Voltage-Gated Sodium Channel Blockers as Immunomodulators

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Received: September 01, 2005; Accepted: September 222, 2005; Revised: October 06, 2005

Abstract: Several Voltage-Gated Sodium Channels (VGSC) are widely expressed on lymphocytes and macrophages but their role in immune function is still debated. Nevertheless, Na^+ influx through VGSC is required for lymphocytes activation and proliferation, since these responses are blocked by Na^+-free medium or by VGSC blockers. These effects may be mediated by the reduced intracellular Na^+ levels, which in turn may impair the activity of Na^+/Ca^{++} exchanger resulting in reduced intracellular Ca^{++} levels during lymphocyte activation. Furthermore, in Jurkat cell line VGSC appear to be involved in cell volume regulation, migration in artificial matrix and cell death by apoptosis. VGSC play a role in macrophage function as well, and VGSC blockers impair both phagocytosis and inflammatory responses. Several VGSC blockers have shown immunomodulatory properties in mice models, skewing the immune response toward a Th2-mediated response, while suppressing Th1-mediated responses, and VGSC already used in clinical practice are known to modulate immunoglobulin (Ig) levels both in mice and in humans. These effects suggest that VGSC blockers may find clinical application in the treatment of autoimmune and inflammatory disease. However, many of these drugs induce a number of severe side effects. The relevance of VGSC function in immune regulation suggest that the testing of newly patented VGSC blockers for their effect on immunity may be worthwhile.

Keywords: Antibody isotypes, immunomodulation, phenytoin, T-helper 2 cells, voltage-gated sodium channel.

VOLTAGE-GATED SODIUM CHANNELS: OVERVIEW

Voltage-gated sodium channels (VGSC) are widely expressed throughout the central and peripheral nervous systems, where they constitute a fundamental requirement for excitability. VGSC are composed of three subunits: a large subunit (about 260 KDa) and two smaller subunits (1 and 2, about 36 KDa). Two additional subunits (3 and 4) have been recently described. Structural analysis of the subunit has revealed six -helical segments spanning (S1 to S6) the membrane in each of the four homologous domains (I to IV), and a re-entrant loop, forming the outer pore region, dipping in the transmembrane region between S5 and S6, which line the ion-permeable channel [1]. The subunits are not involved in the pore structure and play a regulatory role in trafficking and location of VGSC. Several VGSC subunit gene families have been identified by molecular and genetic screenings [1], including at least nine different pore-forming subunits. The subfamily which includes Na_V1.1, Na_V 1.2, Na_V 1.3, and Na_V1.7 is highly expressed in nearly all types of neurons and characterized by elevated sensitivity to Tetrodotoxin (TTX). A second subfamily, including Na_V 1.5, Na_V 1.8, Na_V and 1.9 is abundantly expressed in dorsal root ganglium sensory neurons and in the heart, and shows a variable -to-low sensitivity to TTX. Na_V1.4 and Na_V1.6 constitute a separate set of genes, less closely related to the first two, the former being highly expressed in muscle cells and the latter, largely represented throughout the central nervous system, even including neurons, and glial cells, as well as in several non-neural cell types. A second highly divergent class of VGSC has been described as well, named collectively Na_X and widely expressed in smooth muscle cells, glia peripheral nervous system and heart. Na_X is deemed to be not voltage-gated but to be involved in Na^+ concentration sensing [2]. Structural and electrophysiological properties of VGSC has been recently reviewed elsewhere [3, 4].

VGSC are targeted by several classes of drugs, including anti-arrhythmic drugs, local anesthetics and anticonvulsants. All these drugs share a common preference for the inactive-state of VGSC and a common site of interaction with the subunit, located in correspondence to the inner pore. The location of the interaction site implies that these drugs act, after crossing the membrane, on the cytoplasmic side of the channel [5].

Even if Na^+ channels are heavily expressed in neurons and in other excitable cell types, they are also present on immune cells. However, the role of VGSC in the regulation of Na^+ flux in immune cells with its involvement in pathophysiological mechanisms is still largely unknown. Several VGSC blockers are currently used in clinical practice as anticonvulsants (notably diphenylhydantoine (DPH), carbamazepine (CBZ) and lamotrigine), antiarrhytmics and local anesthetics, and some new molecules provided with VGSC blocker action have been patented recently. Some of these molecules showed interesting effects on immune functions both in mice and in human patients. We review current knowledge about VGSC blockers on immune cells function and suggest possible therapeutic applications of these molecules.

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VGSC EXPRESSION AND FUNCTION IN LYMPHOCYTES

As previously outlined, VGSC are expressed both in normal human T lymphocytes and in lymphoid cell lines. In Jurkat T cells, at least four types of VGSC are expressed at different levels: $Na_V1.5$, $Na_V1.6$ $Na_V1.8$, and $Na_V1.9$. Among these, $Na_V1.6$ is expressed mainly as an inactive splice form, Na_V 1.8 and Na_V 1.9 are expressed at very low levels, as detected by western blot and reverse transcription-polymerase chain reaction, while Na_V 1.5 is the main VGSC in Jurkat cells. Molecular data are in agreement with electrophysiological recordings, showing an inward, fully inactivating Na^+ current, dependent upon membrane depolarization. This current has been proven to be insensitive to TTX, supporting the view that Na_V 1.5 represents the main VGSC in these cells [6]. Interestingly, only a subset of Jurkat cells (about 10%) reveals Na^+ current. Jurkat cells being considered to be clonal, it is conceivable that VGSC expression is not constitutive, but is tightly restricted to a different activation or functional state.

