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THE LYMPHOCYTIC CHOLINERGIC SYSTEM AND ITS MODULATION BY ORGANOPHOSPHORUS PESTICIDES

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Abstract. Clinical and basic mechanism observations reveal interactions between neural and immune systems. These two systems create a complex network for recognizing danger to the host and its protection from outside pathogenic elements as well as from inside overreactions of inflammatory character. Here, we review the interactions of these two systems in relation to the effects of pesticides that clearly involve elements of cholinergic lymphocytic system. We discuss cellular and soluble elements of the immune system, which may be affected by pesticide exposure. We suggest that in-depth studies of the influence of pesticides on lymphocytes may contribute to the development of sensitive methods of measuring early adverse effects appearing in response to pesticide exposure.

Key words:
Pesticide, Lymphocytes, Cholinergic system, Acetylcholine

INTRODUCTION

Environmental toxicants that interact with specific components of the immune system can damage immunocompetence by direct interaction with one or more types of the cell participating in an immune response and adversely affect their function. They may also indirectly affect the immune function through other organ systems (nervous and neuroendocrine). Among different groups of environmental toxicants, organophosphorus (OP) pesticides play a particular role. OP pesticides penetrating the environment enter the human body and exert diversified toxic effects on the immune system. Immunotoxic effects of OP pesticides may be manifested by decreased immunity of the organism (immunosuppression), whereas its overactivation may induce hypersensitivity (allergy, autoimmunization) [1,2]. Relatively not much is known about the immunotoxic effects of OP pesticides [3], generally known as neurotoxins. As such, our current knowledge about their potential toxic effect on the immune system may be hypothetically derived from studies of the nervous system [4].

All OP pesticides have anticholinesterase (AChE) activity and a common mechanism of toxicity. Phosphorylation of AChE causes accumulation of acetylcholine (ACh), overstimulation of cholinergic receptors, and consequently clinical signs of neurotoxicity. However, additional macromolecular targets, e.g., direct action of some OP pesticides on cholinergic receptors, may alter the cascade of the events that follow AChE phosphorylation and thereby

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modify the common mechanism [5]. Because cells of the immune system, mainly lymphocytes, express all cholinergic components found in the nervous system, it is probable that OP pesticides may similarly exert toxic effects on this system. These pesticides may also indirectly affect immune function by acting on the central nervous system (CNS). Cells of the neuroendocrine system can be both the primary target of OP pesticides toxicity and the source of hormones and neurotransmitters possessing immunomodulatory activity. Thus OP pesticide-induced alterations of neuroendocrine function could be an indirect mechanism responsible for an immunotoxic event.

LYMPHOCYTIC CHOLINERGIC SYSTEM

Lymphocytes constitute a cholinergic system that is involved in the regulation of immune function [6]. They express most of the components found in cholinergic nerves, including ACh, choline acetyltransferase (ChAT), high affinity choline transporter (ChT), muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively), and AChE [7]. On that basis, it was widely believed that the lymphocytic cholinergic system can participate in various neuro-immune interactions and contribute to the "cholinergic anti-inflammation pathway". The "cholinergic anti-inflammation pathway", discovered recently [8–10], provides an efficient mechanism for neural inhibition of inflammation and interfaces CNS with immune system [9,11]. The finding that acetylcholine-secreting neurons of the parasympathetic nervous system suppress acute inflammation has coined for such a phenomenon the term "inflammatory reflex" [9]. An electron microscopic study has shown the existence of synaptic-like contact between nerve terminals and lymphocytes in thymic tissue [12]. Sympathetic and vagus innervation of the thymus, liver, heart, lungs, gastrointestinal tract, pancreas, and kidneys may provide the anatomical basis for co-regulation of tissue macrophages, dendritic cells, mast cells, Kupffer cells and other immune and non-immune cytokine-producing cells. The possibility that noradrenaline released from sympathetic varicosities may influence thymocytes is strengthened by the fact that β-adrenoreceptors are expressed in the process of the thymocytes development and the ability of catecholamines to influence the responsiveness of the expression of T allo-antigens on these cells [13]. Thymic epithelial cells (TEC) form a microenvironment that influences maturation and differentiation of thymocytes to T lymphocytes. The demonstration of their capacity to respond to catecholamines suggests that adrenergic stimulation may interfere with the regulation of immune functions. In particular, catecholamines influence the synthesis of IL-6, which is known to affect the T cell proliferative/differentiative program [14]. It has been hypothesized that ACh also plays a role in the regulation of differentiation and maturation of thymocytes. ChAT positive nerve profiles were observed on days 17/18 of gestation [15]. Biological actions of ACh in the thymus are mediated through its interactions with cholinergic receptors. DNA sythesis of thymocytes significantly increases when cells are stimulated with ACh or muscarinic cholinergic agonists [16]. Cholinergic stimulation increases intracellular second messenger, inositol 1,4,5-triphosphate (IP3) and guanosine 3',5'-cyclic monophosphate (cGMP). The increase in IP3 and cGMP concentrations after cholinergic stimulation enhances thymocytes DNA synthesis. This suggests that differentiation and maturation of thymic lymphocytes may be indirectly regulated by TEC stimulated with cholinergic agonists. ACh takes part in the mutual interplay between developing T cells and thymic epithelium, and thereby may influence the generation of T cell repertoire. Moreover, cholinergic agonists may influence T cell maturation affecting negatively thymocite apoptosis [17–19].

A functional role of cholinergic innervation of the hemopoietic compartment was also recently proposed in view of the demonstration of ChAT-immunoreactive nerve fibre-like structures in rat femur bone marrow around hemopoietic islets [20]. However, the fact that ACh released from cholinergic nerve terminals is extremely labile in blood due to the presence of cholinesterases greatly undermines the notion that ACh released from cholinergic nerve terminals can interact directly with AChRs on lymphocytes. It seems far more likely that non-neuronal ACh, released from T cells, and such cells as keratinocytes,

vascular endothelial cells or epithelial cells in the respiratory and gastrointestinal tracts, modulates local antigen presenting cells (APCs) activity via interaction with cell surface molecules [7,21–23]. It was confirmed that a considerable amount of ACh is contained in T lymphocytes and B lymphocytes [24]. T cells contain about three times more ACh compared with B cells, and CD4 positive cells show significantly higher levels of the transmitter compared with CD8 positive cells. Choline uptake via ChT is the rate-limiting step in ACh synthesis catalyzed by ChAT in T lymphocytes. Whether ACh in lymphocytes is stored in vesicles is a matter of dispute [25]. According to some authors no structures histologically resembling synaptic vesicles have been detected in lymphocytes [26]. Probably ACh is synthesized by T lymphocytes when necessary and directly released [27]. However, the possibility that ACh in T lymphocytes is localized within a storage apparatus of some type cannot be ruled out. Tayebati et al. [28] have shown vascular ACh transporter (VAChT) immunoreactivity in both, T and B peripheral blood lymphocytes. An investigation performed with help of confocal laser microscopy pictures showing VAChT immunoreactivity in vesicle-like structures suggest that lymphocytes share with neurons the same ACh storage system. Studies performed to detect the mRNA for ChAT and to measure ACh in thymic, splenic and peripheral blood lymphocytes have shown that cells of the immune system are capable of synthesizing, storing and releasing ACh [29].

