Review article

Vaccination, squalene and anti-squalene antibodies: Facts or fiction?

Giuseppe Lippi a, Giovanni Targher b, Massimo Franchini c,⁎

a U.O. Diagnostica Enzootichica, Dipartimento di Patologia e Medicina di Laboratorio, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy
b Sezione di Endocrinologia, Dipartimento di Scienze Biomediche e Chirurgiche, Università di Verona, Italy
c Servizio di Immunomematologia e Trasfusione, Dipartimento di Patologia e Medicina di Laboratorio, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy

A R T I C L E   I N F O

Available online 29 December 2009

Keywords: Vaccination Squalene Antibodies Autoantibodies Influenza

A B S T R A C T

Squalene, a hydrocarbon obtained for commercial purposes primarily from shark liver oil and other botanic sources, is increasingly used as an immunologic adjuvant in several vaccines, including seasonal and the novel influenza A (H1N1) 2009 pandemic flu vaccines. Nearly a decade ago, squalene was supposed to be the experimental anthrax vaccine ingredient that caused the onset of Persian Gulf War syndrome in many veterans, since antibodies to squalene were detected in the blood of most patients affected by this syndrome. This evidence has raised a widespread concern about the safety of squalene containing adjuvants (especially MF59) of influenza vaccines. Nevertheless, further clinical evidence clearly suggested that squalene is poorly immunogenic, that low titres of antibodies to squalene can be also detected in sera from healthy individuals, and that neither the presence of anti-squalene antibodies nor their titre is significantly increased by immunization with vaccines containing squalene (or MF59) as an adjuvant. This review summarizes the current scientific evidence about the relationship between squalene, anti-squalene antibodies and vaccination.

© 2009 European Federation of Internal Medicine. Published by Elsevier B.V. All rights reserved.

1. Introduction

Squalene is a natural organic compound obtained for commercial purposes primarily from shark liver oil, although it can also be extracted from botanical sources, including olive oil, palm oil, wheat-germ oil, amaranth oil and rice bran. All higher organisms, including humans, produce this natural organic compound, which is an intermediate in the cholesterol biosynthesis pathway, and is essential for the synthesis of cholesterol, steroid hormones and vitamin D [1]. Historically, squalene has been widely used in the formulation of various cosmetics, as an emollient, antioxidant and hydrating, because it can be easily emulsified and has the property of spreading well [2]. Most recently, however, squalene has also been used as an immunologic adjuvant in several vaccines, including malaria, human immunodeficiency virus, herpes virus, cytomegalovirus, human papillomavirus and seasonal and pandemic flu. Basically, immunologic adjuvants are substances, administered in conjunction with the vaccine, which stimulate the immune system and increase the host response. The most common adjuvant comprising squalene is MF59, developed by the ex-Chiron now Novartis Vaccines, which consists in an oil-in-water emulsion, comprising a low content of biodegradable squalene oil (4.3%) as the dispersed phase, which is stabilized by two non-ionic surfactants (Twee 80 and Span 85), and a low ionic strength citrate buffer as the continuous phase [3]. Although the definitive mechanisms supporting MF59 adjuvanticity are still unclear, previous studies (reviewed by Schultz et al.) showed that MF59 administration (i) produces a significant influx of macrophages at the site of injection, a process which is associated with enhanced production of chemokines in cells resident at the injection site, and (ii) induces a strong T-cell response to a variety of different antigens, including bacterial toxins, outer membrane vesicles, polysaccharide conjugates, recombinant and viral antigens (e.g., influenza antigens) [4].

