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適用局所の炎症反応を指標とした医用材料の微生物学的汚染評価に関する予備的検討

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Preliminary study for evaluating microbial contamination of biomaterials focused on the inflammatory response at application sites

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Background: The use of endotoxin-contaminated medical devices may cause harmful effects to patients. Therefore, the endotoxin contamination of medical devices must be evaluated and managed properly. However, the field of risk management in microbial safety for medical devices is not yet developed. The purpose of this study was to collect scientific information to enable proper risk assessment by conducting implant and wound dressing experiments in rats using collagen sheets containing bacterial components and evaluating their effects on tissues and organs.

Results: When collagen sheets containing dried *Escherichia coli* cells were used in a wound dressing study, there was no noticeable histopathological difference between the control and experimental groups. When the same sheets were used in subcutaneous and intraperitoneal implant studies, a dose-dependent inflammatory response was observed. The inflammatory response was not specific for gram-negative bacteria containing endotoxin, as dried *Staphylococcus aureus* cells also induced the response in the subcutaneous implant. However, endotoxin activity may be considered to serve as a good specification for microbial contamination in biomaterials, because of the highest sensitivity for detection among various microbial components.

Conclusions: Our results suggest that the susceptibility to microbe-contaminated biomaterials differs among the sites to which the biomaterials are applied, and that bacterial components other than endotoxin can contribute to topical inflammatory responses. These points should be considered in evaluating microbial contamination of biomaterials.

Keyword: endotoxin, collagen, dermal wound dressing, subcutaneous implant, intraperitoneal implant

1. Introduction

Endotoxin, a surface lipopolysaccharide of gram-negative bacteria, represents one of the most commonly encountered exogenous pyrogens[1)]. In addition to their pyrogenic activity, endotoxins trigger various biological responses such as shock and cytokine production by immunocytes through their activation[2-4)]. As gram-negative bacteria may exist anywhere in the environment, medical devices can become contaminated by endotoxins during the manufacturing process. In particular, controlling the microbial safety of naturally derived biomaterials such as collagen, chitin, chitosan, and alginate is

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especially difficult. Consistent with this, over the past 6 years, 12 medical devices have been voluntarily recalled in Japan because of the possibility of endotoxin contamination, as the use of endotoxin-contaminated medical devices may cause harmful effects to patients. Thus, the endotoxin contamination of medical devices must be evaluated and managed properly. However, the biological effects of endotoxins administered *via* parenteral routes other than intravascular administration have not been studied in detail; furthermore, the effects may differ depending on the entry sites. In addition, although the Japanese Pharmacopoeia (JP) defines the endotoxin limit for injectable drugs according to the sites of application, no official endotoxin limit is currently available for medical devices, which have more complicated compositions than drugs and are used for application in various sites. In many cases, the endotoxin limits for medical devices are independently established in reference to the specifications for injectable drugs and based on the total endotoxin content of the product. Considering the immature state of risk management in microbial safety for medical devices, the collection of scientific information to enable proper risk assessment constitutes an urgent issue.

Several studies on the host response of biomaterials spiked with bacterial components including endotoxins have been reported to date[5-8]; however, detailed information, such as information regarding the dose response of bacterial components and the difference according to application sites or contaminant (*e.g.*, endotoxin, gram-negative bacteria, or gram-positive bacteria), is still lacking. In this context, we have been evaluating the biological effects of microbial component-containing collagen sheets that are applied to various sites in rats. We previously reported that intraosseous implantation of collagen sheets containing dried *Escherichia coli* (*E. coli*) cells, comprising gram negative bacteria producing endotoxin, induced a delay in ossification[9]. Here, we conducted a preliminary study to collect more detailed information regarding the microbial safety of topically applied biomaterials by performing wound dressings, subcutaneous implants, and intraperitoneal implants of collagen sheets containing various amounts of bacterial components, focusing on their effects on tissues and organs in rats.

2. Materials and methods

2.1 Preparation of endotoxin and bacterial cells

E. coli O111 and *Staphylococcus aureus* (*S. aureus*) 209P strains were cultured by shaking in nutrient broth at 37℃ for 16 h, followed by pH neutralization of the culture medium and heat inactivation at 121℃ for 15 min. Heat-killed bacterial cells were collected by centrifugation, then rinsed 3 times with distilled water. Dried cells were prepared by sequential lipid extraction with ethanol, acetone, and diethyl ether. Using the phenol-water method[10], endotoxin from the *E. coli* O3:K2a, K2b:H3 ATCC strain was extracted from dried bacterial cells prepared by the above-described method and purified by repeated ultracentrifugation after deoxyribonuclease and ribonuclease treatment[11]. The endotoxin activities of the purified endotoxin from *E. coli* O3:K2a, K2b:H3 ATCC strain and dried *E. coli* O111 cells were 27.5 and 0.159 EU/ng, respectively. The content of endotoxin present as a contaminant in the dried *S. aureus* cells was 0.48 EU/mg.

2.2 Preparation of samples spiked with bacterial components

Atelocollagen (type I and type III) derived from porcine skin (NH Foods Ltd., Osaka, Japan) was used as the medical material. Different amounts of purified endotoxin from the *E. coli* O3:K2a, K2b:H3 ATCC strain, dried *E. coli* O111 cells, or dried *S. aureus* 209P cells were added to the collagen. Collagen samples were lyophilized in a cylinder with a diameter of 1.6 cm under normal conditions and cut into sheets (thickness: 1.5 mm). All sheets were treated with peracetic acid vapor sterilization for 6 h at 40℃ followed by drying *in vacuo* for 12 h at 50℃ to yield sterilized and weakly crosslinked collagen sheets. Sheets without the addition of endotoxins or bacterial cells were prepared as the control.

In a preliminary study, we also tested collagen sheets crosslinked with heat treatment at 150℃ *in vacuo*, as this material is often used as an implant material. However, the crosslinked collagen sheet itself induced an inflammatory response during the early stage after implantation in rats[12]. Therefore, because this inflammatory response may interfere with evaluation of the actual effects of bacterial components, we selected bio-inert weakly crosslinked collagen

sheets for use in subsequent studies.

