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Rabbit granulomatous enterocolitis induced by injection of muramyl dipeptide emulsified with Freund's incomplete adjuvant

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Abstract: We induced granulomatous enterocolitis in rabbits by injecting them with muramyl dipeptide (MDP), a subunit of the peptidoglycan polymers that endow the bacterial cell wall with structural rigidity, emulsified with Freund's incomplete adjuvant (FIA). Injections of 0.1 ml of a water-in-oil emulsion of MDP and FIA were given submucosally at six sites in the rectum and colon, 10 cm proximal to the anus, using a flexible endoscope. Four rabbits each were sacrificed 1, 2, and 4 weeks after a single injection of the emulsion. Another 4 rabbits each were injected six times at 1- and 2-week intervals, and were sacrificed 1 and 2 weeks after the last injection of the emulsion, respectively. In all 20 rabbits, injected with the MDP emulsion, histological findings of the colon consisted of cellular infiltrations of plasma cells and lymphocytes, granulomatous lesions, and granulomas, although the findings differed in degree. Cellular infiltration in hyperplastic villi and denuded epithelia of the small intestine were seen in 2 of 8 rabbits repeated that received MDP emulsion injections. The histological changes in this animal model may be useful for studying the pathogenesis of inflammatory bowel disease in humans.

Key words: muramyl dipeptide, bacterial cell wall, Freund's incomplete adjuvant, epithelioid granuloma, rabbit granulomatous enterocolitis

Introduction

The intestinal mucosa is continuously exposed to large numbers of proinflammatory bacterial products. The commensal intestinal flora produce or contain a number of potent inflammatory products, such as lipopolysaccharide, peptidoglycan-polysaccharide (PG-PS) complexes, muramyl peptides, and N-formylmethionyl-oligopeptides.

It is possible that the inflow of macrophages and leukocytes into the intestinal wall may result in the inflammation seen in inflammatory bowel disease (IBD), perhaps in response to the chemotactic products of degradable bacterial cell wall fragments that cannot be readily cleared by phagocytes. The immune system against these bacterial products could play an initiating or a perpetuating role in such chronic intestinal inflammation as occurs in IBD.

An animal model of chronic intestinal inflammation could be useful in promoting the understanding of the pathogenesis of IBD and in providing some insight into its etiology. Here, we report rabbit granulomatous enterocolitis induced by the injection of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP), a synthetic, chemically defined subunit of the peptidoglycan polymers that endow the bacterial cell wall with structural rigidity and that exerts adjuvant activity and antigenicity. For injection, MDP was emulsified with Freund's incomplete adjuvant (FIA).

Materials and methods

Male Japanese white rabbits, weighing 2 kg, purchased from a local breeder, were divided into seven groups, A to G (Figs. 1, 2) and used for all experiments.

In order to select an appropriate dose of MDP for inducing adequate local responses in the rabbit colon, MDP (Chemicon, Temecula, Calif.) 1, 10 and 100 μg dissolved in 0.05 ml of phosphate-buffered saline (PBS, pH 7.2) was emulsified with an equal volume of Freund's incomplete adjuvant (FIA; Difco, Detroit,

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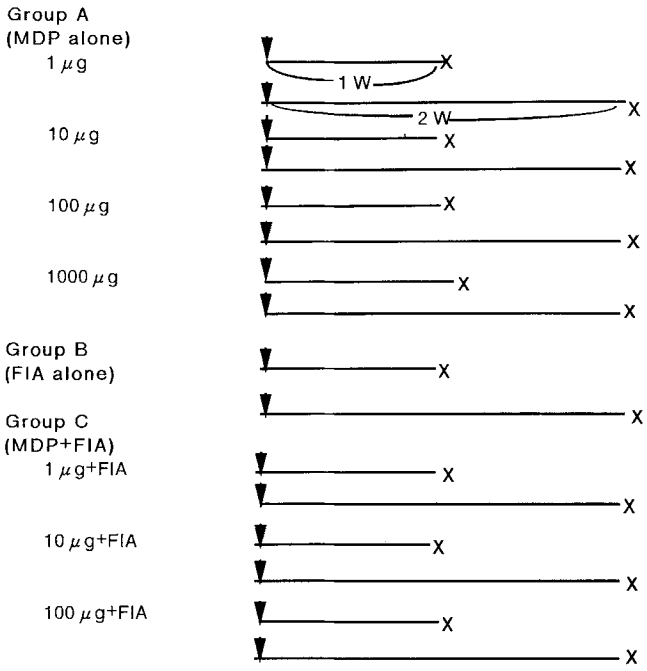

