Immunological Assays for Understanding Neuroimmune Interactions

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The human nervous system, particularly the central nervous system (CNS), enjoys a status of immune privilege owing to the restricted access of cells and molecules originating from the immune system. This protection from immune damage is particularly important for preservation of the CNS, since mature brain cells are terminally differentiated and are not normally replaced after death. There is now new evidence for the presence of stem cells in the brain that may be the source for neuronal cells in adulthood. This phenomenon appears to be very limited and does not compensate for the neuronal loss that occurs during pathological processes. Thus, the brain, unlike other organs that are composed of cell types that have the capacity to divide, is particularly vulnerable to immune-mediated damage.

In the intact mammalian CNS, lymphocytes are rarely detectable, and granulocytes and dendritic cells are absent. The CNS possesses its own specialized resident immune cells (microglia), adapted to serve the brain according to its functional needs and with minimal probability of damage by immune cells. The CNS does not efficiently express class I and class II molecules of the major histocompatibility complex at baseline, thus avoiding deadly immune-related damage. Recently, the existence of a CNS-resident inhibitor factor, named immune privilege factor, has been described. The immune privilege factor also contributes to restrict immune-brain communications at baseline and after CNS injury. The mechanisms underlying the immune-privileged status of the CNS have been partially attributed to the immune privilege factor. When these protective mechanisms fail, the CNS becomes highly vulnerable to immune damage. Untreated rabies infection is an example of total failure to protect the brain from immune-affiliated damage. The rabies virus shuts off critical neuronal genes, including genes encoding informational substances and housekeeping genes, which ultimately leads to deterioration of neuronal function. The neuronal damage is then fatally intensified by powerful humoral and cell-mediated immune reactions.

Cell proliferation, differentiation, and survival are often regulated by growth, differentiation, and survival factors, which have been collectively named cytokines. Cytokines are soluble proteins, or glycoproteins, produced by leukocytes and, in many cases, other cell types. Other characteristics of cytokines include pleiotropy (they have multiple target cells and multiple actions), redundancy (different cytokines may share similar actions), and feedback (they can increase or decrease the level of their own production or of other cytokines). The importance of cytokines in the regulation of immune and inflammatory responses is now clearly recognized. It is well established that cytokines are expressed in resident cells of the CNS and that they play an important role during pathophysiological processes, such as systemic inflammation and stroke. In addition to the many positive stimuli, several mediators act to limit or prevent cytokine gene expression and action. Inhibitors of cytokine production include glucocorticoid hormones, synthetic ste-
The typical effect found signs and symptoms mediated by the CNS. The inflammatory response is also modulated by cytokines that are synthesized in the brain (de novo synthesis in the CNS). Cytokines expressed within the brain have a different role from peripheral cytokines, and may activate the acute phase response and play a role in programmed cell death, neurodegeneration, and neuroprotection.

Cytokines generated in the CNS can regulate the periphery by influencing the neuroendocrine, autonomic, and peripheral nervous systems (Figure 1). Interleukin 1β is known to stimulate the hypo-

**Figure 1.** Schematic of the communication between the immune system and the central nervous system (CNS). Peripheral cytokines stimulate a variety of CNS functions, including neuroendocrine responses, behavioral patterns, sleep, and fever. Peripheral cytokines stimulate the hypothalamus to release corticotropin-releasing hormone (CRH) and the pituitary gland to release adrenocorticotropic hormone (ACTH). Hypothalamic CRH release also stimulates corticotropin release from the anterior pituitary gland. Pituitary adrenocorticotropic hormone stimulates the adrenal gland to release glucocorticoids, which suppress inflammation, completing this counter-regulatory feedback loop between the immune system and the CNS. Neuroimmune interactions are also influenced by the sympathetic nervous system (SNS) and by the release of neuropeptides from the peripheral nerves. PVP indicates the paraventricular nuclei of the hypothalamus; PNS, peripheral nervous system; AVP, arginine vasopressin; LC, locus ceruleus; n., nerve; broken arrows, inhibitory effects; and solid arrows, stimulatory effects. C1, C2, A1, and A2 represent noradrenergic nuclei. Reproduced with permission from Journal of Clinical Investigation.4

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lamic release of corticotropin-releasing hormone. Recently, IL-1β has been postulated to be a multisite regulator of the hypothalamic-pituitary-adrenal axis because of the finding of high levels of IL-1β gene expression in areas such as the paraventricular and arcuate nuclei of the hypothalamus, median eminence, and posterior pituitary gland. Interleukin 1β also stimulates the pituitary gland to release adrenocorticotropic, luteinizing, growth, and thyroid-stimulating hormones and inhibits the secretion of prolactin. Pituitary adrenocorticotropic hormone stimulates the adrenal gland to release glucocorticoids, which in turn suppress inflammation, completing the negative feedback loop between the immune system and the CNS.

