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Investigations on the Mechanism of Stimulation of Non-Specific Immunity by Bacterial Lipopolysaccharides

J. G. HOWARD, D. ROWLEY AND A. C. WARDLAW

The Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, Paddington, London, W.2

Summary. The stimulation of non-specific immunity by lipopolysaccharides could not be correlated with the serum properdin level at the time of challenge. Zymosan was effective in stimulating an increase in properdin without raising resistance to infection. The development of a low properdin level during *Esch. coli* infection did not inevitably lead to death.

The fate of ^{32}P-labelled lipopolysaccharide (^{32}P-LP) after intravenous injection was followed in mice and guinea pigs. Most of it was accumulated rapidly in the reticulo-endothelial system. About a third was removed from the blood more slowly than the remainder, probably owing to a smaller particle size. Some of the ^{32}P was excreted in the urine as inorganic phosphate. Very little radioactivity was found in the circulating leukocytes.

The blood clearance of ^{32}P-LP was slowed by the administration of non-radioactive lipopolysaccharide 30 min. previously. When this interval was 48 hr. the reticulo-endothelial system was found to be hyperactive in terms of rate of clearance and phagocytic capacity. This was accompanied by an increase in the number of active macrophages in the liver and, to a lesser extent, in the spleen. These biphasic changes in the phagocytic activity of the R.E.S. parallel the decreased and increased resistance to infection following the injection of microgram amounts of lipopolysaccharide.

Successive depression and stimulation of the R.E.S. also followed the injection of milligram amounts of Thorotrast. The hyperactive phase was accompanied by increased resistance to *Esch. coli* infection and decreased resistance to *Salm. typhimurium* infection.

The lipid A component of lipopolysaccharide was also taken up by the R.E.S. which it stimulated. Resistance to both *Esch. coli* and *Salm. typhimurium* infections was enhanced.

The basis of lipopolysaccharide-induced non-specific immunity is discussed in relation to changes in the properdin and reticulo-endothelial systems. The superiority of lipopolysaccharide over other colloids in stimulating immunity is considered to be determined by lipid A.

INTRODUCTION

STUDIES on bacterial lipopolysaccharides in this laboratory developed from the observation that the intravenous injection of the cell walls of various Gram-negative bacteria into mice caused first a decrease and then an increase in the resistance of the animals to intraperitoneal challenge with heterologous *Escherichia coli* (Rowley, 1955). Independently,

Field, Howard and Whitby (1955) had found a similar increase in resistance to *Salmonella typhi* infection in mice following intravenous administration of heterologous Gram-negative organisms, either living or dead. That the two investigations were concerned with the same phenomenon was suggested by Rowley, who further postulated that the endotoxin was the active material in both cases. Following this, Field *et al.* (1955) showed that the injection of Shiga O-antigen increased the resistance of mice to *Salmonella typhi*. It was further shown that the protein-free lipopolysaccharide component (LP) of the O-antigen was equally effective (Rowley, 1956). The degradation has subsequently been taken one stage further by our finding that the lipid A component of the lipopolysaccharide retains protective activity while the simultaneously-liberated polysaccharide fragment is inert (Howard, Rowley and Wardlaw, 1957).

Much work has been published recently relating to this phenomenon, and it is clear that the lipopolysaccharide from virtually any Gram-negative organism is effective. The resulting non-specific immunity has been shown to affect infections with *Esch. coli*, *Salm. typhi*, *Brucella melitensis* (Abernathy and Spink, 1956), and *Klebsiella pneumoniae* (Kiser, Lindh and de Mello, 1956). The resistance also extends to Gram-positive bacteria, for Condie, Zak and Good (1955) have claimed an increased resistance to *Streptococcus pyogenes* and *Streptococcus pneumoniae* and Dubos and Schaedler (1956) have found that some degree of immunity to *Mycobacterium tuberculosis* and *Staphylococcus aureus* could be produced. In most papers the resistance described has been transient, lasting 7–10 days.

An initial working hypothesis to explain these changes in resistance was that lipopolysaccharides contain the substrate on which the bactericidal system of serum acts (Rowley, 1956). The initial depression and subsequent increase in resistance could then be explained on the basis of depletion of the serum enzyme followed by its overproduction by the body. In support of this was the finding that following injection of *Proteus vulgaris* cell walls into guinea pigs, the bactericidal power of the sera towards *Esch. coli* first decreased and then increased to a level above normal (Rowley, 1956).

Shortly before Rowley's original paper on this phenomenon, the properdin system was described by Pillemer, Blum, Lepow, Ross, Todd and Wardlaw (1954). Properdin (a newly-discovered serum globulin), the four components of complement and Mg^{++}ions together constitute a bactericidal system (Wardlaw and Pillemer, 1956). Pillemer, Schoenberg, Blum and Wurz (1955) found that properdin combines *in vitro* with yeast cell walls (Zymosan), bacterial cell walls, certain high molecular weight dextrans and levans and bacterial lipopolysaccharides. Further, the *in vivo* injection of cell walls or lipopolysaccharides was shown to produce first a depression and then an elevation of the serum properdin level (Pillemer, *et al.*, 1955; Landy and Pillemer, 1956a,b). The apparent correlation of these biphasic changes with the alterations in immunity was noted by Rowley (1955). The concept of alternate phases of increased susceptibility and increased resistance being determined by changes in the serum properdin level was at first sight attractive. Further investigations of this relationship have revealed it to be both imperfect and inadequate as a basis for all the immunity changes recently described.

An alternative interpretation of the mode of action of lipopolysaccharides in stimulating resistance to infection has been developed from preliminary observations on the *in vivo* fate of these materials after their injection into mice (Rowley, Howard and Jenkin, 1956). For these studies, lipopolysaccharide was labelled with ^{32}P by growing the bacteria in a medium containing this isotope. The labelled lipopolysaccharide (^{32}P-LP) on intravenous

injection was found to be removed rapidly from the blood stream and to become localized in the cells of the reticulo-endothelial system (R.E.S.). Further, the previous administration of non-radioactive LP caused first a decrease and then an increase above normal in the blood clearance rate of ^{32}P-LP. The similarity between these changes in R.E. function and the accompanying changes in non-specific immunity suggested that the primary effect of LP in modifying immunity might be related to its action on the R.E.S. Since many colloidal materials are known to modify R.E. activity, it was desirable to compare some such material with LP in its ability to stimulate non-specific immunity; for this purpose we have studied changes following the administration of Thorotrast.

This communication is concerned with the changes in serum properdin level and R.E. function which accompany the alterations in non-specific immunity following the administration of lipopolysaccharides and other colloids to mice. The fate of intravenously injected lipopolysaccharides is also described.

MATERIALS AND METHODS

Lipopolysaccharide was isolated from *Esch. coli* (strain 2380) by the method of Westphal, Lüderitz and Bister (1952) as modified by Rowley (1956).

The following preparations were kindly donated by Dr. D. A. L. Davies of M.R.E. Porton: *Pasteurella pestis* LP, *Past. pseudotuberculosis* LP, *Shigella shigae* (rough) LP and *Sh. shigae* (smooth) endotoxin.

Radioactive Lipopolysaccharides (^{32}P-LP) were prepared from a variety of organisms by the method described elsewhere (Rowley, Howard and Jenkin, 1956; Rowley, Ali and Jenkin, in press).

Lipid A was isolated from lipopolysaccharide by the method of Westphal and Lüderitz (1954), and suspensions prepared as previously described (Howard, Rowley and Wardlaw, 1957). Radioactive lipid A was isolated from radioactive lipopolysaccharide in the same way. The lipid preparations were non-toxic for mice at 700 μg. intravenously, which was the highest dose tested. On the other hand, parent lipopolysaccharides had an LD_{50} within the range 50–100 μg. intravenously.

Thorotrast obtained from Testagar and Co. Inc., Detroit, Michigan, consisted of 24–26 per cent colloidal thorium dioxide in 25 per cent aqueous dextrin as stabiliser and with 0·15 per cent methyl parasept added as preservative.

Zymosan. Two preparations were used: Batch LE-1, obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; Batch W3, prepared here by the method of Isliker (1957).

ANIMALS

The mice used were the Strong A albino strain obtained from the Royal Army Veterinary Laboratories, Aldershot. In experiments on the clearance of ^{32}P-LP and in histological studies the mice in any experiment did not vary in weight by more than 1 gram. In infection experiments, a weight range of 3 grams was accepted. All animals used were within the range 18–25 g. and in each experiment the mice were of the same sex. They were fed on Diet 41B.

Commercial bred guinea pigs weighing 350–500 g. were used.

INJECTION TECHNIQUES

In mice intravenous injections were made into a tail vein using a 0·25 ml. tuberculin syringe and a gauge 27 needle. The volume injected in blood clearance tests was 0·1 ml., whilst for other purposes the volume never exceeded 0·2 ml.

