Adjuvants have been used for more than 70 yr to enhance the immune response of the host animal to an antigen. Among the mechanisms that adjuvants use to enhance the immune response are the “depot” effect, antigen presentation, antigen targeting, immune activation/modulation, and cytotoxic lymphocyte induction. The immunostimulatory properties of adjuvants result in inflammation, tissue destruction, and the potential for resulting pain and distress in the host animal. The inflammatory lesions produced by adjuvants such as Freund’s complete adjuvant (FCA) have led some to conclude that pain and distress are present, even in cases where the scientific evidence fails to support this conclusion. Recommendations and regulations in the literature, based on available scientific evidence, provide guidance on total adjuvant volumes, volumes per site, routes of injection, booster injections, and adjuvants used for antibody production. Among the numerous adjuvants that are used for experimental antibody production reviewed in this article, many claim to be less inflammatory, tissue destructive, and painful than FCA while producing equal or superior antibody responses. Although no adjuvant surpasses FCA for experimental antibody production against a wide range of antigenic molecules, many produce excellent antibody responses with less inflammation and tissue destruction. Balancing the requisite degree of immunostimulation and the extent of inflammation, necrosis, and potential pain and distress requires consideration of the nature of the antigen, the host immune responsiveness, the adjuvant’s mechanisms of action, and the desired end-product. In cases where the antigen is a weak immunogen or has a very limited availability, the type and role of adjuvant becomes a critical component in producing an acceptable immune response and humoral antibody response.

Key Words: adjuvant; adverse effects; antibody formation; antigen; Freund’s adjuvant; immunization; immunostimulant; antibody production

Introduction

Adjuvants are substances injected along with an antigen that are intended to enhance the humoral and/or cell-mediated immune response to the antigen. Adjuvants generally permit the use of a smaller antigen dose and may modulate the immune response to the antigen. More than 100 adjuvant preparations have been described (Vogel and Powell 1995) although many of these adjuvants are used only rarely due to complexity of preparation, expense, or toxic effects (Stewart-Tull 2000). Adjuvants may be divided arbitrarily into those used for prophylactic purposes, primarily to create therapeutic vaccines that provide protection from infectious organisms or toxins, and those used for experimental purposes, to produce antibodies for further study.

Prophylactic adjuvants must induce a protective immune response against an infectious agent with minimal short- or long-term side effects. Protection may require a humoral antibody response, an activated CD4 cell-mediated response, or a CD8 cytotoxic lymphocyte (CTL) response, depending on the agent. Prophylactic adjuvants should direct antigen presentation and modulate the cytokine network to induce the appropriate protective responses. Adjuvants may produce a number of adverse consequences, both locally at the injection site and systemically (Edelman 2000), and these reactions must be minimized in therapeutic vaccines. Finally, to be useful in clinical human or veterinary medicine, therapeutic vaccines must be economical, stable, and easy to administer. An evaluation of the multitude of therapeutic vaccines is beyond the scope of this paper, and the reader is referred to several excellent books on vaccine development (Hincal and Kaş 1998; O’Hagan 2000; Robinson et al. 2003).

Adjuvants that are used for experimental purposes are used to produce antibodies for use in other experimental or diagnostic procedures. The primary goal in experimental
polyclonal antibody production is to obtain sufficient volumes of high titer and high-affinity antibody economically. Adjuvants used for this purpose are primarily used to enhance reaction to the antigen, and to increase antibody response in both intensity and duration. Enhanced cell-mediated immune response is seldom of significance, although modulation of the immune response may alter the levels of antibody subclasses produced. Concern for the care and well-being of the animals being used to generate antibodies is significant both from the societal impact and the experimental results. The issue of pain and distress has led to governmental regulations and the production and marketing of alternative adjuvants for experimental antibody production. Maximizing the host antibody response and minimizing the pain and distress produced is the combined goal of experimental antibody production. Adjuvants play an important role, but not the only role, in both the immune response and the pain and distress produced.

**Adjuvant Effects**

The mechanisms by which adjuvants promote increased immune response are slowly becoming more defined as the molecular aspects of antigen recognition and immune response become more fully understood. Adjuvants may have up to five of the following mechanisms of action: the “depot” effect, an antigen presentation effect, an antigen distribution or targeting effect, an immune activation/modulation effect, and a CTL induction effect (Cox and Coulter 1997).

One classic mechanism of adjuvant action is the “depot” effect, in which the adjuvant protects the antigen from both dilution and rapid degradation and elimination by the host. By localizing and slowly releasing intact antigen, the adjuvant permits a slow, prolonged exposure of the immune system cells to a low level of antigen. This prolonged exposure results in continued stimulation of antibody-producing cells, resulting in the production of high levels of antibody by the host.

The significance of the depot effect was demonstrated by Herbert (1967), who compared antibody production between a single dose of antigen in a water-in-oil emulsion (mineral oil) with daily injections of a small amount of antigen in saline over 50 days. Although both groups developed high antibody titers, the antibody levels in the emulsion-injected group remained elevated in contrast to the saline groups in which the antibody levels declined after cessation of the daily injections at day 50. The importance of continued low-level antigen stimulation for the production of high-affinity antibody is best explained by the antigen selection hypothesis proposed by Siskind and Benacerraf (1969). They proposed that low-dose antigen exposure resulted in the stimulation of only B cells with high-affinity receptors, whereas at higher doses, B cells with medium- and low-affinity receptors would be stimulated.

Emulsions are the most commonly used depot agents in the experimental production of polyclonal antibodies. Emulsions may be water-in-oil emulsions, oil-in-water emulsions, or more complex types such as water-oil-water emulsions. A variety of oils and emulsifying agents have been used in forming and stabilizing emulsions. Paraffin oil, a crude mineral oil, and the emulsifying agent mannide monooleate (Arlacel A®) were used in the original formulation of Freund’s adjuvant (Freund et al. 1937, 1948). These crude mineral oils contained a variety of organic hydrocarbons as well as reactive contaminants including paraffins, olefins, aromatic hydrocarbons, and cycloparaffins. Changes in oil refining procedures and improvements in purification techniques have resulted in the replacement of the crude oils with highly purified and defined light mineral oils with much lower toxicity. Oils presently used in emulsion adjuvants include highly purified light mineral oils and biodegradable oils such as squalene and squalane.

Aluminum compounds, especially aluminum phosphate and aluminum hydroxide, are commonly used as vaccine adjuvants in human preparations. Unlike emulsions, aluminum adjuvants bind antigens via electrostatic forces between the adjuvant and the antigen with other interactions including hydrophobic interactions, van der Waals forces, and hydrogen bonding (Gupta and Rost 2000). Although generally not as persistent as emulsions, there is evidence of antigen retention up to 7 wk after injection in animals (Harrison 1935).

