Journal of Current Veterinary Research

ISSN: 2636-4026

Journal homepage: http://www.jcvr.journals.ekb.eg

Clinical Pathology

A Review on The Clinical Efficacy of Antitetanic Hyperimmune Serum Prepared in Equine Using Freund Adjuvants in Response to Toxoid and Toxin Immunization

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ABSTRACT

In the industrialized world, tetanus is now a rare disease. However, it is still one of the most dramatic and internationally widespread diseases and a leading cause of death worldwide, with a high case fatality rate, particularly in underdeveloped countries. Immunization to tetanus is antibody-mediated and can be accomplished with tetanus toxoid to elicit an active immunization by forming antitoxin. Consequently, injection of antitetanic hyperimmune serum containing antitoxin can provide passive immunity in those who have a wound that is at high risk and have not been fully vaccinated with tetanus toxoid making it one of the most frequently used treatment agents for tetanus. The production of hyperimmune serum with an adequate neutralizing potency to tetanus is influenced by many factors including adjuvants. Adjuvants are elements that capable of enhancing the antigen-specific immune responses and have proven to be key components in vaccines. Adjuvants come in a variety of forms, each with its own mechanism of action and the immune response elicited by variable making selection of the appropriate adjuvant is a critical issue. Freund's adjuvant is perhaps one of the most commonly used adjuvants that is used as an immunopotentiator (booster) to trigger a humoral antibody response to produce high titer antibodies. There are two types of Freund adjuvants: complete and incomplete.If it contains killed Mycobacterium tuberculosis it is known as Complete Freund Adjuvant (CFA). Without the bacteria it is Incomplete Freund Adjuvant (IFA). Both CFA and IFA are particularly effective adjuvants in producing high-tittered specific antibody but also each of them has its own advantages and disadvantages. Although the efficacy of Freund adjuvants in triggering the humoral immune response and production of high titer antibodies has been proven, there are several concerns that the use of these adjuvants particularly CFA is associated with significant pathological lesions including severe pain at the injection site, abscesses, chronic granulomas, and ulcerating tissue necrosis. However, many recommendations have been published and highlight the necessity of considering factors like the mode of action of adjuvant, antigen's size and composition, the species being immunized, the immunization method and the type of immunity required to help selecting the appropriate adjuvant and thus to mitigate some of these concerns.

Considering the numerous applications of hyperimmune serum in research and clinical fields, the production of hyperimmune serum as a biological reagent will continue to be developed and thus will continue to require the use of adjuvants with continuing concerns about them. Therefore, a greater understanding of their adjuvanticity potential, safety tolerability, and efficacy could help to limit, control, and correct adjuvant-related side effects making them useful products in the vaccine industry and also could help in the creation of new adjuvants that can be used in vaccines against challenging pathogens.
Key words: Equine, Freund adjuvants, Hyperimmune serum, Tetanus and Toxoid.

INTRODUCTION

Tetanus is a deadly bacterial disease that has been known for over 24 centuries. It is one of the most dramatic and internationally widespread diseases of humans and vertebrates. Tetanus is an uncommon but terminal disease caused by *Clostridium tetani* (*Cl. tetani*) that belongs to a class of saprophytic, obligate anaerobic, spore forming gram-positive organisms (Brook, 2018). *Clostridiumtetani* enters the body when it is inserted into the comparatively anaerobic conditions present in wound tissue. Tetanus spores will quickly enter a wound at the time of injury, even though the injury is minor. Contaminated wounds are especially vulnerable, particularly those with devitalized tissue and deep-puncture trauma (Yen and Thwaites, 2019).Because the disease's sporesare still present in the atmosphere and recovery from naturally acquired tetanus does not result in protection against further attacks, tetanus eradication is difficult because and the only prevention approach is immunization (Forstner *et al.,* 2018). Immunization to tetanus is antibody-mediated that can be accomplished with toxoid containing vaccines which stimulate an immune response and production of antitoxin. The currently available antitetanic sera are generally obtained by hyperimmunization of an animal (generally, a horse) by injecting the animal with increasing doses of alum-toxoid (Rosenstein *et al.,* 2020). Injection of antitetanic hyperimmune serum containing antitoxin neutralizes the neurotropic toxin and provides passive immunity in situations when there is a high risk of infection and insufficient time for the body to develop its own immune response, or to reduce the symptoms of ongoing or immunosuppressive diseases such as hypogammaglobulinemia (Lasocka *et al.,* 2021). Freund's Adjuvant is one of the most effective adjuvants currently available, it is essential for the protocols of production of high titer antibodies production and research.There are two types of Freund adjuvants: complete and incomplete depending on if it contains killed Mycobacterium tuberculosis (Complete Freund Adjuvant, CFA) or not (Incomplete Freund Adjuvant, IFA) (Nanishi *et al.,* 2020). Both adjuvantswere proven to be effective in producing high-tittered specific antibody but also each of them has its own advantages and disadvantages. The aim of this review was to highlight the present and up-to-date protective and therapeutic impacts of antitetanic hyperimmune serum prepared in horse by using Freund adjuvants to explore its clinical importance and application.