2.3 Measurement of endotoxin content

To assess the level of endotoxin, 1 mL collagenase (Type IA-S, Sigma-Aldrich Japan, Tokyo, Japan) solution (1 mg/mL in 5 mM HEPES buffer) was added to a test tube containing 100 μL equilibrated EndoTrap® Blue Gel (Hyglos GmbH, Germany) and gently stirred at 4℃ for 8 h using a rotary shaker. The whole mixture was then transferred to another test tube containing 100 μL the EndoTrap® Blue Gel and incubated at 4℃ for an additional 16 h in the same manner. Purified collagenase solution was obtained by collecting the supernatant after centrifugation. The endotoxin activity of the collagenase was reduced from 176.8 to 0.165 EU/mg by purification without the reduction of enzymatic activity.

Precisely weighed collagen sheets (10 mg) were digested with purified collagenase (0.1 mg/mL) in 5 mM HEPES buffer at a pH of 7.3 and kept at 37℃ for 16 h. The collagenase-treated solutions were acidified with hydrochloric acid to pH 3 (2 mL final volume) and ultrasonicated for 10 min at 4℃ (purified collagenase digestion-hydrochloric acid extraction method) followed by serial dilution of the centrifuged supernatants with pyrogen-free 0.1 M Tris buffer (pH 7.3).

Limulus amebocyte lysate (LAL) activity was measured by a kinetic colorimetric assay following the endotoxins test protocol in JP, 17th Edition[13,14]. The endotoxin-specific LAL reagent Endospecy ES-50M (Seikagaku Corporation, Tokyo, Japan) and the JP reference standard endotoxins, lipopolysaccharides (LPS) (from the *E. coli* O55:B5 strain) were used as the assay reagent and standards, respectively. Measurement was performed using a Well Reader SK603 (Seikagaku Corporation, Tokyo, Japan) microplate reader. A test for interfering factors was also conducted in accordance with the JP.

2.4 Release of proinflammatory cytokines from human monocytic cells

Extract induction of proinflammatory cytokines was assayed with an *in vitro* pyrogen test system using human MM6-CA8 monocytic cells, which respond to various pyrogens with high sensitivity[15,16]. Cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Kanagawa, Japan) containing fetal bovine serum (10%), glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), and bovine insulin (9 μg/mL) and primed with 10 ng/mL calcitriol (1α,25- dihydroxyvitamin D_3, Wako Pure Chemical Industries, Ltd., Osaka, Japan). After incubation for 72 h with calcitriol, the cells were plated in a 24-well plate at 1×10^6 cells/0.9 or 1 mL/well, and 0.1 mL JP standard endotoxin at various concentrations or 1 mg collagen sheet was added to each well. The cell suspensions were incubated for 17 h at 37℃ and then the culture supernatants were assayed for interleukin-6 (IL-6) using commercial ELISA kits (Life Technologies Japan, Tokyo, Japan).

2.5 Wound dressing and implant studies

We purchased 7-week old male Fischer rats from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) that were maintained under controlled light and temperature conditions. The rats were given access to food and water *ad libitum*. After a weeklong habituation period, depending on each implantation test described below, the rats were divided into experimental groups A through X and A′ through M′ with 1 or 2 animals housed in each cage. After surgery, the animals were anesthetized with intraperitoneal administration of sodium pentobarbital (30 mg/kg) at the termination of the study. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kanagawa Dental University (approval numbers 24, 25, and 54). The procedures were performed in accordance with the animal experiment guidelines of Kanagawa Dental University. The growth rate and feed consumption during the implant period were almost equal in all groups.

During the coverage of cutaneous wounds with collagen sheet dressing, an incision measuring approximately 1 cm square was made in the dorsal skin of the rats to a depth that reached the dermal layer in 4 locations, followed by suturing of the dressing and skin. For each experimental group (control group and groups A–M), corresponding collagen sheets were applied to two rats (one collagen sheet per rat, n = 2). During subcutaneous implantation, a dorsal medial incision of 3 cm was made on rats followed by blunt dissection of the fascia to create apertures. Next, a single collagen

sheet was inserted into each side of the apertures and the skin was sutured. For each experimental group, corresponding collagen sheets were implanted to two rats (control group and groups A′–M′: one collagen sheet per rat, n = 2; groups U–X: two collagen sheets per rat, n = 4). During intraperitoneal implantation, a 2.5 cm incision was made in the upper abdomen and a collagen sheet was implanted on the surface of the hepatic capsule by visually identifying the liver, followed by suturing of the rectus abdominis muscle and skin. For each experimental group (control group and groups N–T), corresponding collagen sheets were implanted to four rats (one collagen sheet per rat, n = 4).

The cutaneous wound dressing groups were sacrificed after 1 or 2 weeks. The wounded areas were collected and fixed in 10% neutral buffered formalin. To collect the implanted materials along with the surrounding tissue, animals were sacrificed at 1 or 2 weeks (for subcutaneous implantation), or 1 week (for intraperitoneal implantation) after implantation of the collagen sheets, and samples were fixed in 10% neutral buffered formalin. All samples were histopathologically examined after preparation of paraffin-embedded sections using standard procedures and hematoxylin and eosin staining.

2.6 Scanning electron microscopy

The surface structures of collagen sheets were analyzed after gold coating using a JFC-1500 Quick Auto Coater (JOEL Ltd., Tokyo, Japan), and by observation with a JOEL JSM-5800LV scanning electron microscope (SEM) at 40×, 120×, 700×, and 2,000× magnification.

3. Results

3.1 Properties of collagen sheets for implantation

The endotoxin content and IL-6 production ability against MM6-CA8 cells of the collagen sheets used in this study have been reported in our previous study[9] (Table 1, from Haishima *et al.*[9] with modification). Collagen sheets with various endotoxin (LAL) activities were prepared by spiking various amounts of purified endotoxin from *E. coli* O3:K2a, K2b:H3 ATCC strain, dried *E. coli* O111 cells, or dried *S. aureus* cells. The biological activity of the endotoxins in the sheets was confirmed through the detection of substantial IL-6 production from MM8-CA8 cells (Table 1),

Table 1. Endotoxin content and IL-6 production ability against MM6-CA8 cells of the collagen sheets

