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REVIEW ARTICLE

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Xenobiotic immunosuppressive agents: therapeutic effects in animal models of autoimmune diseases

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Abstract An unprecedented arsenal of new xenobiotic immunosuppressive agents has been developed recently. Most of the new immunosuppressants have been tested primarily in the treatment of allograft rejection in experimental models of transplantation, and some of the new drugs have already proven their safety and efficiency in extensive clinical trials on transplant patients. Another field for their potential application is the treatment of autoimmune diseases. This review will give an overview of the therapeutic potential of the new xenobiotic drugs in different animal models of rheumatoid arthritis, systemic lupus erythematosus, myasthenia gravis, multiple sclerosis, diabetes mellitus, thyroiditis and uveoretinitis. The new xenobiotics are either inhibitors of the de novo synthesis of nucleotides, for example mycophenolate mofetil, mizoribine, leflunomide, and brequinar, or are immunophilin-binding agents (cyclosporin, FK506 and rapamycin) that inhibit signal transduction and cell cycle progression in lymphocytes. A different mode of action is likely to account for the immunosuppressive effects of deoxyspergualin, which may interfere with intracellular chaperoning by the heat shock protein HSP70 and the activation of transcription factor NF-kappa B.

Key words Immunosuppressive drugs · Autoimmune disease · Animal models

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Introduction

The increasing knowledge of mechanisms involved in the pathogenesis of autoimmune diseases has provided the basis for opening new therapeutic avenues in pharmacological intervention. New developments in the area of immunosuppressive drugs comprise new synthetic drugs, as well as biological agents. Biological therapies are based on attempts to modulate adverse immune responses by the use of regulatory molecules produced by cells of the immune system, as well as of derivative and recombinant forms of such molecules. Under this operational definition fall monoclonal antibodies (mABs), soluble forms of cell surface receptors, cytokines and their naturally occurring antagonists, antigenic peptides and lymphocyte or DNA vaccines. However, this review will focus on xenobiotics, which are molecules produced by microorganisms or chemically synthesized agents, dissimilar from naturally occurring mammalian molecules. To this group belong several immunosuppressive or immunomodulatory drugs that predominantly interfere with lymphokine synthesis and signal transduction, as well as lymphocyte differentiation and cell cycle progression.

Interference with interleukin-2 expression: cyclosporin and FK506

The immunosuppressive drugs cyclosporin A (CSA) and FK506 (tarcolimus) exert potent antiproliferative effects on activated T cells by interfering with interleukin-2 (IL-2) production, which is required for the transition from the resting G0 state to the G1 phase of the cell cycle [1]. Rapamycin (sirolimus), which has no effect on early cytokine synthesis, inhibits the T-cell response to IL-2, a step required for transition from the G1 to the S phase of the cell cycle. All these substances are of microbial origin and are structurally related. CSA and FK506 bind to intracellular receptors called immunophilins, i. e. cyclophilin and

FK-binding proteins (FKBP), respectively. The drug/immunophilin complex binds to the Ca^{2+}-activated serine/threonine phosphatase calcineurin, thereby inhibiting its activity. Normally, the calcineurin is activated by a rise in intracellular Ca^{2+} upon appropriate T-cell receptor (TCR) signalling; after mediating dephosphorylation of the cytosolic component of transcription factor NF-AT (nuclear factor of activated T cells), the latter translocates into the nucleus and activates the IL-2 gene [reviewed in 2]. Thus, CSA and FK506 inhibit clonal expansion of T cells by blocking IL-2 synthesis.

Interference with IL-2-induced cell cycle progression: rapamycin

While rapamycin and FK506 compete for binding to the same immunophilin FKBP12, the complex rapamycin-FKBP12 does not inhibit calcineurin, targeting instead two proteins (targets of rapamycin [TOR1+2]) associated with the G1 cell cycle progression. Also, rapamycin may inactivate the p70s6 kinase, resulting not only in selective inhibition of the synthesis of several ribosomal proteins, but also in decreased induction of mRNA for new ribosomal proteins [3]. The newly discovered blockade of IL-2-induced expression of proliferating cell nuclear antigen (PCNA) [4] must also be mentioned as a pharmacological effect of rapamycin on IL-2-dependent cell cycle progression. Since rapamycin affects T-cell expansion at several steps that differ from that targeted by CSA, and because both drugs do not interfere at the immunophilin-binding step, they are likely to provide a very effective drug combination.

