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Monitoring Therapeutic mAbs: *IBD Treatments Provide Example For Clinical Laboratory's New Role*

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Therapeutic monoclonal antibodies (mAbs), also known as biologics, are on the forefront of research and development for pharmaceutical companies, with an estimated annual sales growth rate of over 8%. The Food and Drug Administration has approved around 60 therapeutic mAbs, with another 500 in development.

mAb Structure and Metabolism

Human antibodies are composed of two identical heavy chains and two identical light chains, which are held together by disulfide bonds. There are five types of immunoglobulins (defined by their heavy chains: IgG, IgA, IgM, IgD, and IgE) and two types of light chains (κ and λ). The immunoglobulins have a constant, crystallizable fraction (Fc) that interacts with cell receptors. The Fc is formed by constant regions from the heavy chains and a bivalent antigen-binding fraction (Fab) formed by a constant and a variable region from heavy and light chains. The Fab is responsible for defining the antigen specificity of the immunoglobulin.

Therapeutic mAbs are usually an IgG κ isotype. The first-generation mAbs were generated from mouse and rat hybridoma antibodies, with the naming designation "-omab." They found limited clinical success because of their short half-lives and high immunogenicity, which is the property of a drug to induce an immune response by the recipient.

The industry has evolved and developed a number of approaches to humanize rodent antibodies, including chimeric antibodies, which have human constant regions and murine variable regions (-ximab); humanized antibodies, in which 90–95% of the antibody is composed of sequences derived from human IgG, with only the complementarity-determining regions being of murine source (-zumab); and fully human antibodies, which are generated with transgenic animals and phage display (-umab) (Figure 1).

The IgG family of antibodies can be further divided into four subclasses based on the structure of their heavy chains: IgG1, IgG2, IgG3, and IgG4. Structural differences among IgG heavy chains lead to differences in binding of the subclasses to Fc receptors and, consequently, to subclass-specific differences in half-lives and in processes mediated by receptors, including activation of complement (1). Most mAbs are IgG1, but there are also many applications for IgG2 and IgG4 mAbs.

mAb Administration

Many mAbs can be administered intravenously, which allows for infusion of large volumes, lower immunogenicity, and higher bioavailability. Smaller volumes are administered subcutaneously, with self-injection also an option. The absorption mechanism is poorly understood, but appears to occur via lymphatic drainage. Because the skin is adapted to fight foreign substances, immunogenicity is a concern with subcutaneous injections, although the risk is similar with intravenous delivery. Oral mAbs are not yet practical because they would be degraded in the acid pH in the stomach, although researchers are working to overcome this limitation.

The metabolism of mAbs differs from that of other small molecules, and plays a critical role in the length of their half-lives. Human immunoglobulins are synthesized in the rough endoplasmic reticulum of plasma cells and secreted into the extracellular space. The Fc portion of Ig is recognized by Fc receptors (FcRs) on the surface of endothelial cells, and there are several kinds of FcRs. FcR1 and FcR2

(Continued on page 2)

Inside...

Carbon Monoxide Poisoning 6
Carfentanil 9
ACCENT Credit 10

Therapeutic mAbs

Continued from page 1

internalize the immunoglobulins, leading to lysosomal degradation. In contrast, the Brambell FcR recycles the immunoglobulin through endosomes with subsequent secretion back into the circulation, so the pharmaceutical industry has worked to increase the affinity of mAbs for the Brambell receptor.

Most mAbs in clinical use have half-lives of a week or longer, permitting long dosing intervals (2–8 weeks). In binding to its target, the mAb forms an immune complex, which triggers two processes in the immune system. First, it activates the complement system classical pathway, which can lead to different paths of degradation, either via opsonization or chemotaxis. Second, the interaction of the mAb's Fc with the FcR of cells leads to uptake or induction of killing by natural killer cells.

mAbs in Inflammatory Bowel Disease

About 20 years ago, biologics targeting tumor necrosis factor-alpha (TNF)—a cytokine overproduced in autoimmune inflammatory conditions—hit the market, approved by the Food and Drug Administration for use in rheumatoid arthritis, ankylosing spondylitis, and inflammatory bowel disease (IBD).

IBD comprises two major disorders: ulcerative colitis and Crohn's disease. Ulcerative colitis involves the colon and is characterized by inflammation of the mucosal layer, whereas Crohn's disease can involve any component of the gastrointestinal (GI) tract, being characterized by transmural inflammation.

The age of onset of IBD is typically between 15 and 40 years old. Suspicion of IBD generally arises from a combination of symptoms, laboratory tests, and imaging evaluations. GI symptoms include loose stools, bloody diarrhea, abdominal pain, and perianal disease with fistulas or fissures. Extra-GI manifestations include skin rash, jaundice, hepatomegaly, and arthritis. Common laboratory findings include anemia, increased white blood cell and platelet counts, elevated inflammatory markers, hypoalbuminemia, elevated fecal calprotectin, and occult blood in stool. Imaging studies such as colonoscopy and magnetic resonance enterography or computed tomography are often used to visualize the intestinal walls and the inflammation process.