Understanding the complex interactions between the immune system and the CNS requires the dissection of how the brain responds to components of the peripheral immune system, such as cytokines and immune or inflammatory cells, and the analysis of how the CNS response is transduced to the periphery. In this context, quantitation of expression of immune molecules, such as cytokines, within the CNS will shed light on one important component of these interactions. In such analyses, it is not only important to quantitate expression of the range of different cytokines that can be expressed in the brain, but also to determine the anatomical and cellular distribution pattern of such cytokines under different conditions. A variety of sensitive methods are available to accomplish these goals. Herein, we will focus on methods to study cytokine expression within the CNS.

**METHODS**

**In Situ Hybridization Histochemistry (ISHH)**

In vivo studies are generally useful to assess the complexity of the CNS response; therefore, animal models are most suitable to evaluate temporal as well as spatial patterns of CNS response. The brain is anatomically complex, and neighboring regions can express many different genes. The availability of good immunohistochemical techniques for cytokine detection is limited. The limitation of immunohistochemical techniques is in part related to the nature of these molecules: they are secreted extracellularly and rapidly diffuse from the site of secretion; they also generally have a short biological half-life. Hence, ISHH is the method of choice to study neuroimmune interactions, because it provides a high degree of spatial resolution, demonstrating changes at the cellular level, which can be quantified.

In situ hybridization histochemistry is a method that is based on the principle that RNA or DNA sequences will hybridize with probes of complementary sequences. It is used for detecting specific sequences of RNA or DNA in intact tissue or cell preparations. **Figure 2** illustrates the steps for ISHH experimental procedure. The procedure can be briefly summarized into the following steps: (1) freezing and sectioning of samples; (2) prehybridization treatments to optimize probe access to tissue messenger RNA (mRNA) and to reduce nonspecific binding; (3) probe preparation and hybridization; (4) posthybridization washes to remove unbound probe; and (5) detection and quantitation of bound probe. Applications of ISHH include identification of sites of gene expression, analysis of the tissue distribution of transcription, temporal quantification of changes in specific mRNA synthesis, mapping gene localization in chromosomes, and identification and localization of viral infections. A clear advantage of the in situ hybridization technology is its sensitivity: it can detect single-copy genes of individual chromosomes. Thus, the degree of expression of specific RNA sequences in very localized brain regions under different conditions can be quantified. A major advantage of ISHH is that it permits the maximal use of tissues that may be in short supply (eg, clinical biopsy specimens). Several 10- to 20-mm tissue sections can be obtained from a single surgical biopsy specimen, and each tissue section can be hybridized with a different probe.

The selection of probes is a crucial step for the study of neuroimmune interactions, since the specific probe selected will determine the pathways and spatial patterns of mRNA expression that are detected. The ideal probe should have high specificity to the target mRNA and low homology to ribosomal RNA to avoid nonspecific signals. Three main classes of probes can be used: synthetic oligonucleotides, complementary DNA (cDNA), or complementary RNA probes (cRNA, or riboprobes).

Methods such as Northern blot analysis (RNA is separated in a gel, transferred to a nitrocellulose filter, and then hybridized to the target sequence[s]) or solution hybridization (RNA is hybridized to target sequence[s] in solution and then separated in a polyacrylamide gel) that use RNA extracted from tissue can also provide information about changes of specific RNA sequences in tissues. In situ hybridization histochemistry offers a potential advantage in sensitivity over Northern blot hybridization, as well as providing spatial information. In cases in which the transcript of interest is only present in a small number of cells within a large surrounding population, such RNA sequences may be undetectable in tissue extracts owing to dilution by other RNA sequences from the surrounding cells. In situ hybridization histochemistry also offers the advantage of identifying cells on the basis of their contents of specific mRNA sequences encoding products of interest. A caveat about ISHH is that this technique addresses expression of known genes. Other techniques, such as differential display, serial analysis of gene expression, and DNA microarrays (eg, DNA chips), can also address transcriptional changes in unknown genes.

**Western Blot Analysis**

Immunoblotting, also called Western blotting, is a rapid and sensitive assay for the detection and characterization of protein that works by using the specificity of the antigen-antibody recognition. It combines the resolution of gel electrophoresis with the specificity of immunochemical detection. Western blot analysis is a technique that is particularly helpful when one is dealing with anti-
gens that are insoluble, difficult to label, or easily degraded, and therefore less amenable to analysis by immunoprecipitation.

The Western blotting procedure can be divided into 6 steps: (1) preparation of the protein sample (often an extract from cells or tissues); (2) separation of the sample by gel electrophoresis (generally a denaturing gel containing sodium dodecyl sulfate and polyacrylamide is used); (3) transfer and irreversible binding of the separated polypeptides to a membrane support; (4) blocking nonspecific binding sites on the membrane; (5) addition and incubation of the antibody; and (6) detection by chromogenic or luminous assays. Furthermore, separated proteins can be recovered for subsequent studies from polyacrylamide by electroelution.

