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The Influence of D-penicillamine on Granuloma Formation in Implanted Collagen Sponges in Ferritin-sensitized and Non-sensitized Guinea Pigs

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ABSTRACT. In ferritin-sensitized and non-sensitized guinea pigs, using the method of granuloma formation within peritoneally implanted collagen sponges that were either impregnated or not impregnated with ferritin, the effects of D-penicillamine on the cellular aspects of thus created granulomas were quantitatively evaluated by scheduled administration. Daily administration of D-penicillamine (200 mg/kg) for 14 days before and 10, 14 or 18 days after the sponge implantation decreased small mononuclear cell population (SMN) which consisted of lymphocytes, small monocytes and plasma cells, on the 10th-18th days in sponge granulomas in both sensitized and non-sensitized groups, but the administration for 14 days before the sponge implantation higher increased SMN infiltration on the 14th day in sensitized group. This phenomenon disclosed a close association with a marked infiltration of perivascular lymphocytes and plasma cells in the presence of granulomatous inflammation with the involvement of the delayed hypersensitivity reaction. The enhancement of the dominant lymphocytes infiltrating perivascularly by D-penicillamine may be mediated by chemotactic factors such as lymphokines inducing the delayed hypersensitivity reaction. On the other hand, the frequency of foreign body multinucleated giant cells was decreased by daily administration of D-penicillamine before and after implantation in both groups. This decrease in the number of the giant cells is considered to be due to inhibition of accumulation in the sponges of macrophages and/or monocytes to be fused.

Key words : D-penicillamine — Granulomatous inflammation — Sponge implantation

It is a fact already well known pathologically that an insoluble material, if large enough, inserted into organism is absorbed, ingested and eliminated by granulomatous inflammatory reactions. It has also been confirmed that when an insoluble biomaterial containing artificial spongy tissue is intraperitoneally or subcutaneously implanted in animals, the interstices within the spongy tissue become filled with cellular components of the inflammatory reaction, followed by a development of foreign body reactions against the spongy biomaterial, resulting eventually in disappearance of this spongy biomaterial from the organism.[1,2] On the other hand, the intraperitoneal implantation of this spongy biomaterial impregnated with an antigen induces the accumulation of peritoneal exudate cells responding to antigen within the implanted sponge in sensitized

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animals.[3,4] When cellular reaction for irritant material proceeds further in the implanted spongy tissue, the in vivo spongy biomaterial that provided the site of antigen-antibody reaction induces the granulomatous inflammation in which cell-mediated immune responses are involved.[5]

In recent years, it has been proved that D-penicillamine possesses the effect of intensifying accumulation of mononuclear cells at the sites of the delayed hypersensitivity reaction induced in sensitized animals.[4,6,7] However, it has not been attempted to evaluate the effects of D-penicillamine on cellular reactions in the presence of granulomatous inflammation with the involvement of the delayed hypersensitivity reaction.

In this study, using the method of the granuloma formation within cross-linked collagen sponges which are the irritant nature of the sponge itself and allow a stable preservation of its original form in animals for a duration long enough for the development of the granulomatous inflammatory reactions,[8] the collagen sponges were either impregnated or not impregnated with ferritin as an antigen. Thus the involvement of the immune response to ferritin was either induced or not induced at the site of collagen sponge implantation to provoke the foreign body granuloma against the spongy biomaterial, it follows as a consequence that the foreign body granuloma is produced within this collagen sponge tissue. The cytologic analysis of thus created granulomas was undertaken, and a quantitative evaluation of the effects of D-penicillamine on the cell constitution of these granulomatous tissues was also attempted in this study by a scheduled administration.

MATERIALS AND METHODS

Animals

Female English Hartley guinea pigs weighing between 300–450 g were used throughout these investigations.

Immunization

Guinea pigs were sensitized by injecting into the foot pads 800 μg ferritin (cadmium free, Nutritional Biochemicals Co.) emulsified in complete Freund's adjuvant (Difco Lab.). They were challenged 21 days later by implanting collagen sponges ($1 \times 1 \times 1.5$ cm, Koken Co.) containing 100 μg ferritin (ferritin sponge) into the peritoneal cavity.