Bacterial component	Sheet No.	Amount spiked onto the collagen sheet		Measured value (EU/mg)[1]	Recovery (%)	Amount of IL-6 production (pg/mL)[2]	Experimental group		
		ng/mg	EU/mg				Cutaneous wound dressing study	Subcutaneous implant study	Intraperitoneal implant study
Control[3]	–	–	–	0.13	–	12.0	–	–	–
Endotoxin[4]	1	0.039	1.07	1.03	96.5	550	G	G′	–
	2	0.077	2.13	2.13	99.8	3,211	H	H′	–
	3	0.387	10.7	8.8	82.3	13,640	I	I′	–
	4	3.87	107	96.5	90.6	nt[7]	J	J′	–
	5	38.7	1,065	755	70.9	nt	K	K′	–
	6	387	10,650	9,308	87.4	nt	L	L′	–
	7	3,873	106,500	83,283	78.2	nt	M	M′	–
E. coli[5]	8	29.9	4.76	4.70	98.8	2,245	A	A′	N
	9	59.8	9.51	9.60	101	17,370	B	B′	O
	10	299	47.6	33.6	70.7	34,660	C	C′	P
	11	2,991	476	434	91.2	nt	D	D′	Q
	12	8,972	1,427	1,163	81.5	nt	–	–	R
	13	29,906	4,755	3,101	65.2	nt	E	E′	S
	14	299,057	47,550	35,723	75.1	nt	F	F′	T
S. aureus[6]	15	10	4.8×10^{-6}	nt	–	12.2	–	U	–
	16	100	4.8×10^{-5}	nt	–	16.6	–	V	–
	17	1,000	4.8×10^{-4}	nt	–	26.3	–	W	–
	18	10,000	4.8×10^{-3}	nt	–	81.0	–	X	–

[1]Measured by the purified collagenase digestion-hydrochloric acid extraction method. [2]IL-6 background: 4.8–7.5 pg/mL. [3]Control with collagen sheet alone. [4]Purified endotoxin from *E. coli* O3:K2a,K2b:H3 ATCC strain. [5]Dried cells of heat-killed *E. coli* O111 strain. [6]Dried cells of heat-killed *S. aureus* 209P strain. [7]nt, not tested.

although the IL-6 production of collagen sheets spiked with dried *S. aureus* cells was obviously lower than that obtained with endotoxin or *E. coli* cells. SEM observation confirmed that collagen sheets containing the purified endotoxin or dried cells of *E. coli* or *S. aureus* exhibited an identical surface structure to the control sheet[9].

3.2 Cutaneous wound dressing study

Collagen sheets containing dried *E. coli* O111 cells (Nos. 8–11, 13, and 14, Table 1) with endotoxin activity of 4.7 (experimental group A), 9.6 (group B), 33.6 (group C), 434 (group D), 3,101 (group E), or 35,723 (group F) EU/mg, respectively, or collagen sheets containing purified endotoxin (Nos. 1–7, Table 1) with endotoxin activity of 1.03 (experimental group G), 2.13 (group H), 8.8 (group I), 96.5 (group J), 755 (group K), 9,308 (group L), or 83,283 (group M) EU/mg, respectively, were used to cover cutaneous wounds.

At 1 week after surgery, the collagen sheet dressings were adherent to the surface of cutaneous wounds through immersion in exudates in all cases in the control group [collagen sheet alone; Fig. 1(a) and Fig. 2(a) comprise the same image of the control group result], dried *E. coli* cells-containing groups (experimental groups A–F, Fig. 1), and endotoxin-containing groups (experimental group G–M, Fig. 2). In addition, intense neutrophil infiltration from the wounded surface was observed and collagen fibers on the wounded surface appeared to have melted and disappeared. The under dressings were composed of granulation tissue with fibrous hyperplasia accompanied by mild infiltration of inflammatory cells and epithelial regeneration was observed at the margin of the wounded areas. There was no noticeable histopathological difference between the control and experimental groups. At 2 weeks after surgery, in both the control and experimental groups the incision sites were covered by a thin stratified squamous epithelium and collagen fiber hyperplasia was detected beneath the epithelium. Results for the control group and experimental groups A–F and G–M are shown in Figs. 3 and 4; Fig. 3(a) and Fig. 4(a) comprise the same image of the control group result.

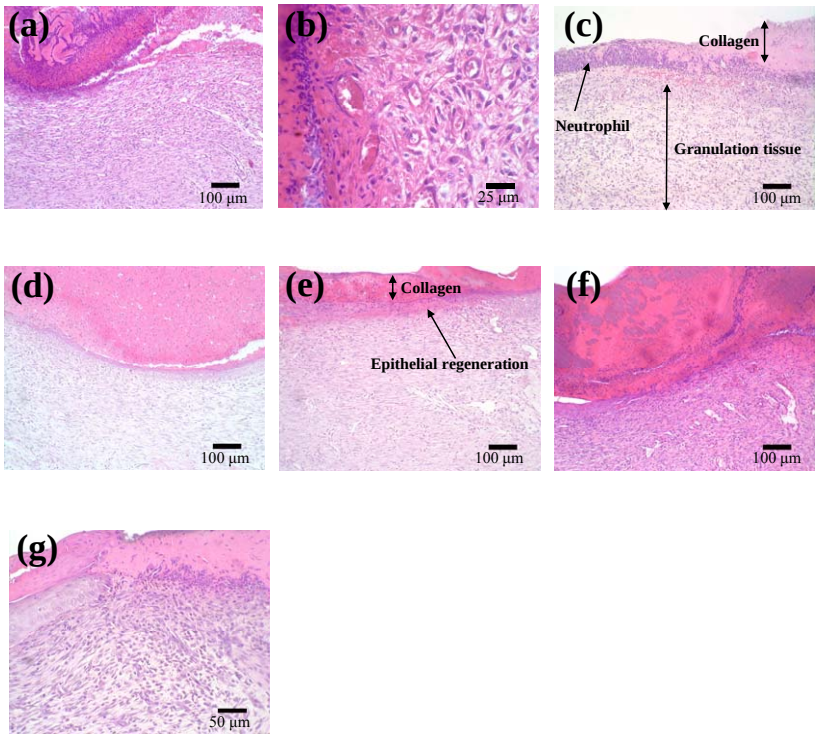

Fig. 1 Histopathology of cutaneous wounds dressed with *E. coli*-containing collagen sheets at 1 week after surgery

At 1 week after surgery, the collagen sheet dressings are adherent to the surface of cutaneous wounds through immersion in exudates in all cases in the control group [collagen sheet alone, (a)] and in the dried *E. coli* cell-containing group [experimental groups A–F, (b–g), respectively). In addition, intense neutrophil infiltration from the wounded surface can be observed and collagen fibers on the wounded surface appear to have melted and disappeared. The under dressings are composed of granulation tissue with fibrous hyperplasia accompanied by mild infiltration of inflammatory cells and epithelial regeneration is observed at the margin of the wounded areas. No noticeable histopathological difference is apparent between the control and experimental groups.