In guinea pigs intravenous injections were made into a saphenous vein.

ASSESSMENT OF CHANGE IN NON-SPECIFIC IMMUNITY

Changes in immunity following the intravenous administration of lipopolysaccharides and other materials to mice were assessed by intraperitoneal challenge with either a natural mouse pathogen (*Salmonella typhimurium* S2/446) or with a non-pathogen (*Esch. coli* strain 145) administered in 1·75 per cent hog mucin (Wilson batch 1701; see Rowley, 1955). The mice were used in groups of 10–15 per dose and change in immunity was assessed by reference to a control group receiving the bacterial challenge only. After infection with *Esch. coli*, results were read at 48 hr. and expressed as mice dead/mice challenged. In mice infected with *Salm. typhimurium* results were expressed as the mean time to death, since complete protection seldom occurred.

ASSESSMENT OF CHANGE IN RETICULO-ENDOTHELIAL FUNCTION

Changes in the phagocytic activity and capacity of the R.E.S. were investigated (*a*) by the clearance of intravenously injected ^{32}P-LP from the blood stream, and (*b*) by histological procedures that are referred to in the text. The clearance tests were made by injecting ^{32}P-LP into tail veins of mice and obtaining blood at accurately timed intervals thereafter.

Radioactivity measurements were made on 0·1 ml. amounts of blood. This volume could not be taken repeatedly and at accurately timed intervals from the same mouse, therefore groups of three or four mice were killed at each interval and the mean count obtained. When the interval was short (1 or 2 min.) blood was obtained by instant decapitation over a paraffined watch glass. For longer intervals, blood was obtained directly from the heart. The blood samples were absorbed by filter paper discs stuck on nickel planchettes by shellac and dried by heating under an infrared lamp. They were counted under a thin mica end-window Geiger counter for such time that the probable error of counting did not exceed 5 per cent. Calculation of the total ^{32}P in the blood was made by taking 9 ml./100 g. as the blood volume in mice.

Washed whole organs were homogenized in a M.S.E. homogenizer and diluted with saline where necessary. 0·1 ml. amounts were taken for counting in the same manner as the blood samples. The total ^{32}P contained in the organ was calculated.

AUTORADIOGRAPHY

Mice were injected intravenously with 100 μg. ^{32}P-LP (= 200,000 counts/min.) and killed 2–6 hr. later. Blood was washed out of the liver with 5 ml. saline via the portal vein before removal. Both the liver and spleen were fixed in formol saline for 24 hr. and paraffin embedded sections made. Contact prints, made using Ilford X-ray film (Industrial G), were developed at intervals for assessing the activity of the sections. It was found that 6–8 days exposure was adequate to produce a clear image.

Autoradiographs were made by the stripping film technique using Kodak autoradiographic plates. The procedure used followed essentially that outlined by Pearse (1953). Deparaffinized sections were coated with the film, dried in a current of air at room temperature and exposed in the dark at 4° for 6–8 days. The developed autoradiographs were counter-stained with 0·1 per cent methylene blue for about 5 min. and differentiated in tap water.

HISTOLOGICAL TECHNIQUES DEMONSTRATING THE RETICULO-ENDOTHELIAL SYSTEM

(1) *Prussian Blue Method*

0·2 ml. colloidal iron saccharate (Ferrivenin-Benger) containing 5 mg. Fe was injected intravenously in mice. After 15 hr. the animals were killed and pieces of liver and spleen fixed in formol saline. Paraffin embedded sections were stained by Perl's method and counter-stained with basic fuchsin. The iron within the R.E. cells was demonstrated by the prussian blue reaction.

(2) *Thorotrast Method*

50 mg. Thorotrast was injected intravenously in mice, which were killed 15 hr. later. The liver and spleen were fixed in formol saline and embedded in paraffin wax. Sections were stained with haematoxylin and eosin, but it was found that the deposits of Thorotrast in phagocytes were most readily seen using dark-ground microscopy.

(3) *Weil-Davenport Method*

The metalophil cells of the liver and spleen are largely composed of R.E. cells, although some related non-phagocytic cells are included as well under this classification. The Weil-Davenport method of silver impregnation was adopted as modified by Marshall (1956) using formalin-fixed sections.

ASSESSMENT OF CHANGE IN SERUM PROPERDIN LEVEL

Properdin was assayed by the zymosan method of Pillemer, Blum, Lepow, Wurz and Todd (1956), but with certain simplifications of procedure. The assays were set up (Table 1) by adding barbital buffer, properdin-deficient serum (RP), test sample and zymosan suspension, in that order, to 10×75 mm. tubes held in an ice bath. Immediately after

TABLE 1

PROTOCOL OF PROPERDIN ASSAY

Tube No.	*Barbital buffer (ml.)*	*RP (ml.)*	*Test sample 1 : 10 (ml.)*	*Zymosan suspension (ml.)*	*Incubate 30–60 min. at 37°*	*Typical results (% haemolysis)*
1	—	0·1	0·1	0·1	Add 1·0 ml. sensitized	0
2	0·05	0·1	0·05	0·1	erythrocytes.	0
3	0·075	0·1	0·025	0·1	Incubate 30 min. 37°.	50
4	0·088	0·1	0·012	0·1	Centrifuge 5 min.	90
5	0·094	0·1	0·006	0·1	1800 r.p.m.	95

The test sample was pipetted with a 0·2 ml. pipette graduated in 0·002 ml. divisions. The zymosan suspension normally contained 8 mg./ml.

addition of zymosan, the tubes were shaken and incubated at 37° for 30–60 min.; the exact time of incubation and most suitable concentration of zymosan were determined beforehand with each batch of RP so as to obtain the steepest possible slope in the dose-response curve (Fig. 1). Then 1·0 ml. of a 1·25 per cent suspension of sheep erythrocytes senstitized with 4 units of haemolysin (Burroughs Wellcome) was added to each tube with an automatic pipette and incubation continued for a further 30 min. The tubes were centrifuged at 1800 r.p.m. for 5 min. and the percentage haemolysis in the supernatants estimated to within 10 per cent by visual comparison with a series of tubes containing known dilutions

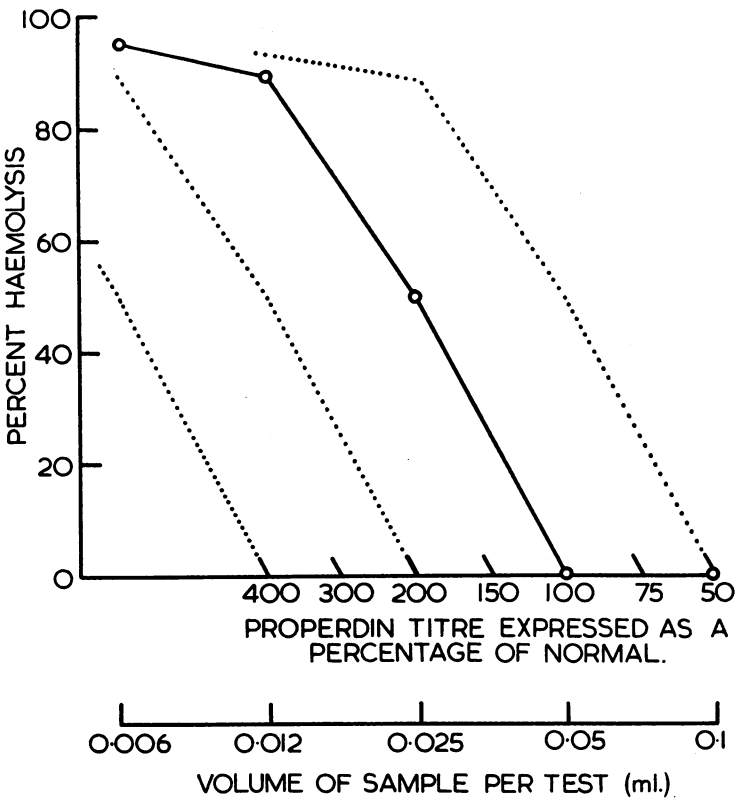

FIG. 1. Properdin assay curve of normal mouse serum (solid line) together with theoretical curves (dotted lines) used to express the titre of unknown sera as a percentage of normal.

of totally lysed erythrocytes. A graph was plotted of percent haemolysis against volume of test sample as shown in Fig. 1, which illustrates typical results obtained with pooled normal mouse serum. The properdin titres of sera from treated mice were expressed as a percentage of the normal serum titre obtained on the same occasion. This was done by fitting the haemolysis values obtained with the sera from the treated animals to the theoretical curves indicated by dotted lines in Fig. 1. In each series of assays, tubes consisting of (*a*) RP+barbital, (*b*) RP+zymosan and (*c*) RP+zymosan+purified human properdin were set up as controls.