Fig. 1. Regimens for the submucosal injection of Muramyl dipeptide (*MDP*) alone, Freunds incomplete adjuvant (*FIA*) alone, and MDP emulsion in preliminary experiments. Time points of injection and sacrifice are indicated by arrows X, respectively. *W*, Week

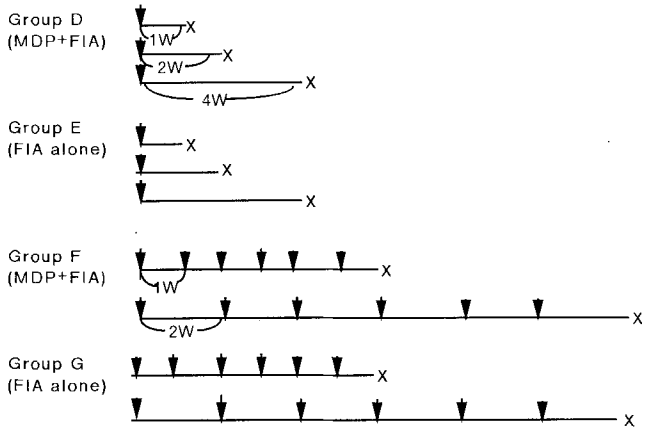

Fig. 2. Regimens for the submucosal injection of MDP emulsion or FIA only into the colon. Time points of injection and sacrifice are indicated by arrows X, respectively

Mich.) and injected submucosally at six sites in the rectum and colon, 10 cm proximal to the anus (Fig. 1, group C). As controls, MDP alone (group A) and FIA alone (group B) were also injected into rabbits. Injection to the rectum and colon was performed using a flexible endoscope (Olympus BF type p10; Olympus, Tokyo, Japan) and a polyethylene catheter with a 23-gauge, 5-mm retractable needle (Sumitomo Bakelite, Tokyo, Japan) for variceal sclerotherapy of the esophagus. Groups A, B, and C comprised a total of 32 rabbits, and 2 rabbits at each time were sacrificed (after being anesthetized with ether) 1 and 2 weeks after a single injection (Fig. 1).

On the basis of the results of these preliminary experiments, the MDP emulsion (100 μg) was injected into 12 rabbits, and 4 rabbits at each time were killed 1, 2, and 4 weeks after a single injection (Fig. 2, group D). In controls, only FIA was injected (group E). MDP emulsion or FIA alone was injected six times at 1- and 2-week intervals and 4 rabbits at each time were sacrificed 1 and 2 weeks after the last injection (groups F and G). Colloidal carbon was infused near the sites of injection of MDP emulsion to confirm the sites. The phagocytic activity of infiltrative cells in the colon was determined in 10 rabbits that had received the injections of MDP emulsion.

The small and large intestine, liver, spleen, and lung were examined macroscopically, and tissue sections were prepared for histological examination. These sections were fixed in buffered formalin and embedded in paraffin. The sections (5 μm) were stained with hematoxylin and eosin.

