Constitutive nuclear NF-κB in cells of the monocyte lineage

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In monocytes, the nuclear factor NF-κB has been invoked as an important transcription factor in the expression of cytokine genes, of cell-surface receptors and in the expression of human immunodeficiency virus. In such cells, DNA binding activity of NF-κB can be detected without intentional stimulation. In our studies, cells of the human monocytic line Mono Mac 6, cultured in medium containing fetal-calf serum and low levels of lipopolysaccharide (LPS), also exhibit such 'constitutive' NF-κB, as demonstrated by mobility-shift analysis of nuclear extracts. This nuclear NF-κB was still present when contaminant LPS was removed by ultrafiltration and when serum was omitted. Protein–DNA complexes of constitutive NF-κB are similar in mobility to the LPS-induced NF-κB and both are recognized by an antibody specific to the p50 subunit of NF-κB. By contrast, treatment of cells with pyrrolidine dithiocarbamate (PDTC) will only block LPS-induced NF-κB, but not the constitutive binding protein. Using LPS-free and serum-free conditions, constitutive NF-κB can be detected in different cell lines of the monocytic lineage (HL60, U937, THP-1, Mono Mac 1 and Mono Mac 6), but not in Molt 4 T cells or K562 stem cells. When ordered according to stage of maturation, the amount of constitutive NF-κB was not increased in more mature cell lines. Furthermore, when inducing differentiation in Mono Mac 6 cells, with vitamin D_3, no change in constitutive or inducible NF-κB can be detected. Analysis of primary cells revealed substantial constitutive NF-κB-binding activity in blood monocytes, pleural macrophages and alveolar macrophages. The constitutive NF-κB appears to be functionally active, since a low level of tumour necrosis factor (TNF) transcript is detectable in monocytes, and this level can be increased by blocking transcript degradation using cycloheximide. The level of constitutive NF-κB in these cells is variable and is frequently found to be lower in the more mature macrophages. Constitutive NF-κB was not maintained by autocrine action of cytokines TNF, interleukin 6, interleukin 10, granulocyte–macrophage colony-stimulating factor or macrophage colony-stimulating factor, since neutralizing antibodies did not reduce constitutive DNA-binding activity. Furthermore, blockade of prostaglandin or leukotriene biosynthesis did not affect constitutive NF-κB. The results show that cells of the monocyte system, under conditions that exclude major external stimuli, do show constitutive NF-κB in the nucleus without an apparent correlation to stage of differentiation.

INTRODUCTION

The nuclear factor NF-κB has been originally described as a DNA-binding protein interacting with the sequence GGGACTTTCC in the mouse Ig κ light-chain enhancer (Sen and Baltimore, 1986) and thereby inducing transcription of the κ light-chain gene. Subsequent work revealed that NF-κB is important for several other molecules expressed by leucocytes, including cytokines like interleukin 2 (IL-2), tumour necrosis factor (TNF), and IL-6 (Hoyos et al., 1989; Liebermann and Baltimore, 1990). NF-κB is a heterodimeric p50/p65 protein that is present in the cytoplasm of resting cells in a complex with an inhibitory protein (IKB). Only after cell activation does IκB dissociate and release NF-κB, which is then transported to the nucleus (Bäuerle and Baltimore, 1988).

The cells of the monocyte system (myelomonocytic stem cells, bone-marrow monoblasts, blood monocytes, tissue macrophages) form a central element in immune defence, in that they are able to phagocytose foreign material, present processed antigen to T cells and orchestrate the immune response by secretion of cytokines, like TNF, IL-1 and IL-6. Expression of these cytokines in cells of the monocyte system can be strongly induced by the bacterial product lipopolysaccharide (LPS) and such LPS stimulation also results in mobilization of NF-κB (Shakhov et al., 1990; Haas et al., 1990).

Quite in contrast with resting T cells, but similar to antibody-producing B cells, constitutive presence of NF-κB in nuclei has been reported for monocytes (Griffin et al., 1989; Haas et al., 1990; Kaufman et al., 1992). In one study it was claimed that constitutive NF-κB will increase with differentiation to mature macrophages (Griffin et al., 1989). Given the sensitivity of monocytes to activation by various signals and, most noteworthy, from trace amounts of LPS (Ziegler-Heitbrock et al., 1986; Taktak et al., 1991), one might assume that such constitutive mobilization is, in fact, induced inadvertently by exogenous stimuli. To address this question, we have studied a set of human cell lines under serum-free and LPS-free conditions. Our data show that in the absence of such exogenous stimuli, cell lines belonging to the monocyte system show constitutive nuclear NF-κB. In contrast with a previous report, we found, however, no apparent increase during differentiation.

MATERIAL AND METHODS

Primary cells

Human peripheral blood monocytes were prepared by using

Abbreviations used: LPS, lipopolysaccharide; PDTC, pyrrolidine dithiocarbamate; TNF, tumour necrosis factor; IL-2, -6, -10, interleukins 2, 6, 10; FCS, fetal-calf serum; PMSF, phenylmethanesulphonyl fluoride; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatability complex; MTT, 3-(4,5-dimethyl-thiazol-2-yl 2,5-di-phenyltetrazolium-bromide); NDGA, nordihydroguaiaretic acid.