A trivalent MF59-adjuvanted seasonal influenza vaccine (Fluad) has been approved by some health agencies, but not the US Food and Drug Administration (FDA), and used in several European countries for seasonal flu shots since 1997. This vaccine was proven to induce higher immune responses to influenza than non-adjuvanted vaccines, and to provide cross-reactive immunity against divergent influenza strains. Similar results have been obtained with a MF59-adjuvanted H5N1 pre-pandemic vaccine, which showed a higher and broader immunogenicity than non-adjuvanted pre-pandemic vaccines [5]. As such, pandemic vaccine antigens against influenza A (H1N1) 2009 containing MF59 are now being manufactured and marketed across Europe. Dormitzer et al. reported that immunizing unprimed mice with a single dose of MF59-adjuvanted pandemic H1N1 influenza subunit antigen elicited functional antibody titres equivalent to those associated with protection of humans from seasonal influenza, whereas two doses of vaccine might be required to elicit a comparable immunological response without the adjuvant [6]. In a preliminary study, 175 individuals (18 to 50 years of age) were randomly assigned...
to receive two intramuscular injections of the monovalent influenza A/California/2009 (H1N1) surface-antigen vaccine, in both MF59-adjuvanted or non-adjuvanted forms. The percentages of subjects with seroconversion and seroprotection 21 days after vaccination as measured by means of the hemagglutination-inhibition assay, were comprised between 70% and 86% for subjects receiving a single dose on day zero, and between 92 and 96% for subjects receiving two doses at days zero and fourteen. The most frequent local reaction was pain at the injection site (70% of subjects). In general, such pain was however not accompanied by redness or swelling. No severe local reactions were reported [7]. Vesikari et al. also reported that MF59-adjuvanted influenza vaccine was well tolerated in healthy children and induced greater, longer-lasting, and broader immune responses than a non-adjuvanted split vaccine [8]. In a recent cost-effectiveness analysis, Khaezi et al. concluded that earlier vaccination of at least 40% of the population against pandemic (H1N1) 2009 flu would be cost-saving. In particular, vaccination in October would have averted 2051 deaths, gain 69,679 quality of life years (QALYs), and save $469 million compared with no vaccination. Likewise, vaccination in November would have averted 1470 deaths, gain 49,422 QALYs, and save $302 million [9]. Regardless of this striking epidemiological and economical analyses, there is widespread concern about the possible side effects of adjuvants of influenza vaccine, especially MF59, which has been boosted further by the attempt to link squalene to Persian Gulf War Syndrome, since this adjuvant was supposedly being present in an anthrax vaccine given to military personnel during the 1991 Persian Gulf War.

2. Squalene and the Persian Gulf War syndrome

The Persian Gulf War syndrome (GWS) or Gulf War illness is a systemic illness afflicting US combat veterans of the 1991 Persian Gulf War typified by a constellation of unexplained symptoms including fatigue, rashes, headache, arthralgias, myalgias, lymphadenopathies, diarrhea, memory loss, autoimmune thyroid diseases, increased allergies, sensitivities to environmental elements and neurological abnormalities [10].