IBD Therapy

For patients with mild to moderate disease, therapy consists of oral medications, but patients with severe symptoms may require hospitalization. The site of Crohn's disease also affects treatment. Initial treatment may include aminosalycilate drugs such as budesonide and mesalamine, antibiotics, and conventional glucocorticoids. Patients whose symptoms return or fail to respond to these therapies likely require treatment with immunomodulators or biologics. Before biologics were available, refractory disease was managed surgically with resection of the inflamed tissue, which had a significant impact on quality of life.

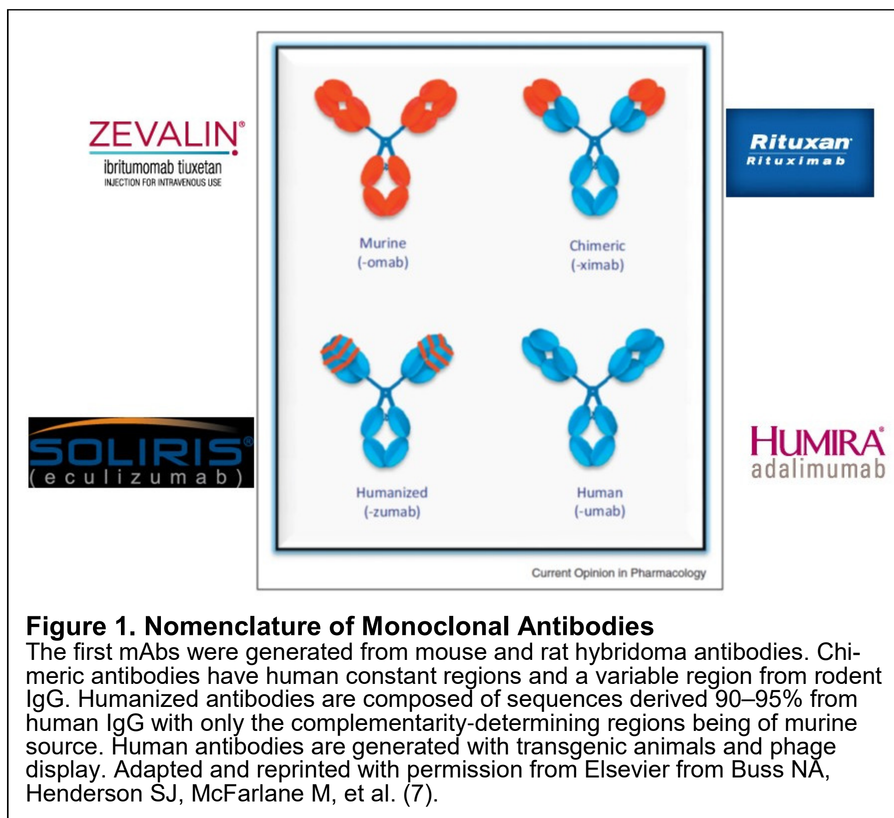

Figure 1. Nomenclature of Monoclonal Antibodies
The first mAbs were generated from mouse and rat hybridoma antibodies. Chimeric antibodies have human constant regions and a variable region from rodent IgG. Humanized antibodies are composed of sequences derived 90–95% from human IgG with only the complementarity-determining regions being of murine source. Human antibodies are generated with transgenic animals and phage display. Adapted and reprinted with permission from Elsevier from Buss NA, Henderson SJ, McFarlane M, et al. (7).

The list of biologics approved for use in IBD is growing, with several options available that target different molecules. TNF inhibitors include infliximab, adalimumab, golimumab, and certolizumab. These agents have different modes and frequency of administration. Another recently approved biologic, vedolizumab, targets the alpha-4-beta-7 integrin receptor located on the surface of gut T-lymphocytes. This targeting means that vedolizumab can be used to reduce inflammation in the small and large intestines with specificity. The most recently approved biologic for Crohn's disease is ustekinumab, which targets IL-12 and IL-23 (Table 1).

Several clinical trials have shown that these biologics are safe and effective, and the decision of which one to prescribe is often based on the patient's insurance coverage and the drug's mode of administration. Infliximab and adalimumab together have over 50% of the market share. For pediatric patients, intravenous infusions are usually preferred over self-administered injections. Some insurance companies re-

Table 1. Monoclonal antibodies approved for use in inflammatory bowel disease

mAbs	Approved uses	Trade name	Target	Delivery mode	Trough therapeutic concentration*	Prevalence of ADAs**
Infliximab	CD, UC	Remicade	TNF	IV	>5 mg/mL	8–61%
Adalimumab	CD, UC	Humira	TNF	SC	5–10 mg/mL	2.6–26%
Golimumab	UC	Simponi	TNF	SC	>0.8 mg/mL	4%
Certolizumab Pegol	CD	Cimzia	TNF	SC	>27.5 mg/mL	8%
Vedolizumab	CD, UC	Entyvio	α4β7 integrin	IV	>40 mg/mL	4%
Ustekinumab	CD	Stelara	IL12/IL23	SC or IV	0.5–5 mg/mL***	3–5%

