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Dissociation between the Adjuvant vs Mitogenic Activity of a Synthetic Muramyl Dipeptide for Murine Splenocytes (39804)

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Dissociation between the Adjuvant vs mitogenic activity of a synthetic muramyl dipeptide for murine splenocytes. Recent studies have revealed that the muramyl dipeptide *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) possesses the minimal structure required for the adjuvant activities of mycobacteria demonstrable by stimulation of enhanced immune responses *in vivo* and *in vitro* (1-4). This compound enhances humoral immunity to soluble antigens even when administered by a route different from that of the immunogen or when given orally (5, 6). In addition, this substance stimulates an enhanced immune response to BSA in LPS-low responder mice (5). Studies concerning the mechanism of action of MDP, as well as other peptidoglycans obtained from bacterial cell walls, indicate that these low-molecular-weight substances may be potent adjuvants because they stimulate T lymphocytes (6) and possibly macrophages (7).

Earlier studies in this laboratory showed that B lymphocytes responding to an antigen such as sheep erythrocytes may be directly stimulated by a synthetic muramyl dipeptide (8). This observation is especially relevant to current concepts of immunogenicity, since it has been postulated that only high-molecular-weight substances with repeating subunits can function as nonspecific stimulators of B lymphocytes responding to T-dependent antigens (9, 10). Many immunologic stimulators are generally considered to be B-lymphocyte mitogens, since often they do not affect the immune response in an antigen-specific manner. In the present study a synthetic muramyl dipeptide was utilized to study in parallel mitogenic activity and antibody-stimulating activities *in vitro*. The muramyl dipeptide was added to normal splenocyte cultures which were tested for antibody formation to sheep RBCs and for [^{3}H]thymidine uptake.

Methods and materials. Experimental animals. Adult male Balb/c mice weighing 18 to 20 g each were used for these experiments. The animals were maintained in plastic mouse cages in groups of 6 to 10 and were fed Purina mouse pellets and water ad libitum.

Spleen cell cultures. The *in vitro* Marbrook culture system was used to determine antibody formation at the single cell level (11). Dispersed cell suspensions were obtained from normal mice, and 5×10^6 viable cells in 1.0 ml of tissue culture medium (MEM plus 10% fetal calf serum) were placed on a dialysis membrane in the inner chamber. The outer chamber contained 11 ml of medium (12).

Spleen cell stimulators. Muramyl dipeptide (MDP) was prepared exactly as described elsewhere (13). *Escherichia coli* LPS was prepared from strain B0127-B8 and obtained from Difco Laboratories, Detroit, Michigan. Phytohemagglutinin-P (PHA) was obtained from Difco Laboratories and used at a 10^{-2} dilution of stock preparation. For use MDP, LPS, or PHA was dissolved in sterile medium and 0.1 ml of an appropriate concentration was added to spleen cell cultures.

In vitro immunization and antibody formation. For immunization, a suspension of freshly washed sheep red blood cells (SRBC) containing approximately 2×10^6 erythrocytes was added to each Marbrook chamber containing spleen cells. The numbers of antibody plaque forming cells (PFCs) appearing in the cultures 4-5 days later were determined by the standard hemolytic plaque assay in agar gel exactly as described elsewhere (14). Zones of hemolysis appear-

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ing in individual plates were considered to be due to 19S IgM antibody. For each experiment PFC assays were performed at least three times with triplicate cultures.

Mitogenicity assay. Spleens obtained aseptically from normal mice were minced with scissors and forceps in RPMI 1640 medium supplemented with 5% fetal calf serum. The cells were washed several times by centrifugation in the cold (4°) and erythrocytes were lysed by hypotonic shock. The cells were then resuspended in physiological saline solution and washed several times with medium, and 0.1 ml containing 10^6 viable nucleated cells was placed into flat-bottomed vials to which was added 0.9 ml of medium and 0.1 ml of the appropriate concentration of MDP or, as a control, LPS or phytohemagglutinin (PHA). The cultures were incubated at 37° in a moist chamber containing 5% CO_2, and after 24 hr were pulsed with 2 μCi of [^{3}H]thymidine and reincubated for an additional 24 hr. The cells were removed, collected on glass-fiber filter paper (Whatman GF/A), air-dried, and resuspended in 10 ml of PCS (Amersham Searle). Radioactivity was determined by 10-min counting in a Packard scintillation counter.

