Lysosomal Disruption during the Development of Endotoxic Shock in the Baboon

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SUMMARY

Lysosomal disruption with release of lysosomal enzymes has been described in shock. Our study was designed to demonstrate the release of lysosomal enzymes in the liver and lung in addition to determining circulating serum lysosomal enzyme values.

Therapeutic means of reducing or inhibiting lysosomal disruption were also investigated. Five groups of baboons were investigated:

Group A: (control group) — no endotoxin or treatment administered.

Group B: (endotoxin only) — liver and lung tissues showed an increase in free lysosomal enzymes, and a similar increase in circulating serum lysosomal enzymes was demonstrated.

Group C: (chloroquine treatment (lysosomal stabiliser) following endotoxin administration) — the liver and lung tissues showed a smaller increase in free lysosomal enzymes and a corresponding lesser increase in circulating serum lysosomal enzymes was demonstrated.

Group D: (dexamethasone treatment (lysosomal membrane stabiliser) following endotoxin administration) — the rise in lysosomal tissue and serum enzyme values was less than that in group B.

Group E: (combination of chloroquine and dexamethasone treatment following endotoxin administration) — the rise in lysosomal tissue and serum values was less than that demonstrated in group B.

The survival of the animals in the various groups correlated well with the lysosomal enzyme tissue and serum values, as did the monitored haemodynamic, haematological and other parameters.

Several problems have hampered a clear delineation of the haemodynamic alterations resulting from Gram-negative bacteraemia. Many of these problems arose because the results of experiments utilising endotoxin injections in the

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dog were extrapolated to man. The peculiar enterohepatic circulation of the dog results in marked splanchnic blood pooling, which is not observed in man.[1] The development of a primate model, in this case the baboon, overcomes many of these difficulties, and makes it ideal for these experiments.

The term 'endotoxin'[2] is usually used to describe the cell wall lipopolysaccharide of Gram-negative bacilli. The administration of purified endotoxin induces a large variety of biological activities in both man and experimental animals.

Lysosomal disruption with release of lysosomal enzymes in shock[3] has been previously described. Various studies on the liver, spleen and kidney have been performed,[4-7] but we could find no references in the available literature to lysosomal changes which occur in the lung during shock. This study was therefore designed to demonstrate the release, if any, of lysosomal enzymes in the liver and the lung after administration of endotoxin, particularly in view of the typical pulmonary changes described in endotoxic shock. In addition, various means of reducing or inhibiting lysosomal disruption were investigated, and the correlation of various parameters of shock with the degree of lysosomal disruption was attempted.

MATERIAL AND METHODS

Experiments were carried out on 22 male and female baboons (*Papio ursinus*), weighing between 10 and 20 kg. After intravenous barbiturate anaesthesia with halothane and O_2 inhalation per endotracheal tube (semiclosed circuit), these baboons were studied by sequential liver and lung biopsies, by laparotomy and thoracotomy respectively.

The baboons were divided into 5 groups:

Group A: 2 baboons served as controls and received no endotoxin, but liver and lung biopsies were performed.

Group B: 5 baboons received purified *E. coli* endotoxin (60 µg/kg) immediately after the control biopsies.

Group C: 5 baboons received endotoxin as above, but were immediately given chloroquine intravenously in a dose of 5 mg/kg.

Group D: 5 baboons received endotoxin as above, and were given dexamethasone in a dose of 2,5 mg/kg immediately after endotoxin administration.

Group E: 5 baboons received both dexamethasone and chloroquine, in the doses described above. Chloroquine was given immediately after endotoxin administration and dexamethasone was given 10 minutes later.

All the baboons were evaluated for haemodynamic, biochemical and haematological changes, as follows: continuous ECG monitoring on an 8-channel recorder,

intra-arterial pressure (1208/B 1208/C pressure transducers, H. P. Sanborn), left ventricular pressure (7F Cordis pigtail catheter), pulmonary arterial pressure (Swan-Ganz catheter), and central venous pressure (right atrial catheter). Catheter positions were controlled by an image intensifier. Blood gases, electrolytes, pH and base excess determinations were carried out. Liver function, SGPT, SGOT, LDH, serum protein, electrophoretic and bilirubin tests were performed, and circulating acid phosphatase levels were determined.

