PERSPECTIVE

Glyphosate-based herbicide: a risk factor for demyelinating conditions of the peripheral nervous system?

Glyphosate is a broad-spectrum herbicide originally introduced to the market in 1974 by the agrochemical company Monsanto. More than 40 years down the line, glyphosate has become one of the most economically meaningful herbicides, with a global use of more than 1.8 million pounds in 2014 (Benbrook, 2016). In non resistant plants, glyphosate is widely believed to exert its herbicidal effect *via* inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme of the shikimate pathway required for the biosynthesis of aromatic amino acids in plants and most microorganisms such as fungi, bacteria and some protozoans. As the shikimate pathway has no known physiological function in mammals, glyphosate was considered safe for humans. However, this view has been challenged by several studies conducted by researchers from various fields, leading to the assumption that agricultural spreading of glyphosate might bear health risks, including but not limited to carcinogenic, inflammatory and endocrine disruptive effects (Mesnage et al., 2015).

While the potential health risks associated with exposure to residues of pure glyphosate from food and environmental contaminations remain a matter of controversial debate, glyphosate-based herbicide (GBH) formulations have almost unequivocally been demonstrated to exert concentration-dependent cytotoxicity. Here, one point particularly worthy of note is that glyphosate is always used as herbicide formulation and not as a pure substance. A significant portion of GBH products contain the isopropylamine salt of glyphosate alongside mostly unspecified mixtures of surfactants and auxiliary agents. These may include petroleum distillates and polyoxyethyleneamines (POEAs) (Defarge et al., 2017). POEAs have been found to contribute substantially to the toxicity of GBH formulations. Following a risk assessment by the European Food Safety Authority, POEAs have been banned as co-formulants for herbicides in the European Union since mid-2016. Nevertheless, outside the European Union, POEAs are still commonly used as surfactants in GBH formulations. Moreover, a recent investigation found several herbicide formulations to contain heavy metals (Defarge et al., 2017).

While numerous studies have focused on general cytotoxicity and carcinogenicity of glyphosate or GBH, very little is known about specific effects of these compounds on the nervous system and no studies have been conducted so far to investigate their impact on the peripheral nervous system (PNS). For this reason, we decided to study and compare the effects of both pure glyphosate and a commercially available GBH product (Roundup LB Plus, Monsanto) on murine embryonic dorsal root ganglia (DRG) explant cultures as an *ex vivo* model of neurite outgrowth and myelination in the PNS (Szepanowski et al., 2018a).

Following explantation, these DRG cultures were initially kept under neurite-stimulating conditions before myelination of the outgrown neurites was subsequently induced. Glyphosate was then added to the culture medium either directly at the start of myelination-induction or following an initial myelination period of 10 days. Finally, the cultures were stained with Sudan Black and the myelin content was determined by measuring the total number of internodes per neurons present in individual culture wells (**Figure 1**). Unexpectedly, we did not find any significant impact of pure glyphosate, even at considerably high concentrations, on the DRG cultures under both culture conditions: Pure glyphosate did not interfere with myelination or cause demyelination in cultures with pre-existing myelin. In sharp contrast, treatment of cultures with the GBH product significantly impaired myelination and was associated with a concentration-dependent demyelinating effect. Of note, the observed effects of GBH on DRG cultures appeared to be exclusively related to an impairment of myelin rather than neurite integrity. Neither glyphosate nor GBH treatment were associated with reduced neurite outgrowth kinetics and neither affected neurite density following exposure times of up to 10 days.

As the GBH contained glyphosate as isopropylamine salt rather than pure glyphosate, we next tested whether a mixture of pure glyphosate and isopropylamine would exert a similar demyelinating effect to that of the GBH formulation. We found a slight, although non-significant reduction in the number of internodes per neurons between pure glyphosate or glyphosate plus isopropylamine. Thus, isopropylamine may have a minor damaging effect on myelin, possibly by saponification of lipids, but its impact was not comparable to that of GBH treatment. These findings suggest that undisclosed additives in the GBH product might be responsible for the observed demyelinating effect.

