MURAMYL-DIPEPTIDE AND ITS SYNTHETIC ANALOG AS POSSIBLE INDUCERS OF INTERLEUKIN-2 PRODUCTION

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Immunologists have devoted great attention in recent years to the search for highly effective inducers of T-cell growth factor, interleukin-2 (IL2). T-cell mitogens concanavalin A (con A) and phytohemagglutin (PHA), and also phorbol myristate acetate [7] have been found to be most effective. However, for various reasons these substances cannot be used to induce IL2. Meanwhile immunomodulators widely used in experimental immunology and in clincial medicine, namely levamisole, muramyl-dipeptide (MDP), and its synthetic analogs, have not been studied as potential inducers of this lymphokine. MDP, a component of the cell wall of mycobacteria, is the active principle of Freund's complete adjuvant [1]. MDP stimulates an increase in phagocytic function of the reticuloendothelial system in vivo [8], promotes production of prostaglandin and collagenase by macrophages [9], induces the cytostatic and cytotoxic action of macrophages against tumors [2], and inhibits macrophage migration [10]. MDP also induces interleukin-1 (IL1) production by macrophages [5].

The aim of this investigation was to study the action of MDP and its synthetic derivative N-acetylglucosaminyl-N-acetyl-muramyl-alanyl-D-isoglutamine (GMDP) on IL2 production in a population of BALB/c mouse spleen cells.

EXPERIMENTAL METHOD

Experiments were carried out on male BALB/c mice weighing 16 g. Mouse spleen cells ($5 \cdot 10^6$ in 1 ml) were incubated with the test substances in medium RPMI 1640 with 5% embryonic calf serum, glutamine (2 mM), and 2-mercaptoethanol ($5 \cdot 10^{-5}$ M) for 24 or 48 h at 37°C in a humid chamber containing 5% CO_2. IA2 was determined in the cultural supernatant after sedimentation of the cells by centrifugation at 400 g. IL2 activity was estimated on T blast cells obtained on the 4th day after stimulation of mouse splenocytes ($5 \cdot 10^6$ in 1 ml) with con A (5 μg/ml) [3]. After washing twice with medium the blast cells, in a volume of 100 μl (10^6 in 1 ml) were added, in wells of flat-bottomed culture panels, to the test samples diluted beforehand with medium (100 μl of each). ^{3}H-thymidine (0.5 μCi/50 μl, specific radioactivity 26 Ci/mmole) was added to the wells in the panels 4 h before the end of culture for 18 h. The radioactivity of the samples was measured on a Tricarb-Packard scintillation beta-counter after deposition of material precipitated with TCA on the filters.

EXPERIMENTAL RESULTS

The supernatant of the culture of activated spleen cells, stimulated by MDP or GMDP induced a proliferative response of the con A blast cells (Table 1). After direct addition to the con A blast cells both reagents raised the level of cell proliferation slightly, whereas the cultural supernatant induced a powerful proliferative response, the intensity of which depended on the dilution of the supernatant. Consequently, the mitogenic effect of the cultural supernatant was due to production of growth factor.

The effectiveness of the stimulating action of MDP and GMDP, and also of the classical IL2 inducer, con A, was compared. The mitogenic action of the supernatant of the splenocyte culture stimulated by MDP or GMDP was much more marked after 24 h than after 48 h. The same rule was observed when the supernatant of a cell culture activated by con A was tested. The supernatant of the 24-h culture of splenocytes stimulated by MDP or GMDP (in optimal concentrations) was about half as effective an inducer of proliferation of

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TABLE 1. Induction of IL2 Formation by BALB/c Mouse Splenocytes under the Influence of MDP and GMDP

Preparation	Dose of preparation, μg/ml	Dilutions of IL2		
		1:2	1:4	1:8
Control		1 430 ± 123	1 069 ± 114	812 ± 74
MDP	1	8 770 ± 768	7 212 ± 562	5 101 ± 569
Control		1 304 ± 110	1 267 ± 134	1 448 ± 98
GMDP	1	7 087 ± 673	8 183 ± 847	5 560 ± 632
Control		1 429 ± 139	1 257 ± 99	1 234 ± 174
MDP	10	12 113 ± 965	10 062 ± 956	7 800 ± 579
Control		1 516 ± 189	1 147 ± 101	1 179 ± 109
GMDP	10	13 813 ± 967	10 547 ± 829	9 075 ± 749
Control		1 465 ± 126	1 652 ± 154	1 394 ± 102
MDP	20	9 942 ± 945	11 562 ± 1013	10 660 ± 859
Control		1 692 ± 134	1 720 ± 147	1 444 ± 119
GMDP	20	13 159 ± 1036	13 564 ± 981	12 279 ± 1459