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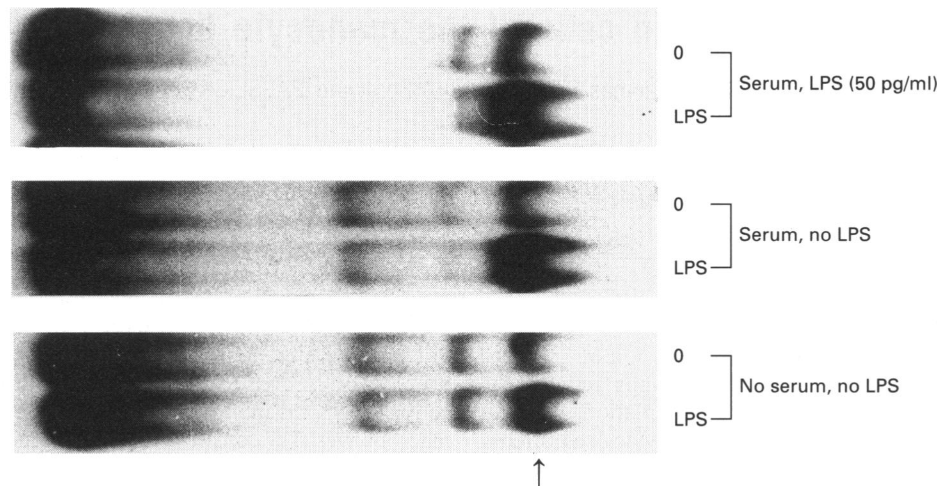

Figure 1 Constitutive NF-κB in Mono Mac 6 cells and LPS-free and serum-free conditions

Mono Mac 6 cells were cultured for 3 days with medium containing either 10% FCS and small amounts of contaminant LPS (lanes 1 and 2), or 10% FCS without detectable LPS (lanes 3 and 4) and without any serum or LPS (lanes 5 and 6). Cells were then either left untreated or were stimulated for 1 h with LPS, followed by preparation of nuclear extracts and gel-shift analysis.

NycoPrep 1.068 solution (Nycomed Pharma AS, Oslo, Norway; cat. no. 223510) according to the manufacturer's description. In brief, 10 vol. of defibrinated blood from healthy volunteers is mixed with 1 vol. of 6% (w/v) Dextran 500 in 0.9% (w/v) NaCl. The plasma layer, containing the leucocytes, is removed after the erythrocytes have settled (30–40 min at room temperature). The plasma is then layered on to NycoPrep 1.068 and centrifuged at 600 **g** for 15 min. The clear plasma, including interface and NycoPrep solution, is collected and washed twice with 0.9% NaCl, containing 0.13% EDTA and 1% fetal-calf serum (FCS). Separated monocytes show a purity of about 85–90% when analysed for CD14 staining in flow cytometry. Except for the initial sedimentation, all procedures were performed at 4 °C and cells were used for preparation of lysates directly unless stimulated in culture medium with LPS at 1 μg/ml for 1 h at 37 °C.

Alveolar macrophages were recovered by broncho-alveolar lavage from healthy volunteers after informed consent was obtained and after the study had been cleared by the Ethics Committee of the University of Munich. Lavage was performed with 100 ml of prewarmed 0.9% NaCl and the fluid recovered was passed through sterile gauze and admixed immediately with an equal volume of RPMI 1640 culture medium at 4 °C containing 10% FCS. These preparations are > 95% macrophages, as assessed by CD14 staining, and they rarely contain bronchial epithelia (< 0.5%). Similarly, pleural lavage preparations contain only a few mesothelial cells and are also > 90% CD14-positive.

Cell lines

Cell lines Mono Mac 1 and Mono Mac 6 were established in this laboratory (Ziegler-Heitbrock et al., 1988). THP-1 (Tsuchiya et al., 1980) and HL60 (Collins et al., 1977) were obtained courtesy of R. Munker, Munich, Germany. U937 (Sundström and Nilsson, 1976) was obtained from K. Nilsson, Uppsala, Sweden; K562 (Lozzio et al., 1976) and Molt4 (Minowada et al., 1972) from L. Gürtler, Munich, Germany. Cells were cultured in RPMI 1640 with 10% newborn-calf serum (Hyclone, Greiner, Nürtingen, Germany), penicillin, streptomycin and L-glutamine. Mono Mac-1 and -6 cells were cultured in RPMI, fortified as described (Ziegler-Heitbrock et al., 1988).

For neutralization of monocyte-derived cytokines, Mono Mac 6 cells were incubated overnight with antibodies raised against TNF (rabbit antibody, 2 μg/ml, kindly provided by Dr. T. Subkowski, BASF-Knoll), IL-6 (goat antibody, 50 μg/ml, Janssen, Hiss, Freiburg), IL-1 (0.5 μg/ml), IL-10 (25 μg/ml), macrophage colony-stimulating factor (M-CSF) (2.5 μg/ml), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (20 μg/ml) (all goat antibodies; Biermann, Bad Nauheim, Germany), followed by culture for 45 min with or without LPS (1 μg/ml) before preparation of nuclear extracts. For induction of cytokine protein, cells were cultured for an additional 4 h.

Prostaglandin and leukotriene biosynthesis was blocked by overnight culture with 1×10^{-5} M indomethacin (cat. no. I-7378, Sigma) or with 5×10^{-6} M nordihydroguaiaretic acid (NDGA) (cat. no. N-1144, Sigma) respectively.