Treatment of guinea pigs

Sensitized and non-sensitized guinea pigs received ferritin sponges or PBS sponges (collagen sponges impregnated with phosphate buffered saline). They were given no D-penicillamine (group 1, served as controls), D-penicillamine (200 mg/kg Takeda Chemical Industries Ltd.) perorally once per day for 14 days before sponge implantation (group 2) or D-penicillamine (200 mg/kg) perorally once per day for 14 days before and 5, 7, 10, 14 or 18 days after sponge implantation (group 3).

Quantification of accumulated cells into the sponges

The guinea pigs were killed 1, 2, 3, 5, 7, 10, 14 or 18 days later. The

collagen sponges were immediately removed, fixed in 10% neutral formalin, and embedded in paraffin. Serial sections at 5 μm thick were stained with hematoxylin and eosin for morphological examination. Five sections per sponge were measured, and the number of cells were counted per unit area in 8 fields or more, as which the superficial areas of sponges were selected. The mean of the cell counts for 6 individual sponges was given.

RESULTS

1) Morphological study on sponge granulomas

1-3 days after implantation :

The collagen sponges implanted intraperitoneally in guinea pigs of all the experimental groups showed the continued accumulation of mononuclear cells (Mono) and polymorphonuclear leukocytes (Poly). The Mono included peritoneal macrophages, monocytes, and lymphocytes. The Poly were predominantly neutrophils and a few eosinophils. There was a variation in intensity of cellular infiltration between the implanted sponges in sensitized and in non-sensitized groups.

5-7 days after implantation :

The presence of Mono was predominant in the superficial interstices of the implanted sponges in sensitized and in non-sensitized groups. These Mono could be divided according to cell size into two populations. Peritoneal macro-

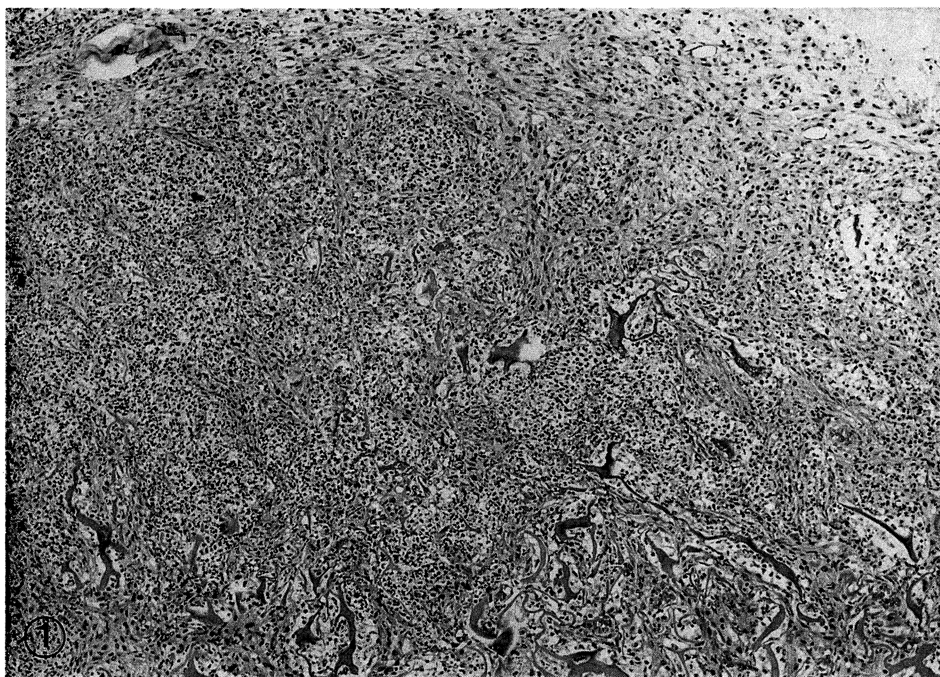

Fig. 1. Ferritin sponge 7 days after implantation in sensitized guinea pigs, showing an accumulation of prominent large mononuclear cells in outside sponge interstices, and old clusters of degenerated polymorphonuclear cells in inner sponge interstices. Hematoxylin and eosin ; ×50.

