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Synergistic Effect of Polyriboinosinic Acid:Polyribocytidylic Acid and Either Bacterial Peptidoglycans or Synthetic *N*-Acetylmuramyl Peptides on Production of Adjuvant-Induced Arthritis in Rats

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Lewis rats developed polyarthritis after a single injection of a water-in-oil emulsion containing various peptidoglycans (PGs) derived from *Lactobacillus plantarum*. A copolymer of polyriboinosinic acid and polyribocytidylic acid markedly potentiated the arthritogenicity of these PGs. The synthetic adjuvants *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln) and MurNAc-L-Ala-D-Gln were non-arthritogenic, but they did produce severe arthritis when mixed in a water-in-oil emulsion with a copolymer of polyriboinosinic acid and polyribocytidylic acid. Substitution of either L-isoGln or D-isoAsn for the D-isoGln in the MurNAc-L-Ala-D-isoGln markedly reduced its capacity to induce the disease. Taken together with the results of skin testing against various PGs and MurNAc-L-Ala-D-isoGln in the diseased rats, the present results suggest that (i) a minimal essential structure required for development of polyarthritis is related to a larger molecule than either MurNAc-L-Ala-D-isoGln or a monomer of PG, probably to a dimer of PG, and (ii) an antigenic determinant(s) for the delayed-type skin hypersensitivity to PGs exists on a common structure shared among these PGs, possibly somewhere on a monomer of PG not on *N*-acetylmuramyl peptides.

Adjuvant-induced arthritis in rats can be induced by a single injection of Freund complete adjuvant containing mycobacterium (18). This disease can also be produced by a single injection of a water-in-oil emulsion containing a variety of bacterial cell walls (7, 9), suggesting that a common factor(s) shared by their cell walls may play an etiological role in the production of this disease.

Our previous reports (11, 12) further demonstrated that a variety of hydrosoluble peptidoglycans (PGs) liberated enzymatically from bacterial cell walls were able to produce severe arthritis similar to that induced by Freund complete adjuvant. We have suggested from these observations that the arthritis-inducing ability of these PGs is closely associated not only with two or more glycan units (*N*-acetylglucosaminyl-*N*-acetylmuramic acid) on the PG subunits (12), but also with their adjuvancy in terms of both enhancing antibody formation and inducing cell-mediated immunity (14).

Recently, Ellouz et al. (4) found that a minimal essential structure of mycobacterial adjuvant is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln). This was confirmed by Kotani et al. (15) in which *N*-acetylmuramyl dipeptide and its various analogs were proved to replace mycobacterium in Freund complete adjuvant. We have, however, failed to demonstrate an arthritis-inducing ability of *N*-acetylmuramyl dipeptide and its analogs such as MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala, MurNAc-L-Ala-D-isoGln-L-Lys, MurNAc-L-Ala-D-isoGln, and L-Ala-D-isoGln-L-Lys-D-Ala (12). On the other hand, we found that a copolymer of polyriboinosinic acid and polycytidylic acid [poly(I:C)], a well-known adjuvant, remarkably potentiated the arthritogenicity of various wax D (10) and lysozyme-solubilized PGs of *Mycobacterium smegmatis*, which by itself was non-arthritogenic (8). These observations led us promptly to investigate the possibility of whether poly(I:C) can exert an adjuvant effect on non-arthritogenic *N*-acetylmuramyl dipeptides and non-arthritogenic PGs for induction of adjuvant arthritis in order not only to elucidate what antigen is responsible for promoting the

development of the disease, but also to understand the immunopathological events of adjuvant arthritis.

MATERIALS AND METHODS

Animals. Female Lewis rats were obtained from either Microbiological Associates, Bethesda, Md., or Charles River Breeding Laboratories, Inc., Wilmington, Mass. They weighed 150 to 175 g at the start of the present experiments.

Synthetic *N*-acetylmuramyl peptides. Synthetic compounds tested were *N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanine (MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala), MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-L-isoGln, MurNAc-L-Ala-D-Gln and MurNAc-L-Ala-D-isoAsn, MurNAc-Gly-D-isoGln, and MurNAc-L-Ser-D-isoGln. The syntheses of these compounds were described in detail elsewhere (16).