Bioassays for TNF and IL-6

TNF was tested in the Wehi164S/actinomycin D cytotoxicity assay as described previously (Ziegler-Heitbrock et al., 1992). In brief, 100 μl of Wehi164S cells were incubated overnight with actinomycin D (1 μg/ml) and were seeded at 1×10^5 cells/well into 96-well flat-bottomed microtitre plates. To this, was added in duplicate threefold serial dilutions of cytokine-containing supernatants. After overnight culture at 37 °C, tetrazolium salt (MTT, cat. no. M-2128, Sigma) was added for 4 h and any crystals formed were dissolved in SDS/HCl. Absorbance was determined in an e.l.i.s.a. reader and units of TNF were calculated with reference to a recombinant TNF standard. IL-6 was measured in the 7TD1 proliferation assay (van Snick et al., 1986). For this purpose 100 μl of 7TD1 cells were seeded at 5000 cells per well in flat-bottomed microtitre plates together with duplicate twofold serial dilutions of 20 μl culture supernatants. After culture for 3 days, viable cells were determined with MTT as given above for the TNF assay. Units of IL-6 were calculated with reference to a recombinant IL-6 standard.

Detection and removal of LPS

LPS was measured using the Pyrogent limulus amoebocyte lysate (LAL) assay (Byk-Santec, Dietzenbach, Germany), which had a

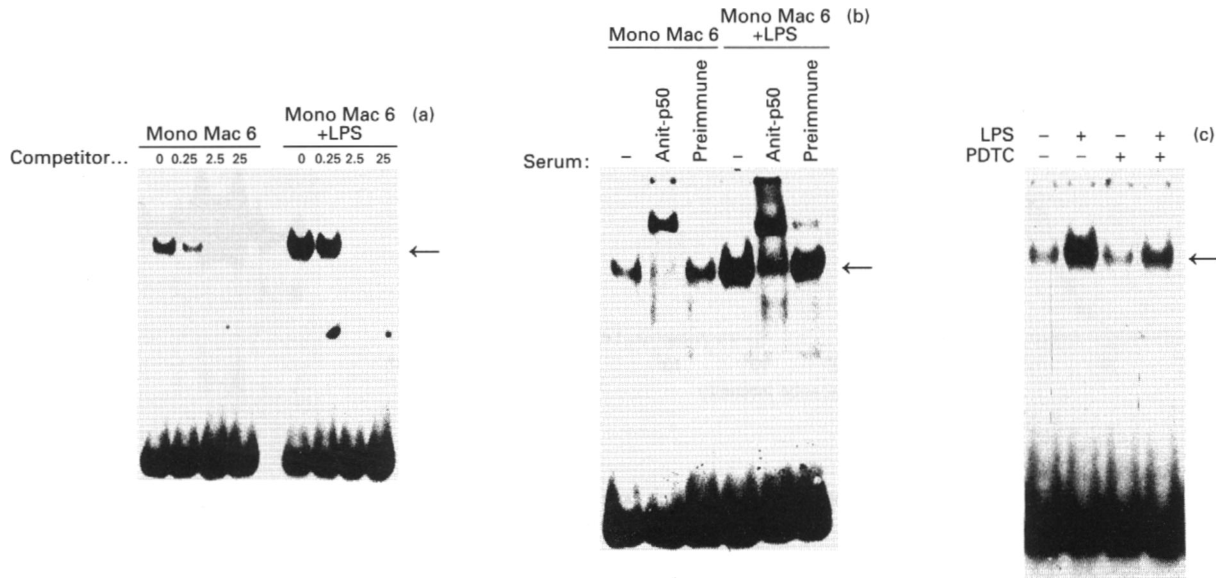

Figure 2 Characterization of constitutive NF-κB

(**a**) Competition by a KB oligonucleotide. Nuclear extracts from untreated or LPS-stimulated cells were assayed in the presence of increasing amounts of competitor derived from the HLA–A_2 enhancer added as 0.25, 2.5 or 25 ng per lane. (**b**) Supershift by anti-p50. Nuclear extracts from untreated or from LPS-treated Mono Mac 6 cells were incubated without serum, with anti-p50 antibody or with preimmune serum. (**c**) Effect of PDTC. Mono Mac 6 cells were cultured for 1 h with or without PDTC at 300 μM followed by 30 min culture with or without LPS.

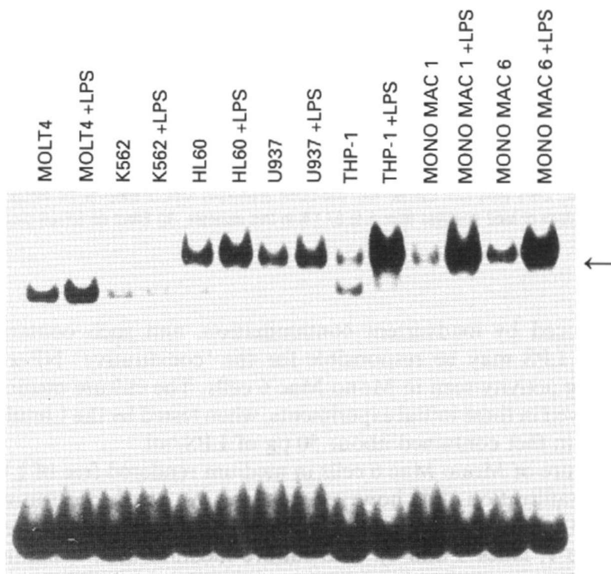

Figure 3 Constitutive NF-κB in different cell lines cultured under LPS-free conditions