RESULTS

CHANGES IN SERUM PROPERDIN LEVEL AND IN NON-SPECIFIC IMMUNITY FOLLOWING INTRAVENOUS ADMINISTRATION OF LIPOPOLYSACCHARIDES

The primary object here was to determine whether increased resistance to infection as a result of previous administration of lipopolysaccharide was necessarily associated with an increased serum properdin level at the time of challenge. For this purpose the lipopolysaccharides in saline were injected intravenously into groups of 15 mice, whilst one group of 15 mice was left, without injection, as normal controls. After 24 or 48 hours, 10 of the mice from each group were challenged intraperitoneally with *Esch. coli* in mucin and the remaining 5 animals anaesthetized with nembutal and bled by cardiac puncture. The pooled blood was held ½–2 hr. at 0° and the sera separated and stored at −20°. In some experiments properdin assays were performed on the sera after centrifugation at 35,000 g. for 2 hr. at 0–5° as recommended by Landy and Pillemer (1956a).

TABLE 2

EFFECT OF ADMINISTRATION OF ZYMOSAN OR LIPOPOLYSACCHARIDE 48 HR. PREVIOUSLY ON THE SERUM PROPERDIN LEVEL OF MICE AND ON THEIR SUSCEPTIBILITY TO ESCH. COLI INFECTION

Number of bacteria in Esch. coli *145 i.p. challenge*	*Material injected i.v. 48 hr. previously*	*Susceptibility to infection (mice dead/ mice challenged)*	*Serum properdin level at time of challenge (% of normal)*	
			uncentrifuged	*centrifuged*
4×10^4	*Esch. coli* 2380 LP 10 μg.	0/10	175	150
	Zymosan LE-1 200 μg.	7/10	125	75
	nil	10/10	100	75
2×10^3	*Sh. shigae* rough LP 10 μg.	1/10	75	100
	Zymosan LE-1 100 μg.	5/10	300	300
	nil	5/10	100	100
2×10^4	*Esch. coli* 2380 LP 25 μg.	0/10	150	150
	nil	9/10	100	100
2×10^4	*Past. pseudotuberculosis* LP 20 μg.	1/10	75	—
	nil	9/10	100	—

The results of four typical experiments given in Table 2 show that lipopolysaccharides from a variety of Gram-negative bacteria were active in doses of 10–25 μg. in stimulating non-specific resistance to infection. However, there was no correlation between these resistance changes and changes in the serum properdin level at time of challenge. In some cases a twofold increase in properdin level was observed, but here the immunity was no more pronounced than in cases where the properdin level was normal or subnormal. Furthermore, the yeast cell-wall preparation 'zymosan' was consistently effective in stimulating an increase in properdin while at the same time failing to increase significantly resistance to infection. Ultracentrifugation of the sera before assay produced usually a 25–50 per cent reduction in properdin titre of all samples without changing their relative values.

PROPERDIN LEVELS DURING INFECTION

A group of 120 mice was injected with 5×10^4 *Esch. coli* in mucin intraperitoneally and groups of 4 killed at intervals in order to measure the mean properdin level. The animals thus taken were representative of the condition of the majority. The dose of

bacteria used produced a 70 per cent mortality with all deaths occurring 7–13 hours after challenge. The properdin level fell to 10 per cent of normal within 1½ hours of challenge, and remained at this low level throughout the period when most mice died. However, apparently well animals taken 15 hours after challenge had properdin levels below 10 per cent of normal, whilst survivors taken 27 hours after challenge had properdin levels varying between this value and 50 per cent of normal. It seems, therefore, that the development of a low properdin level during infection does not inevitably lead to death.

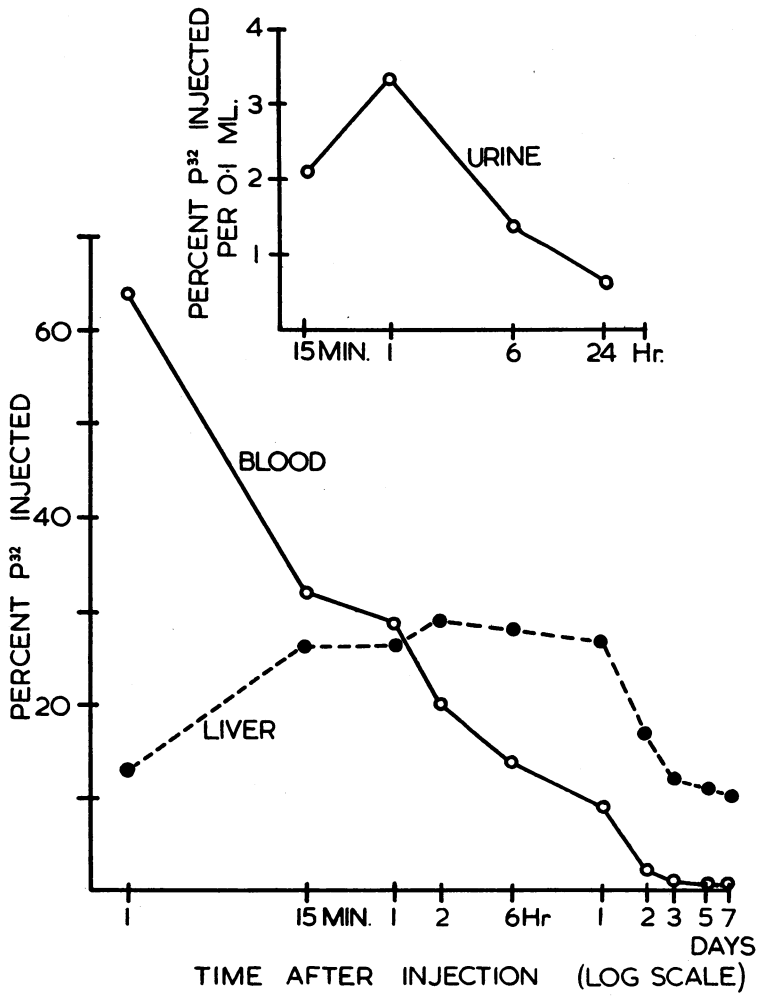

FIG. 2. Concentrations of ^{32}P in blood, liver and urine at various times after intravenous injection of ^{32}P-LP into mice.

THE FATE OF INTRAVENOUSLY-INJECTED LIPOPOLYSACCHARIDE

Removal from the Blood Stream

When ^{32}P-LP was injected intravenously into either mice or guinea pigs, a large proportion was rapidly removed from the circulation (Fig. 2). Generally, the blood level of

radioactivity fell to about 65 per cent in 1 min., to 20–40 per cent in 8–10 min. and to 10–20 per cent in 1 hr. Fourteen different batches of ^{32}P-LP prepared from *Esch. coli* (strains 145, 2206, 2380 and Co18) and *Salm. typhimurium* (strains M206 and C5) were examined. Twelve of these gave clearance rates that fell within the above ranges. Two preparations (*Esch. coli* 2380 batch 5, and *Salm. typhimurium* C5) were removed rather more slowly. With these, the blood level fell to 60 per cent in 8 minutes, to 35 per cent in 30 minutes, and to 25 per cent in 6 hours. The rate of removal of any one preparation did not vary appreciably in several experiments.

TABLE 3

DISTRIBUTION OF RADIOACTIVITY 30 MIN. AFTER INJECTING 100 μG. ^{32}P-LP I.V. INTO A GUINEA PIG

Tissue	*Counts per min. per 0·1 ml.*	*Approx. % ^{32}P-LP in tissues*
Blood	150	30
Urine	685	—
Liver	483	48
Spleen	213	2·5
Lung	110	2
Adrenals	64	0·2
Kidney	123	4
Heart	48	0·7
Brain	2	0

Accumulation in the R.E.S.

The fall in the blood level of ^{32}P-LP in mice was accompanied by a rise in the radioactivity of the liver and spleen which reached a maximum level after about 1 hr. (Fig. 2). As much as one half of the injected lipopolysaccharide appeared in these organs and small amounts were detected in the lungs, adrenals and kidneys. Table 3 shows the distribution of radioactivity in the various organs of the guinea pig. That the clearance from the

TABLE 4

THE EFFECT ON MICE OF PREVIOUS INTRAVENOUS INJECTION OF THOROTRAST ON THE REMOVAL OF ^{32}P-LP FROM THE BLOOD AND ITS ACCUMULATION IN THE LIVER

Previous treatment i.v.	*Counts per min. per 0·1 ml. blood 8 min. after ^{32}P-LP. 4 animals per group.*	*Calculated % ^{32}P-LP*	
		Blood	*Liver*
NIL	217 165 121 135 160 mean	16	65
50 mg. Thorotrast 30 min. previously	538 512 765 714 632 mean	61	21

Dose of ^{32}P-LP: 50 μg. (= 20,600 counts per min.).

