Brief Definitive Report

ADJUVANT POLYARTHRITIS

V. Induction by *N*-acetylmuramyl-L-alanyl-D-isoglutamine, the Smallest Peptide Subunit of Bacterial Peptidoglycan*

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Adjuvant arthritis in the rat can be induced by a single injection of a dispersion of certain dried, heat-killed microorganisms such as mycobacteria, corynebacteria, streptococci, or their cell wall components such as peptidoglycan and certain peptidoglycan subunits in a suitable oily vehicle (1, 2). The pathogenesis of adjuvant arthritis is not yet clearly understood. There is an impressive body of evidence indicating that the disease arises from an aberrant immune response (3–5) primarily involving the cell-mediated (delayed) hypersensitivity (6), although the humoral immune response may also play a role at an early stage of the disease development (7). The identity or nature of the etiologic immunogen responsible for the disease is not known; components of bacterial cell wall and host tissue proteins are two likely possibilities (8, 9). Supporting the view that adjuvant disease in the rat may be an autoimmune phenomenon resulting in the development of autoantibodies and/or specific sensitized lymphocytes acting against the animal's own tissues (3, 6, 8) were the observations that polyarthritis in the rat, similar to the classic adjuvant arthritis, can be induced by oily preparations of type II collagen, and by an apparently nonimmunogenic synthetic adjuvant completely unrelated to materials of bacterial cell wall origin (10).

We now report that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), the smallest peptide subunit of bacterial peptidoglycan (11), having adjuvant activity but not immunogenicity, induced a polyarthritis in the rat when injected in the form of an oily emulsion. On the other hand, its diastereomer, *N*-acetylmuramyl-L-alanyl-L-isoglutamine (12), which has neither adjuvant activity nor immunogenicity, failed to induce the disease.

Materials and Methods

Animals and Materials. Male Lewis rats weighing 235–250 g were purchased from Microbiological Associates, Walkersville, Md.; EL-4 tumor cells were obtained as a gift from Dr. J. Wunderlich (National Institutes of Health, Bethesda, Md.) and were maintained by passage in syngeneic C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine). ^{51}Cr (200–300 mCi/mg sp ac) was purchased from Abbott Diagnostics, North Chicago, Ill. Freund's complete adjuvant (FCA) was prepared by grinding powdered *Mycobacterium butyricum* (10 mg; Difco Laboratories, Detroit, Mich.) with mineral oil (1.01 ml; Primol 355, Hampden Color Chemical Company). *N*-acetylmuramyl-L-alanyl-L-isoglutamine was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

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Adjuvant Arthritis. Adjuvant arthritis was produced by a single intradermal injection of a given preparation of adjuvant into the tail or one of the hind footpads of male Lewis inbred rats. The volume of the uninjected hind footpad was measured by the method of Winter et al. (13) on days 0 and 16, with respect to the injection of adjuvant, unless otherwise noted.

^{51}Cr-labeling of EL-4 Cells. Both the humoral and cellular assays of immune response are based upon the lysis of ^{51}Cr-labeled EL-4 cells. Labeling was carried out according to the method of Canty and Wunderlich (14).

Humoral and cell-mediated Immunity to EL-4 Cells. Humoral and cell-mediated immunity to EL-4 cells were measured according to a previously reported procedure (15).

Results

Adjuvant Effect of MDP. Groups of seven rats were injected intraperitoneally with either saline (0.5 ml), FCA (0.1 ml), a suspension of 5×10^7 EL-4 cells in saline (0.5 ml), a suspension of 5×10^7 EL-4 cells (0.5 ml) plus Freund's incomplete adjuvant (FIA; 0.1 ml), a suspension of 5×10^7 EL-4 cells (0.5 ml) plus FCA (0.1 ml), a suspension of 5×10^7 EL-4 cells (0.5 ml) plus emulsion of MDP in FIA (0.1 ml), or a suspension of 5×10^7 EL-4 cells (0.5 ml) plus an emulsion of the MDP isomer in FIA (0.1 ml). The animals were killed 16 d after sensitization. The spleen was then removed and a blood sample collected. The complement-independent lysis of ^{51}Cr-labeled EL-4 cells by the spleen lymphocytes was determined and used as a measure of cell-mediated immune response to EL-4 cells. The antibody titer of the serum was determined by using ^{51}Cr-labeled EL-4 cells. Results are shown in Fig. 1. Animals that received an intraperitoneal injection of a suspension of EL-4 cells in saline developed both humoral and cell-mediated immune responses to EL-4 cells. Both the humoral and the cell-mediated immune responses were augmented in animals that received an injection of EL-4 suspended in FCA or in an emulsion of MDP in FIA ($P < 0.01$). No enhancement was observed in animals that received an injection of EL-4 cells suspended in an emulsion of *N*-acetylmuramyl-L-alanyl-L-isoglutamine in FIA ($P > 0.1$).

