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Review

Anti-inflammatory effects of macrolide antibiotics

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Abstract

Macrolides are widely used as antibacterial drugs. Clinical and experimental data, however, indicate that they also modulate inflammatory responses, both contributing to the treatment of infective diseases and opening new opportunities for the therapy of other inflammatory conditions. Considerable evidence, mainly from in vitro studies, suggests that leukocytes and neutrophils in particular, are important targets for modulatory effects of macrolides on host defense responses. This underlies the use of the 14-membered macrolide erythromycin for the therapy of diffuse panbronchiolitis. A variety of other inflammatory mediators and processes are also modulated by macrolides, suggesting that the therapeutic indications for these drugs may be extended significantly in future. © 2001 Published by Elsevier Science B.V.

Keywords: Macrolide; Neutrophil; Leukocyte

1. Introduction

Macrolides are a well-established family of antibiotics isolated from streptomycetes. Their main characteristic is a macrocyclic lactone ring with one or two sugar moieties. They experienced a renaissance during the 1980s and 1990s, in which the commercial introduction of several semi-synthetic derivatives significantly expanded their therapeutic importance and utility. Among the most important characteristics of the macrolide antibiotics are a moderately broad spectrum of antibacterial activity, activity by oral administration and a relatively high therapeutic index. The first macrolide introduced into clinical practice was the 14-membered ring compound—erythromycin A. It was isolated from cultures of *Streptomyces erythraea* in 1952 (Washington et al., 1985; for review, see Mazzei et al., 1993; Williams and Sefton, 1993). The 15-membered macrolide antibiotic, azithromycin (a representative of the so-called azalides), has now become one of the most widely prescribed of all antibiotics. Macrolides like tylosine are widely used in veterinary medicine. The most recently synthesized macrolides are in late phase clinical development or approved for clinical use (e.g., the ketolides, ABT-773 and telithromycin). They are called ketolides because instead of sugar (L-cladinosyl moiety) at position 3 of the lactone ring these antibiotics have a keto-group (Wu, 2000). The principle advantage of this series of compounds is their activity against macrolide-resistant streptococci (Resek et al., 2000). For a recent review on anti-infective aspects of macrolides and particularly ketolides see Zhanel et al. (2001).

Structures of the most commonly used antibiotics are shown in Fig. 1. In addition to the chemical (semi-synthetic) approach to the development of new macrolide antibiotics, an alternative approach employing molecular biology techniques has also been used. Biotechnology permits the modification and interchange of specific parts of various biosynthetic gene clusters, like polyketide synthase, to produce new macrolide structures (Katz and Donadio, 1993). This process of artificial natural product formation is called recombinant biosynthesis (Tsoi et al., 1995; Katz and McDaniel, 1999).

The mechanism of macrolide antibiotic action is based on inhibition of bacterial protein synthesis, by interacting with 23S rRNA in the central loop of the peptidyltransferase center as well as with specific ribosomal proteins found in the same region of the ribosome. Macrolides are best known as anti-infectives but also exert other important pharmacological effects such as immunosuppression and immunomodulation. For example, FK506 (tacrolimus) is a highly effective immunosuppressive drug used in organ

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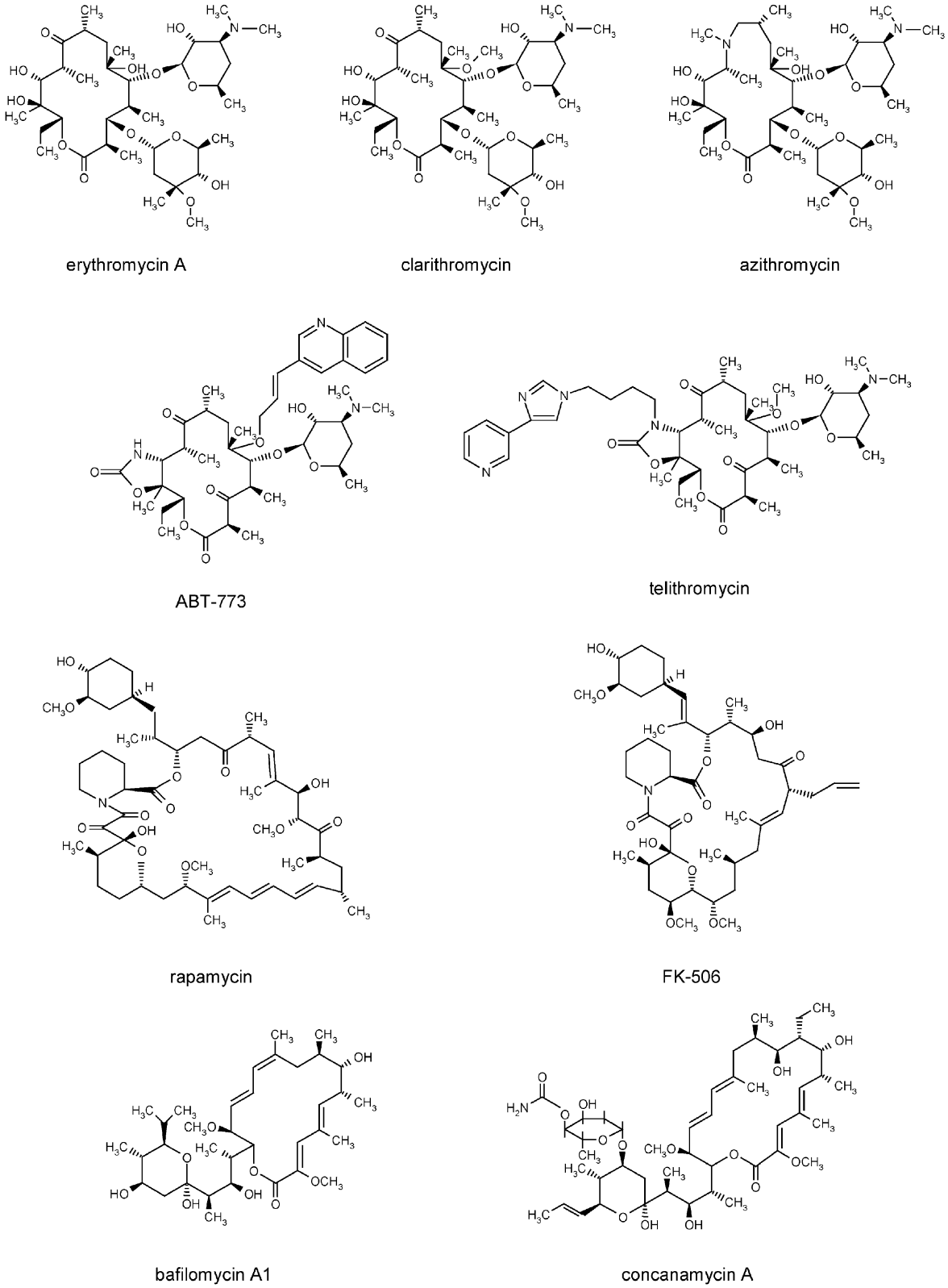

Fig. 1. Chemical structures of some commonly used macrolides.

transplantation and is one of the best immunosuppressive drugs available. This 23-membered macrolide lactone binds to FKBP 12 and modulates the calcineurin pathway. At subnanomolar concentrations it was shown to inhibit the proliferation of T cells stimulated by specific antigens (for review, see Dumont, 2000). Rapamycin (31-membered

ring macrolide), although belonging to the same chemical class causes immunosuppression by a different mechanism. It forms an immunophilin complex that does not bind calcineurin and is therefore devoid of the calcineurin-related effects seen with FK506. Rapamycin was recently shown to inhibit apoptosis in human HL-60 cells (Johnson and Lawen, 1999).

Bafilomycin (16-membered macrolide) and concanamycin (18-membered macrolide) are the most potent specific inhibitors of vacuolar type H^+-ATPase (for reviews, see Droese and Altendorf, 1997; Keeling et al., 1997; Gagliardi et al., 1999). Inhibition of the vacuolar H^+-ATPase indirectly causes apoptotic cell death (Nishihara et al., 1995). Moreover, an intracellular apoptosis-inducing factor with a molecular mass of 33 kDa seems to be induced by concanamycin A treatment of (hybridoma) cells (Hashimoto et al., 2001). Oligomycin and apoptolidin are potent inhibitors of ATP-synthetase (F_0F_1-ATPase) and are among the most cytotoxic drugs for malignant cells (Salomon et al., 2000, 2001). There are also other scattered reports on macrolide actions which could not be classified as anti-bacterial, e.g. gastrointestinal motor stimulating activity (Omura et al., 1987), anti-cancer (Hamada et al., 2000) and anti-angiogenic effects (Yatsunami et al., 1999a,b). Among the most peculiar ones are an anorexigenic reaction associated with weight loss (proposed for obesity treatment) and reduction of serum triglyceride levels with simultaneous increase in serum high-density lipoprotein (HDL) levels (Klein, 1996, 2001).