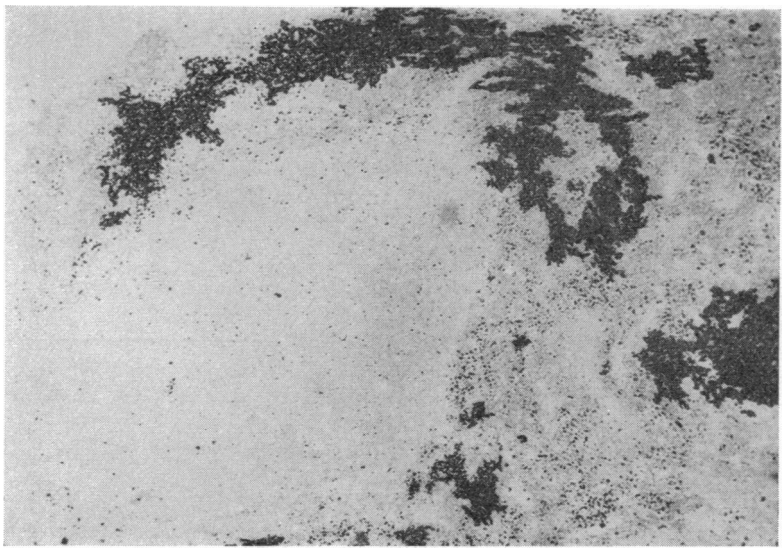

FIG. 3. Autoradiograph of mouse spleen. Section taken 4 hr. after the intravenous injection of 100 μg. ^{32}P-LP. Counterstained with methylene blue. (×192)

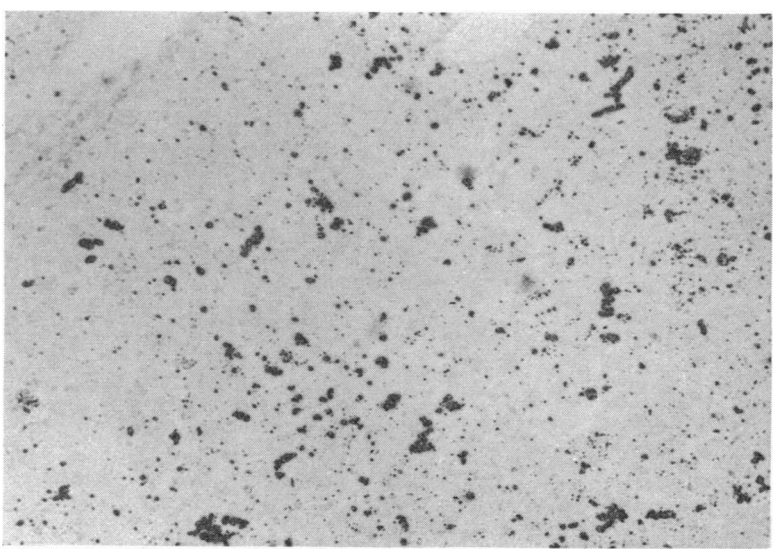

FIG. 4. Autoradiograph of mouse liver. Section taken 2 hr. after the intravenous injection of 100 μg. ^{32}P-LP. Counterstained with methylene blue. (×216)

circulation is due to phagocytosis by the R.E.S. is inferred by the results obtained after 'blockading' with a large intravenous dose of Thorotrast. When this was given 30 min. before the ^{32}P-LP, the rate of fall in blood level of the latter was greatly diminished (Table 4).

The localization of ^{32}P-LP within the R.E. cells was confirmed in mice by the technique of autoradiography. Sections of the spleen (Fig. 3) showed intense precipitation of silver over the pulp at the periphery of the Malpighian follicles, a region where R.E. cells are known to be densely accumulated. The absence of radioactivity from the follicles and the smaller amount in the remainder of the pulp is also in agreement with the distribution of the phagocytes. Sections of liver (Fig. 4) showed a distribution of silver which is closely related to that of the Kupffer cells.

Lipid A prepared from ^{32}P-LP was similarly phagocytosed by the R.E.S. It was removed rapidly from the circulation and a large amount accumulated in the liver. As with LP, the injection of 50 mg. Thorotrast 30 min. previously retarded the blood clearance of ^{32}P-Lipid A.

The Urinary Excretion of ^{32}P

A serum phosphatase has been described which will liberate ^{32}P in a small molecular diffusible form from ^{32}P-LP (Rowley, Howard and Jenkin, 1956). That this enzyme is active *in vivo* may be concluded from the fact that ^{32}P was present in the urine within 15 min. of the intravenous injection of ^{32}P-LP (Fig. 2). The urinary radioactivity was completely diffusible and the ^{32}P in the diffusate was precipitable with ammonium molybate, indicating that the phosphorus was present probably as inorganic phosphate.

An attempt was made to measure the total amount of ^{32}P excreted in the urine of a guinea pig by keeping the animal in a metabolism cage. The urine was collected daily for 3 days, and, although some of the ^{32}P was undoubtedly left in the bottom of the cage, 15·6 per cent of the injected radioactivity was recovered.

The Phase of Slow Elimination

That proportion, about one-third, of the originally-injected ^{32}P-LP which was not removed by the R.E.S. within 1 hr. was found to remain in the blood and to be gradually eliminated in the course of 6–24 hr. (Fig. 2). This gradual elimination from the blood was not accompanied by increased radioactivity in the liver (Fig. 2). When the blood was separated into plasma and cells, almost all the radioactivity was in the plasma. Even in a leukocyte-plasma mixture obtained by treating heparinized blood with 2 per cent dextran, only 0·34 per cent of the total radioactivity was contained in the white cells. This indicated that very little of the ^{32}P-LP had been taken up by the circulating polymorphs.

The majority of the ^{32}P in plasma was probably still present within LP molecules. Serum was obtained from a guinea pig 30 min. after the injection of 250 μg. ^{32}P-LP and the protein and lipopolysaccharide precipitated with 5 volumes of alcohol. By phenol-water extraction of this precipitate, about 3 μg. ^{32}P-LP was recovered.

This material, when injected back into mice, was removed from the blood in the usual way. The radioactive lipopolysaccharide removed by the R.E. cells appeared to be firmly held by them. Thus, only on the second day did the radioactivity of the liver begin to decrease, and at the end of one week about one-third of the amount originally taken up was still present.

The phase of slow elimination could not be attributed to a falling off in R.E. activity,

for 10 μg. amounts of ^{32}P-LP injected three times at 10 min. intervals were all cleared similarly. Secondly, 300 μg. ^{32}P-LP was incubated in 1·5 ml. fresh mouse serum at 37° for 1 hr. and then dialysed against 100 ml. saline with shaking for 2 hr. This serum-treated ^{32}P-LP did not differ from the untreated material when their removal from the circulation was compared.

It was considered at this stage that the slow removal of a component was due to heterogeneity of particle size in ^{32}P-LP preparations. Dobson (1957) found that his colloidal $Cr^{32}PO_4$ contained a fraction of much smaller particle size which was removed more slowly than the remainder by the R.E.S. Two experiments have supported this explanation. Ten minutes after the injection of 150 μg. ^{32}P-LP in mice, blood was removed, the serum separated and dialysed against 100 ml. saline for 3 hr. When 0·1 ml. of this serum (containing *c.* 2 μg. ^{32}P-LP) was injected back into mice, the fall in blood radioactivity was far slower than with the original ^{32}P-LP. Thus, 10 min. after injection, the blood level was 94 per cent as compared with 20 per cent of the original ^{32}P-LP. Secondly, the deposit and supernatant components of ^{32}P-LP were compared after ultracentrifugation at 78,500 g. for 2 hr. The blood clearance of the supernatant was found to be slower than the deposited material, although the difference was less than in the foregoing experiment.