Cell lines were cultured for 3 days in medium depleted of LPS by ultrafiltration, followed by addition of 10% FCS. Before preparation of nuclear extract, one sample of each was stimulated with LPS for 1 h.

sensitivity of 10–25 pg/ml when using *Salmonella minnesota* LPS as a standard. For removal of LPS, culture medium (before the addition of FCS) was passed through a Gambro 2000 ultrafiltration column (Gambro, Hechingen, Germany)(Blumenstein et al., 1988). Cycloheximide (cat. no. C-6255, Sigma) was found to be LAL-negative (< 10 pg/ml)

Treatment with pyrrolidine dithiocarbamate (PDTC)

Mono Mac 6 cells were treated with and without PDTC at 300 μM for 1 h (Ziegler-Heitbrock et al., 1993), followed by 30 min culture with or without LPS at 30 ng/ml.

Preparation of nuclear extracts

Cells with or without 1 h stimulation at 37 °C with 1 μg/ml LPS were extracted essentially as described previously (Haas et al., 1990). In brief, after incubation for 10 min in a hypotonic buffer, they were subjected to cycles of ultrasound (5 s each), such that over 70% of the cells were free nuclei. Nuclei were pelleted and resuspended in high-salt buffer. After 40 min on ice, nuclei were pelleted and the supernatant was diluted with buffer D. Care was taken to add 1 μl of phenylmethanesulphonyl fluoride (PMSF) to the preparation at every step of the procedure. Protein content of the preparation was determined by Coomassie Blue reaction, using the Bio-Rad kit (cat. no. 500-0006; Bio-Rad, Munich, Germany). If required, aliquotted extracts were stored at −80 °C. Contamination of nuclear extracts by cytosolic proteins cannot be completely excluded with the preparation method used. Similar results were, however, also obtained with extracts prepared using a Dounce homogenizer. Furthermore, cytosolic NF-κB is bound to inhibitory proteins (IκB, C-terminal part of p105), which have to be removed by deoxycholate treatment in order to give binding to their cognate DNA.

Gel-shift analysis

Gel shift was performed as described using a ^{32}P labelled κB oligonucleotide corresponding to the NF-κB-binding site of the mouse κB light-chain enhancer (Sen and Baltimore, 1986). For inhibition studies, labelled oligonucleotide was mixed with 0.25 ng, 2.5 ng and 25 ng excess of unlabelled oligonucleotide derived from the HLA-A_2 enhancer. The anti-p50 antibody, kindly provided by A. Israel (Paris, France), was added as 1 μl of undiluted serum to the nuclear extract before addition of the ^{32}P-

