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Selection of a muramyl peptide based on its lack of activation of nuclear factor-κB as a potential adjuvant for AIDS vaccines

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SUMMARY

Activation of the cellular transcription factor nuclear factor-κB (NF-κB) by cytokines and other immunostimulants has been tightly linked with enhanced replication of human immunodeficiency virus-type 1 (HIV-1) in infected cells. Various immunomodulators are currently being examined in animal and human trials for their suitability as adjuvants in potential vaccines against acquired immunodeficiency syndrome (AIDS). It may prove to be beneficial to select adjuvants that do not induce NF-κB activation and particularly if the vaccines are to be aimed at seropositive individuals. We have examined a battery of synthetic immunostimulants of the muramyl peptide family for their ability to activate NF-κB in human and mouse cell lines. In this report, we demonstrate selective activation of NF-κB in different cell lines and by different muramyl peptides possessing immunostimulatory activities. The mechanism of such activation is apparently via production of reactive oxygen intermediates (ROI) since pretreatment of cells with antioxidants blocked subsequent activation of NF-κB. However, among all the molecules tested only one lipophilic, non-pyrogenic adjuvant active muramyl peptide showed a complete lack of NF-κB activation in all cell lines tested. This molecule could well become the adjuvant of choice in future AIDS vaccines.

Keywords muramyl peptides NF-κB adjuvants AIDS vaccines

INTRODUCTION

The cellular transcription factor, nuclear factor-κB (NF-κB) has been shown to play a central role in the activation of several genes involved in the cellular responses to infection or injury [1]. Binding of NF-κB to the enhancer of HIV-1 regulates the transcriptional inducibility of the HIV-1 long terminal repeat (LTR) in activated T lymphocytes [2] and stimulates the HIV-1 enhancer in transiently transfected T cell lines [3]. Moreover, in monocytic cell lines bearing HIV-1 provirus, viral production is stimulated by agents that induce activation of NF-κB [4]. The possibility that NF-κB may even be responsible for maintaining HIV-1 replication has been recently suggested [5]. Thus, it would seem preferable that in the search for a vaccine against AIDS with the aim of administering it prophylactically at a population level or therapeutically to HIV seropositive individuals [6], one should avoid the use of adjuvant components which on their own lead to the activation of NF-κB.

Muramyl peptides are among the candidate adjuvants that have been utilized in experimental AIDS vaccines and with a potential for use in humans [7,8]. Members of this family of synthetic immunomodulators possess potent and diverse biological properties, most of which can be selected for by chemical modification of the parent molecule MDP (NAc-Mur-L-ala-D-isogln). Several muramyl peptides have been shown to act as safe and efficacious adjuvants in experimental vaccines against viruses and to enhance non-specific host resistance against virus infections [9,10]. We have examined the activation of NF-κB in human and mouse cell lines following stimulation with different muramyl peptides possessing or lacking defined immunostimulatory activities. We have also compared and quantified the muramyl peptide activation of NF-κB with that induced by lipopolysaccharide (LPS) and by a synthetic lipid A analog SDZ MRL 953 [11]. Our results demonstrate a lack of NF-κB activation in all cell lines tested, by one new lipophilic, non-pyrogenic, adjuvant active muramyl peptide MDP (thr)-glyceryl-dipalmitoyl (MDP (thr)-GDP). In contrast, all other immunostimulants examined induced activation of NF-κB in one or more of the stimulated cell lines. Furthermore, we present evidence that stimulation of one of the cell lines with MDP (thr)-GDP results in productive interaction with the cells as manifested by the induction of IL-8 gene expression. The novel MDP derivative may therefore be the adjuvant of choice to be incorporated in AIDS vaccines.