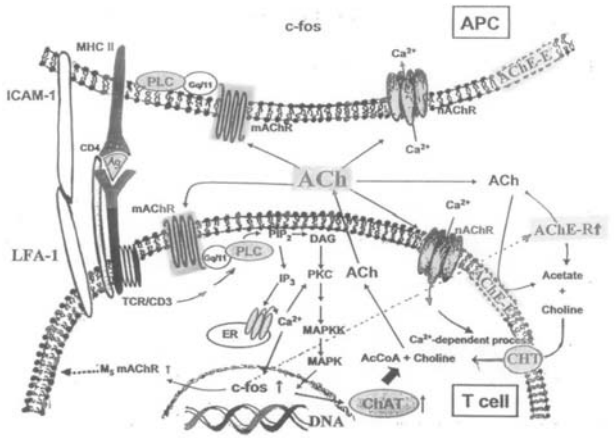
AChE activity and immunoreactivity with anti-AChE antibodies have been detected in lymphocytes. Although Szelenyi et al. [30] detected AChE activity in T cells but not in B cells, Ando et al. [31] and Tayebati et al. [28] found its expression in both subpopulations of lymphocytes. AChE gene is organized and sequentially spliced giving a distinct domains in its protein products. They include sites for alternative splicing of the pre-mRNA at both the 5' and 3' ends. Alternative splicing allows the production of three distinct AChE variants (isoforms), "synaptic" (S), "erythrocytic" (E) and "readthrough" (R), each with a different carboxy (C)-terminal sequence. C-terminal sequences determine homologous assembly into AChE oligomers and their heterologous association with non-catalytic subunits that direct the subcellular localization of the protein. AChE-S forms tetramers that can attach covalently to a collagen-like protein. In AChE-E, a glucosyl bond near the C-terminus undergoes transamidation to attach the glycophosphatidylinositol group to protein, which anchors the mature AChE-E to the outer surface of cells. AChE-R does not seem to have any feature that allows for its attachment to other molecules and remains monomeric and soluble. AChE-R appears in lymphocytes when these cells are induced under chemical or physical stress [32].

Physiological cues that induce AChE gene transcription include cell differentiation, reduced AChE levels, ACh-mediated excitation elicited by exposure to anti-AChE agents, e.g., some OP pesticides, and various traumatic insults. Transcriptional activation of AChE gene is often associated with a shift in its splicing pattern, leading to accumulation of the rare AChE-R variant [33,34]. Recent analyses show that human lymphocytes (T and B) and APCs (dendritic cells and monocytes/macrophages) express both mAChRs and nAChRs. Messenger RNA encoding the M3, M4 and M5 subtypes of mAChRs has been detected in most human mononuclear leukocytes (MNLs), while expression of mRNAs encoding the M1 and M2 subtypes varied substantially among individual subjects [28].

Ligand binding studies demonstrating the presence of nAChRs on human lymphocytes T and B and monocytes/macrophages [35] have been confirmed by examination of functional and metabolic effects of nicotine and other nAChR agonists [25]. Sato et al. [36] analyzed mRNAs encoding nAChR subunits in human MNLs and detected expression of the $\alpha 2$, $\alpha 5$, $\alpha 7$, $\alpha 10$ and $\beta 2$ subunits. It has been shown that CNS can downregulate inflammation by the $\alpha 7$ subunit-mediated inhibition of synthesis of tumor necrosis factor-α (TNF-α). The functional relevance of the macrophage nicotinic receptor $\alpha 7$ subunit has been tested using antisense oligonucleotides [37] and in the $\alpha 7$ subunit-deficient mice [38]. In the presence of nicotine, inhibition of the $\alpha 7$ subunit restores the endotoxin-stimulated TNF-α response; whereas gene knock-out mice are more sensitive to inflammatory stimuli by producing significantly higher levels of serum TNF-α, IL-1 and IL-6 during endotoxemia.

THE EFFECT OF CHOLINERGIC SYSTEM ON LYMPHOCYTIC FUNCTION

According to Kawashima and Fuji [6] stimulation of T and B cells with ACh elicits intracellular Ca^{2+} signaling, up-regulation of *c-fos* expression, increased nitric oxide synthesis and IL-2-induced signal transduction. Acute stimulation of nAChRs with ACh causes rapid and transient Ca^{2+} signaling in T and B cells, probably via α7 nAChR subunit-mediated pathways. Chronic ACh stimulation by contrast down-regulates nAChR expression and suppresses T cell activity. Activation of T cells with phytohemagglutinin or antibodies against cell surface molecules enhances lymphocytic cholinergic transmission by activating expression of ChAT and M3 or M5 mAChR. Activation of M3 or M5 AChRs generally increases phosphoinositide-specific phospholipase C activity and the release of the second messenger – inositol triphosphate. Figure 1 illustrates numerous transduction and regulatory pathways that affect and are affected by the lymphocytic cholinergic system during immunological responses. Stimulation of T and B cells by ACh via mAChRs induces intracellular Ca^{2+} signaling that triggers nuclear signaling and up-regulates gene expression, e.g., *c-fos*. This signal transducing factor has been shown to be induced in cells of central autonomic network by peripheral administration of lipopolysaccharide (LPS) and can be completely blocked by the dorsal vagal complex inactivation [39–41]. In this view, it has been proposed that area postrema may play a role in transducing immune signals relevant to regulation of neural behavior. Area postrema is a component of the dorsal vagal complex (DVC), and has a weak blood-brain-barrier that may provide a contact with circulating mediators induced by LPS. In addition, area postrema contains immune cells that express LPS receptors [42]. There is evidence that area postrema lesion blocks IL-1-induced elevation of plasma adrenocorticotropin and corticosterone as well as *c-fos* expression in the paraventricular nucleus (PVN) [43]. The PVN of DVC-inactivated animals shows the attenuation of LPS-induced c-Fos-IR, compared with controls [44]. Previous studies demonstrated a critical role of catecholaminergic brainstem projection neurons in the mediation of hypothalamic pituitary axis (HPA) activation in response to peripheral immune activation [45]. Taken together, these findings support the hypothesis that neuroimmune activation through acetylcholine mediators and cholinergic system may act through *c-fos* activation. Lymphocytes not only directly react to ACh, but also through stimulation of TCR/CD3 and other cell surface molecules, and enhance this reactivity by the increased expression of both ChAT and mAChRs [6]. Muscarinic AChRs are involved in the enhancement of TCR-induced interleukin (IL)-2 production and IL-2 receptor expression in human T lymphocytes [46]. Thus mAChRs positively modulate cell growth in human lymphocytes by the autocrine mechanism [47,48].