Before deploying to the Persian Gulf in 1990–1991 (and thereafter to the present), all US troops got a standard series of inoculations against infectious diseases, virtually the same one administered to all US citizens travelling to the region. After arrival, however, 150,000 soldiers also underwent anthrax vaccinations according to the Anthrax Vaccine Immunization Program (AVIP), which had already begun in March 1998. Regrettably, squalene was accused to be the experimental anthrax vaccine ingredient that caused the onset of GWS in many Gulf War veterans from the US, UK, and Australia. The original association between GWS and anti-squalene antibodies (ASA) comes from a report by Asa et al. [11], who enrolled individuals immunized for military service in the United States or the United Kingdom or as employees of the U.S. military or their contractors in the Persian Gulf during 1990–1991. The study population included 144 Gulf War-era veterans or military employees, 48 blood donors, 40 systemic lupus erythematosus patients, 34 silicone breast implant recipients, and 30 chronic fatigue syndrome patients. An ASA assay was used to measure the binding of serum immunoglobulin (IgG) to squalene. Briefly, the method was based on drying progressive dilutions of squalene on nitrocellulose membranes, rinsing in wash buffer, and pre-incubating with a blocking buffer prior to adding a 1:400 dilution of human serum. Biotin–avidin-conjugated horseradish peroxidase antibodies were used for detection, employing methanol, 4-chloro-1-naphthol and 0.03% hydrogen peroxide as substrates. Air-dried strips were finally classified using a visual scale from “0” to “4+.” Unexpectedly, up to 95% of overtly ill deployed GWS patients had ASA in their blood and all GWS patients immunized for service in Desert Shield/Desert Storm who did not deploy, but they had the same signs and symptoms as those who did deploy, tested positive for the ASA assay. In contrast, none of the deployed Persian Gulf veterans not showing signs and symptoms of GWS had ASA, likewise patients with idiopathic autoimmune disease and healthy controls [11]. As also recognized by the same authors, however, it is important to note that this study did not establish that squalene was added as adjuvant to any vaccine used in military or other personnel who served in the Persian Gulf War. Suddenly afterwards, Alving and Grabenstein replied with a letter to the editor on the same journal, highlighting several drawbacks that might have flawed the study, including (i) the lack of positive controls that could validate the assertion of detecting ASA (e.g., comparable serum samples demonstrating to contain anti-squalene antibodies after injection with squalene); (ii) the lack of preinjection results to establish that intentional administration of squalene would trigger the development of antibodies to a substance already present in humans; (iii) the lack of elementary negative controls routinely run in enzyme-linked immunoassays to prove that the assay was not detecting cross-reacting substances or other IgG molecules with nonspecific binding to squalene; (iv) the high dilution used to test human samples (i.e., 1:400), so that the presence of antibodies at a higher concentration of serum might have gone undetected in other study groups; (v) the use of aqueous dilutions of squalene for impregnation of nitrocellulose (squalene is an oil, and thereby virtually insoluble in water); and (vi) the lack of a real calibration curve, since antibody reactions were only semiquantitatively reported, so that nonspecific binding of serum immunoglobulin could not be ruled out [12]. Nevertheless, in a further study Asa et al. [11] assessed circulating levels of ASA among individuals participating in the Anthrax Vaccine Immunization Program (AVIP). In this pilot study, 100% recipients of anthrax vaccine with GWS-like symptoms were positive for ASA. In a larger blinded study, however, only 32% of the AVIP personnel (as compared with 15.6% of controls; p = ns) were positive for ASA. Further analysis revealed that ASA were associated with specific lots of anthrax vaccine and that in all but one case ASA were restricted only to personnel immunized with lots of vaccine known to contain squalene. Except for one symptomatic individual, positive clinical findings in 17 ASA-negative personnel were restricted to 4 individuals receiving vaccine from lots containing squalene. Interestingly, ASA were not positive prior to vaccination in preimmunization sera from 4 AVIP personnel. Three of these individuals became ASA positive after vaccination. These results led the authors to conclude that the development of ASA in GWS-like patients was strongly associated with the presence of squalene in certain lots of anthrax vaccine [13].

Few years later, Matyas et al. developed an Enzyme-Linked Immunoassay (ELISA) employing antigen coated on polystyrene ELISA plates and peroxidase-linked monoclonal antibodies (mAbs) that reacted specifically with squalene. The binding of mAbs and anti-squalene serum was dependent upon both the amount of antibody added to the wells and the amount of squalene added to the wells [14,15]. The methodology was further adapted to be used for the measurement of ASA in human serum and plasma using sterile cell culture 96-well plates coated with SQE (20 nmol/well). Phosphate-buffered saline (PBS)-0.5% casein was used as both a blocking agent and dilution buffer. The assay had a high throughput capacity and was proven to be reproducible and quantitative [16]. This assay was used to evaluate samples from three different study groups. The first cohort comprised retired employees of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID alumni); most of them were vaccinated with the U.S. licensed anthrax vaccine and most had received several other vaccines through a USAMRIID special immunization program. The second cohort was enrolled from a healthy population and included subjects who were not vaccinated with anthrax vaccine. The third cohort was enrolled from Camp Memorial Blood Center, United States Army Medical Department Activities, Fort Knox, KY. ASA were detected in all three of the cohorts. In particular, IgG antibodies to squalene were detected in 7.5% and
15.1% of the samples from the USAMRIID alumni and healthy subjects, respectively (p = 0.19), whereas no IgG antibodies to squalene were detected in the Fort Knox cohort. Likewise, IgM antibodies to squalene were detected in 37.5% and 32.3% of the samples from the USAMRIID and healthy groups, respectively (p = ns). Only 19% of the samples from the Fort Knox cohort tested positive for IgM antibodies to squalene. The ASA prevalence was also higher in females and linearly correlated with age. It was thereby concluded that ASA occur naturally in humans and are not correlated with anthrax vaccine [16]. These results were further confirmed by Del Giudice et al. who used a validated ELISA for the quantification of IgG and IgM antibodies against squalene. Serum samples were collected from 43 healthy individuals who had participated in various clinical trials with Chiron vaccines in the US since 1995 (none of these vaccines contained the MF59 adjuvant), 50 healthy adults before the initiation of a trial carried out in western Europe with Chiron subunit influenza vaccines either with or without adjuvant, and in patients undergoing vaccination with the Chiron influenza vaccine with the MF59 adjuvant (n = 48) or with a conventional influenza split vaccine without adjuvant (n = 52). Results of this study showed that immunization with vaccines containing the MF59 emulsion adjuvant did not induce anti-squalene ASA (IgM or IgG), nor enhanced pre-existing ASA titres. Notably, ASA were detected frequently at low titres also in sera from healthy individuals that had never received any vaccine containing squalene [17]. Phillips et al. recently examined the relationship between ASA and chronic symptoms reported by Navy construction workers (Seabees), using the high throughput, i.e., validated assays previously developed by the same study group. Among these, 30.2% were shooters, 7.4% were defined as ill, and 43.5% were positive for serum anti-squalene antibodies. However, no significant association was observed between ASA positivity and chronic multisymptom illness (p = 0.465), and Gulf War veterans had essentially similar proportions of ASA positivity or negative prevalence of chronic multisymptom illness as did non-deployers [18].