Microparticles of 1- to 100-μm diameter composed of biodegradable and biocompatible polymers may serve as depot adjuvants (Eldridge et al. 1991; Jiang et al. 1988; Kreuter 1988). Comprising cyanocrylates and copolymers, these particles with incorporated antigen may persist with long-term antigen exposure and cause little tissue reaction. Liposomes and immune-stimulating complexes (ISCOMs) consisting of lipid honeycomb matrices also serve as depot agents. Finally, alginate-poly-L-lysate microcapsules that incorporate recombinant myoblasts secreting an antigenic protein have been used successfully to provide continuous antigenic stimulation in mice (Gomez-Vargas et al. 2004).

In addition to carrying and protecting the antigen from proteolytic destruction, an adjuvant should preserve the conformational integrity of an antigen and present the antigen to the appropriate effector cells, usually professional antigen-presenting cells and macrophages. Water-in-oil adjuvants maintain the antigen in the aqueous phase while using a surfactant to aid distribution of the antigen to the surface of the aqueous micelles. Oil-in-water adjuvants, conversely, initially localize the antigen in the oil phase and use compounds such as trehalose diester to assist in binding the antigen to the oil phase droplet and to encourage antigen uptake by macrophages (Adam et al. 1973). Oil-in-water emulsion adjuvants tend to work better with hydrophobic or amphipathic antigens, whereas water-in-oil emulsion adjuvants work better with hydrophilic or amphipathic antigens (Hanly et al. 1995). Adjuvants containing amphipathic mol-
ecules or complexes enhance interaction of the antigen with cell membranes and encourage receptor-mediated endocytosis or fluid-phase pinocytosis (Lanzavecchia 1990). Preservation of antigenic epitope conformation requires thoughtful consideration of the antigen composition and conformation as well as knowledge of the adjuvant’s effects on the antigen.

Maintaining the antigen in a single location would prevent exposure of the immune effector cells. The initial reaction at the injection site often leads to encapsulation of the injected material with fibrous tissue, preventing additional antigenic exposure. Studies with both Freund’s adjuvant (Freund 1951) and aluminum compound adjuvants (Holt 1950) have shown that removal of the initial injection site within days of the injection has little effect on the animal’s immune response even though detectable antigen remains localized at the injection site. Many adjuvants form large multimolecular aggregates with the antigen. The aggregates encourage uptake and processing of the antigen and any included immunomodulators by macrophages and professional antigen-presenting cells, typically dendritic cells and Langerhans cells, the dendritic cells of the epidermis. Adjuvants may incorporate carbohydrate moieties or molecular configurations that increase delivery to macrophages and dendritic cells via specific receptors (Abel et al. 1989; Bonifaz et al. 2004; Chinnah et al. 1992; Jiang et al. 1995; Ribi et al. 1984). After initial processing of the antigenic complex, the dendritic cells and macrophages relocate to regional draining lymph nodes or the spleen, where additional processing results in the production of antigen-specific B cells and plasma cells.

Immunomodulators and immunostimulators are used in many adjuvants to recruit, activate, and enhance differentiation of the cells of the immune system. Adjuvants have been shown to alter cytokine gene expression in vivo, directly activating specific T cell subsets and altering antibody isotypes produced (Victoratos et al. 1997). Immunomodulators and immunostimulators have historically been microbial cells, microbial cell components, or chemically modified microbial products. The discovery of mammalian toll-like receptors (TLRs) and the ongoing delineation of their role in both the innate immune response and in regulating the adaptive immune response is clarifying how the microbial compounds used in adjuvants modify the immune response. Toll-like receptors are one mechanism by which the innate immune system cells (phagocytes, macrophages, and dendritic cells) recognize conserved molecular patterns predominantly found in microorganisms and not in vertebrates. Activation of the TLR produces an immediate defensive response through a number of signaling pathways and leading to the production of antimicrobial peptides, chemokines, and cytokines. In addition to the immediate innate immune response, the TLR signaling also lays the foundation for the development of the adaptive immune response. Much of the immunomodulation and immunostimulation attributed to adjuvants is directly attributable to their interaction with TLRs.

A total of 10 TLRs (TLR-1 through TLR-10) have been recognized to date as activating different sets of signaling pathways with different biological effects (Akira 2003; Dalpke and Heeg 2002). TLR-4, the most studied of the TLRs, recognizes the following compounds in addition to others: bacterial lipopolysaccharide (LPS), lipid A, and the less toxic chemically modified derivatives such as monophosphoryl lipid A (MPL®) used in adjuvants (Akira and Hemmi 2003; Dobrovolskaia and Vogel 2002; Persing et al. 2002).

Whole mycobacterial cells, mycobacterial lipoprotein, lipoarabinomannans, and lipomannans, which are integral components of the mycobacterial cell wall and a number of adjuvants, interact with TLR-2 as well as TLR-4 and TLR-6 (Brightbill et al. 1999; Means et al. 1999; Nigou et al. 2002; Quesniaux et al. 2004). Viral double-stranded RNA and polyinosine-polycytidylic acid are recognized by TLR-3 (Alexopoulou et al. 2001), and TLR-9 recognizes 2'-deoxyribo(cytidine-phosphate-guanosine) dinucleotides flanked by specific bacterial DNA sequences (Bauer et al. 2001; Hemmi et al. 2000; Kandimalla et al. 2003; Takeshita et al. 2001).

Recruitment and activation of macrophages and dendritic cells for antigen processing by adjuvant immunomodulators lead to other inflammatory sequelae. Activated macrophages secrete a variety of proinflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-12, and CXCL8 (IL-8). In addition to modulating the adaptive immune response and ultimate antibody production, these cytokines recruit neutrophils, basophils, and lymphocytes; increase vascular permeability; cause local tissue destruction; and result in systemic effects that include fever and the production of acute-phase proteins by the hepatocytes. The local and systemic effects are undesirable sequelae that result from adjuvant use for antibody production and may result in pain and distress to the host animal. Balancing the antibody production needs and the antigen of interest with the inflammatory reactions and potential pain and distress is critical in the choice of an adjuvant.

