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Repeated exposure to bacterial lipopolysaccharide interferes with disposal of pathogenic immune complexes in mice

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SUMMARY

Patients with systemic lupus erythematosus (SLE) experience clinical flares in association with superimposed bacterial infection. To investigate whether heightened immune phenomena during the course of bacterial infections were related to abnormal disposal of immune complexes, we administered bacterial lipopolysaccharide (LPS) to C57BL/6 mice for 5 weeks. Control mice received vehicle only. We then challenged the mice with a subsaturating dose of radiolabelled immune complexes intravenously and determined the localization of immune complexes in liver, spleen and kidney. In comparison to control mice, mice exposed to LPS developed features of polyclonal B cell activation, autoimmune phenomena, delayed removal of immune complexes from the circulation, diminished liver uptake of immune complexes, and enhanced localization of immune complexes in the kidneys. The findings could not be attributed to biological processes dependent on complement concentration. Instead, interferences with Fc receptor function, or with endocytosis of immune complexes may represent likely possibilities. Thus, clinical flares in patients with SLE, in the presence of a superimposed infection, may result from enhanced localization of immune complexes in organs due to altered mechanisms of their disposal.

Keywords lipopolysaccharide lupus nephritis model systemic lupus erythematosus immune complex disease antigen–antibody complex

INTRODUCTION

Systemic lupus erythematosus (SLE) manifests itself with features of polyclonal B cell activation (PBA) (Teodorescu, 1983) which result in autoantibodies to a variety of antigens, and multi-systemic involvement presumed to be related to localization of immune complexes in tissues. Since patients with SLE may experience clinical flares in association with infections (Miescher, Paronetto & Lambert, 1976), an impairment in removal of immune complexes from the circulation, if not preexistent, could conceivably develop.

To investigate the pathogenesis of heightened immune phenomena during the course of infections, we studied the removal and organ localization of radiolabelled immune complexes in a murine model that develops PBA and autoantibodies when exposed to repeated injections of bacterial lipopolysaccharide (LPS) (Izui *et al.*, 1977; Cavallo *et al.*, 1983; Cavallo, Goldman & Lambert, 1984). The choice of *Salmonella* LPS to induce PBA is predicated on various considerations: (i) LPS is commonly encountered in the gut; thus, it may represent a common stimulus (Severinson *et al.*, 1982); (ii) LPS is a typical constituent of the outer membrane of various Gram-negative bacteria (Nurminen *et al.*, 1983); this fact suggests that LPS from organisms other than *Salmonella* could also contribute to PBA and autoimmunity. Indeed, C57BL/6 mice exposed to whole *Escherichia coli* develop autoimmune disease (Fournié *et al.*, 1980); (iii) *Salmonella* bacteraemia is prevalent in patients with SLE (Abramson *et al.*, 1985); and (iv) although B cells derived from SLE patients respond poorly to various exogenous stimuli *in vitro*, it is conceivable that challenged B cells were already activated, and that such cells, as opposed to resting B cells, are poorly responsive (Suzuki & Sakane, 1989). Thus, resting human B cells can be triggered to produce abnormally high numbers of antibody-secreting cells when exposed to *Staphylococcus aureus* (Suzuki & Sakane, 1989) and may also be responsive to LPS.

We show that in mice with features of PBA, the time required for removal of pathogenic immune complexes from the circulation is prolonged, that the increase in removal time may involve mechanisms in addition to decreased uptake by the liver, and that prolonged circulation of such immune complexes enhances their localization in the kidney to result in renal insufficiency.

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MATERIALS AND METHODS

Animals

Mice (C57BL/6) were purchased from Jackson Laboratory (Bar Harbor, ME) and rabbits were purchased from a local supplier. Animals were housed in accordance with federal guidelines, with free access to food and water.