CHANGES IN THE FUNCTIONAL ACTIVITY OF THE R.E.S. FOLLOWING INTRAVENOUS ADMINISTRATION OF LIPOPOLYSACCHARIDES

The effect of previous administration of non-radioactive lipopolysaccharide on the blood clearance rate of ^{32}P-LP in mice is shown in Table 5. It is apparent that when the time interval between the two intravenous injections was 30 min. the blood clearance

TABLE 5

BLOOD CLEARANCE RATES OF ^{32}P-LP IN NORMAL MICE AND IN MICE INJECTED AT VARIOUS TIMES PREVIOUSLY WITH NON-RADIOACTIVE LP

Time between injections	*Dose and batch of ^{32}P-LP*	*Non-radioactive LP (per mouse)*	*Calculated % ^{32}P-LP in blood after 8 min.*
30 min.	5 μg. Batch 3	2 mg. *Past. pestis* LP	74
		100 μg. *Esch. coli* LP	56
		Nil	37
48 hr.	50 μg. Batch 4	100 μg. *Past. pestis* LP	16
		10 μg. „ „ „	15
		Nil	33
	40 μg. Batch 3	10 μg. *Esch. coli* LP	25
		1 μg. „ „ „	26
		Nil	42
8 and 9 days	50 μg. Batch 5	25+50 μg. *Past. pseudotuberculosis* LP	26
		Nil	58

rate was *lower* in LP-treated animals than in normal controls. The minimal dose of LP required to produce a measurable depression fell within the range 10–25 μg. Conversely, if a 48 hr. interval had elapsed, the blood clearance rate was *higher* in those animals that had received 1–100 μg. of LP than in normal controls. These biphasic changes in

the phagocytic activity of the R.E.S. parallel the decreased and increased resistance to infection observed following injection of LP. The duration of R.E. hyperactivity was not studied in detail, but enhanced rates of clearance were still present 8 days after administration of some LP preparations but not of others.

Animals that had received LP 48 hr. previously, in addition to showing an accelerated rate of clearance, also were found to show an increased R.E. capacity. This was investigated by measuring the blood clearance rate of a standard (40 μg.) amount of ^{32}P-LP mixed with various amounts, from 100 to 2000 μg. of non-radioactive LP. Mice treated with LP 48 hr. previously removed a dose of 540 μg. lipopolysaccharide at approximately the same rate as normal animals eliminated 140 μg., i.e. their R.E.S. was about four times as active as normal. (Table 6.)

TABLE 6

BLOOD CLEARANCE RATES OF GRADED DOSES OF ^{32}P-LP IN NORMAL MICE AND IN MICE INJECTED 48 HR. PREVIOUSLY WITH 10 μG. NON-RADIOACTIVE LP

Amount of Esch. coli *LP added to standard dose (40 μg.) of ^{32}P-LP* (μg.)	*Calculated % ^{32}P-LP in blood after 8 min.*	
	Normal mice	*LP-treated mice*
0	31	21
100	38	26
500	59	35
2000	66	51

More directly, the enhanced capacity of the R.E.S. after LP treatment was shown by measuring the amount of LP appearing in the liver. Table 7 shows that 6 hr. after giving ^{32}P-LP, nearly three times as much of it is in the liver of stimulated animals as in the livers of normal controls. The blood clearance rate had increased concurrently. This table also shows the result of an attempt to produce maximal stimulation of the R.E.S. by the repeated injection of increasing quantities of endotoxin. The results obtained with six injections do not differ, however, from those obtained with only two doses of another preparation shown in the same table.

TABLE 7

THE INCREASED CAPACITY OF THE R.E.S. FOR ^{32}P-LP FOLLOWING REPEATED INJECTIONS OF LP OR ENDOTOXIN

Previous treatment (i.v.)	*Calculated % ^{32}P-LP*			
	Liver		*Blood*	
	8 min.	*6 hr.*	*8 min.*	*6 hr.*
20 and 50 μg. *Esch. coli* LP 9 and 7 days previously	45	60	39	11
25, 75, 100, 125, 150 and 200 μg. *Sh. shigae* endotoxin 8, 7, 6, 5, 3 and 2 days previously	39	63	42	4
Nil	19	22	62	24

Dose of ^{32}P-LP (Batch 5): 30 μg. (= 162,000 counts per min.).

HISTOLOGICAL STUDIES ON LP-STIMULATION OF THE R.E.S.

Many observers have noted that after blockading the R.E.S. with metallic oxides and dyes, however intensively, within a few hours phagocytic activity slowly returned to normal and then exceeded it. The phase of hyperactivity has been attributed variously to 'proliferation', 'regeneration' or 'activation' of the R.E.S. (see Jaffé, 1931, 1938). In view of the previously observed cellular basis for this hyperphagocytosis, histological studies have been made of the R.E.S. in LP-treated animals.

Sections of the liver taken from normal mice 15 hr. after the intravenous injection of 5 mg. Ferrivenin showed a characteristic distribution of the phagocytosed iron. Most of the iron was contained within R.E. cells sited at the periphery of the liver lobules, whilst relatively fewer of the centrilobular phagocytes contained any (Fig. 5). When the same amount of Ferrivenin was given to mice which had received 25 μg. *Past. pseudotuberculosis* LP 48 hr. earlier, this typical pattern was found to have disappeared. Not only were a larger number of active phagocytes present, but they were obviously distributed more or less randomly throughout the lobules (Fig. 6). The same results were obtained when 50 mg. Thorotrast was used as a marker material for the R.E. cells instead of Ferrivenin. The livers of normal mice killed 15 hr. after the Thorotrast again showed a perilobular distribution of active phagocytes (Fig. 7). In the lipopolysaccharide-treated animals, not only was there a more diffuse distribution of Thorotrast but the individual phagocytes appeared to contain larger aggregates of it (Fig. 8).

The spleen in normal mice showed dense phagocytosis of Ferrivenin by cells at the periphery of the Malpighian follicles, and further scattered R.E. cells throughout the pulp. The follicles themselves were clearly devoid of phagocytes. In LP-stimulated animals the changes observed in the spleen were less pronounced than in the liver. The Malpighian follicles again showed a complete absence of phagocytic cells, but there was a definite increase in the number of iron-containing cells distributed in the pulp.

COMPARISON OF LIPOPOLYSACCHARIDE WITH OTHER COLLOIDAL MATERIALS THAT ARE TAKEN UP BY THE R.E.S.

Effect of Administration of Thorotrast

Colloidal thorium dioxide (Thorotrast) has been used extensively in recent years for experimental 'blockade' of the R.E.S. and we have found it satisfactory for depressing the blood clearance rate of ^{32}P-LP in mice (Table 4). Moreover, 5 or 50 mg. Thorotrast administered intravenously to mice 30 min. before intraperitoneal challenge with *Esch. coli* markedly lowered their resistance to this infection. It is of interest that a pronounced reduction in serum properdin level also occurred 30 min. after the intravenous injection of Thorotrast (10 mg.) into mice. In these respects Thorotrast resembles bacterial lipopolysaccharides.

Since blockade induced by lipopolysaccharide was followed after 48 hr. by increased immunity and R.E. activity, attempts were next made to see if similar increases would be induced by Thorotrast after its initial blockading effect. Table 8 shows that increased resistance to *Esch. coli* infection and increased blood clearance rate of ^{32}P-LP were observed 48 hr. after either a single dose of 25 mg. of Thorotrast, or after 1, 2 and 4 mg. administered 6, 4 and 2 days previously. Slight protection against *Esch. coli* infection could also be produced with one dose of 5 mg. Thorotrast, but not less. No resistance was obtained

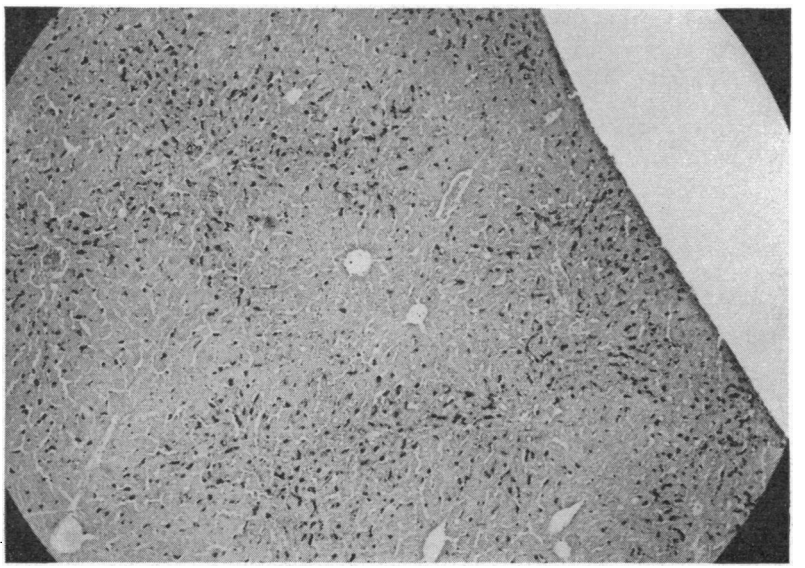

FIG. 5. Liver of mouse 15 hr. after i.v. injection of 5 mg. Ferrivenin. Perl's method. (×100)

