INFECTION AND IMMUNITY, June 1989, p. 1786–1791
0019-9567/89/061786-06$02.00/0

Vol. 57, No. 6

Retention of Bacterial Lipopolysaccharide at the Site of Subcutaneous Injection

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Received 20 December 1988/Accepted 22 February 1989

The tissue distribution of *Klebsiella pneumoniae* O3 lipopolysaccharide (KO3 LPS) was studied in mice injected subcutaneously (s.c.) or intraperitoneally (i.p.) with ^{125}I-labeled KO3 LPS. Marked retention of KO3 LPS radioactivity could be found at the site of s.c. injection for several weeks. On the other hand, about 85% of the radioactivity rapidly disappeared from the peritoneal cavity within 6 h after i.p. injection. The long-term presence of KO3 LPS at the injection site was also supported by experiments with ^{51}Cr-labeled KO3 LPS and immunoblotting and immunofluorescence staining methods. The R-form LPS lacking the O-specific polysaccharide chain of KO3 LPS and the lipid A fraction of KO3 LPS seemed to remain at the site in larger amounts and for longer times than KO3 LPS. There were no marked differences in the retention pattern at the injection site among KO3 LPS, *Escherichia coli* LPS, *Salmonella typhosa* LPS, and *Salmonella enteritidis* LPS. However, much less radioactivity accumulated in the livers and spleens of mice injected with either KO3 LPS or *S. typhosa* LPS compared with the other LPS preparations. It was suggested that retention of LPS at the site of s.c. injection may play an important role in the development of various biological actions of s.c. injected LPS.

The polysaccharide-rich fraction isolated from the culture supernatant of *Klebsiella pneumoniae* strain Kasuya (O3:K1), which has been used as the capsular polysaccharide fraction, exhibits a variety of biological activities, including strong adjuvanticity (12, 14), activity as a polyclonal B-cell activator (16, 17), interferon-inducing activity (10), and activity as a potentiator of the mononuclear phagocyte system (26–28). However, the substance responsible for these biological actions was shown to be *Klebsiella* O3 lipopolysaccharide (KO3 LPS) (11). The potent adjuvant action of KO3 LPS was much greater than that of Freund complete adjuvant or LPS from *Escherichia coli*, other *Klebsiella* spp., and *Salmonella* spp. (12, 14, 29), and severe autoimmune lesions were produced in the respective organs when mice were immunized subcutaneously (s.c.) with the syngeneic tissue antigens together with KO3 LPS (15, 19, 30). Its strong adjuvant action was seen only when antigens were injected s.c. together with KO3 LPS, and the strength of the adjuvant effect of KO3 LPS when injected intraperitoneally (i.p.) or intravenously (i.v.) together with antigen did not differ significantly from that of *E. coli* LPS (18). This finding suggested that the s.c. route of injection had certain advantages for development of the adjuvant action of KO3 LPS. However, there are very few reports on the in vivo distribution of LPS in s.c. injection, since in the great majority of distribution studies LPS has been administered as single, brief i.v. injections (23). In the present study, we compared the tissue distribution of KO3 LPS following s.c. and i.p. administration and studied the retention patterns at the site of the s.c. injection in detail. Furthermore, we compared it with the in vivo distribution of R-form LPS (R-LPS) and the lipid A fraction of KO3 LPS and of various other kinds of LPS.

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MATERIALS AND METHODS

Animals. Male and female SMA mice, approximately 8 weeks of age, were supplied by the Inbred Animal Breeding Laboratory, Fukui Medical School.