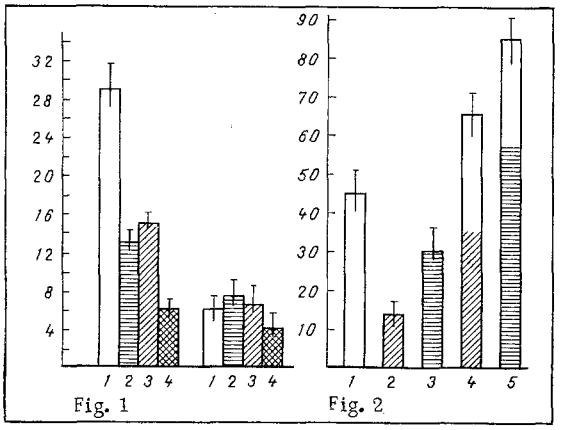

Fig. 1 Fig. 2

Fig. 1. Dynamics of induced IL2 production by BALB/c mouse spleen cells. Abscissa, inducers: 1) con A, 2) MDP, 3) GMDP, 4) levamisole; ordinate, incorporation of ^{3}H-thymidine into T blast cells (in cpm).

Fig. 2. Synergistic effect of MDP and GMDP with con A on IL2 induction. Abscissa, inducers: 1) con A, 2) MDP, 3) GMDP, 4) con A + MDP, 5) con A + GMDP; ordinate, index of stimulation.

con A blast cells as the supernatant of con A-stimulated splenocytes, but activity of supernatants of cultures stimulated by MDP, GMDP, or con A was equalized at the end of culture for 48 h. Since growth and proliferation of con A blast cells in vitro are maintained by IL2 [4], two possibilities can be postulated: Either MDP induces production of IL1 only, and this, in turn, triggers production of IL2 by T amplifier cells, or MDP is at the same time a direct inducer of IL2. Although the problem of the ability of IL1 to stimulate IL2 production in unactivated T lymphocytes has not been finally solved, according to some investigators this monokine stimulates rather than induces IL2 production [6]. Formally speaking it seems possible that MDP-induced IL1 in a population of 4-day-old con A blast cells stimulates IL2 production by IL2 producers, activated by con A and preserved until the time of testing, and this ensures the proliferative response of IL2-dependent T blast cells. However, the following facts make this a questionable possibility: 1) The splenocyte population which we stimulated with MDP is characterized by an extremely low content of Ia^+-macrophages, i.e., cells producing IL1. 2) The kinetics of production of growth factor in the splenocyte population differed from the well-known kinetics of IL1 production, the level of which has a tendency to rise during the first 2-3 days of culture. The most probable explanation in our opinion is thus direct involvement of MDP in the induction of IL2-producing T cells. Additional indirect evidence in support of the action of MDP on lymphocytes is given by the fall in the level of growth factor in the medium after culture for 48 h.

Triggering of the lymphokine cascade with involvement of IL2 may evidently lie at the basis of the action of many immunomodulators and, in particular, of those which increase the efficiency of function of the T system of immunity. We tested this hypothesis, using levamisole as the potential inducer. Levamisole also was found to induce the formation of a factor mitogenic for con A blast cells (Fig. 1). However, it exhibited lower activity as an IL2 inducer than MDP or GMDP. Taken as a whole, our results indicate that MDP and its synthetic analog GMDP can be regarded as possible inducers of IL2 production.

On the basis of the view that MDP is an IL1 inducer [5] we also analyzed this synergistic action of MDP or GMDP with con A. In this case it was decided that the results could best be presented in the form of an index of stimulation (IS), which was determined by the following equation:

$$IS = \frac{\text{counts/min (supernatant with inducer)} - \text{counts/min (inducer)}}{\text{counts/min (control}}.$$

MDP with con A were found to induce higher activity of the factor than could be explained by summation of the effect of the separate inducers. The same result was obtained when GMDP with con A were used as inducers (Fig. 2). Consequently, MDP and GMDP can also be used successfully together with con A as costimulators for more effective IL2 production in vitro. As costimulators of IL2 production, MDP and GMDP have an undoubted advantage over the widely used phorbol myristate acetate, an important disadvantage of which is its ability to potentiate tumor growth.

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