Therapeutic effects of cyclosporin, FK506 and rapamycin

CSA has clear beneficial effects in most of the experimental models of autoimmune diseases; a few instructive examples are selected from the impressive number of studies performed. CSA treatment of a chronic relapsing model of experimental allergic encephalomyelitis (CR-EAE) in SJL/J mice at a non-toxic dosage shortens the length and severity of relapsing paralyses, and decreases the mortality of the animals repetitively challenged with encephalitogenic emulsions [5]. Treatment of non-obese diabetes (NOD) mice successfully prevents the development of insulin-dependent diabetes mellitus (IDDM) when started immediately prior to the onset of glucose intolerance. An earlier treatment schedule completely protects mice from developing insulitis and hyperglycaemia, whereas there is no therapeutic benefit once diabetes is well established [6].

In experimental myasthenia gravis (EMG), CSA given at the time of primary immunization suppresses IgG responses to the acetylcholine receptor (AChR) and prevents the onset of disease; in addition, treated animals respond like naive animals to a secondary antigenic challenge. Treatment of established EMG not only suppresses the ongoing disease (unlike in IDDM) but also prevents the response to secondary immunization; interestingly, this effect of CSA can be overcome by an overwhelming dose of adjuvant [7]. In vitro, exposure to CSA in the presence of AChR induces antigen-specific suppressor populations among AChR-sensitized lymphocytes. In vitro, these suppressor populations inhibit the production of anti-AChR IgG by lymphocytes harvested from rats with EMG [8]. These in vitro studies provide a reasonable model for the explanation of the in vivo effects of CSA in EMG, which is an animal model that is critically dependent on the formation of pathogenic autoantibodies directed towards the neuromuscular junction.

In murine models of lupus, CSA shows clear beneficial effects; however, the degree of efficacy seems to depend on the genetic backgrounds of the animals. Preventive treatment of young (NZB×NZW) F1 mice with CSA leads to suppression of both anti-DNA antibodies and immunocomplexes, thereby protecting against the development of glomerulonephritis; also, treatment of old mice reduces the degree of proteinuria [9]. In contrast, CSA at a dose as high as 25 mg/kg body weight does not reduce the clinical signs of glomerulonephritis in BXSB or MRL-1 mice, although there is some histopathological improvement in the latter. In this strain, the most prominent effect of CSA is a reduction of lymphoproliferation, whereas neither anti-DNA antibody titres nor mortality are reduced [10]. Arthritis and glomerulonephritis in MRL-lpr/lpr mice are clearly ameliorated by CSA therapy, as are the survival rates of these animals; the autoantibody production remains, however, unaltered [11]. CSA also suppresses the signs of ocular and lacrimal gland disease [12].

In preventive treatment of collagen type II induced arthritis (CIA) in DBA/1 mice and in non-human primates, CSA suppresses the development of arthritis. However, as so often happens when treating well-established phases of experimental autoimmunity, there is no amelioration of CIA at later phases of the disease [13, 14].

The effects of CSA, though beneficial in different models of autoimmune disorders, critically depend on the time of administration. According to the proposed mechanism of action of the drug, i. e. the influence on very early IL-2-dependent processes of T-cell activation, CSA must be administered during the sensitization period and for a significant length of time to exert its inhibitory effects. When CSA is administered late or for only a short period of time, the immune responses are often only mildly suppressed or unaffected, or even enhanced. The latter possibility has been described in both EAE and CIA. While CIA in rats is suppressed if CSA therapy is performed at the time of immunization, starting treatment in the preclinical or in the clinical phase of the disease leads to aggravation of arthritis [15]. Similar experiences are reported in acute and chronic models of rat EAE in which CSA precipitates relapses or hyperacute attacks [16, 17]. These apparently paradoxical effects can be explained if one envisages that inactivation in later phases of autoimmunity probably blocks the expansion of suppressor T cells, reversing therefore the

