ELSEVIER

Biochemical Pharmacology 62 (2001) 141–147

Biochemical Pharmacology

Involvement of nuclear factor-kappa B (NF-κB) activation in mitogen-induced lymphocyte proliferation: inhibitory effects of lymphoproliferation by salicylates acting as NF-κB inhibitors

Lucia Cavallini*, Maria Angela Francesconi, Franco Zoccarato, Adolfo Alexandre

Department of Biological Chemistry, C.N.R. Centro di Studio delle Biomembrane, University of Padua, Via G. Colombo 3, I-35121 Padua, Italy

Received 25 August 2000; accepted 20 February 2001

Abstract

The transcription factor nuclear factor-kappa B (NF-κB) is involved in the production of inflammatory cytokines and in the control of the inflammatory response. Some nonsteroidal anti-inflammatory drugs such as acetylsalicylic acid (ASA) or salicylate are known to exert some of their anti-inflammatory pharmacological properties independently of cyclooxygenase inhibition. For ASA and salicylate, an NF-κB inhibitory effect at mM concentrations (pharmacological plasma concentrations reached *in vivo*) has been shown. We studied the action of ASA, salicylate, and several NF-κB inhibitors on the mitogen-induced activation of peripheral blood lymphocytes (PBL) and purified T cells. We showed that ASA and salicylate (1–3 mM) (but not indomethacin, a specific cyclooxygenase inhibitor) as well as a group of chemically unrelated inhibitors of NF-κB (including the sesquiterpene lactone parthenolide, Bay 11-7082, sulfasalazine, the proteasome inhibitor MG-132 and the peptide SN-50, an inhibitor of the nuclear transfer of the p50 subunit of NF-κB), were potent inhibitors of phytohemoagglutinin-activated PBL and T cell proliferation. At the same concentrations, they inhibited NF-κB binding to DNA in nuclear extracts. The inhibition of proliferation was not relieved by exogenous interleukin (IL)-2. We concluded that NF-κB activation has a fundamental role in T cell proliferation independently of IL-2 production. Some pharmacological actions of ASA may be ascribed to the inhibition of immune cell proliferation via the inhibition of the transcription factor NF-κB.

Keywords: Lymphocytes; NF-κB; Aspirin; Salicylates; Proliferation

1. Introduction

Many of ASA's therapeutic effects are clearly due to the inhibition of prostaglandin synthesis [1]. However, not all of ASA's anti-inflammatory actions can be accounted for by simple inhibition of COX 1 and 2. For example, therapeutic serum concentrations of salicylate, the major metabolite of ASA and a poor inhibitor of COX 1 and COX 2, correlate better with clinical anti-inflammation than serum concentrations of ASA [2,3]. It was recently found that ASA and salicylate disrupt signal transduction by inhibiting the translocation to the nucleus of the transcriptional regulator NF-κB [4]. NF-κB is a dimeric protein complex composed of members of the Rel family of transcription factors and is necessary for the transcription of many genes involved in inflammatory and immune responses. In quiescent cells, NF-κB is held in the cytosol by an inhibitory protein, IkB. In stimulated cells, IkB is phosphorylated by IkB kinase (IKK) and targeted for proteasomal degradation, thus releasing the NF-κB dimer (most frequently composed of the p50 and p65 subunits), which moves to the nucleus and binds to DNA (reviewed in [5]). IKK is composed of two kinase subunits (IKKalpha and IKKbeta) and a regulatory subunit (IKKgamma) [5]. ASA and salicylate inhibit the IKKbeta subunit of IKK kinase, thus preventing IkB phosphorylation and its subsequent degradation [6]. In endothelial cells, the salicylate inhibition of the TNF-α-induced

* Corresponding author. Tel.: +39-049-827-6149; fax: +39-049-807-3310.

E-mail address: cavallin@civ.bio.unipd.it (L. Cavallini).

Abbreviations: ASA, acetylsalicylic acid; COX, cyclooxygenase; ERK, extracellular receptor-activated kinase; FBS, fetal bovine serum; IL-2, interleukin-2; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa B; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; and TNF-α, tumor necrosis factor alpha.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00640-2

expression of the adhesion molecules E-selectin, VCAM (vascular cell adhesion molecule), and ICAM (intracellular adhesion molecule) was ascribed to the inhibition of NF-κB signaling [7]. In monocytes, ASA inhibition of NF-κB activation was shown to down-regulate the production of IL-12 [8]. These findings seem to indicate that interference with the NF-κB transcriptional regulation may be an important component of the anti-inflammatory actions of salicylates. However, at similar concentrations, other effects of salicylates have also been reported. They were shown to interfere with signaling via the MAP kinases by inhibiting JNK and activating p38 in fibroblasts [9] and by inhibiting ERK, thus preventing integrin-dependent activation, in neutrophils [10]. The inhibition of leukocyte accumulation *in vivo* at the sites of carrageenan-induced inflammation was ascribed to a salicylate-induced production of adenosine [11].