Poly(I:C), bacterial PGs and BCG. Poly(I:C) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Heat-killed Bacille Calmette-Guerin (BCG) was obtained through the courtesy of the Fisheries and Food Central Veterinary Laboratory, Weybridge, Surrey, England. PGs tested were monomers, dimers and oligomers of PGs which were derived from the cell walls of *Lactobacillus plantarum* (ATCC 8014). The preparation of these PGs was described elsewhere (5, 6, 17). In brief, *L. plantarum* was grown in medium at 30°C for 24 to 27 h (5). The cells were harvested and washed three times with 0.15 M NaCl. The harvested cells were disrupted with a Braun cell homogenizer. The crude cell wall fraction was obtained by differential centrifugation and digested with trypsin. These trypsinized cell walls were further digested with chalaropsis B enzyme (5). This enzyme digest was then applied on an ECTEOLA-cellulose column. The fraction eluted with water from the ECTEOLA-cellulose column was applied to three successive Sephadex columns of G50 (fine), G50 (coarse), and G25 (coarse) gels (5). A disaccharide-peptide subunit dimer (PG dimer) and a monomer of PG were thus obtained (5, 6). An oligomer of the disaccharide-peptide subunit (PG oligomer) was obtained from the L-3 enzyme digest of *L. plantarum* cell walls as described in a previous paper (6, 17).

Production of arthritis in rats. All the rats were injected in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing various amounts of test materials. After inoculation, the rats were examined daily to evaluate time of onset of arthritis and graded from 0 to 4 for each appendage, according to the extent of the swelling, erythema, and ankylosing of the periarticular tissue, including tail, as described in a previous paper (22). In general, we evaluated an arthritogram score below 5 as mild and transient arthritis, 6 to 10 as moderately severe, 11 to 15 as severe, and 16 to 20 as very severe. In some experiments, the rats were injected intradermally in one footpad with 0.05 ml of water-in-oil emulsion.

Preparation of inocula. PGs and synthetic *N*-acetylmuramyl peptides were dissolved in 0.01 M sterile phosphate-buffered saline (pH 7.2). To make a mixture of poly(I:C) and either PG or *N*-acetylmuramyl peptide, 4 mg of poly(I:C) was dissolved in 0.2 ml of phosphate-buffered saline and 4 mg of either PG or *N*-acetylmuramyl peptide was added. A water-in-oil emulsion was prepared by dropwise addition of test materials to an equal volume of the oil vehicle, which consists of 85% mineral oil (E. R. Squibb & Sons, Princeton, N.J.) and 15% Arlacel A (Hilltop Laboratory, Cincinnati, Ohio).

Skin testing. Skin tests were performed 7 and 14 days after inoculation on a shaved area of the flank by intradermal injection with 0.1 ml of phosphate-buffered saline containing 50 μg of test antigen. The diameter of the erythema and the induration of the skin area were carefully recorded at 6 h for immediate reaction and at 24 and 48 h for delayed reaction. An induration of more than 5 mm in diameter was evaluated as positive, according to the report of Bolton and Chorpenning (3).

Histological study. The diseased rats were sacrificed by cardiac puncture under ether anesthesia. Tissues were fixed in 10% Formalin. Histological observations were made on hematoxylin and eosin-stained paraffin sections.

RESULTS

Arthritogenicity of various PGs from *L. plantarum* and synthetic *N*-acetylmuramyl peptides. Our previous report (12) was confirmed in this study in that oligosaccharide-peptide produced very severe arthritis with 100% incidence, disaccharide-peptide-disaccharide (a dimer of disaccharide-peptide) produced very mild to mild arthritis with 50% incidence in one experiment and 20% in another experiment, and disaccharide-peptide (a monomer) did not produce arthritis (Table 1). None of the synthetic *N*-acetylmuramyl peptides, such as MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala, MurNAc-L-Ala-D-isoGln, MurNAc-D-Gln, MurNAc-L-Ala-L-iso-

TABLE 1. *Arthritogenicity of PGs derived from L. plantarum and synthetic N-acetylmuramyl peptides in rats*

Immunization[a]	Polyarthritis incidence/total
PGs from *L. plantarum*	
Oligosaccharide-peptide	10/10
	10/10
Disaccharide-peptide-disaccharide	5/10
	2/10
Disaccharide-peptide	0/10
Synthetic *N*-acetylmuramyl peptides	
MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala	0/10
MurNAc-L-Ala-D-isoGln	0/10
MurNAc-L-Ala-D-Gln	0/10
MurNAc-L-Ala-D-isoAsn	0/10

[a] Each rat was immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing 100 μg of test materials.