Results

No rabbits developed clinically detectable diarrhea or showed weight loss. In groups A to C, gross colonic specimens examined at necropsy demonstrated no abnormal findings. Cellular infiltration of lymphocytes and plasma cells in the lamina propria of the colon, 10 cm proximal to the anus, was negligible with 1 or 10 μg MDP alone, and slight with 100 or 1000 μg (Fig. 1, group A). Moderate eosinophil infiltration in the basal lamina propria and submucosa was seen in 4 rabbits injected with FIA only (group B) and in 4 rabbits injected with MDP 1 μg emulsion with FIA (group C, 1 μg + FIA). Aggregations of foamy histiocytes with eosinophils were observed sporadically in the submucosa, but plasma cells and lymphocytes were not seen around these aggregations. In all rabbits injected with MDP emulsion 10 and 100 μg (group C), histological findings of the colon, 10 cm proximal to the anus, showed well-maintained goblet cell populations, mononuclear cell infiltration, granulomatous lesions composed of histiocytes, mononuclear cells, and small numbers of eosinophils, and granulomas, although the findings differed in degree. The minimal dose of MDP emulsified with FIA in subsequent experiments (groups D to G was 100 μg), since there was greater mononuclear cell infiltration in the lamina propria and submucosa in rabbits injected with 100 μg of MDP than in rabbits receiving injections of 1 or 10 μg MDP (group C).

In the group F rabbits, gross colonic specimens frequently demonstrated intestinal thickening. Extensive thickening of the entire small intestine was also detected in 2 of 4 rabbits in this group. The mucosal surfaces of fixed colons in groups D and F were slightly granular in places. No abnormal findings were seen in the livers, spleens, or lungs of any rabbits. The histological findings in the colon, 10 cm proximal to the anus, in 20 rabbits in the groups injected with MDP emulsion 100 μg (groups D and F) were similar to those in the group C animals (10 μg MDP + FIA and 100 μg MDP + FIA).

As summarized in Table 1, infiltration of plasma cells and lymphocytes (Fig. 3A), and intense focal infiltrations of lymphocytes (Fig. 3B) in the lamina propria and submucosa were more prominent in group F than in group D. Poorly-formed granulomas (Fig. 4A) in the submucosa and subserosa, and epithelioid granulomas (Fig. 4B,C), mainly in the lamina propria and subserosa, were present in groups D and F. Occasionally, multinucleated giant cells were seen in the granulomas. Lymphoid aggregations (Fig. 4D) were present in groups D and F, particularly in the subserosa. Granulomatous lesions, composed of histiocytes, mononuclear cells, and a few eosinophils in the submucosa, accompanying oil cysts of various size, were also observed in groups D and F (Fig. 5A). The accumulation of fat globules and carbon indicated that most of the cells were phagocytic. Some histiocytes had vacuolated or foamy cytoplasm. The colons of rabbits injected with FIA only (groups E and G) showed moderate eosinophil infiltration in the basal lamina propria and submucosa. Granulomatous lesions composed of foamy histiocytes and conspicuous

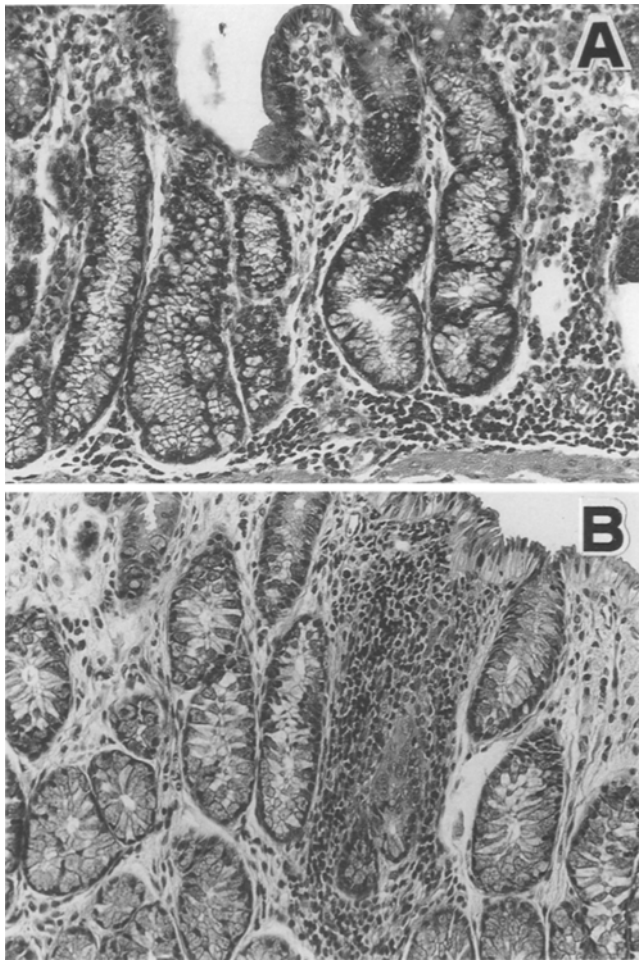

Fig. 3. **A** Infiltration of plasma cells and lymphocytes and **B** intense focal infiltration of lymphocytes in the lamina propria. **A** and **B**, H&E, × 50