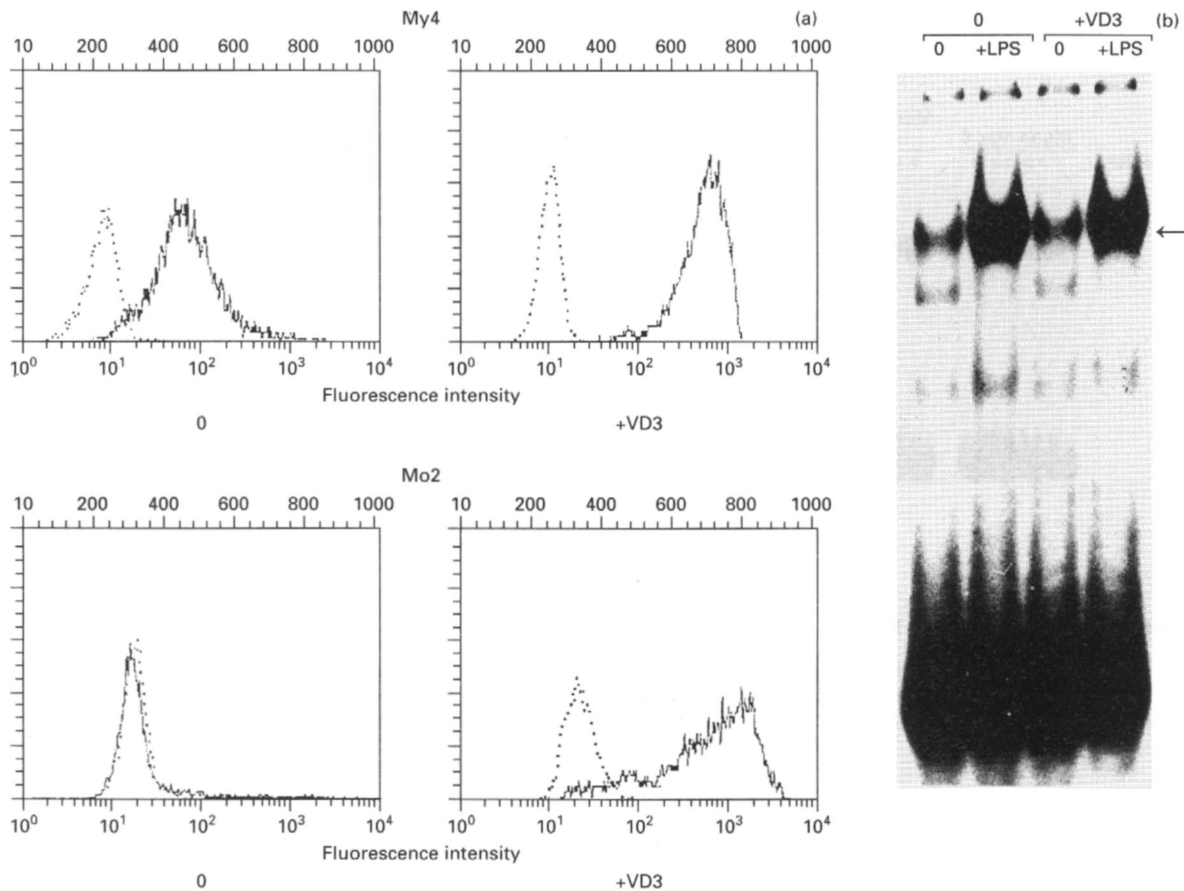

Figure 4 Effect of differentiation induction by vitamin D_3

(**a**) Effect on CD14 expression. Mono Mac 6 cells were cultured for 3 days with or without vitamin D_3 (160 nM). Cells were then stained with anti-CD14 antibodies My4 or Mo2, or the respective isotype controls (dotted lines). Fluorescence intensity for My4 increased from 557 to 716 channels, and percentage of Mo2 positives from 5 to 87.1% in this example. (**b**) Effect on NF-κB binding activity. Nuclear extracts were prepared from the same cells and assayed in gel-shift analysis

labelled oligonucleotide. Samples were run on a 4% polyacrylamide gel and the dried gel was applied to an X-ray film at −80 °C overnight.

Northern-blot analysis

For Northern-blot analysis, mRNA was isolated using RNAzol B (WAK, Bad Homburg, Germany). Samples (10 μg per lane) were then separated on an agarose gel and blots were hybridized with oligonucleotide probes labelled with ^{32}P by poly(A) tailing. The probes used were as follows: TNF (Nedwin et al., 1985) (residues 89–70) 5′-GGTCTGGTAGGAGACGGCGATGCGGCTGATGGTGTGGGTGAGGAGCACATGGGTGGAGGG-3′ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Dugaiczyk et al., 1983) (residues 17–2) 5′-CCCTGGTGACCAGGCGGCCAATACGGCCAAATCCGTTGACTCCGACTTTCACC-3′.

RESULTS

Constitutive NF-κB in Mono Mac 6 cells

Nuclear extracts prepared from Mono Mac 6 cells cultured in standard culture medium with FCS demonstrate 'constitutive' NF-κB-binding activity (Figure 1, lane 1) and stimulation with LPS for 1 h can strongly increase this activity (Figure 1, lane 2). Culture medium may contain substantial amounts of LPS, introduced by inadvertent contamination, and such contaminating LPS may be responsible for the 'constitutive' NF-κB-binding activity seen in Mono Mac 6 cells. The culture medium employed in these initial experiments, when tested by the Limulus assay, in fact contained about 50 pg of LPS/ml.

Culture of Mono Mac 6 cells in medium rendered free of LPS by ultrafiltration did, however, not remove the 'constitutive' NF-κB binding, and the induction by LPS was still apparent (Figure 1, lanes 3 and 4). The culture medium used contained 10% FCS and this may have contained additional factors involved in activation of monocytes. Even with omission of FCS, the NF-κB-binding activity was still clearly evident (Figure 1, lane 5). These results suggest that monocytes may, in fact, be capable of mobilizing NF-κB independent of external signals.

Next we asked whether the constitutive NF-κB detected in Mono Mac 6 cells under these conditions does have properties similar to the LPS-induced nuclear factor. For this purpose, constitutive binding activity was competed for by a prototypic DNA sequence for NF-κB derived from the human major histocompatibility complex (MHC) class I enhancer (Figure 2a). Non-radioactive double-stranded oligonucleotide was added in increasing amounts (lanes 2–4, lanes 6–8) to the NF-κB-binding assay. As shown in Figure 2(a), the constitutive NF-κB, as well as the LPS-induced NF-κB, is competed for, indicating that both signals have similar specificities. Furthermore, an antiserum against p50 equally recognized the constitutive and the LPS-

induced band, as shown by the further shift to higher molecular mass, due to complexing with the antibody (Figure 2b, lanes 2 and 5). Treatment of Mono Mac 6 cells with PDTC did not, however, reduce constitutive NF-κB, while the LPS-induced binding protein was strongly reduced (Figure 2c). In an average of six experiments, LPS-induced NF-κB was reduced by 66.3 ± 29.8 %, while the level of constitutive NF-κB in PDTC-treated cells was reduced by 9.6 ± 12.6 % of the untreated control.

Constitutive NF-κB in cell lines of different degrees of maturation

When testing a panel of cell lines cultured in FCS-containing LPS-free culture medium, we detected no constitutive NF-κB in Molt4 T cells in K562 haematopoetic stem cells. A higher-mobility band seen in these cells and also in THP-1 cells is variable, and in competition analysis turns out to be non-specific (results not shown). The myelomonocytic stem-cell line HL60 does, however, express constitutive NF-κB and the committed U937 line, representing the stage of monoblasts, showed detectable NF-κB binding in untreated cells as well. Furthermore, the more mature promonocytic lines THP-1 and Mono Mac 1 were positive, as was the most mature monocytic Mono Mac 6 line (Figure 3). In contrast with the earlier suggestion that constitutive NF-κB may increase with stage of differentiation (Griffin et al., 1989), we do not see an obvious increase from myelomonocytic stem cells towards monocytic cells. There was, however, a trend towards higher LPS-inducible NF-κB binding with increasing maturation in that the three promonocytic and monocytic lines exhibited high binding compared with the stem-cell line and the immature monoblastic line. A similar pattern was observed when cells were cultured in FCS-free medium. Constitutive NF-κB was still present and there was again no increase with stage of differentiation (results not shown).