The haematological evaluation included Coulter determinations and platelet counts, and fibrinogen levels, prothrombin activity (PA), prothrombin index (PI), partial thromboplastin time (PTT), and fibrinogen degeneration products were sequentially studied. Thrombo-elastography was performed whenever possible. Rectal temperature, pulse rate and hourly urinary output were recorded. Lysosomal enzyme activities were assayed in the control liver and lung biopsy specimens, and on the specimens taken after endotoxin administration. In each case the liver and lung tissue for the post-endotoxin biopsy was taken as far from the site of the control biopsy as possible. The time lapse between the control biopsy and the post-endotoxin biopsy was exactly 6 hours for all baboons.

Tissue fractionation analysis was used to study the distribution of the lysosomal enzymes in free and particulate forms. Lung and liver fractions were obtained by using a Dounce homogeniser (20 strokes, with a loose-fitting pestle) for the fractionation of the tissue in a 0,25M sucrose medium.[8] Supernatant and pellet fractions were prepared by centrifugation of the homogenates. Both fractions were assayed.

Acid phosphatase was assayed according to the procedure described by Shibko and Tappel.[9] Alkaline phosphatase was determined by incubating the enzyme preparation with p-nitrophenyl phosphate as substrate, as described by Garen and Leventhal.[10]

RESULTS

Haemodynamic Evaluation (Table I)

A gradual initial decrease in intra-arterial and left ventricular pressures was observed 1 hour after the first biopsy in group A (Fig. 1 — A1, A2). After 2 hours a gradual recovery of the pressures was noted, which almost attained control values at 5 hours and persisted until the completion of the recording.

Group B (Fig. 1 — B1, B2) showed a gradual decrease in arterial and left ventricular pressures during the first 2 hours, differing slightly from those of the control group A (Fig. 1 — A1, A2). After 2 hours, however, a further decline in arterial and ventricular pressure occurred, reaching a level of 65 mmHg systolic after 6 hours. A constant finding was the rapid increase in systemic peripheral resistance during the first 2 hours which then remained high for the duration of the experiment (Fig. 2). A slight increase in pulmonary arterial pressure was found during the periods of decline in systemic arterial pressure (Fig. 1 — B1, B2).

TABLE I. CHANGES IN THE SYSTOLIC PULMONARY ARTERY PRESSURE (mmHg) IN 4 EXPERIMENTAL GROUPS*

Time	Group B (Endotoxin only)	Group C (Endotoxin, chloroquine)	Group D (Endotoxin, dexamethasone)	Group E (Endotoxin, dexamethasone, chloroquine)
Pre-op.	16 ±2	14 ±2	11 ±2	11 ±1
1st hour	19 ±3	13 ±5	10 ±2	11 ±2
2nd hour	15 ±2	14 ±4	12 ±3	11
3rd hour	23 ±5	13 ±3	8 ±2	9 ±1
4th hour	15 ±3	13 ±7	9 ±2	13 ±1
5th hour	19 ±5	12 ±4	6 ±1	14 ±2
6th hour	20 ±5	14 ±3	8 ±1	13 ±2
7th hour	18 ±4	14 ±2	10 ±2	16 ±4
8th hour	24 ±6	13 ±4	12 ±1	15 ±2

* The values in group A remained constant and are not shown.
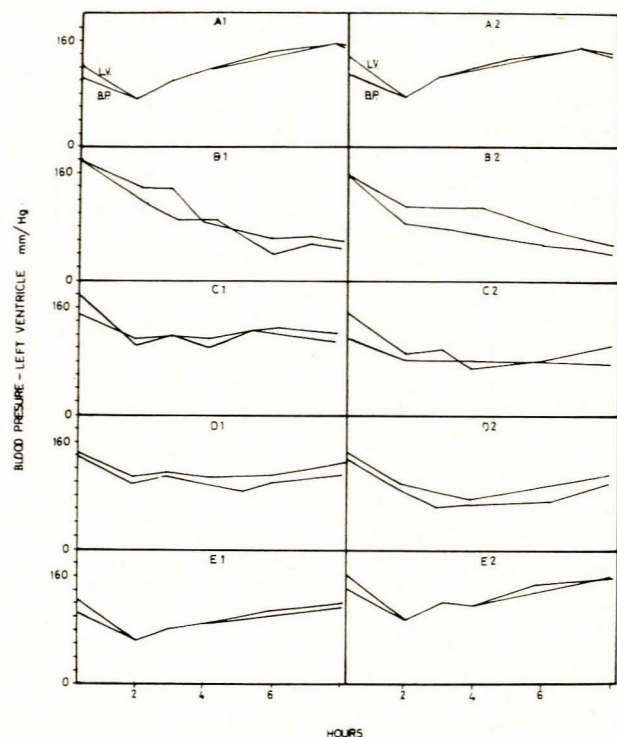

Fig. 1. Haemodynamic changes in the 5 groups. Left ventricular and intra-arterial blood pressure values in 2 representative graphs from each group are shown.