Early studies reported that an increase in Na^+ current was required for proliferation of pig lymphocytes [7], but this response not being inhibited by TTX, it was thought not to be linked to Na^+ channel activation. These conclusions have been partially confuted by more recent findings, including the cloning of TTX-insensitive VGSC. In human, $CD4^+$ T cell clones activated by antigen presenting cells (APC) loaded with streptococcal peptide, voltage-dependent Na^+ current has been detected. In particular, the inward current was detectable in a time window ranging from 10 to 20 min. after T cell contact with APC, being no longer detectable 30 min. after stimulation [8]. This Na^+ current appears to be blocked by high concentration of TTX, suggesting that Na_V 1.5 channel may be responsible for Na^+ influx, by analogy to Jurkat cell line. Na^+ current in lymphocytes was proven to be dependent upon extracellular Na^+ influx, strongly implying VGSC activation; Lai *et al*. (1999) [8] have reported that voltage-dependent Na^+ currents in lymphocytes is completely blocked by amiloride. Even if Lai *et al*. (1999) present electrophysiological recordings showing amiloride inhibitory effect on VGSC-dependent Na^+ currents [8], it should be noted that amiloride may also inhibit Na^+/H^+ exchanger [9]. However, even if Na^+/H^+ seems to play no role in lymphocytes activation [10], the use of more specific VGSC inhibitors might be required to confirm these observations. The Na^+ current appearing in the early phase of T lymphocyte activation plays an important physiological role in antigen(Ag)-specific T cell proliferation. In fact, T cell proliferation is inhibited by Na^+-free culture medium. Interestingly, a similar effect was obtained using Ca^{++}-free culture medium, suggesting that the Na^+ effect could be at least partially mediated by a failure of Ca^{++} influx [8]. Further notions on the role of Na^+ current have arisen from the analysis of Ca^{++} levels in activated T cells. In fact, after TCR engagement, an inositol phosphate-dependent rise in cytoplasmic Ca^{++} levels occurs, due to the release of Ca^{++} ions from cellular pools. However, this scarce and short-lived Ca^{++} increase is followed by a much stronger and sustained Ca^{++} surge, that is required for several transcription factor activation such as NF-AT and NF-kB [11]. When TCR activation occurs in Na^+-free environment, the first wave of Ca^{++} rise is not affected, but the second one is blunted and no proliferation is detected. Notably, the extent of the second wave of Ca^{++} rise is of comparable magnitude in either Na^+-free or Ca^{++}-free conditions [8]. This fact suggests that a VGSC-dependent mechanism is absolutely required for raising Ca^{++} concentration immediately prior to T cell activation. Thus, these data suggest that Na^+ influx and increased intracellular Na^+ levels are required for T cell activation, while their inhibition greatly impairs T cell proliferation.

The reduced Na^+ influx resulting from VGSC blockade might hamper several Na^+-dependent transporters, including prominent Na^+/Ca^{++} antiporter and Na^+/H^+ exchanger. It is well known that the Na^+/Ca^{++} exchanger operates by either decreasing cytoplasmic Ca^{++} levels at expense of the Na^+ gradient or increasing intracellular Ca^{++} extruding Na^+ ions (Fig. **1**). In lymphocytes, the latter modality seems to be likely involved in increasing Ca^{++} levels, while exporting Na^+ ions. Actually, this process should result in a net decrease in intracellular Na^+ levels if no other concurrent mechanisms intervene to render Na^+ ions available. In particular, Na^+ ions are provided to lymphocytes via opening of VGSC. Furthermore, a mitochondrial Na^+/Ca^{++} exchanger has been described, able to increase cytoplasmic Ca^{++} levels, exporting Ca^{++} ions from the mitochondrial matrix [12]. The contribution of the latter is unclear, since in Ca^{++}-free media, as well as in media containing Na^+ channels blockers, Ag-dependent proliferation is blocked. This last evidence proves that Ca^{++} release from intracellular stores is not sufficient to induce a complete Ca^{++}-dependent response, despite the small increase in Ca^{++} concentration detectable after TCR ligation. On the other hand, Na^+ influx may also drive the activity of the Na^+/H^+ exchanger, likely accounting for cytoplasmic acidification. However, there is no direct proof of Na^+/H^+ involvement in lymphocyte function, since in lectin-stimulated peripheral blood mononuclear cells, Na^+/H^+ inhibition does not affect proliferation and DNA synthesis [10]. Thus, it seems likely that sustained Ca^{++} surge required for lymphocytes activation is dependent on the drive provided by the VGSC-mediated Na^+ influx .