Lymphocyte activation represents a central event in the inflammatory/immune response. In this study, we analyze the effects of salicylates on mitogen-induced lymphocyte proliferation. We show that proliferation is potently inhibited by theurapeutic concentrations of ASA and salicylate, but not by the COX inhibitor indomethacin. Furthermore, we show that proliferation is inhibited by a variety of agents that are known to inhibit NF-κB activation. We conclude that the mechanism by which salicylates inhibit lymphocyte proliferation is largely by inhibition of NF-κB signaling and that in general inhibition of NF-κB activation precludes lymphocyte proliferation.

2. Materials and methods

2.1. Materials

RPMI (with L-glutamine), MTT, and PHA were from Sigma Chemical Co.; FBS, penicillin, streptomycin, and Lymphoprep™ from GIBCO BRL; oligonucleotide sense and antisense was synthesized by Amersham Pharmacia Biotech; poly(dI-dC), T4 polynucleotide kinase, and the radiochemicals [^{32}P]ATP and [methyl-^{3}H]thymidine were purchased from Amersham Pharmacia Biotech; parthenolide, MG-132, and SN-50 were from Biomol Research Laboratories, Bay 11-7082 from Calbiochem, and IL-2 from PeproTech EC LTD; human T cell enrichment columns were from R&D Systems. All other reagents were of analytical grade.

2.2. PBL preparation

Peripheral blood mononuclear cells were isolated from buffy coats by gradient centrifugation on Lymphoprep™ following the classical procedure of Bøyum [12]. The isolated mononuclear cells were resuspended in RPMI containing 10% FBS, penicillin, and streptomycin (complete medium), diluted to 2–3 × 10^6 per mL, and seeded in 75-cm^2 tissue culture flasks (Falcon). After monocyte adhesion (at least one day), PBL were removed, washed, and cultured (2–3 × 10^6 cells/mL) in complete RPMI medium in a 37°, 5% CO_2 atmosphere incubator and refed at least every three days. Each preparation was used for a maximum of three weeks.

2.3. T cell preparation

T cells were prepared from PBL by negative selection with the human T cell enrichment columns from R&D Systems following the manufacturer's indications. Cells were equilibrated for at least one day in complete medium before utilization.

2.4. Lymphocyte stimulation

Lymphocyte suspensions were seeded at 1 × 10^6 cells/mL in 6- or 24-well plates. Cells were incubated for at least 1 hr before stimulation with PHA (3 μg/mL). IL-2 when present was 100 U/mL. Anti-inflammatory drugs were added 30 min before stimulation with the exception of SN-50, which was preincubated for three hr prior to stimulation.

2.5. Cell proliferation

Cell proliferation was measured by [^{3}H]thymidine incorporation. [^{3}H]Thymidine (1 μCi/mL, specific activity 25 Ci/mmol) was added at the indicated time of stimulation. Incubations were stopped 6 hr later with 0.1% SDS; solubilized cells were collected and the wells washed with PBS containing 0.1% SDS. The acid-insoluble material recovered after 5% trichloroacetic acid precipitation was washed three times with trichloroacetic acid and counted.

2.6. MTT test

To determine the change in cell number, growth activity was measured by a colorimetric assay using MTT [13]. MTT (0.5 mg/mL) was added to the suspension 48 hr after stimulation and the reaction stopped 1 hr later with 0.5 mL of lysis extraction buffer composed of 20% SDS in 1/1 dimethylformamide/water pH 4.7 as reported in [12]. After overnight agitation, the solubilized formazane was determined at 540 nm.