In many cases, biological targets of macrolides remain unknown and still not sufficiently exploited. The techniques of proteomics and genomics could help to systematically explore targets with which various macrolides are capable of interacting (genes and/or proteins).

The main topic of this review is macrolides that influence targets of relevance for inflammatory processes. This is not a new topic and several excellent reviews have been written about these effects (Gemmell, 1993; Wales and Woodhead, 1999; Labro, 1998a,b; Rubin and Tamaoki, 2000; Labro, 2000; Labro and Abdelghaffar, 2001). The purpose of this review is to emphasize the most interesting newer findings, especially those that reveal the neutrophil as an important cellular target of macrolide action.

2. Inflammation

Most inflammatory diseases whether infective or non-infective in origin, are characterized by abnormal accumulation of inflammatory cells (including monocytes, macrophages, granulocytes, plasma cells, lymphocytes and platelets) that along with tissue endothelial cells and fibroblasts, release a complex array of lipid mediators, growth factors, cytokines and destructive enzymes that cause local tissue damage. Tissue damage in a group of neutrophil-dominated inflammatory diseases (bacterial infections, active phases of rheumatoid arthritis, chronic obstructive pulmonary disease, glomerulonephritis, cystic fibrosis, bronchitis, bronchiecstasis, emphysema, adult respiratory distress syndrome) is mainly caused by activation of neutrophils followed by their release of proteinases and increased production of oxygen species (for an excellent review on functions and pathophysiology of neutrophils, see Witko-Sarsat et al., 2000). Inflammation is the net result of host defense and tissue damaging pathways, which is well illustrated by the dual role of neutrophils as anti-infectious and pro-inflammatory cells.

Mature neutrophils constitute unique weapons to fight infection and play a major role in the host surveillance system against foreign invaders and in inflammation. Neutrophils are vital for host defense, particularly against bacteria and compromise of this defense is hazardous. This is clearly demonstrated by severe neutropenia, as after cancer radiation therapy or a genetic deficiency in the capacity of neutrophils to migrate or in the case of defective production of oxidative species (e.g., chronic granulomatous disease). Neutrophils have evolved an arsenal of anti-microbial weapons including the phagocytically triggered “respiratory burst” which generates toxic reactive oxygen species (superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide, hypohalous acid, *N*-chloramines) and secretory granules which contain potent degradative enzymes and bactericidal proteins like myeloperoxidase, elastase, cathepsin, collagenase, neuraminidase, heparanase, defensins, cationic antimicrobial proteins. Unfortunately, the defensive weapons of the neutrophil can result in “friendly fire” causing damage to normal tissues as well. Consequently, these cells are involved in the pathogenesis of a wide range of the inflammatory diseases.

3. Cellular pharmacokinetics of macrolides

Additional interest in the therapeutic use of macrolide antibiotics has been based on the demonstration of their ability to concentrate within phagocytes. Macrolide antibiotics show unique and favorable cellular pharmacokinetic properties. The concentrations of azithromycin and clarithromycin, for instance, in the epithelial lung fluid normally tend to be at least 10-fold greater than simultaneously measured concentrations in the plasma or serum. These high concentrations often exceed the minimal inhibitory concentration killing 90% of organisms (MIC_{90}) for common respiratory pathogens and may at least partially explain the extraordinary effectiveness of azithromycin and clarithromycin in the treatment of lower respiratory tract infections (Bearden and Rodvold, 1999). Macrolides normally accumulate in inflammatory cells at concentrations up to several hundred-fold higher than those in extracellular fluid (Gladue et al., 1989; Wildfeuer et al.,

1989, 1996; Mtairag et al., 1994, 1995; Vazifeh et al., 1997; Fieta et al., 1997) enabling phagocytic cells to deliver concentrated active drug to sites of infection. This allows, in some therapeutic cases, a daily oral dosing regime (Amsden and Gray, 2001; Schoenwald et al., 1999). The ability of macrolides to penetrate and accumulate in many eukaryotic cell types has important therapeutic implications, particularly with regard to obligatory or facultative intracellular organisms or other microbes that may survive phagocytosis. Based on this property and the fact that cells like granulocytes and macrophages are present at the site of infection in very high numbers, antibiotics can be delivered in a targeted manner to the site of bacterial infection. The presence of macrolides in such high concentrations within cells and their relatively slow efflux from the leukocytes make the possibility of altering some intrinsic function of these cells highly likely. Nevertheless, the mechanism of intracellular accumulation is not clear. Factors such as large interindividual differences in macrolide uptake, temperature- and viability-dependent uptake, and its kinetic characteristics favor the concept of an active (protein-mediated) process (Vazifeh et al., 1997). Actually, the fact itself that macrolide antibiotics are concentrated up to several hundred-fold within cells strongly implies some kind of active process—either with direct or indirect ATP expenditure. Extracellular Ca^{2+} seems to be very important for the uptake, since in its absence, uptake was shown to be greatly impaired (Mtairag et al., 1995; Fieta et al., 1997). In contrast to this finding, it seems that macrolide efflux is not dependent on extracellular Ca^{2+} concentration (Vazifeh et al., 1997). Indirect evidence has been provided for the importance of protein kinase A activity in regulation of macrolide transport process. Protein kinase A seems to play a stimulatory role in macrolide uptake by phagocytic cells (Hand and Hand, 1995). The activation of the transport(er) remains unclear but it does not seem to play an important role in phagocytic precursors (Vazifeh et al., 2000). In addition to this observation, there are some indications that transporters for macrolides might be different in polymorphonuclear leukocytes and macrophages (Vazifeh and Labro, 1999).

Macrolides seem to be concentrated mainly in the cytoplasm and azurophyllic granules of neutrophils, the latter favoring antibiotic delivery to bacteria phagocytosed by leukocytes (Miossec-Bartoli et al., 1999; Vazifeh et al., 1998). The molecular mechanism of macrolide concentration in granules remains unclear at present. For some effects, especially those attributed to actions on the nuclear factor for the expression of immunoglobulin κ light-chain in B lymphocytes (NF-κB) pathway, the nuclear presence of antibiotics could be very important but very little data, in this respect, are available. Limited data on autoradiography and electron microscopy of radioactively labelled azithromycin taken up into leukocytes and monocytes suggest, however, that some macrolide accumulation within nuclei may occur (Wildfeuer et al., 1993).

Much less information is available on intracellular macrolide distribution into cell types other than granulocytes. Martin et al. (1985) reported on accumulation of erythromycin in human cells in culture. Primary cultured cells from bronchus, foreskin, kidney, liver, lung and skin accumulated erythromycin to a similar extent with intracellular/extracellular ratios of antibiotic concentrations ranging from 4.2 to 12.0. A comparison of azithromycin uptake by phagocytic (J774 macrophages) and non-phagocytic cells [rat embryo fibroblasts and normal rat kidney fibroblasts (NRK cells)] has been performed. All cell lines accumulated azithromycin exceptionally well and most of the antibiotic (50–70%) was found to be associated with the lysosomal compartment while the rest was found in the cytosol. Interestingly, as a consequence of drug accumulation, lysosomal buoyant density was decreased (Carlier et al., 1994). Carlier et al. (1987) also demonstrated that about one third of intracellular roxithromycin was associated with azurophylic granules. It appears that fibroblasts serve as a reservoir of drug in tissue and possibly transfer antibiotic to phagocytic cells (McDonald and Pruul, 1991; Gladue et al., 1989). Epithelial-like cell lines also have the capability to accumulate macrolides (azithromycin) but much less so than neutrophils and macrophages (Pascual et al., 1997). The importance of leukocytes as targets for macrolide accumulation is emphasised by the study of Bermudez et al. (1991) who showed that cytokines in vitro stimulate accumulation of macrolides into macrophages, suggesting that at the site of inflammation (infection), cells may accumulate even more macrolide antibiotics than under physiological conditions.

Kinetics of efflux are very different among macrolides, e.g. very fast with erythromycin and clarithromycin, but very slow with azithromycin (Gladue et al., 1989; Fieta et al., 1997), so that cellular accumulation differs between them. Since intracellular concentrations of macrolides in vivo (during therapy), at least in some cellular compartments, may reach high mM values, a number of possible interactions are conceivable. Even reactions with a low affinity constant become possible.

The accumulation of macrolides, particularly of azithromycin, in inflammatory and other cells on the one hand offers the possibility of prolonged activity against invading bacteria. On the other hand, these pharmacokinetic properties suggest that in vivo macrolides may exert more prolonged anti-inflammatory effects than can be observed in short-term cell cultures in vitro.

4. Anti-inflammatory and other in-vitro effects of macrolides

A considerable body of evidence on in vitro effects of macrolides is present in the scientific literature. Nevertheless, these reports are sometimes contradictory. At least partially, the contradictions can be resolved by taking into

account often very different experimental conditions (for review, see Labro and El Benna, 1993). Macrolide antibiotics modulate the functions of inflammatory cells, such as polymorphonuclear leukocytes, lymphocytes and macrophages (Anderson, 1989; Roche et al., 1986). In addition, they directly affect airway secretory cells and epithelial cells (Goswami et al., 1990; Tamaoki et al., 1992).