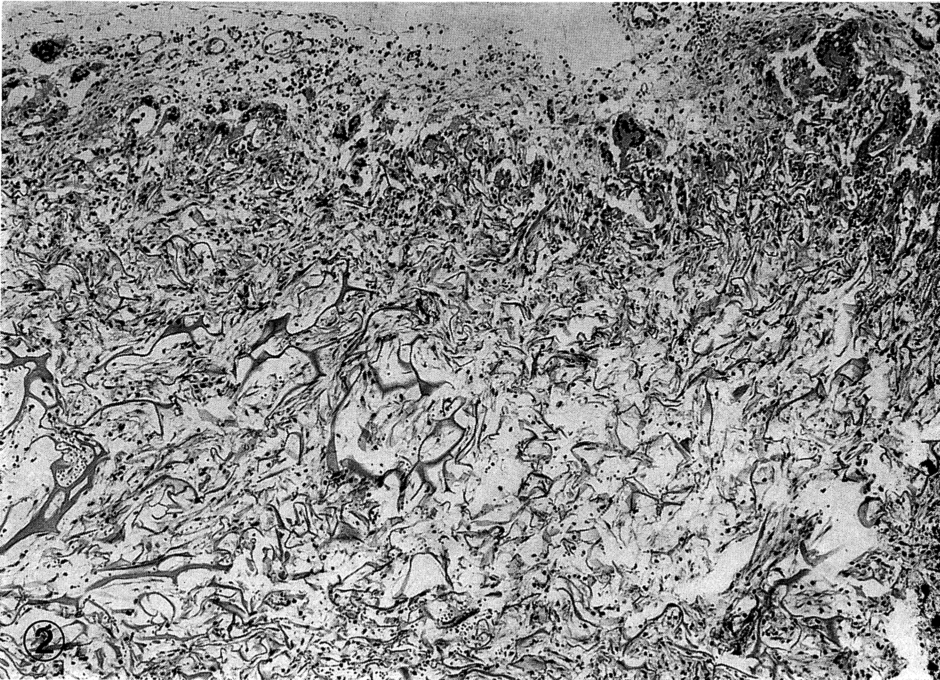

Fig. 2. PBS sponge 10 days after implantation in non-sensitized guinea pigs, showing increased number of multinucleated giant cells in sponge interstices. Hematoxylin and eosin ; ×50.

Fig. 3. Ferritin sponge 14 days after implantation in non-sensitized guinea pigs, showing constructed capillaries, and diffuse lymphocyte and plasma cell infiltration in the perivascular collections. Hematoxylin and eosin ; ×50.

phages were represented in size by the large mononuclear cell population (LMN) which included fibroblasts and epithelioid cells. The exudate lymphocytes were represented in size by the small mononuclear cell population (SMN) which included small monocytes and plasma cells. Multinucleated giant cells containing three or more nuclei appeared approximately the 5th day after implantation in the outermost layer of the sponges and most of them proved to belong to the foreign body type. The Poly that remained on the 5th day after implantation began forming large or small masses with necrotic signs in the depth of the implanted sponges. The interstices outside the deep portion showed an accumulation of Mono in a belt form from the outer layer (Fig. 1).

10–18 days after implantation :

Approximately 10 days after implantation, a capillary proliferation occurred in the surface layer of every implanted sponge and extended into their deeper areas. The surface layer of the implanted sponges presented as fibrosed, appearing as though surrounded by mononuclear and multinucleated giant cell layer, had encapsulated the implanted collagen sponges (Fig. 2). Furthermore, as the granuloma proceeded to develop, in the mature granulomatous tissues situated at the well-vascularized regions, there was a marked infiltration of perivascular small mononuclear cells such as lymphocytes or plasma cells (Fig. 3). This cellular infiltration was most notable 14 days after the implantation in both the sensitized and non-sensitized groups.

On the other hand, approximately 18 days after the implantation, the volumes of implanted sponges, while retaining their original shape, gradually dwindled away to nothing in both groups.