Tetanus

Tetanus is a potentially lethal disease and one of the most dramatic and internationally widespread diseases of nervous system of human and vertebrates that is caused by a neurotropic exotoxin produced by *Clostridium tetani* (*Cl. tetani*). *Cl. Tetani* is a gram-positive, spore-forming non-encapsulated bacillus occurring naturally in soil and the global environment (Brook, 2008). The disease normally happens in people that are not immunized, are only

partially immunized, or are completely immunized but have not had enough booster doses (Pascual *et al.,* 2003). Therefore, this disorder overwhelmingly impacts developed countries due to inadequate immunization coverage. In addition, since *Clostridium tetani* spores are still present in the atmosphere, herd immunity plays no role in tetanus prevention (WHO, 2017). Further, following disease recovery, survivors may confirm long-term consequences (Mertens, 2019 and Randi *et al.*, 2019). For these reasons, tetanus eradication is rare, and cases will continue to occur; therefore, the only prevention approach is immunization and proper wound and traumatic injury care (Forstner *et al.,* 2018). Vaccination programs are now reducing the occurrence and prevalence of tetanus around the world (Bae and Bourge, 2020).

1. Epidemiology of tetanus

While tetanus affects people of all ages, newborns and young children have the highest prevalence (Blencowe *et al.,* 2011).The Centers for Disease Control and Prevention (CDC) in the United States recorded an average rate of 0.1 tetanus cases per million people (Tejpratap and Tiwari, 2008). Most developing nations such as England and Wales have a moderate annual incidence of 0.2 cases/million people with patients above the age of 64 having the largest incidence (Rushdy *et al.,* 2003). On the opposite, tetanus is still prevalent in the developed world, and its occurrence also grows during natural events such as earthquakes and tsunamis. Tetanus also was the second most frequent cause (14%) of patients admitted to a Nigerian hospital with neurologic disorders, following stroke (Talabi, 2003).In Egypt, the Egyptian population's tetanus immunity status was found to be equivalent to that of other nations, with 31.7% vulnerable, 15.7% simple defense, and 52.6% totally protected (Redwan and AL-Awady, 2002). These data may suggest that Egypt's current vaccine campaign is successful, but a more effective vaccination strategy for both adults and children, especially the young, would be advantageous. Tetanus and diphtheria vaccinations should be required for school entry and graduation (Redwan and AL-Awady, 2002). The disorder is most frequent among people that have not been immunized or in the elderly with compromised immunity (Brook, 2008). In (1999), 383 tetanus cases (67.9%) recorded in participant subjects older than 60 years old which might be probably due to inadequate defense and decreased tetanus tolerance (The Egyptian Ministry of Health announcement, Al-Ahram Newspaper, 2001). This trend of tetanus immunity in Egypt is consistent with other countries' records (Redwan and AL-Awady, 2002).

2. Etiology of tetanus

Tetanus is caused by the bacterium *Clostridium tetani*, which can be present in soil, dust, or animal waste. It is a gram-positive, spore-forming, anaerobic bacillus (Berkowitz *et al.,* 2018). These bacteria and their spores can be found all over the world, although they are more common in hot and humid climates where the soil is rich in organic matter (Dong *et al.,* 2019).Tetanus spores are long-lasting and can live in many conditions for extended periods of time. Because the disease's spore can survive in soil for long periods of time, it has a good chance of entering the body through any wound, especially deep lacerated wounds caused by sharp objects penetrating the body (George *et al.,* 2022). The most frequent cause of infection is a superficial wound that goes unnoticed. Surgical operations, intramuscular injections, compound fractures, tooth infections, and insect bites have also been linked to infections (Fava *et al.,* 2020).Tetanus is caused by *C. tetani* toxin, which germinates and produces tetanus neurotoxic protein (TeNT)

(tetanospasmin) when put into the comparably anaerobic circumstances found in wound tissue (South, 2014).

Even if the injury is minor, tetanus spores or bacilli will enter the wound soon. Contaminated wounds are especially vulnerable, especially those with devitalized tissue and deep-puncture injuries (Immunization handbook, 2020).

