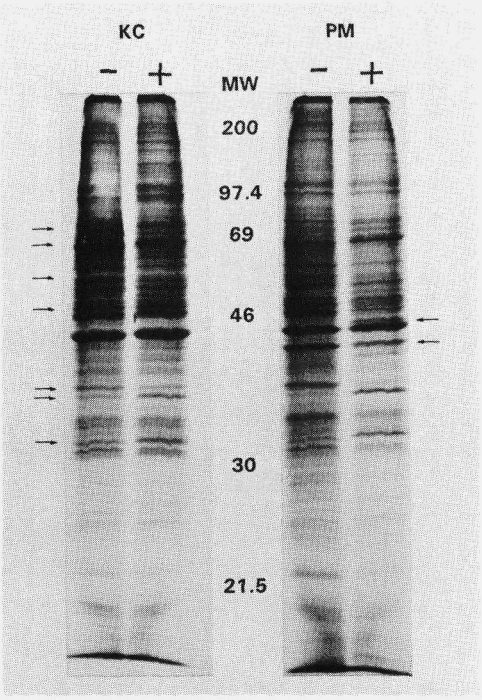


Figure 1. Rat Kupffer cell (KC) and peritoneal macrophage (PM) early response proteins. Cultured cells were treated for 3 hours with (+) or without (-) 100 μg LPS and ^{35}S-methionine added during the final hour of incubation. The pattern of protein synthesis was similar in both cell types. Both cell types showed a similar pattern of response (arrows left), although tissue specific differences in both basal and stimulated proteins were seen (arrows right).

70 and 77 kD and suppression at 38, 51, 59 and 67 kD (Fig. 1). In addition, peritoneal macrophages differed from Kupffer cells in the induction of a 44 kD protein and the suppression of the 40 kD band. Qualitatively similar responses were observed when Kupffer cell early protein synthesis was compared between young and old animals.

However, cells derived from old animals differed markedly from young ones in respect of the timing of induction of protein synthesis and sensitivity to LPS. The response was delayed in cells derived from old animals when compared to young cells exposed to both sub- and supramaximal doses of LPS (Figs. 2a and 2b). These time course experiments showed that in the continued presence of LPS, these proteins continue to be synthesised for at least 12 hours after application of the stimulus, additional new protein synthesis being observed at 4-8 hours, persisting to at least 12 hours, but returning to basal pattern by 24 hours (with the exception of a late-induced 180 kD band) (not shown). The dose sensitivity of this response was different when young and old Kupffer cells were compared (Fig. 3).

Densitometric analysis of time and dose responses (Fig. 4) confirmed the age related delay in protein induction (Fig. 4a) and suggested approximately 2.5 fold decrease in dose sensitivity to LPS in cells from old animals (Fig. 4b).

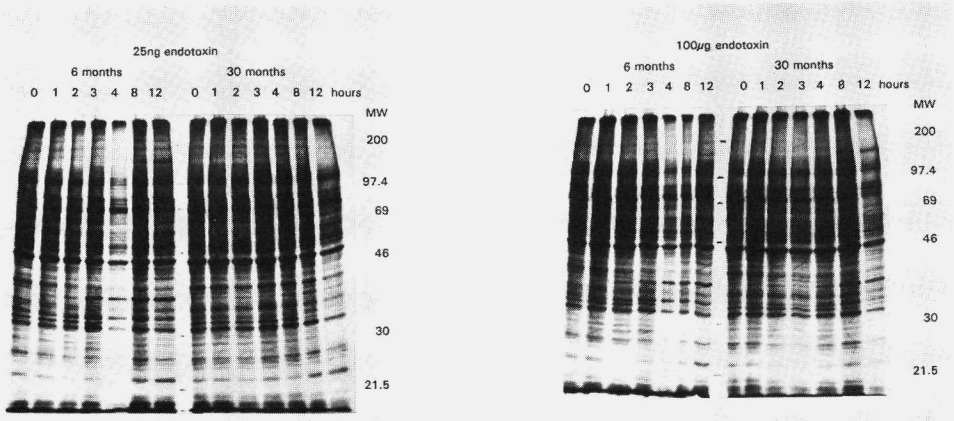


Figure 2. Kupffer cells were isolated from young (6 months) and old (30 months) BN/Rij female rats, cultured overnight and then exposed to 25 ng (a) or 100 μg (b) LPS for the times indicated. During the final hour of each incubation, newly synthesized proteins were labelled with ^{35}S-methionine.Control cells (0 hours) were incubated for 1 hour in the presence of ^{35}S-methioninewithout LPS. SDS/PAGE and autoradiography were performed as described in the text.

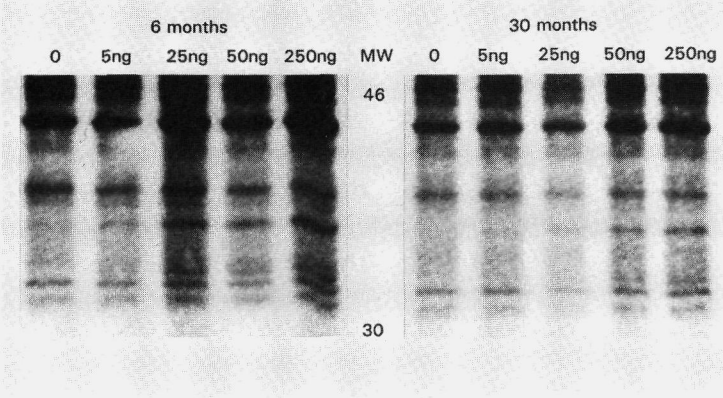


Figure 3. Kupffer cells were isolated from young (6 months) and old (30 months) BN/Rij female rats, cultured overnight and then exposed for 3 hours to various doses of LPS as indicated. During the final hour of each incubation, newly synthesized proteins were labelled with ^{35}S-methionine. SDS/PAGE and autoradiography were performed as described in the text.

The induction of this early response protein set by LPS was explored further by two dimensional electrophoretic analysis using isoelectric focusing in the first dimension and SDS/PAGE in the second dimension. These experiments allowed the identification of over 200 polypeptide spots, 3 of which (77 kD pI 5, 51 kD pI 6.5 and 32 kD pI 5) were

induced by treatment with LPS (Figs. 5a and 5b). Kupffer cells derived from aged rats showed essentially similar patterns of protein synthesis on two dimensional gels, and confirmed that the physical characteristics of the above three LPS induced proteins were not altered by aging (Fig. 5c). These analyses also confirmed that Kupffer cells from aged rats showed reduced sensitivity to LPS (not shown).

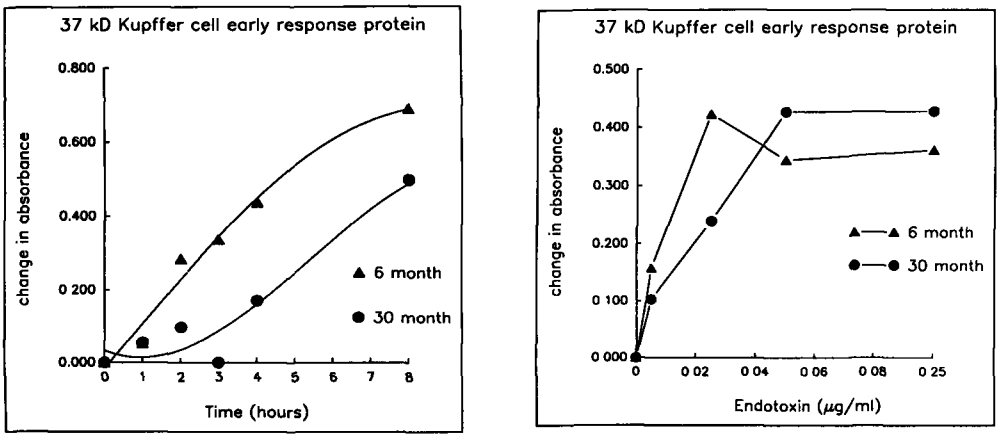
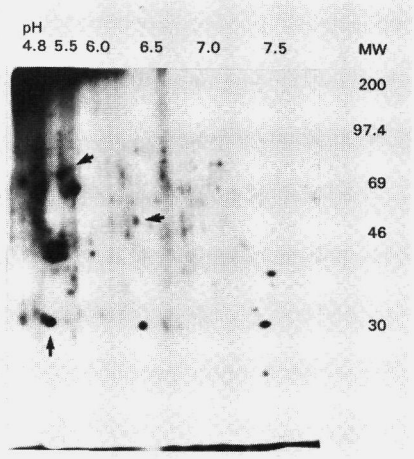


Figure 4. Representative time (a) and dose (b) response curves for the 37 kD Kupffer cell early response protein derived from autoradiographs shown in Figs. 2 and 3. Absorbance readings were standardized to the prominent band seen just below the 46 kD molecular weight marker (which did not change under the experimental conditions).