Th1/Th2 balance in favour of pro-inflammatory effects; alternatively, there might occur selection of particular T cells whose activation is IL-2 independent [18]. In addition, CSA has profound effects on thymocyte maturation and selection. Under normal conditions, cortical thymocytes with an affinity above a given threshold survive positive selection and migrate into the medulla. In the medulla, a second affinity check is required in order to delete autoreactive thymocytes. Under CSA administration, the affinity as experienced by a decrease in signalling intensity upon TCR cross-linking is reduced. More cortical thymocytes will die, resulting in a decreased population in the medulla. The few cells that eventually enter the medulla will undergo negative selection with an increased survival rate. Thus, the absolutely reduced number of medullary thymocytes will contain potentially autoreactive cells that now reach the periphery [19]. In the periphery these autoreactive cells are normally prevented from activation as long as CSA is present. In case of cessation of therapy, the balance between autoregulatory T cells and the CSA-induced autoreactive T cells determines whether CSA-induced autoimmunity will eventually develop.

Similarly to CSA, FK506 is effective in protecting NOD mice against the development of IDDM [20]. In different lupus models, the drug suppresses the diverse disease manifestations, such as skin lesions and glomerulonephritis, significantly prolonging the life span of the affected animals [21–23]. In MRL lpr/lpr mice, high doses of FK506 can even significantly reduce anti-DNA antibody titres, thereby representing an improvement over CSA in this mouse strain [24]. Much the same can be said for glomerulonephritis in MLR/1 mice, which is CSA resistant [10], but is inhibited by FK506 [22]. In CIA, low dose treatment (0.32 mg/kg) is effective only if given within 4 days post-immunization [25], whereas the single administration of a high dose of FK506 (10 mg/kg) suppresses the disease activity even when applied after the clinical onset of arthritis at day 12 or 15 [26]. These data indicate that FK506 may be a more potent immunosuppressant than CSA. This is supported by the notion that FK506 is 10–30 times more effective than CSA in preventing S-antigen-induced experimental uveoretinitis (EAU) [27].

Rapamycin is effective against EAE [28], IDDM [29] and murine lupus in MRL/1 mice [30], similarly to FK506. In adjuvant arthritis, in turn, non-toxic doses of rapamycin suppress established disease; the effect is long-lasting, persisting even after cessation of therapy. This is a major improvement over CSA, which, upon discontinuation, produces an immediate clinical exacerbation [28].

Mycophenolate mofetil, mizoribine, leflunomide and brequinar

Mycophenolate mofetil (MMF), mizoribine (MZR), leflunomide (LFM) and brequinar (BQR) interfere with purine and pyrimidine synthesis. In stimulated lymphocytes, the enzymes responsible for de novo synthesis of purine and pyrimidines must be induced, since nucleotides are required for increased RNA and DNA synthesis and for glycosylation of intracellular proteins. Replication of activated lymphocytes appears to require that all the preceding DNA damage be repaired. Interference with this repair mechanism by an imbalance in the purines or pyrimidines leads, in fact, to an increased rate of apoptosis. Lymphocytes seem to be uniquely sensitive to drugs that block de novo nucleotide synthesis because of the lack of a "salvage" pathway.

The active metabolite of MMF is mycophenolic acid, which inhibits inosine monophosphate dehydrogenase (IMPDH) activity and thus disables de novo purine biosynthesis [31]. The decreased guanine nucleotide levels not only lead to a reduction in mitogen-stimulated T- and B-cell proliferation, but also affect N-linked glycosylation of membrane glycoproteins such as VLA-4 and the ligands of selectins [32]. The reduced T-cell adherence to IL-1-activated endothelial cells and the decreased adherence of monocytes to endothelial cells and laminin may be due to the effect of MMF on adhesion molecules [33, 34].