Experimental results. Incubation of normal spleen cells with graded concentrations of either MDP or *E. coli* LPS resulted in markedly stimulated antibody PFC responses to sheep erythrocytes, as compared to controls. For example, as can be seen in Table I, 10 to 50 μg of MDP resulted in a two- to threefold enhancement of the PFC responses as compared to controls. The same concentration of LPS resulted in a similar PFC stimulation. Likewise, similar concentrations of MDP and LPS resulted in a "polyclonal" stimulation of background PFCs to SRBCs in the absence of antigen (Table I). For example, normal spleen cell cultures incubated *in vitro* without antigen showed a moderate increase in background PFCs. The number of such background PFCs was twice as high in the presence of MDP, similar to the stimulation of background PFCs observed with LPS.

Lipopolysaccharide stimulated a marked enhancement of [^{3}H]thymidine incorporation into normal splenocytes. However, MDP caused a much weaker stimulation. As shown in Table II, the normal background uptake of thymidine into unstimulated splenocyte cultures was increased two- to threefold upon treatment with 10 μg of

TABLE I. EFFECT OF GRADED AMOUNTS OF MURAMYL DIPEPTIDE AND *E. coli* LPS ON SRBC-INDUCED AND BACKGROUND PFC RESPONSES OF NORMAL MOUSE SPLEEN CELLS CULTURED *in Vitro*.

Stimulator added to cultures[a]	Antibody response *in vitro*[b]			
	With antigen[c]		Without antigen	
	PFC/10^6 spleen cells	Percentage of control	PFC/10^6 spleen cells	Percentage of control
None (controls)	318 ± 67	–	23 ± 9	–
MDP (μg)				
1	343 ± 108	108	45 ± 25	196
10	656 ± 214	206	67 ± 23	291
25	728 ± 175	229	76 ± 18	330
50	983 ± 196	309	75 ± 18	326
LPS (μg)				
10	464 ± 40	146	71 ± 12	307
25	531 ± 61	167	61 ± 10	265
50	764 ± 51	240	66 ± 14	287

[a] Indicated dose of stimulator added in 0.1-ml quantities to 5 × 10^6 spleen cells from normal Balb/c mice culture *in vitro* in Marbrook chambers.

[b] Average PFC response per 10^6 viable splenocytes for five to six cultures per group 5 days after culture initiation.

[c] Each culture was immunized on day of culture initiation with 2 × 10^6 SRBC.

TABLE II. MITOGENIC EFFECT OF MDP, LPS, AND PHA ON NORMAL MOUSE SPLEEN CELLS *in Vitro* ASSAYED BY [^{3}H]THYMIDINE INCORPORATION.

Stimulator added *in vitro*[a]	cpm ± SE[b]	Stimulation index[c]
None (controls)	3012 ± 786	–
MDP (μg)		
1	4171 ± 363	1.4
10	8224 ± 622	2.7
50	7039 ± 700	2.3
100	7022 ± 767	2.3
LPS (μg)		
10	26,906 ± 6462	9.5
25	33,290 ± 4280	11.8
50	38,341 ± 11,372	12.6
PHA		
10^{-2}	28,614 ± 3322	9.5

[a] Cultures of 1 × 10^6 normal Balb/c spleen cells were incubated with indicated material for 24 hr and then pulsed with [^{3}H]thymidine for additional 24 hr.

[b] Average response of three to six cultures per group; counts per minute determined from 10-min counts.

[c] Stimulation index = average cpm for treated cultures/average cpm for control cultures.