**Freund’s Adjuvants**

Since its original description (Freund 1956; Freund and McDermott, 1942; Freund et al. 1937), Freund’s complete adjuvant (FCA) has been the most widely utilized and effective adjuvant for experimental antibody production. The general immunostimulatory capabilities of FCA have not been surpassed by any adjuvant (Altman and Dixon 1989; Munoz 1964; Smith et al. 1992; Warren et al. 1986). Unfortunately, the use of FCA has been associated with a variety of lesions, including localized injection site granulomas (Broderson 1989; Johnston et al. 1991; Kleinman et al. 1993; Leenaars et al. 1994, 1998; Leskowitz and Waksman 1960; Steiner et al. 1960; Stills 1994; Stills and Bailey 1991; Toth et al. 1989; Warren et al. 1986); distant sub-
and spleen, where localized small antigenic depots were
transported through the lymphatic system to the draining lymph nodes
was attributed to transport of the antigen-adjuvant emulsion
production, and excision after the first day had little effect
early as 30 min after injection did not eliminate antibody
Interestingly, excision of the injection sites from rabbits as
22 wk after subcutaneous injection (Halbert et al. 1946).
Persisted within the emulsion at the injection site for up to
with water-in-mineral oil emulsions revealed that antigen
phagocytes, macrophages, and dendritic cells. Early studies
injection due to their viscosity. Properly prepared Freund
emulsions are very stable and will not separate into
inocula are prepared by emulsification of an antigen in an
aqueous solution with the oil, producing a water-in-oil
emulsion. The formation of a stable water-in-oil emulsion is
a critical step in the effectiveness of either FCA or FIA as
an adjuvant. The stable water-in-oil emulsion is usually
prepared by forcing the aqueous-phase antigen into an equal
volume of the oil adjuvant through either double-hubbed
small-bore needles or three-way stopcocks (Herbert 1973).
Alternatively, mechanical homogenizers may be used when
larger volumes are needed or antigen is readily available. A
properly prepared water-in-oil Freund’s adjuvant emulsion
is a thick viscous homogeneous white mixture that does not
diffuse when a drop is placed on the surface of water
(Garvey et al. 1977; Herbert 1973). Thick emulsions are
generally more stable than thin emulsions (Lindblad 2000)
but require larger bore needles and increased pressure for
injection due to their viscosity. Properly prepared Freund’s
adjuvant emulsions are very stable and will not separate into
oil and water phases during prolonged refrigerated storage.
The mineral oil used in Freund’s adjuvants has tradi-
tionally had the following three specific mechanisms of ac-
tion: (1) establishing an antigen depot with slow antigen
release, (2) providing a vehicle for antigen transport
throughout the lymphatic system to immune effector cells,
and (3) interacting with antigen-presenting cells including
phagocytes, macrophages, and dendritic cells. Early studies
with water-in-mineral oil emulsions revealed that antigen
persisted within the emulsion at the injection site for up to
22 wk after subcutaneous injection (Halbert et al. 1946).
Interestingly, excision of the injection sites from rabbits as
early as 30 min after injection did not eliminate antibody
production, and excision after the first day had little effect
on antibody production (Freund 1951). This latter effect
was attributed to transport of the antigen-adjuvant emulsion
through the lymphatic system to the draining lymph nodes
and spleen, where localized small antigenic depots were
established (Freund 1951; Osebold 1982; Waksman 1979).
The importance of using an oil that is not readily metabo-
lized and eliminated has been stressed as necessary for con-
tinuous stimulation and was a major reason for the success
of the Freund’s adjuvants (Freund 1956). Although the min-
eral oil of Freund’s adjuvants is generally considered non-
metabolizable, the work of Stetten (1943) and Bollinger
(1970) clearly indicates that approximately 70% of the in-
jected mineral oil is slowly metabolized during the first
year.

The importance of the mineral oils and the water-in-oil
emulsion of FCA was recognized early (Freund 1947, 1951,
1956). The hydrocarbon oils Freund used in the original
formulations were produced by the acid treatment or the
oleum method and were quite crude. Early studies on the
mineral oil component of Freund’s adjuvant identified the
short-chain hydrocarbons as responsible for acute toxicity
and immunosuppression and focused attention on the me-
dium-chain straight and branched hydrocarbons as the best
candidates for less reactive adjuvants (Shaw et al. 1964,
Stewart-Tull et al. 1976; Wilner et al. 1963). The develop-
ment of mineral oil-influenza and mineral-oil-poliomyelitis
vaccines for humans in the late 1940s and early 1950s led
to specific requirements for the mineral oil and surfactant
components for use in human vaccines and the evaluation
of complications associated with the vaccine use (Berlin 1962;
Friedewald 1944; Henle and Henle 1945; Salk 1953; Salk et

After 1970, mineral oils were produced by a single or
double hydrogenation procedure that resulted in more pure
and much less toxic mineral oil (Stewart-Tull 1998). Com-
parisons between the original Freund’s adjuvants and those
produced today are difficult and misleading. The refinement
of the mineral oil component since the original description
of Freund’s adjuvants has led to confusion and many in-
accurate conclusions concerning the pathological effects
from the use of water-in-mineral oil adjuvants designated as
Freund’s but compositionally quite different. This situation
has led some authors to suggest discontinuing the old ter-
minology linking these adjuvants to Freund’s and designat-
ing the newer adjuvants as “immune-stimulating oil
emulsions” (IOEs) (Stewart-Tull 1998).

In the original formulation, Freund used heat-killed
whole cells of virulent Mycobacteria tuberculosis as the
immunostimulant in FCA (Freund and McDermott 1942;
Freund et al. 1937). It was quickly discovered that the in-
clusion of mycobacterial cells increased the humoral anti-
body response and was essential for the delayed-type
hyporesponse reactions (Raffel 1948) typical of FCA but
lacking in FIA. With the discovery of TLRs, the mechanism
of mycobacterial cell recognition and immune response is
becoming better understood. Both TLR-2 and TLR-4 are
critical for the initial recognition and immune response to
mycobacterial infections by both macrophages and dendritic
cells. Activation of different TLRs leads to different cellular
and cytokine responses, which alters both the humoral and
cellular response (Heldwein and Fenton 2002; Means et al.
The many species of mycobacteria vary in their cell wall composition, producing different lipomannans and lipoarabinomannans and interacting with different TLRs that produce both pro- and anti-inflammatory effects (Quesniaux et al. 2004). Although the original FCA used heat-killed and dried virulent Mycobacterium tuberculosis, most of the presently available commercial preparations of FCA use either the relatively avirulent Mycobacterium H37Ra or Mycobacterium butyricum (Stewart-Tull 1995).

Immunological Results

The primary reason for using an adjuvant in the immunization of an experimental animal is to produce a high-titer, high-affinity, and high-avidity antibody for use in other experiments. Both FCA and FIA are very efficient adjuvants in this regard. The mycobacterial components in FCA tend to produce a stronger delayed-type hypersensitivity and skew the response toward a Th1 profile (Linblad 2000). FCA is considered the gold standard for adjuvants by many immunologists, and the general immunostimulatory properties of FCA have not been surpassed by any adjuvant (Altman and Dixon 1989; Munoz 1964). The development of cell-mediated sensitivity to the mycobacterial component of FCA prevents its use in reimmunizations and in species in which a positive tuberculin reaction would be undesirable.