Among the most important findings on macrolide interactions with phagocytes are their inhibitory effects on oxidant production by stimulated cells (Labro et al., 1989; Umeki, 1993; Wenisch et al., 1996) and their modulation of pro-inflammatory and anti-inflammatory cytokine release by these cells (Labro et al., 1989; Khan et al., 1999; Morikawa et al., 1996; Sugiyama et al., 1999).

4.1. Degranulation

Neutrophil-derived microbicidal molecules are packed into granules that are released upon cell activation. Degranulation of granules (vesicles) into phagolysosomes or into the extracellular space is a key event in microbicidal activity. This is illustrated by the Chediak–Higashi syndrome, which is a rare autosomal recessive disorder associated with abnormal azurophilic granules leading to increased susceptibility to infection. Macrolides directly stimulate exocytosis (degranulation) by human neutrophils (Abdelghaffar et al., 1994, 1996, 1997; Vazifeh et al., 1998). Xu et al. (1996) showed that macrolide antibiotics, with the exception of roxithromycin, could stimulate macrophage chemotaxis, phagocytosis and cytocidal activity against *Candida albicans*. In this way, macrolides facilitate their own direct anti-bacterial activity by stimulating host defense reactions against bacteria and other microorganisms. It should, however, be noted that other types of antibiotics are also able to enhance neutrophil bacterial killing and/or phagocytosis, so that the macrolides are not unique in this respect (Sultan et al., 2000; Novelli et al., 2000; Pallister and Lewis, 2000).

4.2. Oxidative burst

Phagocytic cells contain an NADPH-dependent oxidase that transfers electrons from NADPH to O_2 to form O_2^-. Mature neutrophils are fully equipped with an NADPH oxidase and an arsenal of harmful agents stored in granules, ready to be used to destroy phagocytosed microorganisms. The effects of macrolides on the oxidative burst are not completely homogeneous. For instance, the oxidative burst as well as Ca^{2+} influx into neutrophils was inhibited by erythromycin but not by clarithromycin (Mitsuyama et al., 1997). Previous structure–activity studies have shown that 14- and 15-membered macrolides generally impair the oxidative burst of neutrophils, in a time- and dose-dependent manner (Abdelghaffar et al., 1997; Anderson, 1989; Levert et al., 1998, 1999). Erythromycin inhibited the oxidative burst and interleukin-8 secretion (Lin et al., 2000) and azithromycin inhibited phagocytosis of *Escherichia coli* as well as the oxidative burst (Wenisch et al., 1996), both in stimulated granulocytes. Similarly azithromycin and clarithromycin, both of which were shown to be clinically effective in treatment of diffuse panbronchiolitis, inhibited oxygen generation and chemotaxis of neutrophils, while clinically ineffective roxithromycin did not (Sugihura, 1997). Clindamycin, erythromycin, and roxithromycin, on the other hand, were *all* reported to cause dose-dependent inhibition of superoxide production in both unstimulated and stimulated neutrophils (Hand et al., 1990; Ras et al., 1992). Contrasting findings were reported by Bonnet and Van der Auwera (1992) and by Pascual et al. (1995) who failed to detect a modulatory effect of azithromycin on the oxidative burst. The reason for these differences is unclear, but may relate to the different stimuli used and to the activation or primed status of the cells. The various stimuli to the neutrophil oxidative burst lead to activation of different intracellular signal transduction pathways that may be differentially affected by macrolides.

As measured by chemiluminescence assay, 16-membered rokitamycin was also capable of inhibiting the oxidative burst of neutrophils. Washing the cells after incubation with the antibiotic restored the chemiluminescence response (Braga et al., 1997). Dirithromycin also inhibited the oxidative burst in stimulated polymorphonuclear leukocytes, in a time- and intracellular concentration-dependent manner (Hand and Hand, 1993; Levert et al., 1999).

Oxygen species produced by NADPH oxidase and other enzymes downstream of the cascade are damaging, not only to bacteria but also to the host tissue, if generated in excess and for a prolonged period. Attenuation of oxidative burst capability by macrolides, therefore, can be considered to be beneficial in the control of (chronic) inflammatory processes.

4.3. Cytokines / chemokines

In general, macrolides inhibit synthesis and/or secretion of pro-inflammatory cytokines while exerting variable effects on the release of anti-inflammatory cytokines (Table 1). These data have been obtained mainly on cells other than neutrophils.

Interleukin-6 is generally regarded as an inflammation-modulating or even anti-inflammatory cytokine. In human monocytes, both stimulatory and inhibitory effects of macrolides have been observed on interleukin-6. Khan et al. (1999) showed that clarithromycin and azithromycin are capable of inhibiting cytokine production by human monocytes (interleukin-1α, interleukin-6, tumor necrosis factor-α or TNF-α), while Bailly et al. (1991, 1993) claimed an increased production of interleukin-6 in the same cells by erythromycin and spiromycin. Transient stimulation rather than inhibition of interleukin-6 by clarithromycin was also

Table 1
Effects of macrolides on cytokine release in vitro

Cytokine	Cell type	Macrolide	Change	Refs.
Interleukin-6	human monocytes	azithromycin clarithromycin	inhibition	Khan et al. (1999)
		erythromycin spiromycin	stimulation	Bailly et al. (1991, 1993)
	adenocarcinoma	clarithromycin	stimulation	Sassa et al. (1999b)
	bronchial epithelial	erythromycin clarithromycin	inhibition	Takizawa et al. (1995)
	synovial fibroblast	clarithromycin	inhibition	Matsuoka et al. (1996)
Interleukin-1	human monocytes/ macrophages	azithromycin	inhibition	Khan et al. (1999), Morikawa et al. (1996), Takeshita et al. (1989)
		clarithromycin erythromycin		
	human nasal epithelial	clarithromycin	inhibition	Miyanohara et al. (2000)
	synovial fibroblast	clarithromycin	inhibition	Matsuoka et al. (1996)
TNFα	human monocytes (+lipopolysaccharide)	azithromycin	inhibition	Khan et al. (1999), Morikawa et al. (1996)
		clarithromycin erythromycin	inhibition	Iino et al. (1992), Moutard et al. (1999)
		spiramycin dirithromycin		
	adenocarcinoma	clarithromycin	inhibition	Sassa et al. (1999b)
TGFα	adenocarcinoma	clarithromycin	inhibition	Sassa et al. (1999b)
GM-CSF	human monocytes	clarithromycin	inhibition	Morikawa et al. (1996)
	synovial fibroblast	clarithromycin	inhibition	Matsuoka et al. (1996)
	human lung fibroblasts (+TNFα)	erythromycin	inhibition	Sato et al. (2001)
Interleukin-8	nasal fibroblasts (+lipopolysaccharide)	roxithromycin	no effect (inhibition of proliferation)	Nonaka et al. (1998)
	human nasal epithelial	roxithromycin erythromycin clarithromycin josamycin	inhibition	Suzuki et al. (1997b,c)
	human eosinophils	erythromycin	inhibition	Kohyama et al. (1999)
	synovial fibroblast	clarithromycin	inhibition	Matsuoka et al. (1996)
RANTES, eotaxin	human lung fibroblasts (+TNFα)	erythromycin	inhibition	Sato et al. (2001)

reported in cultured adenocarcinoma cells (Sassa et al., 1999a). At the level of RNA, it was shown that erythromycin and clarithromycin suppressed mRNA levels as well as the secretion of interleukin-6 from bronchial epithelial cells (Takizawa et al., 1995). These differences in effects of macrolides on interleukin-6 may reflect differences in cells and stimuli, but also in the changes induced early during cell stimulation.

In any case, effects of macrolides on pro-inflammatory cytokines and chemokines are much more clear-cut: they are almost always inhibitory in vitro (Table 1). Clarithromycin seems to be more potent than erythromycin in inhibiting macrophage synthesis of interleukin-1 (Takeshita et al., 1989) and similar inhibitory effects of spiramycin and dirithromycin were suggested to be due to inhibition of protein kinase C (Moutard et al., 1999). An ex vivo study on human volunteers also revealed an inhibitory effect of erythromycin on chemokine production by leukocytes. After 30 min of intravenous erythromycin infusion (1000 mg), heat-killed *Streptococcus pneumoniae* were used for challenging whole blood and cultures of isolated polymorphonuclear leukocytes. Significant reductions in interleukin-8, epithelial cell-derived neutrophil attractant-78 (ENA-78) and macrophage inflammatory protein-1 (MIP-1) were observed in supernatants after 16-h incubation (Schultz et al., 1999; 2000). An experimental erythromycin derivative, 2001, was also shown to inhibit production of interleukin-8 in the only recent study performed on cytokine production by neutrophils (Yoshimine et al., 1998).