In group C, an initial sharp decrease in arterial and left ventricular pressures was recorded after endotoxin administration; thereafter they stabilised at the levels indicated in Fig. 1 — C1, C2. The pulmonary artery pressures remained constant.

Similar results were obtained in group D, namely an initial decrease in arterial and left ventricular pressures, which subsequently stabilised for the duration of the experiment (Fig. 1 — D1, D2). Pulmonary artery pressures decreased ($P < 0,05$).
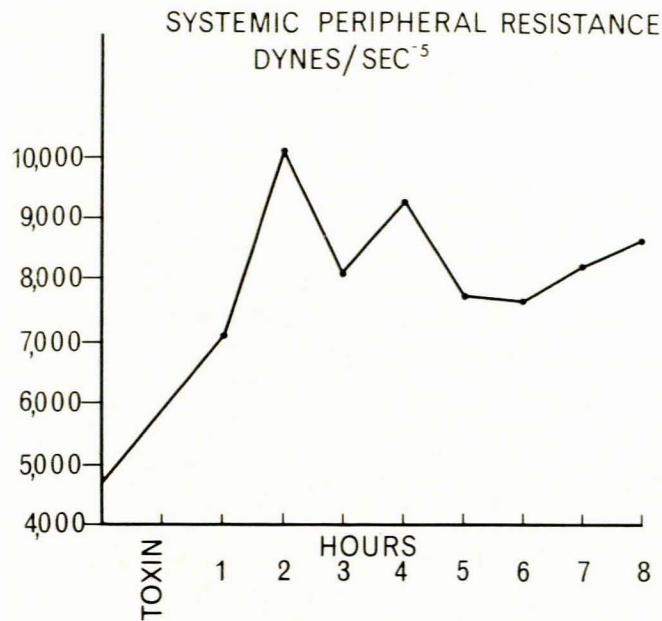
Fig. 2. Average changes in the systemic peripheral resistance for group B only.
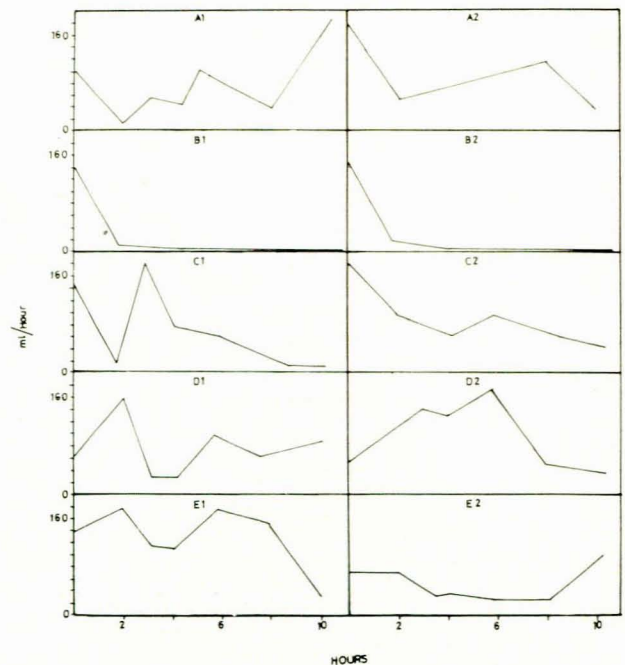

Fig. 3. Urinary output values in the 5 groups. Representative values from each group are shown.
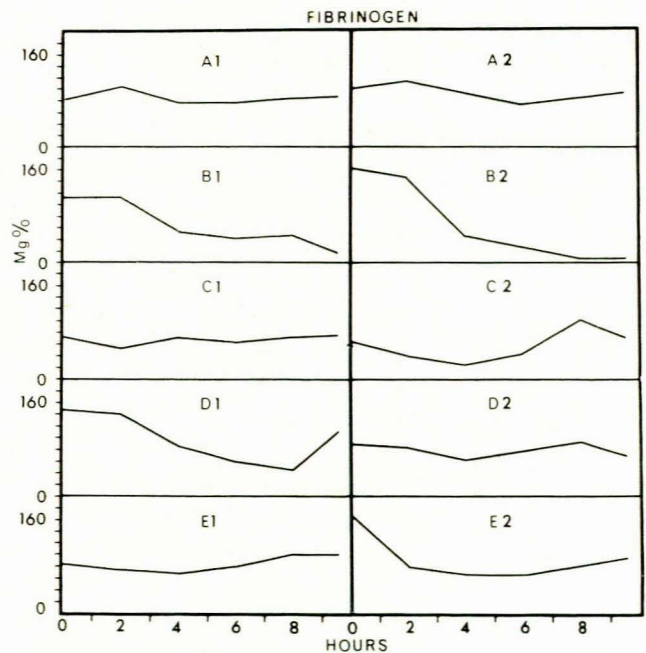
Fig. 4. Fibrinogen values of 2 baboons in each group. Note the rapid decline in group B.