LPS preparation. KO3 LPS was prepared from *K. pneumoniae* LEN-1 (O3:K1−) (22) by the phenol-water method (25). Briefly, the cells were washed by centrifugation in distilled water, and the crude preparation was extracted by adding an equal volume of 90% phenol at 70°C to the cell suspension. The supernatant was dialyzed against distilled water and treated with RNase (20 μg/ml). For deproteinization, it was extracted 10 times with 0.5 volume of a chloroform–*n*-butanol (5:1) mixture. After removal of the precipitate that formed, the supernatant was dialyzed in distilled water. KO3 LPS was precipitated by addition of ethanol containing 0.2% $MgCl_2$ to a concentration of 67% and washed with ethanol. Resolution in distilled water and precipitation with ethanol were repeated three times. The final preparation was dissolved in water and dialyzed against distilled water. Finally, a white powder was obtained by lyophilization. The KO3 LPS used in this study contained neither proteins as measured by the method of Lowry et al. (13) or nucleic acids as measured by UV absorption at 254 nm. KO3 LPS is usually degraded into the polysaccharide moiety, consisting predominantly of mannose (63%), and the lipid moiety (24%) (6). LPS from *E. coli* O111 and O128, *Salmonella typhosa* LPS, and *Salmonella enteritidis* LPS extracted by the method of Westphal were purchased from Difco Laboratories, Detroit, Mich. The R-form of KO3 LPS (R-LPS) was prepared from *K. pneumoniae* LEN-111 (O3−:K1−), a mutant that does not synthesize the O-specific polysaccharide chain derived from strain LEN-1 (21). The lipid A fraction was isolated from KO3 LPS by heating at 100°C for 1 h in 1% acetic acid (9).

Radiolabeling of LPS preparation. Two different methods for radiolabeling LPS with ^{125}I or ^{51}Cr were used. Briefly, 10

TABLE 1. Comparison of tissue distribution of ^{125}I-labeled KO3 LPS injected s.c. and i.p.

Time	Route	Mean[a] retention of ^{125}I-labeled KO3 LPS (% of amount injected) ± SE				
		Injection site	Left leg	Liver	Spleen	Kidney
6 h	s.c.	73.4 ± 3.1	0.3 ± 0	14.4 ± 1.0	0.2 ± 0	3.3 ± 0.2
	i.p.	15.7 ± 1.7	0.8 ± 0.2	42.2 ± 3.3	5.3 ± 0.5	4.2 ± 0.4
3 days	s.c.	46.1 ± 2.4	0.2 ± 0	14.4 ± 0.7	0.4 ± 0	1.4 ± 0.1
	i.p.	2.1 ± 0.1	0.4 ± 0	33.8 ± 1.4	3.1 ± 0.3	1.1 ± 0

[a] Mean for four mice.

mg of protein-free LPS was activated with cyanogen bromide and conjugated with tyramine (2), and the mixture was dialyzed. The tyramine-conjugated LPS (5 mg) was labeled with 1 mCi of ^{125}I by using Iodo-beads (Pierce Chemical Company, Rockford, Ill.). Second, 1 mCi of $^{51}CrCl_3$ was added to a saline solution of LPS (5 mg/250 μl) and kept overnight at room temperature (20). Both radiolabeled LPSs were purified with Sephadex G25 and further dialyzed against an excess volume of saline. The radiolabeled KO3 LPS had the same lethal toxicity to mice as untreated KO3 LPS. Radiolabeling of R-LPS and the lipid A fraction with $^{51}CrCl_3$ was done principally by the same method. The lipid A fraction was suspended in distilled water and solubilized by addition of triethylamine to 1% and warming to 60°C for 5 min. After radiolabeling, the lipid A fraction was then dialyzed against saline for 2 days at 4°C to remove excess triethylamine.

Administration of radiolabeled LPS preparations. The radiolabeled LPS preparations to be injected were diluted in saline. Each mouse was injected with 5 μg of ^{125}I-labeled LPS (about 10^6 cpm) or 50 μg of ^{51}Cr-labeled LPS or lipid A (about 2×10^5 cpm) i.p. or s.c. in the right inguinal region. At various days after injection, four mice were sacrificed, and the right leg (the injection site), left leg, liver, spleen, kidney, lung, heart, thymus, mesenteric lymph node, and thyroid glands were collected. The solution used to wash the peritoneal cavity was collected. The radioactivity in various organs and in the injected volume of LPS was determined at the same time by a gamma scintillation counter. Percent retention was calculated as the radioactivity recovered in the tissue divided by the total radioactivity injected.

