Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 431-439

RESTORATION AND STIMULATION OF THE IN VITRO IMMUNE RESPONSE OF B CELLS TO SHEEP ERYTHROCYTES BY INTERLEUKINS AND MURAMYL DIPEPTIDE (MDP)

Vongthip Souvannavong and Arlette Adam

Institut de Biochimie, Université Paris-Sud, Bât. 432, 91405 Orsay, France

Received October 19, 1984

SUMMARY: MDP, a synthetic muramyl dipeptide, is capable of increasing the primary in vitro antibody response, to sheep erythrocytes, of purified B cells restored with a monokine and helper T cell factors, including Interleukin 2 and the late-acting T cell replacing factor (TRF). First, the possibility that the adjuvanticity of MDP could be due to the elaboration of Interleukin 1, caused by its effect on macrophages, was excluded. In addition, a kinetic study showed that the effect of MDP was greater when added later, concomitantly with or one day after the helper T cell factors.

Therefore, it appears that MDP acts directly on B cells, in a late stage of their differentiation to antibody-producing cells.

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is the smallest effective structure for adjuvanticity of cell wall peptidoglycans (1). Among its various biological activities (reviewed in 2), MDP has been shown to enhance or to depress, both in vivo and in vitro, the antibody production by murine spleen cells in response to sheep erythrocytes (SRBC)(3-8). However, the mechanism of action of MDP for adjuvanticity is not yet well defined. We reported recently that MDP can enhance the IgM response to SRBC in macrophage-depleted spleen cultures restored with either interleukin 1 (IL 1) or a 35.000 M.W. monokine with macrophage replacing activity (named FRM) (9). However, as MDP stimulates macrophages to produce IL 1, it was suggested that its adjuvant effect could be mediated by IL 1 (10,11,12).

We now extend our first results by studying the effect of MDP on the maturation of macrophage precursors; it was indeed quite possible that MDP could

Abbreviations: MDP, muramyldipeptide; FRM, factor having macrophage-replacing activity; IL 1, Interleukin 1; IL 2, Interleukin 2; MLC, mixed lymphocyte culture; TRF, T cell-replacing factor; SRBC, sheep red blood cells; PFC, plaque-forming cell; HEPES,N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid.

0006-291X/84 $1.50

have acted on these cells to induce their maturation, and subsequently the release of IL1 which could then account for MDP adjuvanticity.

A recent report (13) has shown that the production of specific antibody-forming B cells to SRBC requires the synergistic effect of at least three non-specific helper factors, namely a monokine contained in culture supernatants of P388D1 macrophage cell line (probably IL 1), IL 2 and a 30,000 to 50,000 MW factor (IL X) present in Con A-activated spleen cell culture supernatants.

Therefore, we investigated whether MDP also exhibits immunostimulatory activity in B cell cultures, reconstituted with the partially purified monokine FRM and helper T cell factors.