Group E (Fig. 1 — E1, E2) showed an initial gradual decrease in arterial and left ventricular pressures during the first 2 hours, after which the pressure remained remarkably stable. Pulmonary artery pressures remained constant.

Urinary Output

In group A a fairly rapid decrease in the urinary output was noted during the first 2 hours — the output then gradually improved and returned to normal (Fig. 3 — A1, A2). In group B a rapid decline was recorded — after 3 hours the output was less than 10 ml/h (Fig. 3 — B1, B2). A more gradual decrease than that in the untreated group B was recorded in group C (Fig. 3 — C1, C2). After an initial decrease during the first 2 hours, the urinary output improved in group D (Fig. 3 — D1, D2). In group E an initial decrease was noted, after which the output gradually returned to normal values (Fig. 3 — E1, E2).

Clotting Disturbances

Fibrinogen levels showed a minor decrease (Fig. 4 — A1, A2), but stabilised 3 - 4 hours after the first biopsy. In several additional controls no change was observed in PA, PI or PTT, and fibrinogen levels remained almost static. Furthermore, fibrinogen degeneration product levels showed no increase. Other haematological evaluations (WBC, RBC, Hb, MCV, MCH, MCHC and ESR) remained constant. The haematocrit showed a slight decrease due to a haemodilution effect as a result of regular flushing of the arterial and venous catheters to ensure patency. In the control group platelets decreased to values of 200 000/mm³ (Fig. 5a).

A more rapid decrease in fibrinogen levels was demonstrated in group B (Fig. 4 — A1, A2) with values less than 20 mg/100 ml after 5 hours. The PA values declined from 100% to 59% after 5 hours, PI decreased from 68% to 27% and the PTT increased from 39 to 99 sec

during the same period. Platelets diminished markedly, values falling from 200 000 to less than 100 000/mm³ within 5 hours ($P = 0,005$) (Fig. 5b).
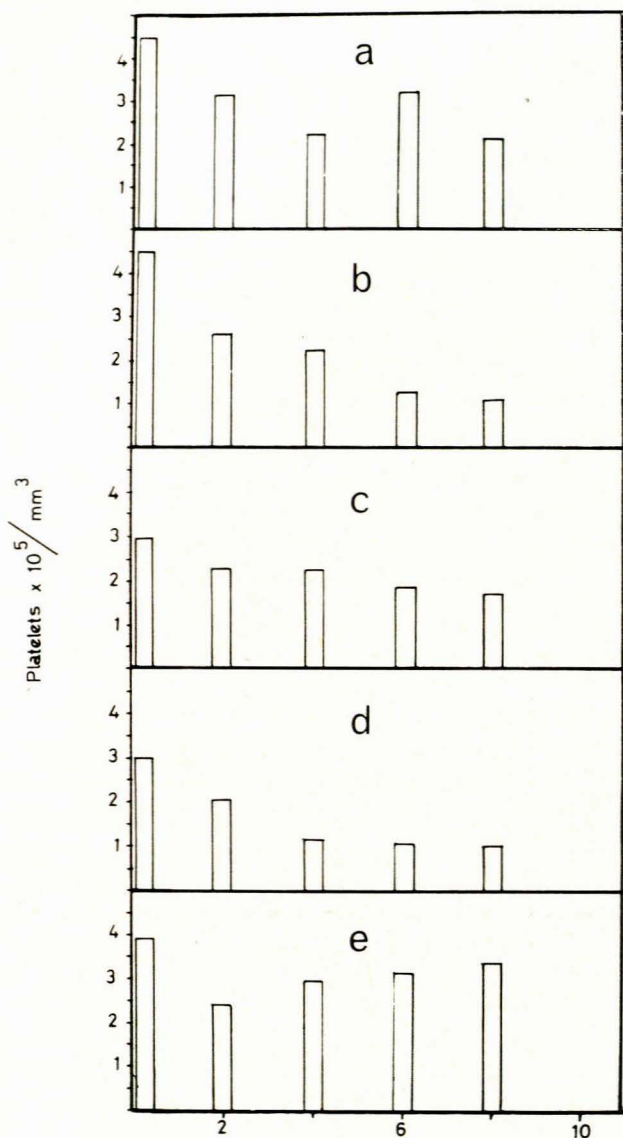

Fig. 5. Average platelet values for the 5 groups.