Immunofluorescence staining. The skin of the inguinal region 4 days after s.c. injection were removed and fixed in 10% Formalin. The paraffin sections were deparaffinized by placing the slide in cold xylene for 10 min, treated in four consecutive baths of cold 95% alcohol. The alcohol was removed by gentle agitation in three baths of fresh cold phosphate-buffered saline. The sections were covered with a 1:100 dilution of rabbit anti-KO3 LPS serum and stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G serum. Control sections were incubated in the same dilution of normal rabbit serum. Immunofluorescence was inspected under a fluorescence microscope.

Gel electrophoresis and immunoblotting. The inguinal region of mice injected with 100 μg of KO3 LPS was collected on various days. Tissue extracts were prepared with a homogenizer, diluted with an equal volume of sample buffer containing 2.5% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol, and boiled for 2 min. Samples were separated by 7.5% or 12.5% polyacrylamide–SDS gel electrophoresis. Antigens in the gel were transferred to a membrane filter by electroblotting as described by Towbin et al. (24). The filters were blocked with 10% skim milk and then incubated with a 1:100 dilution of anti-KO3 LPS or anti-lipid A rabbit serum and subsequently with ^{125}I-labeled protein A (30). Radiolabeled antigen bands were visualized by autoradiography. A low-molecular-weight (MW) standard from Pharmacia (Uppsala, Sweden) was used.

Antisera. Rabbit anti-KO3 LPS serum was prepared by the procedure of Edwards and Ewing (3) with *K. pneumoniae* 2002/49 (K25 reference strain) (O3:K25). Rabbit anti-lipid A serum was prepared by the procedure of Galanos (5) with *K. pneumoniae* LEN-1 and the lipid A fraction extracted from KO3 LPS.

RESULTS

Tissue distribution of ^{125}I-labeled KO3 LPS after s.c. and i.p. injection. ^{125}I-labeled KO3 LPS was injected s.c. or i.p. into mice, and the radioactivity in various organs was counted 6 h and 3 days later (Table 1). There was high radioactivity at the injection site of s.c. injected mice even at 3 days, suggesting retention of radiolabeled KO3 LPS. In the peritoneal cavity, about 15% of the KO3 LPS injected was detected at 6 h, but most of the radioactivity had disappeared after 3 days. In mice injected by the i.p. route, the residual radioactivity in the liver, spleen, and kidney was much higher than in s.c. injected mice. There was no appreciable accumulation of KO3 LPS in the lung, heart, thymus, and mesenteric lymph nodes (less than 2%) after either s.c. or i.p. injection. In addition, the radioactive sample injected migrated to the same position as KO3 LPS on silver-stained SDS-polyacrylamide gels (data not shown), and the homogenate of the regional s.c. tissue contained radiolabeled KO3 LPS 3 days after injection (Fig. 1).

Time course of retention of radiolabeled KO3 LPS at the injection site. Because it became clear that a significant amount of KO3 LPS remained at the injection site, the

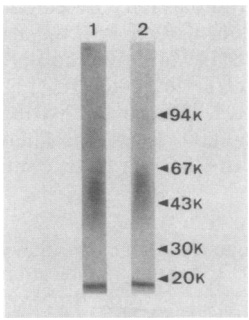

FIG. 1. Detection of ^{125}I-labeled KO3 LPS at the site of injection. ^{125}I-labeled KO3 LPS to be injected (lane 1) and the homogenate of the regional s.c. tissue of mice injected with ^{125}I-labeled KO3 LPS 3 days earlier (lane 2) were analyzed by 10% polyacrylamide–SDS gel electrophoresis, and radiolabeled LPS was detected by autoradiography. The positions of MW markers (in thousands) are indicated.