MATERIALS AND METHODS

Mice: 7 to 12-week-old female BDF1 (C57BL/6xDBA/2), C3H/HeJ, C57BL/6 and DBA/2 mice from CSEAL (Orléans, France) were used.
Adjuvant, antigen and medium: MDP was kindly provided by Dr P. Lefrancier (Choay, Montrouge, France). Sheep erythrocytes were obtained from a selected single donor and kindly provided by the CNRZ (INRA, Brouessy, France). We used RPMI-1640 (Gibco) supplemented with 25mM HEPES, L-glutamine, 8% heat-inactivated fetal calf serum (Gibco), 50μM 2-ME and standard antibiotics.
Preparation of FRM (14): BDF1 resident peritoneal cells were cultured at $1x10^6$/ml for 90 min. to 2 hr and the non adherent cells removed by washing. A further overnight incubation was carried out to prepare the supernatant of adherent cells which was concentrated ten fold with Immersible-CX (Millipore) and then chromatographed on Ultrogel AcA 54 (IBF). FRM eluted in the fraction of an apparent MW of about 35,000.
Production of mixed lymphocyte culture (MLC) supernatants: allogeneic culture supernatants were obtained by mixing whole spleen cells from normal DBA/2 and C57BL/6 mice at 10^7 cells/ml for 24 hr. The MLC supernatant was concentrated 10-fold and chromatographed on an Ultrogel AcA 54 column.
Cell preparations: Macrophages were removed by passage of BDF1 splenocytes through columns of Sephadex G-10 (15). The cells obtained, referred to as G-10 passed cells, were apparently depleted of macrophages as judged by phagocytosis of latex beads, neutral red uptake and esterase staining. Splenocytes were depleted of T cells by incubating 10^7 cells/ml with a monoclonal F7D5 anti-Thy-1.2 antibody (1/500, Olac, Bicester, UK) for 30 min. at 37 °C, then for 45 min. at 37 °C with complement (C, 1/10). To obtain B cells, whole splenocytes were first depleted of macrophages and then treated with anti-Thy-1.2 plus C.
Culture assays (16): 10^6 splenocytes, G-10 passed cells or B cells were cultured in flat bottom wells with 10^6 SRBC (total volume of 200 μl). The IgM anti-SRBC PFC response was determined on day 5. Mean PFC of quadruplicate cultures is given. The cell recovery (about 35%) was not significantly affected by the presence of either supernatant or MDP.
Bioassay for IL 1 activity: C3H/HeJ thymocytes ($1.5x10^6$/200μl) were cultured with samples either alone, or with 2,5 μg/ml of phytohemagglutinin (PHA). The cultures were pulsed with ^{3}H-TdR for the last 6 hr of an incubation of 3 days (1 μCi/well, specific activity 2 Ci/mmole, CEA Saclay). ^{3}H-TdR incorporation was determined and the mean cpm of triplicate cultures is given.
TRF activity: T cell replacing (TRF) activity of supernatants was estimated by their restoring effect on the response of T depleted-splenocytes to SRBC (17).
Bioassay for IL 2 activity: T cell growth promoting activity was assayed by using an IL 2-dependent murine cytotoxic T lymphocyte line (CTLL-2) (18).

$4x10^3$ cells/200µl were cultured in round-bottomed wells with or without various dilutions of the samples to be tested. After 24 hr of culture, ^{3}H-TdR was added for an additional 18 hr. A culture supernatant from Con A-activated mouse spleen cells was used as a positive control (standard IL 2).

RESULTS

Maturation of macrophage precursors in G-10 passed cells. Influence of MDP.

The presence of macrophage precursors in splenocytes after passage through Sephadex G-10 was investigated. Mature macrophages were detected by using the production of IL 1 and quantified by neutral red uptake; the number of positive cells obtained upon incubation of G-10 passed cells with or without MDP is reported on Table 1. Although less than 10 positive cells per 10^6 cultured cells were present at the initiation of cultures, mature macrophages were present at day 5. Their number may vary from experiment to experiment from 500 (exp. 4) to 2,000 (exp. 1) but, in every case, the presence of MDP was without significant effect on the number of newly formed macrophages.

TABLE 1

EFFECT OF MDP ON THE MATURATION OF MACROPHAGES FROM THEIR PRECURSORS IN G-10 PASSED CELLS

Days of incubation of G-10 passed cells (a)	Number of macrophages in G-10 passed cells incubated various times per 10^6 cultured cells (b)				Activity of supernatants from G-10 passed cells (c)					
	experiment 1		experiment 4		on the restoration of the antibody response PFC (d)		on the proliferation of thymocytes Incorporation of ^{3}H-TdR cpm (e)			
MDP (1 µg/ml)	-	+	-	+	-	+	-	-	+	+
								+PHA		+PHA
0	less than 10		less than 10		0 to 40		180	376		
1	280	280	ND	ND	42	41	131	347	141	321
2	820	840	ND	ND	28	48	161	429	178	519
3	700	920	340	400	76	360	133	335	147	313
4	1400	1400	400	460	630	1058	494	1160	1161	2558
5	1200	1600	580	520	1232	1623	2871	5413	3271	5250

a) Macrophage-depleted spleen cells, obtained by passing spleen cells through Sephadex G-10, were cultured in the presence or absence of MDP at 1 µg/ml; supernatants were recovered after various times of incubation.
b) G-10 passed cells were plated at $4x10^6$/4ml in 9 cm^2 tissue culture dishes. The presence of macrophages was determined by counting the neutral red positive cells: results of experiments with the higher level (exp. 1) and the lower level (exp. 4) are given.
c) Supernatants, obtained after 1 to 5 days, were assayed at 100ul and were added at zero time.
d) $1x10^6$ G-10 passed cells were cultured with $1x10^6$ SRBC in microwells (200 µl); PFC per culture on day 5.
e) Activity on the proliferation of thymocytes from C3H/HeJ mice determined either directly or in the presence of PHA (2.5µg/ml). No mitogenic activity could be detected in 3 experiments. Data presented are those of experiment 1; cpm per culture of $1.5x10^6$ cells, on day 3.