3. Pathophysiology of tetanus

Tetanus is normally not harmful when consumed if the organism is kept in the gastrointestinal tract, but when exposed to low oxygen levels, it becomes quite harmful (Farrar *et al.,* 2000). This typically happens at the site of a penetrating wound that has been polluted with feces or soil containing spores (Centers for Disease Control and Prevention, 2004 and Greene and Sykes, 2011). Infections have been also documented to happen because of surgical contamination, inadequate instrument sterilization, and a side effect of poor wound care (Furui *et al.,* 1999; Edlich *et al.,* 2003 and Hahn *et al.,* 2004). Tetanus can also develop after being bitten by a snake, after receiving intramuscular injections, and with significant burns (Karyoute and Badran, 1988 and Abrahamian *et al.,* 2000). Infectious spores can survive in soil for more than 40 years. However, soil is not the organism's sole reservoir but *C. tetani* bacilli and spores can be carried in the intestines of both herbivores and omnivores, and the organism can be easily disseminated in their feces (Hsu and Groleau, 2001). The disease is usually spread by tetanus spores infecting a wound on the skin. In the presence of necrotic tissue with lower oxygen potential, tetanus spores enter the body at the site of inoculation and delivered into a region of injury and convert to tetanus bacilli (Bruggemann *et al.,* 2003 and Cardinal *et al.,* 2020). The germination creates toxins that enter the bloodstream through open, infected skin and reaches deeper than oxygen can reach. Tetanolysin and Tetanospasmin are two toxins produced by C. tetani, resulting in the characteristic "tetanic spasm," a generalized contraction of agonist and antagonistic muscles (Cardinal *et al.,* 2020). Tetanospasmin is an extremely strong neurotoxin which specifically affects the nerve and muscle motor endplate interaction, resulting in a pathological condition of rigidity, muscle spasms, and autonomic dysfunction (Sanford, 1995). Tetanolysin on the other hand can lyse erythrocytes in a test tube, and it can cause tissue damage in living things that creates a favorable environment for bacterial development (Attygalle and Rodrigo, 2004).

Tetanospasmin separates into the light chain and the heavy chain, two disulfide-linked fragments. An area of the toxin's heavy chain promotes adhesion to peripheral neurons and subsequent internalization (Greene and Sykes, 2011). Then the toxin crosses the presynaptic terminals of motor neurons in the neuromuscular endplate and stimulates the neurotransmitter molecules glycine and gamma-aminobutyric acid (GABA).This causes blockage of skeletal muscle neural inhibition, leading to generalized muscular spasm and changes in autonomic function (Farrar *et al.,* 2000).Tetanospasmin can also travel from the neuromuscular endplate through retrograde axonal transport at a pace of 75 to 250 millimeters per day to build up in the central nervous system (brain and spinal cord), where it prevents inhibitory neurotransmitter molecules like glycine and gamma-aminobutyric acid from being released in neural synapses to finally cause neurologic impairment in both humans and animals (Montecucco and Schiavo, 1995; Farrar *et al.,* 2000; Attygalle and Rodrigo, 2004; Yeh *et al.,* 2010 and Greene and Sykes, 2011).

Clinical signs of tetanus

Tetanus is divided into four clinical types: generalized, localized, cephalic, and neonatal tetanus (ZaferBağcı, 2020). Generalized tetanus is the most prevalent form of tetanus, accounting for nearly 80% of cases. Patients have a descending sequence of muscle spasms, starting with lockjaw and rises sardonicus (Bae and Bourge, 2020). Tetanus spasms will last anywhere from minutes to weeks, with spasms beginning in the face and progressing to the rest of the body based on the clinical characteristics. These symptoms are caused by toxins released by the bacterium *Clostridium tetani* (Brook, 2008). Neonatal tetanus is a newborn infection that frequently results in death. The infection results when the umbilical cord becomes polluted due to unhygienic birthing or cord-care procedures. If contamination happens at the time of cord cutting or soon after birth, symptoms appear 3 to 12 days later (Lambo and Anokye, 2013). Localized tetanus is a variation of the illness marked by muscular spasms in a constrained region close to the injury; however, it can lead to the more dangerous generalized form of tetanus (Baumbach and Selvag, 2013).Cephalic tetanus is most often caused by a skull fracture, head laceration, eye damage, dental operations, otitis media, or another injury site. The facial nerve is the most often affected. Other cranial nerves, however, may also be affected (Bae and Bourge, 2020).