TABLE 2. Time course of tissue distribution of ^{125}I-labeled KO3 LPS after s.c. injection

Time	Mean[a] retention of ^{125}I-labeled KO3 LPS (% of amt injected) ± SE			
	Injection site	Liver	Kidney	Thyroid
1 h	70.8 ± 2.5	12.4 ± 2.7	6.3 ± 2.9	0.1 ± 0
1 day	53.0 ± 1.8	17.7 ± 1.2	1.7 ± 0.2	0.7 ± 0.1
2 days	42.4 ± 4.9	12.8 ± 0.5	1.1 ± 0.1	1.6 ± 0.3
4 days	29.5 ± 3.1	11.6 ± 1.4	0.7 ± 0.1	0.9 ± 0.1
7 days	28.0 ± 4.2	8.4 ± 0.5	0.7 ± 0.1	1.3 ± 0.2
9 days	25.6 ± 2.0	6.5 ± 0.1	0.5 ± 0	1.0 ± 0.1
11 days	25.5 ± 1.9	7.6 ± 0.6	0.4 ± 0	0.8 ± 0.1
14 days	23.1 ± 1.3	5.5 ± 0.8	0.4 ± 0	0.7 ± 0.2
19 days	16.1 ± 0.9	4.7 ± 0.3	0.2 ± 0	0.4 ± 0
25 days	8.1 ± 1.6	3.2 ± 0.2	0.1 ± 0	0.4 ± 0
32 days	6.0 ± 1.0	1.6 ± 0.1	0.1 ± 0	0.2 ± 0

[a] Mean for four mice.

detailed time course of its retention was studied by using ^{125}I- and ^{51}Cr-labeled KO3 LPS (Tables 2 and 3). With ^{125}I-labeled KO3 LPS, about 70% of the radioactivity injected remained at the site 1 h after injection (Table 2). About 28 and 6% of the radioactivity remained a week and a month later, respectively. Since the draining (inguinal) lymph node contained less than 1% of the total radioactivity injected (data not shown), the radioactivity seemed to be present in the s.c. tissue of the injection site. The radioactivity in the liver showed the highest count (about 18%) at 1 day. There was no significant accumulation of KO3 LPS in the other organs, such as the opposite leg, spleen, thymus, lung, heart, and mesenteric lymph nodes (less than 0.3%). In addition, the thyroid glands showed a relatively high background, probably due to uptake of free ^{125}I.

Retention of ^{51}Cr-labeled KO3 LPS persisted at the local site longer than ^{125}I-KO3 LPS (Table 3). One-third of KO3 LPS still remained 2 weeks later. On the other hand, the radioactivity in the liver did not exceed 5% of the total radioactivity injected, and accumulation of ^{51}Cr-labeled LPS in all other organs was negligible. With $^{51}CrCl_3$ or $Na^{125}I$, the radioactivity remaining at the site 3 days later was less than 10% of the injected radioactivity, suggesting that free radioisotope was rapidly cleared from the regional site.

Detection of KO3 LPS remaining at the site of s.c. injection by immunoblotting. We showed above the marked retention of radioactivity of KO3 LPS at the injection site. To test the possibility that the radioactivity detected in the tissue was released from labeled KO3 LPS, we tried to detect the remaining KO3 LPS by an immunoblotting method with anti-KO3 LPS or anti-lipid A serum (Fig. 2). The antigen reactive with anti-KO3 LPS serum distributed broadly at an MW of more than 60,000 (Fig. 2A). There was no specific band in the lane of the sample from control mice. Normal

TABLE 3. Time course of tissue distribution of ^{51}Cr-labeled KO3 LPS after s.c. injection

Day	Mean[a] retention of ^{52}Cr-labeled KO3 LPS (% of amt injected) ± SE				
	Injection site	Liver	Spleen	Kidney	Thyroid
1	84.7 ± 4.7	3.7 ± 0	0.1 ± 0	0.4 ± 0	0 ± 0
5	48.0 ± 0.7	3.4 ± 0.2	0.3 ± 0	0.7 ± 0.1	0 ± 0
14	37.6 ± 5.0	4.4 ± 0.3	0.4 ± 0.1	0.6 ± 0.1	0.1 ± 0

[a] Mean for four mice.