ACh – acetylcholine; AChE-E – erythrocytic acetylcholinesterase isoform; AChE-R – readthrough acetylcholinesterase isoform; AcCoA – acetycoenzyme A; ChAT – choline acetyltransferase; CHT – high affinity choline transporter; DAG – diacyl glycerol; ER – endoplasmic reticulum; ICAM – 1 – intracellular adhesion molecule – 1; IP-3 – inositol-1,4,5-triphosphate; mAChR – muscarinic Ach receptor; MAPK – mitogen activated protein kinase; MAPKK – MAP kinase kinase; MHC II – major histocompatibility complex class II; nAChR – nicotinic Ach receptor; PIP2 – phosphatidylinositol 4,5-biphosphate; PKC – protein kinase C; PLC – phospholipase C; TCR – T cell receptor.

Fig. 1. Diagram illustrating numerous transduction and regulatory pathways that affect and are affected by the lymphocytic cholinergic system upon interaction of lymphocytes (T cells) with antigen presenting cells.

Anticholinesterase insecticides that are mainly represented by carbamates and organophosphates produce acute toxicity via inhibition of AChE, a serine hydrolase. Inhibition of AChE occurs as a result of carbamylation or phosphorylation of serine hydroxyl at the active site of the enzyme. Serine hydrolase activity appears to be integral to diverse immune functions including: 1) serum complement activation [49], 2) target cell killing by T cells [50] and natural killer (NK) cells [51,52], 3) antigen stimulated

Ca^{2+} signaling in cytolytic T cells [53], 4) IL-2 signaling in lymphocytes [54], and 5) neutrophil chemotaxis [55], phagocytosis [56], and secretion of TNF-α by monocytes [57]. It has been shown that chemicals able to inhibit AChE activities, like OP and carbamate insecticides, act on lymphocyte proliferation, endotoxin-induced secretion of TNF-α from monocytes and IL-2 driven activity of NK activity, IL-2 driven cells. Carbamylation of cholinesterase and of serine hydrolase also share the same catalytic triad made of serine hydroxyl group, the imidazolium group of a histidine residue and δ-carboxylate of an aspartic acid residue [58]. It is, however, not yet clear if the signal transduction pathway triggered by carbamylation of serine hydrolases and AChE also involves the NF-κB factor. It is reasonable to expect that cross talk or pathway converge occurs when two different signals mediate a common effect. The NF-κB transcription factor pathway may be a convergence point. As a fact, one of the ways by which corticosteroids mediate part of their anti-inflammatory effect is the inhibition of NF-κB activity through the induction of the endogenous inhibitor IκBα [59,60].

Normally, upon interaction of T lymphocytes via TCR/CD3 and CD4 or CD8 with antigen presenting cells, or interaction via cell surface molecules with vascular endothelial cells or inflammatory cells, T cells show enhanced synthesis and release of ACh. This in turn can act, in autocrine way, on mAChRs and nAChRs of T and B cells or other targets in the vascular microenvironment [27]. Acetylcholine is effective in suppressing endotoxin-inducible pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-18. However, the role of anti-inflammatory cytokine IL-10 in this process is doubtful since its release from endotoxin-stimulated macrophages is not affected by acetylcholine. Stimulation of T and B lymphocytes by phytohemagglutinin (PHA) and B-cell activator – Staphylococcus aureus Cowan I – activates the lymphoid cholinergic system as evidenced by increasing synthesis and release of ACh and increased expression of mRNAs encoding ChAT and ACh receptors [7,46,61]. Mitogenic stimulation with PHA increases ACh levels in lymphoid cells and its release in supernatants [24]. Hence stimulation of lymphocytes with PHA activates the lymphoid cholinergic system by inducing an increased synthesis and release of ACh and augmenting the expression of mRNA encoding ChAT and cholinergic receptors.

THE *IN VIVO* EFFECTS OF LYMPHOCYTIC CHOLINERGIC SYSTEM

The expression of mAChRs subtypes was investigated in peripheral blood lymphocytes of bronchial asthma patients [62]. An increased expression of M2 and to a lesser extent of M5 receptors and no changes in M4 receptor were observed in blood lymphocytes of asthmatics compared to the control group. The increase was related to bronchial hyperresponsiveness detected by methacholine challenge test. However, in experiments using guinea pigs, it was shown that chlorpyrifos which inhibits AChE and decreases M2 receptor responsiveness enhances bronchoconstriction [63]. Thus the role and association of M receptors with asthma is still not clear.

Various types of transmitters of the neuroendocrine-immune network, including acetylcholine, may mediate abnormalities in the immune function. Allergic diseases such as allergic rhinitis, atopic dermatitis, gastro-intestinal allergies, and asthma seem to occur through the overproduction of neuropeptides and cytokines [64].

Another evidence that changes in lymphocytic cholinergic activity are related to the immune dysfunction is derived from studies on the immune deficiency rat model, spontaneously hypertensive rats (SHRs) derived form the Wistar Kyoto rats (WKYs), and the immune accelerated mouse model, MRL/MpJ-Ipr/Ipr (MRL-Ipr) [65]. SHRs are known to exhibit immune deficiencies resulting from the emergence of a natural thymocytotoxic autoantibody, an age-related decline of T cell function and morphological changes in immune organs. Fujimoto et al. [29] discovered that ACh content in blood, MNLs (mononuclear leukocytes), thymus and spleen is significantly lower in SHRs than in age-matched WKYs, as is expression of ChAT mRNA in circulating MNLs. Changes in the lymphocytic cholinergic system thus reflect immune deficiency related to T cell dysfunction and support the physiological role of ACh in immunomodulation. MRL-ipr mice spontane-

ously develop a lupus-like autoimmune syndrome, the symptoms of which include nephritis due to production of antinuclear antibodies associated with massive lymphoadenopathy related to expansion of a unique T cell subset expressing Thy-1, CD3 and B220. Fujimoto et al. [66] found that the ACh content in the blood, thymus and spleen of MRL-1pr mice was significantly higher than in the age-matched MRL/MPJ +/+ (wild type) and BALB/c (control) mice.

It has been reported that centrally acting pharmacological agents, like CNI-1493, induce vagus nerve firing [67] and confer anti-inflammatory effects through activation of the cholinergic anti-inflammatory pathway in both local and systemic models of inflammation [68].