Several aspects of VGSC involvement in early T cell response are still unexplained and, in particular, the pathway linking TCR engagement to VGSC activation needs further elucidation. No biochemical data have been reported so far in lymphoid cells, even if NaV1.5 was recently found to be a physiological substrate for the Src family member Fyn [13]. In particular, Fyn is able to phosphorylate a unique tyrosine residue lying in the DIII-DIV cytoplasmic loop, which is involved in Na^+ channel inactivation. At least in HEK cells, tyrosine phosphorylation of NaV1.5 is able to affect its inactivation kinetics, and, being Fyn one prominent kinase involved in T lymphocyte activation, it may play a role also in VGSC regulation. However, one cannot rule out the participation of other pathways in VGSC regulation during T cell activation, most notably PKA and PKC pathways [4].

One major feature of lymphocyte activity in the organism is the ability to adhere to endothelial cells and to migrate through the blood vessel wall into tissues. Cell motility appears to be fundamental both in physiological immune function and in pathological conditions such as those

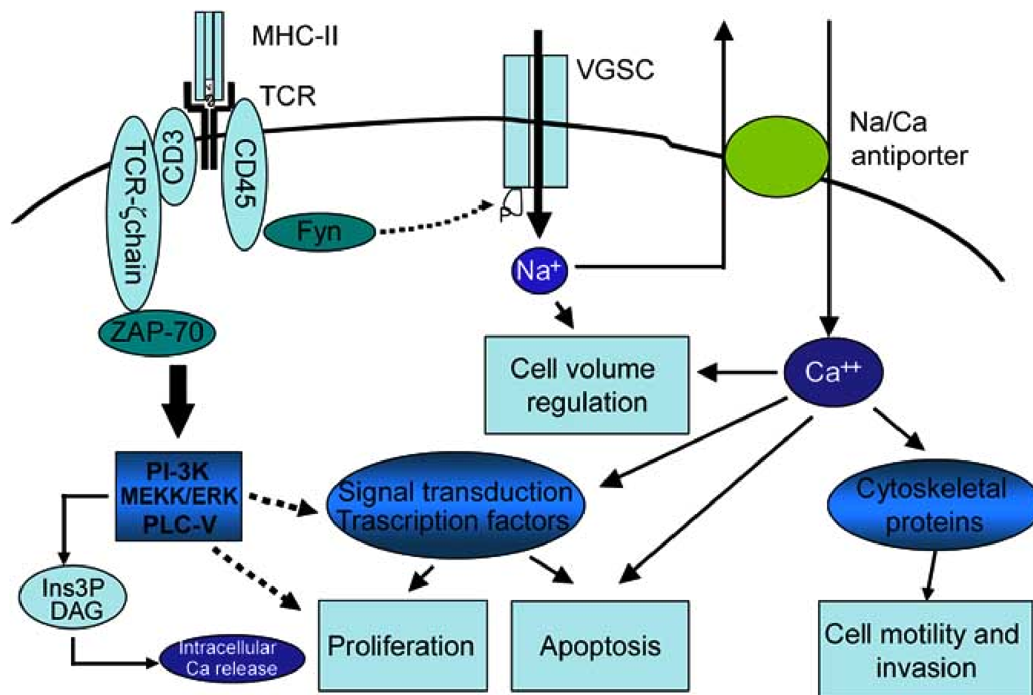

Fig. (1). Cell biology of VGSC in T lymphocytes. VGSC, activated after T Cell receptor (TCR) engagement (speculatively through Src-family dependent phosphorylation), are responsible for Na^+ influx that follows activation. In turn, Na^+ influx provides the drive for Ca^{++} entry trough a Na^+/Ca^{++} exchanger. Sustained Ca^{++} increase regulates several biological responses, including further signal transduction and transcriptional regulation, cell motility and cell death. Dotted lines indicate speculative or indirect links.

observed in multiple sclerosis. In this disease, effective blockade of T cell extravasation results in the improvement of the inflammatory autoimmune process. VGSC have been implied in cell motility of neoplastic cell lines [6]. In this respect, in matrigel invasion assay, about 10% of Jurkat cells showed the ability to penetrate deeply into the artificial matrix. Quite interestingly, same percentage of cells expressed Na^+ current. However, it is not possible to state whether these populations are the same or rather belong to distinct subsets. Furthermore, since TTX was able to reduce greatly Jurkat cells invasiveness (up to 93%), Na^+ current appears to be required for Jurkat cells matrix invasion [6]. Since interesting similarities have been detected between cancer cell motility and T lymphocyte trafficking to distant tissues [14], an involvement of VGSC in lymphocyte extravasation may be speculated. The mechanistical details of this effect are not yet known and, given the complexity of cell trafficking process, multiple targets may be affected. Since Ca^{++} levels are powerful regulators of cell motility and chemotaxis [15], it is postulable that the functional link between VGSC and Na^+/Ca^{++} exchanger may be involved.