DISCUSSION

In these studies we have shown that rat Kupffer cells and peritoneal macrophages demonstrate considerable similarity in both basal and stimulated protein synthesis, although a small number of tissue specific differences are seen. This is in keeping with previous observations of Kupffer cell and peritoneal macrophage protein synthesis by one and two dimensional electrophoresis which also demonstrated considerable similarity between the two cell types and a small number of tissue specific proteins, although no significant differences in protein synthesis after *in vivo* activation by LPS were seen (12).

Both Kupffer cells and peritoneal macrophages responded to stimulation by LPS *in vitro* with an early change in the pattern of protein synthesis similar to that previously described for murine peritoneal macrophages (1). Differences in apparent molecular weight of the proteins described in the present study and those described for murine peritoneal macrophages are likely to be due to species, rather than tissue specific differences as demonstrated by the closely similar pattern of protein induction observed in both Kupffer cells and peritoneal macrophages. A set of proteins induced under



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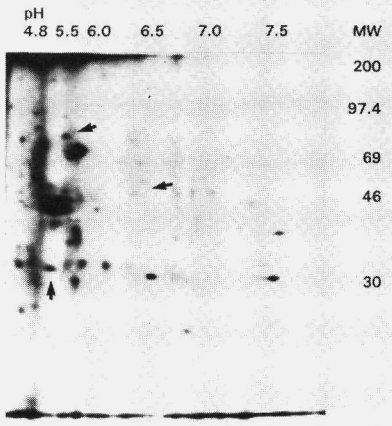

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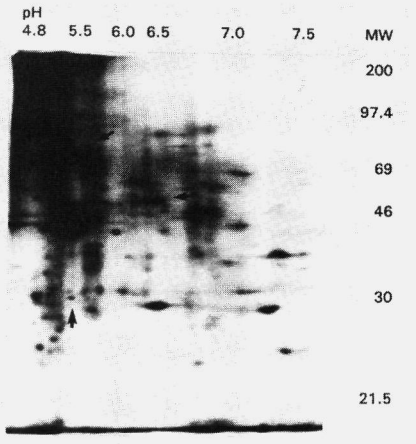

c

Figure 5. Two dimensional IEF/SDS/PAGE of Kupffer cell proteins from unstimulated cells (a), and cells exposed to 100 μg LPS (b), derived from a 6-month-old BN/Rij female rat. The arrows indicate 3 proteins which appear to correspond to the 32, 50 and 77 kD induced bands seen on one dimensional SDS/PAGE. Kupffer cells derived from a 30-months-old BN/Rij female rat (c) showed a similar pattern of response.

similar conditions in murine peritoneal macrophages have been shown to be modulated independently of several of the macrophage 'early competence genes' such as c-myc, c-fos, JE and KC (6,4) and the cytokines interleukin-1α and tumor necrosis factor α (13). Recently one of these proteins has been shown to encode the murine homologue of the human interferon gamma inducible gene IP-10 (14).

In the present study comparison of the pattern of early protein synthesis in response to LPS in Kupffer cells derived from young and aged animals revealed qualitatively similar responses. However further analysis of the doses dependence and timing of this response revealed important differences between the age groups. The responses of Kupffer cells derived from old animals were slower, less sensitive to LPS and less vigorous with respect to some of the specific protein responses.

Interaction of macrophages with bacterial lipopolysaccharide involves binding to one of at least two cell surface receptors. Of these, the CD14 leucocyte differentiation antigen interacts with endotoxin bound to a serum protein (LPS binding protein) (15). The other endotoxin receptor is the so-called 'scavenger' receptor (16), which binds a number of ligands, including LPS, maleyl bovine serum albumin, and, acylated lipoproteins, which are known to induce changes in the state of activation of macrophages and induce the early response protein set in murine peritoneal macrophages (17).

The effects of aging on the expression of these macrophage receptor proteins and subsequent signal transduction events is not known, but there are a number of studies on the effects of age on various other aspects of macrophage function. Several studies have examined the phagocytic capacity of murine macrophages with aging. Aged AB6F1 mice demonstrated a reduced resistance to infection with L. Monocytogenes, but the phagocytic component of this response was considered to be intact on the basis of the growth kinetics of the organism in liver and spleen (18). When measured in NMRI mice *in vivo*, aged animals showed enhanced phagocytic capacity for L. Monocytogenes, the specificity of this observation being confirmed by reticuloendothelial blockade with Dextran sulphate 500 (19).

Observations made in mice *in vitro* have shown no change in responses of adherent cells from mixed spleen cell cultures derived from C57BL/6J and DBA2/J mice to a variety of stimuli (20), however in the same strain, increased phagocytosis, chemotaxis and staining with nonspecific esterase have been observed in thioglycollate elicited peritoneal macrophages (21). Pulmonary alveolar macrophages from optimally aged C57BL/6, BALB/c and DBA/2 mice exhibited increased superoxide production but no change in phagocytic functions.

Studies utilising various rat strains show a more consistent pattern of results. Mononuclear phagocyte cytolytic capacity for various target cells in vitro was said to be reduced in aged Zbz:Cara and DA strains, although in these experiments, the animals were not raised under ideal conditions for aging studies and it is not clear if the oldest group (12-16 months) were truly senescent (22). *In vitro* studies of macrophages from Wistar-Furth rats, examined responses to recombinant IFN-gamma, opsonised zymosan and LPS and suggested a decline during aging, as measured by two criteria of macrophage activation, $O_2^{\cdot-}$ and TNF-α secretion (23). In this model macrophage activation responses were partially or fully restored by implantation of syngeneic pituitary grafts from young rats.

Clearance of LPS by Kupffer cells has been shown to be reduced in aged BN/Rij rats, but not to a major degree (24). However LPS induced mortality is much increased by aging in this model, as is LPS associated tissue injury (25,26).
Comparison with are own data on the induction of acute phase cytokine synthesis and

secretion (27) is illuminating. Induction of the early response protein set and expression of IL-1α and IL-1β occured at low concentrations of LPS (5-25 ng.ml^{-1}). Stimulation of cytokine synthesis and secretion was shown to be similar between cells from young and old rats at high concentrations of LPS near the top of the dose response curve. Similar results were obtained for stimulation of cytokine mRNA synthesis at 100 μg.ml^{-1}, but at the lower concentration of 25 ng.ml^{-1}, the cytokine mRNA response of cells from old rats tended to be somewhat slower. This is in keeping with the observation of a delayed early protein response at this concentration of LPS.

Our observations provide confirmation at the cellular and molecular level that Kupffer cell responses to LPS are less vigorous in old when compared to young animals and are in keeping with our previous observations showing a reduction in the clearance function of these cells with respect to age (28). However, *in vivo* data indicate that old rats display an enhanced sensitivity to intravenous injection of LPS in many respects. This paradox is underlined by the demonstration (in studies performed in the same colony of this rat strain) that the production of eicosanoid inflammatory mediators by Kupffer cells in response to LPS is at least as strong in advanced age.

Despite clear evidence that endotoxin death is mediated via mononuclear phagocyte products and the demonstration of increased endotoxin lethality and tissue damage with aging, some initial responses of the main effector cells of the mononuclear phagocyte system in this context appear to be less vigorous and delayed. The response to bacterial lipopolysaccharide is complex and highly regulated and aging appears to be associated with alteration of the reponse at many levels (29). Kupffer cell early response protein synthesis is one of a number of areas where this important adaptive response appears to be dysregulated in old age.

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CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

Introduction

The experimental results presented in this thesis can be divided into two parts. The first consists of studies in which the effects of aging on the response of the intact organism to endotoxins are analyzed (Chapters 2-6); the second part deals specifically with the possible role of hepatic Kupffer cells in this modified response (Chapters 7-9).

Pathophysiological aspects and mortality

Chapter 2 describes the extent to which aging affects the sensitivity of two rat strains to endotoxin in terms of pathophysiological consequences. Over the dose range of 10-40 mg per kg body weight, all animals showed reduced activity and adopted a hunched posture, with pilo-erection and shivering. After approximately 90 minutes, the blood appeared viscous and hypercoagulable. Blood pressure appeared low and the collection of blood samples from the tail vein was difficult. These phenomena persisted for several hours in young animals, and were then followed by a progressive return to normal appearance and behaviour. Most animals aged 24 months or older, by about four hours, started to uncurl, body temperature declined, often to below 30 ° C; they became increasingly lethargic, and as time progressed they lapsed into coma and died. More detailed dose-related survival studies showed that the LD 50 (of a particular batch of LPS) was about 10 fold different between 6-months-old (± 60 mg.kg-1) and 24-months-old (± 5 $mg.kg^{-1}$) rats (unpublished results). There were no major qualitative changes in the response of the old rats. The characteristics of 'shock' were largely the same as observed in young rats injected with a higher dose.