The effort to find a less reactive adjuvant that produces an antibody response equaling or surpassing that of FCA and FIA has resulted in the publication of a number of articles describing the comparison of alternative adjuvants and FCA. Lipman and colleagues (1992) compared FCA and RIBI adjuvant (RIBI Adjuvant System®). Corixa Corporation, Seattle, WA) in mice using a hapten conjugated to bovine gamma globulin and found the RIBI adjuvant to be superior. Leenaars and coworkers (1995) evaluated four different adjuvants as alternatives to FCA/FIA for weak immunogens in mice and found high antibody titers from FCA/FIA and Specol (a purified water-in-mineral-oil adjuvant) but not from other adjuvants. In another study, Leenaars and colleagues (1998) compared the antibody production with several antigens using a number of adjuvants and several routes of administration in both mice and rabbits. FCA/FIA and two other water-in-mineral-oil adjuvants, Specol and Montanide ISA50, produced high-titered specific antibody in both mice and rabbits regardless of the antigen or route of administration, whereas TiterMax was ineffective in rabbits, and only effective when administered intraperitoneally in mice, and RIBI was ineffective in all cases. Similar results were obtained in rabbits immunized with osteocalcin in FCA/FIA, TiterMax, or RIBI, where the FCA/FIA adjuvant produced six fold-higher antibody titers more rapidly than either TiterMax or RIBI (Smith et al. 1992). The conflicting results in the literature emphasize the importance of considering the size and composition of the antigen, the species being immunized, and the route of immunization when developing an immunization protocol. Although FCA usually produces an acceptable antibody response to an immunogen, other adjuvants may be more effective for certain antigens (Bomford 1980) or equally effective with fewer side effects (Leenaars 1998; Leenaars et al. 1995).

Lesions Associated with the Use of Freund’s Adjuvants

There have been numerous reports on the lesions associated with the administration of FCA and FIA. Focal necrosis and a granulomatous inflammatory response with prominent “foamy” oil-filled macrophages is the hallmark of a FCA injection site (Broderson 1989; Leenaars et al. 1994, 1998; Stills 1994; Stills and Bailey 1991; Wiedemann et al. 1991). Intradermal injections of FCA are associated with large palpable granulomas that often ulcerate (Broderson 1989; Leenaars et al. 1994; Stills 1994; Stills and Bailey 1991). Subcutaneous injections may migrate from the injection site and result in fistulous tracts that eventually open and drain (Broderson 1989), and intramuscular injections are associated with granuloma formation that extends through the muscle planes with possible nerve involvement (Leenaars et al. 1998; Stills 1994). In addition to the injection site lesions, granulomas are frequently detected in the draining lymph nodes, spleen, lung, kidney, and other organs, where microdroplets of the emulsion have been distributed by the lymphatic and circulatory system after injection (Broderson 1989; Waksman et al. 1960).

In numerous studies, the pathological lesions associated with FCA have been compared with those from other adjuvants. Johnston and colleagues (1991) compared FCA, RIBI, and Montanide ISA50 in rabbits and observed no differences in gross appearances between the adjuvants. Other investigators, however, have noted significantly more inflammatory reaction with FCA (Deeb et al. 1992; Leenaars et al. 1994) or a more severe reaction with RIBI (Leenaars et al. 1998) in rabbits. Adjuvant comparisons in other species have yielded, at best, confusing results (Bennett et al. 1992; Leenaars et al. 1995, 1998; Lipman et al. 1992). Although there is general consensus that the use of FCA is associated with significant pathological lesions, it is clear that a variety of factors are involved in determining the extent of the lesions produced with any adjuvant. These factors include but may not be limited to the adjuvant, the antigen, the species, and the route of injection.

Clinical Signs—Pain and Distress

Although the pathological lesions associated with FCA and FIA use are well documented, the issue of clinical signs and pain or distress secondary to the lesions is not nearly as well understood. Amyx (1987) addressed the matter of pain and
distress associated with the use of FCA and concluded that
the local inflammatory lesions likely resulted in pain and
distress due to the use of excessively large inocula. A few
of the articles reporting the FCA-associated pathological
lesions have included some information on clinical assess-
ment for pain or distress (Johnston et al. 1991; Leenaars et
al. 1994; Stills and Bailey 1991). Other reports have as-
sumed the presence of pain or distress based entirely on
pathological lesions and without clinical or behavioral
evaluation (Jennings 1995; Mallon et al. 1991).

Several prospective studies have evaluated both physi-
ological and behavioral changes in rabbits injected with
FCA and FIA emulsions and failed to detect any changes
after the injections (Halliday et al. 2000; Leenaars et al.
1994). Leenaars and coworkers (1998) evaluated the side
effects of several adjuvants, including FCA and FIA, in both
rabbits and mice using clinical assessments, behavioral ob-
servations, and pathological evaluations. Although exten-
sive lesions were reported in the rabbits injected with FCA
and FIA, no clinical or behavioral abnormalities indicative
of pain or distress could be detected, leading Halliday and
colleagues (2000) to conclude that FCA could be used hu-
manely in rabbits.

Leenaars and coworkers (1998), in the same study in
which no clinical signs were reported in rabbits, docu-
mented clinical and behavioral signs indicative of pain and
distress that lasted 2 to 3 days in mice after intraperitoneal
injection of FCA. This documentation was in agreement
with the reports of Toth and colleagues (1989) and Wans-
strup and Christensen (1965). The acute clinical and behav-
ioral signs observed in these studies were comparable to
those in studies using intraperitoneal injection of FCA as a
model for acute inflammation in rats (Geisterfer and Gauldie
1996; Griffen et al. 2003; Olivier et al. 1999). In a
36-hr dose-response study with the FCA dose ranging from
1.25 to 10 mL/kg, adverse clinical signs were not noted,
although body weight losses were noted as early as 5 hr
after injection and increasing through 36 hr after injection.
Time course studies with FCA at a dose of 6 mL/kg indi-
cated significant elevations of acute-phase reactants and in-
flammatory cytokines (IL-6 and IL-1β but not TNFα)
immediately after injection and peaking from 12 to 40 hr
after injection, with most levels returning to normal from 36
to 96 hr after injection (Griffen et al. 2003). The studies
using the intraperitoneal injection of FCA in the rat as a
model of acute inflammation used pure FCA at a dose
well above any recommended dose for polyclonal antibody
production.