Table 1. Histological changes in rabbit colons 10 cm proximal to the anus

	Group D			Group E			Group F		Group G	
	Time after the last injection									
Histological change	1W	2W	4W	1W	2W	4W	1W	2W	1W	2W
Mononuclear cell infiltration[a]										
In the lamina propria	+	++	++	−	−	−	+++	+++	−	−
In the submucosa	++	+	+	−	−	−	+++	+	−	−
Granuloma[b]										
Poorly-formed	+	−	−	−	−	−	+	−	−	−
Epithelioid	−	+	+	−	−	−	+	+	−	−
Lymphoid aggregation	+	−	−	−	−	−	+	−	−	−
Granulomatous lesion with mononuclear cells	+	+	+	−	−	−	+	+	−	−
Transmural infiltration	+	+	+	+	+	+	+	+	+	+

[a] The grade of mononuclear cell infiltration was defined as: −, normal; +, slightly increased in number; ++, moderate; +++, densely increased

[b] The presence or absence of poorly-formed granuloma, epithelioid granuloma, lymphoid agrregation, granulomatous lesion, and transmural infiltration was expressed as + and −, respectively

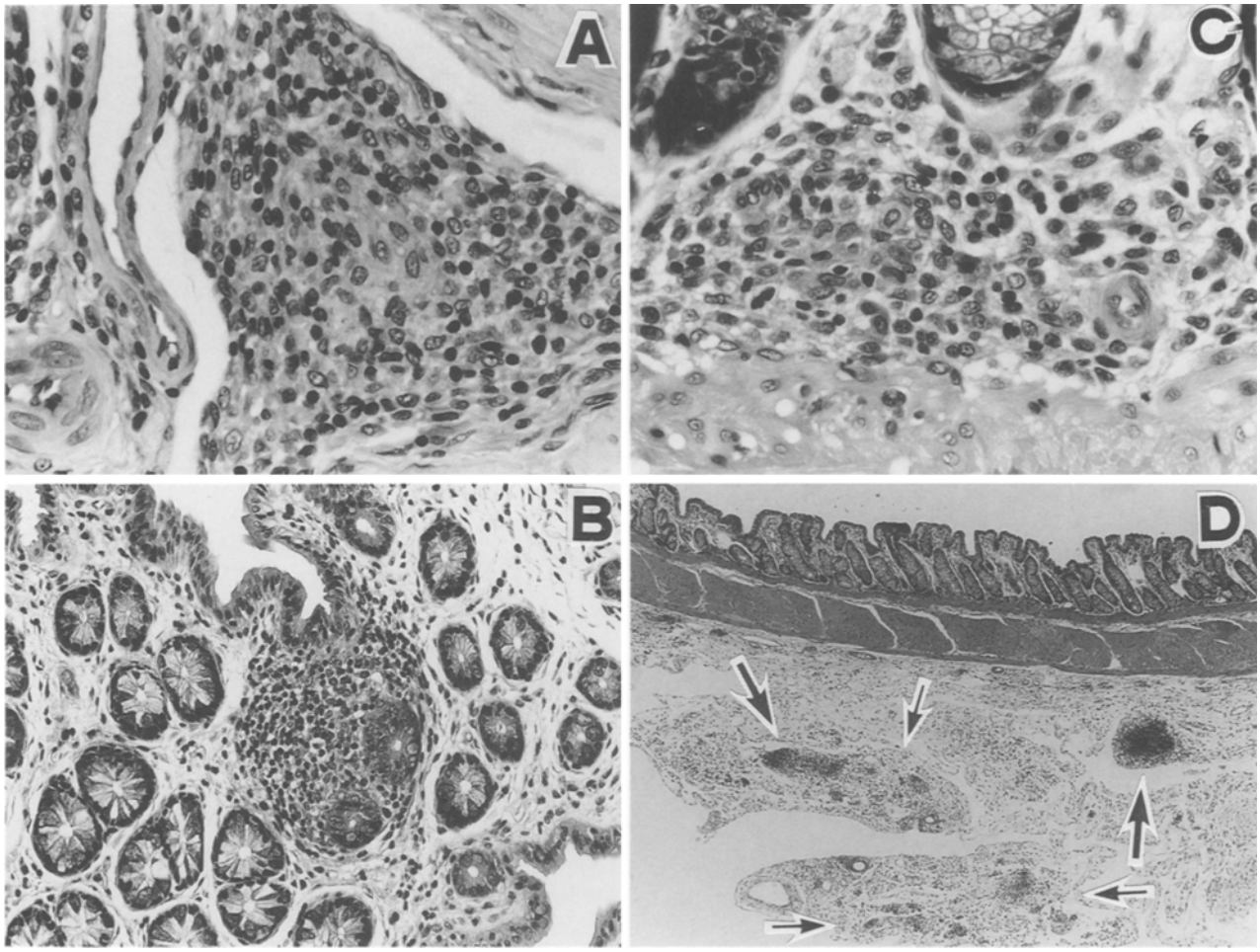

Fig. 4. A Poorly-formed granulomas in the submucosa (H&E, × 100), **B** and **C** epithelioid granulomas in the lamina propria (**B**, H&E, × 50; **C**, H&E, × 100); **D** lymphoid aggregations (*large arrows*) and granulomas in groups (*small arrows*) in the subserosa. H&E, × 10

eosinophils were observed sporadically in the submucosa and serosa, but plasma cells and lymphocytes were not seen around these lesions (Fig. 5B). Transmural infiltration was present in all groups, although the infiltrative cells differed in the groups injected with MDP emulsion and those injected with FIA only.