MZR was isolated from the soil fungus *Eupenicillium brefeldaldianum* in 1974 in Japan [35] and was approved for transplantation rejection in 1984. MZR is a prodrug that has to be phosphorylated to the 5′-monophosphate derivative by adenosine kinase for conversion into the active form. The pharmacological effects of MZR are linked to its reversible inhibitory effect on IMPDH [36], the same enzyme that is also blocked by MMF. In addition to the depletory effect on the guanine ribonucleotide pool in activated T cells, MZR interferes with the cell cycle progression of activated B cells by suppressing the activation of cyclin A [37]. However, in the preventive treatment attempt of CIA, the ameliorating effect of MZR treatment on arthritis scores and joint destruction is due rather to the marked suppression of the delayed type hypersensitivity response to CII than to the mild inhibitory effect on the CII-specific humoral response [38]. Beneficial effects of MZR have more recently been demonstrated in the treatment of pulmonary lesions in the lupus model in MRL/Lpr/lpr mice [39]. The subcutaneous application of 20 mg/kg body weight of MZR on every other day causes a delay in peribronchial and perivascular lymphocytic infiltrations in the lungs when treatment is initiated on 4-week-old MRL/Lpr/lpr mice.

LFM is rapidly converted in vivo to its immunosuppressive metabolite A77 1726, which reversibly inhibits antibody production and proliferation of mitogen-stimulated B and T cells [40, 41]. The proposed mechanism of action of LFM on T cell cytokine production remains as yet controversial [reviewed in 2]. While it has recently been demonstrated that LFM increases the production of TGF-β_1 in stimulated human peripheral blood mononuclear cell cultures [42], the major mechanism of action of LFM seems to be the inhibition of the enzyme dihydro-orotate dehydrogenase (DHOHD) [43], which is required for the de novo synthesis of pyrimidines. Of note, resting lymphocytes possess a relatively small pyrimidine pool; stimulation with mitogen increases dramatically the pyrimidine

pool (eight-fold), especially if compared to the two-fold increase in the purine pool [44].

BQR is a substituted 4-quinoline carboxylic acid analogue originally synthesized for potential anticancer application [reviewed in 31]. BQR also blocks the de novo synthesis of pyrimidines by reversibly inhibiting the enzyme DHODH, the same proposed target of action as LFM. Although both BQR and LFM share the common pathway of DHOH inhibition, there are significant differences between these agents. Whereas therapeutic doses of BQR have been associated with thrombocytopenia, stomatitis and mucositis, only transient thrombocytopenia has been reported with LFM. In animal models of transplantation, BQR has been less effective than LFM in blocking vascular graft rejection [45]. The immunosuppressive effects of BQR on experimental autoimmunity have been tested in a chronic relapsing model of EAE in the Biozzi AB/H mouse [46]. Although BQR actively inhibits peripheral immune responses, it exhibits a rather limited potential to control the ongoing disease of the central nervous system upon systemic administration. The lack of therapeutic benefit in EAE is most likely due to the low penetrance of the blood brain barrier by BQR, since intracerebral injections of the compound significantly inhibit disease progression.

The therapeutic effects of LFM have been demonstrated in EAE, IDDM, EMG, murine lupus and proteoglycan-induced arthritis in Balb/c mice [reviewed in 47]. The effects of this molecule appear best at preventing the development of diseases, although suppression of established diseases is observed in murine lupus glomerulonephritis and antigen-induced arthritis in rats [48]. In the preventive treatment of EAU, LFM is more potent than CSA, as revealed by the comparison of the respective IC50 and IC90 values [49].