Another source of information concerning the pain and
distress associated with the use of Freund’s adjuvant are
cases of human injection. Adjuvants similar to FIA were
used in human influenza vaccines in the 1960s. Recorded
side effects included cystic swellings and muscle indura-
tions persisting up to 1 yr after injection. The histological
picture was a typical oil granuloma. Reports of injection site
pain were uncommon and generally mild (Lindblad 2000;
Salk 1953; Salk and Salk 1977; Salk et al. 1952, 1953). The
use of FIA in human vaccines was discontinued in the mid-
1960s because of concerns of safety. Hughes and colleagues
(1970) reported severe reactions after repeated injections
of FCA in early human cancer immunotherapy studies in hu-
mans. However, these reactions were not seen on the initial
injection and occurred only after patients developed sensi-
tivity to the tuberculin antigen. Chapel and August (1976)
referred severe pain in five of nine cases of accidental FCA
injection in humans. In all of these cases, the patients were
tuberculin sensitive prior to the accidental injection. Finally,
human diseases characterized by chronic granulomatous in-
flammation, including tuberculosis, leprosy, sporotrichosis,
and “Buruli” cutaneous ulcers caused by Mycobacterium
ulcerans, are diseases that are relatively painless and often
devoid of systemic clinical signs until they are quite ad-
vanced (Cotran et al. 1999; Mims et al. 1998). Careful ap-
lication of the human analogy principle (Leenaars et al.
1998) would suggest that the granulomatous lesions would
not likely be very painful.

Other Water-in-Oil Adjuvants

The lesions and perceived pain and distress associated with
the use of FCA and FIA have led to the development of a
number of alternative adjuvants, many of which are water-
in-oil emulsions and should probably be classified as IOEs,
as proposed by Stewart-Tull (1998). The commercially
available water-in-oil emulsions can be divided into two
groups, one that uses poorly metabolizable mineral oils
and the other, metabolizable oils.

Specol

Specol (ID-DLO, Lelystad, The Netherlands) is an adjuvant
composed of a purified and defined light mineral oil
(Markol 52) with the emulsifiers Span 85 and Tween 85
(Bokhout et al. 1981). In comparison studies, Specol has
been reported to produce an antibody response to a number
of antigens comparable to FCA/FIA in rabbits (Leenaars
al. 1998), and parakeets (Beck et al. 2003). In studies evalu-
ating antibody production in mice against the self-antigen
myelin basic protein (Leenaars et al. 1995) and several
small synthetic peptides (Ferber et al. 1999), FCA/FIA was
shown to be superior to Specol. The histological lesions
produced by Specol are generally fewer than those produced
by FCA (Leenaars et al. 1994, 1998) and similar to those of
FIA, although one study (Ferber et al. 1999) reported more
severe lesions with Specol than with FCA/FIA. Information
on pain and distress after Specol injection is limited and
generally similar to that with FCA/FIA. Ferber and cowork-
ers (1999), in comparing a number of adjuvants in mice that
included FCA/FIA, reported pain on palpation only with
Specol, and then only rarely.
Montanide ISA Adjuvants®

The Montanide incomplete Seppic adjuvants (ISAs) (Seppic, Paris, France) are a series of adjuvants composed of a variety of oils, different emulsion characteristics, different emulsifiers, and immunomodulators. The Montanide ISAS50V and ISA70 adjuvants are water-in-oil emulsions based on a purified mineral oil and are similar to FIA. Studies comparing these adjuvants with FCA and FIA have reported similar antibody responses but often with less inflammatory response (Johnston et al. 1991; Leenaars et al. 1994, 1995, 1998). Other Montanide adjuvants are primarily prophylactic adjuvants that utilize a variety of mineral and metabolizable oils and are in various phases of human and veterinary trials (Stewart-Tull 2003).

TiterMax® and TiterMax Gold®

TiterMax® and TiterMax Gold® (CytRx, Norcross, GA) consist of squalene (a metabolizable oil), an emulsifier (sorbitan monooleate 80), a patented block copolymer (CRL8941 or CRL8300, respectively), and microparticulate silica. TiterMax® adjuvants are water-in-oil emulsions with purportedly less tissue toxicity than FCA and equal or better antibody titer production (Jennings 1995). Nonionic block copolymers are composed of linear chains of hydrophilic polyoxyethylene that flank linear chains of hydrophobic polyoxpropylene (Hunter and Bennett 1986). The high molecular weight copolymers have a pronounced adjuvant activity, presumably through antigen presentation, complement activation, chemotactic properties, and macrophage activation (Howerton et al. 1990; Hunter and Bennett 1984). Numerous studies have evaluated TiterMax® adjuvants with a variety of species, immunogens, and routes of administration. Bennett and colleagues (1992) reported titers comparable to those of FCA in rabbits, mice, and goats using luteinizing hormone-releasing hormone conjugated to bovine serum albumin (BSA®) as the antigen with minimal inflammatory responses. Other reports with different antigens have indicated reduced lesions compared with FCA but also inferior antibody titer production compared with FCA in rabbits (Leenaars et al. 1994, 1998; Smith et al. 1992) and guinea pigs (Robuccio et al. 1995). Studies in mice with the same antigen produced comparable antibody titers to FCA but severe lesions after intraperitoneal administration of TiterMax®, as well as minimal lesions and poor antibody production following subcutaneous administration (Leenaars et al. 1998).

Oil-in-Water Adjuvants

Oil-in-water emulsions differ from water-in-oil emulsions in several fundamental aspects. Oil-in-water emulsions are typically formulated with a small overall amount of oil dispersed in an aqueous phase whereas water-in-oil emulsions typically have much higher percentages of oil (≥50%). Unlike water-in-oil emulsions, which form depots at the injection site, oil-in-water emulsions tend to be transported rapidly to draining lymphatic tissues and tend not to form depots at the injection sites. This tendency not to form local depots alleviates the potential of chronic inflammation, tissue destruction, and sequelae at the injection site, making the water-in-oil emulsions more likely candidates for therapeutic vaccines (Allison 1999).

RIBI Adjuvant System®

The RIBI Adjuvant System® (RAS®) (Corixa Corporation, Seattle, WA) is a commercial adjuvant system that has been available for experimental use since 1985. The RAS® system uses a small quantity of metabolizable squalene oil and Tween 80 surfactant in which the antigen is incorporated before emulsification in water (Ribi et al. 1975). The resulting oil-in-water adjuvant is much less viscous than typical water-in-oil adjuvants and can be sterilized by filtration and administered easily by injection (Jennings 1995; Stills 1994). Alone, the emulsion is a poor adjuvant, which requires the addition of immunostimulators (Altman and Dixon 1989). Three immunostimulators are used to produce three different research formulations.