In group C (Fig. 4 — C1, C2) the fibrinogen levels showed an initial decrease, but stabilised after 3 hours. The final values at the conclusion of the experiment were considerably higher than those recorded in the untreated group B (Fig. 4 — B1, B2). The PA values decreased from 60% to 30%, the PI remained constant at 55%, while the PTT increased from 44 to 68 sec. Platelets decreased to a lesser extent than in group B ($P = 0,005$).

In group D, except for an initial decrease in fibrinogen levels, (Fig. 4 — D1, D2), minor variations in PA, PI, PTT and a slight decrease in platelets (less than 50 000/mm³) ($P = 0,005$) (Fig. 5d), no pronounced changes, comparable

to those noted in the untreated group B, could be demonstrated.

In group E an initial decrease in fibrinogen levels occurred; thereafter the values remained constant, and then increased at the termination of the experiment (Fig. 4 — E1, E2). The PA remained normal and the PI showed a slight decrease, while the PTT increased slightly. Platelets were decreased by less than 50 000 mm³ ($P = 0,005$) (Fig. 5e).

Serum Acid Phosphatase Levels and Biochemical Parameters (Table II)

In group A, acid phosphatase activity was at constant levels throughout the duration of the experiment (Fig. 6 — a1, a2). Other biochemical parameters showed negligible variations.
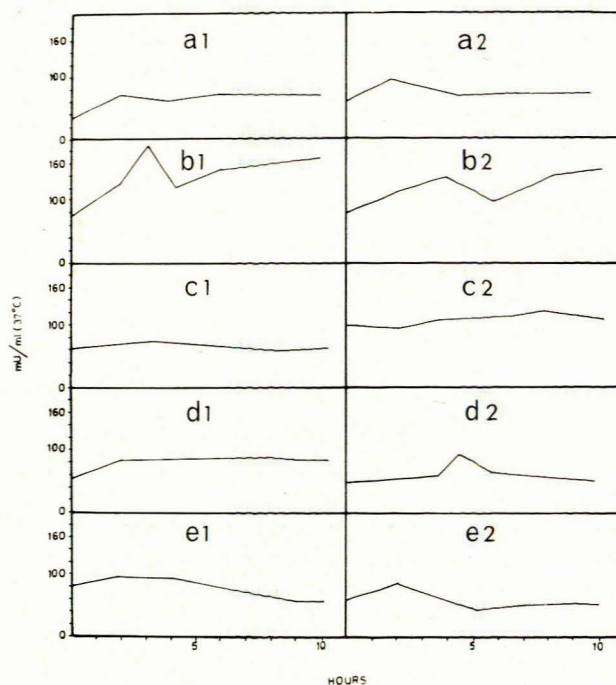

Fig. 6. Serum acid phosphatase values of 2 baboons in each group.

In group B a mild acidosis was recorded. Enzymes analysed showed a slight increase in GPT and GOT, and LDH increased from 243 to 400 units. Circulating acid phosphatase activity doubled after 1 hour. A typical example of acid phosphatase changes is demonstrated in Fig. 7. High levels persisted for the duration of the experiment (Fig. 6 — b1, b2).

A mild acidosis occurred in group C, with a slight increase in LDH. A lesser increase in circulating acid phosphatase was registered (Fig. 6 — c1, c2) ($P = 0,005$).

In group D (Fig. 6 — d1, d2) changes similar to those in group C were noted. Notably, the increase in acid phosphatase levels was less than that demonstrated in group B.

