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IN VITRO IMMUNE RESPONSE TO SHEEP ERYTHROCYTES IN MACROPHAGE DEPLETED CULTURES. RESTORATION WITH INTERLEUKINE 1 OR A MONOKINE FROM RESIDENT MACROPHAGES AND STIMULATION BY N-ACETYL-MURAMYL-L-ALANYL-D-ISOGLUTAMINE (MDP)

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SUMMARY: The involvement of macrophages in the adjuvanticity of N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) has been examined. The stimulation of the in vitro primary immune response to sheep red blood cells (SRBC) has been studied, because it is known that macrophages cooperate through the mediation of soluble compounds for the induction of the anti-SRBC response. The cultures depleted of macrophages by passing spleen cells on Sephadex G-10 were unable to give any response to SRBC. Their immune responsiveness was fully restored by the addition of either Interleukine 1 (IL 1) obtained from P388D1 cells or a factor able to replace macrophages(FRM) obtained from resident peritoneal macrophages. MDP alone, at any dose, was unable to induce any response in such macrophage depleted cultures, but it was able to enhance the antibody response of these cultures reconstituted with monokines, with the same characteristics in dose effect and timing dependence than in whole spleen cells.

Synthetic N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) is the minimal adjuvant active structure capable of replacing mycobacteria in Freund's complete adjuvant (1). Besides its adjuvant activity, MDP can elicit various biological effects both in vivo and in vitro (reviewed in 2,3,4) and structure activity studies have generally shown a good relationship between structural requirements for adjuvanticity and other biological effects; however, it cannot be assumed that a single pathway can account for all the various effects induced by MDP. The mechanism of action of MDP in enhancing the antibody formation is not yet well defined and results obtained on the effect of MDP on the antibody response of mice to sheep erythrocytes (SRBC) have shown that macrophages (5) as well as B cells (6,7) or B and T cells (8) were essential for adjuvanticity. Because MDP is able to activate macrophages directly (reviewed in 9) a simple explanation could be the release of immuno-

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Abbreviations: MDP, muramyldipeptide; IL 1, interleukine 1; FRM, factor able to replace macrophages; MCF, macrophage culture fluid; SRBC, sheep red blood cell; PFC, plaque forming cells; FCS, foetal calf serum; 2-ME, 2-mercaptoethanol.

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stimulatory mediators from macrophages (5, 10-13) and indeed such compounds as IL 1 are able to increase the antibody response in vitro (14).

In the present paper we have examined such a possibility by studying the effect of MDP on the in vitro immune response to SRBC in macrophage depleted cultures because it is known that the antibody response of such non responsive cultures can be reconstituted by the addition of a supernatant obtained from macrophages (MCF) (15,16). Recently (17) we have isolated from the supernatant of resident peritoneal macrophages a monokine with macrophage replacing activity for the anti-SRBC response in vitro (referred to as FRM); thus we have measured the restoration of the immune response in macrophage depleted cultures with either MCF, FRM or IL 1 and determined the effect of MDP in such reconstituted cultures. We have compared the effect of MDP on the antibody response of whole spleen cells and of macrophage depleted cultures by defining the optimal conditions for adjuvanticity in either the presence of macrophages or of their substitutes, having in mind that a non involvement of macrophages for the adjuvant effect of MDP would imply the same characteristics in both cases.

MATERIALS AND METHODS

Animals and antigen: (C57B16 x DBA/2)F1 and C3H/HeJ female mice, 8-10 weeks old, were purchased from CSEAL (Orléans, France); sheep erythrocytes from a selected single donor were kindly provided by the CNRZ (INRA, Brouessy, France); they were kept in serum and washed extensively with saline before use.
Adjuvant and medium: MDP was kindly provided by Dr P. Lefrancier (Choay, Montrouge, France). Silica particles < 3 µm were obtained from Dr L. Le Bouffant (CERCHAR, France). The medium used was RPMI 1640 (Gibco) containing 25 mM Hepes supplemented with 50 uM 2-ME, penicillin, streptomycin (100 U/ml-100 µg/ml) and heat inactivated foetal calf serum (FCS) as indicated.
Preparation of macrophage depleted cultures: Macrophages were removed by passage through columns of Sephadex G-10 according to the method of Ly and Mishell (18) 10^8 spleen cells in 1 ml of 20% FCS containing medium were passed through 10 ml of prewarmed Sephadex G-10 equilibrated with the same medium at 37° C, the cells were collected with 6 ml of RPMI medium. The recovery was about 60% of the initial cell input; the cells obtained after passage through Sephadex G-10 (referred to as G-10 passed cells) were apparently depleted of macrophages as judged by morphologic observation, phagocytosis of latex beads, neutral red uptake and esterase staining.
Culture assays: Cells were cultured according to Mishell and Dutton (19): 10^6 splenocytes or G-10 passed cells were cultured in flat bottom wells of microtiter plates with 5×10^6 SRBC in a total volume of 200 µl of RPMI medium supplemented with 8% FCS and added with various fractions as indicated in results. The direct IgM anti-SRBC PFC response was determined on day 5 by the method of Cunningham and Szenberg (20). The cell recovery was of about 35% and was not significantly affected by the presence of either supernatant or MDP.