Next we asked whether differentiation induction of the Mono Mac 6 cell line will upregulate constitutive NF-κB. Mono Mac 6 cells treated for 3 days with vitamin D_3 do show enhanced phagocytosis (16.6 ± 8.9 in controls, 39.3 ± 31.8 in vitamin D_3-treated cells), and they showed a further upregulation of the CD14 molecule. The staining of vitamin D_3-treated cells with the antibody My4 resulted in an increase of mean fluorescence intensity from 557 to 716 in the example in Figure 4(a), and the antibody Mo2 stained less than 5 % in untreated and 87.1 % in the vitamin D_3-treated Mono Mac 6 cells. These results show that vitamin D_3 can, in fact, induce maturation of Mono Mac 6 cells. Constitutive NF-κB-binding activity was, however, not affected by such treatment (Figure 4b, lanes 1 and 3).

Primary cells

As one source of tissue macrophages, we used cells obtained by pleural lavage. Constitutive NF-κB in such pleural macrophages was found to be lower (Figure 5a, lane 2) or similar (lane 4) to the level found in the blood monocytes from an unrelated control donor (lane 1). Next we tested pairs of primary monocytes and tissue macrophages, obtained from the same donor. In the example in Figure 5b, paired blood monocytes and alveolar macrophages were isolated from two apparently healthy volunteers. Constitutive NF-κB was clearly lower in the alveolar macrophages in both cases. These results show that among primary cells, the tissue macrophages, compared with blood monocytes, do not show higher levels of constitutive NF-κB in most cases. Thus in the monocyte lineage, an increase of constitutive NF-κB with maturation is not apparent from these results.

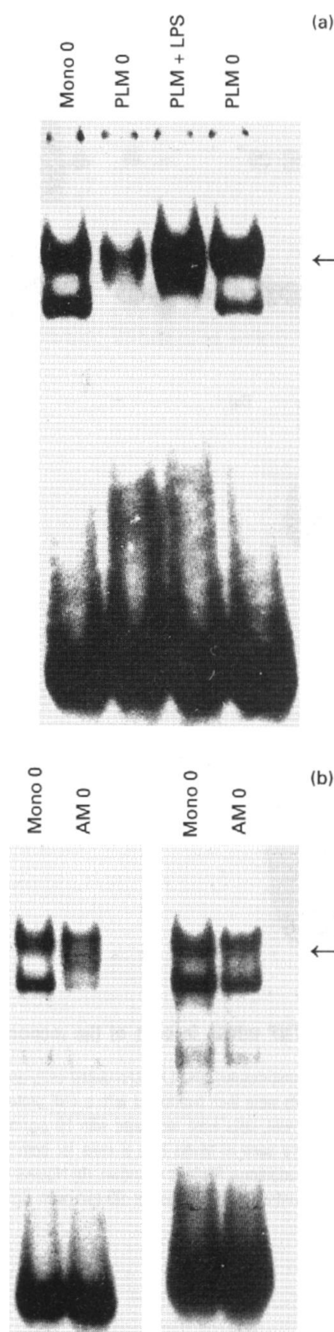

Figure 5 Comparison of NF-κB binding activity in blood monocytes and tissue macrophages

(**a**) Constitutive NF-κB in blood monocytes and pleural macrophages. Monocytes were isolated to < 88% purity by NycoPrep separation from a control donor, pleural macrophages were recovered from patients with bronchial carcinoma before lobe resection. (**b**) Constitutive NF-κB in monocytes and alveolar macrophages. Monocytes were isolated to < 88% purity from blood; alveolar macrophages from the same healthy volunteers were recovered by bronchoalveolar lavage.

Constitutive TNF gene expression

Finally, we asked whether the constitutive NF-κB detectable in nuclei of monocytes might be of functional importance. For this purpose we studied mRNA levels of TNF, a gene which is transcriptionally controlled by NF-κB. Figure 6(a) shows that Molt4 T cells, which express no constitutive NF-κB, also show