Gln, and MurNAc-L-Ala-D-isoAsn, produced arthritis.

Neither disaccharide-peptide nor MurNAc-L-Ala-D-isoGln produced any disease within the wide dose range of 1 to 500 μg/rat (Table 2).

Synergistic effect of poly(I:C) and bacterial PGs on production of arthritis. Poly(I:C) was non-arthritogenic with the doses used (Table 3). Disaccharide-peptide-disaccharide, which by itself was a mild arthritogen (Table 1), produced severe arthritis with 100% incidence when mixed in a water-in-oil emulsion with poly(I:C). Disaccharide-peptide, which was mixed with 0.1 mg of poly(I:C), also produced arthritis with 100% incidence and produced more severe arthritis as the dose of disaccharide-peptide was increased from 0.01 to 0.1 mg. The clinical course of the disease was similar to that produced by BCG with regard to the onset day and the degree of severity of the arthritis (Fig. 1).

Synergistic effect of poly(I:C) and several synthetic *N*-acetylmuramyl peptides. When mixed in a water-in-oil emulsion with poly(I:C), MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala produced severe arthritis with 100% incidence (Table 4). MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala produced severe arthritis with 100% incidence. MurNAc-L-Ala-D-isoGln also produced severe arthritis within its wide dose range. The clinical signs appeared at 9 to 12 days after inoculation, increased to the mean score (more than 15) of the highest arthritogram per group, and gradually subsided but still were apparent until the animals were sacrificed at 61 days after inoculation. MurNAc-L-Ala-L-isoGln produced mild arthritis in four of eight rats. The clinical signs disappeared in about 10.1 days. MurNAc-L-Ala-D-Gln also produced severe arthritis with 100% incidence, comparable to that produced by MurNAc-L-Ala-D-isoGln. MurNAc-L-Ala-D-isoAsn and MurNAc-L-Ser-D-isoGln, however, produced disease that was very mild and rapidly disappeared. MurNAc-Gly-D-isoGln did not produce the disease. The clinical course of the arthritis produced by a mixture of poly(I:C) and various synthetic *N*-acetylmuramyl peptides is

TABLE 2. *Failure of disaccharide-peptide and MurNAc-L-Ala-D-isoGln to induce polyarthritis within wide dose ranges*

Immunization[a]	Polyarthritis	
	Dose (μg/rat)	Incidence/total
Disaccharide-peptide[b]	1	0/6
	10	0/6
	100	0/10
	300	0/10
	500	0/10
N-Acetylmuramyl-L-Ala-D-isoGln	1	0/10
	10	0/10
	100	0/10
	300	0/10
	500	0/10

[a] Each rat was immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing the indicated amounts of test materials.
[b] See text.

TABLE 3. *Synergistic or enhancing effect of poly(I:C) and bacterial PGs on induction of polyarthritis in rats*

Materials	Immunization[a] (route)	Polyarthritis			
		Dose (μg/rat)	Incidence/total	Onset day (mean)[b]	Severity[c]
Poly(I:C)	LN	10	0/7		
	LN	100	0/10		
	LN	200	0/10		
Poly(I:C) + disaccharide-peptide-disaccharide[d]	LN	100 + 100	7/7	10–13 (11.5)	14.6
Poly(I:C) + disaccharide-peptide[d]	LN	100 + 100	7/7	10–13 (11.7)	16.0
	LN	100 + 100	7/7	9–13 (11.9)	11.2
	FP	100 + 1	2/10	19–21 (20.0)	5.0[e]
	FP	100 + 10	3/10	12–14 (12.7)	8.3[e]
	FP	100 + 100	6/9	11–14 (12.4)	10.5[e]
Freund incomplete adjuvant	LN		0/7		
	FP		0/7		

[a] Each rat was immunized either in both inguinal lymph nodes (LN) with 0.01 ml of water-in-oil emulsion or intradermally in one footpad (FP) with 0.05 ml of water-in-oil emulsion containing the indicated amounts of poly(I:C) or a mixture of poly(I:C) and bacterial PGs.
[b] Mean onset day was calculated by averaging the onset day for each rat per group.
[c] This severity was calculated as the arithmetic mean of the highest score of each rat per group.
[d] Derived from *L. plantarum* (see text).
[e] Arithmetic mean of the highest score of each rat per group without scoring the injected foot.

shown in Fig. 2.