5. Clinicopathological effects of tetanus

A full course of clinical laboratory investigations was conducted after tetanus toxoid (TT) successful immunization including a total blood count, electrolytes (calcium, phosphorus, sodium, potassium, and magnesium) and all showed normal ranges (Shaukat and Saeed ,2020).Small sub-lethal doses of tetanus toxin produced secondary anemia indicated by poor red cell count and low hemoglobin, level as well as elevated sedimentation rate (Satué *et al.*, 2022). Guglick *et al.* (1995) found that horses developed clinical or subclinical hepatitis 48 to 87 days after tetanus antitoxin administration, a high GGT levels (ranging from 71 to 206 IU/L) were found in herd members. Determining serum GGT and AP activities could also serve as a functional routine test for determining the status of liver amyloidosis in hyperimmune serum producing horses (Abdelkader *et al.*, 1991).

Sahal *et al.* (2004) reported a case ofhepatitis caused by tetanus toxin administration in hyperimmune serum-producing horses which was evident by the higher levels of liver, enzymatic activities (AST, ALT, LDH, ALP) and overall bilirubin level, when opposed to the control group. The hemogram analyses in the study of Sahal *et al.* (2004) showed that the horses had low hemoglobin levels, low hematocrit levels and leukocytosis.

6. Tetanus in horse

Horses are the most vulnerable of all domestic animals, and the disease can kill more than 80% of infected horses. The incubation period varies from one to three weeks depending on the infective substance inserted into the wound, the wound's anaerobic state, and wound treatment (Bruggemann *et al.*, 2003).Early effects include hyperesthesia and prolapse of the third eyelid. Eating and drinking is difficult due to masticatory muscle paralysis. Spasms of the head muscles resulting in lock jaw. The body is stretched, with a global feature like that of a wooden horse (Popof, 2020). Spastic paralysis progresses quickly from the head to the respiratory muscles and then to the limbs in the acute form.Sweating is a symptom of generalized convulsions. Respiratory failure will cause death in 1–2 days (Van Galen *et al.*, 2017).The revelation that animals, including horses, develop antitetanic antibodies by Von Behring and Kitasato in (1890) was a crucial step in the fight to stop tetanus deaths whereas the

authors confirmed early that the horse was a good source for making antiserum. In addition, army horses were used effectively for generation of antitetanic hyperimmune serum (Descombey, 1930).

Immunity to tetanus

Immunity to tetanus is induced only by immunization because recovery from naturally acquired tetanus does not result in protection against further attacks. This is owing to the tetanospasmin toxin's extreme potency. Tetanospasmin will almost certainly be lethal before it triggers an immunological response (Iqbal and Shahid, 2004 and Tian *et al.,* 2022). Immunity to tetanus is a vaccine-avoidable illness for which both tetanus toxin and toxoid were introduced as vaccines to animals and humans to elicit an active immunization by forming tetanus immune globulin (antitoxin) to cure or fight against toxin-based diseases (Sonobe *et al.,* 2007). A toxin is a poisonous substance that is produced during the biological processes of living organisms often bacteria and fungi and is responsible for various types of acute and chronic diseases that causes the disease tetanus (Martinović *et al.*, 2016).

Toxoid is an attenuated form of toxin produced by removing the toxicity either by chemical (formalin) or heat treatment, while maintaining its immunogenicity (Muniandi *et al.,* 2013). In another word, the key difference between toxin and toxoid is that toxin is a poisonous substance that has both toxic and immunogenic properties while toxoid is an attenuated form of toxin which is immunogenic but non-toxic (Alsarraf *et al.,* 2017).

In active immunization the individual's own immune system is stimulated to produce antibodies. On the other hand, passive immunization is the process of transferring specific antibodies to unprotected persons to protect them against infection or to treat a disease they have been exposed to and they do not have immunity against (Slifka and Amanna, 2018). Passive immunization can occur naturally, when maternal antibodies are transferred to the fetus through the placenta, and it can also be induced intentionally, when high levels of antibodies specific to a pathogen or toxin (obtained from humans, horses, or other animals) are transferred to non-immune persons through blood products that contain antibodies, such as immunoglobulin therapy or antiserum therapy (Lasocka *et al.,* 2021). Injection of antitetanic hyperimmune serum containing tetanus antitoxin provides passive immunity in situations when there is a high risk of infection and insufficient time for the body to develop its own immune response, or to reduce the symptoms of ongoing or immunosuppressive diseases such as hypogammaglobulinemia (Lasocka *et al.,* 2021).

1. T*he main standard modulates the production of hyperimmune serum*

A. Immunoglobulins (Ig) (Antibodies)

Immunoglobulins (Ig) or antibodies are large plasma proteins that have sugar chains attached to amino acid residues via N-linked glycosylation. The numerous roles that antibodies mediate include neutralization, agglutination, fixation with complement activation and activation of effector cells (Wootla *et al.,* 2014).