We examined the supernatants obtained from G-10 passed cells, after various times, to detect the presence of IL 1-like activity. This activity was measured by both its effect on the proliferation of thymocytes, and its helper activity on the antibody response to SRBC. The experiment was repeated five times: in three of them, no IL 1 activity could be detected, even after 5 days of incubation; in the two other experiments, some IL 1-like activity appeared in the supernatants, only after 4 days of incubation; the results of experiment 1 are presented on Table 1. As could be expected, a higher level of activity was observed when cells were incubated with MDP. But these supernatants were active in the reconstitution of an antibody response, only when added at the initiation of cultures of G-10 passed cells: for example, if the addition of day 4-supernatant was delayed till the second day of cultures or later, it was no longer able to reconstitute any response (data not shown). Therefore, IL 1 cannot account for the increased specific IgM response induced by MDP.

Effect of MDP on the IgM response to SRBC in B cell cultures

Enriched splenic B cells, depleted of both T cells and macrophages, do not differentiate into IgM plaque-forming cells (PFC) in the presence of antigen alone (Fig.1). However, their immune responsiveness could be fully restored by

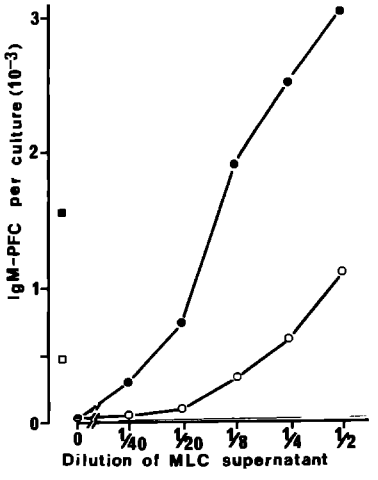

FIGURE 1. Synergy between MDP and MLC supernatant. B cells were cultured with SRBC in the presence of various dilutions of MLC supernatant, without (O — O) or with (● — ●) MDP at a final dose of 1µg/ml. Control whole spleen cells were cultured with SRBC, without (□) or with MDP (■).

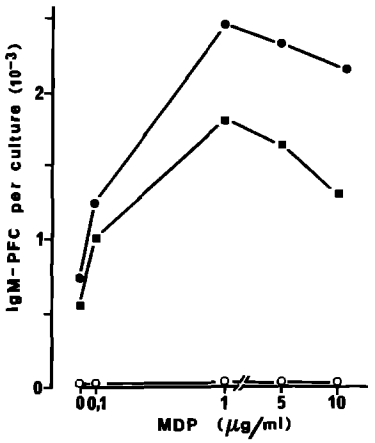

FIGURE 2. Effect of various doses of MDP on the primary response to SRBC in B cell cultures. B cells were cultured with SRBC, without (O—O) or with (●—●) MLC supernatant at a final dilution of 1/4. Whole splenocytes cultured with SRBC in the absence of MLC supernatant, (■—■) .

the addition of mixed lymphocyte culture (MLC) supernatants, which contain the soluble factors providing macrophage and helper T cell signals required for B cells to differentiate into IgM producing cells. MDP alone, at the optimal dose of 1 µg/ml, was without effect on the B cell response; but, it was capable of potentiating the anti-SRBC PFC in cultures supplemented with MLC supernatants. This synergistic effect of MDP could be seen with sub-optimal doses of helper factors, and was even more marked when using higher dilutions of MLC supernatants.

Therefore, MDP is able to promote the IgM PFC response to SRBC, in the apparent absence of both macrophages and helper T cells. In addition, as can be seen in Fig.2, this effect of MDP on the response of B cells, reconstituted with MLC supernatants, has the same profile of dose response as that obtained with whole spleen cell cultures. These results suggest that, with respect to its adjuvant activity, MDP behaves in the same way in the presence of both macrophages and helper T cells, or when these cells are substituted by soluble factors for their helper activity.