Collectively, these findings are consistent with a notion that lymphocytic cholinergic activity is related to the immune system function and ACh, synthesized and released from T lymphocytes, acts as an autocrine and/or paracrine factor regulating immune function. These findings also suggest that a better understanding of the lymphocytic cholinergic system can provide important information about regulatory mechanisms that govern lymphocyte function and the way they may be affected by anticholinesterase compounds such as OP pesticides.

ORGANOPHOSPHOROUS PESTICIDES ARE CAPABLE OF ALTERING LYMPHOCYTIC CHOLINERGIC SYSTEM

All OP pesticides have anticholinesterase properties and a potential common mechanism of immunotoxicity, i.e., phosphorylation of AChE responsible for the accumulation of ACh and overstimulation of lymphocyte cholinergic receptors. At least four steps have to be involved in a cascade of reactions culminating in overt toxicity: 1) binding to and inhibition of an extensive number of AChE molecules with substantial impairment of ACh degradation; 2) accumulation of ACh in lymphocytes or in so called "immunological synapses" formed between lymphocyte and antigen presenting cell; 3) excessive stimulation of lymphocyte cholinergic receptors; and 4) altered lymphocyte cellular functions in response to excessive stimulation of those receptors. Modulation by OP pesticides of any of the processes involved in acetylcholine synthesis, acetylcholine release, cholinergic receptor binding, or signal transduction, concurrent with anticholinesterase exposure, could therefore influence the progression of events from target enzyme (AChE) inhibition to expression of the effects of toxicity.

The mechanism of AChE inhibition in T lymphocytes by OP pesticides may be similar to that observed in nerve cells [5]. Organophosphorus insecticides have been shown to cause a decrease in cholinergic muscarinic receptor mAChR in the brain and peripheral tissues. These changes are believed to be involved in the development of tolerance to OP toxicity. mAChRs identified in circulating lymphocytes have been shown to be modulated similarly to the brain mAChRs following repeated OP exposure. Similarly, lymphocyte AChE activity was significantly inhibited and well correlated with the brain AChE activity during exposure, but the recovery was rapid relative to AChE activity in the brain [69]. OP pesticides act by phosphorlylating active serine site residue on AChE and thereby inhibit the catalytic degradation of ACh. AChE inhibition might evoke increase in the level of ACh in extracellular medium, e.g., blood plasma. However, high levels of butyrylcholinesterase (BChE) in blood and its activity in metabolizing ACh makes unlikely the detection of ACh in blood or plasma. Surprisingly to these suggestions, Kawashima et al. [27], by using sensitive and specific radioimmunoassay for ACh detected significant amounts of ACh in the human blood and plasma (8.66 + 1.02 and 3.12 + 0.36 pmol ACh/ml, respectively). Thus these extra levels of ACh, resulting from inhibition of AChE and BChE by OP pesticides together with those from T lymphocytes, may act as a modulator of immune responses.

T cells recognize antigen peptides through a nanometer scale gap between T cell receptor and major histocompatibility complex (MHC) on APCs, referred to as an immunological synapse [70]. Membrane domains (LFA-1 and ICAM-1) provide an organizational principle for compartmentalization within the immunological synapse that may help account for the longevity and specificity of signaling. Immunological synapse may form a microenvironment

in which T cell-released ACh is restricted from reaching BChE. Thus ACh may achieve higher concentration, in particular when BChE and membrane bound AChE (isoform AChE-E) are inhibited by OP pesticide. ACh released from T cells induces (by autocrine mechanism) intracellular Ca^{2+} signaling in T and B cells via M3 and/or M5 mAChRs, leading to *c-fos*-mediated up-regulation of gene expression [7,25]. The presence of *c-fos*-binding sites in the promoters of key cholinergic genes (e.g., genes encoding AChE or ChAT in T cells) indicated that elevated *c-fos* levels might activate regulatory pathways leading to changes in the expression of proteins, mediating T cell immune response [25,47,71].

Organophosphorus pesticides, first elicit a transient increase in the amounts of ACh by increasing the survival of ACh at the immunological synapse and then increase T cell ChAT activity. This primary phase is connected with enhanced immunological activity of T cells and followed by secondary phase of suppressed T cell activity with stimulation of AChE synthesis and suppression of ChAT synthesis. The secondary phase represents a response of acute activation of AChRs and reduction in the bioavailability of ACh [35]**.** We speculate that in the late phase, an elevated *c-fos* protein level might activate only readthrough AChE (AChE-R) isoform synthesis. After exposure of T cells to OP pesticides (AChE inhibitors), a pronounced increase is observed in the levels of this unspliced mRNA species in which pseudo-intron 4 is retained in the mature transcript, encoding AChE-R isoform. No changes were seen in either the transcript containing the alternative 3' exon 6 and encoding the synaptic form of the enzyme (AChE-S) or in the transcript carrying alternative exon 5 and encoding the hematopoietic form of AChE (AChE-E). Thus in the late phase, OP pesticides mediated not only enhanced transcription AChE gene in T cell, but also modified alternative splicing from this gene, leading to *de novo* synthesis of the unique, secretable AChE-R isoform [33]. T cell-secreted AChE-R appears as monomer and remains soluble in the immunological synapse and thus it may possibly ease ACh hydrolysis.

Increased ACh concentration in immunological synapse after exposure to OP pesticides may be also involved in autocrine activation of T cells through stimulation of mAChRs and thus in the induction of IL-2 production and IL-2 receptor expression. Nomura et al. [48] indicated that mitogen-activated protein (MAP) kinases, extracellular signal-regulated protein kinases (ERK) and the *c-jun* NH_2-terminal kinases (JNK), but not the p38 MAP kinases, are involved in the mAChRs-mediated enhancement of IL-2 production by stimulating AP-1 activity. There are some reports that MAP kinase signal transduction pathways such as: 1) Ras, the JNK cascade, and one or more of AP-1, and 2) Raf-1, MEK1 and ERK, are involved in IL-2 gene transcription in T cells [72,73]. Therefore, ERK and JNK may activate AP-1 via activation of *c-fos* and *c-jun*, respectively [73]. Thus the transcription factor AP-1 and MAP kinase signal transduction pathway seem to be involved in the AChR-receptor-mediated enhancement of IL-2 synthesis by T cells and activation of their proliferation after ACh concentration increase in immunological synapse after inhibition of AChE activity by OP pesticides (Fig. 2).