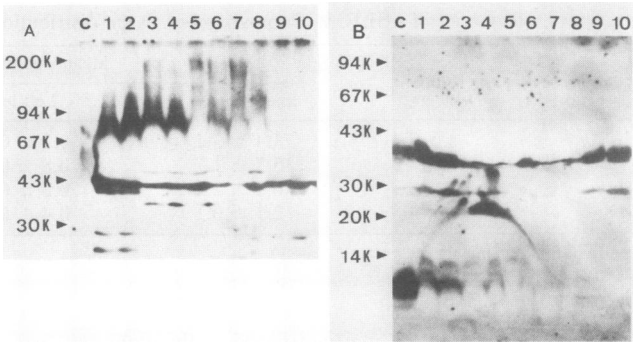

FIG. 2. Detection of KO3 LPS remaining at the injection site by immunoblotting. Extracts of the inguinal s.c. tissue of two mice at 1 (lanes 1 and 2), 3 (lanes 3 and 4), 5 (lanes 5 and 6), and 10 (lanes 7 and 8) days after injection with 100 μg of KO3 LPS were reacted with a 1:100 dilution of anti-KO3 LPS serum (A) or anti-lipid A serum (B). KO3 LPS and lipid A (5 μg) were applied to lane C in panels A and B, respectively. Lanes 9 and 10 were mock-injected control mice.

rabbit control serum could not recognize those bands (data not shown). Anti-lipid A serum also defined a band with an MW of less than 14,000 (Fig. 2B). Even at 10 days after injection, both antibodies definitely recognized remaining KO3 LPS and lipid A. Although both antisera detected the band with a MW of about 43,000, it seemed to be actin, which is nonspecifically reactive with immunoglobulins in the serum (1, 7).

Location of KO3 LPS at the site of s.c. injection. The inguinal skin of mice which had been injected s.c. with 100 μg of KO3 LPS 4 days earlier was stained by the immunofluorescence method. Distinct immunofluorescence was detected widely in the s.c. tissue (Fig. 3). This specific fluorescence was easily distinguishable from a faint background with normal rabbit serum. The fluorescence pattern in the s.c. tissue was homogeneous, not suggesting specific accumulation of KO3 LPS. The normal control tissue stained by anti-KO3 LPS serum did not show any specific immunofluorescence except a faint background, as in Fig. 3B (data not shown).

Difference in tissue distribution of KO3 LPS, R-LPS, and lipid A after s.c. injection. ^{51}Cr-labeled KO3 LPS, R-LPS, and lipid A (1×10^6 to 2×10^6 cpm/50 μg) was used in order to clarify the role of O-specific polysaccharide on the efficiency of retention (Tables 4 and 5). The radioactivity of R-LPS and lipid A also persisted at the site. They decreased at a rate similar to that of KO3 LPS during the first period of 3 days (Table 4), but thereafter KO3 LPS disappeared more rapidly at the site ($P < 0.001$ versus R-LPS and versus lipid A). The radioactivity of R-LPS and lipid A retained in the liver was much less than that of KO3 LPS (Table 5).

Difference in tissue distribution of various kinds of LPS after s.c. injection. The distribution pattern of various ^{51}Cr-labeled LPSs, including KO3 LPS, *S. typhosa* LPS, *S. enteritidis* LPS, *E. coli* O111 LPS, and *E. coli* O128 LPS was compared at 5 and 14 days after injection (Table 6). There were no marked differences in the retention of these various kinds of LPS at the site at either 5 or 14 days. In the case of KO3 LPS and *S. typhosa* LPS, much less radioactivity was measured in the liver and spleen ($P < 0.001$ versus other LPSs). These two LPSs macroscopically induced inflammatory lesions at the site of injection (data not shown).