Effect of MDP on B cells cultured with various combinations of lymphokines

We performed reconstitution experiments in which the antibody response of enriched splenic B cells was restored with a monokine and lymphokines,

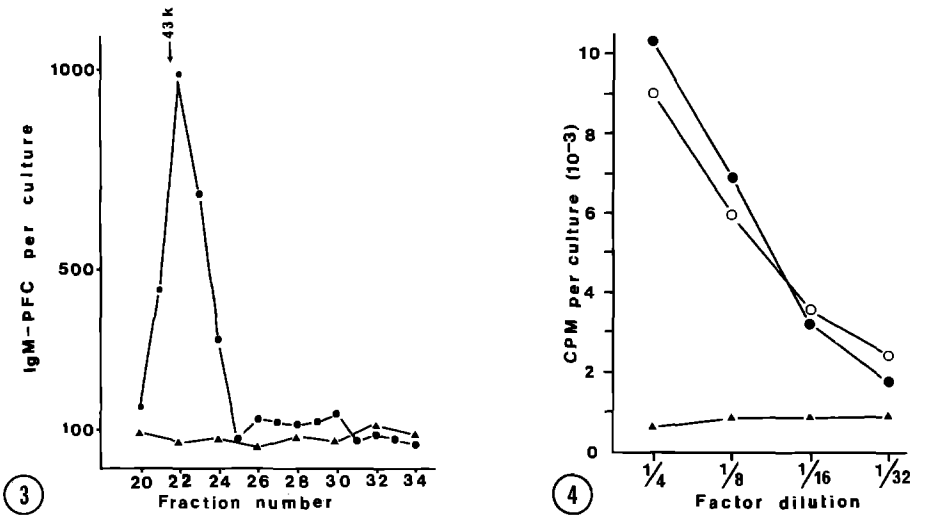

Figure 3. Determination of TRF activity after gel filtration of supernatants on Ultrogel AcA54. 40 µl of each fraction was used for TRF activity. MLC supernatant (●—●) ; FRM preparation (▲—▲) .

FIGURE 4. Determination of IL 2 activity in the preparations of lymphokines used to restore the IgM response of B cells. FRM preparation (▲—▲) , or a pool of 35,000-45,000 M.W. fractions of MLC supernatant (●—●) , were assayed for IL 2 activity. Standard IL 2 (○—○) .

partially purified by chromatography on Ultrogel AcA54. The T cell replacing factor (TRF) activity present in MLC supernatants, as assayed by its ability to support the direct anti-SRBC PFC response of T depleted spleen cells, eluted in 35,000-45,000 M.W. fractions (Fig.3), which were pooled and tested for IL 2 activity. Results reported in Fig.4 show that IL 2 activity of these fractions was as important as that found in standard IL 2. This pool of fractions which contained the helper T cell factors was referred to as IL2-TRF.

The macrophage helper factor was provided by FRM which has neither IL 2 activity (Fig.4), nor TRF activity (Fig.3). As previously shown by a kinetic study, FRM was absolutely required in an early event of the immune induction. In the present study, FRM was added at the initiation of the cultures. As can be seen in Fig.5, FRM either alone, or in addition with MDP, was without effect on the B cell response. Similar results were obtained with IL 2-TRF, either alone or in the presence of MDP. In contrast, when both IL2-TRF and FRM were added, the response of B cells was fully restored and reached the levels obtained when using crude MLC supernatants. However, a greater response was obtained when the helper T cell factors were added on day 1.