2.7. NF-κB activation assay

NF-κB was assayed by electrophoretic mobility shift assay (EMSA) on the nuclear extracts of at least 3 × 10^6 PBL as in [14], after 3 hr of stimulation. Samples were prepared by incubating 5 μg of nuclear extracts with 50 fmol (25,000 cpm) of ^{32}P-labeled double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT

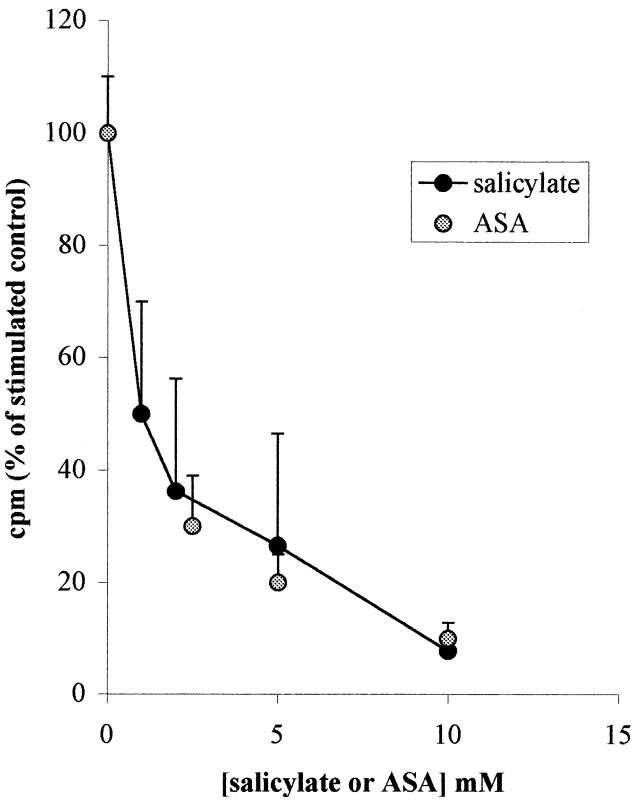

Fig. 1. Inhibition of [^{3}H]thymidine incorporation by ASA and salicylate. PBL preincubated with the indicated amounts of salicylate or ASA were stimulated with 3 μg/mL of PHA. [^{3}H]Thymidine was added 24 hr later. For details see Materials and Methods. Values expressed as % of cpm incorporated in stimulated PBL (approximately 30,000 cpm) represent the means ± SD (bars) of three experiments in duplicate.

CCC AGG C-3′) in the presence of 0.5 μg of poly(dI-dC) in binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2% Nonidet P-40, 5% glycerol, and 50 mM NaCl, final volume 20 μL) for 20 min at 37°. The oligonucleotide–protein complex formed was separated from free oligonucleotide on a 6% native polyacrylamide gel in 50 mM Tris, 200 mM glycine, 1 mM EDTA, pH 8.5 for 1 hr at 20 V/cm. Specificity of the binding was confirmed by using excess unlabeled oligonucleotide. The gels were fixed in 10% acetic acid and dried. Revelation of the radioactive bands and quantitation was performed on a high sensitivity Phospho Screen laser scanning detection system (Cyclone, Packard).

2.8. Statistical analysis

Values are always expressed as means ± standard deviation of at least triplicate experiments. Statistical differences between control and treatments are reported as significant (the probability associated with the Student's *t*-test in a two-tailed distribution of paired data) in the corresponding legends to figures.

Table 1
[^{3}H]Thymidine incorporation in PHA-stimulated PBL. Effect of indomethacin and NF-κB inhibitors

Additions		Concentration	[^{3}H]Thymidine incorporated (%)
—	—		8 ± 5
PHA	none		100
PHA	Parthenolide	1 μM	52 ± 10
PHA	Parthenolide	2.5 μM	15 ± 5
PHA	Parthenolide	5 μM	10 ± 2
PHA	Bay 11-7082	1 μM	37 ± 6
PHA	Bay 11-7082	2.5 μM	10 ± 5
PHA	Bay 11-7082	5 μM	5 ± 3
PHA	Sulfasalazine	1 mM	12 ± 7
PHA	SN-50	18 μM	32 ± 10
PHA	MG-132	5 μM	5 ± 3
PHA	Indomethacin	10 μM	110 ± 10

PBL preincubated with the indicated amounts of agents were stimulated as in Fig. 1. Values are expressed as % of cpm incorporated in stimulated control and represent the means ± SD of three experiments in duplicate. Significance was at least $P < 0.001$ in all treatments compared to stimulated control except for indomethacin (not significant).