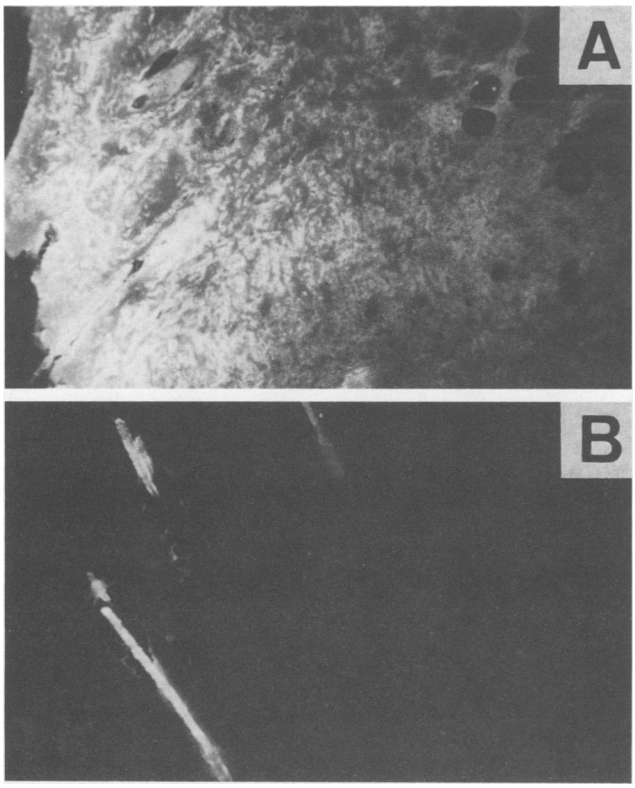

FIG. 3. Immunofluorescence in the s.c. tissue 4 days after injection with KO3 LPS. Staining was done with anti-KO3 LPS serum (A) or normal rabbit serum (B). Spontaneous fluorescence is seen on hair shafts. Magnification, ×100.

DISCUSSION

Marked retention of KO3 LPS at the site of s.c. injection could be found, whereas there was no such retention in the peritoneal cavity after i.p. injection. The possibility that radioisotope released from LPS persisted at the site is unlikely because radioactive molecules with the same MW as the LPS injected still existed at the site (Fig. 1) and free radioisotope seemed to be rapidly cleared from the site. The finding that LPS itself was retained at the site was confirmed by the immunoblotting and immunofluorescence methods. Previously, Noyes et al. (20) reported that the distribution pattern of ^{51}Cr-labeled *E. coli* LPS in mice injected intramuscularly differed markedly from that found in mice injected i.v. or intracranially and that about half the radioactivity remained at the site of injection after 24 h. Our present data demonstrate that a significant quantity of LPS remained for up to several weeks. This finding is inconsistent with the fact that i.v. and i.p. injected LPS is quickly cleared from the bloodstream and retained mainly in the liver (23). The discrepancy may be due to the difficulty in the interaction between s.c. injected LPS and the components in the serum, such as high-density lipoproteins. This retention may cause the differences in the effective dose and the kinetics of induction of biological actions of LPS between i.p. and s.c. injection.

Since there was no marked difference in the retention patterns of various kinds of LPS, it is difficult to speculate on the particular association of retention of KO3 LPS at the injection site with its extraordinary strong adjuvant action. However, retention of KO3 LPS at the site can easily explain the following characteristic phenomena at the regional lymph node: (i) KO3 LPS exhibits a novel adjuvant action on plaque-forming cell response to s.c. injected sheep erythrocytes which develops at a late stage of immunization (7 to 14 days) (29); (ii) major polyclonal activation of B cells develops at a late stage in the regional lymph node after s.c. stimulation with KO3 LPS alone (at 5 to 11 days) (29); and (iii) marked proliferation of macrophages and their precursors can be seen in the regional lymph node at a late stage (7 to 10 days) (27, 28). Probably the main cause for these phenomena is that KO3 LPS remaining at the local site continuously stimulates B cells and macrophage lineage cells at the draining lymph node.