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MATERIALS AND METHODS

Reagents

LPS prepared from *E. coli* serotype 055:B5 was obtained from Sigma Chemical Co (St Louis, MO). SDZ MRL 953 was produced at Sandoz Forschungsinstitut ((SFI), Vienna, Austria) and was dissolved in ethanol/glucose as described previously [11]. The muramyl peptides were of three groups: (1) immunostimulatory and pyrogenic including MDP; MDP-lys (L18) (muroctasin) and MDP-sn-glyceryl dipalmitoyl (MDP-GDP); (2) immunostimulatory but non-pyrogenic including NAc-Mur-L-ala-D-gln-αMe (murametide), NAc-Mur-L-thr-D-isogln-Sn-glyceryl-dipalmitoyl [MDP (thr)-GDP] and NAc-Mur-L-ala-D-gln-*n*-butyl ester (murabutide); (3) non-immunostimulatory including NAc-Mur-D-ala-D-isogln [MDP (DD)]. These molecules have been described previously [12–14] and were provided by SFI and Vacsyn France SA (Paris, France). Stock solutions were made at 1 mg/ml in pyrogen-free isotonic glucose except for MDP-GDP and MDP (thr)-GDP which were solubilized by sonication for 30 min in a 0·5% pluronic F68 (Fluka, Germany) solution in isotonic glucose. Recombinant human tumour necrosis factor-α (TNF-α) with specific activity of 2×10^7 U/mg was purchased from Genzyme (Boston, MA). Phorbol 12-myristate 13-acetate (PMA), pyrrolidine dithiocarbamate (PDTC) and diethyldithiocarbamate (DDTC) were from Sigma.

Cell lines

The human Jurkat T cell (subclone JR kindly provided by Dr T. Hünig, Würzburg, Germany) and the human monocyte–macrophage cell line (Mono-Mac-6) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% (w/v) penicillin/streptomycin (all purchased from GIBCO Laboratories). The mouse pre-B cell line 70Z/3.12 (ATCC No. TIB 158) was cultured in the same medium supplemented with 50 μM 2-mercaptoethanol. Cells were treated with different concentrations of test compounds as indicated in the figure legends.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from cultured cells as described [15]. Equal proportions of cell fractions with 2–8 μg of protein were used in the assays. DNA binding conditions for NF-κB and EMSAs were described in detail elsewhere [16]. Briefly, binding reactions (20 μl) contained 2 μg poly (dI-dC) (Pharmacia), 5–10 000 ct/min (Cerenkov) of ^{32}P-labelled κ enhancer DNA probe (> 3000 Ci/mmol; for sequence see [16]), 1 μl buffer D containing 1% (v/v) Nonidet P-40, 20 μg bovine serum albumin and binding buffer. Reactions were started by the addition of cell extracts and incubated for 30 min prior to electrophoresis. Samples were analysed on native 4% polyacrylamide gels. Dried gels were exposed to Kodak XR5 films on intensifying screens (DuPont) for 5–15 h at −70°C.

Detection of induced IL-8 gene expression in Mono-Mac-6 cells

The procedure described by Chujor *et al.* [17] was followed. Briefly, $5{\cdot}0 \times 10^7$ Mono-Mac cells were cultured in 50 ml medium or in medium containing various immunostimulants. After 2, 6 or 24 h culture, cells were pelleted and total RNA was extracted using 4 M guanidine isothiocyanate buffer followed by ultracentrifugation over caesium chloride cushion. RNA samples were fractionated by gel electrophoresis and blotted on to synthetic membrane filters (Hybond N, Amersham). The filters were baked for 2 h at 80°C and prehybridized for 4 h at 65°C in prehybridization buffer consisting of 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM $C_6H_5Na_3O_7$ $2H_2O$, pH 7·0), 10 × Denhardt's solution (1 × Denhardt's solution is 0·02% bovine serum albumin, 0·02% polyvinyl pyrrolidone, 0·02% Ficoll), 20 mM sodium phosphate, pH 7·0, 7% sodium dodecyl sulphate (SDS), 100 μg/ml sonicated salmon sperm DNA and 100 μg/ml poly(A). Hybridization was then performed for 16 h at 65°C in prehybridization buffer containing 10% dextran sulphate and radiolabelled synthetic oligonucleotide probes specific for either: IL-8: 5′GCTTTACAATAATTTCTGTGTTGGCGC-AGTGTGG3′ or β-actin: 5′GGCTGGGGTGTTGAAGG-TCTCAAACATGATCTGG3′.

The blots were then washed once in 5% SDS, 3 × SSC, 10 × Denhardt's solution, 20 mM sodium phosphate for 30 min followed by another wash in 1 × SSC, 1% SDS at 65°C for 30 min. Filters were exposed for 24–48 h at −70°C to Kodak XAR-5 films using intensifying screens. The synthetic oligonucleotides were labelled at their 3′ ends with (α-^{32}P) deoxyadenosine 5′-triphosphate (Amersham) as previously described [18].