Using TNF-α and interferon-γ-stimulated human lung fibroblast cell line as a model, Sato et al. (2001) showed that erythromycin is capable of inhibiting secretion of eosinophil chemotactic peptides, like RANTES (regulated upon activation, normal T expressed and presumably secreted), eotaxin and granulocyte/monocyte colony-stimu-

lating factor (GM-CSF). Overall eosinophil chemotactic activity by fibroblasts was reduced. Additionally, it was reported that 14-member macrolides inhibit interleukin-8 release by human eosinophils (Kohyama et al., 1999) and cultured human nasal epithelial cells (Suzuki et al., 1997b,c). These results could provide an explanation for the mechanism of beneficial macrolide treatment in allergic airway disorders.

Inhibition of pro-inflammatory cytokines and chemokine, particularly by 14-membered macrolides, could help to prevent phagocytic cells entering the site of inflammation and thereby attenuate undesired inflammatory processes

4.4. Apoptosis

Therapeutic induction of apoptosis (programmed cell death) as a means to resolve chronic inflammation has gained increasing interest (Ward et al., 1999a,b; Chilvers et al., 1998) and macrolides could be blueprints for this approach.

Neutrophils are normally extremely short-lived cells, with a circulating life-time of only 6–7 h (Athens et al., 1961a,b). This means that normal individuals make (and destroy) about 50 billion neutrophils per day, and many more in inflammatory states (Savill, 1992). In other words, at least 50 g of neutrophils are destroyed each day! Many of these neutrophils undergo apoptosis before leaving the bone marrow. Neutrophil apoptosis is accompanied by attenuation of many functional aspects of these cells, including oxidative burst, degranulation, etc., and therefore, presents a very interesting therapeutic target for the development of new anti-inflammatory drugs. In contrast to necrotic neutrophils, apoptotic neutrophils are ingested by macrophages (Haslett, 1999). Granulocyte-induced tissue injury and chronic inflammation may reflect not only excessive granulocyte recruitment but also inhibition of normal apoptosis-based clearance mechanisms.

Several reports have described the pro-apoptotic effects of erythromycin. It was reported to accelerate apoptosis of neutrophils through a mechanism that is at least partially cAMP-dependent (Aoshiba et al., 1995). This action of erythromycin has been confirmed in isolated human neutrophils and extended also to roxithromycin (Inamura et al., 2000). Neutrophils treated with other antibiotics, including the 16-membered macrolide, josamycin, had no effect on human neutrophils. Adachi et al. (1996) extended the pro-apoptotic effect of erythromycin to isolated guinea pig eosinophils stimulated by interleukin-5. A 17-membered tylosine derivative was also reported to cause apoptosis in several different cell lines (Grdiša et al., 1998).

Azithromycin was shown to stimulate apoptosis of neutrophils but, in the presence of *S. pneumoniae*, this effect was abolished, possibly due to bacterial suppression of the process. Furthermore, in this study, azithromycin did not affect interleukin-8 production or the oxidative burst of polymorphonuclear leukocytes (Koch et al., 2000). Both erythromycin and azithromycin showed pro-apoptotic potential in a whole blood model, as determined by flow cytometry (Healy et al., 2000). Tilmicosin, which reduces pulmonary inflammation in calves, was also shown to significantly stimulate apoptosis of peripheral neutrophils when isolated cells were incubated with this macrolide for 2 h (Chin et al., 2000). The tilmicosin-induced apoptosis, in contrast to that described above for azithromycin, occurred regardless of the presence or absence of bacteria (*Pasteurella haemolytica*). In addition, tilmicosin promoted phagocytosis of neutrophils by macrophages. It would appear that either differences in pro-apoptotic effects exist between macrolides or that bacterial actions on this process may differ.

4.5. Adhesion

Leukocyte adhesion is a hallmark of the inflammatory process. The recruitment of these cells to a site of inflammation occurs through a remarkable sequence of events necessary to efficiently and specifically arrest leukocytes on the vascular endothelium and direct their transmigration. There are four phases in the adhesion process—margination, capture, rolling and adhesion. Cell adhesion molecules of the selectin and integrin families are the most common mediators of these events. It is believed that new therapeutic possibilities in inflammatory diseases could be based on (selective) inhibition of adhesion molecules (expression).

Erythromycin was reported to be capable of down-regulating expression of integrins CD11b/CD18 by endotoxin lipopolysaccharide-stimulated neutrophils and therefore could act as an «anti-adhesive drug». In the same study, inhibitory effects of erythromycin were seen on the oxidative burst and interleukin-8 secretion from stimulated granuloctytes (Lin et al., 2000). Erythromycin also inhibited secretion of interleukin-6, interleukin-8 and the soluble integrin, soluble intercellular adhesion molecule-1 (sICAM-1), in cell cultures treated with endotoxin lipopolysaccharide (Khair et al., 1995).

Another study demonstrated significant reduction of Mac-1 on the surface of whole blood cells after 1–3 h treatment with erythromycin or midecamycin, while roxithromycin was ineffective (Okubo, 1997). In contrast to these findings roxithromycin was reported to be effective in reduction of Mac-1 expression in neutrophils from patients with chronic lower respiratory tract disease, including diffuse panbronchiolitis (Kusano et al., 1995). The difference between the results of the two reports might be explained by the fact that Kusano et al. measured expression in patients receiving roxithromycin for a much longer period of time. Roxithromycin caused no changes in lymphocyte function-associated antigen-1 (LFA-1) expression in those patients, which may be a beneficial effect since clearance of bacteria will be maintained. The in vitro

potential of roxithromycin to inhibit neutrophil adhesion to bronchial epithelial cells has also been described (Kawasaki et al., 1998).

Clarithromycin exerts several anti-inflammatory effects on co-stimulatory molecule expression, cytokine production and antigen-specific T cell proliferation (Matsuoka et al., 1996). In synovial (fibroblast-like) cells, clarithromycin markedly inhibited expression of several adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen-3 (LFA-3) and vascular cell adhesion molecule-1 (VCAM-1). Simultaneously, clarithromycin decreased expression of interleukin-1β, interleukin-6, interleukin-8, granulocyte colony stimulating factor (G-CSF) and GM-CSF. Similar results were reported for human bronchial epithelial cells. Clearly, inhibition of adhesion molecule expression makes a notable contribution to the anti-inflammatory effects of macrolides.

4.6. Chemotaxis

Following adherence to the vascular endothelium, leukocytes move between the endothelial cell junctions and enter the tissue along the concentration gradient of mediators that stimulate their directed movement. A wide variety of agents are able to stimulate the non-directed movement (chemokinesis) of inflammatory cells. However, only a limited number of inflammatory compounds (e.g., leukotriene B_4, complement factor 5a, interleukin-8, soluble Fas) are able to stimulate the directed movement (chemotaxis) of neutrophilic leukocytes into the site of inflammation, where they are able to kill microorganisms or initiate tissue damage. Inhibitory effects of macrolides on leukocyte chemotaxis were documented some time ago in vitro (Esterly et al., 1978) as well as in vivo (Nelson et al., 1987). Chemotaxis of neutrophils and their adhesion to endothelial cells was also significantly reduced when neutrophils were treated with macrolide-conditioned medium from endotoxin-stimulated epithelial cells (Tamaoki et al., 1994). Erythromycin, josamycin, miokamycin, roxithromycin and rokitamycin decrease chemotaxis of neutrophils in vitro as well as ex vivo using cells from volunteers that had been treated with these antibiotics (Torre et al., 1991). However, another study performed with neutrophils isolated from patients with cystic fibrosis found no evidence for a significant effect of erythromycin on interleukin-8 induced neutrophil chemotaxis in vitro (Brennan et al., 2001). Presumably, the inhibition of chemotaxis by macrolides is dependent upon processes that are deficient in cystic fibrosis.

4.7. Nitric oxide

Nitric oxide (NO) shows a wide variety of effects in healthy and diseased tissues. It exerts bactericidal activity through the formation of the intermediate NOO^-, but also causes vasodilation, and modulates the activities and release of other inflammatory mediators (Nathan and Xie, 1994; Nathan, 1992). The NO generated by constitutive endothelial NO synthase (eNOS) is thought to be involved in housekeeping physiological processes. The increased NO production following stimulation by cytokines, among other stimuli, i.e. inducible NOS (iNOS), particularly in inflammatory cells, is thought to be important in host defense and inflammatory mechanisms. Bacterial infection causes up-regulation of NO production, inducing NOS activity in neutrophils and macrophages (Wheeler et al., 1997; Nathan and Hibbs, 1991). An anti-adhesive effect of NO has been proposed, mainly through inhibition of leukocyte rolling and adhesion to endothelial cells (for a review, see Hickey, 2001).