The study of toxic mechanisms of OP pesticides has earlier focused on their interaction and irreversible inhibition of BChE and AChE, and little was known about interaction with ACh receptors (AChRs). Such an action of OP pesticides was evaluated by Pope [5]. While it is difficult to compare relative potencies between reversible (i.e., receptor binding) and irreversible (i.e., BChE or AChE phosphorylation) interactions, extremely low concentrations of OP pesticides required to inhibit T cell AChE compared to those necessary for interaction with nAChRs suggest that such additional actions on nAChRs may have, under most conditions, little practical relevance. In contrast, several studies [5] have reported that some OP pesticides can

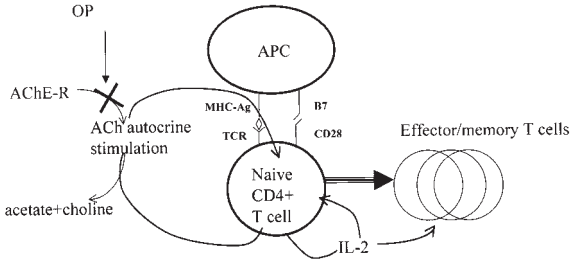


Fig. 2. Diagram illustrating the effect of OP pesticides on T cell differentiation.

interact directly with mAChR at much lower concentrations and may change the amount of ACh present in immunological synapse.

Modulation of ACh synthesis by T cell and its release to immunological synapse may play an important role in toxicity of some OP pesticides. High-affinity choline uptake, the rate-limiting step in ACh synthesis in lymphocytes, may be reduced by some OP pesticides which possess a similar anti-cholinesterase potency but different acute toxicity. Choline uptake by T cell is regulated by intracellular cAMP levels, which in turn can be affected by activation of M4 mAChR. There is some evidence that such a receptor is also present in T cells [6,25]. We might speculate that some OP pesticides, e.g., parathion and chlorpyrifos (their oxons), can selectively activate these subtypes of muscarinic receptors, and cAMP formation could be reduced with a concomitant reduction in choline uptake and ACh synthesis. The direct activation of M4 receptor by both chlorpyrifos oxon and paraoxon could therefore reduce ACh synthesis and indirectly limit the amount of acetylcholine accumulated in the immunological synapse following BChE and AChE inhibition. Chlorpyrifos oxon is more potent at this limiting action and its toxicity is less acute than that of paraoxon. Li et al. [74] found that some of OP pesticides, e.g., dimethyl 2,2-dichlorovinyl phosphate and diisopropyl methylphosphonate markedly inhibit the activities of natural killer cells and cytotoxic T lymphocytes. These pesticides probably inhibited granzyme activity by decreasing choline uptake and ACh synthesis in lymphocytes and killer cells.

The presented information provide a compelling picture in which lymphocytes constitute a cholinergic system involved in the regulation of immune function. Activation of T cells mediated by APCs (dendritic cells or macrophages) or PHA enhances lymphocytic cholinergic transmission by activating ACh synthesis. This function of T cell cholinergic system may be disturbed by OP pesticides. Organophosphorus pesticides may directly modulate this system by increasing ACh concentration in immunological synapse (when T cell interact with APC) or directly act on muscarinic ACh receptors.

ORGANOPHOSPHOROUS PESITICDES MAY INDIRECTLY AFFECT LYMPHOCYTES

It has been suggested that OP pesticides may also indirectly influence the immune T cell function by affecting CNS regions that possess high AChE activity and clearly detectable mAChRs activity. The hippocampus-limbic system is the region of the brain especially susceptible to OP pesticides. This system directly affects hypothalamus and brainstem involved in immune regulation [75,76]. The hypothalamus releases corticotropin-releasing factor (CRF) involved in the regulation of adrenocorticotropic hormone (ACTH) secretion from the pituitary gland. Then ACTH, through an endocrine action, regulates the release of adrenal glucocorticoids. These steroids have been known to exert strong effects on various metabolic and immunologic functions of T cells and other immune cells. CRF appears to exert also a more direct effect on the brainstem centers as well as effects on immune regulation through direct actions on the autonomic nervous system that involve neurotransmitters, epinephrine and norepinephrine. These catecholamines interact with lymphocytes to mediate suppression of the immune response by beta-adrenergic receptors [77]. The diffusible immuno-modulatory network, which includes glucocorticoids and catecholamines is rather slow, distributed in differen organs and dependent on concentration gradients. By contrast, the cholinergic immunomodulatory pathway by the vagus nerve is discrete and localized in tissues where immunological response originate [8,78]. The notion that parasympathetic nerves (cholinergic nerves) interact directly with inflammatory cells via nAChR-mediated pathways was proposed by Tracey [9] following his observations on effectively inhibited release of TNF-α from macrophages *in vitro* by ACh and nicotine.

The existence of such a functional link between the immune system and CNS suggests that the interaction of OP pesticides and/or its metabolites with a component of the hippocampus-limbic system, resulting in its changed function, may indirectly affect T cell immune functions. Such an interaction could conceivably result in either

an enhanced or suppressed release of neurotransmitters (e.g., hormones, glucocorticoids, catecholamines or ACh) possessing T cell immunomodulatory activity. Thus if a certain level of neurotransmitter or hormone influences the development and/or magnitude of an immune response, OP pesticide-induced alteration of neuroendocrine function would be an indirect mechanism responsible for an immunotoxic event. It has been suggested that the immune-to-brain communication could be achieved through two main pathways, neural and humoral. The former signals the occurring inflammation to the brain through the activation of vagus nerve sensory fibers. Immunogenic stimuli activate nervous afferents directly by releasing cytokines from dendritic cells, macrophages, and other vagal-associated immune cells, or indirectly through the chemoreceptive cells located in vagal paraganglia [78]. The transmission of cytokine-dependent signals to the brain through the vagal sensory neurons takes place according to the magnitude of the immune challenge [79–82]. It is likely that the vagal afferent neural pathway plays a dominant role in mild to moderate peripheral inflammatory responses, whereas acute, robust inflammatory responses signal the brain primarily via humoral mechanism. The humoral pathway is preferred by the immune system to communicate with the brain, especially in case of systemic immune challenge [83–86]. It is, however, still unclear how the circulating cytokines interact with brain structures involved in the anti-inflammatory response, and how they can affect synthesis of cytokines originating from CNS.

Conversely, the brain-to-immune communication proceeds via β-adrenoreceptors, catacholamines and glucocorticoids; the latter mainly through the suppression of nuclear factors.

Carbamate insecticides, like OP pesticides, inhibit ChE and induce immunosuppression by downregulating T-cell proliferation, IL-2 production, and IFN-γ production [87]. It could be speculated whether modulation of the immuno-neuro-endocrine system through the common target – AChER – is shared by both classes of pesticides.