This dramatically increased sensitivity to the lethal effects of endotoxin occurred irrespective of sex and strain in that it was also observed in 24-months-old male BN/BiRij rats and 24-months-old WAG/Rij rats. It is remarkable that this changed sensitivity is already quite apparent at 24 months, while the 50 % survival age of these

rats is at least 30 months. At the age of two years very little disease and pathology has yet developed (1). Since our main aim was to study the effects of aging per se, not of disease, most of the studies were done with rats of this age.

Thermoregulation

Old rats were much more prone to develop hypothermia in response to LPS than young ones (Chapter 2). Since prevention of hypothermia had a marked beneficial effect as it extended survival time of the old rats given a lethal dose of LPS, hypothermia may play an important role in the progressive deterioration of the general condition of the rats in the hours following the initial response.

Altered thermoregulation is a well described feature of aging in mammals. Significant changes in normal body temperature regulation and response to cold and heat stress (2-5), and in meal-induced thermogenesis (6-8) have been reported in elderly humans. Also in rodents, age-related reduction in the ability to maintain normal body temperature, particularly at low ambient temperatures, is well documented (9-16; see also Chapter 2). These changes were most likely caused by age-related alterations in metabolic heat production (17-19) and cold-induced thermogenesis (20,21), particularly in brown adipose tissue (22,23), which is largely responsible for metabolic heat production in rodents (24). The decreased thermogenic response to norepinephrine observed in BN/BiRij rats (22) supports the idea that intrinsic alterations in brown adipose tissue may contribute to the increased intensity of LPS-induced hypothermia in old rats.

Habicht (25), Hoffman-Goetz and Keir (26) and Deeter (27) have reported on age-related changes in the fever response to endotoxin in rodents. In a recent study, Refinetti et al (13) found no differences in the fever response of young and old rats to Candida administration.

In a recent detailed study in mice by Strijbos et al (28) it was shown that old (27-36 months) mice had a strongly diminished fever and metabolic response to endotoxin, noradrenaline, IL-1α, and, IL-1β (and not to PGE_2), whereas middle aged (15-18 months) mice had the same response as young adult ones. Both the metabolic and fever responses of old rats to these stimuli were normalized when the mice were treated with a glucocorticoid antagonist, or with antibodies to lipocortin, which mediates the inhibitory effects of glucocorticoids on prostaglandin (E_2) synthesis. The role of the hypothalamic-pituitary-adrenal axis was not investigated in our studies and little is known about the effects of aging on this system in our rat strain. The apparent suppression of the inflammatory response by glucocorticoids in aged mice (28) may not present in the 24-month-old rats used in our studies. In a small pilot experiment, in which BN/BiRij rats were injected with a low dose of 1 μg LPS/kg, 24-month-old rats displayed the same degree of fever (2.2 $\pm$ 0.5°C (S.D.) (n = 3); basal 36.5 $\pm$ 0.6°C) as 6-month-old ones (1.7 $\pm$ 0.2°C (n = 2); basal 37.7 $\pm$ 0.2°C) (unpublished results). This indicates that age-

related changes in temperature response in these rats are less significant at lower than at higher concentrations of endotoxin.

Morphogenesis of hepatic and pulmonary injury

Damage to cells and tissues is a critical component of the multi-organ failure resulting from administration of endotoxin. The following studies were done to investigate the sequence of morphological changes after a dose of LPS that was lethal in old rats, but not in young ones, in search of the mechanisms responsible for the increased tissue damage in old rats.

The early (15 min to 7 hrs) hepatic and pulmonary ultrastructural and biochemical changes induced by endotoxin in young and aged rats are summarized in chapters 3 and 4. Aged rats which were given endotoxin had significantly increased numbers of polymorphonuclear leucocytes in hepatic sinusoids at 30 minutes and thereafter, as compared to endotoxin-treated young rats receiving the same dose (1 mg per 100 g).

Morphologic evidence of coagulation within hepatic sinusoids, namely aggregates of fibrin enmeshed among polymorphonuclear leukocytes and platelet aggregates, were frequently observed after endotoxin treatment in aged but not in young rats. In contrast, Kupffer cells of endotoxin-treated young rats frequently contained phagocytized neutrophils and platelets, whereas this phenomenon was rarely observed in Kupffer cells of endotoxin-treated aged rats. Hepatocellular morphologic injury was more pronounced and occurred earlier in endotoxin-treated aged rats, and was accompanied by significant increases in serum transaminase levels. Endotoxin-treated aged rats had an earlier onset and greater severity of endothelial cell injury than did endotoxin-treated young rats. Pulmonary endothelial cell injury was of greater severity and occurred earlier in aged rats as compared with young rats. Platelet sequestration and aggregation were observed only in aged rats in this study, and occurred in conjunction with the initial degenerative changes of the endothelium. Morphological evidence of granulocyte degranulation and fragmentation was also observed only in aged rats.

Endothelial cell injury also occurred earlier in aged rats, in both liver and lungs. Additionally, endothelial cell injury not associated with adjacent intravascular coagulation was observed only in aged rats. This observation suggests that the endothelium of aged rats is more susceptible to the detrimental effects of endotoxin as compared with young rats.

The extent of injury to the endothelial cell population may be an important factor in accounting for differences in endotoxin-induced mortality between young and aged rats.

Increased platelet aggregability in aged rats is another likely contributing factor. Other investigators have documented an age-related increase in the reactivity of platelets as determined by a lower threshold to an aggregating agent (collagen), greater amplitude of the aggregation curve, and higher thromboxane B_2 formation upon exposure to

collagen (29,30). A compensatory mechanism of higher vascular prostacyclin production in aged rats to counteract the greater platelet aggregability was also suggested. However, the endotoxin-induced injury to the endothelium observed in our study would most likely impair normal prostacyclin production, so that platelet aggregability can occur less inhibited in aged rats. The platelet may be the cell population initially responsible for the marked differences in the severity of endothelial cell injury observed between young and aged rats.

The extensive neutrophil degranulation observed in this study indicates that neutrophils also further aggravate the severity of endothelial cell injury in aged rats. The recruitment of neutrophils is not surprising when viewed in the context of the pronounced and diffuse endothelial cell injury.

Coagulation and fibrinolysis

The results of chapters 2-4 indicate a greater aggregation of blood elements in hepatic sinusoids and pulmonary capillaries of endotoxin-treated aged rats. Others have demonstrated the importance of sinusoidal thrombosis in the development of endotoxin-induced hepatic injury and the reduction or abrogation of hepatic damage by anti-coagulant therapies (31,32). Numerous pathophysiologic mechanisms could partially account for the observed difference in occurrence of endotoxin-induced intravascular coagulation between young and old rats, including differences in onset and severity of endothelial cell injury or platelet aggregability, alterations in hepatic blood flow and microcirculation, or differential activation of the intrinsic coagulation system.

The much more severe increase in blood viscosity and in fibrin deposition in old compared with young endotoxin-treated rats could be due to a stronger induction of procoagulants, but also to inhibition of fibrinolytic activity. Administration of endotoxin resulted in little or no changes in circulating plasminogen and anti-plasmin levels, which can not be the cause of the observed changes in fibrinolysis. Fibrinogen decreased slightly soon after injection, probably due to fibrin formation. There were no clear differences between young and old rats in levels of plasminogen, anti-plasmin and fibrinogen, before or after endotoxin treatment.

Figure 1 gives a schematic overview of coagulation and fibrinolysis. Tissue type plasminogen activator (t-PA) and its inhibitor (PAI-1) are major regulators of the blood fibrinolytic system, and their appearance in the blood circulation is strongly stimulated by LPS administration, already at low doses of LPS (Chapter 5). Intravenous injection of endotoxin into rats induced a rapid increase in fibrinolytic activity, due to an increase of t-PA, which lasted for about 1 hr. This was followed by a period of decreased activity caused by increased PAI activity. A very similar response has been observed in man after endotoxin injection (33,34). The response to endotoxin of the fibrinolytic system is thus highly comparable between man and rat.

