Effect of isopropyl methylphosphonofluoridate (IMPF) poisoning on selected immunological parameters of angiogenesis

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In Memory of Professor Janusz Bany

Abstract

Introduction and objective. Acetylcholinesterase (AChE) and cholinergic receptors play an important role in the immune system, including lymphocyte-induced angiogenesis. However, their exact role is not fully understood. The presented work tests the influence of isopropyl methylphosphonofluoridate (IMPF), an irreversible inhibitor of AChE, on selected immune parameters associated with angiogenesis in mice. The levels of VEGF, bFGF, TNF-α, and IFN-γ production were measured, together with the ability of lymphoid spleen cells to induce local GvH reaction after a single dose of IMPF.

Materials and method. Experiments were performed in male BALB/c mice. Acetylcholinesterase activity in erythrocytes was determined by the Ellman’s procedure. Levels of cytokines were measured in serum using standard commercial ELISA kits. Influence of IMPF intoxication upon angiogenesis was examined by the LIA test, according to the Sidky and Auerbach procedure.

Results. The results showed a 6- and 8-fold increase in VEGF at days 1 and 7 of the experiment, respectively, as well as a decrease (at days 14 and 21 after administration) followed by a significant increase (day 1) in bFGF levels. A statistically significant decrease in the concentration of IFN-γ was observed throughout all experiments. The maximum decrease in the level of TNF-α was found at days 1 and 7 after administration of IMPF. Additionally, a significant decrease was found in the ability to form new blood vessels following IMPF administration.

Conclusions. This study revealed that IMPF has a significant effect on the regulation of lymphocyte-induced angiogenesis, which is related with the modulation of angiogenic and pro-inflammatory cytokines secretion. The observed differences suggest a possible derangement of certain elements of the neuronal and/or non-neuronal cholinergic system.

Key words

non-neuronal cholinergic system, cytokines, immunotoxicology, organophosphates

INTRODUCTION

The notion that the immune system of animals is largely autonomous has been widely accepted in previous years. Currently, it is known that the response of this system is regulated not only by antigens, but also via influences of the nervous and endocrine systems. These systems are parts of the complex network in which communication and control are executed through common mediators and receptors [1, 2, 3], and interactions occur at many levels [4, 5]. The lymphoid tissue is mostly innervated by the autonomic nervous system (ANS). Thymus, bone marrow, lymph follicles, lymph nodes, spleen, and tonsils are innervated by the cholinergic nerve endings [3]. The nerve endings are also able to create a special type of connection with lymphocytes and macrophages, resembling that of a synapse [6].

For a long time it has been known that damage within the central nervous system (mainly hypothalamus and limbic system) can affect functioning of the components of the immune response [7]. Lesions within the anterior hypothalamus cause suppression of T cell responses to mitogens and reduced NK cell activity [8]. Additionally, acetylcholine (ACh) has a potential to modulate the immune system, affecting cytotoxicity, activation, and mobility of T cells, as well as production of antibodies by lymphocytes [7, 9, 10, 11, 12].

Recently, acetylcholine, acetylcholinesterase (AChE), and cholinergic receptors have been discovered in non-neuronal cells and shown to be involved in many processes, including angiogenesis [13], inflammation [14, 15, 16], secretion of cytokines [17, 18, 19], neoplasia [20], and autoimmune diseases [21, 22].

The neurotoxic action of organophosphate compounds (OPs), including isopropyl methylphosphonofluoridate (IMPF), a main representative of this group, has been described many times. OPs act by the strong blocking of acetylcholinesterase and thus disturb long-term signal transmission by cholinergic pathways. However, little is
known about their neuroimmunomodulatory properties. Unfortunately, the mechanisms underling the relationships between neurotoxic and immunomodulating activities of OPs still remain unclear [12].

The aim of the presented study was to investigate the effects of poisoning with an irreversible inhibitor of AChE – isopropyl methylphosphonofluoridade – on selected immune parameters associated with angiogenesis in mice. The experiments aimed to determine the activity of acetylcholinesterase, cytokine levels (VEGF, bFGF, TNF-α, and IFN-γ), and the ability of lymphoid spleen cells to induce local GVH reaction following IMPF intoxication.