CONCLUSIONS

Comprehension of the direct and indirect effects of organophosphorous pesticides on the immune system could lead to a better understanding of pesticide toxicity so that more effective preventive measures could be taken. In neurotoxicology, there is a need for sensitive indicators reflecting subclinical nervous system insult especially due to environmental chemicals causing neurological impairment and illness after chronic low-dose exposure. Now we can state that interaction of OP pesticides with neural cholinergic system is often accompanied by similar changes involving components of such system present in lymphocytes. On this basis, indirect strategies may be developed to investigate neural cell function parameters by methods using accessible cells like lymphocytes. The validity of surrogate markers of biochemical events occurring in the nervous system has been documented by studies performed on laboratory animals and in humans [88]. Applicability of this approach in conventional population studies of environmental OP pesticides remains to be demonstrated. However, data on the effect of OP pesticides on receptors and signal transduction pathways in peripheral lymphocytes suggest useful applications of certain surrogate markers in mechanistic *in vivo* studies of neurotoxicity of these compounds and, possibly, in assessing early biochemical effects of OP pesticides in humans. The use of peripheral lymphocytes as indicators of effects exerted by OP pesticides would offer obvious advantages. Most methods for measuring neurotoxicity of OP pesticides are highly invasive. Blood lymphocytes can be obtained in a relatively noninvasive manner from subjects who have been exposed to given OP pesticides and their use may circumvent ethical and feasibility constrains precluding direct assessment of neurotoxicity in the intact organism.

In our opinion, characterization of lymphocytic cholinergic markers may be a useful starting point for finding out whether their assessment can be used for exploring the status of homologous brain markers. The development of the same bands of immunoreactivity in lymphocytes and stratum suggests that markers in question are the same in both types of tissue [28]. The observed expression of

ACh, ChAT, AChE and VAChT by all blood lymphocytes as well as in brain cholinergic areas suggest that they may represent a more reliable marker of cholinergic neurotransmission than muscarinic receptors, of which M1 subtype is mostly diffused in the brain, but not expressed by peripheral blood lymphocytes [89–91].

REFERENCES

1. Thomas PT, Busse WW, Kerkvliet NI. *Immunologic effects of pesticides*. In: Baker SR, Wilkinson CF, editors. *The Effects of Pesticides on Human Health*. New York: Princeton Scientific Publisher; 1990. p. 261–95.
2. Zabrodskii PF, Kirichuk VF, Germanchuk VG. *Role of anticholinesterase mechanism in suppression of antibody formation during acute poisoning with organophosphorus compounds.* Bull Exp Biol Med 2001; 131: 467–9.
3. Burns LA. Meade BJ, Munson AE. *Toxic responses of the immune system*. In: Klaassen CD, Mc Graw-Hill, editors. *Casarett and Doull's Toxicology*. New York: The Basic Science of Poisons; 1996. p. 355–402.
4. Ehrich M. *Organophosphates*. In: P. Wexler, editor. *Encyclopedia of Toxicology*. San Diego, CA: Academic Press; 1998. p. 467–71.
5. Pope CN. *Organophosphorus pesticides: do they all have the same mechanism of toxicity?* J Toxicol Environ Health B Crit Rev 1999; 2: 161–81.
6. Kawashima K, Fujii T. *The lymphocytic cholinergic system and its contribution to the regulation of immune activity.* Life Sci 2003; 74: 675–96.
7. Fujii T, Kawashima K. *An independent non-neuronal cholinergic system in lymphocytes.* Jpn J Pharmacol 2001; 85: 11–5.
8. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. *Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin.* Nature 2000; 405: 458–62.
9. Tracey KJ. *The inflammatory reflex.* Nature 2002; 420: 853–9.
10. Tracey KJ, Czura CJ, Ivanova S. *Mind over immunity*. Faseb J 2001; 15: 1575–6.
11. Blalock JE. *Harnessing a neural-immune circuit to control inflammation and shock.* J Exp Med 2002; 195: F25–8.
12. Bulloch K, Pomerantz W. *Autonomic nervous system innervation of thymic-related lymphoid tissue in wildtype and nude mice.* J Comp Neurol 1984; 228: 57–68.
13. Singh U, Owen JJ. *Studies on the maturation of thymus stem cells. The effects of catecholamines, histamine and peptide hormones on the expression of T cell alloantigens.* Eur J Immunol 1976; 6: 59–62.
14. Le JM, Fredrickson G, Reis LF, Diamantstein T, Hirano T, Kishimoto T, et al. *Interleukin 2-dependent and interleukin 2-independent pathways of regulation of thymocyte function by interleukin 6.* Proc Natl Acad Sci U S A 1988; 85: 8643–7.
15. Singh U, Fatani JA, Mohajir AM. *Ontogeny of cholinergic innervation of thymus in mouse.* Dev Comp Immunol 1987; 11: 627–35.
16. Tominaga K, Hato F, Kinoshita Y, Tominaga M, Yamada M. *Enhancement of DNA synthesis in rat thymocytes by stimulating their muscarinic acetylcholine receptors.* Cell Mol Biol (Noisy-le-grand) 1992; 38: 815–22.
17. Rinner I, Kukulansky T, Felsner P, Skreiner E, Globerson A, Kasai M, et al. *Cholinergic stimulation modulates apoptosis and differentiation of murine thymocytes via a nicotinic effect on thymic epithelium.* Biochem Biophys Res Commun 1994; 203: 1057–62.
18. Rinner I, Kukulansky T, Skreiner E, Globerson A, Kasai M, Hirokawa K, et al. *Adrenergic and cholinergic regulation of apoptosis and differentiation of thymic lymphocytes.* Adv Exp Med Biol 1994; 355: 113–7.
19. Rinner I, Globerson A, Kawashima K, Korsatko W, Schauenstein K. *A possible role for acetylcholine in the dialogue between thymocytes and thymic stroma.* Neuroimmunomodulation 1999; 6: 51–5.
20. Artico M, Bosco S, Cavallotti C, Agostinelli E, Giuliani-Piccari G, Sciorio S, et al. *Noradrenergic and cholinergic innervation of the bone marrow.* Int J Mol Med 2002; 10: 77–80.
21. Grando SA, Kawashima K, Wessler I. *Introduction: the non-neuronal cholinergic system in humans.* Life Sci 2003; 72: 2009–12.
22. Fujii T, Ushiyama N, Hosonuma K, Suenaga A, Kawashima K. *Effects of human antithymocyte globulin on acetylcholine synthesis, its release and choline acetyltransferase transcription in a human leukemic T-cell line.* J Neuroimmunol 2002; 128: 1–8.
23. Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ. *The non-neuronal cholinergic system in humans: expression, function and pathophysiology.* Life Sci 2003; 72: 2055–61.
24. Rinner I, Kawashima K, Schauenstein K. *Rat lymphocytes produce and secrete acetylcholine in dependence of differentiation and activation.* J Neuroimmunol 1998; 81: 31–7.
25. Kawashima K, Fujii T. The lymphocytic cholinergic system and its biological function. Life Sci 2003; 72: 2101–9.
26. Fujii T, Yamada S, Watanabe Y, Misawa H, Tajima S, Fujimoto K, et al. *Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator.* J Neuroimmunol 1998; 82: 101–7.
27. Kawashima K, Fujii T, Watanabe Y, Misawa H. *Acetylcholine synthesis and muscarinic receptor subtype mRNA expression in T-lymphocytes.* Life Sci 1998; 62: 1701–5.