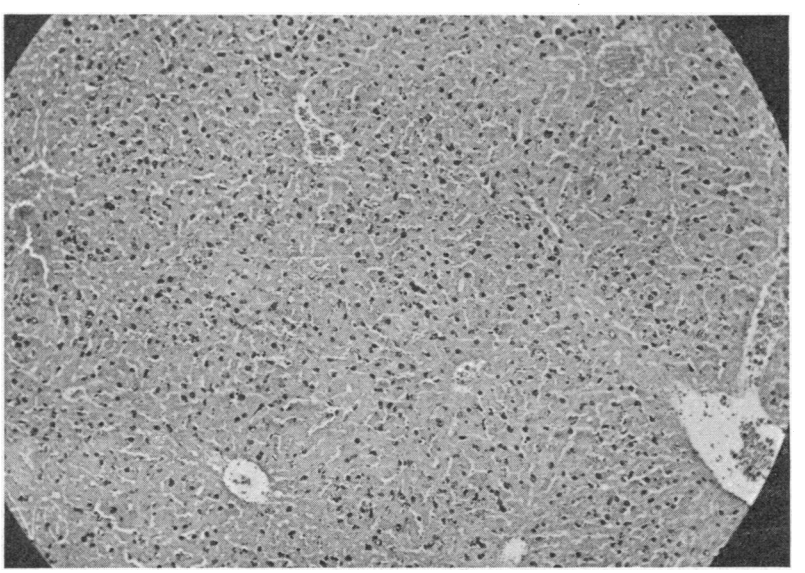

FIG. 6. As Fig. 5, but 25 μg. *P. pseudotuberculosis* LP given i.v. 48 hr. previously. (×100)

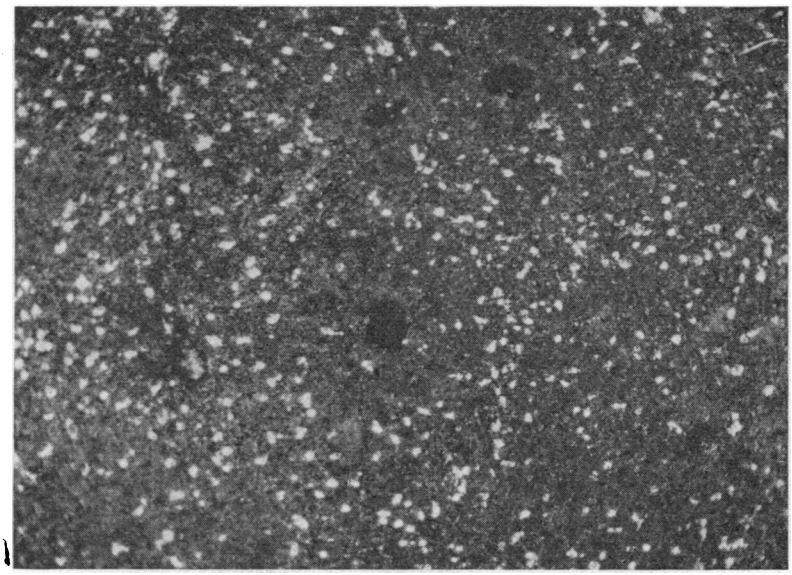

FIG. 7. Liver of mouse 15 hr. after i.v. injection of 50 mg. Thorotrast. Dark-ground illumination. (×140)

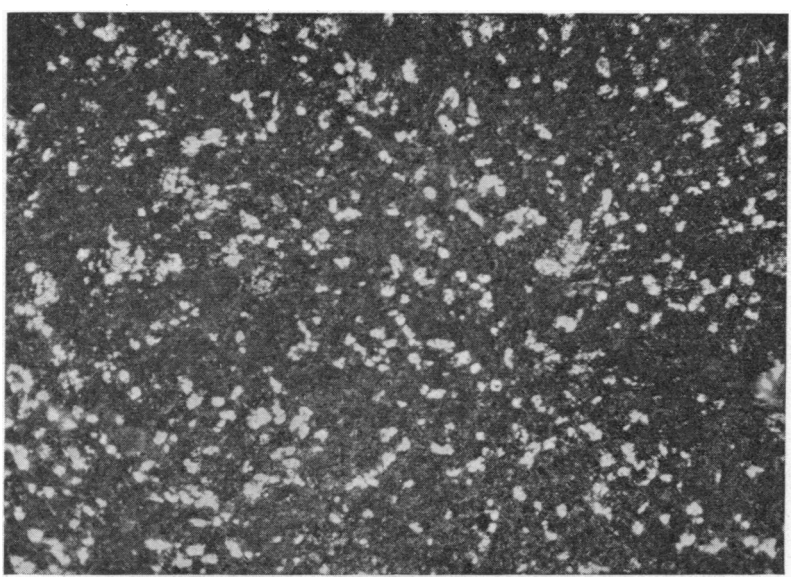

FIG. 8. As Fig. 7, but 25 μg. *P. pseudotuberculosis* LP given i.v. 48 hr. previously. (×140)

when the interval between the Thorotrast and challenge was reduced to either 6 or 24 hr. Histological evidence of later activation of the R.E.S. by a blockading dose of Thorotrast was also obtained. A comparison of Figs. 9 and 10 shows that 48 hr. after 10 mg. Thorotrast there was an increase in the number of argyrophilic cells in the splenic pulp.

TABLE 8

CHANGES IN R.E. FUNCTION AND IN NON-SPECIFIC IMMUNITY FOLLOWING INTRAVENOUS ADMINISTRATION OF THOROTRAST AND LIPID A TO MICE

Treatment (i.v.)			*R.E. Function*	*Non-specific Immunity*		
Material	*Dose*	*Time previously*	*Calculated per cent ^{32}P-LP in blood*	Esch. coli *in mucin:* 10^4	10^5	Salm. typhimurium
			(after 60 min.)			(10^3 organisms) Expt. A Expt. B
Thorotrast	25 mg.	48 hr.		5/30*		4·5†
	1, 2 and 4 mg.	6, 4 and 2 days	4	5/20*		5·5†
	Nil		10	38/50*		8·5† 7·5†
			(after 2 min.)			(10^2 organisms) Expt. A Expt. B
Lipid A	60 μg.	48 hr.				19†
	50 μg.	48 hr.		1/10*		
	50 μg.	24 hr.	36			14†
	10 μg.	48 hr.			3/10*	
	Nil		68	6/10*	8/10*	8·5† 7·5†

* Mice dead / Mice challenged

† Mean time to death (days).

It will be observed in Table 8 that groups of animals from the same batch of Thorotrast-treated mice that showed an increased immunity to *Esch. coli* and increased R.E. function showed a *decreased* immunity to *Salm. typhimurium* infection, i.e. the median survival time following infection with this mouse pathogen was shortened by Thorotrast treatment. The possibility was envisaged that after a few days the R.E.S. had become again depressed by the physical presence of Thorotrast, thus allowing more rapid multiplication of phagocytosed bacteria within the cells. However, even 8 days after a total of 100 mg. Thorotrast, the phagocytic activity of the R.E.S. was found to be enhanced in a ^{32}P-LP clearance test. Another possibility was that the mice were not dying from *Salm. typhimurium* infection, but from some side effect of the Thorotrast. Following an intraperitoneal challenge of 1000 organisms, however, animals which had received 25 mg. Thorotrast 2 days previously showed blood counts of *Salm. typhimurium* 50–100 times higher than the normal animals (Fig. 11). The total bacterial populations in the liver and spleen were also found to be persistently higher in the Thorotrast-treated group.

Effect of Administration of Lipid A

When similar experiments were made with lipid A, certain differences between the effects of this material and of Thorotrast were observed (Table 8). As already reported elsewhere (Howard *et al.*, 1957), increased resistance to both *Esch. coli* and *Salm. typhimurium* infections followed the injection of lipid A 24–28 hr. previously. Another point is that the lipid gave protection with doses as low as 10 μg. per mouse, whereas Thorotrast was effective (in *Esch. coli* infection) only with doses well above 1 mg.