Histological examination of the colon at sites distant from the injection sites, i.e., approximately 20 cm from the anus, revealed slight mononuclear cell infiltration, particularly in the lamina propria, but no granulomatous lesions, in rabbits that received repeated MDP injections (group F). In 2 of 4 rabbits that had received MDP emulsions at 2-week intervals for a total of six times, hypertrophy of the small intestine was observed. Moreover, infiltration of plasma cells and lymphocytes in the lamina propria of hyperplastic villi, denuded epithelium (Fig. 6), villus destruction, and regenerative mucosa were also seen. Aggregations of histiocytes were present in the subserosa.

Discussion

Several animal models of chronic intestinal inflammation have been developed via a variety of techniques, such as the administration of trinitrobenzene sulfonic acid,[1] carrageenan,[2] indomethacin,[3,4] and immune complexes.[5] Recently, Sartor et al.[6] and Yamada et al.[7] reported PG-PS-induced enterocolitis, i.e., chronic transmural, granulomatous enterocolitis, which had several unique features as a model of chronic intestinal inflammation. This model demonstrated that bacterial cell wall fragments were capable of producing chronic granulomatous inflammation in the intestine and mesenteric lymph nodes. However, PG-PS is troublesome to purify and not commercially available, and laparotomy is required for injection. We have developed granulomatous enterocolitis in rabbits by injecting an emulsion of MDP and FIA. Our model is easily induced by submucosal injection with an endoscope, and MDP is commercially available.