Experimental protocol

At 8 weeks of age, 44 mice were randomly assigned to experimental ($n=22$) and control ($n=22$) groups. The experimental mice received 50 μg of LPS of *Salmonella minnesota* Re595 (Calbiochem-Behring, La Jolla, CA) dissolved in 0·2 ml of sterile saline twice per week for 5 weeks, *via* an i.p. route (Cavallo *et al.*, 1983, 1984). Control mice received saline only. The rough mutant Re595 was selected for our studies because its LPS is rich in lipid A which is mitogenic and minimally antigenic, and it can induce long lasting production of IgG and IgM antibodies (Anderson *et al.*, 1973; Anderson, Coutinho & Melchers, 1978; Izui, Eisenberg & Dixon, 1981). At the completion of the injection period, at about 3·5 months of age, the mice were challenged with a standard preparation of radiolabelled immune complex and the removal of immune complexes from the circulation and their localization in liver, spleen, and kidney were determined. From 2 to 3·5 months of age, periodic specimens of blood and urine were obtained for various analyses.

Sample collection

Blood specimens were collected under ether anaesthesia by puncture of the retro-orbital sinus. Plasma was removed immediately and kept frozen at −70°C until assayed. Urine specimens were collected in metabolic cages as previously described (Kelley & Cavallo, 1978).

Preparation and characterization of immune complexes

Antibodies to bovine serum albumin (BSA) elicited in rabbits were purified by affinity chromatography. BSA rendered monomeric by gel filtration was labelled with ^{125}I (Amersham, Arlington Heights, IL) by the lactoperoxidase method (Marchalonis, 1969). Unincorporated iodine was removed by dialysis and the integrity of labelled protein was verified by thin-layer chromatography. The efficiency of radioiodination ranged between 80 and 95%, and the specific activity of labelled protein ranged between 3 and 4 μCi/μg. Cold BSA was mixed with radiolabelled BSA to prepare immune complexes at five-fold antigen excess using the protocol of Mannik *et al.* (1971). The equivalence point for BSA–anti-BSA complexes was determined by a quantitative precipitin curve and $22Na^+$ (Amersham) was used as a volume marker. The protein concentration of prepared immune complex was adjusted to 12·5 mg/ml. We verified, by ultracentrifugation, that about 50% of the radioactivity was contained in an area of the curve that was greater than Ag_2Ab_2 (Fig. 1), and that our preparation of immune complexes activate complement in sera of mouse, rabbit, and guinea pig. Before injection, immune complexes were centrifuged at 1000 ***g*** for 20 min to remove insoluble aggregates (Haakenstad, Striker & Mannik, 1982).

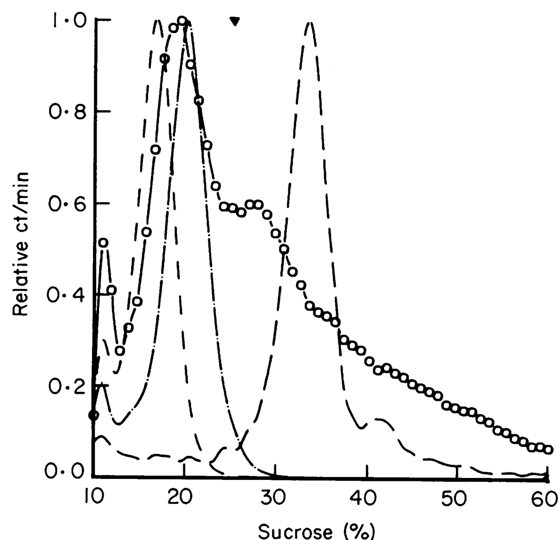

Fig. 1. Sucrose density ultracentrifugation of BSA–anti-BSA immune complexes. The solid line with open circles (O—O) represents analysis of a typical preparation of IC; each circle represents a fraction of the gradient. The following marker proteins were included in the analysis: BSA, (- - - -); IgG, (- · - ·); IgM, (— — —). Relative ct/min normalizes the ct/min of each fraction relative to the maximum ct/min for the sample. (▼), Estimated position for Ag_2Ab_2 complexes.