3. Results

Resting PBL were stimulated with the mitogen PHA. DNA synthesis was measured as [^{3}H]thymidine incorporation after about 24 hr of stimulation. As shown in Fig. 1, thymidine incorporation was inhibited by ASA and salicylate. Semimaximal inhibition was obtained at approximately 1 mM of either agent, well within the concentrations (2–5 mM) required for the anti-inflammatory action of salicylate. The inhibition of PBL proliferation does not appear to depend on COX inhibition, first because salicylate, a poor COX inhibitor, was as effective as ASA, and second because inhibiting COX with indomethacin was without effect on lymphocyte proliferation. Indomethacin actually induced a slight, although not significant, potentiation of lymphocyte proliferation in accordance with [15] (Table 1).

Lymphocyte proliferation is known to depend on the stimulus-induced production of IL-2 and on the up-regulation of CD25, the high-affinity α-chain of the IL-2 receptor [16]. It was therefore possible that the effects of salicylates could be overcome by supplementing IL-2. However, exogenous IL-2 (100 U/mL) did not alleviate the salicylate inhibition of lymphocyte proliferation (not shown).

Some effects of ASA and its metabolite salicylate have been ascribed to the disruption of signal transduction between cell surface receptors and the transcription of inflammatory cytokines via inhibition of IkB phosphorylation, resulting in the prevention of NF-κB signaling. To assess the importance of NF-κB inhibition in the salicylate effect on PBL proliferation, we tested if other NF-κB inhibitors would similarly affect lymphocyte proliferation (Table 1). Parthenolide, a sesquiterpene lactone from the medicinal plant *Tanacetum parthenium* used in traditional medicine for the treatment of fever and migraine, was recently re-

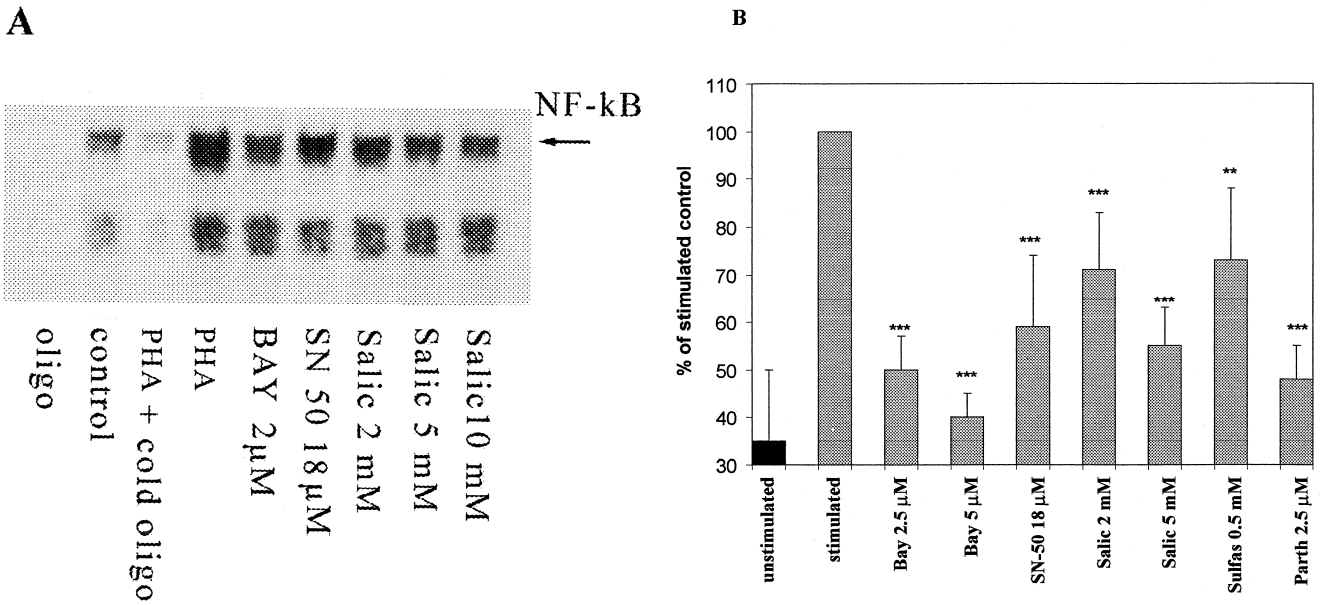

Fig. 2. Inhibition of NF-κB activation by salicylates or NF-κB inhibitors. Aliquots of 3-mL suspensions of PBL seeded in 6-well plates were incubated as in Fig. 1. Cells were removed after 3 hr of stimulation and washed in PBS. Nuclear extracts were prepared as indicated in Materials and Methods. (A) A typical [^{32}P] image of the EMSA pattern of nuclear NF-κB acquired with Cyclone (Packard). Where indicated, a 100-fold excess of unlabeled oligonucleotide (cold oligo) was included to confirm the specificity of the binding. (B) The intensity values of the NF-κB-linked [^{32}P]oligonucleotide band areas (total lux) are reported as % of the PHA-stimulated control and represent the means of three different experiments. ***, $P < 0.001$; **, $P < 0.01$. Bay, Sulfas, and Parth represent Bay 11-7082, sulfasalazine, and parthenolide, respectively.