In normal lymphocytes, cellular size and overall viability are regulated by Na^+ ,K^+ and Cl^- fluxes [16], suggesting a role for VGSC in this process. In fact, in Jurkat cells, VGSC-dependent current appears to be relevant to cell volume regulation. In this respect, these cells cultured either in Na^+-free or in coline-for-Na^+ replaced medium appear to inflate and to increase their forward scattering in flow cytometry analysis. In normal culture conditions, VGSC are not required for cell survival, since their complete inhibition by TTX or by saxitoxin does not modify cell viability or the appearance of apoptosis markers [6,16]. VGSC-mediated Na^+ current requirement is extremely essential for lymphocyte apoptosis setting. Upon anti-Fas antibody treatment, lymphocytes undergo apoptosis, displaying classical morphological features: cell shrinkage and fragmentation, phosphatidyl serine exposure, Poly(ADP-rybose) polymerase (PARP) cleavage, cromatin condensation and DNA fragmentation. All these events follow intracellular Na^+ levels surge. However, when cultured in Na^+ free medium, lymphocytes exhibited a swollen phenotype (instead of a common shrinked morphology), while retaining every other apoptotic feature. This effect has been proven reversible, since Na^+ reintroduction allowed for apoptotic cell shrinkage [16].

Even more surprising is the intimate relationship between VGSC function and apoptosis induction in Jurkat cells. In fact, VGSC blockade by saxitoxin treatment completely abrogated Fas-dependent apoptosis (including failure to lose intracellular K^+, to degrade DNA and to undergo shrinkage) , suggesting that Na^+ current may be involved both in early stages of apoptosis induction as well as in downstream events. However, when cultured in very-low Na^+ content media, caspase 9 and caspase 3 activation as well as PARP cleavage after Fas crosslinking are not modified, even if cell shrinkage does not occur [16]. Thus, it seems possible that apoptosis induction and subsequent morphological changes have a different requirement for Na^+ fluxes. In fact, complete VGSC blockade abolishes apoptosis induction, but very low levels of VGSC activation are enough to ensure activation of caspases and allow for cellular demise, even in the absence of evident morphological changes.

VGSC BLOCKERS EFFECTS ON B LYMPHOCYTES AND IMMUNOGLOBULIN ISOTYPE

Current studies on the immune system function have been focusing on the regulatory cross-talk among T and B lymphocyte subsets, dendritic cells, macrophages and several other non-immune cells. Virtually, nothing is known about VGSC expression across different subpopulations of T, B lymphocytes and antigen-presenting cells (APC). Since TTX is highly toxic when administered to mice, studies on immune effect of VGSC have relied on pharmacological agents. In this respect, DPH is the most extensively studied drug in this context. Several cellular targets have been reported for DPH , including VGSC, Na-K-ATPase, sigma opioid receptors, GABA-A receptor complex and ionotropic glutamate receptors. Nevertheless, at concentration used in experimental and clinical settings, DPH is considered a VGSC blocker [17,18]; however, the multiplicity of targets should be considered in the evaluation of experimental results. Both in mice and in human patients, DPH exerts profound effects on different parameters of the B cells immune function. In fact, when administered to mice along with Trinitrophenyl (TNP)-Ovalbumin or TNP-Ficoll, DPH markedly increased the number of antibody-secreting cells in popliteal lymph nodes (PN) compared with vehicle treated mice, thus affecting both IgM- and IgG-producing cells [19]. In rats immunized against Keyhole Lymphet Haemocyanin (KLH), DPH treatment skewed antibody response increasing IgE production, without affecting IgG levels [20]. In mice immunized against TNP-OVA, DPH treatment greatly affected B cell activity, causing an increase in IgM, IgE and IgG1 antibody-secreting B cells. Moreover, in the same experimental model, DPH treatment significantly decreased T/B cell ratio in peripheral lymph nodes and mainly through the increased proliferation of $CD19^+$ B lymphocytes. At histological examination, the architecture of lymph nodes appears to be conserved with an increase in both size and number of germinal centers, thus indicating B cell proliferation and differentiation toward memory cells [21]. Notably, no *in vitro* studies have been performed so far to confirm these results using more specific VGSC blockers (i.e. TTX or saxitoxin) or knock-out mice.