Trehalose 6,6′-dimycolate (TDM®), the lipid component of mycobacterial cord factor, is a nonimmunogenic compound found in virulent strains of mycobacteria (Altman and Dixon 1989). TDM augments both humoral and cell-mediated immune responses when combined with other immunostimulators and antigens (Azuma et al. 1974; Ribi et al. 1975, 1982). The immunostimulatory effectiveness of TDM has been reported to be relatively less in the rabbit than in rodents (Altman and Dixon 1989). TDM is found in all three of the RAS® formulations.

Monophosphoryl lipid A (MPL®) is a chemically modified form of lipid A, the portion of bacterial LPS that mediates the endotoxic effects. MPL® retains much of the immunostimulatory activity of lipid A with toxicity reduced by a factor of 2,000 to 10,000 (Ribi et al. 1984). MPL® serves as an agonist for TLR-4, increasing both humoral and cellular immune response. MPL® is found in two of the RAS® formulations and is absent only in the formulation recommended for use in mice with strong antigens.

Cell wall skeleton (CWS®) is a second mycobacterial-derived immunostimulator used in the RIBI Adjuvant System®. The immunostimulatory activity of CWS is due primarily to muramyl dipeptide (MDP®), which is responsible for much of the adjuvant activity of whole mycobacterial cells (Ellouz et al. 1974). CWS, with MPL® and TDM, is present in the RAS® formulation recommended for use in rabbits, goats, and primates.

Studies that have compared and evaluated the pathological lesions induced by RIBI adjuvant have yielded varying results. Johnston and coworkers (1991) and Leenaars and colleagues (1994) found lesions comparable to those in-
duced by FCA in rabbits, whereas others have noted only minimal lesions with RIBI adjuvant (Deeb et al. 1992; Leenaars et al. 1998). Studies comparing intraperitoneal FCA and RIBI in mice consistently report fewer pathological lesions with RIBI (Lipman et al. 1992, 1995, 1998). With the exception of the report of Lipman and coworkers (1992) in mice, studies comparing the antibody response between RIBI and FCA/FIA have generally indicated a much higher antibody production with the FCA/FIA regimen (Johnston et al. 1991; Leenaars et al. 1995, 1998).

**Syntex Adjuvant Formulation (SAF)®**

The Syntex Adjuvant Formulation (SAF, SAF-1, SAF-m) (Chiron Corporation, Emeryville, CA) is a microfluidized oil-in-water emulsion adjuvant composed of threonyl muramyl dipeptide (t-MDP) in an emulsion vehicle consisting of 5% squalane, 2.5% Pluronic® L121, 0.2% polysorbate 80 (Tween 80), and phosphate-buffered saline (Vogel and Powell 1995). Microfluidization preforms the stable emulsion with lipid particle sizes of 160 nm to enhance transportation from the injection site to the draining lymphatic tissues. Squalane differs from squalene in that it is fully saturated and not subject to autoxidation (Allison 1999). Pluronic® L121 (poloxymers 401) is a synthetic block copolymer of ethylene oxide and propylene oxide that has adjuvant activity presumably through antigen presentation, complement activation, chemotactic properties, and macrophage activation (Allison 1999; Allison and Byars 1986; Howerton et al. 1990; Hunter and Bennett 1984). t-MDP is a synthetic analog of MDP, the adjuvant active component of mycobacterial cell walls. The inclusion of t-MDP augments both the cell-mediated immune response and cytotoxic T cell responses to antigens (Allison 1999). The synthetic modification reduces both pyrogenicity and hypersensitivity in comparison with either MDP or CWS. Developed as a prophylactic vaccine adjuvant, SAF has been evaluated in humans and a number of other species for efficacy and safety. In a human cancer immunotherapy study, SAF reactions consisted of injection site erythema, tenderness, induration, myalgia, arthralgia, and pyrexia (Hsu et al. 1997). Influenza virus vaccination studies with SAF in mice and guinea pigs have shown that SAF induced higher and more uniform antibody levels than virus alone (Byars et al. 1990); however, studies in guinea pigs with recombinant herpes simplex GD-2t (Byars et al. 1994) and cotton-top tamarins with gp340 viral surface antigen from Epstein-Barr virus resulted in high levels of cytotoxic T cells and protection (Morgan et al. 1989). A study comparing SAF and FCA in mice with influenza virus showed SAF to be equal to or superior to FCA (Hjorth et al. 1997), although a study in guinea pigs with influenza virus and several adjuvants found SAF inferior to FCA, TiterMax, RIBI, and aluminum phosphate for antibody production (Robuccio et al. 1995).

**Immune-stimulating Complexes**

ISCOMs are 30- to 40-nm honeycombs composed of an antigen, cholesterol, phospholipids, and a saponin derived from the South American soap tree (*Quillaja saponaria*) (Dalsgaard 1978; Dalsgaard et al. 1995; Morein et al. 1984). A number of semipurified or purified *Quillaja* saponins are commercially available for use in ISCOM formulation, and include Auil-A (Superfos AS), Spikoside (AdVet), and Iscoprep 703 (Iscotec AB). The three-dimensional structure of the ISCOM is classically formed when the immunostimulatory *Quillaja* saponin, cholesterol, phospholipids, and a hydrophobic or amphipathic antigen are allowed to react in a suitable detergent (Dalsgaard et al. 1995; Stewart-Tull 1996). Hydrophilic antigens such as small peptide monomeric chains do not incorporate into ISCOMs and must be modified by incorporating hydrophobic regions. The ISCOM matrix formation and immunogenicity is dependent on a number of factors (e.g., the purity of the *Quillaja* saponin, the hydrophobicity of the antigen, the ratio of *Quillaja* saponin to protein antigen, the phospholipids used), which makes proper formulation of the adjuvant problematic. The difficulty of formulating ISCOMs was alleviated when it was reported that preformed antigen-free ISCOMs or ISCOMs with an unrelated but immunostimulatory antigen (ISCOM-matrix or preformed ISCOM) could be mixed with an antigen and could serve as an adjuvant. Basic antigens with high isoelectric points may absorb to the ISCOM-matrix whereas others either require covalent attachment (Lövgren-Bengtsson and Morein 2000) or are delivered as unassociated compounds. The immunogenicity of ISCOMs or ISCOM-matrix formulations with incorporated or attached antigens is generally greater than ISCOM-matrix formulations with unassociated antigen, and higher levels of the *Quillaja* saponin, antigen, or additional immunostimulatory compounds may be required for the latter ISCOM-matrix formulations (Lövgren-Bengtsson and Sjölander 1996). The ISCOM-matrix formulation, even when used with unassociated antigen, has the advantage of greatly reduced saponin-related toxicity compared with the use of free saponin (Quil-A) as the adjuvant (Lövgren-Bengtsson and Morein 2000).