Table 1. Summary of immunological assays in plasma of lipopolysaccharide (LPS) and of control mice at 3 months of age

Groups	IgG (mg/ml)	IgM (mg/ml)	C3 (% of normal)	ssDNA*	C1q-BA†
LPS	34·2±1·3‡	7·2±0·2§	94·4±9·9	95·0±1·9§	3·0±0·1§
Control	2·8±0·1	0·2±0·0	100·0±8·3	18·7±0·6	0·7±0·2

Mean ± s.e.m.
* Percentage DNA binding by 10·0 μl of plasma.
† mg equivalent of aggregated human IgG/ml.
‡ $P \leq 0·005$.
§ $P \leq 0·001$.

Table 2. Urinary protein and plasma urea and creatinine concentrations in lipopolysaccharide (LPS) and in control mice at 3 months of age

Groups	Urinary proteins (mg/24 h)	Urea (mg/dl)	Creatinine (mg/dl)
LPS	5·4±0·1	22·6±0·2*	0·23±0·02*
Control	5·5±0·2	25·6±0·2	0·10±0·00

Mean ± s.e.m.
* $P \leq 0·001$.

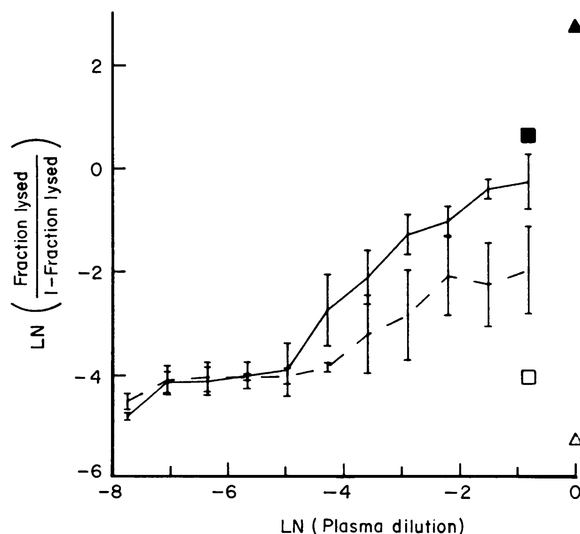

Fig. 2. Curve of haemolytic activity in plasma of LPS and control mice at 3 months of age. Data points are mean ± s.e.m. (- - - -), data from mice which received LPS; (——), data from control mice. There are three mice in each group. (■), haemolytic activity of fresh mouse plasma; (□), haemolytic activity of an aliquot of the same specimen after heat inactivation. (▲), 100% lysis; and (△), background leakage of radiolabel.

Removal of circulating immune complexes

We added KI to drinking water at least 24 h before studies. Ten mice in the LPS group and 10 mice in the control group were studied at 3·5 months of age. We administered immune complexes (0·2 ml; about 1·0 μCi bound radioiodine), under ether anaesthesia, into the tail vein of mice, by a single injection. Blood specimens were collected from the retro-orbital sinus at 1, 2·5, 5, 10, 15 and 30 min and at 1, 2, 4, 12, 24 and 30 h after immune complexes were injected. Exactly 10·0 μl of each specimen were transferred immediately into 1·25 ml of 10 mM EDTA and centrifuged to separate plasma and blood cells. A 1-ml aliquot of supernatant was transferred into an equal volume of saturated ammonium sulphate, centrifuged, and counts in the pellet fraction, representing precipitable immune complexes in half-saturated ammonium sulphate, were used to plot percent remaining *versus* time. The value for zero time (= 100%) was extrapolated by linear regression of log ct/min against time for the first three or four time-points. All measured values for subsequent timed collections represent the immune complexes remaining in the circulation as a percent relative to the value attributed to zero time (= 100%). Each curve was analysed by curve peeling, and the half-life ($t_{\frac{1}{2}}$) attributed to each component of the curve was determined.