MATERIALS AND METHOD

Isopropylmethylphosphonofluoridade (IMPF) was obtained from the Military Institute of Chemistry and Radiometry in Rembertów (Poland).

The experiments were performed in male mice of BALB/c (BALB/CanNcrlCmd) strain obtained from the Institute of Experimental and Clinical Medicine at the Polish Academy of Sciences in Warsaw. Additionally, F1 generation of male hybrides (BALB/c x DBA/2) were used for cutaneous angiogenesis test. Animals were kept in rooms with standard cages and bedding, in a 12-hour light cycle. A constant temperature of 22±2 °C was maintained. Adjustable ventilation (according to needs) and humidity were provided. The animals had access to food (standard pellets) and water ad libitum. The environment and animal health were monitored on a daily basis by an attending veterinarian.

The number of animals used in the experiments was 164 mice, including 134 mice of BALB/c strain and 30 mice of hybrid (BALB/c x DBA/2) F1 generation.

The study was approved by the Local Ethics Committee (Decision No. 17/04).

Experimental procedures. The animals were divided into two study groups: a control group with saline administered, and the studied group, which received a 0.5 LD50 dose of IMPF (100 mg/kg b.w.). Both saline and IMPF were administered intraperitoneally. Analysis of selected parameters was performed at four time points: 1, 7, 14 and 21 days following intoxication.

Acetylcholinesterase activity in erythrocytes was determined in blood collected from the retrobulbar plexus into heparinized tubes. The procedure described by Ellman [23] was followed regarding the use of 5,5'-dithio-bis (2-nitrobenzoic) acid and acetylcholine iodide. Absorbance was read in a spectrophotometer (Lambda 35, Perkin Elmer) after 1 and 6 minutes at the wavelength of 412 nm.

For the immunoassay test, blood was collected from the retrobulbar plexus (after animals were anaesthetized) and allowed to clot for 4 hours at the temperature of 4 °C. Serum was obtained by centrifugation of samples for 20 minutes at 4 °C at 2,000 g, and frozen at -80 °C for further tests.

Levels of cytokines in serum samples were determined using a suitable ELISA kit (Quantikine ELISA Kit, R&D Biosystems, USA) according to the manufacturer’s protocol. In order to eliminate random errors, each sample collected from the animal was determined in duplicate using the arithmetic mean of the two measurements as the test result. The absorbance of samples was measured spectrophotometrically (Spectra Count™, Canberra–Packard) at the wavelength of 450 nm; the reference wavelength of 570 nm was used and converted to the concentration units of the parameter based on a multi-point calibration curve.

To determine the influence of IMPF intoxication on angiogenesis, the ability of a lymphoid cell (isolated from the spleens of tested mice) to form new vessels was examined. This procedure is based on the GVH reaction (LIA method – lymphocyte-induced angiogenesis), in which transplanted cells recognize foreign antigens and produce numerous immunological mediators, including pro-angiogenic factors [24, 25]. Isolated cells were administered intradermally into ‘recipients’, as stated by the procedure described by Sidky and Auerbach [26], according to our own modification.

Spleens were collected from euthanized mice at the time points selected for each animal group. Dissected organs were cut into small pieces and macerated with a sterile metal sieve. Next, samples were washed twice in PBS (Biomed, Poland), centrifuged, and resuspended in CM medium (10% FBS in a solution of RPMI 1640 containing antibiotics and L-arginine (PAA Laboratories, Austria). After counting, cells were diluted to an appropriate concentration (2 × 10^6/mL), stained (0.1% Trypan blue solution; Sigma Aldrich, Poland), and thereafter given to the inbred strains of hybrid F1 (BALB/c x DBA/2) (in the semi-allogenic system).

Previously inactive, 6- to 8-week-old mice of F1 generation were shaved on both sides of the torso. 50 µL of previously prepared suspension of lymphoid cells was administered intradermally to the areas with no hair (three points on each side of the body). After 72 hours, the animals were euthanized, and after separating the skin from the muscle, the inner surface of the skin was examined.