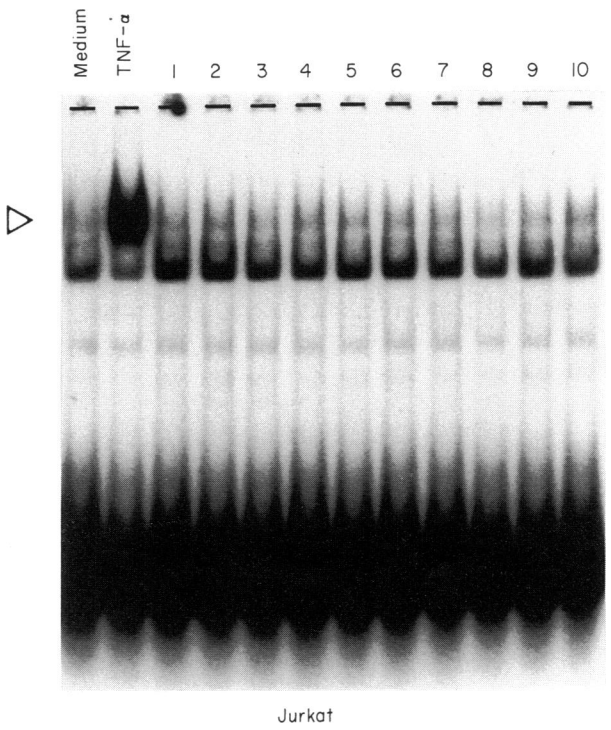

Fig. 1. The effects of muramyl peptides and SDZ MRL 953 on the activation of NF-κB in Jurkat T cells. Cells of the Jurkat cell subclone JR were incubated for 2 h in the presence or absence of the following compounds: 270 U/ml human recombinant TNF-α, 10 μg/ml of the muramyl peptides MDP (1), murametide (2), MDP-DD (3), murabutide (4), muroctasin (5), 0·5% pluronic (6; the solvent control for No. 7 and 8), MDP-GDP (7), MDP (thr)-GDP (8), ethanol/glucose (9; the solvent control for No. 10) and SDZ MRL 953 (10). For abbreviations of the compounds, see Materials and Methods. Nuclear extracts were prepared and equal amounts of protein analysed for κB-specific DNA binding in EMSAs using a ^{32}P-labelled κ enhancer DNA probe. A fluorogram of a native gel is shown. The open arrowhead indicates the position of the specific NF-κB DNA complex.