Organ localization of immune complexes

The quantity injected, the preparation, and delivery of immune complexes to mice was the same as outlined in the previous section. Twelve mice in the LPS group and 12 mice in the control group were studied at 3·5 months of age. The localization of labelled immune complexes was determined in liver, spleen and kidneys at 1, 4, 12 and 24 h after injection. Ten minutes before harvesting of organs, each mouse received 0·2 ml of homologous red blood cells (RBC) labelled with $^{51}CrO_4$ (Amersham) to estimate the vascular volume for each collected organ. Immediately before the organs were excised, 10·0 μl of blood were obtained. Radioactivity was determined in the specimen of blood and in the excised organs. The ct/min due to radioiodine was corrected for spill-over of $^{51}CrO_4$ into the ^{125}I window. The contribution of the vascular compartment was subtracted from the gross radioactivity of the organ (Bockow & Mannik, 1981), and the corrected value was expressed in μg immune complexes per g of tissue.

We labelled RBC with $^{51}CrO_4$ according to the protocol of Finbloom & Plotz (1979). Unincorporated $^{51}CrO_4$ was eliminated by repeated rinsing of RBC, and the volume was adjusted so that 0·2 ml (about 10^7 RBC) would deliver about 2×10^5 ct/min. For all experiments, labelled RBC were prepared shortly before use.

Laboratory determinations

Plasma concentrations of mouse IgG and IgM were determined by solid-phase radioimmunoassay (Andrews *et al.*, 1978). Antisera to mouse IgG and IgM were purchased from Litton Bionetics (Charleston, SC). The antibody (Dako, Santa Barbara, CA) used to develop the assay was labelled with ^{125}I by the lactoperoxidase method (Marchalonis, 1969) and is polyvalent for mouse immunoglobulins. The specific activity of labelled antibody was about 3 μCi/μg.

Circulating immune complexes were estimated by the C1q binding assay (Zubler *et al.*, 1976). The purity of C1q (Sigma Chemical Co., St Louis, MO) was verified by immunoelectrophoresis. C1q was labelled by the lactoperoxidase method (Marchalonis, 1969). The specific activity of labelled C1q was about 2·2 μCi/μg. The C1q-reactive material was referred to a standard curve comprised of aggregated human gamma globulin at concentrations in the range of 0·1 to 2·5 mg/ml.

Antibodies to ssDNA were estimated according to the method of Izui, Lambert & Miescher (1976). The DNA (Sigma) was labelled with ^{125}I by the method of Commerford (1971). The specific activity of labelled DNA was about 1 μCi/μg. Results were expressed as percent binding relative to the counts in 20 ng of labelled DNA precipitated by 12% trichloracetic acid and were corrected for non-specific interaction between plasma proteins and DNA. Where binding exceeded 90%, the sample was diluted and assayed again.

Complement (C3) was determined in plasma samples by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965). The values of individual plasma samples were compared with a pool of 10 normal plasma samples, and the results were expressed as percentage of normal. Antiserum to mouse C3 was purchased from Cooper Biomedical (Malvern, PA). In addition, total complement activity was determined through a haemolytic assay using the general procedure and the controls established by De Waal *et al.* (1988), except that haemolysis was assessed by release of $^{51}CrO_4$. Sheep red blood cells were labelled with $^{51}CrO_4$ by the method of Hardy (1986). Preserved sheep blood and antibodies against sheep red cells (IgG, 7S) were purchased from Diamedix (Miami, FL). CH_{50} U/ml were estimated for each plasma sample by linear regression of Ln $\{\gamma/(1-\gamma)\}$ against Ln (plasma dilution), where γ is the lysed fraction of indicator cells.

Creatinine and plasma urea nitrogen were determined by standard automated methods (Ektachem 700, Eastman Kodak, Rochester, NY).

Urinary proteins were determined by the method of Lowry *et al.* (1951) using BSA as protein standard. This procedure intercalates a step in which proteins are precipitated and washed