Development of Arthritis after the Injection of FCA, MDP in FIA, or N-acetyl-L-alanyl-L-isoglutamine in FIA. A subplantar injection of either FCA (0.1 ml), emulsion of MDP in FIA (5.0 mg/ml, 0.1 ml), emulsion of *N*-acetylmuramyl-L-alanyl-L-isoglutamine in FIA (5.0 mg/ml, 0.1 ml), or saline (0.1 ml) was administered to Lewis rats (seven animals per group), and the volume of the uninjected hindfootpads of each rat was measured at regular intervals for 16 d. The results are plotted in Fig. 2. The development of arthritis in animals given MDP followed the same time course as animals injected with FCA. The disease had become well established by day 16.

Dermal Hypersensitivity Reactions in Rats with MDP-induced Polyarthritis. Groups of seven rats were given a subplantar injection (0.1 ml) of saline, an emulsion of MDP in FIA (5.0 mg/ml, 0.1 ml), an emulsion of MDP in FCA (5.0 mg/ml), or an emulsion of *N*-acetyl-L-alanyl-L-isoglutamine in FIA (5.0 mg/ml, 0.1 ml). On the 16th d after injection, all animals were given intradermal injections of 0.1 ml of saline solutions of MDP at concentrations of 6.25, 12.5, 25, and 50 μg/0.1 ml, and solutions of *N*-acetylmuramyl-L-alanyl-L-isoglutamine at concentrations of 6.25, 12.5, 25, and 50 μg/0.1 ml. Observations were made at 4, 6, 24, and 48 h after intradermal injection. No significant inflammation was observed at any injection site throughout the observation period.