New blood vessels present at the sites of injection were counted. The mean number of vessels was determined using the criteria of Sidky and Auerbach [26], by means of a surgical microscope. The numbers of thin or tortuous vessels were counted, as well as branched vessels originating in the area surrounding the site of injection. The sites were scored within the inner circle at a radius of 1/3 of the field view, the centre of which was the site of injection [27].

Statistics. All results were presented as mean values and standard errors (SEM). The character of the distribution of the data was checked using the Shapiro-Wilk test. For comparisons between separate groups, t-test was used when the distribution of the collected data was normal and the homogeneity of variance was maintained. In other cases, the Mann-Whitney U-test was used. The level of significance was set at p < 0.05.

RESULTS

Determination of acetylcholinesterase activity in mice after IMPF intoxication. Table 1 shows the activity of AChE (as the quotient of the difference in absorbance of the test sample and background) for the control and IMPF group at selected time points. The statistically significant decrease in the enzyme activity in the poisoned group occurred during the first 14 days, compared to control. The AChE activity returned to the level of activity observed in the control group approximately 21 days after intoxication. The mean absorbances of controls were show as 'pooled' because of lack of significant differences at each time point.
Quantification of VEGF, bFGF, TNF-α and IFN-γ after IMPF intoxication in sera. Results from the control group were presented as one mean value for the factor, because there were no significant differences in each tested time point (1, 7, 14, 21 days).

The results for the vascular endothelial growth factor (VEGF) are shown in Figure 1, and for the basic fibroblast growth factor (bFGF) in Figure 2.

An approximately 6- to 8-fold significant increase in the levels of VEGF in the sera of IMPF intoxicated animals on days 1 and 7 of the experiment was observed (Fig. 1). Statistical analysis of the results showed that this increase was statistically significant compared to the control group (p < 0.001). At day 14 following intoxication, a decline was found in the level in the control group. This level was maintained until the end of the follow-up (day 21).

Much stronger fluctuations were recorded for the second pro-angiogenic factor – bFGF. After the initial, statistically significant, strong (about 4-fold) increase at day 1 (p < 0.001) (Fig. 2) following the IMPF administration, a decrease was found in the concentrations of bFGF in the sera of poisoned animals at days 7 and 14. At day 14, bFGF concentrations returned to the minimum values observed for the control group (p < 0.05). Furthermore, concentrations of bFGF significantly increased again at day 21, in comparison to the control group (p < 0.05).

Similar trends were observed for the other two pro-inflammatory factors (IFN-γ, TNF-α). After significant decrease at the beginning of the experiment, both factors slowly returned to the baseline.

Figure 3 shows the mean concentration of interferon-γ (IFN-γ), examined at selected time points in the sera of animals intoxicated with IMPF compared to the control group. A statistically significant decrease in the concentrations of IFN-γ was observed throughout the experiment (p < 0.001). The largest decrease in comparison to the control group was found at day 7 after administration of IMPF.

Figure 4 shows the mean concentrations of tumour necrosis factor α (TNF-α), identified at the selected time points. Similarly as for IFN-γ, there was a statistically significant decrease in the levels of this cytokine in the serum, compared to the control group (p < 0.001).

For TNF-α, the maximum decrease in the levels of this cytokine was recorded at day 1 after administration of IMPF. At the subsequent time points, mean concentrations of examined cytokine increased to reach at day 14 the level comparable to that observed in the control group. This level was maintained until the last day of the study.

### Table 1. Erythrocytes AChE activity (mean ± SEM) determined by Ellman’s colorimetric method [23] at selected time points in the control group and after IMPF administration

<table>
<thead>
<tr>
<th>Time</th>
<th>Control ± SEM</th>
<th>IMPF ± SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hours</td>
<td>0.0327 ± 0.0021</td>
<td>0.0116 ± 0.007</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>n = 18</td>
<td></td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.0327 ± 0.0021</td>
<td>0.0171 ± 0.009</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>n = 18</td>
<td></td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>0.0327 ± 0.0021</td>
<td>0.0195 ± 0.0016</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>n = 18</td>
<td></td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>0.0327 ± 0.0021</td>
<td>0.0210 ± 0.0015</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>n = 18</td>
<td></td>
<td>n = 11</td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>0.0327 ± 0.0021</td>
<td>0.0309 ± 0.0015</td>
<td>n.s.</td>
</tr>
<tr>
<td>n = 18</td>
<td></td>
<td>n = 11</td>
<td></td>
</tr>
</tbody>
</table>

p – level of significance; bold font – statistically significant differences; n.s. – statistically insignificant difference; n – number of tested animals