Na^+ channel involvement in antibody isotype regulation is supported by clinical data. Early reports claimed that DPH treatment not only affected IgA levels without changes in IgG or IgM levels, but actually increased IgE levels [22]. A recent cross-sectional survey of IgA, IgM and IgG levels in epileptic patients has revealed that DPH treatment reduced IgA levels without affecting IgG and IgM classes [23]. Quite interestingly, low serum IgA levels were a common finding in patients who had been treated with DPH but were not taking the drug at the moment of the survey, despite a partial recovery in IgA levels after DPH withdrawal. In a different clinical sample, DPH treatment clearly reduced serum IgA and IgG concentrations without affecting B lymphocyte count and IgM levels [24]. Previous studies on B cells showed that during DPH treatment, the number of IgA bearing lymphocytes was not affected even when serum IgA levels were reduced [25]. However, more recent and accurate data suggest that DPH may impair Ig secretion globally, with IgA subtype more severely affected, because of a direct impairment on B cell proliferation [26]. In this respect, in a clinical trial involving patients affected by rheumatoid arthritis (RA), DPH also caused a reduction in serum IgM levels [27]. Of note, data obtained in murine models are not in full agreement with clinical findings, except for the increased IgE levels. For instance, with particular reference to the DPH-induced reduction of IgA levels in humans, more complex mechanisms than that investigated so far may be invoked. Since intracellular Ca^{++} surge is a fundamental step induced by B cell receptor activation [28], one can postulate the same mechanism responsible for VGSC blocade-induced T-cell Ca^{++} response impairment. On the other hand, since B lymphocytes homing the mucosa-associated lymphoid tissue appear to be a relevant stage in IgA isotype switching and migration to distant sites [29], VGSC might be involved in B cell trafficking. DPH is not the only VGSC blocker endowed with immunomodulatory properties. Carbamazepine (CBZ), an anticonvulsant drug, has been shown to affect antibody production when administered to human patients. In this respect, CBZ was proven to reduce IgA and IgM production [24,30] and, more recently, in one case CBZ suppressed mainly IgG, and, to a lesser extent, IgM and IgA production [31]. In the latter, a selective inhibition in the maturation of B cells from $sIgM^+$ to IgG^+ has been identified, while IgA^+ B cells were reduced to lower-normal range. Similar effects of CBZ upon antibody production have been reported in other patients (for review see [31]) with variable involvement of Ig subclasses but with constant sparing of IgE levels. These clinical data are supported by previous observations in mice since CBZ treatment reduced markedly splenic plaque forming cell units (a marker of antibody-producing B cell count) and depressed delayed-type hypersensitivity (a marker of T-cell mediated immunity). Apparently, both these effects were likely mediated by T cell populations, since they could be transferred by splenic cells into lethally irradiated mice [32]. CBZ effects are in part similar to those of DPH, since both DPH and CBZ are able to reduce IgA levels and Th1-dependent reactions. However, CBZ-mediated inhibition on the IgM class appears to be higher than that of DPH and apparently no increase of IgE levels has been detected.

VGSC ROLE IN T CELL FUNCTION

The role of VGSC in T cell activation following TCR engagement has been described *in vitro* [8]. Several data suggest a role for VGSC in T cell function *in vivo*. After TNP-OVA immunization in DPH-treated mice, an increase in $CD4^+$ and $CD8^+$ T lymphocytes was observed [21]. Also clinical data suggest an effect of DPH on lymphocyte subsets since in DPH treated epileptic patients, an increase in $CD4^+$ levels and in $CD4^+/CD8^+$ ratio has been reported [24]. In this framework, several experimental findings suggest that DPH may induce a preferential Th2-type response. In fact, the IgE-skewing effect of DPH has been related to an increase in interleukin(IL)-4 levels detectable after PN lymphocytes restimulation *in vitro* with Concanavalin A. However, in similar conditions, DPH treatment did not affect Interferon (IFN)- or lypopolysaccaride (LPS)-stimulated tumor necrosis factor (TNF)- secretion [21]. Likewise, DPH alters cytokine secretion by spleen cells from DPH-treated KLH-immunized mice challenged *in vitro* with KLH. IL-4 levels were increased in DPH-treated mice compared with controls, whereas IFN- , IL-2, IL-1 levels were decreased. *In vivo* administration of LPS to DPH-treated mice resulted in a

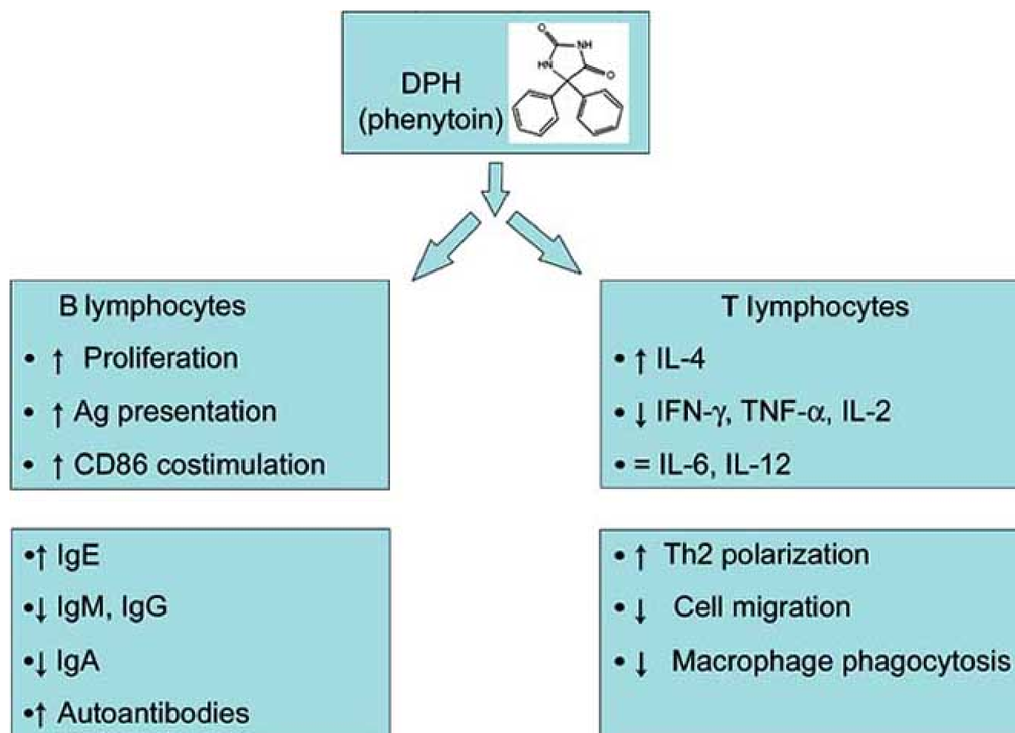

Fig. (2). Summary of DPH effects on B and T lymphocytes according to *in vitro* and *in vivo* cinical trials. Question marks indicate still unexplored areas of investigation.

