MULTIPLEX IMMUNOASSAYS AS AN EFFECTIVE METHOD TO SIMULTANEOUSLY ANALYZE INFLAMMATORY MEDIATORS IN CENTRAL NERVOUS SYSTEM (CNS) CELLS: HUMAN ASTROCYTES STIMULATED WITH SARIN (GB).

Carmen M. Arroyo, Elisa D. Purcell, David W. Kahler and Clarence A. Broomfield and Brennie E. Hackley, Jr.‡
‡Drug Assessment Division
*Scientific Advisor
£ORISE, Oak Ridge Institute for Science and Education, Oak Ridge, TN
*‡1 U.S. Army Medical Research Institute of Chemical Defense
3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010

The central nervous system (CNS) is an immune-privileged site where the role of immune cells and mediators in brain injury caused by organophosphates (OP) is poorly understood. Many mediators have been identified in nervous system tissue. For instance, interleukin-6 (IL-6), a cytokine that acts on a wide range of tissue influencing cell growth and differentiation, is an agonist for vascular endothelial growth factor (VEGF). CNS cells producing IL-6 include astrocytes, macrophages, microglia, neurons, and brain endothelial cells. Here we describe the response of five different mediators, human interleukin-1beta (hIL-1β), hIL-6, hIL-8, tumor necrosis factor – alpha (hTNF-α), and human granulocyte macrophage-colony stimulating factor (hGMCsF) associated with human astrocytes incubated with an OP, sarin (GB). Human astrocytes (~10^6 cell density) were stimulated with a high concentration of GB (0.8 mM) for 48 hours at 37°C. The expressed mediators in human astrocytes were detected by using the Luminex® 100™ protein multiplex immunoassay. Constitutive non-stimulated human astrocytes secreted hGMCsF (0.59 ± 0.03 ng/mL), hIL-1β (0.33 ± 0.05 ng/mL), hIL-6 (1.43 ± 0.02 ng/mL) and hIL-8 (0.39 ± 0.02 ng/mL). hTNF-α secretion was not detected or observed. GB decreased the endogenous secretion of these mediators as follows: hGM-CSF (0.51 ± 0.02 ng/mL), hIL-1β (0.28 ± 0.03 ng/mL), hIL-6 (1.00 ± 0.02 ng/mL). Meanwhile, the induction chemokine hIL-8 was increased 0.42 ± 0.03 ng/mL by GB as measured by Luminex® 100™. Up and down-regulation of these mediators in human astrocytes promises a non-intrusive mechanism for assessment of the role of individual mediators in brain cell development, function and response to insult, such as that caused by the OP.

Many neurodegenerative disorders are associated with inflammatory processes in the central nervous system (CNS). The use of animal models to examine these phenomena and to develop therapeutic agents is frequently problematic, since regulation of many inflammatory mediators including hormones and cytokines in the CNS are fundamentally different between humans and rodents. Significant limitations exist in the application of rodent animal models in the evaluation of candidate anti-inflammatory or anti-viral therapies for human CNS diseases. We have developed isolation and primary culture systems for all major types of neural cells derived from human brains. Both neurons and astrocyte cells can be kept or grown in culture at high purity to allow a variety of cell biological approaches. Respecting the complexity of a human brain, it is our conviction that many fundamental processes in neural cells can be productively approached by first examining purified cells in culture, as is customary in many other fields of biomedical research. Cultured cells allow for experimental interventions that would be impossible to perform in vivo. These interventions include modulation of pro- or anti-inflammatory responses by defined compounds. Cultured neural cells are amenable to a variety of techniques to study functional genomics and proteomics.

Cytokine and chemokine signaling by astrocytes

Astrocytes play a key role in signaling networks in the brain that control development, growth, inflammatory processes and repair. Like astrocytes in vivo, cultured adult human astrocytes produce a wide range of cytokines, chemokines, and neurotrophic factors. Many molecular triggers influence this production. Thus, cultured astrocytes can be used to monitor the effects of candidate compounds that are designed to modulate signaling by cytokines, chemokines, or neurotrophic factors.