2) Quantitative study on sponge granulomas

The quantitative determination was attempted on the sequential behaviors of each cell population that constituted the granulomatous tissues per unit area

TABLE 1. The accumulation of mononuclear cells and polymorphonuclear leukocytes into the intraperitoneally implanted collagen sponges

Group		number of mononuclear cells and polymorphonuclear leukocytes		
		time after implantation		
		1 d	2 d	3 d
Sensitized animals				
Ferritin sponge implants	[a]Mono	1042±74[1]	1515±88	1531±97
	[b]Poly	2577±169	1115±93	704±73
Non-sensitized animals				
Ferritin sponge implants	Mono	440±64	793±82	843±91
	Poly	1072±110	601±75	442±32
PBS sponge implants	Mono	715±67	1036±80	1095±87
	Poly	683±72	328±24	114±12

Each group consisted of 6 guinea pigs. [a]Mononuclear cells (Mono) were counted as the the total number of macrophages, monocytes, and lymphocytes. Dominant neutrophils were regarded as [b]polymorphonuclear cells (Poly). Results[1] are expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges $\pm$SE. All values are significantly different ($p<0.025\sim p<0.001$) compared to sensitized animals.

TABLE 2. Effect of D-penicillamine on the accumulation of large mononuclear cells into the intraperitoneally implanted collagen sponges

Group No. and treatment	number of large mononuclear cells[1] time after implantation 5 d	7 d	10 d	14 d	18 d
Sensitized animals					
Ferritin sponge implants					
1. Control no treated	577±46[2]	720±72	1333±106	1280±99	1119±83
2. [a]D-penicillamine treated	623±65	778±73	1150±83	1075±113	1011±89
3. [b]D-penicillamine treated	514±43	696±65	1045±112	984±118	863±78
Non-sensitized animals					
Ferritin sponge implants					
1. Control no treated	588±40	702±63	1317±98	1224±154	985±110
2. [c]D-penicillamine treated	567±50	722±36	1190±103	1222±158	1006±81
3. [d]D-penicillamine treated	520±42	669±54	1025±106	1154±112	947±117
PBS sponge implants					
1. Control no treated	620±41	660±44	1005±89	1020±86	894±66
3. [d]D-penicillamine treated	608±59	683±61	922±57	973±54	919±74

Each group consisted of 6 guinea pigs. Large mononuclear cells[1] were counted as the total number of mononuclear cells which ranged in size larger than 12 μm in diameter. [a]D-penicillamine administered perorally at a dose of 200 mg/kg daily for 14 days before challenge. [b]D-penicillamine administered perorally at a dose of 200 mg/kg daily for 14 days before and 5, 7, 10, 14 or 18 days after challenge. [c]D-penicillamine administered perorally at a dose of 200 mg/kg daily for 14 days before collagen sponge implantation. [d]D-penicillamine administered perorally at a dose of 200 mg/kg daily for 14 days before and 5, 7, 10, 14 or 18 days after collagen sponge implantation. Results[2] are expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges ±SE.

of implanted sponges in no treated and in treated groups.

1. Time-associated changes in cellular accumulation in no treated groups

In the sensitized animals, the numbers of Mono and Poly per unit area of the sections of ferritin sponges were greater than in the non-sensitized animals 1-3 days after the implantation due to delayed hypersensitivity reaction (Table 1). After 5 days, however, LMN became predominant in every implanted sponge, and no significant differences were observed between the two groups in the number of cells per unit area of the sponge sections (Table 2). In the sensitized and non-sensitized animals, LMN in the PBS sponges showed a lower accumulation than these in the ferritin sponges only on the 10th day. On the other hand, the accumulation of SMN in every sponge granuloma reached their maximum peak on the 14th day after implantations (Table 3). This resulted from a summation of an increase in the amount of lymphocytes and plasma cells infiltrating perivascularly. However, this summation included the total accumulation of exudate lymphocytes and small monocytes in addition to the dominant perivascular lymphocytes. The amount of SMN was greatest in the ferritin sponges of the non-sensitized animals.