KO3 LPS seemed to disappear from the injection site more quickly than R-LPS and the lipid A fraction and was retained in greater amounts in the liver. The difference in the

TABLE 4. Retention of radioactivity at the injection site after s.c. injection of ^{51}Cr-labeled KO3 LPS, R-LPS, and lipid A

Day	Mean[a] retention (% of amt injected) ± SE		
	KO3 LPS	R-LPS	Lipid A
1	70 ± 9	84 ± 4	79 ± 2
3	73 ± 6	77 ± 4	68 ± 1
5	43 ± 2	74 ± 3	66 ± 5
10	35 ± 5	64 ± 7	58 ± 6
15	35 ± 4	63 ± 2	55 ± 3

[a] Mean for four mice.

TABLE 5. Retention of radioactivity in the liver after s.c. injection of ^{51}Cr-labeled KO3 LPS, R-LPS, and lipid A

Day	Mean retention (% of amt injected) ± SE[a]		
	KO3 LPS	R-LPS	Lipid A
1	4.5 ± 0.5	0.4	0.6
3	3.1 ± 0.1	0.5	1.0
5	3.8 ± 0.8	0.6	1.1
10	5.4 ± 0.6	0.8	1.0
15	4.3 ± 0.3	0.9	0.8

[a] Mean for four mice. For R-LPS and lipid A, the SE was less than 20%.

TABLE 6. Comparison of tissue distribution of various kinds of ^{51}Cr-labeled LPS after s.c. injection

LPS	Day	Mean retention (% of amt injected) ± SE[a]			
		Injection site	Liver	Spleen	Kidney
KO3	5	48 ± 1.7	1.3 ± 0.1	0.2	0.3
	14	37 ± 5.1	0.7 ± 0.1	0.2	0.3
S. typhosa	5	57 ± 4.0	1.0 ± 0.1	0.3	0.3
	14	53 ± 4.0	0.7 ± 0.1	0.3	0.3
E. coli					
O111	5	57 ± 3.0	2.9 ± 0.1	0.8	0.4
	14	45 ± 4.5	2.8 ± 0.2	0.6	0.3
O128	5	59 ± 8.2	3.0 ± 0.4	1.6	0.6
	14	52 ± 0.2	2.9 ± 0.1	0.7	0.2
S. enteritidis	5	47 ± 5.8	2.7 ± 0.3	0.7	0.4
	14	42 ± 4.1	2.6 ± 0.2	0.7	0.3

[a] Mean for four mice. For the spleen and kidney, the SE was less than 10%.

retention of these LPS preparations at the site after s.c. injection may be due to the difference in their solubility, because R-LPS and lipid A show much lower solubility and make larger aggregates than S-form LPS. The presence of the hydrophilic polysaccharide may play some role in this phenomenon. However, it has been reported that blood clearance and liver uptake of highly aggregated R-preparations were extremely fast after i.v. injection (23).

There were some differences in the distribution patterns between ^{125}I- and ^{51}Cr-labeled KO3 LPS. The polysaccharide portion of LPS could be labeled by ^{125}I (2), while the lipid A fraction could be labeled by ^{51}Cr. This difference in the radiolabeled portions could explain the different patterns in their tissue distribution. Furthermore, the low radioactivity of ^{51}Cr-labeled preparations in the liver may be explained by the idea that release of ^{51}Cr from lipid A occurs rapidly there. This idea is consistent with the finding reported by Freudenberg et al. (4) that on in vivo degradation of LPS in rats, LPS first undergoes partial degradation of its fatty acid and that LPS in the liver has a lower content of fatty acids. On the other hand, it has been reported that *Klebsiella* polysaccharide remained in the liver for many weeks (8).

There are few reports on the distribution of LPS after s.c. injection. The present study demonstrated retention of LPS at the site of s.c. injection. This long-term retention of LPS may explain its characteristic biological actions after s.c. injection.

ACKNOWLEDGMENT

We thank H. Ito for supplying SMA mice.

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