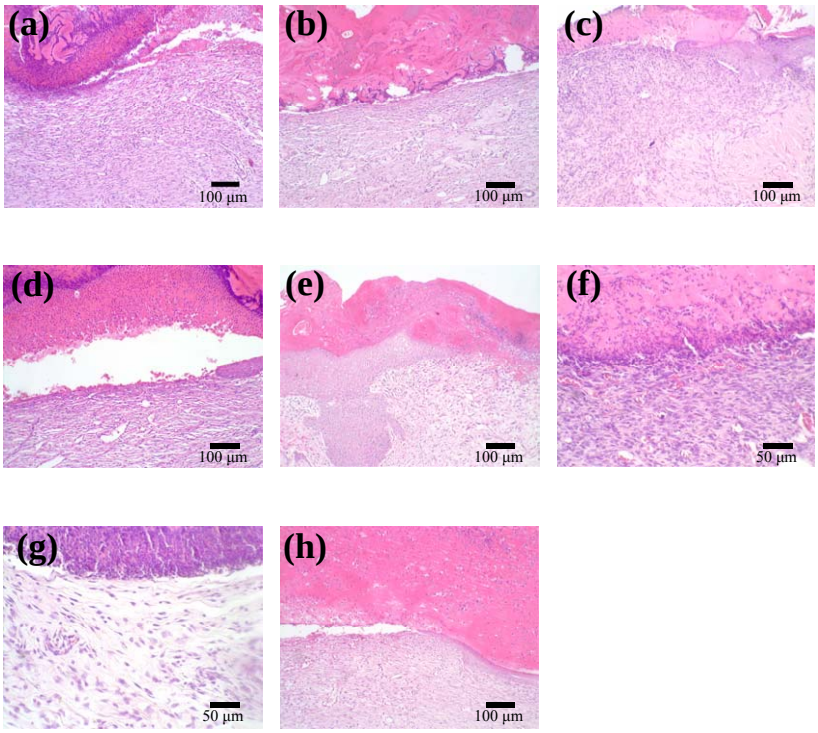

Fig. 2 Histopathology of cutaneous wounds dressed with purified endotoxin-containing collagen sheets at 1 week after surgery

At 1 week after surgery, the collagen sheet dressings are adherent to the surface of cutaneous wounds through immersion in exudates in all cases in the control group [collagen sheet alone, (a)] and in the purified endotoxin-containing group [experimental groups G–M, (b–h), respectively). In addition, intense neutrophil infiltration from the wounded surface can be observed and collagen fibers on the wounded surface appear to have melted and disappeared. The under dressings are composed of granulation tissue with fibrous hyperplasia accompanied by mild infiltration of inflammatory cells and epithelial regeneration is observed at the margin of the wounded areas. No noticeable histopathological difference is apparent between the control and experimental groups.

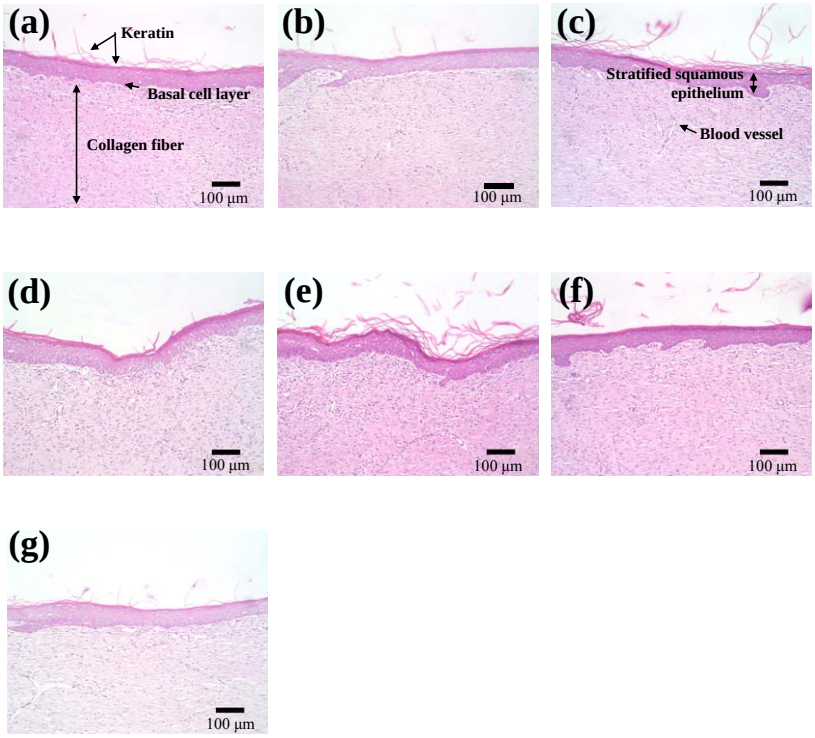

Fig. 3 Histopathology of cutaneous wounds dressed with *E. coli*-containing collagen sheets at 2 weeks after surgery

At 2 weeks after surgery, in both the control and experimental groups, the incision sites are covered by a thin stratified squamous epithelium and collagen fiber hyperplasia can be detected beneath the epithelium; results for the control group (a) and experimental groups A–F (b–g, respectively) are shown.