marked reduction of serum IFN- levels [20], while IL-6 and IL-12 levels were not affected. A similar downregulation of IFN- secretion was observed in lymphocytes obtained from cervical lymph nodes of DPH-treated mice bearing glioma [33]. In synthesis, the cytokine pattern of DPH-treated mice is consistent with Th2 polarization of immune responses.

Mechanistically, DPH effects appear to be highly dependent upon CD40-CD154 activation; in fact, anti-CD154 antibody administration during TNP-OVA immunization under DPH treatment greatly reduced lymph nodes cellularity (up to 65% compared to controls). B cells, CD4+ and CD8+ T cells appear to be equally affected, showing about 50%, 60% and 40% decrease, respectively. DPH did not affect CD4+/CD8+ cell ratio, but greatly affected T/B cell ratio both in lymph nodes (see above) and in peripheral blood lymphocytes (PBL) [21].

DPH effects on IL-4 secretion and antibody production appear to be highly related to CD40-CD154 function as well. In fact anti-CD154 antibody was able to block IL-4 increase (withouth affecting other cytokine release) and to reduce greatly the number of IgE-secreting B cells and serum IgE levels in mice [21].

According to Nierkens and associates [21], DPH exposure greatly increased the capacity of B cells to behave as an efficient APC and to provide costimulation. In fact, CD80, CD86 and CD54 expression on DPH-exposed B cells amounted to nearly 90% of the total expression of these molecules in PBL. Costimulatory molecule expression appears to be dependent upon CD154 ligation, since anti-CD154 antibody supplementation completely abolished DPH-induced increase in the percentage of CD86 and CD54 expressing cells [21]. Taken together, these changes seem to be responsible for B-cell dependent Th2-polarization of immune response. Quite interestingly, all these studies have dealt with murine models, while no investigations have been undertaken in the human model following DPH exposure. Surprisingly, immunomodulatory properties have been discovered into a structurally unrelated Na^+ channel blocker lidocaine, widely used in clinical practice as local anesthetic and antiarrythmic drug. When exposed to lidocaine in culture, T cells from asthma patients exhibited a marked reduction in proliferative response after challenge with Dermatophagoides Ags, purified protein derivative or phorbol esther. Furthermore, lidocaine treatment of lymphocytes resulted in the downregulation of IL-5 and IFN- both at mRNA and protein levels. Lidocaine effects were not overcome by phytohaemagglutin and the Ca^{++} ionofore ionomycin , thus suggesting complex effects of Ca^{++} entry through Na^+/Ca^{++} exchanger. Interestingly, lidocaine effects appear to be dependent upon activation state, since lymphocytes preincubation with IL-2 decreased its inhibitory response [34]. These data may suggest that VGSC, which are targeted by lidocaine, are not constitutively expressed but are regulated by lymphoid cells activation state and cytokine environment.

Until now just a single report has investigated the effects VGSC blocker (namely DPH) upon human natural killer (NK) cell, evidencing a reduction in cytotoxicity against neoplastic cell line and reduced IFN- stimulation of NK activity [35]. However, since those data were obtained using not fractionated PBL, at the moment a critical appraisal of these results is not allowed.

An alternative mechanism explaining the effects of DPH on immune activity has been put forward. In fact, *in vivo* DPH treatment resulted in increased plasma ACTH and corticosterone levels in mice [20]. Similar DPH-dependent hormonal alterations were not detected in humans [36], even if they may have contributed to some cutaneous side effects observed in certain patients under DPH treatment [37]. To date, no experimental or clinical data are available about other VGSC blockers already used in clinical practice and notably about Lamotrigine.

VGSC ROLE IN MACROPHAGES AND MICROGLIAL FUNCTION

Voltage gated Na^+ channels have been detected in microglial cells and in macrophages by electrophysiological recordings [38] and initially identified as TTX-sensitive. Na^+ current in microglial cells appears to be linked to their activation state. In fact, a significantly larger fraction of microglial cells in the ramified state showed detectable Na^+ current as compared to microglial cells in the ameboid state. VGSC expression appears to be highly related to the morphological state, since co-culture of microglia and astrocytes resulted in an increase in Na^+ current along with the acquisition of ramified morphology [39]. More recently, NaV 1.2 and NaV1.6 were identified as the most abundant VGSC expressed in murine microglial cells, with almost not significant expression of NaV 1.1, 1.3, 1.8, 1.9 channels [40]. In agreement with early electrophysiological data, Na^+ current in microglial cells appears to be completely blocked by TTX, confirming the role $Na_V1.6$ channel [40]. VGSC are involved in several physiological functions of microglial cells: in fact, TTX is able to reduce latex bead phagocytosis in resting microglial cells, and to impair LPS-induced upregulation of phagocytic activity [40]. These findings are supported by futher experiments in *med* mice, which lack functional $Na_V1.6$ [41]. In fact, LPS-stimulated microglial cells and macrophages from *med* mice showed a lower phagocytic activity when compared with control mice [40]. However, residual phagocytic activity in *med* macrophages is completely TTX-resistant, ruling out the contribution of NaV 1.2 in this process. Taken together, these results suggest that $Na_V1.6$ is required for full expression of phagocytosis; mechanisms that not require TTX-sensitive VGSC still enable phagocytosis, although at a lower extent.