Antibodies that exist naturally are a critical part of the humoral immune response. They shield the host from infectious bacteria, viruses and infections which stimulates the development of antibodies that bind directly to the different antigens (Wootla *et al.,* 2014). They can be polyclonal or monoclonal based on how they are produced (Singh *et al.,* 2020). Antibodies can regulate and get rid of infections by different mechanisms, even though blocking pathogen entry is one possible defense mechanism. Using a remarkable diversity of antimicrobial processes that are locked

within immune system, antibodies not only bind and directly neutralize pathogens but also promote the clearance of bacteria, viruses, fungi, and parasites through their interaction with the innate and adaptive immune systems (Lu*et al.,* 2018). The development of serum treatment for diphtheria and tetanus by vonBehring and Kitasato in the late 19th century laid the foundation for the use of antibodies to mitigate the negative clinical effects of microbial infection (Walker and Burton, 2018). Consequently, antibodies were documented to offer prophylactic and therapeutic intervention options for disease prevention and control which raise their clinical, diagnostic, and testing applications especially in high-risk populations (Berry, 2018 and Rosenstein *et al.*, 2020).Traditional immunization methods have created an antibody-based humoral response that is highly efficient against contagious diseases that are sensitive to it.

Immunization involves introducing an outside material to trigger a certain immune response in the host (Rosenstein *et al.*, 2020).Nowadays, the use of vaccination in the prevention and treatment of autoimmune illnesses, cancer, addictions, allergies, and infectious diseases is extensively being studied. Additionally, vaccination is employed to create physiologically active substances like polyclonal and monoclonal antibodies (Schunk and Macallum, 2005). The hyper immune serum is a serum that contains exceptionally large quantities of specific antibodies providing an unusual degree of immunization and can be produced by repeated immunizations i.e., repeated injections of antigens.

Production of hyperimmune serum containing antibodies is one of the most frequently used successful treatment for various types of bacterial and viral diseases (Zhang *et al.,* 2015 and Putri *et al.,* 2022). Therefore, the hyperimmune serum as a non-antibiotic biological reagent continues to be developed for research needs, immunodiagnostic tools as well as is considered an alternative treatment to lower the disease recurrence rates. Additionally, the specific antibodies in hyperimmune serum can be used to treat and control diseases in the event of an outbreak (Rosenstein *et al.*, 2020)

Considering the numerous applications of hyperimmune serum in research and clinical fields, the preparation method for developing hyperimmune serum against pathogens is essential. There are two methods of hyperimmune serum production, monoclonal antibody production and polyclonal antibody production (Qiu *et al.*, 2016).

a. Monoclonal antibodies (mAbs)

Monoclonal antibodies are a class of therapeutic molecules that are currently being studied in clinical trials to treat a wide range of diseases, including inflammatory, autoimmune, oncologic, cardiovascular, and infectious diseases to reduce the disease morbidity (Liu, 2014 and Pelletier *et al.*, 2020).

Monoclonal antibodies are produced from a cell line made by cloning a unique white blood cell(they are exact copies of one antibody) and have the affinity of binding only to the same epitope of an antigen that is recognized by the antibody (Tsumoto *et al.*, 2019). Because of their high specificity, homogeneity and low degree of cross-reactivity, monoclonal antibodies are an appealing choice for the development of new therapeutics and molecular drug targets against a wide range of common diseases (Liu, 2014).Monoclonal antibodies are produced *ex vivo* using tissue-culture techniques. Hybridoma technology and phage presentation are the two most common methods for manufacturing monoclonal antibodies. Unlike polyclonal antibody processing which depend on hyperimmune serum production from the animals (Santos *et al.,* 2018).

b. Polyclonal antibodies

Polyclonal antibodies (pAbs) are mixture of heterogeneous immunoglobulin molecules (IgG, IgM, IgE, IgA, and IgD) which are usually produced by different B cell clones in the body. They can recognize and bind to many different epitopes of a single antigen. This property is particularly helpful in detecting low-expression antigens or denatured proteins (Peltomaa *et al.,* 2022). The primary goal in experimental polyclonal antibody production is to obtain sufficient volumes of high titer and high-affinity antibody economically.