Six wells were plated for each determination, duplicate wells were pooled and assayed for PFC responses. Results are expressed as the mean ± SEM.

Preparation of macrophage culture fluids and of monokines: Resident peritoneal cells were obtained from non stimulated mice; the yield was usually $5x10^6$ cells/mouse. 10^6 cells/ml were cultured in RPMI medium supplemented with 8% FCS; after 90 min. non adherent cells were removed and adherent cells were cultured overnight in the same medium. The macrophage culture fluid (MCF) was recovered after the removal of cells by centrifugation and kept at 4°C until used. In some experiments, T lymphocytes were removed by treatment of peritoneal cells with anti-Thy-1.2 monoclonal antiserum (Olac Bicester UK) for 30min at 4°C followed by incubation with guinea pig complement for 45 min at 37°C.

IL 1 containing supernatants were obtained the same way except that macrophages were incubated in silica containing medium (50 µg/ml) (21). Standard IL 1 was obtained from P388D1 cells: $2x10^6$ cells/ml of RPMI medium, containing 1% FCS but no 2-ME were incubated 6 days at 37°C.

Supernatants were concentrated 15 times with Immersible-CX (Millipore) and applied on Ultrogel AcA54 (IBF) as previously described (17); FRM (for factor able to replace macrophages) with 35,000 apparent molecular weight was obtained from MCF; IL 1 was obtained from either P388D1 cells or from peritoneal macrophages treated with silica (22).

RESULTS

Restoration of the antibody response to SRBC of macrophage depleted splenocytes

The removal of macrophages by one or even several cycles of adherence greatly reduced the antibody response but still enabled the non adherent cell population to develop an immune response to SRBC. In contrast, the passage of splenocytes through Sephadex G-10, under well defined conditions, resulted in a population that apparently did not contain macrophages as judged by morphological observation and phagocytosis experiments; furthermore the antibody response of these lymphoïd populations was completely abrogated, even in 2-ME containing media. As described by others (16), we have observed that the antibody response of macrophage depleted cultures could be reconstituted fully by adding an optimal amount of peritoneal macrophages (about 1%) or a culture fluid obtained from these cells (MCF). The obtention of an active supernatant was not dependent on the presence of either T cells or FCS, because MCF was obtained after anti-Thy-1.2 plus C treatment of peritoneal cells and in RPMI medium deprived of both 2-ME and FCS; but nevertheless, MCF was usually obtained with the 8% FCS containing medium further used in culture assays.

We have recently reported (22) that the activity of MCF, spontaneously released from unstimulated cells, was linked to the presence of FRM, a 35,000 apparent molecular weight compound, whereas the addition of silica to these macrophages induced the release of IL 1 instead of FRM. A standard of IL 1 was