3. Conclusions

The huge clamour raised on the high prevalence of ASA detected by Western blotting in the sera of US military personnel affected by the so-called Persian Gulf War syndrome, was strongly criticized on technical grounds, and was also considered inconclusive by the Institute of Medicine (IOM) [19]. Although it has been also suggested that the basis for this illness may be an altered immune system, compelling evidence is lacking, and the in vitro immunological responses are not abnormal in symptomatic Gulf War veterans [20]. Even if the anthrax vaccine or other vaccines had contained squalene in biologically active amounts, it is very unlikely they would have induced a natural or even pathological antibody response. Therefore, the real aetiology of the Persian Gulf War syndrome remains still largely unknown, but it should not involve ASA status since the available clinical data are consistent with the hypothesis that squalene is poorly immunogenic, that low titres of ASA can be also detected in sera from healthy individuals, but that neither the presence ASA, nor their titre is increased by immunization with vaccines containing squalene (or MF59) as an adjuvant. The Persian Gulf War syndrome and its medically unexplained health-related symptoms shares much with other medically unexplained disorders encountered in clinical practice, raises the questions whether or not they exist, and which are their real cause, such as yet-to-be-discovered medical problem or psychogenic condition [10].

Microfluidized squalene or squalene emulsions are efficient adjuvants, eliciting both humoral and cellular immune responses. Although some experimental reports suggested an association of MF59 with various autoimmune diseases in mice [21], only a few of them were confirmed epidemiologically, and even less in humans. Mice are inbred, of limited age, and live in a relatively sterile environment, so that they cannot be considered the panacea to predict human responses. On the other hand, a huge amount of recent publications along with a large safety database clearly attests that MF59 is a safe and potent vaccine adjuvant in humans that has been already licensed in more than 20 countries. Its main strengths are represented by the increased immunogenicity, greater than that achievable with aluminum-based adjuvants of influenza vaccines, especially in the elderly and in chronically ill. MF59 also allows for a broader cross-reactivity against viral strains not included in the vaccine [4,22,23]. Extraordinary efforts are being undertaken worldwide to develop plans to mitigate the dire public health consequences of the new 2009 (H1N1) influenza pandemic. Routine influenza immunization practices with MF59-adjuvanted vaccines are well tolerated and are currently considered among the most effective strategy for limiting the burden of this infection, providing substantial benefits in terms of reduction of morbidity, complications, hospitalizations and deaths. Whether the antigens contained in these vaccines might cause disease later in life is as yet uncertain, but denying vaccination because of the treat of developing ASA is apparently unjustified.

4. Learning points

• Squalene is increasingly used as an immunologic adjuvant in several vaccines, including seasonal and the novel influenza A (H1N1).
• Nearly ten years ago, antibodies to squalene have been detected in veterans of the Gulf War and associated with the Gulf War syndrome, thereby raising widespread concern about the safety of squalene containing adjuvants of influenza vaccines.
• Further clinical evidence however revealed that squalene is poorly immunogenic, that low titres of antibodies to squalene can also be detected in healthy individuals, and that neither the presence of anti-squalene antibodies nor their titre is significantly increased by immunization with vaccines containing squalene (or MF59).
• Taken together, the current scientific evidences point out that denying vaccination because of the risk of developing antibodies to squalene is probably unjustified.

References