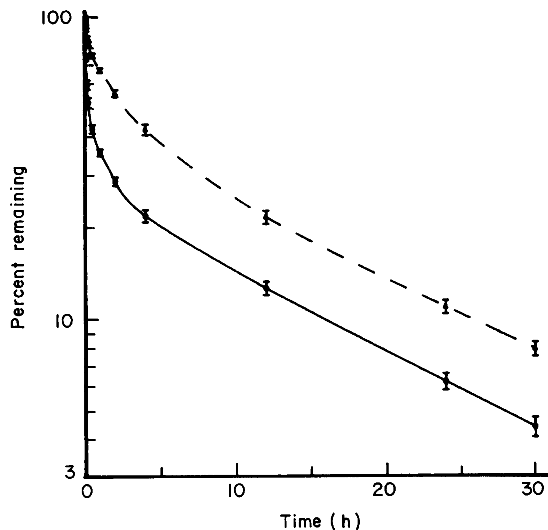

Fig. 3. Computer-fitted curve of disappearance of radiolabelled immune complexes in LPS and in control mice at 3·5 months of age. Data points are mean ± s.e.m. (– – – –), data from mice which received LPS; (——), data from control mice. There are nine mice in each group. Percent remaining refers to the content in the circulation relative to time zero (= 100%).

to eliminate interference by compounds such as uric acid (Pesce, 1974).

For all determinations, fluids from LPS and control mice were assayed in parallel.

Statistical analysis

Data were analysed by the Mann–Whitney *U*-test. $P < 0{\cdot}05$ was considered significant.

RESULTS

At 3 months of age, after eight injections of LPS, the concentrations of the following plasma analytes (Table 1) were increased in LPS, in comparison to control mice: IgG, 12-fold ($P \leq 0{\cdot}005$); IgM, 36-fold ($P \leq 0{\cdot}001$); anti-ssDNA, five-fold ($P \leq 0{\cdot}001$); and C1q-reactive material, four-fold ($P \leq 0{\cdot}001$). Complement concentrations and urinary protein excretion (Table 2) were comparable in LPS and control mice. Total complement activity, judged by the haemolytic assay (Fig. 2), was comparable ($P > 0{\cdot}05$) in LPS ($0{\cdot}9 \pm 0{\cdot}9$ CH_{50} U/ml) and in control ($3{\cdot}0 \pm 1{\cdot}2$) mice. Plasma urea concentration (Table 2) was slightly decreased and creatinine concentration 2·3-fold increased ($P \leq 0{\cdot}001$) in LPS, in comparison to control mice.

The disappearance of radiolabelled immune complexes from the circulation (Fig. 3) was best described by the summation of three exponential components (Haakenstad & Mannik, 1977): first (transient) component, which represents equilibration of immune complexes between intra- and extravascular compartments; second (intermediate) component, which represents removal of pathogenic immune complexes larger than Ag_2Ab_2; and third (persistent) component, which represents metabolism of small size immune complexes equal or smaller than Ag_2Ab_2.

The $t_{\frac{1}{2}}$ and the relative percentage (%) of radiolabelled immune complexes attributable to each component of the curve are shown in Table 3. The percentage of radiolabelled immune complexes remaining in the circulation of LPS mice was statistically different from control mice for all three components of the curve ($P \leq 0{\cdot}003$). However $t_{\frac{1}{2}}$ was increased for the intermediate component only in LPS mice ($P \leq 0{\cdot}003$), compared with control mice. One mouse from the control group died before sample collections were completed, and data from one mouse from the experimental group could not be used because of technical failure.

Liver uptake of radiolabelled immune complexes was decreased at 4 and at 24 h in LPS ($P \leq 0{\cdot}049$), in comparison to control mice. Kidney localization of radiolabelled immune complexes was 3·3-fold increased in LPS mice at 12 h, compared with control mice ($P \leq 0{\cdot}049$). However, kidney localization of radiolabelled immune complexes at 4 and 24 h was apparently diminished in LPS mice, compared with control ($P \leq 0{\cdot}049$). The uptake of radiolabelled immune complexes by the spleen was negligible (less than 0·5% of immune complexes administered) and was not statistically different ($P > 0{\cdot}05$) between LPS and control mice (data not shown).