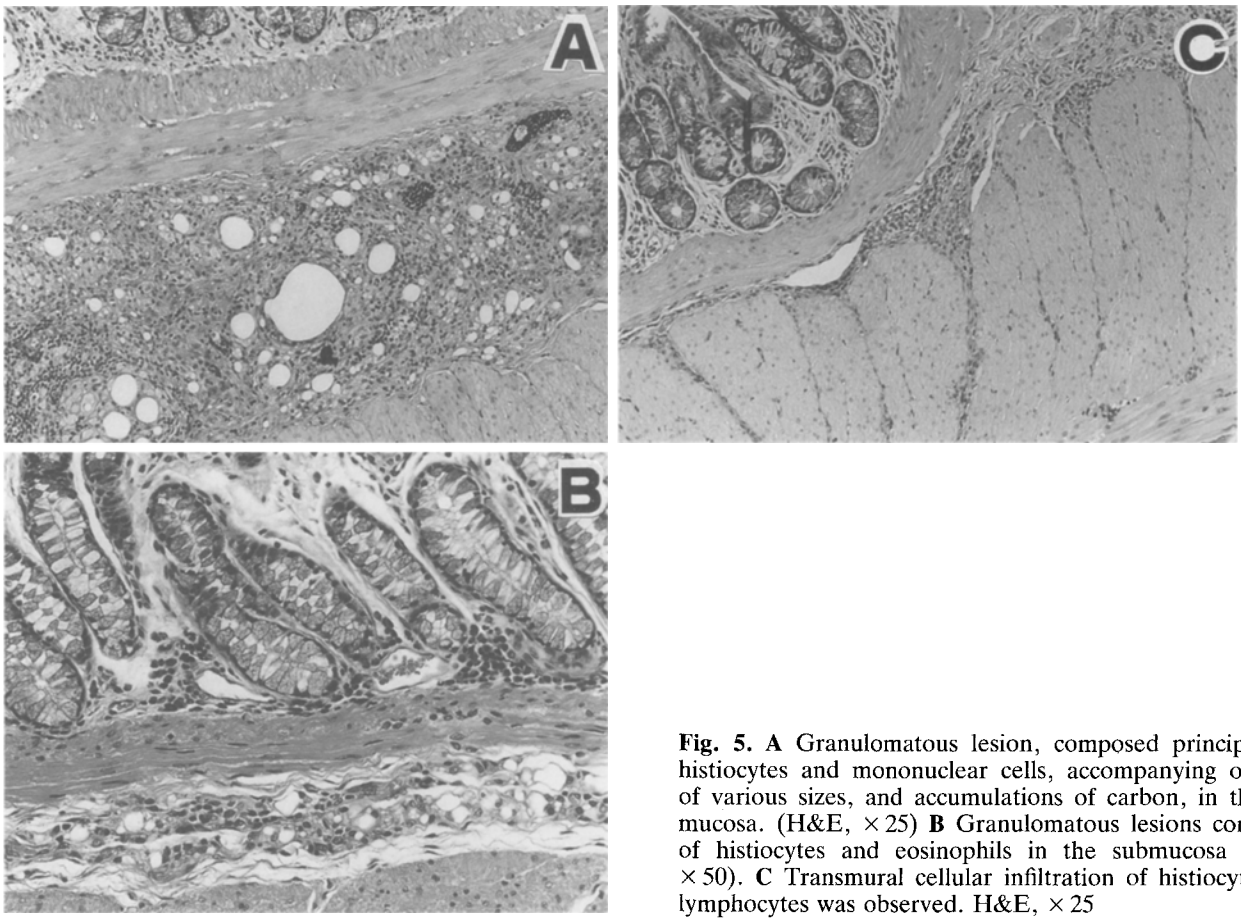

Fig. 5. A Granulomatous lesion, composed principally of histiocytes and mononuclear cells, accompanying oil cysts of various sizes, and accumulations of carbon, in the submucosa. (H&E, ×25) **B** Granulomatous lesions composed of histiocytes and eosinophils in the submucosa (H&E, ×50). **C** Transmural cellular infiltration of histiocytes and lymphocytes was observed. H&E, ×25