To better understand relevant mechanisms that might explain the observed differential effect of pure glyphosate and GBH on myelin integrity, we next assessed the influence of GBH on markers of oxidative/nitrosative stress and cell damage, such as malondialdehyde, nitric oxide and lactase dehydrogenase activity. However, we could not find specific effects of GBH on any of these markers.

Since these measures for cellular damage did not yield any results specifically related to GBH, we focused on another mechanism that might contribute to demyelination and also prevent myelination of unmyelinated cultures, namely inflammatory Schwann cell activation.

Schwann cells, as myelin-forming cells of the PNS, are generally accepted to be immunocompetent cells, resembling several features of classical innate immune cells (Ydens et al., 2013). Similar to macrophages, Schwann cells express a wide range of pattern recognition re-

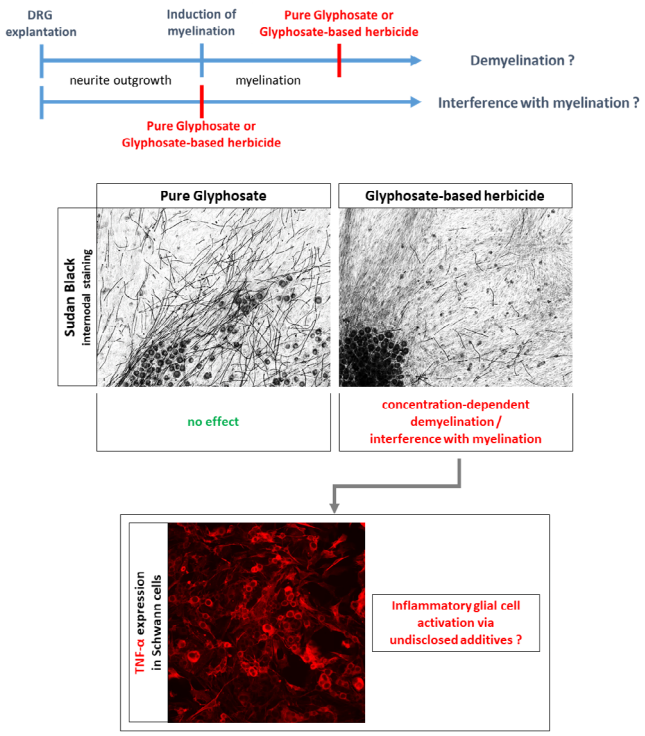

Figure 1 GBH, rather than pure glyphosate, may cause PNS demyelination *via* undisclosed additives propagating inflammatory glial cell activation.
In an *ex vivo* model of PNS myelination, freshly explanted murine embryonic DRGs were kept under neurite-stimulating conditions before myelination was induced. In order to test whether pure glyphosate or GBH may interfere with myelination or cause demyelination, either compound was added to cultures directly at the start of myelination-induction or to cultures with pre-existing myelin, respectively. Quantification of myelination was performed by Sudan Black staining to determine the number of internodes per neurons in individual cultures wells. Whereas pure glyphosate did not affect internodal density, GBH treatment was associated with a concentration-dependent reduction in the number of internodes. The demyelinating effect of the GBH was associated with a marked induction of TNF-α in Schwann cells. These findings suggest that GBH might impede the formation and maintenance of myelin sheaths *via* a mechanism involving inflammatory glial cell activation. GBH: Glyphosate-based herbicide; PNS: peripheral nervous system; DRGs: dorsal root ganglions; TNF-α: tumor necrosis factor-alpha.

ceptors including toll-like receptors (TLRs) and receptors for advanced glycation endproducts, and are thus involved in the immune surveillance of the PNS. While TLRs have classically been recognized for their role in defending against microbial pathogens, TLRs can also be activated by endogenous ligands such as mRNA or heat shock proteins that leak into the extracellular space, *i.e.* after tissue injury. As a consequence of TLR stimulation, Schwann cells secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 (Ydens et al., 2013). However, with regard to myelin integrity, this enormous plasticity of Schwann cells takes its toll. The adoption of an inflammatory phenotype is preceded by Schwann cell dedifferentiation, characterized by the downregulation of myelination-associated genes, which will finally result in the breakdown of myelin sheaths (Jessen and Mirsky, 2008; Stettner et al., 2014; Szepanowski et al., 2018b). Indeed, Schwann cell dedifferentiation may represent a central pathomechanism in several demyelinating conditions of the PNS, including mechanical nerve injury and inflammatory diseases such as the Guillain-Barré-Syndrome and chronic inflammatory demyelinating polyneuropathy (Jessen and Mirsky, 2008; Hutton et al., 2011).