CD, Crohn's disease; UC, ulcerative colitis; TNF, tumor necrosis factor alpha; IV, intravenous infusion; SC, subcutaneous infusion; ADA, anti-drug-antibodies
* For many mAbs, therapeutic concentrations are not well-defined, and target concentrations vary among the conditions treated..
** Includes both binding and neutralizing antibodies. Different methods for ADA testing, time-points in measurement, disease populations, and diverse clinical trials contribute to the wide range of reported ADAs.
*** These concentrations are from clinical trials for treatment of psoriasis. Concentrations in CD are not well-established.

quire evidence of failure of TNF inhibitors before covering vedolizumab or ustekinumab.

Assessing Reponse to Therapy

Assessing the response to therapy in IBD requires a biopsy, endoscopy, or colonoscopy—all invasive procedures—because the symptoms do not usually correlate with the presence of mucosal lesions or with elevated biomarkers of inflammation. Recently, fecal calprotectin has been used as an indirect assessment of response to therapy. Calprotectin comprises 60% of the total cytoplasmic protein content in neutrophils. When neutrophils are activated in the lumen of the GI mucosa as part of the inflammatory process of IBD, their intracellular content is released, so the amount of calprotectin present in the feces is proportional to the number of neutrophils. However, in patients who have neutropenia or who are taking nonsteroidal anti-inflammatory drugs or proton-pump inhibitors, results can be falsely decreased or only borderline elevated.

Assessing a patient's response to biologics therapy is critical because the therapies are expensive and side effects include a greater risk for infections, such as reactivation of latent tuberculosis or hepatitis B; infusion- or injection-site reactions; cutaneous reactions; hepatoxicity; and demyelinating disease. Higher incidences of mortality and hospitalization in heart failure patients have been documented.

Despite the therapeutic efficacy of TNF inhibitors in some patients, more than one-third of patients show no response, and therapy becomes ineffective over time in up to 50% of the responders. The reasons for the lack of response are not well understood, but may include disease processes mediated by proinflammatory molecules other than TNF. The loss of response over time is associated with low albumin, high body mass index, the degree of systemic inflammation, and the degree of immune response to therapy (2).

Another important factor is immunogenicity, in this case the development of autoantibodies to infliximab. Laboratory testing of patients to quantitate infliximab and assess immunogenicity can help optimize therapy in cases of partial response or loss of response.

As a side note, TNF itself is not the analyte of choice for monitoring therapy with TNF inhibitors because TNF tests do not distinguish between free TNF and TNF bound to the mAb, either in the extracellular or membrane-bound form of the cytokine.

Impact of Immunogenicity of mAbs in IBD

Immunogenicity can lead to the production of anti-drug antibodies (ADAs). The risk factors associated with the development of ADAs include which disease the patient has and whether the patient has received prior or concomitant immunosuppressive therapy. Characteristics of the monoclonal antibody also play a role. Although ADAs are often directed against murine sequences present in chimeric monoclonal antibodies, ADAs can also form against humanized or fully human monoclonal antibodies. In addition, dosing frequency, duration of treatment, route of administration, and interruption in treatment can all affect the risk of ADA formation.

ADAs can be classified based on how they affect the function of the monoclonal therapeutic. A neutralizing ADA prevents the binding of the mAb to its target ligand, essentially rendering the drug non-functional. In contrast, a non-neutralizing ADA binds to the mAb in a way that does not inhibit the drug/target interaction. The presence of ADAs can also affect the availability of the mAb. Some ADAs accelerate clearance of the drug, likely through immune complex metabolic pathways, whereas others reduce clearance, leading to increased concentrations.

Infliximab Quantitation

Currently, infliximab quantitation using a commercially available assay is one of the most commonly performed tests for a mAb in routine practice. It is generally performed in conjunction with an assessment of immunogenicity looking for antibodies-to-infliximab (ATIs). These tests are often ordered in IBD patients on infliximab therapy who are experiencing a loss of response. The results play an im-

portant role in patient management. For patients who have undetectable or low concentrations of infliximab (measured at trough) but no detectable ATIs, the physician may increase the dose in an attempt to increase the amount of the drug in circulation. If the patient has low infliximab in the presence of an ATI, the physician may switch the patient to another TNF inhibitor. For patients with high trough concentrations of infliximab, whether or not an ATI is present, the physician may to switch to a therapy with a different mechanism of action, such as the anti-alpha4-beta-7-integrin antibody vedolizumab.

Clinical tests for adalimumab, certolizumab, and vedolizumab are also available commercially. For many biologics, there is little evidence supporting the link between trough concentrations, ADAs, and loss of response.