28. Tayebati SK, El-Assouad D, Ricci A, Amenta F. *Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes.* J Neuroimmunol 2002; 132: 147–55.
29. Fujimoto K, Matsui M, Fujii T, Kawashima K. *Decreased acetylcholine content and choline acetyltransferase mRNA expression in circulating mononuclear leukocytes and lymphoid organs of the spontaneously hypertensive rat.* Life Sci 2001; 69: 1629–38.
30. Szelenyi JG, Bartha E, Hollan SR. *Acetylcholinesterase activity of lymphocytes: an enzyme characteristic of T-cells.* Br J Haematol 1982; 50: 241–5.
31. Ando T, Fujii T, Kawashima K. *Expression of three acetylcholinesterase mRNAs in human lymphocytes.* Jpn J Pharmacol 1999; 79: 289P.
32. Soreq H, Seidman S. *Acetylcholinesterase – new roles for an old actor.* Nat Rev Neurosci 2001; 2: 294–302.
33. Kaufer D, Friedman A, Seidman S, Soreq H. *Acute stress facilitates long-lasting changes in cholinergic gene expression*. Nature 1998; 393: 373–7.
34. Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H. *Structural roles of acetylcholinesterase variants in biology and pathology.* Eur J Biochem 1999; 264: 672–86.
35. Kawashima K, Fujii T. *Extraneuronal cholinergic system in lymphocytes.* Pharmacol Ther 2000; 86: 29–48.
36. Sato KZ, Fujii T, Watanabe Y, Yamada S, Ando T, Kazuko F, et al. *Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines.* Neurosci Lett 1999; 266: 17–20.
37. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. *Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation.* Nature 2003; 421: 384–8.
38. Orr-Urtreger A, Goldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M, et al. *Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents.* J Neurosci 1997; 17: 9165–71.
39. Krahl SE, Clark KB, Smith DC, Browning RA. *Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation.* Epilepsia 1998; 39: 709–14.
40. Naritoku DK, Terry WJ, Helfert RH. *Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve.* Epilepsy Res 1995; 22: 53–62.
41. Marvel FA, Chen CC, Badr N, Gaykema RP, Goehler LE. *Reversible inactivation of the dorsal vagal complex blocks lipopolysaccharide-induced social withdrawal and c-Fos expression in central autonomic nuclei.* Brain Behav Immun 2004; 18: 123–34.
42. Quan N, Stern EL, Whiteside MB, Herkenham M. *Induction of proinflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat.* J Neuroimmunol 1999; 93: 72–80.
43. Lee HY, Whiteside MB, Herkenham M. *Area postrema removal abolishes stimulatory effects of intravenous interleukin-1beta on hypothalamic-pituitary-adrenal axis activity and c-fos mRNA in the hypothalamic paraventricular nucleus.* Brain Res Bull 1998; 46: 495–503.
44. Gaykema RP, Dijkstra I, Tilders FJ. *Subdiaphragmatic vagotomy suppresses endotoxin-induced activation of hypothalamic corticotropin-releasing hormone neurons and ACTH secretion.* Endocrinology 1995; 136: 4717–20.
45. Ericsson A, Kovacs KJ, Sawchenko PE. *A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons.* J Neurosci 1994; 14: 897–913.
46. Fujino H, Uehara T, Murayama T, Okuma Y, Ariga H, Nomura Y. *Extracellular signal regulated protein kinase and c-jun N-terminal kinase are involved in ml muscarinic receptor-enhanced interleukin-2 production pathway in Jurkat cells.* Biol Pharm Bull 2000; 23: 1198–205.
47. Okuma Y, Nomura Y. *Roles of muscarinic acetylcholine receptors in interleukin-2 synthesis in lymphocytes*. Jpn J Pharmacol 2001; 85: 16–9.
48. Nomura J, Hosoi T, Okuma Y, Nomura Y. *The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system*. Life Sci 2003; 72: 2121–6.
49. Arlaud GJ, Colomb MG. *Modelling of C1, the first component of human complement: towards a consensus?* [letter]. Mol Immunol 1987; 24: 317.
50. Young JD, Cohn ZA. *Cell-mediated killing: a common mechanism?* Cell 1986; 46: 641–2.
51. Hudig D, Redelman D, Minning LL. *The requirement for proteinase activity for human lymphocyte-mediated natural cytotoxicity (NK): evidence that the proteinase is serine dependent and has aromatic amino acid specificity of cleavage.* J Immunol 1984; 133: 2647–54.
52. Lavie G, Leib Z, Servadio C. *The mechanism of human NK cell-mediated cytotoxicity. Mode of action of surface-associated proteases in the early stages of the lytic reaction.* J Immunol 1985; 135: 1470–6.
53. Utsunomiya N, Nakanishi M. *A serine protease triggers the initial step of transmembrane signalling in cytotoxic T cells.* J Biol Chem 1986; 261: 16514–7.
54. Wong RL, Gutowski JK, Katz M, Goldfarb RH, Cohen S. *Induction of DNA synthesis in isolated nuclei by cytoplasmic factors: inhibition by protease inhibitors.* Proc Natl Acad Sci USA 1987; 84: 241–5.
55. Becker EL. *Phosphonate inhibition of the accumulation and retention of K+ by rabbit neutrophils in relation to chemotaxis.* J Immunol 1971; 106: 689–97.

56. Musson RA, Becker EL. *The role of an activatable esterase in immune-dependent phagocytosis by human neutrophils.* J Immunol 1977; 118: 1354–65.

57. Scuderi P. *Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor p-toluenesulfonyl-L-arginine methyl ester (TAME).* J Immunol 1989; 143: 168–73.

58. Casale GP, Vennerstrom JL, Bavari S, Wang TL. *Inhibition of interleukin 2 driven proliferation of mouse CTLL2 cells by selected carbamate and organophosphate insecticides and congeners of carbaryl.* Immunopharmacol Immunotoxicol 1993; 15: 199–215.

59. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. *Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis.* Science 1995; 270: 286–90.

60. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS Jr. *Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids.* Science 1995; 270: 283–6.