TABLE II. SERUM ACID PHOSPHATASE VALUES (mU/ml) AT 37°C IN 4 EXPERIMENTAL GROUPS*

Time	Group B (Endotoxin only)	Group C (Endotoxin, chloroquine)	Group D (Endotoxin, dexame-thasone)	Group D (Endo-toxin, dexame-thasone, chloro-quine)
Pre-op.	60 ± 8	56 ± 7	72 ± 23	48 ± 14
1st hour	126 ± 18	84 ± 6	137 ± 80	37 ± 13
2nd hour	110 ± 45	38 ± 7	156 ± 101	35 ± 12
3rd hour	108 ± 62	65 ± 7	122 ± 41	36 ± 15
4th hour	187 ± 17	68 ± 8	116 ± 31	36 ± 13
5th hour	142 ± 17	69 ± 3	258 ± 167	33 ± 14
6th hour	134 ± 28	67 ± 2	137 ± 42	31 ± 13
7th hour	158 ± 19	60 ± 3	128 ± 14	30 ± 9
8th hour	170 ± 24	62 ± 1	131 ± 31	31 ± 11

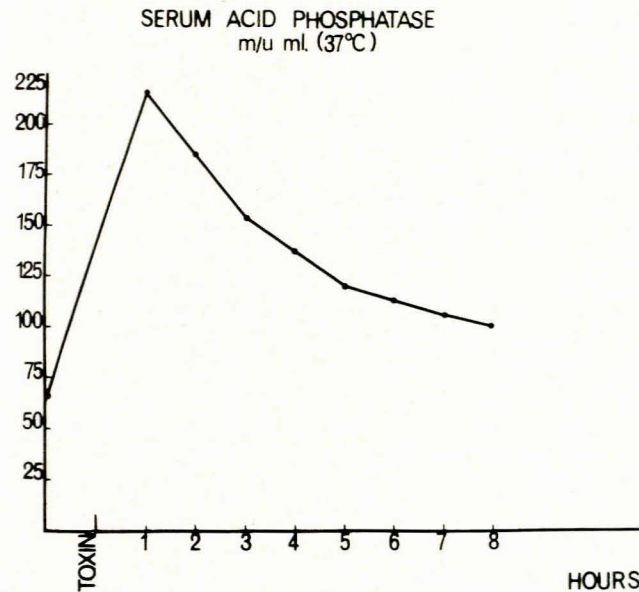* The values in group A remained almost constant and are not shown.

Fig. 7. Serum acid phosphatase values in a group B animal which received endotoxin only.

In group E a moderate increase in LDH, GPT and GOT was demonstrated. Acid phosphatase levels remained constant, after a slight initial increase ($P = 0,001$) (Fig. 6 — e1, e2).

Lysosomal Enzymes (Table III)

In group A no marked increase in free or intraparticulate lysosomal enzyme activities could be demonstrated.

Group B, however, showed considerable increases in the free and intraparticulate lysosomal enzyme activities.

Values of both free and intraparticulate lysosomal enzymes in group C were less than those demonstrated in group B.

It was also found that the free and intraparticulate enzyme activities of group D were not increased to the same extent as those of the untreated group B.

In group E, free and intraparticulate lysosomal enzyme levels were lower than the levels demonstrated in group B, and also lower than activities registered for groups C and D.

Temperature

Group A (Fig. 8 — A1 and A2) showed an initial decrease in temperature following the period of anaesthesia, after which the temperature remained stable. In group B (Fig. 8 — B1 and B2) an increase of 1°C was recorded after 3 hours, followed by an increase of 3° - 4°C. In group C (Fig. 8 — C1 and C2) a total increase in temperature
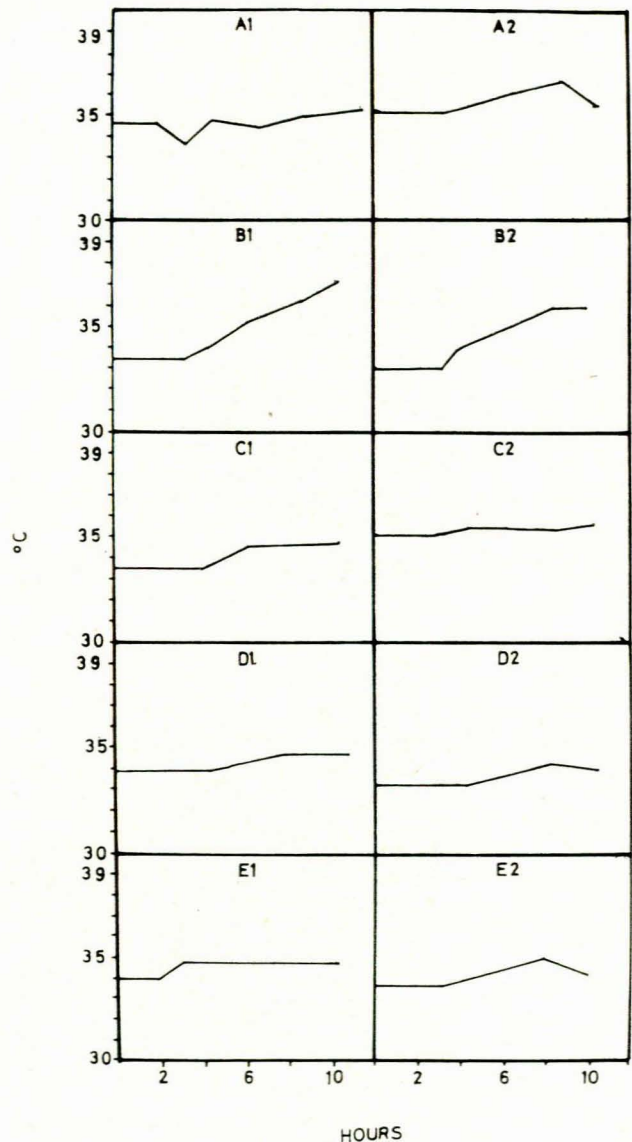