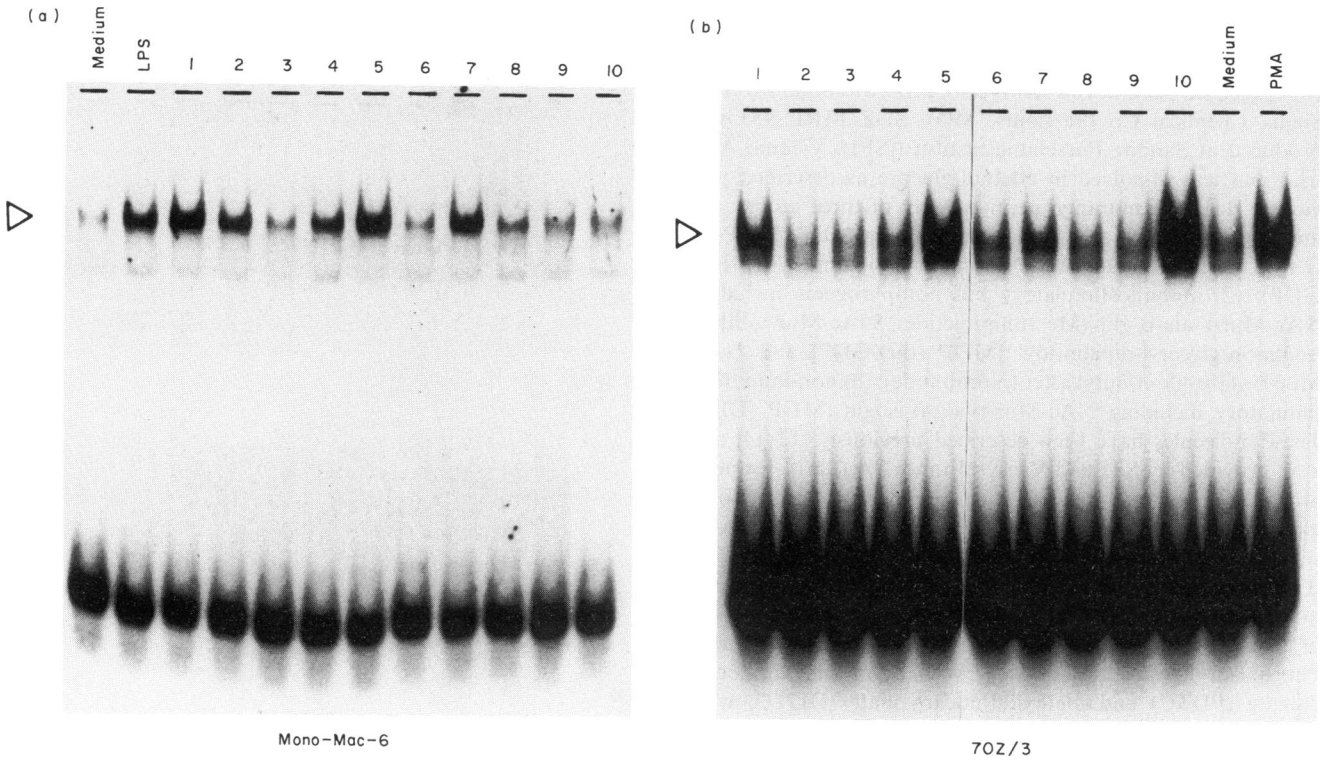

Fig. 2. The effects of muramyl peptides and SDZ MRL 953 on the activation of NF-κB in a human monocyte–macrophage cell line (Mono-Mac-6) and mouse pre-B cells (70Z/3). Cells of the cell line Mono-Mac-6 (a) or 70Z/3 (b) were incubated for 140 min in the presence or absence of 10 μg/ml of various muramyl peptides, SDZ MRL 953, lipopolysaccharide (LPS) or 50 ng/ml phorbolester (PMA). Nuclear extracts were analysed on EMSAs. A fluorogram of a native gel is shown. The open arrowhead indicates the position of the specific NF-κB–DNA complex. For details see Fig. 1 legend.

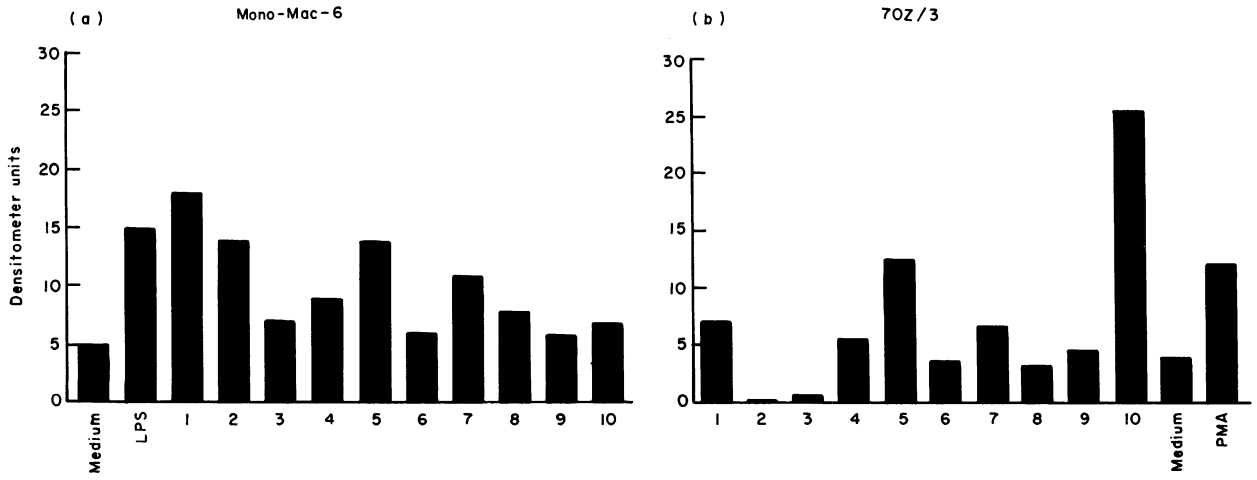

Fig. 3. Quantification of the effect of muramyl peptides and SDZ MRL 953 on the activation of NF-κB in Mono-Mac-6 and 70Z/3 cells. The amount of the specific NF-κB-DNA complex was determined by densitometric analysis of the fluorograms shown in Fig. 2 using an Hirschmann densitometer (elscript 400) and is indicated in arbitrary densitometer units.