The relationship between Na^+ channel expression and morphological and functional activation of microglial cells has been studied *in vivo* mainly in the experimental allergic encephalomyelitis (EAE) setting, a condition induced by mice immunization with myelin AGs and deemed to mimick autoimmune demyelinization. During EAE, $Na_V1.6$ levels are upregulated up to four-fold both as protein and as mRNA, an increase that closely follows the trend of CD45 and OX-2 activated-immunophenotype marker expression, as well as the characteristic morphological changes. A smaller but significant upregulation of $Na_V1.2$ could also be detected [40].

The intracellular Ca^{++} increase and subsequent activation of signaling pathways have been implicated in microglial cell activation and morphological changes [42]. VGSC might be involved in this pathway, since VGSC-dependent Na^+ influx may increase intracellular Ca^{++} levels through the Na^+/Ca^{++} exchanger (see above).

This view is supported by the effect *in vivo* of DPH on microglial cells during an inflammatory process. Without affecting microglial cell number *per se*, DPH is able to reduce up to fourfold the number of activated microglial cells in EAE mice spinal cord, corresponding to an improvement in murine neurologic impairment [40].

These data have potential relevance to human diseases since in multiple sclerosis brain samples an increase in NaV1.6 expression can be traced in microglial cells in the outer rim of demyelinating plaques and up to seven-fold upregulation is detectable in macrophages located inside plaques [40].

VGSC BLOCKERS AS IMMUNOMODULATORS: CLINICAL EVIDENCES

The evidence that VGSC are relevant to immune cell function through the availability of inhibitors of VGSC, attributes a putative role of DPH and related substances as an immunomodulator in inflammatory and autoimmune diseases. A few preliminary reports have provided evidence for a possible therapeutic application of DPH in RA. In a small open trial, 35 patients affected by RA were treated with oral DPH and a significant improvement in several clinical parameters was observed [43]. Similar results were reported by a small open study in which 18 RA patients were recruited [44]. A larger, double-blinded trial involving 100 RA patients, randomized to receive DPH, auranofin or chloroquine showed that DPH was as effective as chloroquine in improving clinical endpoints. In particular, DPH efficacy was even higher than that of chloroquine when patients with a disease history shorter than 6 months were compared. Interestingly, DPH effect was somewhat lower in patients with longer disease history [27]. Notably, the number of patients experiencing DPH side effects was comparable to that of chloroquine and auranofin-treated patients [27]. Although these as well as other preliminary data suggest a possible role for DPH in clinical practice [37], to our knowledge, no randomized, double-blinded clinical trials have been performed to date.

On the other hand, several immune-mediated adverse effects have been reported during DPH treatment and with other VGSC blockers. DPH treatment has been related to subacute lupus appearance [45], dermatomyositis [46], vasculitis [47], and systemic lupus erythematosus [48]. Similar lupus-like reaction , with detection of antibodies directed against chromatin and double-stranded DNA, have been described in patients taking procainamide, a VGSC blocker used as an antiarrhythmic drug. Likewise, a similar autoimmune reaction could be induced in mice injected intrathimycally with procainamide active metabolites [49]. It is not clear so far, how much of these effects may be due to VGSC blockade *in vivo*. However, when considering those *in vitro* studies which support VGSC involvement in several immune cell functions, it is possible to speculate that both immunosuppression and autoimmune induction may arise from a common pathogenic denominator involving the amplification of Th2-mediated responses at the expense of Th1-polarized responses.

NEW PATENTS FOR VGSC BLOCKERS

VGSC blockers are already used in several clinical settings and some of them have a very long clinical record. However, many of these molecules are known to induce frequently side effects (notably rash) that may hinder further clinical applications as immunomodulators. In this respect, some recently patented molecules should be noted, in particular [50], [51] and [52]. They have been developed as derivatives of aryl substituted pyridines, pyrimidines, pyrazines and triazines carbocyclic [50] and heterocyclic substituted semicarbazones and thiosemicarbazones [51] and [52]. Both groups have been tested *in vivo* for anticonvulsants ability or local anesthetic properties; however, none has been tested for immunomodulatory functions. It is suggested that these molecules might be worth testing as immunomodulators. In particular, *in vitro* preliminary assay using flow cytometry might be useful for screening their ability in blocking Ca^{++} surge in B and T cell after activation. Thereafter, investigations about their effect on T cell cytokine pattern may be worthwhile. These molecules might be tested in animal models of autoimmune disease to detect any clinically relevant action. It might be anticipated that molecules designed to avoid blood-brain barrier crossing may be devoid of CNS side effects common to VGSC blockers used as anticonvulsants.