Foreign body multinucleated giant cells began to appear from about 5 days after the implantation in both sensitized and non-sensitized animals, and their numbers per unit area of the sections became largest after 10 days

TABLE 3. Effect of D-penicillamine on the accumulation of small mononuclear cells into the intraperitoneally implanted collagen sponges

Group No. and treatment	number of small mononuclear cells[1] time after implantation 5 d	7 d	10 d	14 d	18 d
Sensitized animals					
Ferritin sponge implants					
1. [a]Lymph-mono	154±22[2]	165±19	330±39	453±41	327±48
[b]Plasma cell	18±3	24±6	63±9	202±34	159±22
2. Lymph-mono	184±15	213±30	502±49*	800±74***	636±60**
Plasma cell	21±4	29±7	45±6	189±25	145±33
3. Lymph-mono	139±17	140±21	138±17**	295±24*	157±24*
Plasma cell	14±3	20±7	27±4*	99±23*	63±14*
Non-sensitized animals					
Ferritin sponge implants					
1. Lymph-mono	192±29	203±28	501±54	978±137	706±69
Plasma cell	26±5	41±11	188±20	495±66	385±59
2. Lymph-mono	181±20	209±25	452±39	1002±155	627±61
Plasma cell	22±5	30±9	129±24	327±59	297±42
3. Lymph-mono	167±20	196±23	272±73*	400±94**	320±74***
Plasma cell	22±6	29±9	53±13***	190±45**	155±22***
PBS sponge implants					
1. Lymph-mono	120±16	129±15	239±18	297±35	246±37
Plasma cell	15±3	15±4	19±3	36±9	32±8
3. Lymph-mono	119±18	125±19	127±15*	137±20*	119±19*
Plasma cell	14±3	21±6	25±4	29±9	31±7

See Table 2 for each group. Small mononuclear cells[1] which ranged in size 8 to 12 μm and smaller in diameter were separately counted as the amount of [a]lymphocytes and small monocytes (Lymph-mono), and the number of [b]plasma cells (Plasma cell). Results[2] are expressed as mean counts per 1(=0.0625×16) mm^2 of 30 sections from 6 sponges ±SE. *$p<0.05$, **$p<0.025$, ***$p<0.001$ versus control group 1.

(Table 4). The frequency of giant cells was affected by the presence or absence of ferritin in the sponge rather than the sensitization or non-sensitization of the hosts. The number of nuclei per giant cell varied from 3 to 89.

2. Effects of D-penicillamine on cellular accumulation

D-penicillamine administration did not affect the number of LMN per unit area of the sections of the sponges 5–18 days after the implantation in either the sensitized or non-sensitized group (Table 2). It, however, affected the infiltration of SMN that consisted of lymphocytes, monocytes, and plasma cells, on the 10th–18th days in the sponge granulomas (Table 3). Daily administration of D-penicillamine for 14 days before and 10, 14 or 18 days after the implantation decreased the amount of lymphocytes and small monocytes as well as plasma cells in both sensitized and non-sensitized animals, but the administration for 14 days before the implantation increased lymphocyte-small monocyte infiltration in the sensitized animals.

The frequency of foreign body multinucleated giant cells per unit area of the sponge sections was decreased by daily administration of D-penicillamine

TABLE 4. Effect of D-penicillamine on the formation of multinucleated giant cells into the intraperitoneally implanted collagen sponges