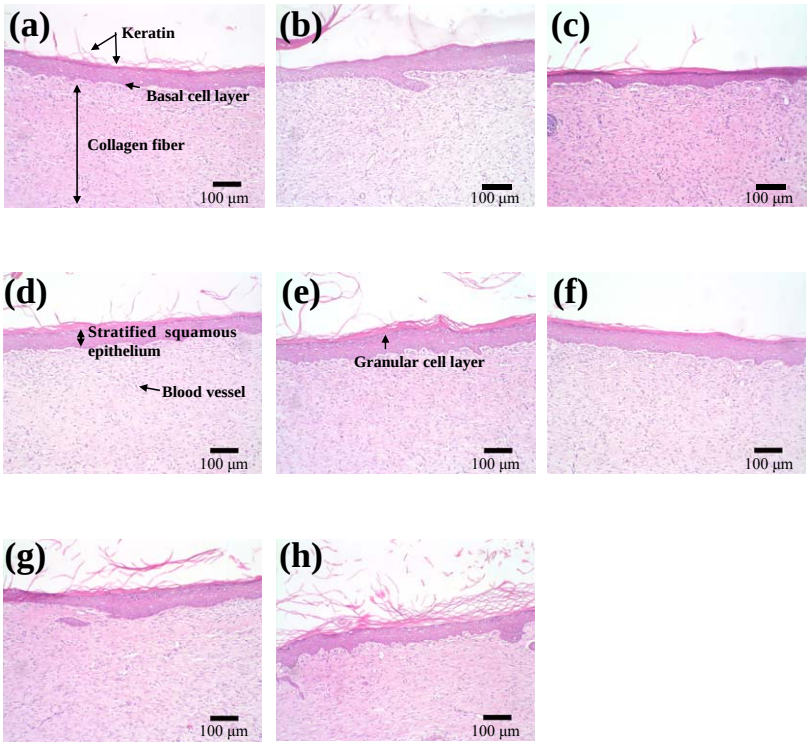

Fig. 4 Histopathology of cutaneous wound dressed with purified endotoxin-containing collagen sheets at 2 weeks after surgery

At 2 weeks after surgery, in both the control and experimental groups, the incision sites are covered by a thin stratified squamous epithelium and collagen fiber hyperplasia can be detected beneath the epithelium; results for the control group (a) and experimental groups G–M (b–h, respectively) are shown.

3.3 Subcutaneous implant study

The same collagen sheets used for the cutaneous wound dressing study (Nos. 1–11, 13, and 14; experimental group A′–M′) were used for the subcutaneous implant study.

At 1 week after implantation, the collagen sheets of the control group [Fig. 5(a) and Fig. 6(a) comprise the same image of the control group result] and experimental groups A′–D′ [Fig. 5(b–e)] were encapsulated in a thin layer of fibrous connective tissue with occasional invasion by fibroblasts and capillary vessels. No particular inflammatory response was observed except for a minor population of infiltrating lymphocytes. Conversely, in experimental group E′, infiltration of numerous neutrophils and some lymphocytes was observed and the sheet was encapsulated in fibrotic inflammatory granulation tissue [Fig. 5(f)]. In group F′, a higher level of neutrophil infiltration was observed in the sheet [Fig. 5(g)]. In comparison, in the endotoxin-containing groups (experimental groups G′–M′), only a few fibroblasts and low levels of infiltrated neutrophils, lymphocytes, and plasma cells were observed inside the collagen sheets even in experimental group M′, which contained the highest dose of endotoxin (Fig. 6).

At 2 weeks after implantation, capillary vessel invasion and an increase in fibroblasts were observed in control group [Fig. 7(a) and Fig. 8(a) comprise the same image of the control group result] and experimental group A′–D′ collagen sheets [Fig. 7(b–e)]. Lymphocyte infiltration into these sheets was only very slight with no inflammatory response observed. Conversely, in experimental group E′, infiltration of numerous macrophages around the implanted sheet was observed [Fig. 7(f)]. In experimental group F′, infiltration of neutrophils was observed as well as macrophages, in addition to degeneration of the implanted collagen fibers [Fig. 7(g)]. In comparison, in the endotoxin-containing groups (experimental groups G′–M′), no noticeable inflammatory response was observed whereas invasion of the capillary vessels and an increase in fibroblasts could be seen in the implanted sheets (Fig. 8).

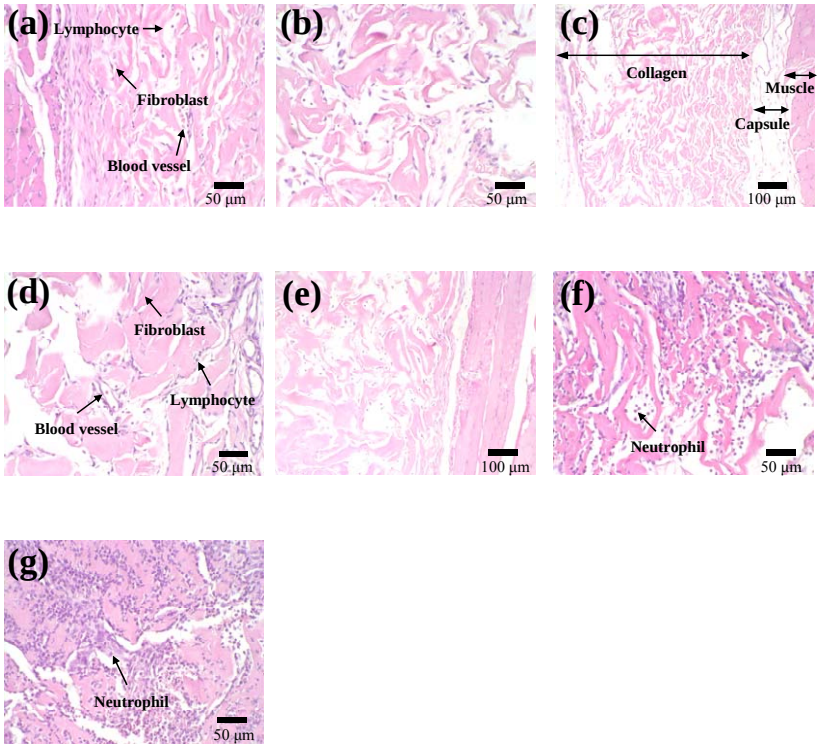

Fig. 5 Histopathology after 1 week from subcutaneous implantation of *E. coli*-containing collagen sheets

At 1 week after implantation, collagen sheets of the control group (a) and experimental groups A′–D′ (b-e, respectively) are encapsulated in a thin layer of fibrous connective tissue with occasional invasion by fibroblasts and capillary vessels. No particular inflammatory response can be observed except for a minor population of infiltrating lymphocytes. Conversely, in experimental group E′ (f), infiltration of numerous neutrophils and some lymphocytes can be observed and the sheet appears encapsulated in fibrotic inflammatory granulation tissue. In the case of experimental group F′ (g), a higher level of neutrophil infiltration is apparent in the sheet.