CURRENT AND FUTURE DEVELOPMENTS

VGSC appear to be widely expressed throughout the immune system and a number of experimental data seem to support their involvement in the regulation of both innate and adaptive immune responses.

However, in the light of the great development of cellular and molecular immunology in recent years, VGSC's role is still obscure for the largest part. In particular, there is a lack of data regarding VGSC function in cellular signaling in lymphoid cells and no details are known about both upstream and downstream events during lymphocytes activation as well as VGSC modulation by cytokines. Finally, VGSC's influence on the function of APC, $CD8^+$ lymphocytes, regulatory T cells and B lymphocytes subsets has been poorly investigated. Moreover, many VGSC roles are inferred by experiments using drugs (such as DPH and amiloride) which targets more several cellular targets (beyond their estabilished action as VGSC blockers); these results should be complemented by experiments using more specific blockers (TTX, saxitoxin) or gene-knockout techniques.

At least three classes of widely used drugs are known as VGSC blockers: anticonvulsants (phenytoin, and carbamazepine), local anesthetics (lidocaine), antiarrhythmics (procainamide); new molecules have been patented more recently [50, 51] and [52]. Yet, data about their activity on immune system are limited. Despite many reports describing immunological dysfunctions in patients treated with DPH, CBZ and other VGSC blockers, very few studies have been conducted to investigate properly these effects. Moreover, many of these researches have been run over a period exceeding 30 years, sometimes using methodology now largely outdated and producing data not easily interpretable in the light of current immunological models. Furthermore, several VGSC blockers (e.g. lamotrigine, oxcarbazepine) have been not studied yet for their immunomodulatory properties.

It is hypothesized that VGSC modulators might find suitable application in clinical practice in several settings. In autoimmune disease therapy, VGSC inhibitors might be used to reduce Th1 detrimental responses and macrophage inflammatory response. In particular, these effects might be even more appealing in neurological conditions such as multiple sclerosis or traumatic spinal cord injury, in the light of recently described direct neuroprotective effects of VGSC blockers [53]. In particular, VGSC phenytoin and flecainide were proven effective in ameliorating axonal loss both in chronic-relapsing and progressive models of EAE [53,54] and in a model of autoimmune demyelinating neuritis, an inflammatory pathology affecting peripheral nervous system myelin [55]. However, multiple mechanisms have been proposed to account for axonal damage in MS. Na^+ channels expression increased on demyelinated axons in optic nerve and VGSC, usually restricted to Ranvier's node, are diffusely detectable on axonal membrane. Thus, axonal damage might result from increased intracellular Ca^{++} load resulting from Na^+/Ca^{++} antiporter activation driven by VGSC-dependent Na^+ influx [56,57]. On the other hand, DPH can also downregulate also inflammatory reaction in EAE, resulting in reduced macrophages infiltration in CNS [40]. Thus, it is conceivable that DPH and other VGSC blockers may exert therapeutic action in MS targeting both VGSC expressed on axonal membranes, reducing axonal Ca^{++} overload, and VGSC expressed on macrophages and T lymphocytes, reducing inflammatory infiltration and macrophage activation in CNS. Possible side effects due to excessive blockage of axonal VGSC and consequent reduction of axonal excitability have been anticipated for VGSC blocker use in MS. However, given the low dose of VGSC required for therapeutic effects in mice models, these effects seem unlikely. Moreover, several MS patients already use VGSC blockers at therapeutic doses without any side-effects [57].

Inflammatory bowel disease (IBD) may offer another target for immunomodulation by VGSC blockers. According to our own experience on IBD [58,59] and Hepatitis C virus (HCV) infection[59,60] , where an exaggerated Th1 response is responsible for a chronic inflammatory status, the use of DPH as well as other VGSC blockers could be envisaged. At the same time, the possibility to analyze the inflammatory and regulatory cytokine content of PBL and monocytes by flow cytometry could be very useful for understanding the mechanisms of action of these drugs [61,62]. Finally, it was previously reported that in some instances, PBL from both IBD and HCV patients may undergo an increase in intracellular Ca^{++} concentration through an exaggerated Ca^{++} influx [63]. It would be very interesting to evaluate either the role of VGSC in the immune cells of these patients or the potential use of VGSC blockers when PBL have lost immunocompetence because of an exaggerated Ca^{++} influx.

DPH may be considered as a prototype VGSC blocker because of its very long clinical record. However, considerable side effects have reduced its use in recent years. Nevertheless, DPH is the only VGSC blocker for which

several experimental investigations concerning its effect on immunological parameters have been performed. Even if DPH may not enter in clinical practice as immunomodulatory drug, its actions suggest that a throughout analysis of immunomodulatory effects of other VGSC blockers (including molecules with better side-effects profile e.g. oxcarbazepine, lamotrigine and newly patented molecules) may be worthwhile.

ACKNOWLEDGEMENTS

Paper supported in part by grants from MIUR and Ministero della Salute (Rome, Italy).

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