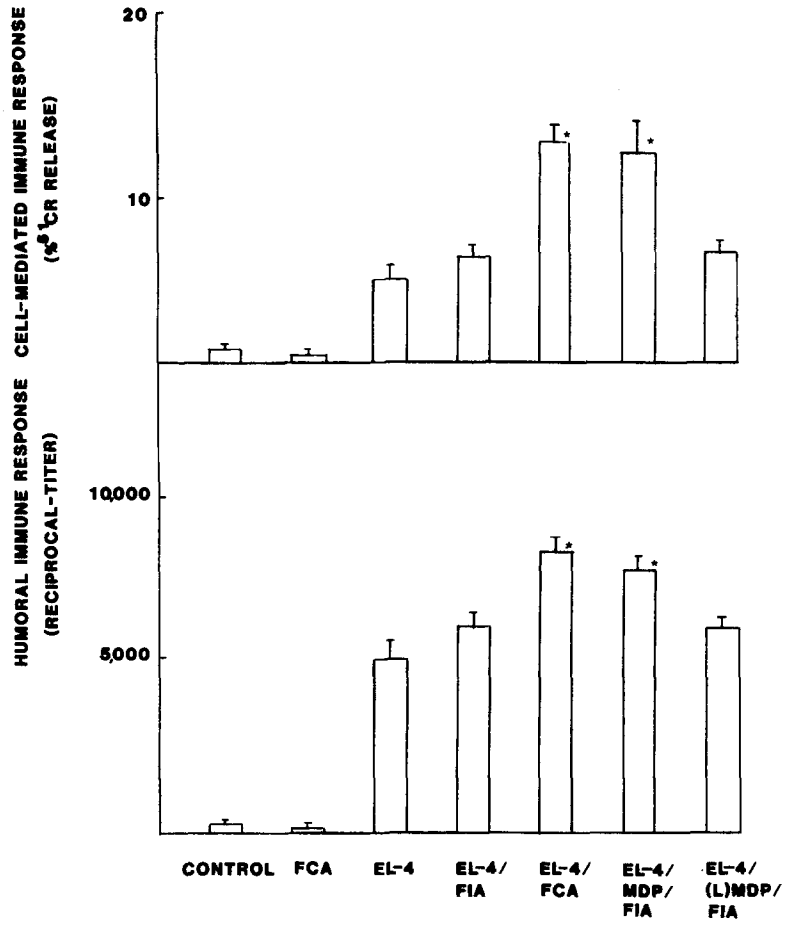

FIG. 1. The development of the humoral and the cell-mediated immune response to EL-4 cells in Lewis rats injected intraperitoneally with saline, FCA, or a suspension of EL-4 cells in: 1) saline; 2) FIA; 3) FCA; 4) an emulsion of MDP in FIA; and 4) an emulsion of the isomer of MDP in FIA. The bars represent standard deviations. Antibody titers shown are means of four determinations with pooled sera. *Significantly different from the control values ($P < 0.01$).

Discussion

Adjuvant arthritis in the rat, as well as rheumatoid arthritis in humans, is believed to arise from an aberrant immune response to antigen(s) whose identity remains elusive. The disease may be (*a*) the result of a delayed hypersensitivity response to bacilli or their constituents, such as peptidoglycans (2), which resist degradation by mammalian lysosomal enzymes (17) and may remain in macrophages to serve as a persistent source of immunogen; or (*b*) an autoimmune phenomenon brought about by the injection of adjuvant and resulting in the development of autoantibodies and/or specific sensitized lymphocytes acting against the animal's own tissues (3, 6, 8).

In an effort to identify the substances in the bacterial cell wall responsible for the induction of adjuvant disease, we and others have, during the past decade, isolated and identified many chemical moieties in the bacterial cell wall and tested for their ability to induce arthritis when mixed with an oil vehicle. Bacterial cell wall can be

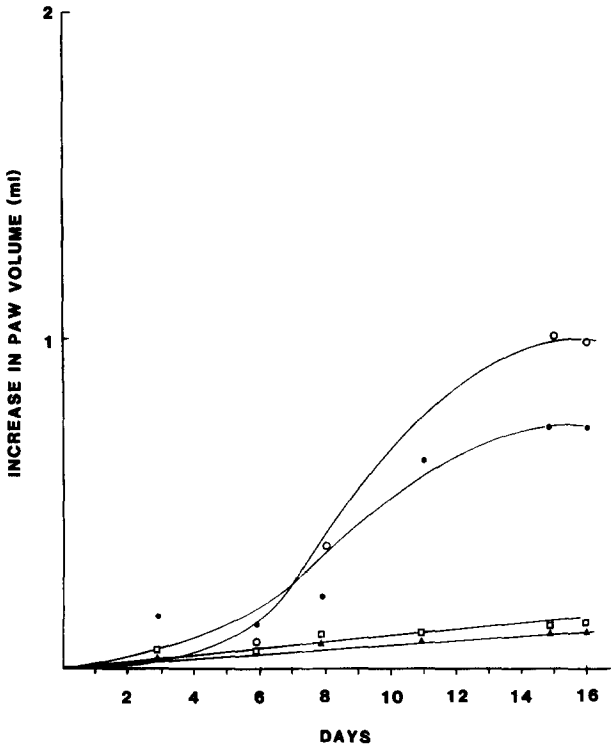

FIG. 2. Kinetic study on the development of arthritis after a single subplantar injection of saline (▲), FCA (○), an emulsion of MDP in FIA (●), or an emulsion of the isomer of MDP, FIA (□).