Cell culture and chemical treatments

Primary human astrocytes were obtained from Clonexpress Inc. (Gaithersburg, MD, USA). These cells did not differentiate into neuron. Human astrocytes were isolated from fetal brain tissue obtained from agencies authorized to procure and distribute such tissues for research. Brain tissue samples digested with collagenase and trypsin were plated in a proprietary medium that
Report Documentation Page

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

<table>
<thead>
<tr>
<th>1. REPORT DATE</th>
<th>2. REPORT TYPE</th>
<th>3. DATES COVERED</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 DEC 2004</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

4. TITLE AND SUBTITLE

Multiplex Immunoassays As An Effective Method To Simultaneously Analyze Inflammatory Mediators In Central Nervous System (Cns) Cells: Human Astrocytes Stimulated With Sarin (Gb).

6. AUTHOR(S)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

ORISE, Oak Ridge Institute for Science and Education, Oak Ridge, TN; U.S. Army Medical Research Institute of Chemical Defense 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:

<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>unclassified</td>
<td>unclassified</td>
<td>unclassified</td>
</tr>
</tbody>
</table>

17. LIMITATION OF ABSTRACT

UU

18. NUMBER OF PAGES 19a. NAME OF RESPONSIBLE PERSON

2

2
supports growth of both neuronal precursor cells and astrocytes. Following two weeks of growth in this medium, neuronal precursor cells were separated away from an enriched population of astrocytes. Cells were supplied as cryopreserved vials containing $2 \times 10^6$ viable cells. The cells were plated in tissue culture dishes. The second passage astrocytes were subcultured in 150 cm$^2$ flasks at a seeding density of $\sim 2.5 \times 10^3$ cells per cm$^2$ in astrocyte growth medium for seven days.

**Chemical treatments**

Human astrocytes in 150 cm$^2$ culture flasks containing fresh media were exposed to different concentrations of the organophosphorus nerve agent sarin (military designation: GB; isopropyl methylphosphonofluoridate) 0.80 to 0.22 mM per flask. Cell viability experiments (trypan blue exclusion) of controls (non-stimulated) and GB-stimulated cells showed that the cell viability for controls was greater than 95% of surviving cells and approximately 85% or lower with 0.8 mM GB under similar culture conditions. The culture flasks were maintained at room temperature in a chemical fume hood for approximately an hour and then transferred to a CO$_2$ incubator at 37°C for 24 or 48 h.

**Luminex$^{100\text{W}}$ Analysis System and Multiplex Antibody Reagent Kits**

The general immunocytologic assay protocol is very similar to standard cytokine enzyme-linked immunosorbent assays. This novel method allowed the simultaneous measurement of 10 different biomarkers (i.e., human cytokines/chemokine/growth factors) and was designed to work in conjunction with the Luminex LabMAP$^{\text{TM}}$ system.

**hIL-6 ELISA quantification**

The standard cytokine ELISA was applied as previously described (Arroyo et al., 2003).

**Summary**

In the CNS, astrocytes are a major inducible source of interleukin 6 (IL-6). Furthermore, increasing evidence supports an essential role of IL-6 in the development, and differentiation as well as in de-and re-generation of neurons in the CNS. We show that IL-6 is released from constitutive non-stimulated human astrocytes. In addition, we show that stimulation of cultured astrocytes with GB decreases the secretion of hIL-6 as well as hIL-1β. We find that GB reduces hIL-6 in a dose-dependent manner. Our results indicate that IL-6 regulates specific expression in GB-stimulated astrocytes as shown by RT-PCR (Figure 1).

![Figure 1. Differential expression of hIL-6 on control astrocytes and after stimulation with GB (0.09 mM).](image)

IL-6 appears to have multiple effects, some neuroprotective and others damaging. The effects depend on the duration of exposure and the cell type (Hull et al., 1999; Orzylowska et al., 1999). Therefore, further studies are in progress to elucidate the role of IL-6 in diverse CNS functions such as protection of neuron from insults, neuronal survival, and neuro-immune responses caused by OP stimulation.

**References**