ISCOMs and ISCOM-matrix formulations have been evaluated extensively as prophylactic adjuvants, particularly for viral infectious agents (Morein and Lövgren-Bengtsson 1999; Rimmelzwaan and Osterhaus 1995). ISCOMs and ISCOM-matrix formulations are rapidly transported to the draining lymphoid tissues after administration and do not form antigen depots at the injection site (Morein et al. 1993). Studies comparing ISCOM formulations with several oil-in-water adjuvants similar to SAF and TiterMax™ have shown that the ISCOM formulation produces a similar humoral antibody response to influenza virus and a much higher CD8 cytotoxic lymphocyte response than either of the emulsion adjuvants (Coulter et al. 1998). The intraperitoneal injection of ISCOMs, either with an antigen or alone, induces an intense local acute inflammatory reac-
tion in mice with a rapid influx of neutrophils and mast cells (3.5 hr), followed by macrophages, dendritic cells, and lymphocytes peaking at 48 hr after injection. The levels of IL-1, IL-6, IL-12, and interferon-γ peaked at 24 to 72 hr after intraperitoneal injection, corresponding to the cellular influx and the stimulation of CD4 and CD8 T lymphocytes (Smith et al. 1999). The ability of ISCOMs to target dendritic cells leading to the priming of CD4 and CD8 T lymphocytes and a TH1 response is similar to that of FCA and in distinct contrast to the aluminum adjuvants, with which an exclusively TH2 humoral response occurs (Beacocks-Sharp et al. 2003; Villacres et al. 1998).

The use of ISCOMs as adjuvants for experimental polyclonal antibody production is quite limited. Leenaars and coworkers (1995) compared three antigens with a number of adjuvants, including a preformed ISCOM that contained rabies virus glycoprotein to which the antigens were covalently bound. Antibody titer levels produced by the ISCOM-matrix formulated antigens were equivalent to or slightly higher than FCA/FIA, and significantly higher than the levels produced with Specol for myelin basic protein. The histological lesions produced by the ISCOM in this study were minimal by the subcutaneous route, although lesions were seen after intraperitoneal administration. Studies comparing ISCOMs and other adjuvants in rabbits are limited to studies that have evaluated therapeutic vaccination strategies. The results have varied greatly, ranging from producing significantly better virus neutralizing antibody (Browning et al. 1992), being comparable to FCA (Sjölander et al. 1993a), to producing a significantly lower antibody response (Levi et al. 1999; Lockyer et al. 1993, Sjölander et al. 1993b). None of these studies reported significant lesions after injection of ISCOMs.

The use of ISCOMs in the experimental polyclonal antibody production has the additional drawback of a complicated adjuvant preparation. The formation of ISCOMs, as mentioned above, is not without difficulty, and the covalent linking of antigen to preformed ISCOM-matrix is labor intensive. Antigens must also be capable of covalent linking by one of the standard chemistry methods without denaturation and loss of antigenicity. As stated by Leenaars and colleagues (1995), these are significant disadvantages.

**Aluminum Salt Adjuvants**

Absorption of antigens onto aluminum phosphate or aluminum hydroxide is the most common method of producing vaccines for human and veterinary medical use (Gupta and Rost 2000). Aluminum-adjuvanted vaccines have been used in humans for more than 60 yr, with an excellent safety record (Altman and Dixon 1989). Aluminum salt adjuvants bind antigens via electrostatic forces, providing a short-lived depot effect. Aluminum adjuvants exhibit some immunostimulatory properties, and they promote an almost exclusive TH2 response to the antigen (Linblad 2000). Granulomas consisting initially of macrophages are produced at injection sites, and there is a lymphocytic influx. Aluminum-adjuvanted antigens are rapidly cleared after injection, which leads to peak antibody titers 3 to 4 wk after injection with a rapid decline, although repeated injections can lead to prolonged antibody responses (Altman and Dixon 1989). Although not extremely useful for primary immunizations for experimental antibody production, aluminum salt adjuvants have a number of desirable characteristics that warrant their consideration as adjuvants for booster injections (Stills 1994).

**Gerbu® Adjuvants**

The Gerbu Adjuvants® (GERBU Biochemicals GmbH, Am Kirchwald 6, 69251 Gaiberg, Germany) are a group of adjuvants all based on the use of the immunomodulator N-acetyl-glucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutamine (GMCP®), a soluble glycopeptide from the cell wall of *Lactobacillus bulgaricus*. All Gerbu adjuvants also contain cimetidine as an immune enhancer, and most contain saponin, another immunomodulating compound. A specialized Gerbu adjuvant for mouse monoclonal immunization (Gerbu Adjuvant MM®) contains a poly(adenylic)poly(uridylic) acid heteroduplex (poly A:U) and theophylline, in addition to the GMCP and cimetidine. Gerbu adjuvants are aqueous adjuvants with suspended solid ultrafilterable particles composed of a paraffin mixture that contains dimethyl(dioleoyl)ammonium chloride (Esterquat 1). Other components include mannide monooleate, glycerol, L-proline, and ciprofloxacin.

The Gerbu adjuvants were primarily developed and tested as adjuvants for human and veterinary therapeutic use, and the formulations have evolved over time. The early Gerbu adjuvants contained dimethyl dioctadecylammonium chloride (DDA), zinc L-proline, and GMCP (Vogel and Powell 1995). The DDA was replaced by Esterquat 1 to reduce tissue irritation, and the other immunomodulators were added to improve immune response. Evaluation and comparison of studies using Gerbu adjuvants are complicated by the evolution of the adjuvant system and the tendency to refer to all products as “Gerbu adjuvant.”

Studies with Gerbu have consistently found the adjuvant to produce few lesions and to be tolerated well, although failing to produce polyclonal antibodies that equal those of other adjuvants. Therapeutic vaccination studies in sheep and avians with Gerbu and other adjuvants have shown Gerbu to be effective for immunization and protection but deficient in antibody production compared with FCA, FIA, and others (Beck et al. 2003; Shu et al. 2001). Similar results were obtained in mice immunized with the extracellular domain of the thyrotropin receptor, an autoantigen, where both FCA and TiterMax produced high titers although Gerbu did not (Seetharamaiah et al. 1996). In the same study Gerbu, along with FCA and TiterMax, produced high titers to BSA, but unlike FCA and TiterMax, the antibodies produced with Gerbu were restricted to the immu-
noglobulin G1 subclass. Ferber and coworkers (1999) compared Gerbu and a number of other adjuvants for subcutaneous immunization of mice with a number of synthetic peptides for monoclonal antibody production. Gerbu produced antibody titers nearly equivalent to those of FCA/FIA and superior to those of TiterMax, RIBI, or PolyA:PolyU. A study comparing a number of adjuvants with several antigens in rabbits found Gerbu to be extremely well tolerated but ineffective at generating adequate polyclonal antibody titers (Leenaars et al. 1998).