Fig. 8. Temperature changes of 2 baboons in each group.

TABLE III. LYSOSOMAL ENZYME VALUES IN 2 BABOONS OF EACH GROUP

	Group	First biopsy*								Second biopsy (8 hours after first biopsy)							
		Acid phosphatase activity (μmol/mg/min)				Alkaline phosphatase activity (μmol/mg/min)				Acid phosphatase activity (μmol/mg/min)				Alkaline phosphatase activity (μmol/mg/min)			
		Lung		Liver		Lung		Liver		Lung		Liver		Lung		Liver	
		Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet	Super-natant	Pellet
First baboon	A	18	28	14	26	4	3	3	5	16	28	15	24	4	3	3	7
	B	16	53	26	41	9	11	13	13	20	100	30	47	9	36	18	20
	C	12	18	40	32	6	16	8	8	15	20	50	29	9	13	21	9
	D	30	97	35	75	10	23	8	8	35	120	39	63	13	21	3	11
	E	19	78	25	40	13	19	12	12	18	72	28	14	14	20	2	7
Second baboon	A	21	102	42	65	7	16	13	12	23	86	47	67	6	14	14	12
	B	21	107	38	93	10	11	4	14	28	258	50	110	14	22	6	29
	C	14	20	17	24	5	11	9	8	12	39	18	24	4	14	12	9
	D	11	33	13	29	9	3	4	12	10	34	23	40	9	3	5	22
	E	14	45	18	32	9	3	18	12	13	21	17	33	8	3	26	12

* The first biopsy values indicate the enzyme levels before the administration of endotoxin. The second biopsy values indicate the enzyme levels 6 hours after the administration of endotoxin.

of 1°C, compared with more than 3°C in group B, was observed. The temperature increase in group D was similar to that in group C. In group E a temperature increase of 1°C was registered (Fig. 8 — E1 and E2).

Survival

The survival periods of the experimental animals were as follows:
Group A — both animals survived indefinitely.
Group B — all the animals died within 36 hours.
Group C — 3 animals survived beyond 48 hours for an indefinite period; 1 animal died after 48 hours and 1 at 72 hours.
Group D — 3 animals survived, and 2 died — both after 48 hours.
Group E — 1 animal died at 96 hours. Four animals survived indefinitely.

Postmortem Findings

In all the animals which died from endotoxic shock, the postmortem macroscopic and microscopic findings were remarkably similar irrespective of treatment. Pulmonary oedema was a constant finding. Reactive germinal centres in the spleen were noted. Fatty changes in the liver and intraglomerular hyaline thrombi (typical of disseminated intravascular coagulation) were constantly found. Renal tubular necrosis was seen, and all these animals died within 36 hours.

DISCUSSION

Both the supernatant and the particulate fractions, prepared from specimens from animals which had been given endotoxin, showed elevated lysosomal enzyme activity in comparison with those from animals not given endotoxin. The rise in enzyme activity correlated well with the clini-

cal, haemodynamic, haematological and blood chemistry analyses and with the ultimate survival or death of the experimental animals. Lysosomes contain a variety of hydrolytic enzymes, which are shielded from the cytoplasm by the lysosomal membrane.[6,7] Disruption of this membrane, as a result of some abnormal event, liberates the enzymes and allows them to hydrolyse cellular proteins, nucleic acids and polysaccharides.

Janoff *et al.*[6] proposed that liberation of these enzymes may occur in the livers and visceral organs of shocked animals as a consequence of the hypoxia and the acidosis which develop in these tissues, and that further release of active lysosomal hydrolases from damaged tissue into the bloodstream might be a potential factor in the fatal outcome of traumatic shock.