61. Fujii T, Watanabe Y, Inoue T, Kawashima K. *Upregulation of mRNA encoding the M5 muscarinic acetylcholine receptor in human T- and B-lymphocytes during immunological responses.* Neurochem Res 2003; 28: 423–9.

62. Ricci A, Amenta F, Bronzetti E, Mannino F, Mariotta S, Tayebati SK. *Expression of peripheral blood lymphocyte muscarinic cholinergic receptor subtypes in airway hyperresponsiveness*. J Neuroimmunol 2002; 129: 178–85.

63. Fryer AD, Lein PJ, Howard AS, Yost BL, Beckles RA, Jett DA. *Mechanisms of organophosphate insecticide-induced airway hyperreactivity.* Am J Physiol Lung Cell Mol Physiol 2004; 286: L963–9.

64. Frieri M. *Neuroimmunology and inflammation: implications for therapy of allergic and autoimmune diseases.* Ann Allergy Asthma Immunol 2003; 90: 34–40.

65. Fossati L, Takahashi S, Merino R, Iwamoto M, Aubry JP, Nose M, et al. *An MRL/MpJ-lpr/lpr substrain with a limited expansion of lpr double-negative T cells and a reduced autoimmune syndrome.* Int Immunol 1993; 5: 525–32.

66. Fujimoto K, Fujii T, Kawashima K. *Increased acetylcholine contents in blood and lymphoid organs of MRL/MpJ-Ipr/Ipr (MRL-Ipr) mice, an immune accelerated model.* J Pharmacol Sci 2003; 91: 286P.

67. Borovikova LV, Ivanova S, Nardi D, Zhang M, Yang H, Ombrellino M, et al. *Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation.* Auton Neurosci 2000; 85: 141–7.

68. Bernik TR, Friedman SG, Ochani M, DiRaimo R, Ulloa L,Yang H, et al. *Pharmacological stimulation of the cholinergic antiinflammatory pathway.* J Exp Med 2002; 195: 781–8.

69. Fitzgerald BB, Costa LG. *Modulation of muscarinic receptors and acetylcholinesterase activity in lymphocytes and in brain areas following repeated organophosphate exposure in rats.* Fundam Appl Toxicol 1993; 20: 210–6.

70. Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, et al. *The immunological synapse.* Annu Rev Immunol 2001; 19: 375–96.

71. Fujii T, Kawashima K. *Calcium signaling and c-Fos gene expression via M3 muscarinic acetylcholine receptors in human T- and B-cells.* Jpn J Pharmacol 2000; 84: 124–32.

72. Dong C, Davis RJ, Flavell RA. *MAP kinases in the immune response.* Annu Rev Immunol 2002; 20: 55–72.

73. Schaeffer HJ, Weber MJ. *Mitogen-activated protein kinases: specific messages from ubiquitous messengers.* Mol Cell Biol 1999; 19: 2435–44.

74. Li Q, Nagahara N, Takahashi H, Takeda K, Okumura K, Minami M. *Organophosphorus pesticides markedly inhibit the activities of natural killer, cytotoxic T lymphocyte and lymphokine-activated killer: a proposed inhibiting mechanism via granzyme inhibition.* Toxicology 2002; 172: 181–90.

75. Webster JI, Tonelli L, Sternberg EM. *Neuroendocrine regulation of immunity.* Annu Rev Immunol 2002; 20: 125–63.

76. Dantzer R, Konsman JP, Bluthe RM, Kelley KW. *Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent?* Auton Neurosci 2000; 85: 60–5.

77. Carlson SL, Fox S, Abell KM. *Catecholamine modulation of lymphocyte homing to lymphoid tissues.* Brain Behav Immun 1997; 11: 307–20.

78. Goehler LE, Gaykema RP, Hansen MK, Anderson K, Maier SF, Watkins LR. *Vagal immune-to-brain communication: a visceral chemosensory pathway.* Auton Neurosci 2000; 85: 49–59.

79. Ishizuka Y, Ishida Y, Kunitake T, Kato K, Hanamori T, Mitsuyama Y, et al. *Effects of area postrema lesion and abdominal vagotomy on interleukin-1 beta-induced norepinephrine release in the hypothalamic paraventricular nucleus region in the rat.* Neurosci Lett 1997; 223: 57–60.

80. Hermann GE, Emch GS, Tovar CA, Rogers RC. *c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve.* Am J Physiol Regul Integr Comp Physiol 2001; 280: R289–99.

81. Van Dam AM, Bol JG, Gaykema RP, Goehler LE, Maier SF, Watkins LR, et al. *Vagotomy does not inhibit high dose lipopolysaccharide-induced interleukin-1beta immunoreactivity in rat brain and pituitary gland.* Neurosci Lett 2000; 285: 169–72.

82. Hansen MK, Nguyen KT, Fleshner M, Goehler LE, Gaykema RP, Maier SF, et al. *Effects of vagotomy on serum endotoxin, cytokines, and corticosterone after intraperitoneal lipopolysaccharide.* Am J Physiol Regul Integr Comp Physiol 2000; 278: R331–6.

83. Elmquist JK, Scammell TE, Saper CB. *Mechanisms of CNS response to systemic immune challenge: the febrile response.* Trends Neurosci 1997; 20: 565–70.

84. Hopkins SJ, Rothwell NJ. *Cytokines and the nervous system. I: Expression and recognition.* Trends Neurosci 1995; 18: 83–8.

85. Szelenyi J. *Cytokines and the central nervous system.* Brain Res Bull 2001; 54: 329–38.

86. Rivest S, Lacroix S, Vallieres L, Nadeau S, Zhang J, Laflamme N. *How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli.* Proc Soc Exp Biol Med 2000; 223: 22–38.

87. Jeon SD, Lim JS, Moon CK. *Carbofuran suppresses T-cell-mediated immune responses by the suppression of T-cell responsiveness, the differential inhibition of cytokine production, and NO production in macrophages.* Toxicol Lett 2001; 119: 143–55.

88. Costa LG, Manzo L. *Biochemical markers of neurotoxicity: research strategies and epidemiological applications.* Toxicol Lett 1995; 77: 137–44.

89. Caulfield, MP. *Muscarinic receptors – characterization, coupling and function.* Pharmacol Ther 1993; 58: 319–79.

90. Tayebati, SK, Codini M, Gallai V, Mannino F, Parnetti L, Ricci A, et al. *Radioligand binding assay of M1-M5 muscarinic cholinergic receptor subtypes in human peripheral blood lymphocytes.* J Neuroimmunol 1999; 99: 224–9.

91. Bany U, Ryzewski J, Maslinski W. *Relative amounts of mRNA encoding four subtypes of muscarinic receptors (m2-m5) in human peripheral blood mononuclear cells.* J Neuroimmunol 1999; 97: 191–5.