ported to be a potent inhibitor of IkB phosphorylation and NF-κB signaling [17], although it also inhibits tyrosine phosphorylations as well as the activities of the MAP kinases ERK, JNK, and p38 [18]. As shown in Table 1, parthenolide was a powerful inhibitor of lymphocyte proliferation (IC_{50} about 1 μM). Another powerful inhibitor of PBL proliferation was BAY 11-7082 (IC_{50} about 0.5 μM), a recently introduced inhibitor of the TNF-α-induced nuclear translocation of NF-κB and anti-inflammatory agent [19], which acts by preventing IkB phosphorylation. Unlike parthenolide, BAY 11-7082 is not an inhibitor but rather a potentiator of MAP kinases JNK and p38 [19]. Lymphocyte proliferation was also strongly affected by sulfasalazine, an anti-inflammatory agent that was recently reported to inhibit NF-κB signaling [20]. Proteasome inhibitors affect NF-κB activation by preventing the degradation of phosphorylated IkB [21]. One such compound, MG-132 [21,22], was also a powerful inhibitor of lymphocyte proliferation, as shown in Table 1. The NF-κB inhibitor SN-50, a 26 aa cell-permeable peptide containing a sequence that interferes with the nuclear translocation of the p50 subunit of NF-κB, has been reported to be a rather specific inhibitor of NF-κB signaling [23]. As shown in Table 1, SN-50 was also an inhibitor of lymphocyte proliferation. All the reagents tested were equally effective in the presence of exogenous IL-2 (not shown). We evaluated salicylate and the other inhibitors of lymphocyte proliferation for their effects on the nuclear translocation of NF-κB in PBL. The degree of NF-κB binding to nuclear extracts from PHA-treated PBL in the presence of proliferation inhibitors was evaluated with the electrophoretic mobility shift assay (EMSA) technique. PHA induced a strong nuclear translocation of the NF-κB dimer, which was significantly decreased by salicylate, BAY 11-7082, parthenolide, and SN-50. A representative experiment is reported in Fig. 2A. The % inhibition of NF-κB association with DNA from three experiments is reported in Fig. 2B.

MTT reduction is a measure of cell-associated reducing power, which is in turn a function of cell number and viability. An MTT reduction test is reported in Fig. 3. It shows that PHA-induced proliferation (measured with the MTT test 2 days after stimulation) was strongly affected by all the NF-κB inhibitors except for indomethacin, which had only a slight tendency to increase proliferation. Salicylate inhibition at low concentrations was less powerful in these experiments than in Fig. 1, probably because salicylate at longer incubation times is metabolized to non-inhibitory compounds. Some decrease in MTT reduction was also visible in unstimulated cells, an indication of cytotoxicity. This is in agreement with a recent report showing that a basal level of NF-κB activity is required for the survival of unstimulated primary lymphocytes [24]. To better assess the cytotoxic effects, we monitored the action of NF-κB inhibitors on Trypan blue exclusion in unstimulated and stimulated PBL in parallel experiments. We found that the number of cells that did not exclude Trypan blue (a sign of cytotoxicity) was higher in resting than in stimulated PBL. In fact, the ratio of Trypan blue-negative/positive cells was 3.5 ± 0.3, 1.5 ± 0.2, and 2 ± 0.2 ($N = 3$) in unstimulated, parthenolide, and BAY-treated PBL, respectively, increas-