Infliximab Test Methods

In the many clinical laboratories that have implemented tests for infliximab and ATIs, the methodologies vary widely.

The traditional sandwich enzyme immunoassay (EIA) was one of the first methods available. There are several variations of this method that stem from differences in the capture and detection reagents. For the capture reagent, either TNF or an anti-infliximab antibody can be used. For detection, the EIA may incorporate an enzyme-labeled anti-human IgG or a labeled anti-infliximab antibody. Regardless of the detection antibody, quantitation is based on the absorbance of a colored product, which is proportional to the amount of infliximab (3).

Two other methods, the mobility shift assay and the cell-based reporter assay, are based on the interaction of infliximab with TNF. In the mobility shift assay, a labeled form of TNF is incubated with a patient sample containing infliximab. Through size-exclusion high-performance liquid chromatography (HPLC), the larger infliximab/TNF complex is separated from the free TNF. The fluorescence of the labeled TNF is monitored and the area under the curve for the bound TNF is proportional to the concentration of infliximab in the sample (4).

In the cell-based reporter assay, a defined amount of TNF is added to the patient sample, after which the patient sample is added to a cell line in which a cloned luciferase reporter gene is under control of a TNF response element. In the absence of infliximab, TNF binds to its receptor on the cell surface, leading to transcription and translation of the luciferase reporter gene. If infliximab is present, it binds to TNF and prevents the target from binding to its receptor, thereby producing less luciferase. The concentration of infliximab in the sample is inversely proportional to the luminescent signal (5).

The final method for infliximab quantitation is a tandem mass spectrometry method (LC-MS/MS). This method begins with the addition of a digestion standard to the sample, followed by immunoglobulin enrichment. The immunoglobulin fraction is subjected to trypsinization, which produces a light-chain variable region peptide that is unique to infliximab. After addition of isotopically labeled peptides, the mixture is subjected to liquid chromatography and selective reaction monitoring using a triple quadrupole mass spectrometer. Fragment ions from the infliximab-specific peptides are monitored and compared to a standard curve for quantitation (6) (Table 2).

Table 2. Laboratory methods for measurement of biologics, using infliximab as an example

Method	Mechanism of action	Quantitation	Challenges
Mobility shift assay	Labeled TNF binds to infliximab. Complexes have different sizes and are measured by size-exclusion high performance chromatography and detected using fluorescence.	Area under the curve of immune complex by fluorescence detection.	Measurement of functional mAb. May not distinguish one TNF inhibitor from another.
Enzyme immunoassay	TNF is coated on a solid surface. Detection antibody is labeled anti-human IgG antibody or an anti-infliximab antibody is used as detection antibody.	Signal intensity (absorbance) is proportional to amount of TNF inhibitor bound to its target or ADA.	Measurement of functional mAb. Other endogenous molecules can bind to TNF and generate nonspecific signals.
Bridging immunoassay	Antibodies-to-infliximab are labeled with 2 different molecules, one is usually biotin. Biotin-labeled molecule is captured on a streptavidin surface and the other label serves as detection trigger.	Signal intensity (such as chemiluminescence) is proportional to amount of TNF inhibitor bound to the anti-drug-antibody.	The ADAs may bind to the mAb-binding epitope or elsewhere. Form of the mAb detected may vary.
Cell-based reporter gene assay	Sample is added to a cell line in which a cloned luciferase reporter gene is under control of a TNF response element. Assay can theoretically be used for any TNF inhibitor.	TNF inhibitor amount is inversely proportional to the amount of luciferase emitted luminescence.	Measurement of functional mAb. May not distinguish one TNF inhibitor from another.
Liquid chromatography-tandem mass spectrometry	Trypsin digestion yields peptides unique to infliximab variable region.	Peak area intensity is proportional to total infliximab.	Peptides unique to the mAb are harder to identify as mAbs become more humanized.

TNF, tumor necrosis factor-alpha; ADA, anti-drug antibodies

Differences Among Assays

There are some important differences among the various infliximab quantitation assays. One difference is related to which form of infliximab is measured. In a patient sample, infliximab can exist free in the circulation, or it can be bound to either endogenous TNF or an ADA. The LC-MS/MS method is unique because it is based on the tryptic-digest approach, so it measures total infliximab, both bound and free. In contrast, any method based on ligand binding measures infliximab in a form that could still bind to TNF. Theoretically, elevated concentrations of endogenous TNF could negatively interfere with the ligand-binding based assays. However, this interference is not likely to be relevant in practice because the concentration of TNF is less than 1/1000th of the standard trough concentration of infliximab. Any neutralizing ADA present could also interfere in these assays because it would prevent infliximab from binding to TNF.

The ligand-binding assays are also subject to positive interference from other TNF inhibitors. These ligand-binding assays are based on the binding of any drug to TNF, and not specific for infliximab compared to adalimumab, for example. Although it may seem advantageous to have a single assay that could detect multiple TNF inhibitors, these results could be confusing, especially when patients transition from one therapy to another.