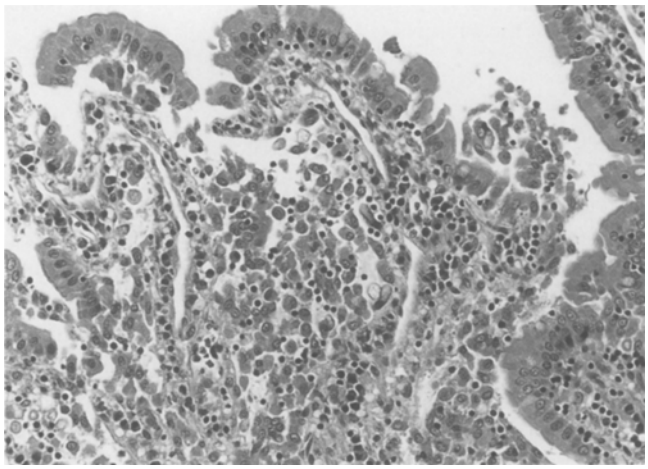

Fig. 6. Infiltration of plasma cells and lymphocytes in the lamina propria of hyperplastic villi and denuded epithelia. H&E, ×25

MDP is a subunit of the peptidoglycan polymers that endow the bacterial cell wall with structural rigidity. Many commensal bacteria in mammals possess glycopeptides similar to MDP in structure and bioactivity. Bacterial peptidoglycan can be degraded by various enzymes in the gut to release muramyl peptides. The gut plays the role of a reservoir of MDP-like molecules. Although MDP is a small molecule and cannot initiate an immune response unless first bound to a carrier molecule, it has adjuvant activity and antigenicity as a hapten, and is granulomagenic with mineral oil or branched fatty acids. Emori and Tanaka[8] and Tanaka and Emori[9] have reported that MDP produced a massive granuloma at the sites of injection (footpad) and in the draining lymph nodes of guinea pigs and rats. Emori et al.[10] reported that MDP alone did not induce epithelioid granuloma, but that it induced extensive granulomas when chemically conjugated with branched fatty acids. They indicated the importance of a close association of MDP with some lipids, including mineral oil, in the formation of epithelioid granuloma. FIA itself, which does not contain a mycobacterial component, is reported to induce macrophages infiltration with no plasma cells and inconspicuous lymphocytes.[11–13]

In our experiments, MDP alone induced slight infiltration of lymphocytes and plasma cells in the lamina propria of the colon, but no granulomas and

granulomatous lesions. FIA alone induced granulomatous lesions composed of histiocytes and eosinophils, but no lymphocyte or plasma cell infiltration, lymphoid aggregation, or epithelioid granuloma. MDP emulsion with FIA induced mononuclear cell infiltration, epithelioid granulomas with occasional giant cells, poorly-formed granulomas, and lymphoid aggregations. These results suggest that MDP emulsified with FIA has important effects related to chronic inflammatory changes. In addition, the MDP we used is very soluble in water and may disappear from the injected tissues. MDP incorporated in a water-in-oil emulsion may be released slowly and elicit a local, sufficient inflammatory response.

The granulomatous lesions with mononuclear cells and oil cysts seemed to occur when MDP emulsion was deposited in large amounts, while it is possible that granulomas and mononuclear cell infiltration without oil cysts could occur when the MDP emulsion is deposited in smaller amounts. Tissue reactions to MDP emulsion could be seen more clearly at sites with small amounts of deposits than at sites with large amounts. The injection of small amounts of MDP emulsion is appropriate for evoking histological changes in rabbits. MDP emulsified with FIA may exert a chemotactic activity for macrophages[14] and lymphocytes (some of which can product cytokines) and contribute to the formation of lymphoid aggregations and epithelioid granulomas.

In 2 rabbits that received repeated injections of MDP emulsion, infiltration of plasma cells and lymphocytes was also present in the hyperplastic villi of the small intestine, in areas beyond the injected sites. It is conceivable that some MDP emulsion may enter the lymphatics and systemic circulation from the colon to stimulate gut-associated lymphoid tissues.

In regard to the injection of MDP incorporated with FIA, Wakasa et al.[15] have reported the formation of granulomas by a single shot after laparotomy. However, to the best of our knowledge, no attempt to induce chronic enterocolitis by intraluminal injection of MDP emulsion has yet been made.

The histological changes evoked in our animal model in this study suggest that bacterial products may be involved in chronic intestinal inflammation. Further long-term studies are necessary to elucidate the mechanisms underlying the involvement of bacterial products in chronic enterocolitis.

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