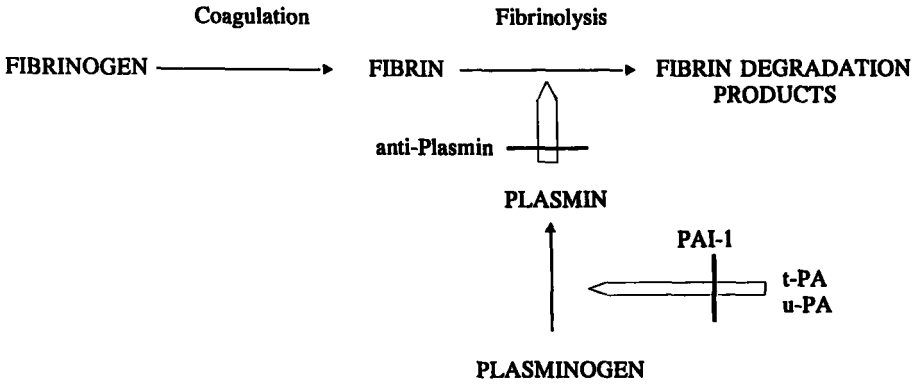

Figure 1. Schematic representation of fibrinolysis.

At a low dose of 1 μg LPS.kg^{-1}, no differences in stimulation were observed between young and old rats. In old endotoxin-treated rats, both the initial increase in t-PA (up to 1 hour) and the subsequent decrease in fibrinolytic activity due to increased PAI, were more pronounced than in young rats at higher doses of LPS. Aged rats also show larger increases of PAI in response to injection of interleukin-1 and tumor necrosis factor. However, experiments in which the formation or action of cytokines were inhibited showed that the LPS-induced stimulations of t-PA and PAI-1 were not dependent on either IL-1 or TNF-α. This makes it unlikely that these mediators play an important role in the disturbed regulation of fibrinolysis in old rats.

Studies that examined the effects of aging on blood coagulation indicate that a greater thrombotic potential exists in elderly humans and aged rats (35,36). Our study in rats demonstrated increased plasminogen activator inhibitor levels and more strongly suppressed fibrinolytic capacity in aged rats treated with endotoxin, than in young ones. This may largely explain, in the presence of ongoing coagulation activation, the continued deposition of fibrin aggregates and their persistence in liver and lungs of the old rats. This deposition is likely to result in persistence of compromised tissue perfusion, which will strongly contribute to the observed damage in various organs. In addition, metabolic activity and distribution of nutrients to the tissues will be inhibited.

Basal activities of t-PA were lower and those of PAI-1 were higher, resulting in a somewhat lower normal fibrinolytic activity in old rats. This was also observed in studies

in humans by others. In a recent workshop on fibrinolysis and aging (37), the evidence presented generally indicated that plasma levels of t-PA antigen increase with age, although t-PA activity does not change and global plasma fibrinolytic activity appears to decrease, presumebly due to increased inhibition by PAI-1 (38). Since high basal PAI-1 levels are considered to indicate a risk of a myocardial infarction (39), these changes, although moderate, may still be significant for the elderly population. However, a recent study from our laboratory showed that basal levels of t-PA antigen and PAI-1 activity were the same in healthy volunteers aged 75-85, which fulfill the strict health criteria described in the so-called SENIEUR protocol (40), as in 18-35-year-old controls (Table 1).

Table 1. Activities of fibrinolytic factors in plasma of young and old healthy volunteers.*

	Age (years)	
	18-35	75-85
t-PA antigen	100 ± 27 (21)	120 ± 34 (19)
PAI-1 activity	100 ± 31 (23)	105 ± 37 (20)

Values are given as percentage of the average of the young control group (means ± S.D. (n)). T-PA antigen was expessed as $ng.ml^{-1}$. PAI-1 activity activity as ng activase.ml^{-1}.

* Data obtained in a collaborative study with Dr G.J. Ligthart and J.G.J.M. Maters, M.D (Section of Gerontology, Medical Faculty, Leiden University) and Dr J.J. Emeis and Dr T. Kooistra (TNO-IVVO).

Glucose homeostasis

The results presented in Chapter 6 demonstrate that old rats display much more severe disturbances in glucose homeostasis in response to endotoxin than young ones. This is primarily due to increased dose-sensitivity, but there are also qualitative differences.

Glucoregulation and LPS. Insulin and glucagon are the major regulators of glucose homeostasis in mammals (41-44), but most of the endotoxin-induced mediators, like eicosanoids (notably PGD_2 (45) and leukotrienes (46)), TNF (47-49), IL-1 (50-52), platelet-activating factor (PAF) (53,54) and α- and β-adrenergic hormones (55,56) also affect plasma glucose levels and hepatic and peripheral glucose metabolism.

The plasma glucose response to LPS consisted of 3 to 4 phases, dependent on dose and nutritional status. During the first 30 min after endotoxin administration (5-40 $mg.kg^{-1}$), a brief, small decrease in plasma glucose was noted, probably due to increased glucose utilisation by peripheral tissues (57). During the next 90 min, glucose levels rose sharply, up to severe hyperglycaemia. This phase is characterized by high hepatic glucose output,

which exceeds peripheral glucose utilization. Beyond two hours after injection, all rats displayed a decline in glucose levels, resulting in mild to severe hypoglycaemia at between 2 and 6 hours, at sufficiently high doses of LPS. Between about 8 and 24 hours, plasma glucose might, or might not, return to normal levels, dependent on the dose and age-group. These phases have also been described during sepsis and endotoxemia in humans (58,59) and experimental animals (60,61).

Age-related changes in glucose response to LPS. The dose dependency was different for young and old rats. Non-fasted old rats injected with 1 mg endotoxin per 100 g had a response similar to that of young ones receiving 7.5 mg endotoxin per 100 g. The response of the old rats was much more pronounced than in young ones receiving the same dose, both during the hyper- and the hypoglycaemic phase. All animals that did not survive had a blood glucose concentration less than 2 mmol when moribund.

In non-fasted rats, the initially large quantities of glycogen present in the liver of both young and old rats were almost depleted at three hours after LPS administration. Plasma levels of neither insulin nor glucagon were altered during the hyperglycemic phase. The higher hyperglycemia in old rats appeared to be dependent on glycogenolysis, since it was much less pronounced in fasted rats. The stronger early stimulation of hepatic glucose output in non-fasted rats might be related to activity of monokines, several of which are known to induce hepatic glycogenolysis. Kupffer cell-derived mediators, particularly prostaglandins (mainly D_2), rather than TNF, are responsible for the induction of glycogenolysis by LPS in perfused rat liver (62,63). Increased production of these mediators may, however, not be the cause of the more severe hyperglycaemia in old rats, at least not as a result of intrinsic age-related changes in Kupffer cells (see below).

The increased hypoglycaemia which occurred after the depletion of hepatic glycogen was accompanied by very high levels of plasma glucagon and an apparently diminished hepatic gluconeogenesis. Insufficient glucose output by the liver during the hypoglycemic phase may largely be a secondary phenomenon, resulting from malperfusion, hypoxia and serious damage to liver parenchyma and endothelium. Hypothermia is not likely to be a direct cause of the hypoglycemia, since experimental hypothermia in rodents results in increased blood glucose levels, probably because hepatic gluconeogenesis is less sensitive to inhibition by lower body temperature than peripheral glucose utilisation (64,65). However, the increased tendency of old rats to develop hypoglycemia was also observed when the two age groups were treated with a dose that induced similar degrees of shock. This indicates that the maximal rate of gluconeogenesis may be reduced in old rats.

The glucoregulatory response of old rats to endotoxin was also strongly affected in studies on the effect of aging on hepatic carbohydrate metabolism in a rat model of septic shock based on experimental peritonitis, induced by cecal incision (60,61). The hyperglycemia noted at 5 hours after cecal incision was stronger in aged rats, in spite of relatively low glycogen levels due to fasting. At 7 hours, old rats were severely

hypoglycemic. Hepatic concentrations of glucose and hexosephosphates were markedly decreased by endotoxin in our study, without significant age-related differences. We did not determine fructose-2,6-diphosphate, which was found to be increased in liver of endotoxin-teated rats by Miller et al. (66). F-2,6-dP, a potent allosteric activator of phosphofructokinase, which stimulates the phosphofructokinase/F-1,6-dP cycle in the direction of glycolysis, is likely to be the factor responsible for decreased gluconeogenesis in endotoxemia (66). We observed no changes in fructose-1,6-diphosphate (F-1,6-dP), except for an increase at 5 h in old rats, while Schumer (61) found an increase in hepatic F-1,6-dP in young, but not in old rats. Hexosemonophosphates were decreased, in both our young and old rats, while Schumer described an increase in old but not in young rats. Neither the inhibition of glucose output by the liver at the level of glucose-6-phosphatase, nor the suppression of phosphofructokinase-1 activity that were suggested to be present in old septic rats by Schumer and Kuttner (60) were evident in our LPS treated old rats. Although the changes in metabolite concentrations may differ, both our study and those of others indicate that the rate of gluconeogenesis may be reduced in endotoxemic old rats (60,67). Inhibited gluconeogenesis has been shown to augment the severity of endotoxin shock in rats (68).