replaced by wax D, a chloroform-soluble fraction of cell wall (18), and by water-soluble cell wall fractions (2, 19). Both wax D and the water-soluble fractions contain a peptidoglycan moiety that was found to be arthritogenic. Specific enzymatic degradation of cell-wall-derived peptidoglycan produced a group of peptidoglycan subunits (20). Two of the peptidoglycan subunits, an oligosaccharide-hexapeptide[1] and a disaccharide-hexapeptide polymer[2] were found to be capable of producing adjuvant arthritis. An oligosaccharide-hexapeptide monomer was nonarthritogenic. Whether these active materials induce adjuvant arthritis by providing the necessary etiologic antigen (immunogenicity) or by stimulating the immune response to certain intrinsic antigen(s) within the host (adjuvanticity) has not been clearly established. Certain peptidoglycan subunits, such as disaccharide-hexapeptide, shown to have adjuvant activity in the guinea pig, were found to be incapable of inducing adjuvant arthritis in the rat (2, 20). This apparent lack of correlation may be due to a difference in structural requirement between adjuvanticity and arthritogenicity. Alternatively, it may be due to a species difference between the guinea pig and the rat in structural requirements for adjuvanticity. The adjuvant activity of these materials in the rat has not been determined. MDP has been reported to be arthritogenic by Nagao and

[1] (*N*-acetylglucosaminyl-*N*-acetylmuramyl)$_{3\ (or\ 4)}$-*N*-(L-alanyl-D-isoglutaminyl-*N*-(Glycyl-glycine)-L-lysyl-D-alanine.

[2] (*N*-acetylglucosaminyl-*N*-acetylmuramyl-*N*-(L-alananyl-D-isoglutaminyl-*N*-(glycyl-glycine)-L-lysyl-D-alanine)$_n$.

Tanaka (21) and nonarthritogenic by others (2, 12). These differences could perhaps be related to some genetic or environmental (viral) factors.

There is some suggestive evidence that supports the view that the immunogen(s) responsible for the development of adjuvant arthritis is intrinsic, e.g., a constituent of host tissue, or a viral host protein complex. First, an oily preparation of an apparently nonimmunogenic synthetic compound, *N*,*N*-dioctadecyl-*N'*,*N'*-bis(2-hydroxyethyl)-propane-diamine, completely unrelated to bacterial cell wall materials, was found to be capable of inducing a polyarthritis in rats that was almost indistinguishable from classic adjuvant arthritis induced by FCA both in morphology and in the time course of disease development (10). Second, Trentham et al. (22) showed that a single injection of an oil emulsion of native type II collagen induced a chronic arthritis in Wistar rats that was similar to the classic adjuvant arthritis and appeared concurrently with the development of hypersensitivity to type II collagen. Third, there is circumstantial evidence that the immunogen(s) responsible for the development of adjuvant arthritis might be a latent virus or virus-host-protein complex (10, 23). For example, administration of interferon-inducing substances, interferon-containing plasma from Sindbis virus-infected rats, or interferon prepared in vitro from cultures of rat embryo fibroblasts (23), was reported to suppress the development of adjuvant arthritis.

The present finding that an apparently nonimmunogenic, bacterial peptidoglycan-derived small peptide was capable of inducing adjuvant arthritis lends further support to the view that intrinsic immunogen(s) play a role in the development of this disease. The development of autoimmunity to such immunogen(s) requires adjuvant stimulation because *N*-acetylmuramyl-L-alanyl-L-isoglutamine, which does not possess adjuvant activity, failed to produce the disease.

Torisu et al. (24) reported that 6% of the patients with cancer who had received bacille Calmette-Guérin immunotherapy developed arthritis that was considered a side effect of bacterial adjuvant. The present results and the previous finding of arthritogenicity of an alkyldiamine (10) suggest that the arthritogenesis as a side effect in some situations may not be limited to bacterial adjuvant.

Summary

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), an apparently nonimmunogenic bacterial peptidoglycan-derived small peptide, was found to induce a polyarthritis in the rat similar to that induced by Freund's complete adjuvant when injected in the form of an oil emulsion. An oil emulsion of its isomer, *N*-acetylmuramyl-L-alanyl-L-isoglutamine, which unlike MDP has no immunostimulatory activity, failed to induce the disease.

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