RESULTS

Activation of NF-κB in human cell lines by immunostimulants

Stimulation of the human T cell line Jurkat with any of the muramyl peptides or with SDZ MRL 953 did not result in detectable activation of NF-κB (Fig. 1). This was in contrast to stimulation of the same cells with TNF-α (270 U/ml) which induced a marked activation of the transcription factor. However, activation of NF-κB was noted in the human Mono-Mac-6 cell line following stimulation with 10 μg/ml of LPS, MDP, murabutide, murametide, muroctasin or MDP-GDP (Fig. 2a). The induced protein complex co-migrated with that of purified NF-κB and showed κB-specific binding in competition experiments (data not shown). No activation of NF-κB could be detected under the same conditions following incubation with SDZ MRL 953, MDP (DD), MDP (thr)-GDP or solvent controls. Analysis of the intensity of the bands by densitometric measurement revealed that the highest activation of NF-κB was

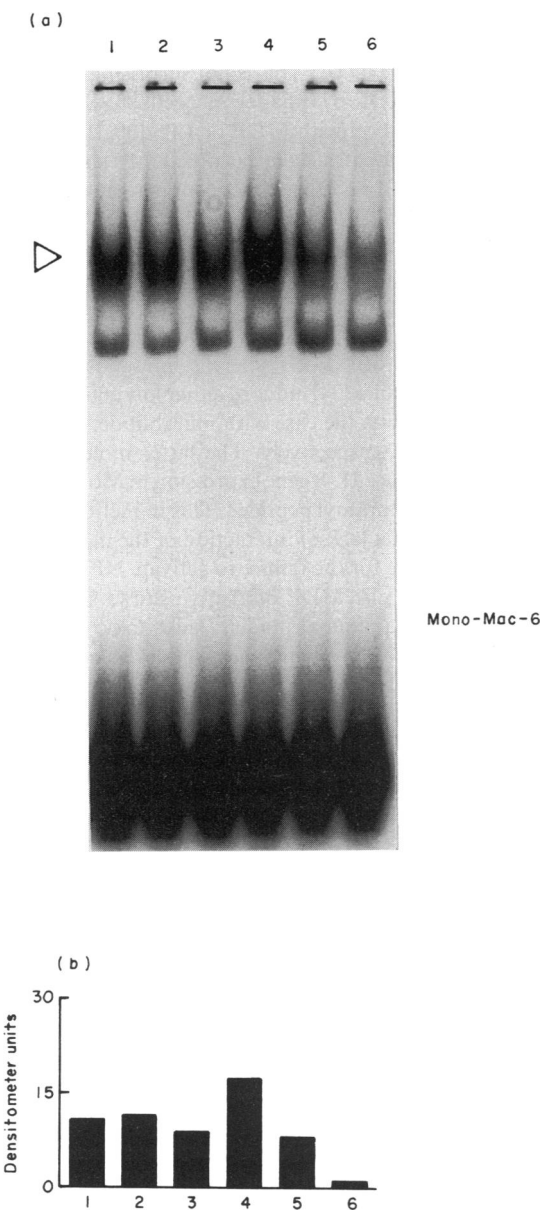

Fig. 4. The effect of dithiocarbamates on the activation of NF-κB in Mono-Mac-6 cells by muroctasin. (a) The inhibiting effect of DDTC and PDTC on the muroctasin-induced NF-κB activation. Mono-Mac-6 cells were preincubated for 90 min with 250 μM DDTC (lanes 2, 5) and 250 μM PDTC (lanes 3, 6) or left untreated (lanes 1, 4) followed by stimulation with 7·5 μg/ml muroctasin for 105 min (lanes 4–6). Nuclear extracts were analysed by EMSA. A fluorogram of a native gel is shown. The open arrowhead indicates the position of the specific NF-κB-DNA complex. (b) Quantification of the inhibitory effect of DDTC and PDTC by densitometric analysis.

induced by MDP followed in descending order by LPS, murametide, muroctasin, MDP-GDP and murabutide (Fig. 3a). Incubation of Mono-Mac-6 cells with 1 μg/ml of muramyl peptides revealed strong NF-κB activation by MDP, muroctasin and MDP-GDP, a weak activation by murametide and no inducible activation by MDP-(DD), murabutide or MDP (thr)-GDP (data not shown).