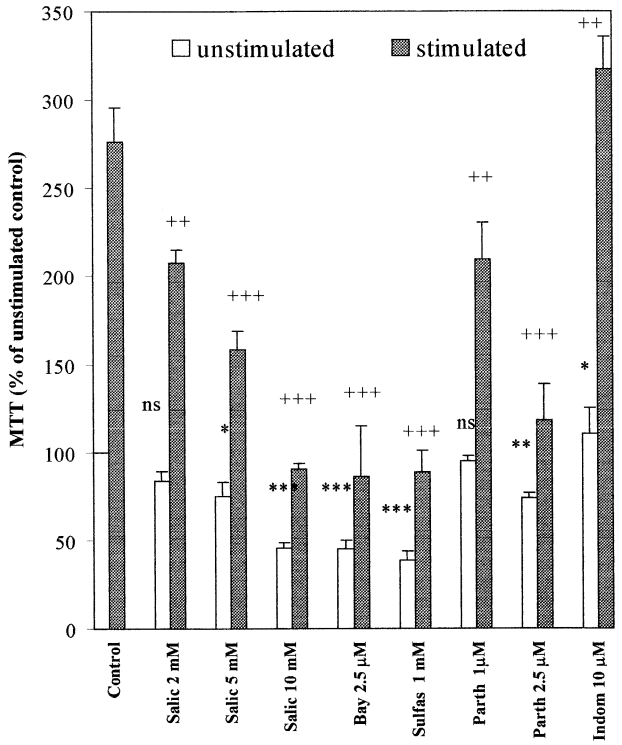

Fig. 3. Inhibition of cell growth by salicylates and NF-κB inhibitors. Conditions as in Fig. 1. MTT was added at 48 hr. The values are expressed as % of the unstimulated control and represent the means ± SD of three experiments in duplicate. Statistical differences are indicated by: *** or $^{+++}$, $P < 0.001$; ** or $^{++}$ $P < 0.01$; * $P < 0.1$; ns, not significant, and are related to unstimulated or stimulated controls, respectively. Abbreviations as in Fig. 2 except for Indom, which stands for indomethacin.

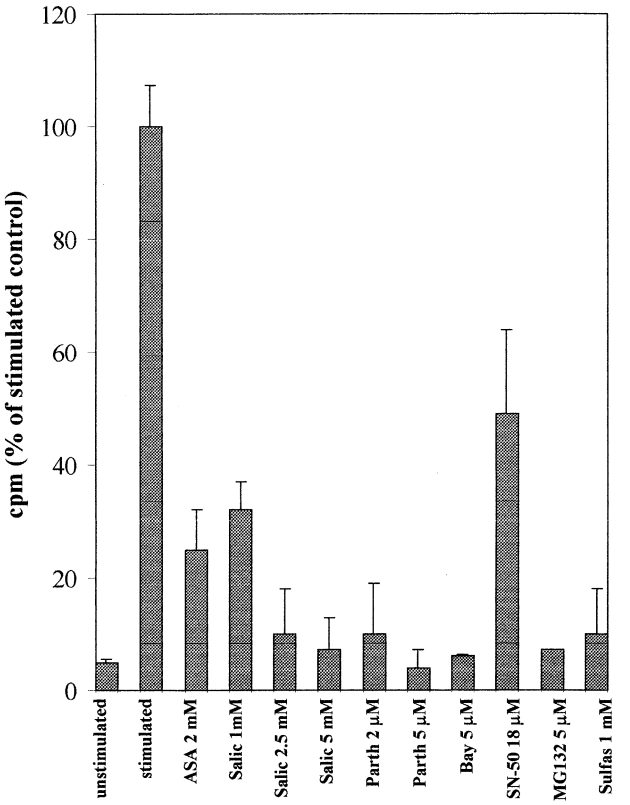

Fig. 4. Inhibition of [^{3}H]thymidine incorporation by salicylates and NF-κB inhibitors in purified T lymphocytes. Purified T cells were incubated as in Fig. 1. [^{3}H]Thymidine was added 48 hr later. Values in triplicate are expressed as % of the cpm incorporated in stimulated T cells (15,000 cpm). Significance was at least $P < 0.001$ in all treatments compared to stimulated control.

ing to 4.7 ± 0.3 in all cases in stimulated PBL. Thus, the cytotoxicity associated with the inhibition of NF-κB activity decreased in the presence of mitogen, in accordance with [24].

T lymphocytes are likely the largest contributors to cell proliferation in the experiments described, since PHA is a relatively specific stimulator of T lymphocytes. Experiments performed on purified T lymphocytes confirmed the results obtained in PBL. The initiation of [^{3}H]thymidine incorporation was somewhat delayed in purified T cells, but the pattern of inhibition was unchanged. Data of a representative experiment on purified T cells are reported in Fig. 4.