Polyclonal antibody production is a quick and inexpensive process which involves the repeated immunization of an animal with a desired antigen to produce a hyperimmunized serum with heterologous polyclonal antibodies (Dixit *et al.,* 2016). Additionally, a major advantage of polyclonal over monoclonal antibody is that it can be used for a variety of testing applications that do not require high precision or a systematic diagnostic procedure (Ascoli and Aggeler, 2018).Passive antibody treatments with polyclonal antibodies derived from xenographic reservoirs have been used for a long time in the nineteenth century to cure infections and to guard against infectious agents and toxins (Pelletier *et al.,* 2020). Further, for the past forty years, polyclonal antibodies have been employed in kidney transplantation. They could be utilized for induction, correcting acute rejections and perhaps conferring some allotolerance to lessen chronic allograft nephropathy (Sidhu *et al.,* 2007). With an annual US market worth over $20 billion, antibody therapies are one of the pharmaceutical classes with the quickest growth. They were created to treat a wide range of illnesses, including cancer, autoimmune, and infectious diseases (Wang *et al.,* 2013).

2. Using equine as animal model for production of antitetanic serum

Equine model for hyperimmune serum production is a well-known and easily scalable technology proven to generate high titers of neutralizing antibodies and has been used to treat many diseases such as tetanus.

Equine serum products have evolved into essential, effective therapeutic medicines that are widely acknowledged by most equine practitioners and used in equine clinics throughout the last few decades (Roberts's *et al.,* 2012). In comparison to the more expensive production procedure of specific mAbs, the cheaper manufacturing costs of hyperimmune equine antisera provide an appealing alternative therapeutic option, especially for developing and third-world countries (Wang *et al.,* 2019). Anti-SARS-CoV-2 hyperimmune globulin preparations generated in horses have extraordinarily high ELISA titers as well as highly potent neutralizing activity (Cunha *et al.,* 2020). Equine polyclonal antibodies (EpAbs) are easy to make, allowing for rapid treatment expansion and scaling. Also, after immunizing horses with the receptor-binding domain of the viral Spike glycoprotein, a new therapeutic drug was designed based on these principles (Zylberman *et al.,* 2020).

3. Using of adjuvants for production of hyperimmune serum

Adjuvants, which are named after the Latin root adjuvare, which means "to benefit or assist," are chemicals, microbial components, or mammalian proteins elements that may improve the immune response to antigens. Adjuvants also may be useful for improving responses in susceptible populations with poor immune systems (Etsuro *et al.,* 2020). They are important components of vaccinations that have proven to be particularly efficient in improving the immunogenicity of antigen-specific vaccines by increasing the protective humoral immunity against infectious illnesses and reducing mortality

and morbidity (Wu and Liu, 2021). Attention to adjuvants began in (1925) by Ramon who discovered that substances that cause sterile inflammation at the injection site could improve tetanus and diphtheria antisera development (Di Pasquale *et al.,* 2015).

Glenny (1926) discovered that alum improved antibody responses and from that date it has been commonly used as an adjuvant in a number of human vaccines. While water-in-oil emulsions were discontinued in the 1960s due to their high reactogenicity, they were quickly replaced by the invention of oil-in-water emulsions. In the 1970s, liposomes and virosomes that adsorb or encapsulate antigen were created (Di Pasquale *et al.,* 2015). The discovery of pattern recognition receptors (PRRs) and their agonists in the 1990s and early 2000s opened new pathways for adjuvant discovery and growth as many adjuvants were found to actively or indirectly activate PRRs (Plotkin *et al.,* 2012). In (2009), the US Food and Drug Administration (FDA) approved the first novel adjuvanted vaccine (AS04) against human papillomavirus, which is made up of alum and TLR4A MPLA, Following that, multiple vaccines containing novel adjuvants were approved globally (Etsuro *et al.,* 2020). Including adjuvants in vaccine seems to enhance the efficacy of weak antigens, to induce appropriate immune responses not sufficiently induced in the absence of adjuvant. Further, there is now an increased appreciation of the ability of adjuvants to increase not just overall antibody titer but greater numbers of functional antibodies, antibodies with higher affinity for vaccine antigens or both (Reed *et al.,* 2013).

A. Types of adjuvants

Hundreds of compounds with adjuvant action have been discovered since the discovery of Ramon in (1925). The immune response elicited by adjuvants differs therefore, the selection of appropriate adjuvant is influenced by its mechanism of action, animal species, specific pathogens, vaccine antigens, vaccination technique, and type of immunity required (Spickler and Roth, 2003). Adjuvants come in a variety of forms, each with its own mechanism of action. Adjuvants in development or used in experimental and commercial vaccines include aluminium (alum) and calcium salts, Oil Emulsions, liposomes, saponins, nanoparticles, microparticles, immune-stimulating complexes (ISCOMs), non-ionic block copolymers, derivatized polysaccharides, carrier proteins and a variety of bacterial products and their derivatives (Spickler and Roth, 2003). Various water-in-oil emulsions in which water droplets are retained within a continuous mineral oil process were first tested in humans in the mid-twentieth century (Plotkin *et al.,* 2012).