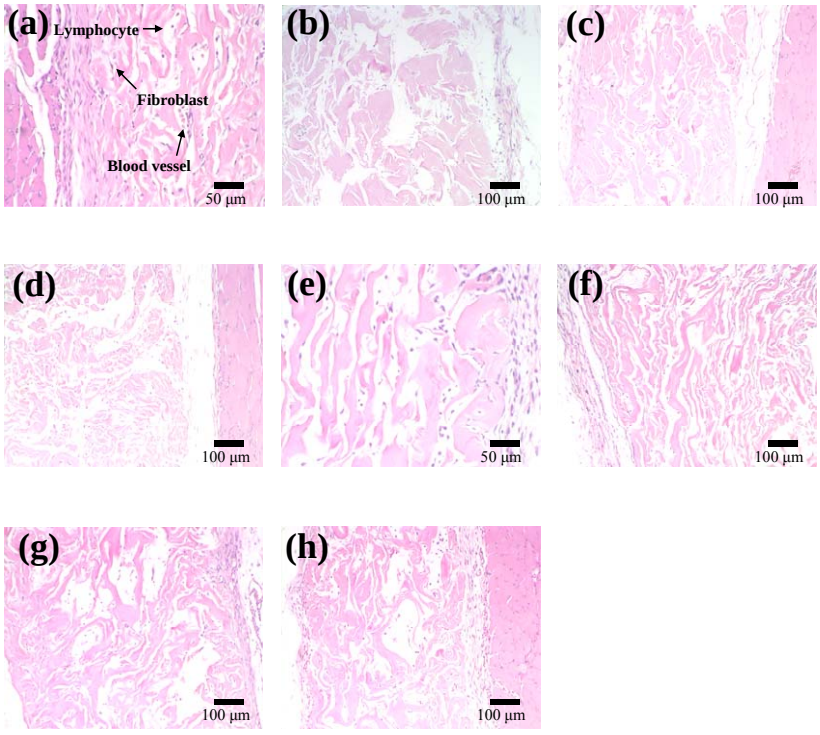

Fig. 6 Histopathology after 1 week from subcutaneous implantation of purified endotoxin-containing collagen sheets

At 1 week after implantation, only a few fibroblasts and low levels of infiltrated neutrophils, lymphocytes, and plasma cells were observed inside the collagen sheets; results for the control group (a) and experimental groups G′–M′ (b–h, respectively) are shown.

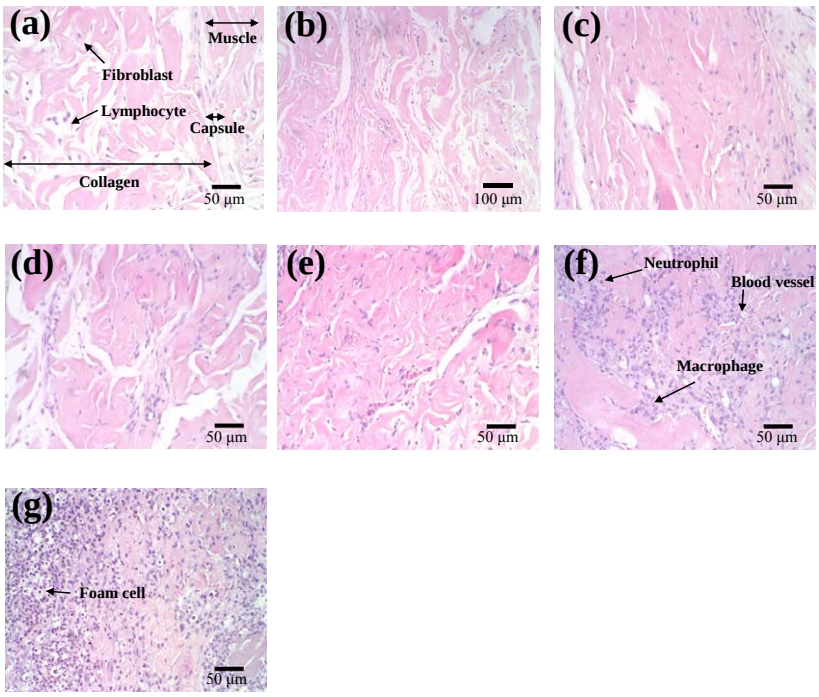

Fig. 7 Histopathology after 2 weeks from subcutaneous implantation of *E. coli*-containing collagen sheets

At 2 weeks after implantation, for collagen sheets of the control group (a) and experimental groups A′–D′ (b–e, respectively), invasion of the capillary vessels and an increase in fibroblasts can be observed. The infiltration of lymphocytes into these sheets is only very slight with no inflammatory response observed. Conversely, in experimental group E′ (f), infiltration of numerous macrophages around the implanted sheet is apparent. In experimental group F′ (g), infiltration of neutrophils can be observed as well as macrophages, in addition to degeneration of the implanted collagen fibers.

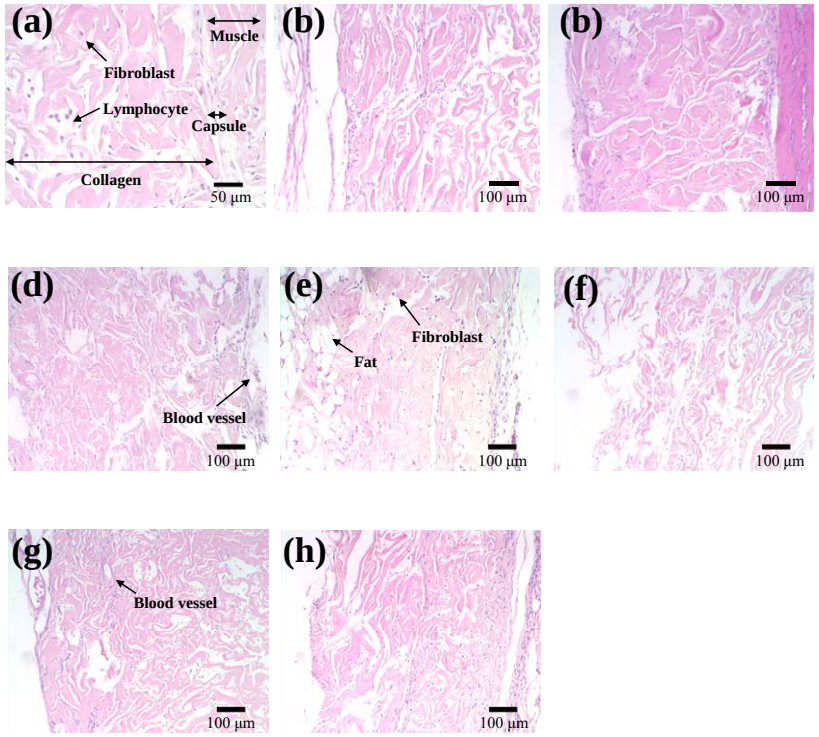

Fig. 8 Histopathology after 2 weeks from subcutaneous implantation of purified endotoxin-containing collagen sheets

At 2 weeks after implantation, no noticeable inflammatory response is apparent whereas invasion of the capillary vessels and an increase in fibroblasts can be observed in the implanted sheets; results for the control group (a) and experimental groups G′–M′ (b–h, respectively) are shown.