4. Discussion

In addition to its well-established role in activating the transcription of genes involved in immunological responses, studies indicate that NF-κB also functions in promoting cell proliferation. For instance, lymphocytes lacking the p50, p65, or c-Rel subunits of NF-κB exhibit defects in mitogenic responses [25–28], and p50/p52 double-knockout animals fail to generate mature osteoclasts and B cells [29,30]. Furthermore, deregulation of NF-κB activity has been associated with oncogenesis. In fact, NF-κB is activated by oncogenic Ras and is required for Ras to induce foci in NIH 3T3 cells [31], while constitutive NF-κB activation is required for proliferation and survival in Hodgkin's disease lymphoma cells [32]. In lymphocytes, constitutive NF-κB activation appears necessary for survival [24]. The inhibition of cell proliferation following inhibition of NF-κB signaling has recently been reported in 3T3 fibroblasts. It was shown that cyclin D1 was down-regulated under these conditions and that pRb was maintained in a hypophosphorylated, active state [33]. Whether this is also the case in T cells will be the subject of future research.

The observations presented in this study show that mitogen-stimulated T cell proliferation is prevented by salicylates (observations on the antiproliferative action of salicylates in lymphocytes have previously been reported [34, 35]), as well as by a number of other unrelated agents whose common property is the inhibition of NF-κB activity. The main determinant of the salicylate inhibition of lymphocyte proliferation is clearly not the inhibition of COX, since indomethacin is without effect.

NF-κB activity appears to be a major player in the induction of PBL proliferation. Although salicylate and

some of the NF-κB-interfering agents described in this research have also been reported to affect MAP kinase signaling, the effects are different for salicylate (inhibition of ERK and JNK, activation of p38, [9,10]) BAY 11-7082 (no effect on ERK, activation of JNK and p38, [19]), and parthenolide (inhibition of all the MAP kinases, [18]), whereas they have the same effect on proliferation. Furthermore, inhibition of proliferation is induced by SN-50, which specifically prevents the nuclear translocation of the p50 subunit of the NF-κB dimer [23], and by inhibition of the proteasome-mediated degradation of phosphorylated IkB. The inhibition of mitogen-induced proliferation as described here is not alleviated by exogenous IL-2, although we have evidence that IL-2 production, as well as the production of some chemokines, is strongly depressed by NF-κB inhibitors.[1] This is an indication that NF-κB intervenes both at the level of IL-2 synthesis and at the level of IL-2-induced mitogenesis. In terms of proliferation, inhibiting NF-κB appears to be just about as effective as inhibiting the Ca^{2+}/calmodulin-dependent phosphatase calcineurin with cyclosporine A or FK506. The different mechanisms of action of the two classes of drugs open new possibilities for using NF-κB inhibitors as immunodepressants as an alternative to or more likely in association with cyclosporine or FK506 so as to decrease the dosages of these toxic compounds.

It is interesting that salicylate and ASA are powerful inhibitors of lymphocyte proliferation at concentrations that are readily attained *in vivo*. It is likely that a significant component of the anti-inflammatory action of salicylates may be ascribed to the effect described in this paper. Among the agents studied in this research, a very effective inhibitor of lymphocyte proliferation is parthenolide. This sesquiterpene lactone, together with a similar compound, helenalin (from *Arnica montana*) which is also an NF-κB inhibitor [22], is widely used in traditional medicine. Parthenolide was reported to be an inhibitor of IkB phosphorylation, while helenalin was shown to alkylate the p65 subunit of NF-κB, thus inhibiting the binding to DNA of a regularly translocated NF-κB dimer. In [22], it was suggested that parthenolide may in fact work similarly to helenalin.

The highly effective action of parthenolide (and likely of other sesquiterpene lactones) warrants a further study of such compounds for widespread medical utilization as anti-inflammatory agents and immunomodulators/immunodepressants, and for possible application in the treatment of some forms of cancer.

Acknowledgments

This work was supported by a CNR strategic project contribution on apoptosis (No. 04960.ST74, 04899.ST74) and MURST grants 40 and 60%.

[1] Cavallini L, Francesconi MA, Zoccarato F, Alexandre A. Manuscript in preparation.