There are two forms of water-in-oil emulsions being in common use. Complete Freund's Adjuvant (CFA) a water in oil emulsion which contains inactivated mycobacteria mostly Mycobacterium tuberculosis and incomplete Freund's Adjuvant (IFA) which is the same water in oil emulsion but without mycobacteria pathogen. Both adjuvants offered potent immunogenicity and were used in early influenza vaccines (Etsuro *et al.,* 2020). Alum and oil emulsions were both used in animal vaccinations. Complete Freund's Adjuvant initially used was discontinued due to toxicity, but the Incomplete Freund's Adjuvant is still used where a strong adjuvant is needed (Spickler and Roth, 2003). As an alternative to water-in-oil emulsions, oil-in-water emulsions showed dramatically improved reactogenicity profiles (Galli *et al.,* 2009). Oil-in-water emulsions have been widely used in many seasonal and pandemic influenza vaccines for adults which improved both cell-mediated and humoral Th1 and Th2 responses (Garcon *et al.,* 2012).

B. Mechanisms of action of adjuvants

T helper cells and humoral immunity are successfully stimulated by the majority of adjuvants. Adjuvant processes have recently been tried to fit into more general immune function ideas. This activation is thought to happen when antigen-presenting cell (APC) pattern recognition receptors bind to conserved motifs in bacterial lipopolysaccharides, sugars, or other compounds (Gallucci *et al.*, 1999). Antigens appeared to be delivered to pathways that the antigen-presenting cells (APCs), together with macrophages and B cells, process antigens and display epitopes in major histocompatibility complex class I (MHC I) molecules and the development of cytotoxic T-lymphocytes (CTL) response. Other signals essential to activate immunization are provided by dendritic cells, macrophages, and B cells, such as costimulation by the B7 family of chemicals.This mechanism has been related to some adjuvants such as liposomes (Alving, 1995).

Some adjuvants can reduce antigen absorption in the liver and thus increase the quantity of antigen reaching APCs. High-molecular-weight sulfated dextrans have been linked to this mechanism (Cox and Coulter, 1997). Particulate adjuvants like aluminium salts encourage the development of aggregates, which are easier to phagocytize. Moreover, carbohydrate polymers like mannan and acemannan may be able to direct antigens to APCs by interacting with carbohydrate receptors (Cox and Coulter, 1997). Some immunomodulatory adjuvants, either directly or through cytokine induction, boost the expression of costimulatory molecules or MHC molecules on APCs (Oxenius, 1999). T-helper cell type 1 (Th1) responses and cell-mediated immunity (CMI) are linked to some cytokines such as interferon gamma (INF-g), interleukin-2 (IL-2), and interleukin-12 (IL-12). T-helper type 2 (Th2) responses and humoral immunity are related with IL-4, IL-5, IL-6, IL-13, and perhaps IL-10. Some adjuvants often impact the kind of immunity by altering the balance of these two sets of cytokines increasing the concentrations of some cytokines while decreasing the concentrations of others (Gradonand Lutwick, 1999).

Schijns (2000) and Vogel (2000) showed that Chemicals, microbial components, and mammalian proteins are the most important common adjuvants. They mostly act by promoting antigen presentation, antigen stability or serving as immunomodulators in general. The mycobacterial components of CFA were found to signal T lymphocytes to adopt a Th1 profile, resulting in high delayed-type hypersensitivity to autoantigens. T-lymphocyte differentiation continues to take on a Th2 profile in the absence of mycobacteria. Additionally, CFA-mediated innate immune compartment activation was critical not only for controlling the early induction process, but also for providing a surplus of effector and receptor cells in the late phase (Billiau and Matthys, 2001). Seubert *et al.* (2008) found that the MOA of MF59, a squalene-containing oil-in-water emulsion, tended to involve rising monocyte and dendritic cell antigen uptake. AS03, an oil-in-water emulsion containing squalene, polysorbate 80, and tocopherol (a form of vitamin E) was used in a variety of influenza vaccines. In mice, tocopherol in AS03 appeared to modulate cytokine and chemokine expression, increase antigen loading in monocytes, increase granulocyte recruitment in draining lymph nodes and improve antibody responses (Morel *et al.*, 2011). By encasing the antigen, nanoparticles can increase the stability of the antigen and can also enhance the immune response by precisely targeting dendritic cells and macrophages (Karandikar *et al.*, 2017).