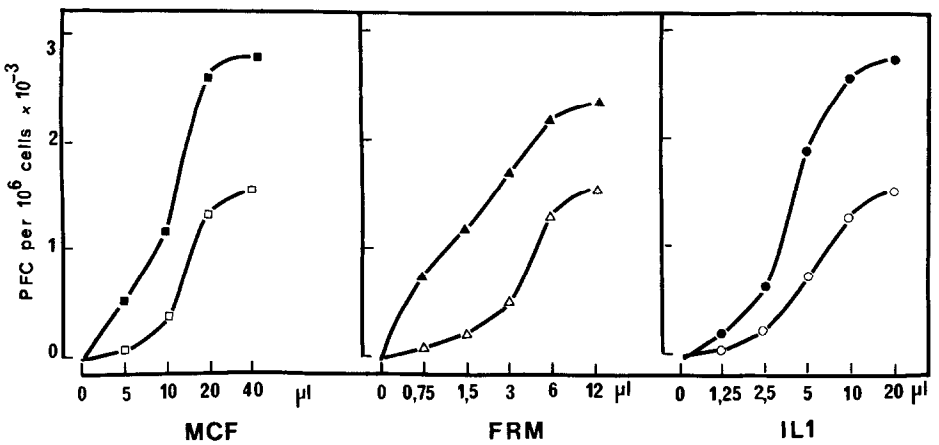

Figure 1- 10^6 macrophage depleted spleen cells obtained by passing spleen cells through Sephadex G-10 columns were cultured with SRBC and increasing amounts of macrophage supernatants in the presence or absence of an optimal dose of MDP (1µg/ml).
--□-- Supernatant obtained from resident peritoneal macrophages or MCF; --■--: +MDP.
--Δ-- FRM, the 35,000 MW compound obtained by AcA 54 column chromatography of MCF; --▲--: +MDP.
--○-- IL 1, the 15,000 MW compound obtained from P338D1 cells stimulated with silica after chromatography on AcA 54; --●--: +MDP.
PFC were determined on day 5. Values given are mean PFC from 3 experiments.

prepared from P388D1 cells. As can be seen on Fig.1 the reconstituting activity of supernatants was dose dependent: the antibody response increased progressively to a plateau which was usually obtained with 10% dilutions of MCF and both IL 1 and FRM showed the same profile of activity. However, in contrast with the results obtained in the restoration of the immune response with macrophages there was no inhibition of the PFC response even at high concentration of supernatants, showing the inability of these monokines to mediate all regulatory functions of macrophages.

Effect of MDP on the antibody response of splenocytes and G-10 passed cells.

We have compared the effect of MDP on the antibody response to SRBC of whole spleen cells and of macrophage depleted splenocytes. As can be seen on Fig.2, MDP alone, at any dose, was unable to reconstitute any PFC reponse to SRBC in G-10 passed cells, but when these unresponsive splenocytes were reconstituted with MCF, MDP was able to increase the response. Dose response relationship has shown that the same dose of 1 µg/ml was required for an optimal effect in either the splenocyte cultures or in the macrophage depleted cultures

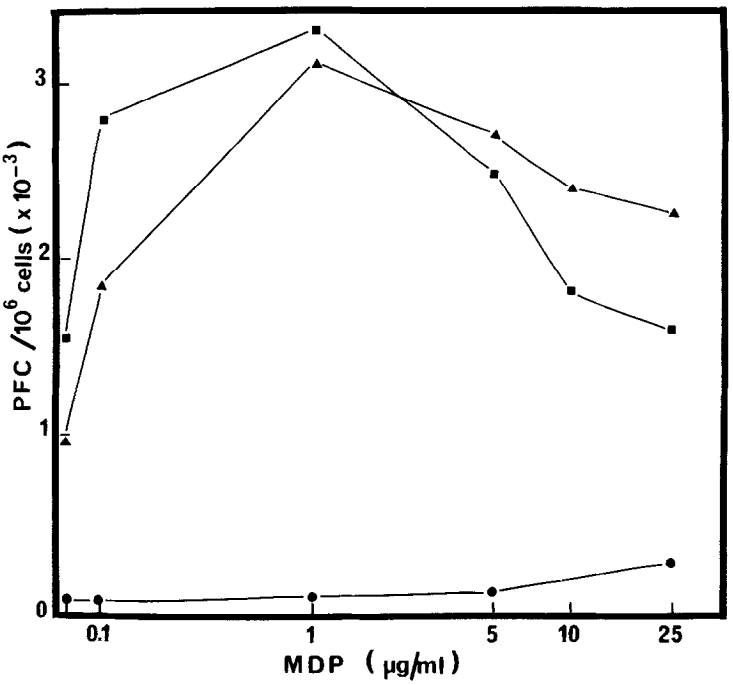

Figure 2- 10^6 BDF1 unfractionated spleen cells or 10^6 BDF1 spleen cells passed over Sephadex G-10 columns were cultured with SRBC and various amounts of MDP in a total volume of 200 µl.
--■-- whole spleen cells; --●-- G-10 passed cells; --▲-- G-10 passed cells with MCF added at 1/16 dilution.
Values given are mean PFC ± SEM for six replicates pooled by two.

reconstituted with MCF. As can be seen on Fig.1, when this optimal dose of 1 µg/ml of MDP was added to cultures reconstituted with increasing amounts of MCF, both unstimulated and MDP stimulated responses showed a similar profile.