3.4 Intraperitoneal implant study

Collagen sheets containing dried *E. coli* O111 cells (Nos. 8–14; experimental groups N–T, respectively) were used for the intraperitoneal implant study.

Representative images used for the pathological diagnosis of biological effects at 1 week after surgery are shown in Fig. 9. The pathological observations for groups N and O were the same as for the control group. In these groups, the implanted sheets were adhered to the hepatic capsule *via* fibrous connective tissue and the adjoining areas exhibited invasion of small numbers of capillary vessels and fibroblasts and mild lymphocyte infiltration [Fig. 9(a–c)]. Group P pathological observations were also comparable, except that lymphocyte infiltration was observed slightly more frequently [Fig. 9(d)]. Additionally, a slight increase in capillary vessel and fibroblast invasion was observed in groups Q and R with infiltration of lymphocytes and a few plasma cells [Fig. 9(e, f)]. Abscess formation was observed in groups S and T. The implanted sheets in these groups were adhered to the surface of the liver by chronic inflammatory granulation tissue that was accompanied by lymphocyte, neutrophil, and macrophage infiltration [Fig. 9(g, h)].

3.5 Effect of dried *S. aureus* cells

To evaluate the difference between gram-negative and gram-positive bacteria, a subcutaneous implant study was conducted using collagen sheets containing 10–10,000 ng/mg dried *S. aureus* cells (Nos. 15–18; experimental groups U–X, respectively). Evaluation of the biological effects at 1 week after surgery (Fig. 10) revealed that the pathological appearance of experimental groups U and V was the same as that of the control group [Fig. 10(a–c); Fig. 5(a), Fig. 6 (a), and Fig. 10(a) comprise the same image of the control group result]. All implanted sheets were encapsulated in a thin layer of fibrous connective tissue with occasional invasion of fibroblasts and capillary vessels. No particular inflammatory response was observed except for a minor population of

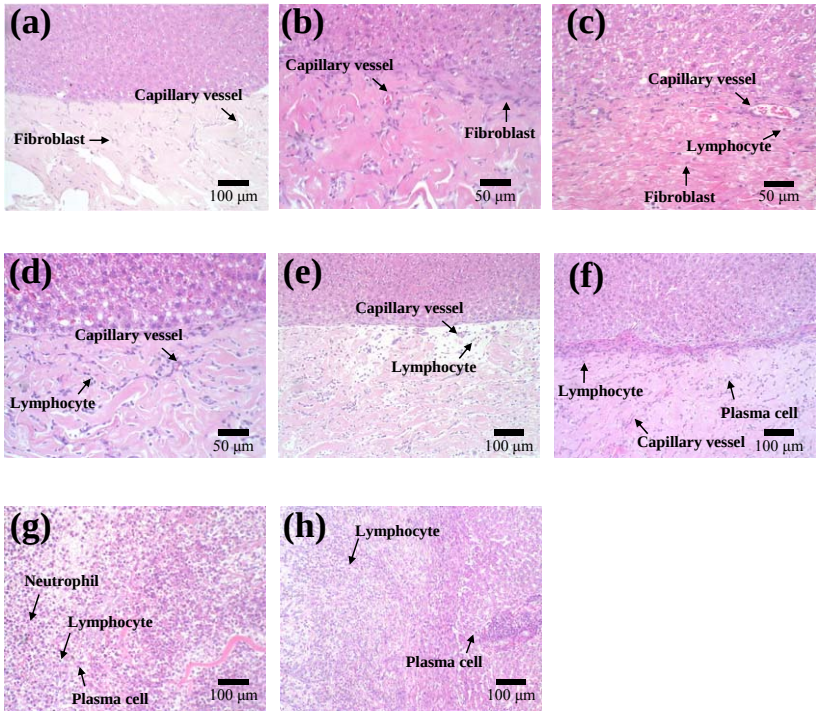

Fig. 9 Histopathology after 1 week from intraperitoneal implantation of *E. coli*-containing collagen sheets

The pathological observations for groups N (b) and O (c) were the same as for the control group (a). In these groups, the implanted sheets have adhered to the hepatic capsule *via* fibrous connective tissue and the adjoining areas exhibits invasion of small numbers of capillary vessels and fibroblasts and mild infiltration of lymphocytes. The pathological observations for experimental group P (d) are mostly similar to those of experimental group N and O except that lymphocyte infiltration can be observed slightly more frequently. Additionally, in the case of the experimental groups Q (e) and R (f), a slight increase in the invasion of capillary vessels and fibroblasts and infiltration of lymphocytes and a few plasma cells can be observed. Abscess formation is apparent in experimental groups S (g) and T (h). The implanted sheets in these groups are adhered to the surface of the liver by chronic inflammatory granulation tissue along with accompanying lymphocyte, neutrophil, and macrophage infiltration.