Erythromycin, but not clarithromycin, was reported to ameliorate neutrophil-induced endothelial cell damage, affecting neutrophil functions as well as release of NO from endothelial cells. Erythromycin apparently stimulates eNOS-mediated NO production by a protein kinase A-dependent mechanism (Mitsuyama et al., 1997). An additional report from the same group indicated that erythromycin was capable of enhancing NOS expression, an effect that might contribute to the enhancement of NO release from endothelial cells (Mitsuyama et al., 1998). Contrary to these findings, iNOS expression and NO production by endotoxin lipopolysaccharide and interferon-γ stimulated alveolar macrophages were shown to be reduced by clarithromycin treatment in vitro (Tamaoki et al., 1998). Similarly, erythromycin, clarithromycin and josamycin, in a concentration-dependent manner, inhibited induction of iNOS mRNA as well as NO production by alveolar macrophages (Kohri et al., 2000), suggesting that erythromycin at least may exert differential effects on eNOS and iNOS.

Erythromycin, roxithromycin and josamycin were reported to be active in suppressing iNOS in pulmonary alveolar macrophages together with inhibition of lL-1β and TNF-α (Tamaoki et al., 1999). A very recent report indicates that erythromycin can cause release of NO at synaptic neurons, indicating that neuronal NOS is also affected by this macrolide (Sarna et al., 2000).

Interestingly, NO was shown to inhibit NF-κB activation in macrophages, suggesting an inverse relationship between NO and NF-κB, in vitro as well as in vivo (Baisakhi et al., 1999).

The effects of macrolides on neutrophil function in vitro are summarized in Fig. 2.

4.8. Other cells / effects

Although lymphocytes do not accumulate macrolide antibiotics to the extent seen in macrophages, monocytes and granulocytes, it was reported that azithromycin could

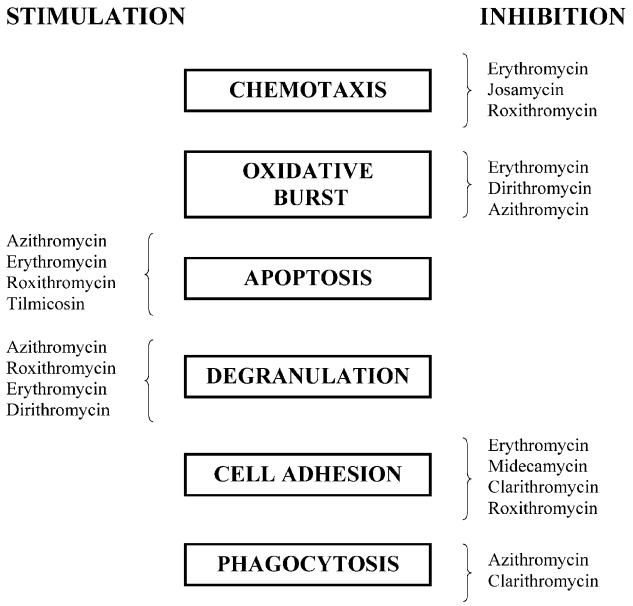

Fig. 2. Effects of macrolides on neutrophil function in vitro, apart from cytokine production.

stimulate the proliferative lymphocyte response to pokeweed mitogen (Tomažić et al., 1993). On the other hand, Keicho et al. (1993) described an anti-proliferative effect of erythromycin, which, in terms of the proposed mechanism was distinct from that of the macrolides rapamycin or FK506 (tacrolimus) on lymphocytes. Erythromycin also caused an impairment of the T cell response to interleukin-2 (Roche et al., 1986). Clarithromycin has been also shown to inhibit mouse thymocyte proliferation (Takeshita et al., 1989) and roxithromycin inhibits the capacity of T-lymphocytes to produce interleukin-5 (Konno et al., 1994). Roxithromycin also promotes growth, differentiation and activation of eosinophils as well as the appearance of interleukin-3, interleukin-4 and TNF-α in aqueous lung extracts (Adachi et al., 1996). However, this does not represent a general growth-promoting action, since the same macrolide also suppressed the growth of nasal polyp fibroblasts (Nonaka et al., 1999; Yamada et al., 2000). An azalide derivative of tylosine (100 μM) was also reported to exert a potent inhibitory effect on normal and carcinoma cell lines, most probably inducing apoptotic cell death (Grdiša et al., 1998).

A stimulatory effect of erythromycin on proliferation of mouse macrophages has been observed. The effect was seen in the absence of any exogenous growth factors. It is possible that erythromycin causes synthesis and/or secretion of some autocrine macrophage growth factors (Kita et al., 1993a,b). Other macrolide-induced modifications of mammalian cell functions or metabolism have been described. They include, for example, inhibition of the growth (Yamada et al., 2000; Nonaka et al., 1998, 1999) and differentiation of promyelocytic leukemia cells (Hoshina et al., 1997; Nagai et al., 1995), anti-tumor effects (Yatsunami et al., 1999a,b; Hamada et al., 2000), inhibition of V-Type ATPases (Gottlieb et al., 1995), decreased glycoconjugate secretion by cultured human airway cells, gastrointestinal motor activity (Omura et al., 1987) and perturbation of early stages of endocytosis in phagocytic and non-phagocytic cells (Tyteca et al., 1999). It was reported that 14/15-membered macrolides were capable of binding to phospahatidylinositol-containing liposomes as well as of inhibiting lysosomal phospholipase A_1 (Montenez et al., 1999).

A beneficial effect of clarithromycin as adjuvant chemotherapy of cancer has been reported (Hamada et al., 2000) and another report describes an anti-angiogenic effect of roxithromycin, or more precisely inhibition of TNF-α-mediated vascular endothelial growth factor (VEGF) induction (Yatsunami et al., 1998). This action of roxithromycin was associated with inhibition of the activation of NF-κB, activation protein-1 (AP-1) and simian-virus-40-protein-1 (SP-1) transcription pathways (Oyama et al., 2000). Inhibition of angiogenesis could (in addition to reversal of multidrug resistance) provide a potential explanation for anti-tumor effects of macrolides. At the same time, the observed anti-angiogenic effects could be a basis for use of macrolides in the treatment of chronic inflammatory conditions, especially those in which VEGF plays an important role. It is interesting to note in this connection that neutralizing antibodies to interleukin-8 reduce tumorigenesis in mice while transfection-induced overexpression of interleukin-8 stimulates angiogenesis as well as tumorigenesis (Kitadai et al., 1999; Yatsunami et al., 1997). Interleukin-8, therefore, seems to be a key parameter linked to inflammation on one side and tumorigenesis on the other (Arenberg et al., 1996). Its inhibition by macrolides could provide a key to a number of different therapeutic uses.

Macrolides are also known to be capable of interacting with P-glycoprotein (-like proteins). Expression of multidrug resistance by eukaryotic cells leads to reduced intracellular concentrations of macrolides and to impairment of activity against intracellular bacteria (Nichterlein et al., 1998). While it seems clear that ATP-binding cassette (ABC) transporters act as pumps through which cells release accumulated macrolides, it is not clear whether the same protein(s) serve(s) as an "entrance door" to the cell for macrolides. Nevertheless, the fact that macrolides are substrates for some of the P-glycoprotein-like drug pumps could be used practically to maintain intracellular concentrations of some other substances, like cytostatic drugs, sufficiently high. Indeed it was demonstrated that administration of erythromycin, in addition to drugs like actinomycin D, doxorubicin, vinblastine or cyclosporine could reverse multi-drug resistance phenotype (Hofsli and Nissen-Meyer, 1989; Wang et al., 2000; Terashi et al., 2000). Therefore, macrolides could therapeutically help to overcome the problem of multiple drug resistance in some cases.

5. Effects of macrolides on animal models of inflammation

5.1. Healthy animals

There appear to be some differences between the effects of macrolides on inflammatory models and their effects on potentially inflammatory parameters in healthy animals. In the healthy guinea pig, roxithromycin increased airway ciliary activity after 14 days of oral administration. In addition, neutrophils in these animals exhibited increased superoxide production but their phagocytic activity remained unchanged (Sugiura et al., 1997). Ex vivo, it was shown that in healthy mice, a 28-day (but not a 7-day treatment) with erythromycin (10 mg/kg) resulted in increased interleukin-1 production by isolated macrophages and interleukin-2 by isolated splenocytes (Kita et al., 1990). Similar to these findings, roxithromycin (10 mg/kg) in mice after 28 days (and not 7 days) caused increased interleukin-1 and TNF-α production by macrophages as well as interleukin-2, interleukin-4 and interferon-γ by isolated splenocytes (Kita et al., 1993a,b), pointing towards immunoenhancing properties after repeated administration. In contrast to these enhancing effects of macrolides in healthy animals, the same compounds have been shown to have inhibitory effects in inflammation models, in which inflammatory cells are primed by cytokines (Table 2).

5.2. Classical inflammation models

The model of carrageenin pleurisy revealed that macrolide antibiotics, like roxithromycin, clarithromycin, and erythromycin have anti-inflammatory activity that likely depends on their ability to prevent the production of pro-inflammatory mediators and cytokines. In this model of acute inflammation, NO production, TNF-α levels, and prostaglandin E_2 were significantly reduced by the macrolide pretreatment (Ianaro et al., 2000).