To test the hypothesis of whether GBH might be associated with peripheral glial cell activation in DRG cultures, we analyzed TNF-alpha expression in S100-positive cells, suggestive of Schwann and satellite cells, *via* immunocytochemistry. Here, we recognized a significant increase in TNF-alpha expression with GBH, compared to vehicle. Furthermore, this effect appeared to be concentration-dependent.

In order to corroborate these findings, we prepared pure Schwann cell cultures which were kept under differentiating conditions. These cultures were subsequently incubated with GBH for 72 hours. GBH treatment caused a marked induction of TNF-alpha expression in Schwann cells as demonstrated both by immunocytochemistry and ELISA from Schwann cell lysates. In addition, we found a significant elevation of nitric oxide. These findings further support our previous assumptions that implicate inflammatory Schwann cell activation as a mechanism underlying GBH-driven demyelination.

Since the composition of the GBH product used in this study is not fully declared, the actual compound responsible for demyelination and Schwann cell activation remains to be elucidated. The GBH product used in our study is commonly available to non-professional users in Germany. As such, it seems rather unlikely for this particular GBH to contain cytotoxic POEAs. However, a recent study has screened 22 pesticide formulations, including 11 GBH products, for different heavy metals. All of these products except for one contained a variable mixture of heavy metals such as arsenic, chromium and lead. Heavy metals were found in GBH products regardless of whether they had been obtained from markets in North America, Europe or Asia (Defarge et al., 2017).

Interestingly, heavy metals have been implicated in the inflammatory activation of cultured cells. Their inflammatory potency may even be comparable to stimulation with lipopolysaccharide or cytokines such as TNF-alpha or IL-1beta (Wagner et al., 1998). Heavy metal-driven cell activation and cytokine release may involve the transcription factors nuclear factor κB and activating protein-1 (Wagner et al., 1998). The activating protein-1 transcription factor family includes several members of the JUN subfamily. Interestingly, Schwann cell dedifferentiation following mechanical nerve injury, but also under inflammatory conditions is widely considered to be dependent on the induction of c-Jun expression (Jessen and Mirsky, 2008). As such, a heavy metal-induced regulation of activating protein-1/c-Jun may underlie or contribute to GBH-driven Schwann cell activation. Additionally, inflammatory cytokines released following herbicide exposure may further propagate Schwann cell dedifferentiation or death in an auto- and paracrine fashion. Therefore, the demyelinating effect of GBH in our study might, at least in part, be attributable to heavy metals in herbicide formulations. Further investigations will be required to test this hypothesis.

Our findings raise the question as to whether GBH residues ingested with food or as environmental contaminations may contribute to or even trigger demyelinating conditions of the PNS. While glyphosate is detectable in urine samples from the general population, it appears unlikely that the concentrations of glyphosate, comparable to those used in our study, would be reached by the consumption of contaminated foods or drinking water (Niemann et al., 2015). However, there have been occasional reports of GBH intoxication following acute exposure.. With regard to the PNS, a recent case report has linked acute GBH exposure (after the handling of herbicides without protective wear) to the development of peripheral neuritis (Kawagashira et al., 2017). Although this report lacks causative evidence, auxiliary agents in GBH may have contributed to inflammation in this particular condition. As such, individuals chronically exposed to GBH or related chemicals, especially those who do not take adequate precautionary measures, may be at an increased risk of developing inflammatory diseases, including demyelinating neuropathies.

In summary, our study suggests that undisclosed additives in GBH formulations may impact myelin integrity via a mechanism involving inflammatory Schwann cell activation. However, further investigations and epidemiological studies are urgently required to corroborate the assumption that GBH leads to an increased risk of demyelinating neuropathies.

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