Glucose metabolism and aging. Age-related changes of several components of the glucoregulatory system have been described, both in humans and in experimental animals, although the nature and magnitude of the reported changes vary between studies (see below). Much of the research on the effects of aging on glucose metabolism has been carried out in search of explanations for the high prevalence of non-insulin dependent (type II) diabetes mellitus (NIDDM) in the elderly (41,69-73).

NIDDM and aging in humans. In western countries, the prevalence of NIDDM is almost 10 % in the age groups older than 65 years (74). NIDDM is characterized by: a. mild, or severe, fasting hyperglycemia, which is accompanied by increased, or normal, plasma insulin levels, respectively (75); and, b. glucose intolerance, i.e. increased hyperglycemia after glucose loading (venous plasma glucose > 11.1 mM after oral glucose tolerance test (OGTT) (74). The plasma insulin response (PIR) to glucose is not altered consistently in NIDDM. PIR is mostly decreased, but it is also frequently normal, or even increased. These differences appear to be related to the degree of fasting hyperglycemia (41). Mild fasting hyperglycemia (up to 7 mM) is associated with a stronger insulin response to glucose, but patients with severe hyperglycemia (> 7.8 mM) have a low or negligible response. It appears that the primary defect in patients with NIDDM can be impairment of insulin secretion by pancreatic β-cells, but also impairment of the sensitivity of muscle (76) and liver (69,77) to insulin. The former defect would occur mainly in lean, the latter mainly in obese patients (41). Obesity is a strong risk factor for the development of NIDDM (78), and so are mild glucose intolerance (OGTT between 7.8 and 11.1 mM) and insulin insensitivity (74). Whatever

the primary cause, both defects are present by the time glucose intolerance has developed. Both decreased uptake of glucose by muscle and liver, and decreased suppression of hepatic gluconeogenesis and glucose output in response to insulin contribute to the glucose intolerance observed, but to different degrees in different patients and stages of the disease (41,73,79).

Glucoregulation in healthy elderly. Although aging does not generally cause glucose intolerance or elevated fasting glucose levels (80) some age-related changes that are similar to those observed in (early) NIDDM have been also observed in aged human volunteers (77,81-83), particularly in obese subjects (84-86). In apparently healthy elderly volunteers, OGTT revealed mild glucose intolerance (81) and slower, but later higher, PIR, as well as delayed suppression of hepatic glucose output (HGO) (77). Glucose clamp studies showed that basal, as well as hyperglycemically stimulated glucose disposal rates were always lower in the elderly than in young adults (77). Another study (87) used the technique of euglycemic insulin clamp, in which a constant infusion of insulin is applied, and glucose is infused at a rate necessary to maintain normal plasma glucose levels. This revealed also reduced stimulation of glucose disposal by insulin, but the suppression of HGO at low physiological insulin levels was even more rapid in the elderly. The PIR to glucose appeared not to be affected by aging in this study.

Glucoregulation in aging experimental animals. In experimental animals, alterations have been described in base-line hepatic glucose output, due to an apparent increase in basal gluconeogenesis (89). Niedermüller (90) found age-related changes in the metabolism of ^{14}C-labeled glucose that are indicative of a decrease in the relative contribution of the pentose pathway (next to glycolysis) in the oxidation of glucose. Khandarwal et al (91) reported only insignificant differences between plasma insulin levels and activities of glycogen synthase and glycogen phosphorylase in liver of 12- and 24-months-old rats.

Kalant et al (92) observed an increased peak glucose and decreased PIR after a glucose tolerance test in aging rats. Increased insulin resistance was described in aging genetically obese mice (93). Euglycemic clamp studies in rats revealed some decrease in insulin-induced glucose disposal rate, but no decrease was observed in insulin binding by liver, muscle and fat between the age of 10 and 20 months (94).

In rodent liver, age-related changes were described in the metabolic response of hepatocytes to insulin (95) and glucagon (96). However, stimulation of urea synthesis by glucagon or epinephrine (97) and insulin binding to the cell membrane (95) was the same in hepatocytes from young and old rats. Significant changes have been described in the α- and β-adrenergic responses of the liver, but the major age-related changes occur during development (98,99). Regulation of hepatic enzymes of intermediary metabolism may be altered (100,101).

In rat adipocytes, changes in glucose metabolism and insulin receptor recycling have

been described (102-104).

There were no indications for major alterations in the normal glucose homeostasis in our 24-months-old rats. Both fasting and non-fasting plasma glucose levels were the same as in young rats. Pilot experiments with an oral glucose tolerance test (Fig. 2) showed that the increase in plasma glucose levels after a glucose load was similar in 6- and 30-months-old rats. The concomitant rise in plasma insulin was, however, stronger in the old rats (Fig. 3). In another experiment, the response to insulin (0.5 U/kg; i.p.) was tested and the same degree of hypoglycemia (< 2 mM) was induced in 6- and 36-months-old BN rats. However, the return to normal values was delayed in old rats (unpublished results, H. Van de Berg et al., TNO-ITV Zeist). These changes in insulin responsiveness and insulin sensitivity are similar to those that are considered to be characteristic for the earliest stages in the development of non-insulin dependent diabetes mellitus (NIDDM) (41). These data indicate that, in the BN strain used, age-related changes in glucoregulation occur, which were not strong enough to be interpreted as functional deteriorations, but are likely to be more significant under challenge.

Conclusions. In conclusion, subtle intrinsic changes in the glucoregulatory system were observed, but they appear to play only a minor role in the increased sensitivity of old rats to LPS. However, alterations in glucose response to LPS contribute to the increased severity of shock, both as a result of intrinsic changes in glucoregulation (gluconeogenesis) and as a secondary reaction to pathophysiological changes and secreted mediators.

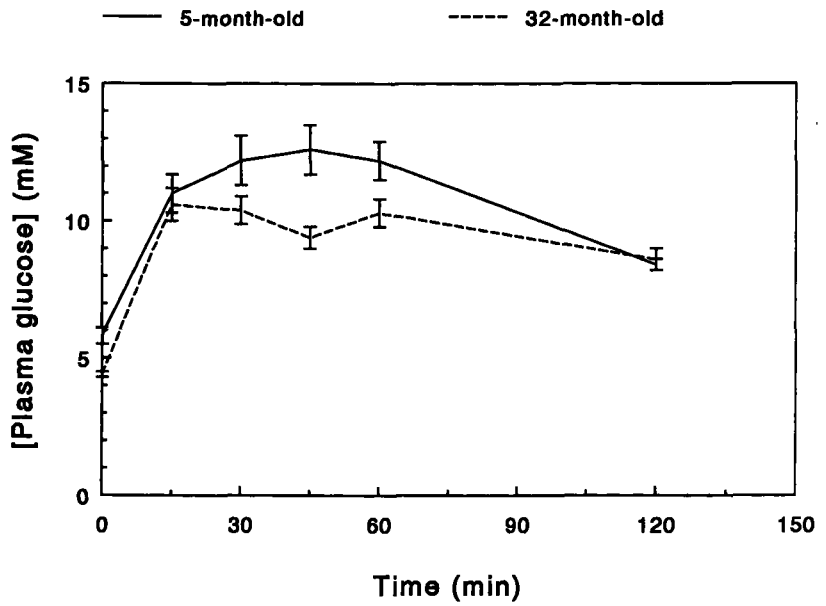

Figure 2. Plasma glucose levels in young and old rats after an oral glucose tolerance test. Values are mean ± SEM (young: n = 3; old: n = 2).