MDP. Addition of 50 or 100 μg of MDP per culture resulted in no greater stimulation. On the other hand, LPS at a concentration of 10 to 50 μg per culture resulted in a 10-fold or greater increase in thymidine incorporation. PHA stimulated a 9- to 10-fold increase.

Discussion. Results of this study revealed a marked stimulation of antibody responses to sheep red blood cells by splenocytes incubated with a relatively low-molecular-weight synthetic muramyl dipeptide, even though this material had only weak effects on the blastogenic response of the cells during a 72-hr incubation *in vitro*. Such results contrast with some of the current views in cellular immunology postulating that the adjuvant activity of a bacterial cell wall product is intimately related to mitogenicity, both *in vivo* and *in vitro* (15–18). For example, it is felt by some investigators that receptors on the surface of B lymphocytes react directly with a mitogen, resulting in a nonspecific polyclonal activation of lymphoid cells (18). In the presence of a specific antigen, increased numbers of antibody-forming cells to the immunogen develop, but the major signal which activates an immunocyte is thought to be due to a mitogenic not an antigenic stimulus (19). In this context, it has been postulated that only those substances which can directly trigger proliferation of lymphocytes function as stimulators of immune responses (9).

Recent studies in a number of laboratories, including this one, have indicated that low-molecular-weight substances which are naturally present in the cell walls of bacteria known to have "natural" adjuvant properties can also stimulate enhanced immune responses *in vitro*, yet lack mitogenic activities (4, 8). For example, the water-soluble polysaccharide-rich material derived from bacterial LPS has been found to stimulate an *in vitro* anti-RBC response similar to that of the intact LPS, despite the fact that the low-molecular-weight detoxified material was not mitogenic (10). Similarly, other recent studies have shown that synthetic low-molecular-weight substances related to natural bacterial cell wall products may enhance immune responses without being mitogenic. For example, Rosenstreich *et al.* (20) reported that the synthetic glycolipid palmitoyl-β-glucosamine was not mitogenic for B lymphocytes unless heated and sonicated. This indicated that the physical state and a certain degree of dispersion were necessary for mitogenicity. Similarly, Azuma *et al.* (4) recently reported that water-soluble but polymerized peptidoglycolipids of several bacterial strains were not mitogenic for lymphocytes.

Other studies have shown that water-soluble adjuvants consisting of galactans and peptidoglycans which react with macrophages were also B-cell mitogens (21). In this study, Damais *et al.* (21) reported that a monomer prepared from *E. coli* which had potent adjuvant activity for spleen cells of rabbits and normal or nude mice was not mitogenic. Therefore, the adjuvant and mitogenic activities of cell walls of certain bacteria may be dissociated in the low-molecular-weight peptidoglycans or muramyl dipeptides. The higher-molecular-weight substances or the aggregated form of the low-molecular-weight materials may be necessary for mitogenicity, but not adjuvanticity.

Thus low-molecular-weight substances commonly found in the cell walls of bacteria may serve as stimulators of immune responses even though they lack the ability to directly induce marked blastogenic transformation of immunocytes, comparable to that obtained with known control T- and B-cell mitogens. This supports the less prevalent view that mitogenicity may not be a necessary prerequisite for the adjuvant activity of an immunologic stimulator.

Summary. A relatively low-molecular-weight synthetic muramyl dipeptide was found to stimulate antibody formation by normal mouse splenocytes immunized with sheep red blood cells *in vitro*. This material also resulted in a marked stimulation of "background" antibody-forming cells to the erythrocytes in cultures incubated without antigen. In contrast, the muramyl dipeptide, regardless of concentration, did not induce a marked blastogenic response *in vitro*, as occurs with adjuvants such as bacterial lipopolysaccharides. These results suggest that the nonspecific immunostimulatory properties of a low-molecular-weight substance such as the muramyl dipeptide tested, which is commonly found in bacterial cell walls, may not be dependent upon mitogenicity.

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