Routes and Volumes of Adjuvant Administration

A number of publications have listed acceptable or recommended routes of administration for FCA, FIA, and other adjuvants (Amyx 1987; CCAC 2002; Grumstrup-Scott and Greenhouse 1988; Johnston et al. 1991; Leenaars et al. 1999). The subcutaneous and intradermal routes are commonly used to take advantage of the presence of Langerhans cells (Harlow and Lane 1988; Stills 1994). Intradermal injections have the advantage of being easily visualized and monitored (Halliday et al. 2000; Johnston et al. 1991; Stills 1994; Stills and Bailey 1991), although the potential for ulceration exists and the injection into the closed space of the dermis may be painful (CCAC 2002). Subcutaneous injections avoid the potential of pain from the injection but permit migration of injected adjuvant and potential fistulous tracts (Broderson, 1989; Stills 1994; Stills and Bailey 1991). Intramuscular injections have the advantage of ease of injection and the ability to administer large-volume injections at a single site. The disadvantages include the inability to monitor the injection site with the known inflammatory lesions closely and the potential of pain on injection (Broderson 1989; CCAC 2002; Johnston et al. 1991; Leenaars et al. 1998, 1999; Stills 1994; Stills and Bailey 1991). Intradermal, subcutaneous, and intramuscular injections are common routes of adjuvant administration and are generally accepted by institutional animal care and use committees (IACUCs) in the United States (Stills 2000).

Intraperitoneal adjuvant injections are frequently used in mice and other small rodents but are known to induce a local and acute inflammatory reaction, behavioral changes, and peritonitis (CCAC 2002; Griffen et al. 2003; Leenaars et al. 1995, 1998, 1999; Lipman et al. 1992; Toth et al. 1989; Wanstrup and Christensen 1965). Intraperitoneal injections are generally not recommended for polyclonal antibody production (CCAC 2002; Leenaars et al. 1999). In spite of these recommendations, a survey of US IACUCs in 2000 indicated that few committees restricted the use of the intraperitoneal route of adjuvant administration (Stills 2000).

The injection of adjuvants into the “footpad” of rabbits has long been considered inappropriate and is prohibited by most, if not all, authors and institutions (Amyx 1987; Jackson and Fox 1995; Stills 2000). Footpad injections in rodents have previously been justified based on the need to localize the immune response to the popliteal lymph node (Amyx 1987; Jackson and Fox 1995); however, this injection site currently is strongly discouraged and generally replaced with injections at the base of the tail, dorsum of the foot, or other areas in the popliteal area (CCAC 2002; Jackson and Fox 1995; Leenaars et al. 1999; Stills 2000). Intrasplenic and intra-lymph node injections may be appropriate in specific situations but require extensive justification by the investigator for approval (CCAC 2002; Leenaars et al. 1999).

Numerous publications also list recommended maximum total and single site injection volumes for different routes of adjuvant administration (Amyx 1987; CCAC 2002; Grumstrup-Scott and Greenhouse 1988; Johnston et al. 1991; Leenaars et al. 1999). These recommendations generally apply to water-in-oil emulsion adjuvants, although this limitation is not generally stated specifically. Increasing the volume of FCA does not appear to increase the antibody response (Leenaars et al. 1994), although the extent of the lesions produced increases (Stills 1994; Stills and Bailey 1991) and volume is believed to be a major influence on the lesions produced (Amyx 1987; Grumstrup-Scott and Greenhouse 1988; Johnston et al. 1991). Dividing the inocula into multiple sites potentially increases immune cell exposure and antibody production (Amyx 1987; Grumstrup-Scott and Greenhouse 1988; Stills and Bailey 1991) and decreases the lesion volume of individual sites (Stills and Bailey 1991). As with the recommendations on injection routes, there is a wide range of maximum total volumes and site volumes for FCA injections accepted by IACUCs in the United States (Stills 2000).

Discussion and Concluding Remarks

In the foreseeable future, the production of polyclonal and monoclonal antibodies will continue to require the use of adjuvants, with continuing concerns for animal health and welfare. Although numerous recommendations have been published, the ultimate selection of immunological adjuvant, immunization route, and schedule requires careful consideration of the antigen, the effect of the adjuvant, and the desired end-product.

FCA is a very potent and effective adjuvant with a wide range of antigens, and it remains the de facto gold standard for adjuvants. The present formulations of FCA are much less reactive and toxic than the older formulations, but they still induce considerable granuloma formation and pathological lesions. The lesions produced by FCA can be limited and minimized by using the newer FCA formulations containing purified mineral oils and limited mycobacterial content, preparing stable antigen-adjuvant emulsions, minimizing both total volume and single injection site volumes, and using aseptic preparation and injection techniques.

The extensive lesions produced by FCA have led some to suggest that pain and distress are invariably associated with the use of FCA. With the notable exception of intra-
peritoneal FCA injection in rodents, the scientific evidence does not support a conclusion of pain or distress after injection of the newer FCA adjuvants. This perception has, however, led to the development of numerous recommendations and regulatory requirements concerning the use of FCA. It has also encouraged the use of other adjuvants, some of which have been documented to produce more acute inflammation, pain, and distress than FCA. Although the pathological lesions associated with FCA are well documented, the general issue of pain and distress after FCA injection by routes other than intraperitoneal is not well documented.

The intraperitoneal administration of FCA in rodents is clearly associated with several days of pain and distress that can be observed both clinically and behaviorally. The use of intraperitoneal FCA in rats to produce a model of acute inflammation further supports the conclusion that intraperitoneal FCA is associated with pain and distress. With the additional potential complication of granulomatous peritonitis and the availability of other adjuvants that produce equal or better antibody responses via the intraperitoneal route, the intraperitoneal use of FCA is not recommended.

The lesions produced by FCA should foster the continuing search for less damaging replacement adjuvants. Replacement water-in-oil adjuvants (e.g., FIA, Specol, and Montanide ISA adjuvants) should be considered as alternatives to FCA that may produce equivalent or better antibody responses with less tissue destruction and granuloma formation. Intraperitoneal injection of FCA in mice for monoclonal antibody immunization has been shown to cause pain and distress although other adjuvants have produced equal or better antibody levels and fusion efficiencies. Other commercially available adjuvants (e.g., TiterMax®, RIBI®) may be appropriate in specific cases, but they are not without side effects. As newer adjuvant formulations are developed and tested, alternatives to the presently available adjuvants will permit greater antibody production with less lesion development.

References