DISCUSSION

All mice that received LPS developed typical features of PBA including hypergammaglobulinaemia, increased autoantibody formation, and increased amounts of C1q-binding material in the plasma. These findings are comparable to those reported previously (Cavallo *et al.*, 1984). It is interesting that the concentration of C3 in the circulation was not statistically different when data from LPS and control mice were compared. Since LPS mice had substantial amounts of C1q-reactive material in the circulation, it is likely that complement activation was countered by complement synthesis sufficient to maintain circulating C3 at near normal concentrations. That total complement activity, required for opsonization, was intact was verified through a haemolytic assay. The concentration of plasma creatinine was increased in LPS mice. Since these mice had no evidence of muscle disease, it is likely that the raised creatinine reflects impairment of renal function due to localization of immune complexes in kidneys. Indeed, previous studies from our laboratory using the same experimental approach demonstrated glomerular deposits of immunoreactants and a proliferative type glomerulonephritis in mice (Cavallo *et al.*, 1983, 1984). Surprisingly, however, dysfunction of glomerular filtration in LPS mice was not associated with increased urinary protein excretion. Since urea concentration is not a good measure of renal function, because it is influenced by nitrogen intake, this may explain the paradoxical lower plasma urea concentration in LPS mice in the face of renal insufficiency, as indicated by plasma creatinine values.

Our experimental protocol allowed us to estimate the contribution of each component of the curve of disappearance of immune complexes from the circulation. Since we measured cell-free immune complexes precipitable in half-saturated ammonium sulphate, we could discriminate between free and complexed antigen and minimize the contribution of degraded antigen, which increases with time. Additionally, this approach takes into account the fact that removal of immune complexes from the circulation in mice is not affected by complement-dependent binding of immune complexes to blood cells (Bockow & Mannik, 1981; Miller *et al.*, 1975), and the notion that cell-free immune complexes localize in tissues more readily.

Table 3. Half-life values ($t_{\frac{1}{2}}$) and percentage (%) for components of curve of disappearance of radiolabelled immune complexes in lipopolysaccharide (LPS) and in control mice at 3·5 months of age

	Transient		Intermediate		Persistent	
Groups	$t_{\frac{1}{2}}$ (h)	%	$t_{\frac{1}{2}}$ (h)	%	$t_{\frac{1}{2}}$ (h)	%
LPS	0·1±0·0	19·3±1·2*	2·1±0·2*	41·5±2·5*	13·2±0·5	39·2±2·4*
Control	0·1±0·0	51·1±2·5	1·0±0·1	23·8±2·3	12·0±0·6	25·8±1·8

Mean±s.e.m.
* $P \leq 0{\cdot}003$.

The attribution of three exponential components to the curve of disappearance of immune complexes from the circulation is in keeping with results of other investigators who used comparable immune complexes and experimental protocols (Bockow & Mannik, 1981; Finbloom & Plotz, 1979; Haakenstad & Mannik, 1974; Haakenstad, Case & Mannik, 1975; Haakenstad & Mannik, 1976; Rifai & Mannik, 1983; Jimenez, Haakenstad & Mannik, 1983). Also $t_{\frac{1}{2}}$ values and the relative percentage of immune complexes removed by each respective component of the curve in control mice are in general agreement with published data (Bockow & Mannik, 1981; Haakenstad & Mannik, 1974; Haakenstad *et al.*, 1975; Haakenstad & Mannik, 1976), when the quantity of immune complexes administered and technical variables are taken into account. Indeed, for the first component in our experiments $t_{\frac{1}{2}}$ was 0·1 h (*versus* 0·08–0·15 h), for the second $t_{\frac{1}{2}}$ was 1·0 h (*versus* 1·8–7·0 h), and for the third $t_{\frac{1}{2}}$ was 12·0 h (*versus* 35–58 h). The wide range of values in published data probably reflect such variables as composition, intrinsic properties, or quantity of radiolabelled immune complexes administered to mice. Lower values for $t_{\frac{1}{2}}$ in our control mice for the second and third components of the curve could be explained by the lower quantity of immune complexes we injected: about 1·3 mg of antibody protein *versus* 2·0–5·0 mg in referenced experiments (Bockow & Mannik, 1981; Haakenstad & Mannik, 1974; Haakenstad *et al.*, 1975; Haakenstad & Mannik, 1976), and by the fact that our protocol measured only precipitable, cell-free, immune complexes.