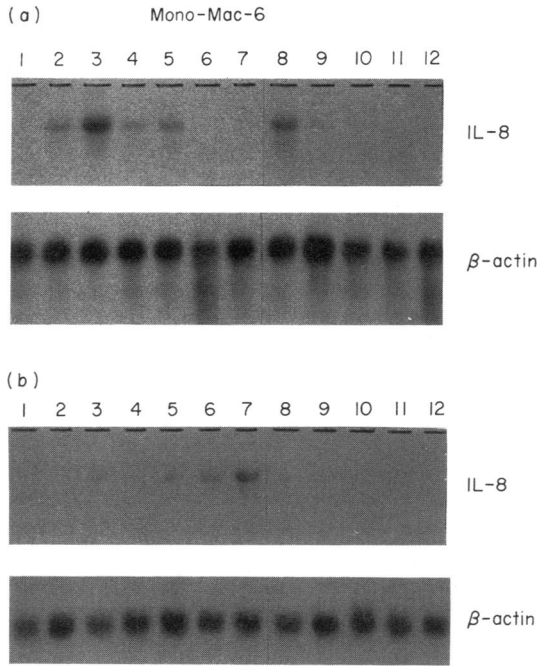

Fig. 5. Northern blot analysis of the induction of IL-8 mRNA accumulation in Mono-Mac-6 cells. Analysis was done using 10 μg total RNA from 6 h (a) and 24 h (b) cultured cells with medium alone (lane 1), 1 ng and 10 ng/ml LPS (lanes 2 and 3) or with 10 μg/ml of SDZ MRL 953 (lane 4), murabutide (lane 5), MDP-GDP (lane 6), MDP (thr)-GDP (lane 7), MDP (lane 8), muroctasin (lane 9), MDP (DD) (lane 10), ethanol/glucose (lane 11) as solvent control for lane 4, and pluronic/glucose (lane 12) as solvent control for lanes 6 and 7. Hybridization with a synthetic ^{32}P-labelled oligonucleotide probe for IL-8 and washing were performed under stringent conditions. Filters were stripped and re-hybridized to an oligonucleotide of β-actin to control for equal RNA amounts.

Activation of NF-κB in a mouse cell line

Stimulation of the pre-B cell line 70Z/3 with 10 μg/ml of either MDP, murabutide, muroctasin, MDP-GDP, SDZ MRL 953 or 50 ng/ml PMA induced a variable extent of NF-κB activation (Fig. 2b). The highest level of activation as measured in densitometer units was induced with SDZ MRL 953 and the lowest with murabutide (Fig. 3b). No detectable NF-κB activation was noted in the 70Z/3 cell stimulated with murametide, MDP (DD), MDP (thr)-GDP or solvent controls (Figs 2b and 3b). When stimulation was carried out with a compound concentration of 1 μg/ml, a strong activation of NF-κB was only observed with LPS, SDZ MRL 953 and muroctasin (data not shown).

Effect of pretreatment of cells with anti-oxidants on the subsequent activation of NF-κB by muroctasin

To elucidate the mechanism of activation of NF-κB by muramyl peptides, cells were pretreated with the antioxidants PDTC or DDTC prior to stimulation with 5 μg/ml of muroctasin. Such pretreatment has been recently shown to inhibit IL-1, TNF-α, PMA or LPS induced activation of NF-κB by inhibiting production of ROI in stimulated cells [19]. The results in Fig. 4 clearly demonstrate that pretreatment of Mono-Mac-6 cells with either of the antioxidants blocked the muroctasin induced NF-κB activation. Similar effects of the dithiocarbamates were

noted using muroctasin-stimulated 70Z/3 cells (data not shown).

Induction of IL-8 mRNA in Mono-Mac-6 cells by various stimulants

One possible explanation for the lack of activation of NF-κB by MDP (thr)-GDP could be that the molecule does not productively interact with the cells and, therefore, is not able to induce any detectable stimulation. To rule this out, we examined the induction of IL-8 mRNA in Mono-Mac-6 cells following stimulation for 2, 6 or 24 h with 1 and 10 ng/ml LPS or with 10 μg/ml of each of the other stimulants. Accumulation of IL-8 mRNA was not observed following stimulation for 2 h with any of the six muramyl peptides tested. However, a strong accumulation of IL-8 mRNA was detected in Mono-Mac-6 cells following stimulation for 6 h (Fig. 5a) with LPS (lanes 2, 3), MDP (lane 8), SDZ MRL 953 (lane 4) or murabutide (lane 5). A weak induction could be detected after 6 h stimulation with muroctasin (lane 9) or MDP (thr)-GDP (lane 7) and no detectable IL-8 message was observed in cultures stimulated with MDP-GDP (lane 6), MDP (DD) (lane 10) or medium and solvent controls (lanes 1, 11, 12). In contrast, IL-8 mRNA accumulation in 24 h cultures (Fig. 5b) was only detected following stimulation with MDP (thr)-GDP (lane 7) and to a lesser extent with MDP-GDP (lane 6), murabutide (lane 7) and LPS (lane 2).

