

REVIEW

Microbial Cell-Wall Contaminants in Peptides: A Potential Source of Physiological Artifacts

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MAJDE, J. A. *Microbial cell-wall contaminants in peptides: A potential source of physiological artifacts.* PEPTIDES 14 (3) 629-632, 1993.—Microbial cell-wall products (MCWP) such as endotoxins are easily introduced into peptides produced under standard laboratory conditions. Because these products stimulate the induction of cytokines and other mediators, which, in turn, trigger a broad range of physiological responses, MCWP in peptide preparations are potential sources of artifacts. This brief tutorial outlines the physical/chemical nature of MCWP, some of their sources, their physiological effects, and a simple method to control for them in some peptide preparations.

Pyrogen	Endotoxin	Lipopolysaccharide	Peptidoglycan	Bacteria	Fungi	Mycoplasma
Microbial cell-wall product	Cytokine	Hormone	Nitric oxide	Eicosenoid	Physiology	Contamination
Artifact						

MICROBIOLOGISTS have long recognized that bacteria and related organisms contain physically stable cell-wall components with potent physiological effects. In recent years we have learned that many of the effects of these chemically unique MCWP (21) are related to their induction in immune system phagocytes of the peptide hormones termed cytokines (9). Concurrently, an intimate relationship has been demonstrated between cytokines and classical neuroendocrine hormones, particularly proopiomelanocortin products (4), both within and without the immune system.

Microbial contaminants, dead or alive, enter this complex web by inducing cytokines (and other potent physiological mediators discussed below) in unexpected situations. If a peptide under investigation, adrenocorticotropin (ACTH) for example, is contaminated with MCWP, those products may induce cytokines in the system in which ACTH is being examined. The induced cytokines, in turn, may interact with ACTH in ways that will confound the response examined. A specific example is the cytokine transforming growth factor- β , which at picomolar concentrations inhibits steroidogenesis by ACTH (12). Whenever a complex physiological response that requires several hours to occur is being examined, in vivo or in vitro, MCWP-induced cytokines can potentially contribute to that response.

Unless they are removed by certain procedures described below, MCWP are essentially ubiquitous contaminants of materials that are not processed using the aseptic good manufacturing practice (GMP) conditions employed by the pharmaceutical industry. The GMP conditions are defined by the U.S. Food and Drug Administration (6) and are designed to achieve a final endotoxin level of no more than 0.25 endotoxin units/ml. The

GMP-grade or U.S. Pharmacopeia (USP)-grade products certified for human use are generally free of detectable MCWP, but comparable manufacturing conditions are not commonly used for production of research reagents. While many investigators working with cytokines have learned (sometimes the hard way) to control for MCWP in their materials, this source of artifacts is not broadly recognized by peptide biologists outside of the immunology community. In this tutorial I will briefly describe the unique chemical and physical properties of these problematic MCWP and the ways in which they intrude into peptide research. I will also outline methods for removing them, and a simple method to control for them in experimental design.

SOURCES AND CHARACTERISTICS OF MICROBIAL CELL-WALL PRODUCTS

The pharmaceutical industry has long recognized that microbial contamination of any reagent or container used in the manufacture of a drug or biologic can result in the introduction of pyrogens (i.e., materials that cause fever in rabbits through induction of cytokines such as interleukin-1). Pyrogens commonly are comprised of MCWP left behind on the walls of the container, in the water used for production, in the chromatography columns used for purification, or in the product itself, especially if the product is derived from tissue that was not handled aseptically. For example, fetal calf serum is a common source of pyrogens in cell culture. Sterilizing a product, either by steam or filtration methods, after it is contaminated does not reliably eliminate pyrogens—most MCWP introduced prior to sterilization are stable to autoclaving and will pass through standard filters, thus remaining in the product (31).