**Figure 1.** VEGF concentrations (mean ± SEM) at selected time points in sera of animals intoxicated with IMPF

n – number of tested animals

* Statistically significant differences compared to control group, p < 0.001

** Figure 2.** Concentrations of bFGF (mean ± SEM) at selected time points in sera of animals intoxicated with IMPF

n – number of tested animals

* Statistically significant differences compared to control group, p < 0.001

** Statistically significant differences compared to control group, p < 0.05

** Figure 3.** Concentration of IFN-γ (mean ± SEM) at selected time points in sera of animals intoxicated with IMPF

n – number of tested animals

* Statistically significant differences compared to control group, p < 0.001

** Figure 4.** Concentration of TNF-α (mean ± SEM) at selected time points in sera of animals intoxicated with IMPF

n – number of tested animals

* Statistically significant differences compared to control group, p < 0.05

n.s. – statistically insignificant difference.
Determination of the ability of lymphoid cells to induce local GvH reaction. Table 2 shows the average number of newly-created vessels in the cutaneous angiogenesis test performed with LIA assay. There was a significant decrease in the number of blood vessels formed at days 1, 7 and 14 following administration of IMPF.

Table 2. Average number (±SEM) of newly created vessels in mice cutaneous angiogenesis test (see material and methods)

<table>
<thead>
<tr>
<th></th>
<th>Control ± SEM</th>
<th>IMPF ± SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>15.545 ± 0.450</td>
<td>14.125 ± 0.464</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 21</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>15.545 ± 0.450</td>
<td>12.136 ± 0.281</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 21</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>17.417 ± 0.329</td>
<td>15.545 ± 0.450</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>n = 24</td>
<td>n = 22</td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>16.708 ± 0.410</td>
<td>16.188 ± 0.265</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>n = 24</td>
<td>n = 21</td>
<td></td>
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</table>

* Statistically significant differences compared to control group. p < 0.001

DISCUSSION

Organophosphates constitute an exceptionally diverse group of substances, marked by varying degrees of toxicity [28]. This group includes military agents, considered to be highly toxic Ols, including particularly dangerous isopropylmethylphosphonofluoridate (IMPF, SARIN). The main mechanism of toxicity of these compounds is the irreversible inhibition of acetylcholinesterase [29], with the degree of enzyme inhibition depending on the route of administration and dose [30, 31].

The results obtained in this study show that the selected dose of IMPF (0.5 LD$_{50}$) significantly inhibits the activity of acetylcholinesterase until day 14 after intoxication (Tab. 1).

Due to the fact that organophosphates easily cross the blood-brain barrier [32], their effects are not limited to peripheral structures. Studies conducted using the tritiated compound ('H) allowed for the detection of high concentrations of IMPF in some structures of the central nervous system [33]. IMPF intoxication significantly reduced the activity of AChE in some regions of the brain, e.g. hippocampus [34] which, together with the hypothalamus, is the most important region for the neuroimmunomodulation processes [5, 35]. It can be safely assumed that IMPF influences AChE activity in brain regions necessary for neuroimmunological interaction; thereby significantly impacting these processes. It should be emphasized that these processes take place primarily through the autonomic nervous system, cholinergic neurons being the crucial elements of ANS (parasympathetic system) [36]. At the same time, it is known that neuroimmunological disturbances may directly or indirectly lead to changes in certain immune parameters [13, 37, 38], also influencing the processes of angiogenesis (for example, angiogenic cytokines).

The presented study shows that acute IMPF intoxication in mice leads to disturbances in the secretion of cytokines, depending on their type and the time after intoxication. This applies not only to the cytokines generally regarded as proangiogenic, VEGF and bFGF, but also to proinflammatory factors (TNF-α and IFN-γ).