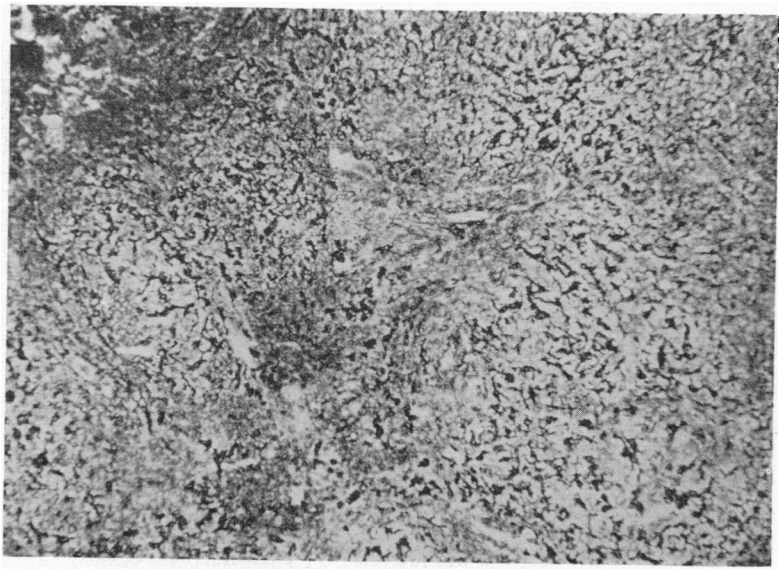

FIG. 9. Spleen of mouse. Modified Weil-Davenport method. (×200)

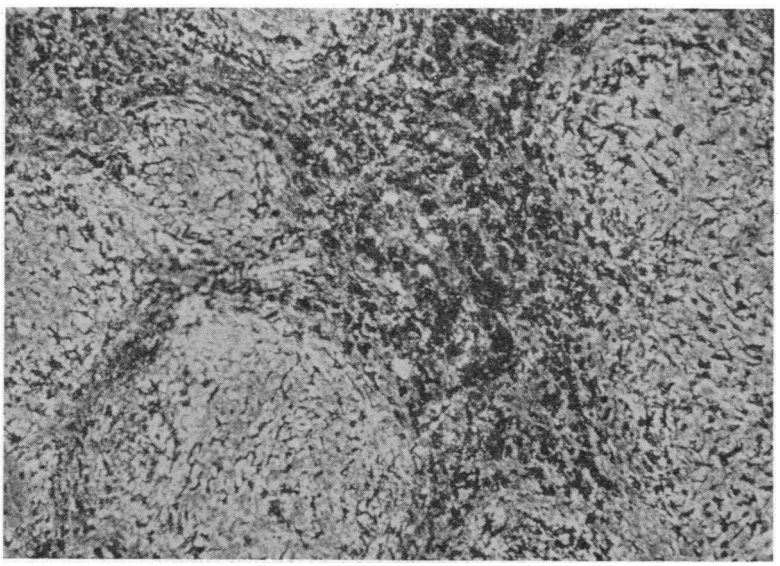

FIG. 10. As Fig. 9 but 10 mg. Thorotrast given i.v. 48 hr. previously. (×200)

The rate of clearance of ^{32}P-LP was increased by the injection of 50 μg. lipid A 24 hr. previously. Histological confirmation of activation of the R.E.S. was also obtained by following the distribution of Ferrivenin. When mice were given 25–50 μg. lipid A 24–48 hr. previously, their livers showed an increased number of active phagocytes. These were scattered diffusely throughout the entire lobule, whilst the phagocytes in normal livers showed the characteristic perilobular distribution previously described.

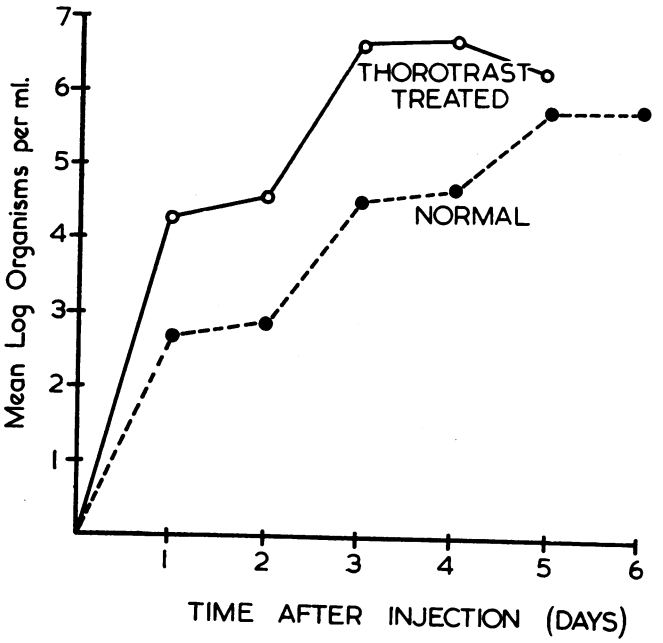

FIG. 11. Concentration of viable bacteria in the blood of normal and Thorotrast-stimulated mice at various times after intraperitoneal infection with *Salm. typhimurium*.

Lipid A resembles lipopolysaccharide, therefore, in stimulating both non-specific immunity and the reticulo-endothelial system when injected in microgram amounts. Although it is not as effective as the parent LP in terms of minimal effective dose it is far superior to other colloids so far examined. Further, the resistance it induces, unlike that following Thorotrast, extends to *Salm. typhimurium* as well as *Esch. coli* infections.

DISCUSSION

The hypothesis that lipopolysaccharides stimulate non-specific immunity by increasing the serum properdin level originated in observations on the biphasic changes in properdin level produced by injection of zymosan (Pillemer and Ross, 1955) and the apparently parallel changes in immunity elicited by bacterial cell walls (Rowley, 1955). Later and more detailed investigations suggest, however, that although changes in properdin level and in non-specific immunity may parallel each other after the injection of certain materials, the properdin change may not be the cause of the change in immunity. Our own data and that of Landy (1956) and Landy and Pillemer (1956a,b) indicate that whereas most, if not all, lipopolysaccharides are capable of stimulating a non-specific increase in resistance to infection, only some preparations regularly elicit a concurrent elevation in

serum properdin level. Our data are also in agreement with the finding that zymosan given intravenously is an excellent agent for stimulating an increase in serum properdin level (Pillemer and Ross, 1955), although it stimulates immunity poorly, and inconsistently (Kiser, Lindh and de Mello, 1956).

The hypothesis has been advanced (Landy, 1956; Landy and Pillemer, 1956b) that resistance to infection is determined rather by the ability of animals to maintain a normal properdin level during the course of the infection than by the properdin level at the time of challenge. Thus, these authors observed a progressive fall in the serum properdin level of sick animals up to the time of death, whereas surviving animals maintained their properdin at levels close to normal. Quite apart from the difficulty of distinguishing cause from effect here, our experience has been that a low properdin level during infection does not necessarily lead to death. For example, we have found that mice surviving an infection with 5×10^4 *Esch. coli* intraperitoneally have had properdin levels which were only 10 per cent of normal, even 27 hr. after challenge.

However, even if one accepts that in certain instances there is a correlation between the degree of immunity and the serum properdin level, there still remain several theoretical difficulties. Firstly, the mouse is peculiar among laboratory animals in having serum that appears to be devoid of bactericidal activity (Marcus, Esplin and Donaldson, 1954; Muschel and Muto, 1956; personal observations). Secondly, lipopolysaccharides produce an increase in non-specific resistance to organisms such as *Mycobacterium tuberculosis* (Dubos and Schaedler, 1956), *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Condie, Zak and Good, 1955) that are not killed even by the highly bactericidal serum of man. Finally, unequivocal evidence that resistance to experimental infection may be raised by the parenteral administration of purified properdin has never been published. It is thus difficult to accept that immunity changes in mice are related to changes in serum properdin level unless one postulates for properdin a rôle other than as a participant in a humoral bactericidal mechanism. A possibility remains that properdin in the mouse may have some other property such as that of an opsonin. In this connection, it is perhaps significant that the intravenous injection of Thorotrast into mice is accompanied by marked reduction in serum properdin level concurrently with removal of the colloid by the R.E.S.

The observation that intravenously injected lipopolysaccharides become localized in the R.E.S. and, moreover, produce a biphasic change in R.E. phagocytic activity suggested that the parallel changes in immunity could be explained in terms of this cellular defence mechanism. We have taken as our index of R.E. activity the rate of clearance of intravenously injected radioactive lipopolysaccharide from the blood stream. Activation of the R.E.S. by the injection of LP was produced far more regularly than elevation of the properdin level and furthermore was closely correlated with the degree of resistance to infection. Other investigators who used colloidal radioactive gold (Cornwell and Good, 1953) and colloidal carbon (Biozzi, Benacerraf and Halpern, 1955) have also observed alterations in R.E. activity as a result of previous injections of endotoxin. Indeed, the notion of R.E. 'blockade' followed by hyperactivity is almost as old as Aschoff's original description of this system. In 1923, Siegmund discussed the stimulating effect of the injection of such dyes as carmine, and metallic suspensions. Further, this 'irritation-therapy' was found to be correlated with an increased immunity to pneumococcal infections in mice. More recently, Kiser, Lindh and de Mello (1956) have produced enhanced resistance to *Klebsiella pneumoniae* infection in mice by previous intravenous injection of colloidal sulphur.