Deoxyspergualin

Deoxyspergualin (DSG) is an analogue of the bacterial product spergualin from the soil commensal *Bacillus lactosporous* [50]. In contrast to the xenobiotics described so far, the molecular mechanisms underlying its pharmacological effects remain elusive. DSG interacts with HSP70, a member of the heat shock protein 70 family, thereby blocking the nuclear translocation of HSP70 in the heat shock response [51]. In a murine pre-B-cell line, exposure to DSG inhibits the expression of the kappa light chain that normally follows lipopolysaccharide-induced NF-κB activation, thereby suggesting suppression of humoral immune responses during certain stages of B-cell development [52]. Also, treatment of normal mice with DSG appears to block an early step of T-cell differentiation in the thymus, namely, the transition from the double-negative $CD4^-$ $CD8^-$ phenotype into the $CD4^+$ $CD8^+$ double-positive phenotype, which is associated with the expression of a pre-TCR complex; in contrast, DSG does not seem to affect later stages of T-cell differentiation or activation of mature T cells in vitro and in vivo. Interestingly, pre-B-cell differentiation is also blocked at a similar checkpoint that is controlled by the expression of a pre-receptor complex containing the immunoglobulin heavy chains, but not the light chains. Mature B cells, similarly to mature T cells, seem insensitive to DSG [53]. Whether or not this effect on pre-B- and pre-T-cell differentiation is due to the interference of DSG with the action of an NF-κB family member, whose action is specifically required at parallel stages in B- and T-cell development, awaits further investigation.

DSG is a potent immunosuppressant in EAE, IDDM, CIA, adjuvant arthritis, EMG, murine lupus, experimental thyroiditis (EAT) and EAU [reviewed in 54]. However, the dramatic effect of the drug on pre-B- and pre-T-cell development [53] appears to cause serious problems with the physiological replacement of B and T lymphoid compartments; these effects should be carefully considered in designing and monitoring long-term treatment. The advantage of this drug is its lack of nephrotoxicity, which renders it attractive not only for treatment of rapidly progressive glomerulonephritis, but also as part of a combined immunosuppressive therapy.

Combination of xenobiotics

Synergy of different xenobiotics in combination is very attractive because it reduces the need for high doses of each single drug, reducing thereby drug toxicity, while maintaining an effective degree of immunosuppression. Combination therapies have been tested in different models. S-antigen-induced EAU, for example, can be completely prevented by a combination of CSA and rapamycin. The synergy approach in this study allows a nine-fold reduction in the rapamycin dose and a five-fold decrease in CSA [55]. In antigen-induced arthritis, a combination of subtherapeutic doses of LFM and CSA administered after disease onset effectively reduces clinical and histopathological signs of the disease [48]. Interestingly, subtherapeutic doses of CSA and the immunomodulatory hormone 1,25-dihydroxyvitamin D3 [56] can also exert cooperative immunosuppressive effects in EAE and EAT [57, 58].

Progress in understanding the mechanisms of action of the new xenobiotics will facilitate the future development of rational combination therapies. Hopefully, such combinations will prove to be more effective in the therapy of already ongoing autoimmune diseases than the established protocols; most of the therapeutic benefits reported in this review remain confined to preventive treatment approaches. On the way towards clinical application in humans, experimental models of autoimmune diseases remain valuable tools to elucidate at least some of the remaining uncertainties with the new drugs. Thus, it is possible that the enzyme levels responsible for de novo or salvage pathway synthesis of nucleotides may vary between individuals and in dependency on different diseases states. Such variabilities could critically affect therapeutic efficacy and the toxicity of agents that interfere with the nu-

cleoside metabolism of the lymphocytes. In addition, the pathogenetic aspects of the various autoimmune models clearly indicate a diversity of mechanisms relevant in different pathologies. The contribution of lymphocyte subsets to tissue pathology varies considerably among models: in NOD mice with IDDM there is a clear involvement of both $CD8^+$ and $CD4^+$ T-cell subsets; in EAE $CD8^+$ T cells are relevant, however, Th1-polarized $CD4^+$ T cells appear to predominate; in CIA, in turn, the most obvious feature seems the cognate interaction between $CD4^+$ T cells and B cells; finally, in EMG the production of antibodies to AChR seems to dictate the development of the disease. For these reasons, particular features that may act as selective or specific hooks for immunotherapeutic intervention are most likely to differ among the disease models. It is nonetheless conceivable that the targeting of common pathogenic pathways by combinations of drugs that interfere with basic mechanisms of lymphocyte biology, such as cell cycle progression, may prove equally effective and convenient.

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