Despite these potentially confounding factors, initial studies have demonstrated reasonable comparability of infliximab quantitation by these various methods. However, more studies are needed to understand the performance of each method in routine clinical practice.

ATI Assessment

In addition to infliximab quantitation, assessment for ATIs may also be needed for optimal patient management, and several methods are currently available. In solid-phase EIAs, immobilized infliximab is used as the capture antigen, with a labeled anti-human IgG as the detection antibody. The bridging immunoassay is another test related to the solid-phase EIA in which two forms of labeled infliximab are added to the patient sample—one biotinylated and the other with a fluorescent or luminescent label. Any ATI present forms a “bridge” between the two forms of infliximab. The biotinylated infliximab captures the bridging complex using a streptavidin-coated solid phase, followed by fluorescent or luminescent detection.

Mobility shift and cell-based reporter assays have also been adapted for ATI assessment. The mobility shift assay uses a fluorescently labeled infliximab, which is added to the patient sample. The principle is then similar to the infliximab mobility shift assay: Free infliximab is separated from any infliximab/ATI complexes using size-exclusion HPLC, with the amount of fluorescence proportional to the amount of ATI in the sample.

In the cell-based reporter assay, both TNF and infliximab are added to the patient sample. In the absence of ATIs, infliximab binds to TNF, thereby preventing transcription of the luciferase gene. However, in the presence of an ATI, the antibody binds to infliximab and prevents interaction with TNF. TNF is free to bind to its cell-surface receptor and induce transcription of the luciferase gene. The amount of light produced is directly proportional to the concentration of ATIs (Table 3).

Interference in ATI Tests

In all the ATI methods, interference by infliximab in the patient sample can be an issue. Most ATI methods are based on detecting an interaction between a labeled form of infliximab and the ATI. When infliximab is present, the unlabeled drug competes with the labeled form, leading to a potential false-negative result. One way to mitigate this interference is to use an acid-dissociation protocol, in which the endogenous infliximab/ATI complexes are dissociated in acidic pH, followed by neutralization in the presence of labeled drug. For this approach to be successful, preferential binding of the ATI to the labeled infliximab is needed, which is usually achieved by increasing the concentration of the labeled drug. Methods that use acid-dissociation are sometimes

Table 3. Methods for measurement of anti-drug-antibodies (ADAs), using infliximab as an example

Method	Mechanism of action	Type of ADA measured
Mobility shift assay	Complexes of ADA with fluorescent-labeled infliximab.	ADAs that bind to infliximab, forming an immune complex.
Enzyme immunoassay	Immobilized infliximab is used to coat the solid surface plate, with labeled anti-human IgG as the detection antibody.	ADAs that bind to infliximab, forming an immune complex.
Bridging immunoassay	Label A and label B forms of infliximab are used in a bridging format.	ADAs that bind to 2 molecules of infliximab form a “bridging” immune complex. Species and immunoglobulin isotype independent.
Cell-based reporter gene assay	Sample is saturated with infliximab, then added to a cell line in which a cloned luciferase reporter gene is under control of a tumor necrosis factor-alpha (TNF) response element.	Neutralizing antibodies to infliximab.

referred to as "drug-tolerant" assays. However, it is unlikely that acid-dissociation completely overcomes endogenous drug interference. Even drug-tolerant methods can underestimate the ATI in the presence of high concentrations of infliximab.

Interpretation of ATI results can be challenging, given the diversity of methodologies. Results are often reported in different units, making quantitative comparisons almost impossible. However, most clinical management decisions are made based simply on the presence or absence of an ATI, in conjunction with infliximab trough concentrations.

Conclusion

Monoclonal antibody therapeutics are among the fastest growing classes of pharmaceuticals. Although testing for them is currently limited, the use of drug quantitation and the assessment of ADAs in patient management will continue to expand. With all the methodologies available, and the caveats associated with each of them, it is critical for clinical laboratorians to expand our expertise in this field. Although there are many challenges, this field presents another opportunity for the clinical laboratory to impact management and improve patient outcomes.

Learning Objectives

After reading this article, the reader will be able to recognize the types of monoclonal antibody therapeutics used for inflammatory bowel disease. The reader will be able to list methods used in the clinical laboratory to monitor therapeutic monoclonal antibodies and to summarize the indications for testing, as well as the main challenges with the available approaches.

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The authors report an intellectual property/royalty income interest in an LC-MS/MS-based method for measurement of therapeutic monoclonal antibodies.

Carbon Monoxide Poisoning

A Signaling Molecule Going Rogue

By Carmen Gherasim, PhD

Carbon monoxide (CO) is a colorless, odorless, and nonirritating gas whose signaling properties become toxic at high concentrations in ambient air. CO is often dubbed the "silent killer" because its effects are imperceptible but potentially lethal—with a fatality rate as high as 30%.