Erythromycin administration caused anti-inflammatory effects in zymosan-induced peritonitis in rats (Agen et al., 1993). The anti-inflammatory effect of eythromycin was most obvious after 28 days of (pre)treatment and it was comparable to 2-day treatment with dexamethasone (Mikasa et al., 1992) indicating that the anti-inflammatory protective effect of macrolides is a rather slow process that needs a very long time in order to become fully developed. Similar findings were obtained on mouse endotoxin lipopolysaccharide-induced inflammation (Suzaki et al., 1999). Surprisingly, the anti-inflammatory effect after 5 weeks of pretreatment with roxithromycin was very weak, but after 7 weeks a dramatic suppression of cytokine secretion (interleukin-1β and TNF-α) was observed. Roxithromycin was reported to be active in reducing the acute inflammatory reaction through a mechanism different from conventional anti-inflammatory agents such as indomethacin.

In another study, several macrolide antibiotics were demonstrated to be effective in carrageenin-induced paw oedema, the standard animal model used for evaluating anti-inflammatory drugs (Scaglione and Rossoni, 1998). Erythromycin and azithromycin were also shown to be anti-inflammatory in reducing circulating lysosomal enzyme activities in vivo in adjuvant–induced arthritis in rats (Carević and Djokić, 1988).

5.3. Airways inflammation

Rats pretreated with erythromycin or roxithromycin were protected from airway inflammatory reactions caused

Table 2
Effects of macrolides on experimental inflammatory models in vivo

Model	Species	Macrolide	Change	Refs.
Healthy	guinea-pig	roxithromycin	↑ ciliary activity ↑ neutrophil oxidative burst	Sugiura et al. (1997)
	mice	roxithromycin erythromycin	↑ macrophage interleukin-1 ↑ splenocyte interleukin-2	Kita et al. (1990, 1993a,b)
Carrageenin pleurisy	rat	roxithromycin erythromycin clarithromycin	↓ NO, prostaglandin E_2, TNFα	Ianaro et al. (2000)
Zymosan peritonitis	rat	erythromycin	↓ inflammation	Agen et al. (1993)
Lipopolysaccharide inflammation	mouse	roxithromycin	↓ interleukin-1, TNFα	Suzaki et al. (1999)
Adjuvant arthritis	rat	erythromycin azithromycin	↓ lysosomal enzymes	Carević and Djokić (1988)
Lipopolysaccharide airway inflammation	rat	erythromycin roxithromycin	↓ vascular leakage	Tamaoki et al. (1994)
	guinea-pig	erythromycin clarithromycin	↓ neutrophil accumulation	Tamaoki et al. (1997)
Immune complex lung inflammation	rat	erythromycin josamycin	↓ neutrophil accumulation ↓ NO in exhalate	Tamaoki et al. (1999)
Otitis media	rat	erythromycin	↓ leukotriene B_4, leukotriene C_4, ↓ prostaglandin E_2, neutrophil adhesion	Enomoto et al. (1996)

by injection of *E. coli* endotoxin lipopolysaccharide (Tamaoki et al., 1994). Vascular leakage was much less pronounced in macrolide-treated rats. Importantly, no protection was observed in neutropenic rats, indicating that the main target for the anti-inflammatory activity of the macrolides was the neutrophil. This is supported by the finding in another study that clarithromycin and erythromycin inhibit endotoxin lipopolysaccharide-induced recruitment of neutrophils into guinea pig trachea (Tamaoki et al., 1997). A similar anti-inflammatory action, targeting the neutrophil, was seen in the rat model of immune complex-induced lung injury. Erythromycin and josamycin both inhibited neutrophil accumulation and reduced the concentration of NO in exhaled air (Tamaoki et al., 1999).

The ketolide antibiotic, HMR 3004, also showed protective properties in the mouse model of pulmonary infection caused by heat-killed *S. pneumoniae*. It inhibited release of interleukin-6, interleukin-1β and NO in bronchoalveolar lavage fluid and also down-regulated neutrophil accumulation (Duong et al., 1998). However, it is difficult to determine whether this was due to antibacterial and/or anti-inflammatory effects of the compound.

5.4. Otitis

The importance of neutrophils for the anti-inflammatory actions of macrolides was further supported by studies on the rat model of otitis media. Erythromycin inhibited production of leukotriene B_4, leukotriene C_4 and prostaglandin E_2 and the expression of adhesion molecules on neutrophils (L-selectin and Mac-1). As a consequence, erythromycin also inhibited accumulation of macrophages and neutrophils in middle ear effusions (Enomoto et al., 1996).

5.5. Tumor models

Oral administration of erythromycin (1–10 mg/kg) increased the survival times of tumor-bearing mice in both allogeneic and syngeneic systems by 2- to 3-fold compared to vehicle. It was proposed that erythromycin caused an indirect antineoplastic activity by enhancing the production of interleukin-4 which acted in a stimulatory manner on tumoricidal activity of macrophages (Hamada et al., 1995). Inhibition of expression of interleukin-6 and TGF-β, TNF-α and matrix methalloproteinase-9 was observed with clarithromycin in a model of transplanted tumors in rats (Sassa et al., 1999b), indicating that the use of clarithromycin in cancer may be worth investigating.

5.6. Relationship between plasma concentration and anti-inflammatory action

We have studied the relation between erythromycin plasma concentrations and anti-inflammatory effects in rats treated 2 h earlier with intraperitoneal endotoxin lipopolysaccharide. Erythromycin was administered intravenously (Rusić-Pavletić et al., 2000). A single i.v. administration of erythromycin (50 mg/kg) resulted in rapid plasma clearance of erythromycin followed by a slower reduction of serum concentrations of interleukin-6 and interferon-γ. These inhibitory effects were subsequently found to be preceded by a decrease in circulating TNF-α concentrations. Delayed effects of erythromycin were also observed in that the endotoxin lipopolysaccharide-induced increases in plasma levels of the acute phase proteins, C-reactive protein (CRP) and α-macroglobulin, were also reduced by the single macrolide administration. These data further confirm the anti-inflammatory effects of erythromycin, indicating that the time course of these effects of erythromycin can be followed sequentially with specific inflammatory markers.

6. Molecular targets of anti-inflammatory macrolides

In a recent milestone report (Aoki and Kao, 1999) erythromycin in vitro was shown to inhibit activation of the transcription factor NF-κB through a calcineurin-independent pathway. This is one of the first successful attempts to define the anti-inflammatory targets of the macrolide antibiotics at the molecular level. In a reporter gene assay, erythromycin at a concentration of 10^{-5} M inhibited interleukin-8 NF-κB transcription by 37%. It remains unclear whether the basis for inhibition lies in the interaction of erythromycin with the NF-κB protein or with the NF-κB binding promoter region.

Desaki et al. (2000) showed that erythromycin in human bronchial epithelial cells is capable of inhibiting activation of both NF-κB and AP-1 transcription factors, which are crucial regulators of interleukin-8 expression. Since interleukin-8 has been recognized as one of the major regulators of neutrophil recruitment in airway diseases and is one of the most important regulators of polymorphonuclear leukocyte apoptosis (Dunican et al., 2000), this finding supports the concept that NF-κB and AP-1 are potentially the most important molecular targets for at least some anti-inflammatory effects of the macrolides. Clarithromycin is capable of reducing gene expression of pro-inflammatory interleukin-1β in fibroblasts and epithelial cells from nasal mucosa by NF-κB inactivation (Miyanohara et al., 2000). It was also reported that clarithromycin caused interleukin-8 gene repression in bronchial epithelial cells, apparently via the AP-1 binding site (Abe et al., 2000). Most recently, in pulmonary epithelial and blood mononuclear cells, it was shown by flow cytometry that clarithromycin attenuates NF-κB activation induced by TNF-α. Interestingly, this inhibition is not linked to preservation of IκB-α protein. Furthermore, NF-κB-dependent reporter gene expression was inhibited in cells pretreated by clarithromycin (Ichiyama et al., 2001).

Collectively, these findings place NF-κB and AP-1 transcription factors at the center of the mechanism of action of macrolides on the inflammatory process. NF-κB is the most extensively described transcription factor that regulates activity of at least 160 different genes and seems to play a role in a variety of inflammatory diseases of bacterial as well as non-bacterial origin (Baeuerle and Baltimore, 1996; Baeuerle and Baichwal, 1997). The role of NF-κB seems to be particularly important in the pathogenesis of various lung diseases, including adult respiratory distress syndrome, respiratory viral infections, cystic fibrosis, occupational and environmental lung disease (Christman et al., 2000). Immunodeficiencies were observed when distinctive members of the NF-κB family were inactivated by gene disruption (Sha et al., 1995; Kontgen et al., 1995). NF-κB-regulated targets among many include: cytokines (TNF-α, interleukin-1β, interleukin-6, GM-CSF, G-CSF), chemokines (interleukin-8, growth regulatory oncogene-α, GRO-α, monocyte chemoattractant proteins, MCP, MIP), cell adhesion molecules (CD11b, VCAM, E-selectin, ICAM), and acute phase proteins (C-reactive protein, serum amyloid A precursor, endotoxin lipopolysaccharide-binding protein). It was recently reported that local oligonucleotide gene "knock-out" of NF-κB efficiently prevents carrageenin-induced inflammation and TNF-α-induced renal inflammatory responses, respectively (D'Acqisto et al., 2000; Tomita et al., 2000).