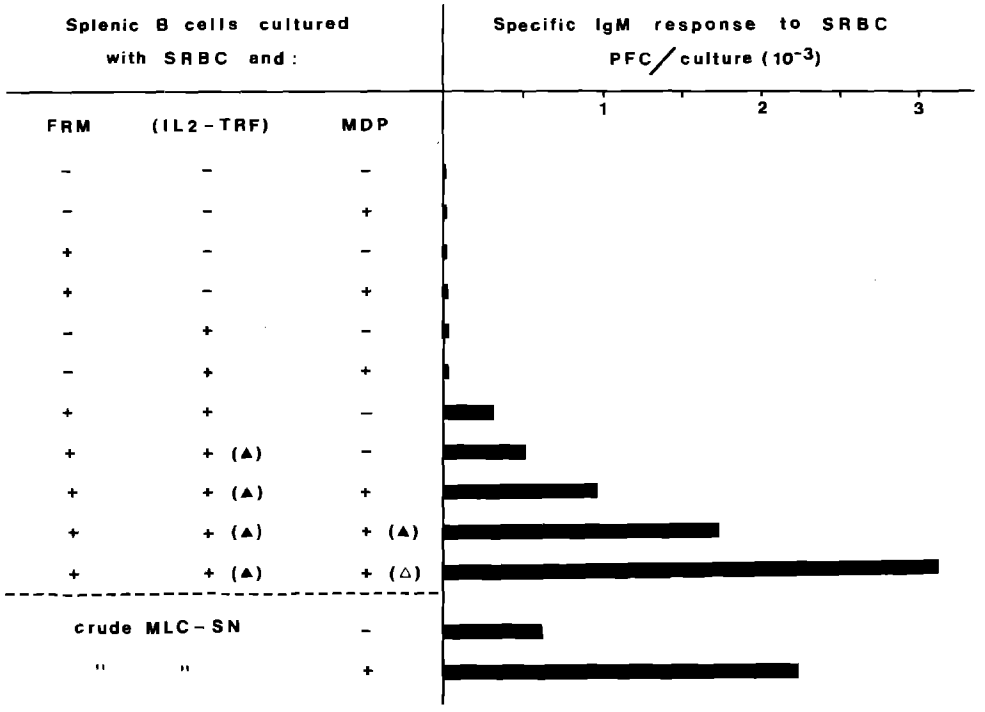

FIGURE 5. Ability of MDP to enhance the IgM response of B cells reconstituted with helper factors. B cells were cultured with SRBC, in the presence of 40 µl of crude MLC supernatant or various combinations of helper factors. Factors added on day 1, (▲); on day 2, (△); on day 0, if not indicated. FRM:10 µl; IL 2-TRF:20 µl; MDP:1µg/ml.

A kinetic study was performed to determine the optimal time for adding MDP. An enhancing effect of MDP can be seen when it is added either on day 0, or on day 1 concomitantly with IL2-TRF, but the best adjuvant activity is obtained when MDP was added on day 2, one day after the helper T cell factors. These results show that MDP is not required at an early step of the activation of B cells, but in a later stage of B cell response.

DISCUSSION

The data reported here establish the capacity of MDP to enhance the primary antibody response to SRBC of purified B cells.

Watson and Whitlock (6), by using a limiting dilution analysis in nude splenocyte cultures, suggested that MDP could act directly on B cells for its adjuvant effect, by mimicking the T helper cell signal; however, they did not exclude the possibility that MDP could stimulate macrophages to produce helper

factors. Leclerc et al.(8) also made this hypothesis, because the response of spleen cells of nude mice on the one hand, and on the other, of splenocytes of normal mice depleted of macrophages by carbonyl iron ingestion restored with 2-mercaptoethanol (2-ME), were stimulated by MDP.

We recently reported that MDP is without effect on the antibody response of macrophage-depleted splenocytes, unless a monokine is added to cultures, even in the presence of 2-ME (9). Our present study on the effect of MDP on the maturation of macrophage precursors eliminates the possibility that macrophages could mediate the adjuvanticity of MDP via the release of IL 1.

The B cells, obtained by depletion of both Thy-1.2-bearing and SephadexG10 adherent cells, are unresponsive by themselves and require the presence of interleukins. Their response was restored with a 35,000 M.W. monokine with macrophage replacing activity called FRM (14), and a 35,000-45,000 fraction of MLC supernatants, containing at least IL 2 activity and a TRF-like activity such as that described by Schimpl and Wecker (17). In such restored cultures, MDP is able to increase the antibody response, thus suggesting its direct effect on B cells for adjuvanticity. Furthermore, MDP may be added late in the B cell activation process, thus providing little opportunity for helper factors to be released from either macrophage precursors or immature T cells which could eventually contaminate our B cells. However, we do not yet know the nature of the signal provided to B cells by MDP. It is quite possible that our T helper preparation contains other helper factors than IL 2 and TRF, such as B cell growth and differentiation factors (BCGFs,BCDFs,TRFs). These factors were identified in supernatants from various cell lines, and their presence in MLC supernatants has been suggested (19, 20, 21). It is possible that MDP could replace, or act in synergy with, one of them. The use of highly purified interleukins will be essential to determine whether or not MDP provides a lymphokine-like signal to B cells for immunoadjuvanticity.

Acknowledgements. We are grateful to Prof. E. Lederer for helpful comments and to Dr Martine Papiernik for the generous determination, in her laboratory, of IL 2 activity in all factor preparations. We also thank Nicole Esquirol for her excellent technical assistance. This work was supported by CNRS (E.R.141), PIRMED and Ligue Nationale Française contre le Cancer.

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