Group No. and treatment	number of multinucleated giant cells (Giant cell) and nuclei per cell — time after implantation				
	5 d	7 d	10 d	14 d	18 d
Sensitized animals					
Ferritin sponge implants					
1. Giant cell	6.9±1.8[1]	14.8±3.0	75.4±19.7	59.5±12.7	27.2±4.6
(Nuclei/cell)	(5.4±0.3)[2]	(6.1±0.4)	(7.6±0.4)	(9.4±0.5)	(8.5±0.6)
2. Giant cell	5.7±1.2	17.7±4.7	42.1±11.8	48.5±8.1	35.9±7.3
(Nuclei/cell)	(5.4±0.4)	(6.5±0.5)	(7.0±0.4)	(9.8±0.4)	(8.8±0.4)
3. Giant cell	1.1±0.3***	1.1±0.3***	6.1±1.4***	22.5±4.7*	14.4±2.4*
(Nuclei/cell)	(4.1±0.4*)	(4.7±0.4*)	(6.0±0.5*)	(7.9±0.5*)	(7.7±0.5)
Non-sensitized animals					
Ferritin sponge implants					
1. Giant cell	3.9±0.9	11.4±2.5	69.3±11.3	42.3±8.9	29.1±5.4
(Nuclei/cell)	(6.9±0.9)	(9.3±0.7)	(16.2±0.9)	(12.5±1.1)	(8.1±0.6)
3. Giant cell	2.3±0.9	4.5±1.0*	27.8±6.1**	20.1±4.9*	14.5±3.7*
(Nuclei/cell)	(4.6±0.8*)	(7.3±0.7*)	(12.5±1.1**)	(12.2±0.8)	(8.6±0.5)
PBS sponge implants					
1. Giant cell	38.6±7.5	62.3±10.1	286.1±40.6	149.7±33.3	82.4±18.5
(Nuclei/cell)	(5.2±0.2)	(7.8±0.5)	(12.2±0.7)	(11.0±0.6)	(9.3±0.5)
3. Giant cell	29.5±5.2	36.3±5.2*	63.9±8.3***	69.0±10.0*	33.7±7.4*
(Nuclei/cell)	(4.3±0.4*)	(6.3±0.4*)	(8.8±0.6***)	(10.7±0.4)	(9.0±0.6)

See Table 2 for each group. Results[1] are expressed as mean counts per 100 (10×10) mm^2 of 30 sections from 6 sponges ±SE. (Results)[2] were expressed as mean counts of nuclei per giant cell ±SE. $*p<0.05$, $**p<0.025$, $***p<0.001$ versus control group 1.

before and after the implantation in both sensitized and non-sensitized animals (Table 4). This decrease occurred in association with a decrease in the mean number of nuclei in a giant cell. However, the D-penicillamine administration for 14 days before the implantation alone had no effect on the occurrence of foreign body multinucleated giant cells in the sensitized animals.

DISCUSSION

In the model experiments studying the formation of inflammatory granulomas, a practical and simple method has yet to be provided that permits comprehensive, comparative investigation on the behaviors of cell groups that compose granulomatous tissues. This report includes studies on granulomas experimentally produced under various circumstances usually by either the coexisting effect of immunologic and foreign body reactions during granulomatous inflammation or by separating the effect of immunologic reactions. This procedure was performed on a ferritin-sensitized or-non-sensitized animal by implanting one piece of ferritin-impregnated or-non-impregnated collagen sponge into its peritoneal cavity.[8)] Therefore, when granulomas were produced in the intraperitoneally implanted ferritin sponges in ferritin-sensitized guinea pigs,

the granuloma formation was founded in effect upon the situations of prolonged delayed hypersensitivity reactions. In this report, the induction of the delayed hypersensitivity was assessed by the higher enhancement of Mono accumulation at the peritoneal reaction site in the sensitized guinea pigs than in the non-sensitized guinea pigs on the 1st–3rd days after the implantation. However, in such in vivo situations, variation in intensity of LMN accumulation was not produced at the site of cell-mediated immune response of the delayed hypersensitivity type throughout the duration of granulomatous inflammation on the 5th–18th days after the sponge implantation (see control group 1 in Table 2).

In a previous paper we reported the enhancing effect of D-penicillamine administration (200 mg/kg/day for 14 days before the challenge) on the delayed hypersensitivity reaction using the sponge implant method, which was related to the increase in Mono emigration at the immune reaction site. Nevertheless, the phenomenon of LMN accumulation in the ferritin sponges within sensitized guinea pigs did not conform between the D-penicillamine treated and untreated groups on the 5th–18th days after the challenged implantation. Our results suggested the possibility that D-penicillamine administered in a dose of 200 mg/kg for 14 days prior to the challenge just acted at the beginning time of onset of the delayed hypersensitivity reaction but not a prolongation of emigration of exudate peritoneal macrophages.