Additionally, it seems that NF-κB activation plays the most important role in apoptosis of granulocytes. Inhibition of the NF-κB pathway seems to act pro-apoptotically in human granulocytes (Ward et al., 1999a,b). It is interesting that apoptosis of neutrophils is inhibited by stimuli that are capable of up-regulating the vacuolar H^+-ATPase (Gottlieb et al., 1995). Therefore, it is likely that macrolides, like concanamycin and bafilomycin, which are potent inhibitors of H^+-ATPase, would act pro-apoptotically on granulocytes. It is tempting to further speculate that other "classical" macrolides (antibiotics) could, at least partially, act in an inhibitory manner on H^+-ATPase, shortening the lifespan of granulocytes.

Interestingly, *Chlamydia pneumoniae* and *Helicobacter pylori* also activate NF-κB (AP-1) in human vascular smooth muscle and gastric cells, respectively (Miller et al., 2000; Isemoto et al., 2000). Some of the beneficial effects of the macrolide therapy in treatment of atherosclerosis and ulcer might, therefore, at least partially be explained by this mode of action.

Since some of the macrolides (FK-506, rapamycin) are strong immunosuppressants, it is interesting to point out that FK-506 (tacrolimus), for example, acts by tethering the calcium-dependent phosphatase—calcineurin that acts primarily on transcription factors belonging to the nuclear factors of activated T-cells. FK 506 can only partially inhibit NF-κB activation (Schreiber and Crabtree, 1992). Therefore, immunosuppression is mainly independent of NF-κB, and vice versa, immunomodulatory effects of macrolides do not seem to be immunosuppressive in "classical terms". Further investigations are necessary to determine whether NF-κB is a target of macrolides as a class or only specific compounds.

According to another scenario, the transductional pathway by which macrolides interfere with neutrophil function seems to involve phospholipase D. Abdelghaffar et al. (1997) showed that macrolide antibiotics directly stimulate phospholipase D activity in resting neutrophils.

A recent report indicated that macrolides (erythromycin) could also inhibit Ca^{2+} oscillations and Ca^{2+} influx in epithelial cells (Kanoh et al., 2000; Zhao et al., 2000).

7. Macrolides and inflammatory diseases

7.1. Diffuse panbronchiolitis

In contrast to the large numbers of in vitro studies on anti-inflammatory effects of macrolide antibiotics, only a very limited number of clinical trials have been reported, and few in-vivo studies deal with the anti-inflammatory potential of macrolides. Some macrolide antibiotics like erythromycin, clarithromycin and roxithromycin have already been used as anti-inflammatory drugs, especially for the treatment of diffuse panbronchiolitis. Reports on the use of macrolides for diseases like rheumatoid arthritis and cystic fibrosis are also available (Arayssi, 1998; Singh, 1989; Jaffe et al., 1998).

When used for the low-dose, long-term treatment of diffuse panbronchiolitis, erythromycin resulted in a very good improvement rate (Kadota et al., 1993; Shoji, 1998; Nagai et al., 1991). Diffuse panbronchiolitis is an endemic chronic airway disease characterized by massive infiltration and excessive activation of neutrophils in lungs (for review, see Tsang, 2000). Although erythromycin inhibits hypersecretion due to inhibition of mucus and water secretion from epithelial cells, its major target, once again, is the neutrophil. Neutrophil accumulation at the inflamed site (due to inhibition of cell attachment to the capillary vessels), interleukin-8 secretion from the epithelial cells and production of interleukin-8 and leukotriene B_4 by the neutrophils are all inhibited by erythromycin. A central role of interleukin-8 in the pathogenesis of diffuse panbronchiolitis was proposed by Sakito et al. (1996). High interleukin-8 and interleukin-1 concentrations were detected in bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis while erythromycin therapy caused a decrease in interleukin-1β and interleukin-8 concentrations. Significant positive correlation between interleukin-8 levels and percentage of neutrophils in lavage was also documented.

Administration of macrolides to protect against neutrophil-associated airway hypersecretion was proposed based on the finding that erythromycin was capable of

inhibiting goblet cell secretion and mucosal infiltration caused by inhalation of interleukin-8 (Tamaoki et al., 1996). Interleukin-8 expression by human bronchial epithelial cells was reduced when the cells were treated with therapeutic concentrations of erythromycin or clarithromycin (Takizawa et al., 1997). The effect was not observed with 16-membered macrolides (josamycin). Cryptogenic pneumonia patients receiving erythromycin for 2–3 months (600 mg daily) showed decreased levels of interleukin-8 in bronchoalveolar lavage fluid while the level of TNF-α remained unchanged. Erythromycin at the same time reduced the chemotactic activity of the fluid. This was most probably due to a decreased concentration of chemokines, including interleukin-8 (Hotta, 1996). Oishi et al. (1994) reported that the beneficial effect of erythromycin in chronic airway disease patients could be partially mediated through reduced interleukin-8 production, diminishing neutrophil accumulation and elastase release in the airways. The beneficial effects of erythromycin in diffuse panbronchiolitis also include a reduction of superoxide production, and a reduction of the proteolytic enzyme levels in lungs, which is in good accordance with a number of in-vitro effects observed with this macrolide (Ichikawa et al., 1992). Other mediator alterations in this disease are not altered by erythromycin. For instance, nasal NO is reduced in patients with diffuse panbronchiolitis, but 2 weeks' treatment with erythromycin had no effect on this variable (Nakano et al., 2000). A recent paper reported a lack of effect of azithromycin on interleukin-6 and interleukin-8 concentration in sputum of human volunteers with ozone-induced airway inflammatory reaction (Criqui et al., 2000), once again emphasising that all macrolides may not have a similar spectrum of activity. With regard to the role of macrolides in the inflammatory process, it was suggested that the antioxidant properties of macrolides could be beneficial in airway inflammation by protecting the ciliated epithelium against damage inflicted by bioactive phospholipid-sensitized phagocytes (Feldman et al., 1997, 1999).

7.2. Asthma

Roxithromycin has been reported to exert some anti-asthmatic effects, based on inhibition of bronchial hyperresponsiveness and polymorphonuclear leukocyte superoxide production (Konno et al., 1994; Kamoi et al., 1995). An anti-inflammatory bronchial effect of roxithromycin was recently reported in a clinical trial. When asthma patients were treated for 8 weeks with roxithromycin (150 mg), the patients' symptoms, eosinophil count, serum eosinophilic cationic protein (ECP), as well as sputum eosinophils and sputum ECP were significantly decreased (Shoji et al., 1999). No antileukotriene effect was observed. Erythromycin was also claimed to exert beneficial effects in asthma (Miyatake et al., 1991). However, these results should be regarded with some reservation because inhibition of superimposed infections could improve airways function in asthmatic patients. For more detailed reviews on macrolides and asthma see Cazolla et al. (2000) and Avilla and Boushey (2000).

7.3. Chronic sinusitis

In 1987, results of a clinical trial using erythromycin for the treatment of acute bronchitis were published, but from this trial it was not clear whether beneficial effects were primarily anti-infective or anti-inflammatory (Dunlay et al., 1987). Comparable findings in a similar study were reported by Shirai et al. (1995), while Suzuki et al. (1997a) observed reduction of interleukin-8 and neutrophil recruitment in nasal smears of chronic sinusitis patients upon long-term low-dose roxithromycin treatment (150 mg, once a day). Clinical effects of roxithromycin used in long-term therapy of patients with chronic respiratory tract infections were claimed at least partially to be attributable to the suppression of excess release of chemotactic mediators (leukotriene B_4, interleukin-8, neutrophil elastase) from inflammatory cells (Nakamura et al., 1999). In parallel experiments in vitro, roxithromycin showed inhibitory effects on interleukin-8 release from macrophages and neutrophils. Similar beneficial anti-inflammatory effects were reported in studies by Suzuki et al. (1999) and Kimura et al. (1997) in which potential anti-inflammatory effects of erythromycin and roxithromycin, respectively, were addressed in chronic sinusitis.

Clarithromycin has also been reported to be efficient in the treatment of chronic sinusitis (Hashiba and Baba, 1996a,b). In a clinical trial (Phase IV), 25 non-infected patients with clinically stable chronic sinusitis and persistent maxillary sinus inflammation, received 500 mg clarithromycin for 14 days (Hamid et al., 2000). As a result, statistically significant reductions in interleukin-6, interleukin-8 (22 out of 25), TNF-α, elastase and edema score were observed. Another Phase IIIb/IV double-blind, randomized study performed on 120 subjects addressed the mucolytic effect of clarithromycin in chronic bronchitis (MacLeod et al., 2000). After 7 days, neutrophil activity, interleukin-8 and mucus viscosity were (on average) 32%, 42%, and 50% lower, respectively. Furthermore, 2–3 months' treatment of patients with chronic rhinosinusitis with clarithromycin or roxithromycin caused significant improvement that was inversely correlated with the eosinophil counts in blood, nasal smears and sinus mucosa (Suzuki et al., 2000).