Endotoxin is the best recognized pyrogen and is the MCWP detected by the *Limulus* amoebocyte lysate test [(34), chapter 85] that has essentially replaced rabbit pyrogen testing [(6), part 610.13]. Endotoxin is derived from the cell walls of gram-negative bacteria, which include the genetic engineer's workhorse, *Escherichia coli*, and the extremely versatile pseudomonads—organisms infamous for their capacity to grow in distilled water and at refrigerator temperatures. Pseudomonads are thought to be the primary source of pyrogens in water systems and chromatography columns stored wet under nonsterile conditions.

Endotoxins consist of lipopolysaccharide (LPS) and LPS-associated proteins (LAP) that are principally released upon lysis of dead or dying bacteria. The LPS component with its unique sugars and fatty acids has been characterized in detail. In particular, the terminal diphosphorylated lipid A moiety responsible for the electronegativity and the toxicity of LPS (mediated in part through cytokines), as well as its role in gram-negative septic shock, is chemically defined (25). Less is known about LAP, but its practical significance in certain endotoxin detection assays has been recently discussed (22). Central to this discussion is the fact that LPS at subnanogram quantities induces cytokines, eicosenoids, and other mediators. It is also remarkably heat stable and is unaffected by boiling or autoclaving (31). Another important physical property is its negative charge, which causes it to bind tenaciously to glass. One of the routine GMP procedures that copes with both of these physical features is baking glassware and other system components that can tolerate it in dry heat at no less than 170°C for 2 h [(6), part 600.11; (34), chapter 87].

Although the terms pyrogen and endotoxin are often used interchangeably, *Limulus*-negative MCWP that may be detectable in the rabbit pyrogen assay or in the more sensitive leukocytic pyrogen test (11) also induce cytokines. These include peptidoglycans (36) found in the cell walls of all bacteria (both gram-negative and gram-positive) and notable for their D-amino acid content; the complex carbohydrates of fungi, such as glucan (yeasts being the most common contaminants); and the lipoglycans of mycoplasma (14). While the best characterized peptidoglycan, muramyl dipeptide, is far less toxic on a weight basis than endotoxin, some larger forms of peptidoglycan appear to be comparable in toxicity to endotoxins (10). Like LPS, peptidoglycans are extremely heat stable and are unaffected by autoclaving (15). The significance of peptidoglycans, as well as heat-stable fungal and mycoplasmal derivatives (the latter common in products made in vitro by cell lines) as contaminants of peptides, is undocumented because testing for *Limulus*-negative MCWP is not routinely performed.

DOCUMENTATION OF MICROBIAL CELL-WALL CONTAMINANTS

A casual survey of any medical literature database for the term "endotoxin contamination" reveals dozens of clinical problems associated with pyrogens; it is beyond the scope of this article to review these.

Pyrogen contamination of research products is less obvious without extensive testing unless the bioassay used specifically examines the fever response in a sensitive species such as the rabbit. Nevertheless, there are many sources of MCWP in common laboratory reagents and processing procedures that can introduce pyrogens into the peptide of interest.

Water systems [particularly deionization resins and reverse osmosis membranes (19)] and chromatography columns probably are responsible for most pyrogen contamination of laboratory products. Glass surfaces exposed to contaminated water bind LPS and introduce it into materials, especially lipophilic materials, that are subsequently added to the contaminated glass container.

Other MCWP sources are diluents or other additives that were contaminated during their preparation. Significant concentrations of MCWP have been documented in such common reagents as isolated enzymes (16), plant mitogens (13), monoclonal antibodies (37), non-USP serum albumins (5), recombinant hormones (11), and cytokines themselves (26). The cytokine-inducing activity of human immunodeficiency virus gp120 (24) may be due to MCWP contamination of this recombinant product. The neurotoxic properties of β -amyloid peptide are batch dependent (an important clue) and may be an artifact of *Limulus*-negative MCWP contamination (23).

Microbial cell-wall products induce cytokines, in part, because they are potent signalling molecules; it has been suggested that some of the signalling action of phorbol myristate (27) may be due to MCWP contamination. Because only a few laboratory reagents are routinely tested for MCWP, these contaminants often are not recognized.