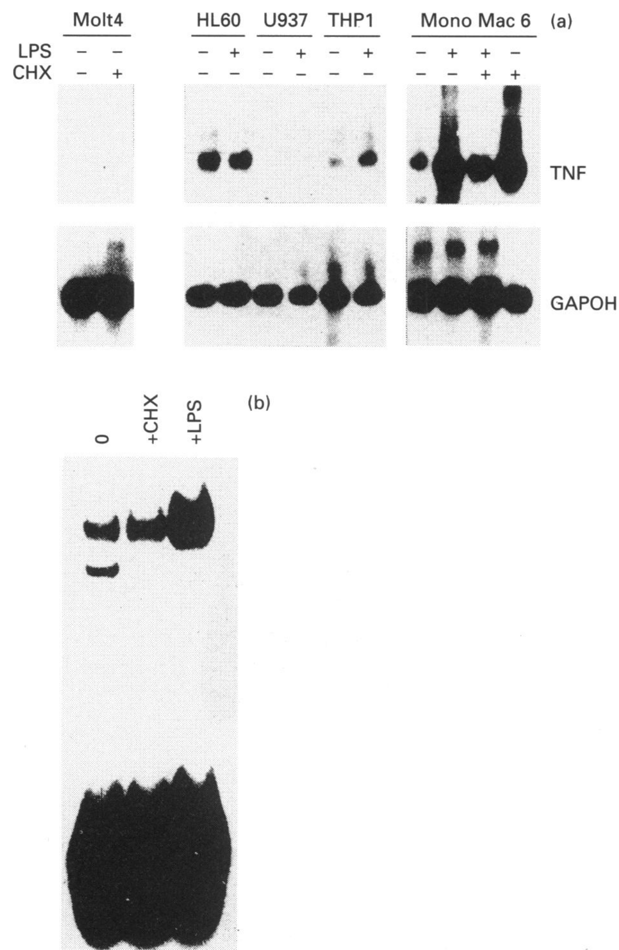

Figure 6 Constitutive TNF mRNA in cells of the monocyte lineage

(**a**) mRNA was isolated from unstimulated and LPS-stimulated cells and 10 μg per lane was separated by agarose gels. Blots were then hybridized with a TNF probe and a GAPDH probe. For cycloheximide (CHX) treatment, cells were incubated with 10 μg/ml CHX for 2 h. Higher-molecular-mass transcripts were seen in Mono Mac 6 cells for both TNF and GAPDH. The biological significance of these additional RNAs is unknown. (**b**) Nuclear extracts were prepared from Mono Mac 6 cells, left untreated or treated for 90 min with CHX at 10 μg/ml or, for comparison, with LPS.

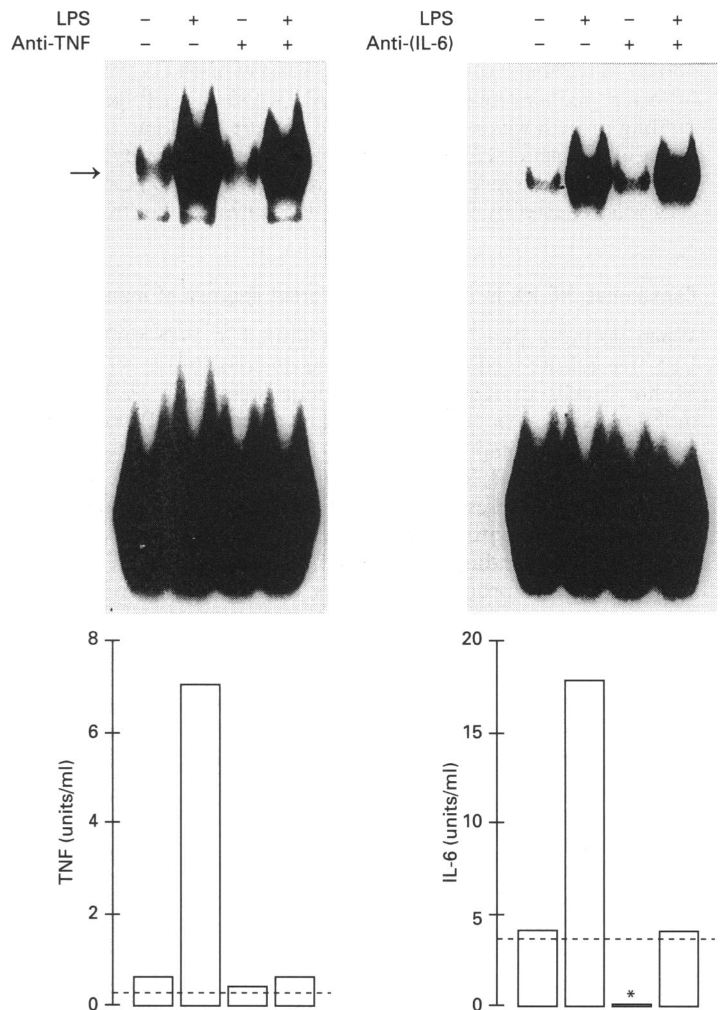

Figure 7 Lack of effect of anti-TNF and anti-IL-6 on constitutive NF-κB

Mono Mac 6 cells were treated overnight with neutralizing antibodies, and nuclear extracts were isolated after an additional 45 min with or without LPS (upper panel). Supernatants were recovered 4 h after stimulation with or without LPS and TNF was tested in the Wehi-164S/actinomycin D cytotoxicity assay, and IL-6 was measured in the 7TD1 bioassay (one of two experiments each).

no evidence of constitutive TNF transcripts. HL60 myelomonocytic stem cells and THP-1 monoblastic cells, both of which express constitutive NF-κB, also exhibit constitutive TNF transcripts. Furthermore, the monocytic Mono Mac 6 cells also clearly show constitutive TNF mRNA. Only in the monoblastic U937 cell line, was no TNF mRNA detectable. In addition, in Mono Mac 6 cells, LPS stimulation substantially increased TNF mRNA prevalence. Since cytokine transcripts are subject to rapid degradation by protein-synthesis-dependent mechanisms, we asked whether constitutive levels of mRNA might be enhanced by blockade of degradation. For this purpose, we treated Mono Mac 6 cells with cycloheximide at 10 μg/ml for 90–120 min. Such treatment did not affect NF-κB-binding activity in nuclear extracts (Figure 6b), but TNF mRNA was enhanced 2.5-fold (Figure 6b). These results suggest that constitutive NF-κB may, in fact, drive transcription, an effect that is counteracted by rapid degradation of cytokine mRNA.