The predominantly perilobular site of cells which phagocytose Thorotrast and Ferrivenin in the liver of normal animals as compared with the diffuse distribution in LP-treated animals merits some attention, for Kupffer cells are known to be present throughout the entire lobule. Lison and Smulders (1948) and Smulders (1951) found, however, that R.E. cells existed in the liver in two forms. Type G consists of amoeboid macrophages found only in the perilobular region and ingesting particles with a diameter greater than 80Å. Type F is the flattened histiocyte distributed throughout the liver sinusoids and normally only taking up material with a particle size less than 80Å. Thorotrast and Ferrivenin would therefore be expected to appear in cells of type G only. Smulders (1951) also found that on irritation by large doses of dyes, or after the injection of choline and histamine, the inactive stellate cells in the centrilobular region changed into the amoeboid type and would then take up larger colloidal materials. It seems likely, therefore, that injection of LP also activates the R.E.S. in the same manner. This response is clearly obtained following various stimuli, for Gabrieli, Goulian and Cutler (1955) found that after infection or whole body irradiation in rats, the uptake of chromic phosphate by R.E. cells in the liver was likewise diffuse rather than of perilobular distribution.

One difference, to be emphasized, between lipopolysaccharides and such materials as Thorotrast and colloidal sulphur is their relative potencies. Whereas lipopolysaccharides are effective in stimulating immunity at dosages sometimes as low as 0·1 μg. per mouse, Thorotrast, colloidal sulphur and native dextran have to be administered in 1000-fold higher amounts to produce comparable effects. The peculiarly high activity of lipopolysaccharides in stimulating immunity is due, we believe, to their content of the lipid A component (Howard *et al.*, 1957). The fact that the isolated lipid A is active at doses of 10 μg. per mouse in both stimulating immunity and in increasing the activity of the R.E.S. suggests that, like the parent lipopolysaccharide, it possesses some special attribute apart from its colloidal nature. Since R.E. cells are known to take up preferentially colloids of particle size within a certain limited range (Jancso, 1955) it is possible that optimal potency in stimulating immunity depends both on a certain particle size and on the possession of lipid A or similar material distributed throughout the macromolecule.

Although stimulation of the R.E.S. by Thorotrast increases resistance to *Esch. coli* infection, it simultaneously enhances susceptibility to *Salm. typhimurium*. As this mouse pathogen can successfully parasitize the R.E. cells of the animal, hyperphagocytosis alone may, in fact, be to the detriment of the host. Most studies on the interrelationship between bacteria and R.E. cells have been concerned with the first stage-phagocytosis. Little work has been done on the second stage-digestion of the organism; and in fact the nature of the bactericidal mechanism(s) within fixed macrophages is largely unknown. An attractive possibility to be considered on the present data is whether LP or lipid A within R.E. cells stimulate not only their phagocytic activity but their bactericidal activity as well. On the other hand, inert colloids such as Thorotrast may stimulate solely phagocytosis. Thus, if an organism is normally killed by the R.E. cells (e.g. *Esch. coli*) hyperphagocytosis alone may be enough to produce a measure of immunity. Alternatively an R.E. parasite such as *Salm. typhimurium* would require more effective intracellular bactericidal mechanisms for an increased resistance to infection to become apparent. This theory is clearly difficult of proof, but merits further investigation.

The present research supports the participation of the R.E.S. in non-specific immunity, although the evidence cannot yet be considered conclusive. Further work may bring to light some more intimate connection between these cells and the properdin system.

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REFERENCES

Abernathy, R. S. and Spink, W. W. (1956). 'Protection against Brucella with heterologous endotoxins.' *J. clin. Invest.*, **35**, 687.

Biozzi, G., Benacerraf, B. and Halpern, B. N. (1955). 'The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo-endothelial system in mice.' *Brit. J. exp. Path.*, **36**, 226–35.

Condie, R. M., Zak, S. J. and Good, R. A. (1955). 'Effect of meningococcal endotoxin on resistance to bacterial infection and the immune response of rabbits.' *Fed. Proc.*, **14**, 459–60.

Cornwell, S. and Good, R. A. (1953). 'Studies of reticulo-endothelial function with colloidal gold.' *Anat. Rec.*, **115**, 295.

Dobson, E. L. (1957) in 'Physiopathology of the Reticulo-endothelial System,' p. 80. Blackwells, Oxford.

Dubos, R. J. and Schaedler, R. W. (1956). 'Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins.' *J. exp. Med.*, **104**, 53–65.

Field, T. E., Howard, J. G. and Whitby, J. L. (1955). 'Studies on the rapid production of a non-specific type of immunity to *Salmonella typhi* infection in mice.' *J.R. Army med. Cps.*, **101**, 324–34.

Gabrieli, E. R., Goulian, D. and Cutler, J. L. (1955). 'Hepatic radioautography following intravenous injection of radioactive chromium phosphate as a further contribution to reticulo-endothelial functional tests.' *Yale J. Biol. Med.*, **28**, 63–71.

Howard, J. G., Rowley, D. and Wardlaw, A. C. (1957). 'Stimulation of non-specific immunity by the "lipid A" component of bacterial lipopolysaccharide.' *Nature, Lond.*, **179**, 314–15.

Isliker, H. (1957). *Vox Sanguinis* (in press).

Jaffé, R. H. (1931). 'The reticulo-endothelial system in immunity.' *Physiol. Rev.*, **11**, 277–327.

Jaffé, R. H. (1938). In H. Downey's *Handbook of Haematology*, Vol. 2, p. 973. Hoeber, New York.

Jancso, N. (1955). '*Speicherung. Stoffanreichung in Reticuloendothel und in der Niere.*' Akademiai Kiado, Budapest. Abridged English translation in 'RES Abstracts', Pfizer, New Jersey.

Kiser, J. S., Lindh, H. and De Mello, G. C. (1956). 'The effect of various substances on resistance to experimental infection.' *Ann. N.Y. Acad. Sci.*, **66**, 312–27.

Landy, M. (1956). 'Increase in resistance following administration of bacterial lipopolysaccharides.' *Ann. N.Y. Acad. Sci.*, **66**, 292–303.

Landy, M. and Pillemer, L. (1956a). 'Elevation of properdin levels in mice following administration of bacterial lipopolysaccharides.' *J. exp. Med.*, **103**, 823–33.

Landy, M. and Pillemer, L. (1956b). 'Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides.' *J. exp. Med.*, **104**, 383–409.

Lison, L. and Smulders, J. (1948). 'Discriminating "athrocytes" in the reticulo-endothelial system.' *Nature, Lond.*, **162**, 65–6.

Marcus, S., Esplin, D. W. and Donaldson, D. M. (1954). 'Lack of bactericidal effect of mouse serum on a number of common microorganisms.' *Science*, **119**, 877.

Marshall, A. H. E. (1956). '*The Cytology and Pathology of the Reticular Tissue*', p. 258. Oliver and Boyd, Edinburgh.

Muschel, L. H. and Muto, T. (1956). 'Bactericidal reaction of mouse serum.' *Science*, **123**, 62–3.

Pearse, A. G. E. (1953). '*Histochemistry*', p. 488. Churchill, London.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W. and Wardlaw, A. C. (1954). 'The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena.' *Science*, **120**, 279–85.

Pillemer, L., Blum, L., Lepow, I. H., Wurz, L. and Todd, E. W. (1956). 'The properdin system and immunity III. The zymosan assay of properdin.' *J. exp. Med.*, **103**, 1–13.

Pillemer, L. and Ross, O. A. (1955). 'Alterations in serum properdin levels following injection of zymosan.' *Science*, **121**, 732–3.

Pillemer, L., Schoenberg, M. D., Blum, L. and Wurz, L. (1955). 'Properdin system and immunity. II. Interaction of the properdin system with polysaccharides.' *Science*, **122**, 545–9.

Rowley, D. (1955). 'Stimulation of natural immunity to *Escherichia coli* infections. Observations on mice.' *Lancet*, *i*, 232–4.

Rowley, D. (1956). 'Rapidly induced changes in the level of non-specific immunity in laboratory animals.' *Brit. J. exp. Path.*, **37**, 223–34.

Rowley, D., Howard, J. G. and Jenkin, C. R. (1956). 'The fate of ^{32}P-labelled bacterial lipopolysaccharide in laboratory animals.' *Lancet*, *i*, 366–7.

Siegmund, H. (1923). 'Reizkorpertherapie und aktives

REFERENCES (*continued*)

mesenchymatisches Gewebe.' *Münch. med. Wschr.*, **70**, 5–6.

SMULDERS, J. (1951). 'Recherches sur l'athrocytose discriminante dans le système reticulo-endothelial. Etude qualitative et quantitative.' *Arch. Biol. (Liège)*, **62**, 133–93.

WARDLAW, A. C. and PILLEMER, L. (1956). 'The properdin system and immunity V. The bactericidal activity of the properdin system.' *J. exp. Med.*, **103**, 553–75.

WESTPHAL, O. and LÜDERITZ, O. (1954). 'Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien.' *Angew. Chem.*, **66**, 407–17.

WESTPHAL, O., LÜDERITZ, O. and BISTER, F. (1952). 'Uber die Extraktion von Bakterien mit Phenol/Wasser.' *Z. Naturf.*, **73**, 148–55.