Immune response in rats immunized with a mixture of poly(I:C) and either MurNAc-L-Ala-D-isoGln or bacterial PGs. The rats were immunized with oligosaccharide-peptide, disaccharide-peptide, a mixture of poly(I:C) and disaccharide-peptide, or a mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln. Skin tests were performed on day 7 (data not shown) and on day 14 after immunization. Seven days after immunization with oligosaccharide-peptide, the rats did not develop arthritis but had already developed delayed-type hypersensitivities (DTH) to monomers, dimers, and oligomers of PGs which were 13.2 ± 1.0, 9.4 ± 0.2, and 10.5 ± 0.02 mm, respectively. On day 14, all the rats developed the disease and DTH to a monomer, a dimer, and an oligomer of PG which were 10.4 ± 0.2, 8.4 ± 0.5, and 9.3 ± 0.4 mm, respectively (Table 5). Disaccharide-peptide by itself did not induce arthritis but induced weak DTH to a monomer and an oligomer of PG on days 7 and 14. A mixture of poly(I:C) and disaccharide-peptide induced DTH to a monomer and an oligomer of PG which were 8.0 ± 0.0 and 10.0 ± 0.0 mm on day 7 (not shown) and 8.0 ± 0.4 and 10.0 ± 0.0 mm on day 14, when all the rats developed arthritis. Neither poly(I:C) nor MurNAc-L-Ala-D-isoGln induced DTH to either MurNAc-L-Ala-D-isoGln or bacterial PGs. A mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln induced DTH to a monomer, a dimer, and an oligomer of PG but not to MurNAc-L-Ala-D-isoGln. BCG as a positive control induced DTH similar to those induced by a mixture of poly(I:C) and either MurNAc-L-Ala-D-isoGln or disaccharide-peptide. Freund incomplete adjuvant as a negative control did not induce DTH to any of the PGs tested. No immediate reaction was observed in any rats. Delayed skin reactions on day 7 were apt to be slightly stronger than those on day 14.

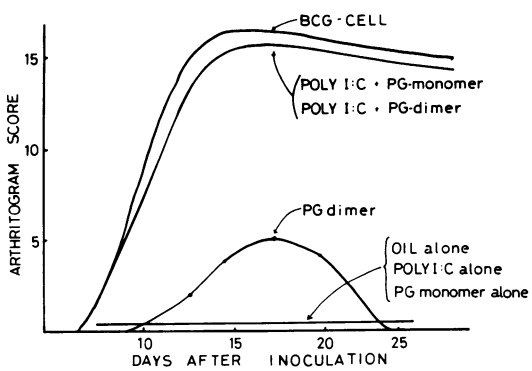

FIG. 1. *Typical clinical course of arthritis more than 3 weeks after immunization with BCG or a mixture of poly(I:C) and PGs.*

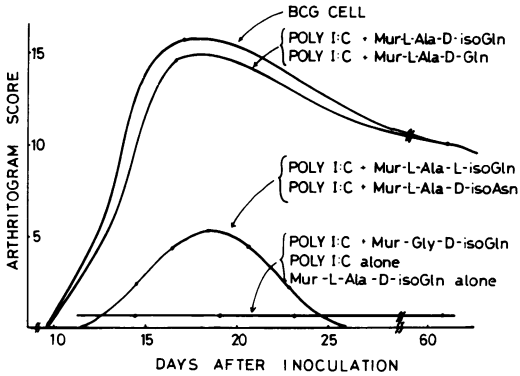

FIG. 2. *Comparison of latency period and severity of arthritis in rats immunized with a mixture of poly(I:C) and various N-acetylmuramyl peptides over 8 weeks.*

TABLE 4. *Synergistic effect of poly(I:C) and synthetic N-acetylmuramyl peptides on induction of polyarthritis in rats*[a]