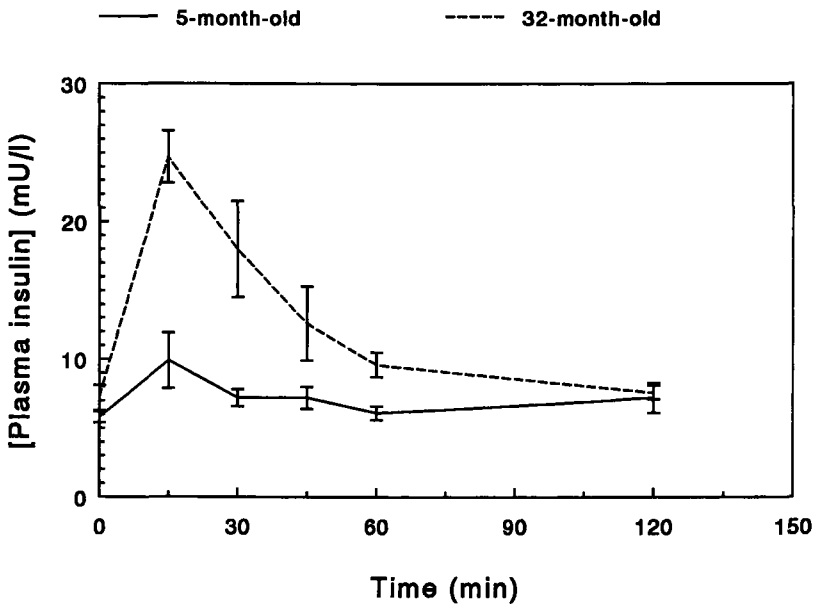

Figure 3. Plasma insulin levels in young and old rats after an oral glucose tolerance test. Values are mean ± SEM (young: n = 3; old: n = 2).

Role of age-related changes in Kupffer cells

Endocytosis. Kupffer cells from old rats displayed a reduced endocytic capacity, both for colloidal albumin (chapter 7) and for LPS (Chapters 2), but only to a moderate degree. The decreased capacity of Kupffer cells of old rats to clear endotoxin from the circulation was apparent only during the elimination phase and had little or no effect on circulating endotoxin levels. The mechanisms of uptake of colloidal albumin and LPS are still poorly described, and little can be said about the causes of the age-related decrease in endocytic capacity. The receptors or binding sites for LPS on Kupffer cells are different from the 80 kD protein identified on other types of macrophages, not much is known about their nature and function (105,106). In recent studies, Dini et al (107,108) observed that the expression and the activity of the mannose-specific receptors on sinusoidal liver cells and the galactose recognition system of Kupffer cells were lower in old rats.

The results of the present study demonstrated marked differences in Kupffer cell phagocytosis of platelets and neurophils between the young and aged LPS-treated rats. This suggests that the phagocytosis of inflammatory cells by Kupffer cells may be a mechanism which affords protection against endotoxin-induced lethality more effectively in young rats than in old ones.

Mediator production. Many of the features of endotoxin shock are mediated by cytotoxic mediators, like TNF, interleukins and eicosanoids. Macrophages, particularly

Kupffer cells, are a likely source for most of these biological response modifiers (for reviews see: 109). The literature on cytokine production and secretion is difficult to interpret. For many years there was no consistency in nomenclature for cytokines and this confuses the issue up until about 1988 when international agreement on cytokine nomenclature was reached (110). Another confounding factor is that various sorts of activity, upon which biological assays were based, are shared between more than one cytokine (for a recent overview see 111).

The basal production of biological response modifiers was very low in cells of both young and old rats indicating that the resident macrophages of the liver were in an inactivated state in both age groups. Upon stimulation by LPS, the same types of mediators were produced as described for other types of macrophages, although the patterns were different.

Eicosanoids. Incubation of isolated Kupffer cells with endotoxin in vitro showed that these cells can be induced to produce large quantities of prostaglandins D_2 and $F_{2\alpha}$ within one hour, and little or no thromboxane (Tx) B_2, PGE_2, 6-keto-$PGF_{2\alpha}$ and leukotrienes. The production of PGD_2 and $PGF_{2\alpha}$ by rat Kupffer cells was described earlier by others (63,112-114). The absence of PGE_2, and particularly of 6-keto-$PGF_{2\alpha}$ (which is formed after prostacyclin production) and TxB_2, is still remarkable in view of other studies in which Kupffer cells produced considerable quantities of these cyclooxygenase products when activated with phorbol ester, PAF, zymosan, and also LPS (109,112,114,115). The pattern is also different from our own data on Ca-ionophore stimulated eicosanoid production by Kupffer cells, which included a large proportion of PGE_2, 6-keto-$PGF_{2\alpha}$ and leukotrienes (116). Apparently, LPS induces a specific selection of eicosanoids, while Ca-ionophore non-specifically triggers the metabolic conversion of arachidonic acid into the different eicosanoids. It is well documented that regulation of eicosanoid synthesis is complex (112,117). The pattern depends greatly on the stimulating agent, the type of macrophage, its activation state, and, the species. The synthesis of eicosanoids is strongly modulated by the presence of cytokines, hormones and eicosanoids themselves (112,117). Human Kupffer cells secreted more types of eicosanoids in response to endotoxin, particularly TxB_2 and PGE_2, than did rat Kupffer cells, but human Kupffer cells appeared already to be activated before incubation with LPS (118).

Tumor necrosis factor and interleukins 1 and 6. As reported by several others (see: 109,112) IL-1 and TNFα were also induced by endotoxin, but Kupffer cells especially produced large amounts of IL-6 in response to endotoxin, which was also described by Busam et al (119) and Decker (112), and by Feder et al after in vivo treatment with endotoxin (120). The secretion of IL-1, IL-6 and TNFα by Kupffer cells occurred later, and was longer lasting than that of eicosanoids. Endotoxin stimulated the synthesis of IL-1, IL-6 and TNF-α mRNAs in Kupffer cells in vitro, an observation which has not

previously been reported. The degree of induction, both for gene expression and secretion of the cytokines is striking, and emphasizes the role of these cells as producers of important autocrine and endocrine biological response modifiers. Of particular interest is the dichotomy observed between the IL-1 response and the IL-6/TNFα response at the mRNA level. Kupffer cells from both young and aged animals appear to be exquisitely sensitive to endotoxin in respect of expression of mRNA for both IL-1α and IL-1β, and less sensitive in this regard with respect to IL-6 and TNFα gene expression. These results indicate that Kupffer cells respond to LPS primarily by transcriptional regulation of cytokine gene expression. The induction of cytokine mRNAs by LPS was also described for monocytes, and not for mature macrophages (121). The post-translational regulation of TNF induction in (activated) peritoneal macrophages, resulting in secretion within one hour after exposure to LPS (122), was not found in Kupffer cells and very little TNFα mRNA was present in unstimulated Kupffer cells.

Mediator production in old rats. The effects of aging on the induction of any of these mediators were minimal. Kupffer cells isolated from the 24-months-old rats produced similar quantities of the endotoxin-induced eicosanoids as those of young ones, although older, 38-months-old, rats displayed some increase in $PGF_{2\alpha}$ and leukotriene production. Also, no changes were observed in the secretion of IL-1, IL-6 and TNFα by Kupffer cells from 6- and 30-months-old rats, only a possible tendency for a slower induction of mRNA.

Macrophages and aging. There are few literature data available for direct comparison, but these results on Kupffer cells from old rats may be compared to reported age-related changes in other types of macrophages. Several biochemical and functional characteristics of rodent macrophages have been reported to change, generally to decrease, with aging, but unchanged activities of the same or similar properties were also found (for reviews, see: 111,123,124). In the past years, information on aging changes in macrophages and their significance for the older organism has considerably increased. In recent in vitro studies with rat peritoneal macrophages, the responses to recombinant interferon-gamma, opsonised zymosan and endotoxin were examined. The results suggested a reduced response during aging, as measured by two criteria of macrophage activation, $O_2^{\cdot-}$ production and TNFα secretion (125). Lavie and Gershon (126) observed only a lower $O_2^{\cdot-}$ production by peritoneal macrophages of old mice after stimulation with opsonized zymosan. After stimulation with latex, phorbol myristate acetate or unopsonized zymosan, the oxygen free radical production was similar in cells from young and old mice. Chen et al (127) described that the aging defects in yeast phagocytosis, $O_2^{\cdot-}$ production and lysozyme activation of murine macrophages could be restored by treatment with detoxified lipid A, an important component of LPS. No changes in phagocytosis and membrane fluidity were observed in peritoneal macrophages between adult (6-m) and aged (24-m) mice by Hamm et al. (128). Seth et al. (129) reported that spleen

macrophages, and not B lymphocytes, from aged mice are defective in stimulating autoreactive T cells in vitro, and this defect could be corrected by addition of IL-1. Other evidence on the role of macrophages in age-related decreases in immune function is found in the decreased number and size of tingible body macrophages in germinal centers of immunized aging mice (130). Eposito et al. (131) observed no decrease in the respiratory burst and bactericidal activity of alveolar macrophages from senescent mice. Even increased natural resistance of alveolar macrophages (and killer cells) was found, resulting in decreased pulmonary metastases of mammary carcinoma in older rats (132). On the other hand, macrophages seem to be primarily responsible for the increased susceptibility of aging mice to infection with Brughia pahangi (133) and respiratory infections by Klebsiella (134). Other recent studies have resulted in evidence that defects in macrophage function may be an important factor in the impaired cutaneous wound healing (135), in macular degeneration (136) in senescent mice. Macrophages also play an important role in atheroma formation and atherosclerosis (137-139).