DISCUSSION

The absence of effective treatment in AIDS has prompted a vast interest and effort in the development of vaccines against the disease. Candidate HIV and analogous simian immunodeficiency virus (SIV) vaccines have been developed and formulated with several types of adjuvants or delivery systems for studies in animals and humans [20,21]. In addition to prophylactic vaccines against AIDS, attempts are being made to develop immune-based therapies including immunotherapeutic vaccines aimed for HIV-seropositive individuals (reviewed in [22]) as advocated by Salk [6]. The considerations for efficacy and safety of the adjuvant component in an HIV-vaccine may be different from the general rules observed in traditional vaccine development. In particular, one must take into account the possible elicitation of the undesirable side effect of HIV activation by the adjuvant [23]. Cytokines (IL-1β, TNF-α) and immunostimulants (LPS) are known to enhance HIV-1 expression through the activation of the cellular transcription factor NF-κB which then binds to two motifs in the HIV-1 LTR and consequently activates the LTR-driven RNA transcription [2,4,24]. Thus, it would seem preferable not to induce this pathway by components of the vaccine although there is yet no evidence to suggest that the overall level of HIV replication in a host is limited by the extent of NF-κB activation.

Muramyl peptides have already been utilized as adjuvants in experimental vaccines against SIV [7,25] and HIV [8,26] with promising activities. The target immune cells for these immunostimulants were found to be macrophages or B lymphocytes [27,28] but not T lymphocytes [29]. Our results showing activation of NF-κB by some muramyl peptides in the pre-B and the monocyte–macrophage cells but not in the Jurkat T cells support these earlier findings and supplement the data base of activation markers induced by MDP. The muramyl peptide-induced activation of NF-κB in the two responsive cell lines could be blocked by pretreatment of the cells with the antioxidants PDTC and DDTC. This is not surprising since enhanced production of ROI by phagocytic cells has been reported following stimulation with MDP or LPS [30,31]. On the other hand, dithiocarbamates have been recently shown to inhibit production of ROI and the subsequent activation of NF-κB in LPS-stimulated cells [19,32].

The degree of activation of NF-κB by the immunostimulants under study can be associated with a certain profile of biological effects. Immunostimulatory and pyrogenic molecules like LPS, SDZ MRL 953, MDP, MDP-GDP and muroctasin [33] were strong activators of NF-κB whereas immunostimulatory but apyrogenic molecules [34] induced either low activation levels or no activation as was the case with murabutide, murametide or MDP (thr)-GDP, respectively. The latter molecule, however, was shown to induce IL-8 gene expression in Mono-Mac-6 cells, similar to other muramyl peptides. This induction is in contrast to the notion that a lack of interaction of the molecule with the cells is responsible for the failure to activate NF-κB. This novel apyrogenic lipophilic MDP derivative shows strong adjuvant activity in classical animal models, lacks toxic synergism with LPS [14,35] and selectively induces high levels of IL-6 and IL-8 but not IL-1β or TNF-α gene expression and secretion in whole human blood cultures (Bahr, in preparation). Moreover, the lipophilic nature of the molecule renders it an adjuvant for enhancement of both humoral and cell-mediated immunity in contrast to hydrophilic muramyl peptides which can only enhance humoral responses [36].

Activation of HIV-1 expression from a latent or low level state of replication is dependent, in part, on the state of activation of host cells. On this basis, vaccine strategies for HIV-1 should ensure that the elements giving rise to protective immunity are present while those eliciting undesirable side effects are excluded. This becomes even more critical in vaccines aimed at post-exposure prophylaxis where side effects such as strong activation of NF-κB by vaccine components may contribute to enhanced HIV replication instead of protective immunity. Our present screening of potential adjuvants for human use points to the unique safety of MDP (thr)-GDP and places it on top of the list of adjuvants to be seriously assessed in AIDS vaccines.

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