The current study shows a strong increase in the concentration of vascular endothelial growth factor (VEGF) in the first week after intoxication (days 1 and 7), compared to the control group (Fig. 1). In the case of basic fibroblast growth factor (bFGF), the greatest increase in serum concentration was observed on days 1 and 21 following intoxication (Fig. 2). In general, other examined cytokines were found to decrease their concentrations after intoxication. There was a statistically significant decrease in the concentrations of interferon-γ (IFN-γ) for the entire duration of the experiment; the largest decrease in comparison to the control group was noted at day 7 after IMPF administration (Fig. 3). For tumour necrosis factor (TNF-α), the maximum reduction in concentration was observed at day 1 after intoxication (Fig. 4). The average concentrations gradually returned to the level comparable with the control group (without intoxication), starting at day 14.

It appears that secretion of cytokines, both angiogenic and proinflammatory, as well as the balance between them, is one of the main factors determining the mechanism of angiogenesis which was disturbed by the action of the toxic agent.

Angiogenic cytokines, with their signaling pathways and the process of the spatial rearrangement of endothelial cells leading to the formation of new capillary networks, can also be regulated by other cytokines, such as TNF-α and IFN-γ [39, 40].

Furthermore, the presented study shows that IMPF intoxication causes changes in the activity of spleen lymphoid cells (mainly T cells) involved in local graft versus host reaction (GvH).

In general, it was found that the number of newly-created vessels in the cutaneous angiogenesis assay (LIA method) was significantly decreased in the group of animals treated with IMPF, in comparison with the control group (Tab. 2). T helper cells are primarily responsible for formation of new blood vessels in the LIA assay, secreting a variety of cytokines, including VEGF stimulating growth of new blood vessels [41, 42, 43]. TNF-α may also be an important factor in the pathogenesis of GvH reaction [41]. This can act as a secreted autocrine factor enhancing cell clonal expansion [44, 45, 46]. It was found that the donor cells secreting TNF-α inhibit GvH reaction by reducing the level of IFN-γ-dependent immune response type 1, essential for the increase in the level of TNF-α in the recipient [47].
As shown in the presented study, the changes observed in animals after IMPF intoxication lead not only to the disruption of the 'cytokine network', but also influence the activity of T lymphocytes. These observations confirm previous studies showing inhibition of T cell proliferative activity in mice treated with IMPF [48, 49, 50, 51]. It is unclear, however, what the mechanism is behind IMPF influencing T cell activity. It seems that the autonomic nervous system is probably involved. Kalra et al. [52] showed that rats intoxicated with subclinical doses of IMPF inhibit T cell proliferative activity in response to concavalin A, independently of the hypothalamic-pituitary-adrenal (HPA) axis. It was also found that the levels of serum corticosteroids in rats treated with IMPF were significantly lower compared to controls. These observations suggest that the immune suppression induced by IMPF is not dependent on the activation of the HPA axis, but involves the pathways of the autonomic nervous system. This was also confirmed by chlorizondamin activity, which blocks transmission in the autonomic nervous system, abolishing the effect of IMPF. In addition, other studies found that chemical disruption of the sympathetic nerve transmission influences T cell dependent antibody response, IL-2 production, cytotoxic T lymphocytes, and delayed-type hypersensitivity [53, 54].

The general agreement is that IMPF intoxication causes disturbances in certain processes influencing the development and severity of angiogenesis in mice, and the observed differences suggest a possible deregulation of certain elements of both the neuronal and non-neuronal cholinergic systems. The mechanisms of action require further investigation, which may be a natural continuation of the findings of the presented study. This applies particularly to the analysis of the expression of receptors for the factors involved in formation of vascular abnormalities, and mechanisms of cytokine secretion and their interrelationships.

CONCLUSION

The authors' experience and analysis of the available literature, lead to the following conclusions:

1. IMPF intoxication in animals has a significant impact on the regulation of lymphoctic angiogenesis, which seems to be associated with the modulation of pro-inflammatory and angiogenic cytokines secretion.

2. The observed differences in parameters determining angiogenesis in mice poisoned with IMPF suggest a possible deregulation of certain elements of both the neuronal and non-neuronal cholinergic systems.

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REFERENCES