References

[1] Vane JB. Towards a better aspirin. Nature 1994;367:215–6.
[2] Cronstein BN, van de Stouwe M, Druska L, Weissmann G. Nonsteroidal anti-inflammatory agents inhibit stimulated neutrophil adhesion to endothelium: adenosine-dependent and -independent mechanisms. Inflammation 1994;18:323–35.
[3] Abramson SB, Leszczynska-Piziak J, Clancy RM, Philips MR, Weissmann G. Inhibition of neutrophil function by aspirin-like drugs (NSAIDS): requirement for assembly of heterotrimeric G proteins in bilayer phospholipid. Biochem Pharmacol 1994;47:563–72.
[4] Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 1994;265:956–9.
[5] Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998; 16:225–60.
[6] Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents ASA and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 1998;396:77–80.
[7] Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. J Immunol 1996;156:3961–9.
[8] Mazzeo D, Panina-Bordignon P, Recalde H, Sinigaglia F, D'Ambrosio D. Decreased IL-12 production and Th1 cell development by acetyl salicylic acid-mediated inhibition of NF-kappaB. Eur J Immunol 1998;28:3205–13.
[9] Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad Sci USA 1997;94:2869–73.
[10] Pillinger MH, Capodici C, Rosenthal P, Kheterpal N, Hanft S, Philips MR, Weissmann G. Modes of action of aspirin-like drugs: salicylates inhibit erk activation and integrin-dependent neutrophil adhesion. Proc Natl Acad Sci USA 1998;95:14540–5.
[11] Cronstein BN, Montesinos MC, Weissmann G. Salicylate and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of the inhibition of prostaglandin synthesis and p105 of NFkB. Proc Natl Acad Sci USA 1993;96:6377–81.
[12] Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;21:77–99.
[13] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989;119:203–10.
[14] Chaturvedi MM, LaPushin R, Aggarwal BB. Tumor necrosis factor and lymphotoxin. Qualitative and quantitative differences in the mediation of early and late cellular response. J Biol Chem 1994;269: 14575–83.
[15] Tanaka K, Tanaka H, Kanemoto Y, Tsuboi I. The effect of nonsteroidal anti-inflammatory drugs on immune functions of human peripheral blood mononuclear cells. Immunopharmacology 1998;40: 209–17.
[16] Nelson BH, Willeford DM. Biology of the interleukin-2 receptor. Adv Immunol 1998;70:1–81.
[17] Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, Schulze-Osthoff K, Droge W, Schmitz ML. Sesquiterpene lactones specifically inhibit activation of NF-κB by preventing the degradation of IkB-β. J Biol Chem 1998;273:1288–97.
[18] Hwang D, Fisher NH, Jang BC, Tak H, Kim JK, Lee W. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with

the inhibition of MAP kinases. Biochem Biophys Res Commun 1996;226:810–8.
[19] Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IkB-α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. J Biol Chem 1997;272:21096–103.
[20] Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998;101: 1163–74.
[21] Palombella VJ, Conner EM, Fuseler JW, Destree A, Davis JM, Laroux FS, Wolf RE, Huang J, Brand S, Elliott PJ, Lazarus D, McCormack T, Parent L, Stein R, Adams J, Grisham MB. Role of the proteasome and NF-kappaB in streptococcal cell wall-induced polyarthritis. Proc Natl Acad Sci USA 1998;95:15671–6.
[22] Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. J Biol Chem 1998;273:33508–16.
[23] Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995;270:14255–8.
[24] Kolenko V, Bloom T, Rayman P, Bukowski R, Hsi E, Finke J. Inhibition of NF-κB activity in human T lymphocytes induces caspase-dependent apoptosis without detectable activation of caspase-1 and -3. J Immunol 1999;163:590–8.
[25] Doi TS, Takahashi T, Taguchi O, Azuma T, Obata Y. NF-kappa B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. J Exp Med 1997;185:953–61.
[26] Kontgen F, Grumont RJ, Strasser A, Metcalf D, Li R, Tarlinton D, Gerondakis S. Mice lacking the c-*rel* proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev 1995;9:1965–77.
[27] Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell 1995;80:321–30.
[28] Snapper CM, Zelazowski P, Rosas FR, Kehry MR, Tian M, Baltimore D, Sha WC. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. J Immunol 1996;156:183–91.
[29] Franzoso G, Carlson L, Xing L, Poljak L, Shore EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U. Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 1997;11: 3482–96.
[30] Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 1997; 3:1285–9.
[31] Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ, Baldwin AS. Oncogenic Ha-Ras-induced signaling activates NF-kappaB transcriptional activity, which is required for cellular transformation. J Biol Chem 1997;272:24113–6.
[32] Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 1997;100:2961–9.
[33] Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 1999;19:5785–99.
[34] Crout JE, Hepburn B, Ritts RE. Suppression of lymphocyte transformation after aspirin ingestion. N Engl J Med 1975;292:221–3.
[35] Conti P, Cifone MG, Alesse E, Ianni G, Angeletti PU. Effect of salicylates on lymphocyte blastogenesis *in vitro*: association with other non-steroid anti-inflammatory drugs. Clin Rheumatol 1983;2: 127–32.