CO poisoning remains the leading cause of unintentional poisoning worldwide despite efforts to raise awareness of its preventable nature. In the U.S., the Centers for Disease Control and Prevention estimates that nearly 500 people die annually of unintentional CO poisoning not related to house or other fires, a number that is likely an underestimate because many cases are not reported or not diagnosed. An estimated 15,000 people a year are admitted to emergency departments and miss at least one day of normal activity because of CO poisoning (1).

The diagnosis of CO poisoning is difficult because the symptoms are nonspecific and there is often a poor correlation between carboxyhemoglobin levels and the severity of the exposure. These challenges can delay treatment.

CO's Role in Signaling

Low concentrations of carbon monoxide are produced physiologically as a catalytic byproduct of heme degradation by heme oxygenase enzymes. Heme oxygenase-2 generates small quantities of CO, which acts as a signaling molecule to modulate key cellular processes such as inflammation, apoptosis, and antioxidant defense. CO levels can increase sub-

stantially in response to hypoxia, iron starvation, and oxidative stress conditions, which can cause tissues to induce the expression of heme oxygenase-1 (2).

Exogenous administration of low concentrations of CO has been shown to have cytoprotective effects in various diseases, including acute lung injury, ischemia reperfusion injury, graft rejection, and sepsis. Molecular targets modulated by interaction with CO include soluble guanylate cyclase, ion channels, nitric oxide synthase, NADPH oxidase, xanthine oxidase, and cytochromes (2). Despite its complex nature as a gasotransmitter, carbon monoxide is best known for its toxicity at high concentrations.

Etiology of CO Poisoning

CO can be generated as an environmental toxin from incomplete combustion of organic materials when there is not enough oxygen present for the complete oxidation to carbon dioxide. The most common indoor sources of carbon monoxide in homes include malfunctioning heating systems, water heaters, generators, fireplaces, ranges, ovens, and other carbon-based-fuel devices operated in spaces without adequate ventilation. Industrial settings such as boiler rooms, breweries, warehouses, petroleum refineries, and car repair shops are also high-risk environments for CO exposure. Recreational activities that involve a risk of carbon monoxide poisoning include diving, boating, water skiing, and teak surfing (hanging on to a platform behind a motorboat) because of dangers such as a faulty diving tank or exposure to motorboat fumes. Although smoking tobacco is unlikely to generate enough CO to be a problem, CO poisoning secondary to hookah smoking has been reported.

The incidence of CO poisoning increases in the winter months in colder climates because of the greater use of heating sources. More home insulation to maintain comfort and fuel efficiency also increases the risk of CO accumulation. CO poisoning can be prevented through periodic inspections of furnaces and water heaters to ensure proper functioning and identify leaks. Additionally, carbon monoxide detectors can warn inhabitants of increasing CO levels.

CO Poisoning Symptoms

Toxic CO concentrations can induce a wide range of nonspecific symptoms depending on the concentration and duration of exposure (Table 1). Common symptoms of mild and moderate CO poisoning include dizziness, fatigue, weakness, and headache that, in the absence of a clear source of exposure, can often be confused with other diagnostic possibilities such as heart disease, respiratory illness, and anemia. Other signs that can help narrow the diagnosis include flushed checks, the absence of fever, and a history of exposure to a CO source.

Severe CO poisoning induces headache, malaise, chest pain, shortness of breath, nausea, irritability, ataxia, altered mental status, loss of consciousness, coma, and death. The most common clinical signs include tachycardia, hypotension, cognitive and sensory disturbances, metabolic acidosis, myocardial ischemia or infarction, and pulmonary edema due to cardiac toxicity. Prompt diagnostic testing to initiate treatment is critical for reducing the neurological damage following CO exposure.

Mechanism of CO-Induced Toxicity

CO readily diffuses into pulmonary capillaries, where it binds to hemoglobin with an affinity 200 to 300 times greater than that of oxygen to form carboxyhemoglobin (COHb). COHb impairs the ability of hemoglobin to carry oxygen in the bloodstream, which can lead to cellular hypoxia (3). The organs most sensitive to oxygen deprivation are the heart and brain.

Although COHb was once thought to be the main pathway of CO poisoning, it is now widely accepted that the pathophysiological effects of CO extend beyond its interaction with hemoglobin. Dissolved CO in the plasma can enter cells, bind to other heme-containing proteins such as myoglobin and cytochromes, and alter their function (Figure 1).

Table 1. Symptoms Associated with Increasing Concentrations of Carbon Monoxide (6,7)

% CO Atmospheric	PPM CO Atmospheric	% COHb In Blood	Signs and Symptoms (time to onset)
0.001	10	2	No appreciable effects
0.007	70	10	Shortness of breath on vigorous exertion; dilation of cutaneous blood vessels
0.01	120	20	Slight headache; shortness of breath on moderate exertion
0.02	220	30	Headache; irritability; easily fatigued; impaired judgment; dizziness; blurred vision (2–3 hours)
0.035–0.052	350–520	40–50	Pounding headache; confusion; collapse; fainting on exertion (1–2 hours)
0.08–0.122	800–1220	60–70	Unconsciousness; intermittent convulsion; respiratory failure; eventual death if not removed from CO (25–45 minutes)
0.160–0.195	1600–1950	80	Rapidly fatal (10–20 minutes)
>0.32	3200		Fatal (<10 minutes)

The very same proteins and pathways targeted by CO-based signaling are aberrantly regulated by high concentrations of CO, which induce nitric oxide and reactive oxygen species generation and impair cardiac and neuronal ion channels function. These "extra-hemoglobin effects" can explain the extreme variability between patients admitted for CO poisoning and support the need for multiple therapies (4).