Western blotting does not require high-affinity antibodies, and it has been useful in identifying specific antigens that are recognized by polyclonal or monoclonal antibodies. It is highly sensitive, and it can detect as little as 1 ng of antigen. Protein blotting has important clinical applications; eg, it is a confirmatory test for human immunodeficiency virus type 1. Virus proteins that have been separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis are blotted onto nitrocellulose membranes and processed with patient serum. After incubation with an antihuman IgG, the antigens are identified by chromogenic detection.

Enzyme-Linked Immunoabsorbent Assay

Different cytokines may share similar actions, and this redundancy of action contributes to the lack of absolute specificity of cultured mammalian cells for the activities of a specific cytokine. Cytokine bioassays have relatively poor reproducibility. Immunoassays, which fill the need of a more specific and reproducible assay method, are regularly used for cytokine quantification in plasma and other body fluids and secretions. Immunoassays are based on antibodies that are specific to each different cytokine; the amount of antibody binding will determine the amount of cytokine measured. Im-

Figure 2. Steps for in situ hybridization histochemistry (ISHH) procedure designed for a time course study. Frozen rat brain tissues obtained from 4 experimental groups (control, 2 hours after lipopolysaccharide injection [LPS-2h], LPS-6h, and LPS-24h) were sectioned and mounted on microscope slides. The sections were stored at −70°C until they were used for ISHH. Sections were fixed, delipidated, dehydrated, and hybridized with a radiolabeled nucleotide riboprobe to determine the presence of interleukin-1β (IL-1β) messenger RNA (mRNA). Hybridized slides were exposed to film for quantitation. Autoradiographs showing the paraventricular nuclei of the hypothalamus (PVN) are displayed for each of the experimental groups. Note that IL-1β mRNA is upregulated in the PVN after intraperitoneal injection of LPS, and peaks 6 hours after the injection. Hybridized slides are then dipped in emulsion for cellular localization. RNase indicates ribonuclease.
munoassays can be calibrated by cytokine concentration or biological activity when suitable cytokine standards of known biological potency are available.

There are broadly 3 types of immunoassays: radioimmunoassay (RIA), immunoradiometric assay (IRMA), and enzyme-linked immunosorbent assay (ELISA). The enzyme-linked immunosorbent assay is very commonly used for cytokine quantification. It is a type of sandwich assay that requires the availability of monospecific antibodies. The use of 2 antibodies is necessary: the first is an anticytokine monoclonal antibody that is bound to a solid substrate (generally, the bottom of each well of a 96-well microtiter plate), and the second is an anticytokine IgG antibody that is either conjugated to an enzyme or subjected to detection with a third antibody–enzyme complex or variable combinations of biotinylated antibodies and streptavidin–enzyme complexes.

The following steps are usually performed: (1) the 96-well enzyme-linked immunosorbent assay plate is prepared, and the monoclonal anticytokine antibodies are bound to the wells; (2) the samples are delivered to the wells and incubated (diluted or undiluted samples can be used, depending on the protocol and level of cytokine expected in the sample), and the cytokine of interest will attach to the bound monoclonal antibody; (3) the samples then undergo several washes to remove any excess antibody remaining; (4) an anticytokine IgG antibody is then added to the wells and incubated; (5) several washes are performed to remove any excess of the second antibody; (6) the substrate solution is added; and (7) optical densities readouts are used to detect color development and quantification. Commercially available kits generally have good sensitivity and reproducibility and are relatively easy to use.

**RELEVANCE OF THE STUDY OF NEUROIMMUNE INTERACTIONS OF NEUROSCIENCE**

Understanding the dialogue between the CNS and the immune system will unlock many enigmas in neurobiology. Among these enigmas are mechanisms that could explain some unique CNS proprieties, such as the failure of the CNS to generate inflammation that can be associated with systemic inflammation, the phenomenon of viral persistence in the CNS, and the minimization of immune affiliated damage in the CNS during confrontation with microbes.

**APPLICATIONS**

Various types of CNS disorders are now known to have inflammatory mediators as key elements of their pathogenesis. These disorders include not only multiple sclerosis, but also ischemic diseases (eg, stroke), traumatic disorders (eg, acute brain trauma), and neurodegenerative conditions (eg, Alzheimer disease). Inflammation is treatable. There are now a variety of established and emerging new approaches to the management of inflammation that can be applied to the treatment of disorders with an inflammatory component. Thus, the identification of expression of cytokines, chemokines, and other inflammatory mediators in the pathogenesis of various types of neurological disorders opens up avenues for novel therapeutic approaches in neurology. Controlled clinical trials will indicate the extent to which new approaches may be of benefit to patients with neurological conditions.

**REFERENCES**