Materials	Polyarthritis			
	Dose (μg/rat)	Incidence/ total	Onset day (mean)[b]	Severity[c]
Poly(I:C)	100	0/7		
Poly(I:C) + MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala	100 + 100	8/8	10–14 (11.3)	15.6
Poly(I:C) + MurNAc-L-Ala-D-isoGln	100 + 10	8/8	10–12 (11.8)	15.0
	100 + 100	8/8	9–12 (10.5)	16.3
	100 + 300	6/6	10–12 (11.2)	17.0
Poly(I:C) + MurNAc-L-Ala-L-isoGln	100 + 100	4/8	12–17 (15.4)	6.8
Poly(I:C) + MurNac-L-Ala-D-Gln	100 + 100	8/8	9–13 (11.0)	17.9
Poly(I:C) + MurNAc-L-Ala-D-isoAsn	100 + 100	6/8	13–18 (14.2)	6.5
Poly(I:C) + MurNAc-L-Ser-D-isoGln	100 + 100	2/7	16 (16.0)	7.5
Poly(I:C) + MurNAc-Gly-D-isoGln	100 + 100	0/8		

[a] Each rat was immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing the indicated amounts of poly(I:C) or a mixture of poly(I:C) and synthetic *N*-acetylmuramyl peptides.

[b,c] See Table 3, footnotes *b* and *c*.

TABLE 5. *DTH to various PGs and MurNAc-L-Ala-D-isoGln 14 days after immunization in rats*

Immunization[a]	Dose (μg/rat)	48-h skin reaction[b] (avg ± standard error [mm])			
		Monomer	Dimer	Oligomer	MDP
Oligosaccharide-peptide[c]	100	10.4 ± 0.2	8.4 ± 0.5	9.3 ± 0.4	Neg.[d]
Disaccharide-peptide[c]	100	9.6 ± 0.4	ND[e]	7.8 ± 0.4	Neg.
Poly(I:C) + disaccharide-peptide	100 + 100	8.0 ± 0.4	ND	10.0 ± 0.0	Neg.
Poly(I:C)	100	Neg.	Neg.	Neg.	Neg.
MurNAc-L-Ala-D-isoGln	100	Neg.	Neg.	Neg.	Neg.
Poly(I:C) + MurNAc-L-Ala-D-isoGln	100 + 100	10.5 ± 0.5	8.2 ± 0.15	10.5 ± 0.2	Neg.
BCG	100	9.2 ± 0.4	ND	10.0 ± 0.3	Neg.
Freund incomplete adjuvant		Neg.	Neg.	Neg.	Neg.

[a] Each rat was immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing the indicated amounts of test materials.

[b] Skin tests were performed 7 (data not shown) and 14 days after immunization. One group consisted of six rats. Monomer, Disaccharide-peptide; dimer, disaccharide-peptide-disaccharide; oligomer, oligosaccharide-peptide (see text). MDP, MurNAc-L-Ala-D-isoGln.

[c] See text.

[d] Neg., Negative skin test.

[e] ND, Not done.

Histological studies of joint and adjacent tissues. Histological studies of the joints and periarticular tissues were carried out in the fully developed arthritic rats 21 days after inoculation of a mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln. The rats immunized with BCG also were examined histologically as a positive control of adjuvant-induced arthritis. There was synovial hyperplasia, which was characterized by proliferation of the synovial villi and by infiltration of mononuclear cells in the tarsal joint, of the rats immunized with a mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln (Fig. 3). A mass of fibrin was attached to the synovial stroma and extended into the joint space. Similar chronic mononuclear cell infiltration and connective tissue pannus invasion were observed in subchondral bone and tendons as well as growth of pannus from synovial margins out over the surfaces of the articular cartilage. These histological changes were indistinguishable from those induced by either BCG or Freund complete adjuvant (18).

DISCUSSION

The present study confirmed our previous report (12) that (i) neither MurNAc-L-Ala-D-isoGln nor its various analogs induced arthritis and that (ii) a monomer of PG (disaccharide-peptide) was non-arthritogenic. When mixed in a water-in-oil emulsion with poly(I:C), a well-known adjuvant, MurNAc-L-Ala-D-isoGln and MurNAc-L-Ala-D-Gln induced very severe arthritis with 100% incidence, whereas substitution of either L-isoGln or D-isoAsn for the D-isoGln in the synthetic MurNAc-L-Ala-D-isoGln markedly reduced its capacity to induce arthritis. Substitution of L-Ser for the L-Ala in MurNAc-L-Ala-D-isoGln also reduced its arthritogenicity, whereas MurNAc-Gly-D-isoGln did not induce arthritis. The clinical signs and course of the disease (Fig. 1 and 2) were similar to those induced by BCG. The lesions of the disease were clinically and histologically (Fig. 3) indistinguishable from those induced by Freund complete adjuvant and included skin lesions, ear nodules, the extension of the erythema, and swelling of the periarticular tissue, including tail (21).