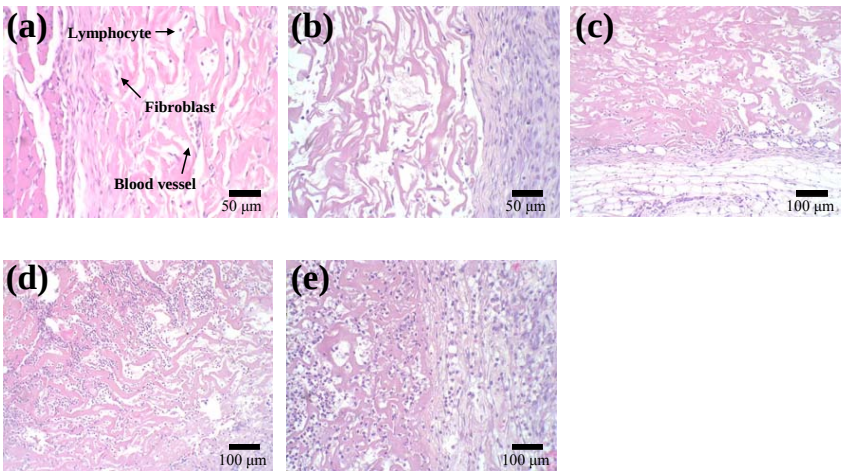

Fig. 10 Histopathology after 1 week from subcutaneous implantation of *S. aureus*-containing collagen sheets

The pathological observations of the experimental groups U (b) and V (c) are the same as those of the control group (a). All implanted sheets are encapsulated in a thin layer of fibrous connective tissue with occasional invasion of fibroblasts and capillary vessels. No particular inflammatory response is apparent except for a minor population of infiltrating lymphocytes. In experimental group W (d), 3 cases exhibited mild infiltration of lymphocytes into the collagen sheets, which were encapsulated in chronic inflammatory granulation tissue, whereas the remaining case (d) exhibited infiltration of numerous neutrophils into the collagen sheet encapsulated in chronic inflammatory granulation tissue. A similar inflammatory response can be observed in all cases in the experimental group X (e).

infiltrating lymphocytes. In group W, 3 cases exhibited mild lymphocyte infiltration into the collagen sheets, which were encapsulated in chronic inflammatory granulation tissue, whereas the remaining case exhibited infiltration of numerous neutrophils into the encapsulated collagen sheet [Fig. 10(d)]. A similar inflammatory response was observed in all cases in experimental group X [Fig. 10(e)].

4. Discussion

The present study constitutes the first report to evaluate the biological responses of biomaterials with microbial contamination at several sites in rats. It is very preliminary with respect to providing non-quantitative data with small numbers of samples; nevertheless, the results yielded in this study may be useful as basic data for the risk assessment of naturally derived biomaterials such as collagen, chitin, chitosan, and alginate that are often contaminated with microbial components including endotoxin.

4.1 Cutaneous wound dressing study

The melting and disappearance of collagen fibers observed in the experimental cutaneous wound dressings were likely to have been caused by enzymatic reactions accompanying neutrophil necrosis. In addition, the relatively low level of inflammatory change at the wounds suggested that the collagen sheets covering the wounds effectively protected them against outside infection. There were no noticeable histopathological differences between the control and experimental groups in regard to endotoxins or bacterial cells, indicating that bacterial components did not affect cutaneous wound healing. As body fluid at a wound infiltrates from the inside tissue to the wounded surface, the likelihood of absorption of bacterial components by the body is considered to be low even if dressing materials are contaminated by endotoxins or dead bacteria.

4.2 Subcutaneous and intraperitoneal implant studies

In the subcutaneous implant study in rats, similar to the control group, experimental groups implanted with collagen containing endotoxins (experimental groups G′–M′) or containing low concentrations of bacterial cells (groups A′–D′) did not exhibit induction of inflammatory changes. However, collagen containing high bacterial cell concentrations (groups E′ and F′) induced strong suppurative inflammation with visible neutrophil infiltration, even at 2 weeks after implantation in experimental group F′. The macrophage infiltration observed in these 2 groups was considered to represent a foreign body reaction against neutrophil residue and denatured collagen fibers.

In comparison, in the intraperitoneal implant

study, a slight increase of lymphocyte infiltration was observed in the experimental groups implanted with collagen sheets containing dried *E. coli* cells with LAL activity of 33.6–1,163 EU/mg (groups P–R). However, mild infiltration of lymphocytes was observed in the control group and we therefore concluded that the observed increase of lymphocyte infiltration did not constitute a morbid condition. Conversely, a substantial inflammatory reaction was induced in the experimental groups implanted with collagen sheets containing dried *E. coli* cells with LAL activity of 3,101 EU/mg or higher (groups S and T). Overall, the substantial inflammatory response observed in the subcutaneous and intraperitoneal implant studies could be explained by the effects of bacterial components because the collagen sheet itself is bio-inert and does not induce substantial inflammatory responses. Considering that a dose-dependent inflammatory response was observed in subcutaneous and intraperitoneal implant studies, these experimental systems may be useful as evaluation methods for the microbial safety of biomaterials corresponding to specific applications.

As discussed in our previous study[9], it is speculated that purified endotoxin is a hapten and therefore could not be recognized as a foreign substance in the experimental condition, which may explain in part why little difference was observed in the histopathological features between the experimental groups implanted with sheets containing purified endotoxins and control groups in the subcutaneous implant study. In comparison, a substantial inflammatory reaction was induced in the experimental groups subcutaneously implanted with collagen sheets containing dried *S. aureus* cells at amounts of 1,000 ng/mg or higher (groups W and X). This result suggests that contamination of materials applied *in subcutis* with not only gram-negative bacteria but also gram-positive bacteria can induce inflammatory response in the region. Accordingly, the indicator chosen to identify the biological contamination of medical devices should be able to discern contamination of various types of microbes including gram-negative and gram-positive bacteria. Endotoxin contamination in medical devices and materials, for example, always indicates the presence of live or dead gram-negative bacteria. However, it also may account for the presence of other microbes, such as gram-positive bacteria and fungi, because gram-negative bacteria generally co-exist with various other types of microbes. Therefore, the detection of endotoxin activity in medical materials should be interpreted as a reflection of the level of general bacterial cell contamination. Thus, endotoxin appears to represent a promising marker for evaluating the microbial contamination of medical materials.

Notably, the above results suggested that the susceptibility to microbe-contaminated biomaterials differs among the sites to which the biomaterials are applied. Thus, the endotoxin limits of medical devices or materials should be established with consideration of the site of application. Microbial contaminants can induce not only local but also regional or systemic histological effects as well. Therefore, to obtain a better understanding of the microbial safety of biomaterials and establish endotoxin limits, systemic effects should be evaluated in future studies.

5. Conclusions

This study preliminarily clarified several critical points in evaluating the microbial contamination of biomaterials as focused on inflammatory responses at application sites: (1) susceptibility differs among the sites of application, and (2) bacterial components other than endotoxin contribute to the topical inflammatory response. A dose-dependent inflammatory response could be observed in both subcutaneous and intraperitoneal implants. Furthermore, wound sites appeared to be highly resistant to the microbial contamination of dressing materials.

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