CONSEQUENCES OF MICROBIAL CELL-WALL CONTAMINATION

Assuming that you are not concerned with meeting Food and Drug Administration regulations for human use, what are the consequences of MCWP contamination of peptide X produced under standard laboratory conditions for the peptide biologist?

Lipopolysaccharide rapidly and potently induces nitric oxide (29), a powerful physiological mediator (32), as well as eicosenoids such as prostaglandins and leukotrienes in macrophages (1).

Cytokines are induced in many cell types by MCWP (9), not just in phagocytes. The MCWP in general, through inflammatory cytokines induced peripherally or in the brain, activate (or inhibit) the hypothalamic-pituitary-adrenal axis, insulin release, fever, sleep, blood coagulation, hypotension, acute phase proteins, and anorexia (9), to list the best recognized physiological responses. Cytokines synergize with each other (35) and with MCWP (28) to function at remarkably low concentrations.

Sensitivity to MCWP (most studied with LPS) varies substantially among species: rabbits, guinea pigs, swine, sheep, and humans respond to ng/kg levels of LPS while mice, rats, and dogs require mg/kg amounts (25). However, the sensitivity of all species can be greatly increased by prior priming with cytokines [particularly with interferons (33)] that may be induced by MCWP in cell culture media, or in the case of in vivo studies, by subclinical infections in the animals (7,8). The priming effect of even normal flora is illustrated by the resistance of germ-free mice to LPS when compared to conventional mice (25). It is highly likely that cytokine priming plays a significant role in experimental variability between animal sources and between laboratories.

The route of administration of MCWP affects both the sensitivity and the kinetics of the response, intracerebroventricular inoculations being 20- to 500-fold more active (if slower in effect) than intravenous inoculations (20). While such observations may represent primarily a dilution effect, they may also represent differential access to MWCP-reactive cells.

In view of the potent physiological activities of MCWP-induced mediators, it is highly likely that MWCP contaminants contribute significantly to variability within numerous bioassays used by peptide researchers.

MWCP CONTROL

The use of aseptic processing conditions and sterile, pyrogen-free reagents for peptide X isolation is the wise, if sometimes impractical, approach to MWCP control. Alternatively, MWCP

can be removed downstream. Separation techniques such as high pressure liquid chromatography can separate X from MCWP, and subsequent sterile packaging in pyrogen-free diluent and pyrogen-free containers (including tissue culture-grade plastics) can prevent recontamination. Ultrafiltration methods that remove all MCWP also exist (30), although they are not yet practical for routine use. Commercial endotoxin removal products based on the affinity of LPS for positively charged surfaces (17) or for *Limulus* anti-LPS factor are available (3). Electric charge-based approaches can be improved by detergent treatment to remove neutralizing LAP (18) or detergents can be used to simply phase-separate LPS from water-soluble proteins such as albumin (2). Such approaches are limited to endotoxin removal, however, and will not cope with other MCWP.

If you purchase peptide X, query the manufacturer as to whether X has been manufactured under GMP conditions or at least tested for endotoxin by the *Limulus* test. If the manufacturer is not familiar with these terms, then you must assume that X has been produced under conditions that could lead to MCWP contamination. Batch inconsistencies are a strong indication that MCWP may be involved in the peptide response (23), regardless of source.

Tests for MCWP are complex and expensive. A simple MCWP control procedure can be used to test heat-susceptible peptides from any source: simple heat inactivation. Boiling peptide X for at least 30 min will denature most larger peptides but will leave the MCWP contaminants unaffected. If the response you are investigating does not disappear upon heat inactivation, then you must consider the very real possibility that the response is due, at least in part, to contaminating MCWP. Alternatively, if the peptide under test is also heat stable, *Limulus* testing or pyrogen testing (the preferred method) may be required to assure that the effects of the peptide are attributable to the peptide alone.

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