Dysfunction of the cardiovascular system is the most common cause of mortality associated with CO poisoning. In addition to impairing myoglobin function, CO can displace nitric oxide from platelets. Nitric oxide mediates relaxation of the smooth muscle and decreased vascular resistance, and the presence of CO can induce these processes to a degree that can result in cardiogenic shock and dysrhythmia. Patients exposed to CO also exhibit a high risk for morbidity due to neuropsychological sequelae such as cognitive and affective changes, peripheral and cranial neuropathies, psychosis, and dementia. These conditions can develop days or weeks after exposure, and can be persistent, disabling, and even permanent.

CO Poisoning Testing

Confirmatory laboratory tests are required after the initial evaluation of a patient with a history that indicates possible CO poisoning. Elevated COHb on co-oximetry with multiwave pulse oximetry is the most common laboratory finding, but the result does not always correlate with the severity of exposure or other clinical presentations. If the patient left the toxic environment and started breathing normal air for several hours, the levels of COHb can diminish and make the test results confounding. In general, an elevation of 3% COHb for nonsmokers and >10% COHb for smokers strongly supports a diagnosis of CO poisoning (5). False elevation of the COHb levels can occur in sickle-cell anemia patients when endogenously generated CO increases due to hemolytic anemias or hemolysis. Therefore, treatment of a patient with CO poisoning should not be based solely on COHb levels.

Treatment for CO Poisoning

Oxygen therapy is the current standard care for CO poisoning because it addresses the hypoxic toxicity and prevents the development of neurological sequelae. Normobaric oxygen therapy (breathing 100% oxygen) or hyperbaric oxygen therapy (exposure to 1.5 atmospheres of 100% oxygen) reduces the average half-life of COHb from the 320 minutes (in patients breathing normal room air) to 71 and 21 minutes, respectively. In addition to accelerating COHb elimination, hyperbaric oxygen also increases the partial-pressure of oxygen in the plasma, which allows oxygen to bind to free-binding sites on hemoglobin. However, extended hyperbaric therapy can potentially enhance the CO-induced production of reactive oxygen species.

In patients exposed to fire, the presence of other toxic products such as cyanide and hydrogen sulfide generated by the combustion of synthetic materials should not be overlooked because they can lead to similar symptoms. High concentrations of hydrogen sulfide and cyanide directly inhibit mitochondrial function, leading to lactic acidosis, cardiovascular instability, and impaired mental state. Concomitant treatment for CO poisoning with cyanide and hydrogen sulfide scavengers should be considered when their presence is suspected.

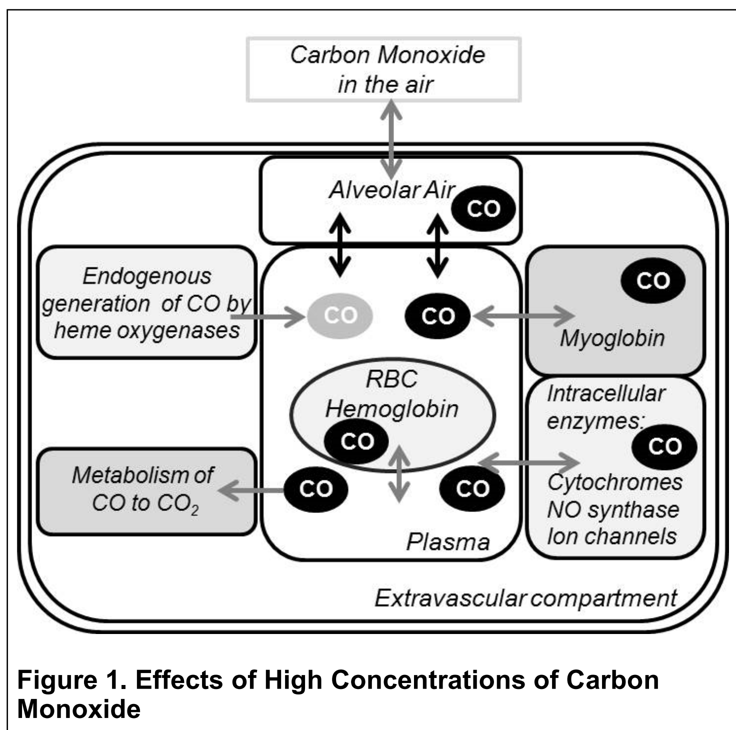

Figure 1. Effects of High Concentrations of Carbon Monoxide

Learning Objectives

After reading this article, the reader will be able to distinguish between the signaling and toxic properties of carbon monoxide as well as recognize that carbon dioxide poisoning symptoms are nonspecific, making the diagnosis challenging. The reader will also be able to describe the toxic effects of carbon monoxide not related to hemoglobin.