Our previous study (12) demonstrated that (i) oligomers and polymers of PGs induced severe arthritis with 100% incidence, (ii) a dimer of PG, such as GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-diaminopimeryl-D-Ala *meso*-diaminopimeryl-D-isoGln-L-Ala-MurNAc-GlcNAc, also induced mild arthritis with low incidence, and (iii) monomers of PGs such as GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-diaminopimeryl-D-Ala and GlcNAc-MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala, which were proved to serve as adjuvants (15). From these observations, we have suggested that (i) adjuvancy is needed, but not enough to induce adjuvant arthritis, and arthritogenicity requires a specific antigen(s) in addition to the presence of an adjuvant-active agent and (2) PG subunits with two or more glycan units (repeating unit of disaccharide: GlcNAc-MurNAc) may play an important role in providing an antigenic determinant(s) responsible for induction of adjuvant arthritis, since adjuvancy of these PGs is attributable to MurNAc-L-Ala-D-isoGln (4, 13–15).

It is thus considered that MurNAc-L-Ala-D-isoGln and its analogs, as well as a monomer of PG, serve as adjuvants but are not enough to induce arthritis, probably because of a lack of an

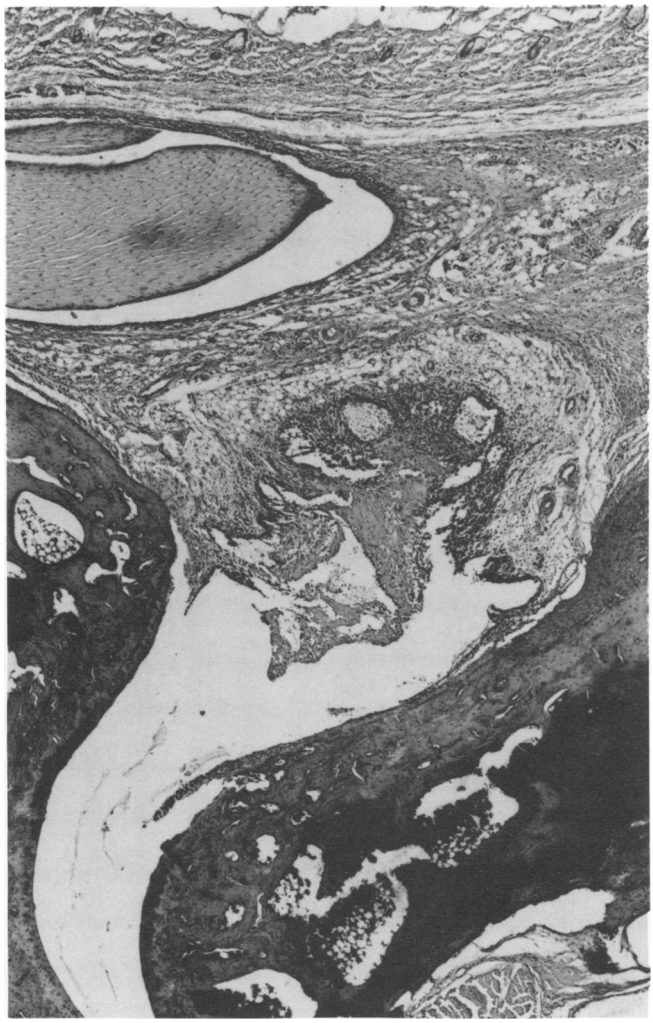

FIG. 3. *A tarsal joint, 14 days after inoculation of a mixture of poly(I:C) and N-acetylmuramyl dipeptide. Synovial hyperplasia, subsynovial edema, and mononuclear cell infiltration are shown. Peritendonitis, tendonitis, intense inflammatory reaction, granulation tissue growth, and synovial villus are also shown. Stained with hematoxylin-eosin.* ×40.