On the other hand, a continuous increase in the SMN throughout the duration of granulomatous inflammation is accompanied by an increase in the perivascular mononuclear cells with their maximum increase on the 14th day after the sponge implantation in the sensitized and no-sensitized guinea pigs (see control group 1 in Table 3). The perivascular monouclear cells corresponded with predominant lymphocytes and some plasma cells from the aspect of morphological characteristics. The perivascular infiltration of both lymphocytes and plasma cells further increased in the ferritin sponges of non-sensitized guinea pigs than in these of sensitized guinea pigs. Such a phenomenon may give support to the fact that a prolongation of the cell-mediated immune responses in delayed hypersensitivity to ferritin suppresses the production of serum antibodies.[9]

The effect of D-penicillamine on granulomatous inflammation in rats was suggested by a report of a reduction in the number of inflammatory cells invading the subcutaneously implanted polyurethane sponge impregnated with dead tubercle bacilli in the granulation tissue produced by the inflammatory reaction attributable to the sponge itself and the impregnated irritant.[10] The effect of the agent on chronic synovitis was also demonstrated in rabbits with antigen-induced experimental arthritis by a reduction in the infiltration of mononuclear cells.[11] The inhibition by D-penicillamine of the mononuclear cellular infiltration in granulomatous inflammation may be related to a reduction in the number of peripheral blood lymphocytes or B-lymphocytes (surface IgG- and surface IgM-bearing cells) observed after the administration of the agent to patients with rheumatoid arthritis.[12,13] We observed, in both sensitized and non-sensitized guinea pigs treated daily with D-penicillamine before and after the implantation of collagen sponge, a reduction in the numbers of SMN such as lymphocytes and plasma cells accumulating around the vessels of the granulomatous tissue formed within the sponge. However, the D-penicillamine

administration for 14 days before the implantation increased the dominant lymphocytes infiltrating around the vessels in the sensitized animals which first developed delayed hypersensitivity reaction but granulomatous inflammatory reaction later. This enhancement of lymphocyte chemotaxis by D-penicillamine may be mediated by lymphokines such as the lymphocyte chemotactic factors isolated from the sites of PPD-induced delayed-type hypersensitivity skin reaction in the guinea pig, which act on human as well as guinea pig lymphocyte subpopulations.[14]

Formation of foreign body multinucleated giant cells, which is characteristic of inflammatory response to irritant spongy biomaterials, was suppressed in the presence of ferritin. This phenomenon may be ascribed to an inhibition of the fusion of macrophages due to the cytotoxic effects of ferritin.[15] Our observation that the number of LMN per unit area was greater in the ferritin sponge than in the PBS-sponge 10 days after the implantation, when the formation of foreign body multinucleated giant cells reached a peak, appears to support this speculation. This result also suggests the presence of unknown factors that prevent the fusion among macrophages. Daily administration of D-penicillamine before and after the implantation, on the other hand, suppressed the appearance of foreign body multinucleated giant cells in both the sensitized and non-sensitized groups. However, this inhibition occurred unrelated to the differences in the changes in the number of LMN per unit area between the treated and untreated animals in both the two groups (Table 2). Therefore, the decrease in foreign body multinucleated giant cells during D-penicillamine administration is considered to be due to inhibition of accumulation in the sponge of macrophages and monocytes to be fused.[16,17] A decrease in the macrophage subpopulation due to the inhibitory effect of D-penicillamine on the macrophage chemotaxis may be compensated for by an increase in fibroblast infiltration, resulting in no perceivable changes in terms of the total LMN number. Differential numeration of macrophages and fibroblasts in ordinary hematoxylin and eosin preparations is not technically feasible. Therefore, a decrease in the local accumulation of macrophages caused by D-penicillamine can be indirectly observed only by a decrease in foreign body multinucleated giant cells. The efficacy of D-penicillamine in granulomatous inflammation, therefore, may be explained by the inhibitory effects of the agent on chemotactic lymphokines for macrophage and/or monocyte infiltration.[18]

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