Eicosanoids and aging. Only few studies on age-related changes in the regulation of eicosanoid production are available (140-143). No changes were seen in prostanoid production by human aorta (140). Changes in normal levels of prostanoids in plasma may contribute to the increased hypertension of older individuals (141), although no changes were observed in a Japanese study (142). Renal prostanoid synthesis is altered in the elderly, as deduced from lower 6-Keto-PGF1 alpha, higher TxB2, but unchanged PGE_2 and $PGF_{2\alpha}$, levels in urine (143).

Cytokines and aging. The literature on the effects of aging on the synthesis and secretion of cytokines is limited. The message from animal studies is fairly clear: macrophages derived from aged mice and rats synthesise and/or secrete reduced quantities of interleukin 1 compared to those derived from young animals. Many of the studies used the relatively unspecific LAF assay (144-146), but those which utilised specific antisera (147) or cDNA probes (148) confirmed this age associated defect. Studies in man do not show the same age related decline in interleukin 1 production by peripheral blood monocytes, using unspecific bioassays (149-152). Two recent studies suggest a decline in the ability of aged peritoneal macrophages to synthesize and secrete TNF (125,147). No data on IL-6 and aging have been published. It is clear that despite a huge expansion of research into the general area of acute phase cytokines in the past ten years, our knowledge of the effect of aging on the signal molecules of the acute phase response in aged man is still deficient.

Kupffer cells and aging. Taken together, our data and those of others show that overt functional defects are not necessarily the rule or a predictable entity for macrophages of old individuals, but that, dependent on genetic background and conditions of aging, some functional changes that contribute to the decreased plasticity of old age are likely to occur.

Our own data on early response protein synthesis provide confirmation at the cellular and molecular level that Kupffer cell responses to LPS are less vigorous in old when compared to young animals (chapter 10). This observation is more in keeping with our previous observations showing an age-related reduction in the clearance function of these cells regarding both LPS and colloidal albumin.

However, the in vivo experiments showed that old rats display an over-reaction, rather than a less vigorous one, after intravenous injection of LPS in many respects (c.f. chapter 3). Tissue responses such as pulmonary, hepatic and renal injury were more severe and activation of the fibrinolytic system was more pronounced. It seems, therefore, unlikely that Kupffer cells, despite displaying age-related changes in their response to LPS, are a key factor in the increased susceptibility of old rats to LPS.

General conclusions

The studies presented in this thesis comprise the partial characterization of the sequence of pathophysiological events occurring in aged endotoxin-treated rats, and of the cells and molecules involved in the dysregulation of homeostasis of these animals.

Major alterations in the response of old rats were already noted within half an hour after endotoxin administration, and these were likely to contribute strongly to the dramatic final consequences, in terms of tissue damage, hypothermia, hypoglycaemia and death. These early events include platelet aggregation and degranulation, adherence of polymorphonuclear leukocytes, fibrin deposition, endothelial damage, and, metabolic disturbances. Each of these processes is known to be mediated by its own set of interreactive biological response modifiers, rather than by endotoxin directly (1). The response of old rats to endotoxin has undergone numerous quantitative and quantitative changes, none of which could be singled out as being primarily responsible. In conclusion, the increased susceptibility of old rats to endotoxin is probably not due to a reduced capacity of any particular function, but rather the result of a diminished homeostatic control, which may reflect intrinsic hyperreactivity of responsive cells (not Kupffer cells), both to endotoxin itself and to secondary mediators.

The Kupffer cells of the liver are generally considered to be of primary importance for the removal of endotoxin from the circulation, and for the initiation of many of the body's reactions through the production of monokines. Our studies show that these functional properties of Kupffer cells are quantitatively affected by aging in several respects, but these changes are not likely to be dominating the critical early phases of the response of the old rats to endotoxin. The excessive primary response to endotoxin appears to be primarily localized in platelets, neutrophils and in vascular and liver endothelial cells. This results in the altered release of mediators, including pro-coagulants and anti-fibrinolytic factors. In addition, the response to secondary mediators such as interleukin 1 and TNF is increased, which further contributes to the cascade of

events that causes collapse of the homeostatic control mechanisms in old rats under relatively mild challenges.

The age-related changes that underlie the increased sensitivity of old rats to LPS are so generalized and manifold, that it is very difficult to translate them into possibilities for improvement of the treatment of septic shock in elderly humans. Our results suggest that increased emphasis on existing supportive therapy, with maintenance of body temperature, blood pressure, and normoglycemia is warranted. Strategies involving prevention of thrombosis and disseminated intravascular coagulation by anti-coagulation, as well as stimulation of fibrinolysis by plasminogen activators might be particularly important in the elderly. However, the risks associated with an increased bleeding tendency appear to outweigh the possible benefits. Most importantly, early intervention in the primary response to LPS, which could prevent much of the excessive secondary reactions is of crucial importance. In this respect new developments which enable the moderation of cytokine production and of their effects, such as specific antibodies and antagonists, but even more the use of detoxifying anti-LPS antibodies, could dramatically increase the survival rates of elderly in shock.

Our studies have also revealed additional information on the effects of aging on normal or basal regulation of homeostasis, particularly regarding glucose metabolism and fibrinolysis. It is strongly suggested by the results of our studies, that, in spite of the fact that such changes should not be considered as abnormal or defective, they are highly indicative for a generalized, progressive, deterioration of the subtle interplay between cells and molecules that keeps organisms alive and in good health.

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SUMMARY

The studies described in this thesis are focussed on the effects of aging on the reactions of laboratory rats to systemic administration of bacterial endotoxins. Endotoxins are lipopolysaccharides which are largely responsible for the acute phase reaction evoked by infection with gram-negative bacteria. The experiments were intended to gain insights into the causes and mechanisms of reduced resistance of older individuals for extreme challenges.

In chapter 2 the general pathophysiological effects of various dosages of endotoxin are described, using rats of different age, genetic background and gender. The results show that mortality, tissue and organ damage and induction of hypothermia increase drastically and progressively with increasing age. In addition, the clearance of endotoxin from the circulation was reduced, but much less, by comparison.

In the morphological studies presented in chapters 3 and 4, the time sequence of the events that resulted in the damage in lung and liver of old rats, which is much more severe than in young ones. These experiments showed that older rats have a much stronger tendency for intravascular coagulation, with stronger activation of platelets and granulocytes, more extensive adhesion of granulocytes to endothelial cells. Primary damage to these organs occurred in the endothelium. In lungs, this was followed by haemmorhage, extravascular coagulation and oedema, while extensive parenchymal damage was found in liver. These phenomena were not, or to much lesser extend, observed in young adults treated with the same dose.

In chapter 5, the effects of endotoxin treatment on biochemical parameters of fibrinolysis of young and old rats were compared. Basal plasma levels of plasminogen activators (t-PA and u-PA) were lower, and those of the plaminogen activator inhibitor PAI-1 were higher, in old than in young rats. Administration of endotoxin resulted in more pronounced changes in in some but not all components of fibrinolysis in old rats. Especially the higher increase in the concentration of PAI-1 observed one hour after of injection, could explain the progressive deposition of fibrin aggregates in old rats. Older rats also reacted with a stronger increase in plasma PAI-1 concentration to the injection of interleukin-1 and tumor necrosis factor.

Endotoxins also evoke extensive changes in metabolism. In chapter 6, a comparison is made between the effects of endotoxin on glucose metabolism of young and old rats, also in dependance of nutritional conditions (fasted/non-fasted). In non-fasted animals, both the hyperglycemic reaction of the first hour, and the hypoglycemia occurring later, were more severe in old rats, while only an increased tendency to develop hpoglycemia was observed in fasted old rats. These phenomena may be related to a decreased capacity of hepatic gluconeogenesis, partly caused by damage to liver cells. There were,

however, only minor differences in the time-dependent changes in the concentrations of glucose metabolites after endotoxin treatment of young and old (fasted) rats.

The Kupffer cells of the liver are the major cell type involved in the clearance of endotoxin from the circulation. They are also considered to be responsible for a important part of the secundary reactions of the body, through the secretion of cytokines. In chapter 7, investigations into the possible age-related changes in endocytotic capacity are decribed, using colloidal albumin as a model substrate. Although Kupffer cells of old rats display a reduced uptake of colloidal albumin, it seems unlikely that the increased sensitivity of old rats to endotoxin is to be ascribed to reduced clearance by Kupffer cells.