antigenic determinant responsible for induction of arthritis. If this is the case, the question arises as to why a mixture of poly(I:C) and either MurNAc-L-Ala-D-isoGln or a monomer of PG induced arthritis. Also, why do adjuvant-inactive MurNAc-L-Ala-L-isoGln and MurNAc-L-Ala-D-isoAsn (13) induce arthritis when injected together with poly(I:C)? One possible explanation is that either *N*-acetylmuramyl peptides or disaccharide-peptide may be adsorbed on the surface of the poly(I:C) molecule, making up an arthritogenic molecule similar to PGs in terms of providing an antigenic determinant(s) responsible for induction of arthritis. In other words, poly(I:C) can serve as an adjuvant and either *N*-acetylmuramyl peptides or disaccharide-peptide can provide an antigenic determinant(s) on the surface of the poly(I:C) molecule similar to PGs with two or more glycan units (disaccharide), which were very potent arthritogens (Table 1).

The present study on the immunogenicity of PG subunits revealed that non-arthritogenic disaccharide-peptide (a monomer of PG) induced weak DTH to a monomer of PG, whereas a mixture of poly(I:C) and a monomer of PG induced weak DTH to a monomer of PG but strong DTH to an oligomer of PG. Oligosaccharide-peptide (an oligomer of PG) induced strong

DTH to a monomer, a dimer, and an oligomer of PG. The present study further demonstrated that MurNAc-L-Ala-D-isoGln by itself did not induce any skin hypersensitivity to either MurNAc-L-Ala-D-isoGln or other PGs, whereas, surprisingly, a mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln induced DTH to a monomer, a dimer, and an oligomer of PG but not to MurNAc-L-Ala-D-isoGln. BCG also induced DTH to these PGs, but MurNAc-L-Ala-D-isoGln did not. It is thus possible that a common structure shared among these PGs, possibly a monomer of PG (*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-Ala-D-isoGln-*meso*-diaminopimeric acid-D-Ala) (5, 6) is related to an antigenic determinant responsible for the DTH. At this time, we could not determine whether this antigenic determinant is located in the peptide portion of this PG or glycan unit or both. The reason why a mixture of poly(I:C) and MurNAc-L-Ala-D-isoGln induced the immune response to bacterial PGs remains uncertain. This may, however, be explained by the hypothesis described earlier that MurNAc-L-Ala-D-isoGln may be making up an antigenic molecule similar to the bacterial PGs on the surface of poly(I:C). Further studies would be required to prove this hypothesis. In any case, it seems that *N*-acetylmuramyl peptides cannot serve as antigens in terms of eliciting DTH or, probably, producing humoral antibody (1).

Taken together with our previous findings that a dimer of PG induced arthritis whereas a monomer of PG did not (12), the present study implies that (i) a minimal essential structure required for development of arthritis is related to a larger molecule than either *N*-acetylmuramyl peptides or a monomer of PG, probably to a dimer of PG, and (ii) an antigenic determinant for DTH to these PGs exists on a common structure shared among the PGs, possibly somewhere on a monomer of PG not on *N*-acetylmuramyl peptides. It is unclear whether the DTH to this antigenic determinant is involved in the development of arthritis in rats, since a monomer of PG by itself was non-arthritogenic.

Berry et al. (2) postulated that endogenous antigens which were produced locally in tissues due to an alteration induced by adjuvant or adjuvant linking with altered tissue products, or both, may play an important role in adjuvant arthritis. Steffen and Wick (19) demonstrated a DTH reaction to collagen in rats given adjuvants. Trentham et al. (20) found that type II collagen induced polyarthritis in rats similar to adjuvant arthritis. Hence, the possibility cannot be ruled out that poly(I:C) and either *N*-acetylmuramyl peptides or PGs react synergistically as adjuvants in causing alteration of endogenous antigens and eventually participate in the immunopathological events of arthritis. Further studies would be required to elucidate the exact nature of endogenous antigens and the antigenic relationship between endogenous antigens and either bacterial PGs or *N*-acetylmuramyl peptides. The combination of poly(I:C) and MurNAc-L-Ala-D-isoGln or its analogs would provide a useful experimental model either in elucidating more precise structural requirements for development of arthritis or in studying the immunopathological events of arthritis.

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