The removal of pathogenic immune complexes (intermediate or second component of the curve) was prolonged in LPS mice $t_{\frac{1}{2}} = 2{\cdot}1$ h), compared with values in control mice ($t_{\frac{1}{2}} = 1{\cdot}0$ h; $P \leq 0{\cdot}003$). This difference in $t_{\frac{1}{2}}$ could not be attributed to either biologic characteristics or to the quantity of immune complexes administered. In fact, data from Table 3 indicate that the referenced findings are due, at least in part, to a shift of immune complexes from the first to the second (and third) components of the curve. Thus, it appears that less immune complexes left the circulation in LPS mice during the initial phase of their removal. This phase of removal is due to increase in vascular permeability (Haakenstad & Mannik, 1977), as it can be abolished by complement depletion (Bockow & Mannik, 1981), or by pretreatment of mice with corticosteroids (Haakenstad *et al.*, 1975). Since complement concentration in plasma was comparable in LPS and control mice, factors other than complement concentration (?modulation of vascular changes during LPS/PBA) may be involved and need to be investigated.

Table 4. Organ localization of radiolabelled immune complexes in lipopolysaccharide (LPS) and in control mice at 3·5 months of age

Organ	1 h	4 h	12 h	24 h
Liver uptake				
LPS	125·0±18·8	41·4±3·0*	27·1±6·0	7·4±0·5*
Control	170·8±20·6	67·8±8·7	20·0±1·2	26·7±2·7
Kidney localization				
LPS	57·0±5·2	22·8±0·2*	36·3±3·4*	4·7±0·5*
Control	61·8±12·2	32·7±2·8	11·1±1·8	25·9±0·5

Values (μg immune complex per g) are mean±s.e.m.
* $P \leq 0{\cdot}049$ compared to controls matched for tissue and for age.

Although a shift of immune complexes to the second component of the curve could explain the prolonged $t_{\frac{1}{2}}$ and increased amount of immune complex to be removed through this component, a decreased uptake of immune complex by the mononuclear phagocyte system could also be a contributory factor. Indeed, data from Table 4 indicate that, compared with values in control mice, there was less liver uptake of immune complexes in LPS mice and that differences between the two groups attained statistical significance at 4 h post-administration of immune complexes. Thus, the prolonged $t_{\frac{1}{2}}$ and the increased amount of immune complexes removed by the second component of the curve must be due to a shift of immune complexes from the first to the second component, and to decreased uptake of immune complexes by the liver. The extent of contribution of each mechanism, however, cannot be determined from our experiments. That it is reasonable to equate liver uptake of soluble immune complexes with overall mononuclear phagocyte function is indicated from data in the literature (Haakenstad & Mannik, 1977), and from our own studies in which we determined that less than 0·5% of immune complexes administered was found in the spleen. Whether the decreased liver uptake is due to increased occupancy of Fc receptors, to dysfunction of Fc receptors (Magilavy, Rifai & Plotz, 1981), or to defective endocytosis of immune complexes (Finbloom, 1985), is under investigation.

As judged by results of liver uptake studies, the period of increased circulation of pathogenic immune complexes lasted more than 4 h, but less than 12 h, in LPS mice (Table 4). Prolonged circulation of pathogenic immune complexes facili-

tates their deposition in tissues (Haakenstad & Mannik, 1977) and, in keeping with this notion, a larger quantity of immune complexes was detected in kidneys of LPS mice at 12 h, compared with control mice. Table 4 also suggests that 4 h after administration of immune complexes, when liver uptake was decreased, the quantity of immune complexes detected in the kidneys of LPS mice, surprisingly, was decreased. A review of experimental protocols and related data failed to uncover a technical cause to explain this finding.

In mice chronically exposed to bacterial LPS, hepatic uptake of immune complexes is decreased, removal of immune complexes from the circulation is delayed, and prolonged circulation of pathogenic immune complexes enhances their localization in kidneys. These findings may parallel immunologic changes likely to occur in patients with SLE as they undergo clinical flares in the presence of superimposed bacterial infections.

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