The reactions of isolated Kupffer cells to endotoxin were further studied with in vitro incubations. The influence of age on the production of some important cytokines by endotoxin treated Kupffer cells is described in chapter 8. There were minor changes in the production of prostaglandins and leukotrienes only at very high age. No age-related changes were obsreved in the secretion of interleukins-1 and-6, and of tumor necrosis factor. There were some changes in the time- and dose-dependency of the induction of the m-RNA's of these cytokines. There were, however, no indications for an increased reactivity of cells from old rats to endotoxin. The experiments described in chapter 9 deal with the effects of endotoxin on protein synthesis, which was analyzed by one- and two-dimensional gel electrophoresis. The results confirm that Kupffer cells from old rats react less, rather than more strongly to endotoxin.

In chapter 10 a summary of the most important results is given, and their significance is discussed in the frame of the effects of aging on homeostatic systems, under normal conditions, as well as under stress. The general conclusions include that the age-dependent changes that form the basis of the decreased capacity to maintain homeostasis under challenge do not appear to be limited to some specific control mechanisms, but constitute a scala of changes in the regulation of haemostasis and metabolism and in the response to injury.

SAMENVATTING

De in dit proefschrift beschreven studies zijn gewijd aan de effecten van veroudering op de reacties van laboratorium ratten bij blootstelling aan bacteriële endotoxinen. Endotoxinen zijn lipopolysacchariden die voor een belangrijk deel verantwoordelijk zijn voor de acute-fase-reactie die optreedt bij infecties met gram-negatieve bacteriën. De experimenten waren erop gericht inzicht te krijgen in de oorzaken en mechanismen van de verminderde weerstand van oudere individuen voor extreme belasting.

In hoofdstuk 2 worden de algemene pathofysiologische effecten bij verschillende doses endotoxine beschreven in ratten van verschillende leeftijden, genetische achtergrond en sexe. Het blijkt dat de mortaliteit, de schade aan weefsels en organen en de inductie van hypothermie drastisch en progressief toenemen met de leeftijd. Daarnaast nam verwijdering van endotoxinen uit de bloedbaan af, doch naar verhouding in veel mindere mate.

Bij het morfologisch onderzoek beschreven in de hoofdstukken 3 en 4 werd bestudeerd op welke manier de ernstiger beschadiging van longen en lever in oudere ratten ontstaat. Uit deze experimenten is gebleken dat oudere ratten een veel sterkere neiging tot intravasale stolling vertonen, waarbij activatie van bloedplaatjes en granulocyten, alsmede de hechting van granulocyten aan endotheelcellen in versterkte mate optraden. Beschadiging van deze organen vond primair plaats in het endotheel. In de longen werd dit gevolgd door bloedingen, extravasale stolling en oedeem, terwijl in de lever uitgebreide beschadiging van het parenchym werd gevonden. Deze verschijnselen deden zich bij jong volwassen ratten, behandeld met dezelfde dosis endotoxine, niet of in veel mindere mate voor.

In hoofdstuk 5 worden de effecten van endotoxine op biochemische parameters van de fibrinolyse bij oudere ratten vergeleken met die bij jonge dieren. Basale plasmagehalten van plasminogeen-activatoren (t-PA en u-PA) waren lager, en die van de remmer van plasminogeen-activatoren (PAI-1) hoger in oude dan in jong volwassen dieren. Bij oude ratten werd na endotoxinebehandeling een sterkere verandering in de plasmanivo's van enkele, maar niet alle, fibrinolyse factoren gevonden. Met name de sterkere toename in de concentratie van PAI-1, die vanaf ca. 1 uur na injectie werd gevonden, zou de toenemende depositie van fibrine-aggregaten in de oudere ratten kunnen verklaren. Oudere ratten reageerden ook op injectie van interleukine 1 en tumor necrose factor met een sterkere inductie van PAI-1.

Endotoxinen veroorzaken ook uitgebreide veranderingen in het metabolisme. In hoofdstuk 6 wordt een vergelijking gemaakt tussen de effecten van endotoxine op het glucose metabolisme van jonge en oude ratten, mede in afhankelijkheid van de voedingstoestand (gevast/niet gevast). In niet gevaste dieren waren zowel de

hyperglycemische reactie tijdens het eerste uur, als de later optredende hypoglycemie ernstiger bij oude ratten, terwijl in gevaste dieren alleen een verhoogde neiging tot hypoglycemie werd gevonden. Deze verschijnselen hebben mogelijk te maken met een verminderde capaciteit van de gluconeogenese in de lever, mede veroorzaakt door schade aan levercellen. Er werden echter slechts geringe verschillen gevonden tussen jonge en oude ratten wat betreft de tijdsafhankelijke veranderingen in de concentraties van glucosemetabolieten in de lever.

De Kupffercellen van de lever zijn de belangrijkste cellen bij de verwijdering van endotoxinen uit de bloedbaan. Zij worden door de uitscheiding van cytokines ook verantwoordelijk geacht voor een belangrijk deel van de secundaire reacties van het lichaam op endotoxinen. In hoofdstuk 7 staat onderzoek beschreven naar de mogelijke leeftijdsafhankelijke veranderingen in de endocytotische capaciteit van deze cellen, waarbij colloïdaal albumine als modelsubstraat is gebruikt. Hoewel Kupffercellen van oude ratten een verminderde opname van colloïdaal albumine vertoonden, lijkt het om andere redenen onwaarschijnlijk dat de verhoogde gevoeligheid voor endotoxinen kan worden toegeschreven aan een verminderde klaring door Kupffercellen.

De reacties van geïsoleerde Kupffercellen op endotoxinen werden verder bestudeerd met *in vitro* studies. In hoofdstuk 8 wordt de invloed van de leeftijd op de productie van enkele belangrijke cytokines door met endotoxine behandelde Kupffercellen geanalyseerd. In de productie van prostaglandinen en leukotriënen traden slechts zeer geringe verschillen op bij zeer oude ratten, terwijl geen verschillen werden gevonden in de secretie van de interleukines 1 en 6, en van tumor necrose factor. Wel waren de dosis- en tijds-afhankelijkheid van de inductie van m-RNA's van deze cytokines enigszins veranderd. Er was echter geen sprake van een verhoogde gevoeligheid van de cellen die de sterkere reacties van oude ratten op endotoxine zouden kunnen verklaren. De in hoofdstuk 8 beschreven experimenten waarin de effecten van endotoxine op de synthese van eiwitten met één- en twee-dimensionale gel electroforese werden bestudeerd, bevestigen dat Kupffercellen van oude ratten eerder minder sterk, dan sterker reageren op endotoxine.

In hoofdstuk 10 wordt een samenvatting gegeven van de belangrijkste resultaten en wordt de betekenis ervan besproken in het kader van de effecten van veroudering op homeostatische systemen, zowel onder normale condities, als bij blootstelling aan extreme omstandigheden. Als algemene conclusie wordt gesteld dat de leeftijdsafhankelijke veranderingen die ten grondslag liggen aan het verminderde vermogen om de homeostase te bewaren onder belasting niet beperkt zijn tot enkele aanwijsbare controlemechanismen, doch bestaat uit een scala van veranderingen in de regulatie van haemostase en (energie)metabolisme, en in de reacties op beschadiging van weefsels.

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CURRICULUM VITAE

Adriaan Brouwer werd geboren op 3 mei 1949 te Nieuwer Amstel, het huidige Amstelveen. Op het Spinoza Lyceum te Amsterdam werd in 1966 het HBS-B diploma behaald. Het kandidaatsexamen Scheikunde werd in 1970 afgelegd op de Universiteit van Amsterdam, in 1974 gevolgd door het doctoraalexamen Biochemie. Na een korte periode op de Afdeling Nierziekten van het Radboudziekenhuis trad hij op 1 december 1975 in dienst van het toenmalige Instituut voor Experimentele Gerontologie TNO te Rijswijk, dat in 1991 fuseerde met twee andere TNO instituten en onder de naam Instituut voor Verouderings- en Vaatziektenonderzoek in het nieuwe Gaubius Laboratorium te Leiden werd gevestigd. Momenteel is hij hier werkzaam als hoofd van de sectie Voeding en Veroudering van de Afdeling Dementie en Celfysiologie. Als wetenschappelijk medewerker werd tijdens de eerste jaren onder leiding van Prof. Dr. D.L. Knook gewerkt aan biomedisch proefdieronderzoek op het gebied van veroudering van levercellen. Het huidige werkterrein omvat, naast het hier beschreven werk, calorische restrictie en het effekt van veroudering op het metabolisme van voedselbestanddelen.