In bacterial endotoxaemia it appears that lysosomes also play an important role. Elevated activities of circulating acid phosphatase and β-glucuronidase in rats, following endotoxin administration, were demonstrated by Janoff *et al.*[6] who reported further that the increased levels of circulating lysosomal enzymes correlated well with changes in the total and free activity of lysosomal enzymes in liver homogenates. They also found that pretreatment of the rats with cortisone 30 minutes before injection of endotoxin resulted in smaller increases in plasma acid phosphatase, presumably owing to an increased stability of the lysosomal membranes. Filkins,[4] after experiments on rats, suggested that the lysosomes of the hepatic macrophages are mediators of endotoxin inactivation and that this may play a crucial role in the prevention of resultant shock, and he suggested, in contrast to Weissmann,[7] that chloroquine had a labilising effect on hepatic lysosomes *in vivo*.

Our study illustrated, firstly, that there is an increase in circulating lysosomal enzyme activity after the administration of endotoxin. In the control group no increase in circulating enzyme activity could be shown.

Secondly, our results indicated that chloroquine administration after endotoxin reduced the amount of free lysosomal enzyme activity, with an improvement in the investigated parameters of shock, in the baboons. These

results support the view of Weissmann[7] that chloroquine has an *in vivo* effect of lysosomal membrane stabilisation.

Furthermore, through a process of selective uptake of chloroquine by the lysosomes the intralysosomal pH is altered and shifts from a low to a high pH. This depresses the activity of these acid hydrolytic enzymes.

Thirdly, we illustrated that dexamethasone given immediately after endotoxin had a similar beneficial effect, namely reduced lysosomal enzyme activity. These findings illustrate the stabilising effect of corticosteroids on lysosomal membranes.

Fourthly, this study showed that a combination regimen — chloroquine followed 10 minutes later by dexamethasone — has an even greater effect on reducing the amount of free lysosomal enzyme activity, and these results correlated well with the monitored parameters and the ultimate survival of the animal. These experiments also indicated, and it has been reported by other authors,[11] that there is a considerable individual tolerance to shock among primates, and that it is impossible to predict how a particular animal will react to a specific dose per weight of endotoxin, although death occurred within 36 hours in all animals given only endotoxin.

We found that the haemodynamic evaluation of endotoxicosis is not necessarily an accurate criterion of the state of shock, as was also reported by Hinshaw *et al.*[12] but we did find that a rapid change in the fibrinogen levels and consequent alteration in coagulability of blood was a valuable parameter in defining the subsequent course of endotoxicosis.[13,14]

Furthermore, our experiments illustrate that clotting disturbances and a rise in lysosomal enzyme activity occur simultaneously. This correlates with the observations made by Horwitz *et al.*[14] Results indicate that the disseminated intravascular coagulation cannot be attributed to hypercoagulability resulting from hypoperfusion and acidosis, but that it may be a direct consequence of the endotoxaemia. At this stage the hypothesis seems to be that lysosomal enzymes might be primarily incriminated in the development of the changes in coagulability typically seen in endotoxic shock, and more specifically in disseminated intravascular coagulation. Our results illustrate that a lesser increase in circulating lysosomal enzymes correlates well with a concomitant slower fall in fibrinogen level in the treated animals.

CONCLUSIONS

These findings indicate that an experimental basis has been established for the administration of corticosteroids in

shock.[15-18] Chloroquine has been shown experimentally to have a lysosomal stabilising effect, and in combination with dexamethasone it had an even greater stabilising effect. Obviously, the early administration of chloroquine and steroids is of prime importance, as they are of little value once lysosomal disruption has taken place. Results indicate that lysosomal disruption, with consequent enzymatic release, takes place well before the typical disturbances in haemodynamic criteria[18] are observed. In the clinical situation, endotoxic shock frequently develops with devastating rapidity, with little indication of the impending catastrophe being evident in the usually monitored haemodynamic parameters. Our studies would suggest that a sequential serum assay for circulating acid phosphatase and β-glucuronidase might be used as a parameter in conjunction with monitoring of coagulation changes,[19] to detect the early phase of endotoxicosis and to indicate effectiveness or ineffectiveness of treatment.

These experiments also demonstrate an increase in free and particulate lysosomal enzyme activity in lung fractions, thus emphasising the crucial role played by the lungs in the syndrome of shock, as has been mentioned by other investigators.[20]

The suppression of lysosomal enzyme activity modified the course of endotoxic shock in these experiments, and resulted in the survival of 10 of the 15 treated baboons.

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