Blockade of autocrine pathways

Since cytokines like TNF are capable of inducing a mobilization of NF-κB, one might speculate that constitutive NF-κB is under the control of such autocrine cytokines. Hence, we have performed blocking studies in order to exclude a role for monocyte-derived cytokines and arachidonic acid metabolites.

Treatment of Mono Mac 6 cells overnight with anti-TNF antibody did not affect constitutive or LPS-induced NF-κB (Figure 7, upper panel), while at the same time the antibody efficiently neutralized LPS-induced TNF activity (Figure 7, lower panel). This suggests that the antibody was capable of inactivating TNF protein that might be produced at low but undetectable levels in unstimulated cells. The same applied to anti-IL-6 antibody, which efficiently neutralized IL-6 activity but had no effect on constitutive or induced NF-κB. Additional anti-cytokine antibodies that were found not to affect constitutive NF-κB

either were anti-(IL-1β), anti-(IL-10), anti-(M-CSF) and anti-(GM-CSF) (results not shown). In addition, blockade of cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism had no effect. Hence, although not all monocyte products have been studied, this set of major monocyte-derived cytokines and arachidonic acid metabolites can be excluded as inducers of constitutive NF-κB.

DISCUSSION

NF-κB is a transcription factor central to a variety of genes involved in immune defence. The genes regulated by NF-κB can be grouped into (a) cytokines, (b) receptors and (c) acute-phase proteins. In addition, some viruses, like human immunodeficiency virus and cytomegalovirus, have picked up NF-κB-binding sequences and exploit this transcription factor for their propagation (Müller et al., 1993). Activation of leucocytes by different types of signals will lead, in the cytoplasm of the cell, to the release of the p50/p65 heterodimer from the inhibitor IκB. The p50/p65 complex then translocates to the nucleus and binds to regulatory sequences with the consensus sequence GGGRNNTYCC.

Constitutive binding activity in nuclear extracts of cells without deliberate activation has been noted for B lymphocytes (Sen and Baltimore, 1986) and more recently also for cells of the monocyte system (Griffin et al., 1989; Haas et al., 1990; Kaufman et al., 1992). Monocytes are, however, very sensitive to external signals. Adhesion, for instance, can trigger gene expression (Haskill et al., 1988), and very low levels of LPS (in the pg/ml range) may induce expression of genes, like IL-6 (Taktak et al., 1991). LPS is a frequent contaminant of tissue-culture media, where it can be introduced by medium ingredients, by FCS or by contaminated glassware (Weinberg, 1981). Hence, it is possible that a monocyte function considered constitutive is actually induced by unrecognized signals. Therefore, we have cultured the cells in the absence of serum and we have removed LPS by ultrafiltration. With this approach, we could show that monocytes do, in fact, mobilize a substantial amount of NF-κB independent of external signals.

It was suggested recently that constitutive NF-κB within the monocyte lineage may increase with increasing stage of differentiation (Griffin et al., 1989). The cell lines and primary cells studied herein represent different stages of maturation, and it is apparent from our analysis that constitutive NF-κB does not increase with differentiation.

When looking at the different cell lines, there appears to be, however, a trend to higher levels of inducible NF-κB in the more mature cells, which may explain higher levels of 'constitutive' NF-κB under conditions of inadvertant activation. This may have been the case in studies by Griffin et al. (1989), but it is also possible that these investigators employed different sublines of the original cell lines.

Autocrine mechanisms may be involved in maintaining the constitutive level of NF-κB, since monocyte-derived cytokines like TNF are efficient inducers of this transcription factor (Müller et al., 1993). By using neutralizing antibodies against this and several other cytokines and by using blockade of arachidonic acid metabolism, we were, however, unable to remove or reduce constitutive NF-κB. Hence, an autocrine mechanism can be excluded with regard to these major monocytic products.

The question remains what the functional role of such constitutive NF-κB might be: in other studies we have shown that the constitutive NF-κB mainly consists of p50 (Ziegler-Heitbrock et al., 1993, 1994). This complex may be related to the nuclear factor KBF1, which was described to regulate MHC class I expression (Israel et al., 1989). In fact affinity cross-linking of a similar high-mobility band in uninduced U937 cells demonstrates a p50 and a p55 band within this complex (Kaufman et al., 1992). As to the functional role of constitutive NF-κB in monocytes, we do see significant cytokine expression in untreated monocytic cells but not in T cells, which express no constitutive NF-κB. The evidence linking constitutive NF-κB and constitutive TNF is, however, only circumstantial. To resolve this question, studies with stable reporter gene transfectants are required. Constitutive TNF transcripts were also reported to occur in various human tissues (Tovey et al., 1988), and one might speculate that tissue macrophages are the source of such cytokine mRNA. Furthermore, whole blood cultures blocked in transcription by actinomycin D could still be triggered by LPS to release TNF protein (Hofsli et al., 1988), indicating that preformed TNF transcripts were present in these cells. Our studies *in vitro* show that blocking of mRNA degradation by cycloheximide enhances TNF transcript levels, while no NF-κB is induced. Others have reported that cycloheximide does induce NF-κB (Collart et al., 1990). These contrasting findings may be due to an LPS contamination of cycloheximide or to the use of different cell lines. However, the enhancement of TNF transcripts in the absence of a cycloheximide effect on constitutive NF-κB in our study argues that cycloheximide may simply prevent the degradation of constitutively transcribed TNF mRNA. Such an ongoing transcription, probably driven by constitutive NF-κB, may allow monocytes to react extremely rapidly to external stimuli like invading micro-organisms.

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