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Carfentanil

An Extremely Potent Opioid Has Begun Making an Appearance in Street Drugs

By Robert A. Middleberg, PhD

In this new age of designer drugs of abuse, both newer and older compounds are making their way into street drugs. One such recently identified substance is carfentanil, which is a carbomethoxy-analogue of fentanyl with a molecular weight of 394.5066 g/mol (Figure 1). It is purportedly 10,000 time more potent than morphine and 100 times more potent than fentanyl.

First synthesized in 1974 by Janssen Pharmaceutica, its legitimate use was as a tranquilizer or general anesthetic for large, often exotic, animals. It was sold under the brand name Wildnil (1). It is a Schedule II controlled substance. In mid-2016, carfentanil was found as a street drug in combination with heroin or in tablets by itself (2).

Mechanism of Action and Effects

Carfentanil acts mainly on mu receptors as a competitive agonist leading to both positive and negative regulation of G-protein complexes. As with other opioids, the primary effects include analgesia and sedation. Its toxic sequelae also mirror those of other opioids, including respiratory depression, coma, and death (1).

It is estimated that as little as 20 µg of carfentanil can be fatal to an adult human; however, these are estimates based on its relative potency. Because of this potency, laboratory personnel must take care to avoid contacting airborne particles, such as when handling suspected bulk powders. Opioid overdose antidotes, such as naloxone, are effective in treating exposure; however, due to its rapid action and potency, treatment is time-sensitive (3).

The substance has been linked with the October 2002 Nord-Ost siege in Moscow, when 170 of 850 people died during a hostage situation in a movie theater (4). Since mid-2016, carfentanil has resulted in numerous hospitalizations and deaths in the U.S. The Midwest, especially Ohio, has been one of the hardest hit areas.

The drug is believed to be synthesized in China or Mexico and make its way to the U.S. via drug traffickers in Mexico and South America (5).

Although no human-based studies are available on the metabolic profile of carfentanil, predictive models and urine from a victim of the Nord-Ost siege indicate that norcarfentanil is a primary metabolite. This pathway would be in keeping with the known metabolic profile of fentanyl. Additionally, the half-life of carfentanil is predicted to be relatively long, making identification of the compound and metabolites possible for an extended period after exposure (4,6).

Tests

Carfentanil can be identified in biological specimens using mass spectrometric-based screening tools, such as liquid chromatography/time-of-flight mass spectrometry, as well as analyte-specific procedures, such as liquid chromatography/tandem mass spectrometry.

At NMS Labs, patient samples that ultimately tested positive for carfentanil did not have positive fentanyl immunoassay results, but we did not have enough information to determine whether the negative results were related to low concentrations or a general lack of cross-reactivity with fentanyl immunoassays. Reference materials for both carfentanil and norcarfentanil are available (Toronto Research Chemicals, Toronto, Canada).

Since we became aware of its appearance as a drug of abuse, NMS Labs has identified carfentanil in 27 postmortem cases, often in conjunction with other common drugs of abuse and designer opioids,

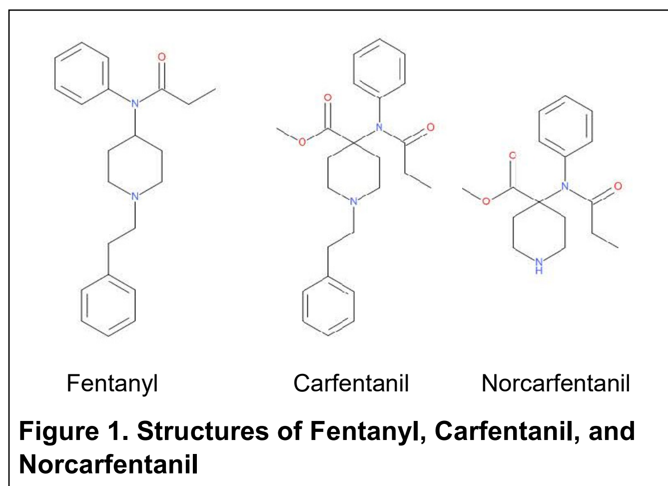

Figure 1. Structures of Fentanyl, Carfentanil, and Norcarfentanil

including U-47700 and furanyl fentanyl. This number is only one lab's experience, so there is no doubt that dozens more deaths have resulted from this potent, deadly opioid. Both clinical and forensic laboratories must be keen to the recent appearance of this substance and be able to recognize its presence.

Learning Objectives

After reading this article, the reader will be able to describe the presence of carfentanil in street drugs as well as summarize its pharmacology.

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