The benefit of long-term, low-dose macrolide treatment in chronic sinusitis and nasal polyps, in addition to antibacterial activity, also includes down-regulation of the host immune response by affecting a number of chronic inflammation parameters (e.g., granulocyte apoptosis, increased mucociliary transport and reduced secretion) (see review of Cervin, 2001). However, another set of data strongly indicates that clarithromycin's inhibitory effect on (endo-

toxin lipopolysaccharide-induced) proliferation of rat nasal epithelium is independent of the presence of neutrophils. This means that other cell types could also be targets for anti-inflammatory effects of macrolides. Since relatively long pre-incubation (2 weeks) is necessary in order to fully reveal this anti-proliferative effect of clarithromycin on nasal epithelium, a different time course of action to that on neutrophils would seem to be involved (Tohnai et al., 1998).

7.4. Cancer

A randomized clinical trial performed on 49 patients, showed that clarithromycin treatment significantly increased the median survival time (MST) for non-small-cell lung cancer patients. Clarithromycin-treated patients (400 mg/day) had an MST of 535 days while those not treated with this macrolide antibiotic had MST of 277 days. Therefore, long-term treatment using clarithromycin appears to support previously mentioned in vitro data, suggesting that it may be beneficial for unresectable non-small-cell lung cancer patients (Mikasa et al., 1997).

7.5. Coronary artery disease

Another therapeutic application of azithromycin has been intensively checked in various clinical trials. The rationale for the use of azithromycin therapy in coronary artery disease (atherosclerosis, myocardial infarction), lies in the finding that atherosclerotic plaques are "infected" by *C. pneumoniae*. This pathogen seems to cause, or at least very much contribute to pathological changes in walls of arteries. As already mentioned, *C. pneumoniae* has been shown to upregulate the NF-κB signalling pathway, causing a number of different effects, including a proliferative effect on cells forming the atherosclerotic plaque (macrophages, smooth muscle cells, fibroblasts). It is also tempting to link this process with the proatherogenic effect of CXCR2 chemokine receptor ligands, like interleukin-8 and GRO-α (Terkeltaub et al., 1998). As already mentioned, macrolides seem to be potent inhibitors of interleukin-8 secretion. Azithromycin was reported to accumulate in vivo in high concentrations in atherosclerotic plaques (Schneider et al., 2000). Therefore, if *C. pneumoniae* is a target (micro)organism, it would be expected that antibiotics with antichlamydial activity may be able to reduce or ameliorate plaque formation (for a review on inflammation and infection as components of coronary artery disease see Brull et al., 2000). This approach, after the breakthrough in treatment of gastric ulcer (caused by *H. pylori*) could be the second major example in which a paradigm of primary inflammatory disease is turned into primarily infective disease. Nevertheless, clinical results collected so far on atherosclerosis seem to be much less satisfactory and the working hypothesis still has to be checked in clinical practice. A number of trials (already performed as well as ongoing) have failed to reach a consensus on the usefulness of azithromycin in the prevention and treatment of atherosclerosis and related diseases.

The idea of atherosclerosis as an infective disease, treatable with azithromycin, came from two animal studies. In a rabbit model of atherosclerosis (cholesterol-free chow; nasopharyngeal inoculation with *C. pneumoniae*) early treatment with azithromycin was highly effective in preventing atherosclerotic plaques. Delayed treatment with the antibiotic was ineffective (Fong et al., 1999). In a similar rabbit model, in which animals were fed with moderately cholesterol-enhanced diet, it was shown that *Chlamydia* infection accelerates atherosclerosis development, while treatment with azithromycin showed a clear protective role (Muhlestein et al., 1998).

The ACADEMIC trial (Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with *Chlamydia*) was performed on 320 coronary artery disease patients who were seropositive for *C. pneumoniae* (Anderson et al., 1999). The dosage was 500 mg for 3 days, followed by 500 mg/week for 3 months. Although clinical markers of inflammation (serum C-reactive protein, interleukin-1, TNF-α and interleukin-6) improved following azithromycin treatment, no differences in antibody titres and clinical events were observed. Therefore, a larger study including several thousand patients and a longer follow-up period was proposed (Muhlestein et al., 2000).

The WIZARD trial (Weekly Intervention with Zithromax (azithromycin) for Artherosclerosis and its Related Disorders) is being performed on 3300 patients who experienced myocardial infarction and had positive *C. pneumoniae* IgG titers. Results of this study are not yet known (Dunne, 2000).

Another randomized double-blind study—Azithromycin and Coronary Events Study (ACES) is under way. Participants (stable coronary artery disease patients) receive 600 mg once a week for 1 year with a 4-year follow-up period (Jackson, 2000). A much smaller study focussed on cell adhesion molecules in serum as markers of endothelial cell activation. Cell adhesion molecules have an important role in recruitment of inflammatory cells during plaque development and are expressed on endothelial cells upon activation. Circulating levels of ICAM-1 and E-selectin, and VCAM-1 were not affected by treatment with azithromycin (500 mg/week for 3 months) (Semaan et al., 2000). A review on the (potential) use of azithromycin in treatment of coronary artery disease and various other diseases has been published recently (Duran and Amsden, 2000).

7.6. Healthy volunteers

To be able to distinguish between anti-bacterial actions and effects on inflammation-relevant cells, we initiated a pilot study on the effects of azithromycin in healthy human volunteers. Twelve healthy volunteers received standard azithromycin therapy (3 consecutive days 500 mg azithro-

mycin, orally) and a battery of some 40 hematological and biochemical parameters was chosen according to the relevance they play in the inflammatory process. Parameters were measured 2 h before azithromycin treatment (baseline), 3 h after the last dose (peak plasma concentration), 24 h after last dose (peak intracellular concentration) and 28 days after the last dose (wash-out period).

We observed an initial neutrophil degranulating effect of azithromycin, reflected in a rapid decrease, between 2 and 24 h, in the activities of β-glucuronidase, *N*-acetyl-β-D-glucosaminidase and myeloperoxidase and a corresponding increase in serum activities of these enzymes (Novak-Mirčetić et al., 2001). This action could contribute towards the anti-bacterial effects of azithromycin. A significant drop in interleukin-8 serum concentration in all volunteers was seen 3 and 24 h after the last dosage of azithromycin and a similar, but more moderate trend was seen for GRO-α and sVCAM concentrations in serum, as well as acute phase proteins (Čulić et al., manuscript in preparation). All these proteins are known to be NF-κB-regulated. According to morphologic findings and in agreement with previously published in vitro data, apoptosis of polymorphonuclear leukocytes appeared to be stimulated for up to 28 days after the last azithromycin dosage. Apoptosis is usually accompanied by ubiquitous functional down-regulation of the cell and its stimulation can be viewed as a beneficial anti-inflammatory result. Interleukin-8 and NF-κB are known to be negative regulators of apoptosis. Moreover, TNF-α-induced apoptosis of polymorphonuclear leukocytes was reported to be mediated by interleukin-8 production (Dunican et al., 2000). In our study neutrophils isolated from blood also showed a delayed down-regulation of the oxidative burst, meaning that their capability for production of reactive oxygen species was attenuated due to antibiotic treatment (Čulić et al., manuscript in preparation). Serum interleukin-6 concentration additionally showed a decrease during treatment, while serum interleukin-1, TNF-α, GM-CSF showed an increase, although the latter cytokines are also known to be NF-κB regulated.

In contrast, a 3-day course of azithromycin (500 mg/day) given to healthy volunteers did not alter the pro-inflammatory cytokine profile in blood and alveolar macrophages (Aubert et al., 1998). Superoxide generation also remained unchanged in that trial.

It would be interesting and necessary to monitor these parameters in some kind of inflammatory non-infective disease, particularly since, as described earlier, effects of macrolides in healthy animals tend to differ from those in infected or inflamed conditions.

8. Conclusions

Over many years, macrolides have proven to be very effective antibacterial agents. Clinical and experimental data now indicate that the effects of macrolides are not just restricted to direct action on bacteria, but also involve modulation of host defense mechanisms. Phagocytes in particular appear to be important targets for macrolides. This is indicated by a decade of use of erythromycin in clinical treatment of diffuse panbronchiolitis. Effects of macrolides on host defense mechanism and/or tissue damage may underlie additional therapeutic effects in a number of infective diseases and may help to account for beneficial therapeutic activity in atherosclerosis and chronic sinusitis. Experimental studies in vitro certainly support the possibility that other 14-membered macrolides than just erythromycin exert direct anti-inflammatory effects possibly through effects on transcriptional factors. Data on other macrolides and studies in vivo in general suggest that there may be differences between individual macrolides. It is likely that in the near future, it will become more apparent whether such potentially beneficial effects on chronic inflammatory processes are a property of the macrolides as a class, or whether specific compound are more effective than others.

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