4. Freund adjuvants

Freund adjuvants are, in a way, historic adjuvants and for many researchers the first and most obvious association to the word adjuvant at all. Freund adjuvants are water-in-mineral oil emulsions (W/O emulsions) that are used as immunopotentiators (booster). They were First designated by Jules Freund in the 1940's, for the purpose of providing continuous release of antigens necessary for stimulating a strong, persistent immune response (Di Pasquale *et al.,* 2015). Freund adjuvants are considered among the most commonly and effective adjuvants currently available, that are used to trigger a humoral antibody response to produce high titer antibodies. Therefore, they constitute irreplaceable components of induction protocols in experimental immunology due to their high efficacy (Etsuro *et al.,* 2020). There are two types of Freund adjuvants: complete and incomplete.If it contains killed Mycobacterium tuberculosis it is known as Complete Freund Adjuvant (CFA). Without the bacteria it is Incomplete Freund Adjuvant (IFA) (Dubé *et al.,* 2020).

A. Complete freund adjuvants (CFA)

Complete Freund's adjuvant (CFA) is one of the strongest adjuvants for producing hyperimmune serums and has long become one of immunologists' most valuable resources (Dubé *et al.,* 2020). CFA is a water-in-oil emulsion containing mineral oil, a surfactant, and heat-killed mycobacteria that enhances cellular and humoral antibody responses to immunogens. CFA's mycobacterial components cause more delayed-type hypersensitivity, skewing the response toward a Th1 profile so it was considered a gold standard for adjuvants with greater immunostimulatory capabilities (Reed *et al.,* 2013). In addition, CFA may be a good adjuvant for certain types of antigens, which are difficult to obtain, of small molecular weight, weakly immunogenic or only available in very small quantities (Putri *et al.,* 2022). Leenaars *et al.,* (1998) compared the antibody production with several antigens using a number of adjuvants and several routes of administration in both mice and rabbits. The authors found that Freund adjuvants produced high-tittered specific antibody in both mice and rabbits regardless of the antigen or route of administration. Similar results were obtained in rabbits immunized (Smith *et al.,* 1992). However, CFA cannot be used in reimmunizations or in species where a positive tuberculin reaction is undesirable (Lipman *et al.,* 1992).

Unfortunately, there are several concerns about the use of CFA including severe pain at the injection site and its potential to cause serious complications, such as abscesses, chronic granulomas, and ulcerating tissue necrosis (Broderson, 1989; Johnston *et al.,* 1991; Kleinman *et al.,* 1993 and Leenaars *et al.,* 1998).

Becauseof the adverse reactions to FCA, its usage has been limited to antibody research. Furthermore, several regulatory guidelines have been established because of the lesions generated and the possibility for pain and discomfort (CCAC, 2017). According to Institutional Animal Care and Use Committee Guidebook,CFA should only be used for the first (priming) dose. Unless expressly justified, subsequent immunizations should be completed with Incomplete Freund's Adjuvant or another adjuvant (Stills, 2000).

B. Incomplete freund adjuvants (IFA)

Incomplete freund adjuvant (IFA) is similar to CFA in structure, but it lacks the mycobacterial portion, making it making well tolerated, less inflammatory, less distressful, and capable of being administered several times and that repeat vaccination at the same site induces the formation of tertiary lymphoid structures in the vaccine site microenvironment (Pollack *et al.,* 2020). The antigen is continuously released from the oily deposit, the antigen lifespan is prolonged, and the innate

immunity of the local area is stimulated, which increases phagocytosis, leukocyte infiltration, and cytokine production (Mussener *et al.*, 1995).Following the dendritic cell absorption of adjuvant elements IFA was reported to induce many immunological changes including increased phagocytosis, cytokine secretion by mononuclear phagocytes, and temporary activation and proliferation of CD4+ lymphocytes (Billiau and Matthys, 2001).In comparison to the same formulation without the adjuvant, using IFA as an adjuvant increased the long-lived antibody titers in a human influenza vaccination (Apostólico *et al.* 2016).

CONCLUSIONS AND FUTURE PROSPECTS

Considering the numerous applications of hyperimmune serum in research and clinical fields, the production of hyperimmune serum as a biological reagent will continue to be developed and thus will continue to require the use of adjuvants with continuing concerns about them. Therefore, a greater understanding of their adjuvanticity potential, safety tolerability, and efficacy could help to limit, control, and correct adjuvant-related side effects making them useful products in the vaccine industry and also could help in the creation of new adjuvants. Also, it is clear that a variety of factors may contribute to the careful, and proper selection of an adjuvant such as the antigen, the species, and the route of administration. Nevertheless, further numerous studies are still needed to develop full criteria about these factors under different experimental conditions.

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