We had previously observed that the addition of MDP could be delayed, since it was still active when added several days after the initiation of splenocyte cultures (23). Data presented on Table 1 show that the same phenomenon is observed when MDP is added to macrophage depleted cultures reconstituted with MCF. Although an optimal effect was obtained when MDP was added at zero time it was still efficient when added 3 days after the initiation of cultures. In contrast, MCF had to be added within the first hours of experiments, because even after 1 day, it was incapable of reconstituting the antibody response in macrophage depleted cultures. These kinetic experiments show that MCF, like macrophages, is absolutely required in an early event of the immune induction; MDP must be acting later, only when this first event has been initiated.

TABLE 1
Restoration and stimulation of the PFC response
Time dependence

Cultured cells	Delayed addition		PFC per culture				
			Time of addition				
	MCF	MDP	0	4h	24h	48h	72h
G-10 passed cells	-	-	0 - 20				
(a)	+	-	1034 ± 163	689 ± 79	116 ± 24	53 ± 10	14 ± 15
G-10 passed cells	-	-	1109 ± 172				
plus MCF (b)	-	+	3114 ± 410	2920 ± 205	2715 ± 284	2200 ± 505	1506 ± 160
Unfractionated	-	-	1283 ± 147				
spleen cells (c)	-	+	2856 ± 597	3398 ± 480	3300 ± 452	2660 ± 505	1900 ± 416

10^6 unfractionated spleen cells or G-10 passed cells were cultured with SRBC in a total volume of 200µl, with MCF at 1/16 dilution and/or MDP at 0.2 µg/well.
a) MCF was added at various times after the initiation of cultures.
b) MCF was added at zero time and MDP was added latter.
c) MDP was added to unfractionated spleen cell cultures after various times.
Values given are mean PFC ± SEM for six replicates pooled by two.

Similarly, MDP was able to increase the PFC response of G-10 passed cells reconstituted with either FRM or IL 1 (Fig.1) stressing the relationship between these two monokines.

DISCUSSION

The present study was realized to determine if macrophages directly, or by the way of mediators, were essential for the adjuvant effect of MDP. It has been reported that the immune response of macrophage depleted cultures to SRBC can be reconstituted by the addition of a supernatant obtained from peritoneal cells (15,16), the compound responsible for this effect was not identified but it was suggested that IL 1 could play such a role (24). We have found recently (22), that FRM, a 35.000 M.W. compound, was spontaneously released from normal macrophages whereas the addition of silica to these cells led to the production of IL 1. FRM could be a precursor of IL 1 expressing its differentiative but not its proliferative properties, although the relationship between the two compounds is not yet well defined. The present results show that the 2 compounds apparently behave the same way in reconstituting the immune response, with a similar increase by the subsequent addition of MDP. The ability of soluble compounds to replace the requirement for macrophages is in contrast with the results obtained in other examples of T cell activation showing the

requirement for two distinct signals: one being provided by monokines but the other requiring the presence of accessory cells (25). Our data are consistent with the hypothesis of the mediation of the action of macrophages by soluble compounds, because the anti-SRBC PFC response of Sephadex G-10 passed cells could be reconstituted with either macrophage culture supernatants or IL 1 as well as FRM. However, we cannot exclude the possibility that despite sufficient macrophage depletion to abrogate the antibody response, even in 2-ME containing media, some residual, highly effective accessory cells are still present. These cells cannot alone permit the PFC response but they could act in conjunction with the added monokine. If this second type of accessory cells is necessary, then it must be admitted that MDP could act on them. It can be assumed that these hypothetical cells are unable to release monokines because no antibody response occurs without addition of MCF. Our results clearly show that MDP is without effect in such macrophage depleted cultures unless a substitute is added to the culture. As the only missing component is MCF (or IL 1) it is clear that MDP must be acting by another way than by the release of monokines. An analysis of the enhancing effect of MDP in whole spleen cells or in G-10 passed cells reconstituted with MCF has shown the parallelism in kinetics and dose response in both cases; these facts suggest that the adjuvanticity of MDP can be explained by an action on lymphoïd cells in a late event of the immune response in disagreement with reports on the mediation of the adjuvant effect by macrophages (5). The mechanism whereby MDP potentiates the immune response is not yet elucidated, but considering the results obtained by Watson et al. (6) showing the T helper activity of MDP, it can be suggested that pre-T cells or B cells are the targets for the adjuvant effect of MDP. The obtention of the pure interleukines mediating the helper effects of other cells in the transformation of B cells in antibody forming cells will hopefully help in understanding the mechanism of action of MDP at the molecular level.

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