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Dossier: Autoimmunity and Biotherapy

Autoimmunity induced by adjuvant hydrocarbon oil components of vaccine

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Abstract

Adjuvant oils such as Bayol F (Incomplete Freund's adjuvant: IFA) and squalene (MF59) have been used in human and veterinary vaccines despite poor understanding of their mechanisms of action. Several reports suggest an association of vaccination and various autoimmune diseases, however, few were confirmed epidemiologically and the risk of vaccination for autoimmune diseases has been considered minimal. Microbial components, not the adjuvant components, are considered to be of primary importance for adverse effects of vaccines. We have reported that a single intraperitoneal injection of the adjuvant oils pristane, IFA or squalene induces lupus-related autoantibodies to nRNP/Sm and -Su in non-autoimmune BALB/c mice. Induction of these autoantibodies appeared to be associated with the hydrocarbon's ability to induce IL-12, IL-6, and TNF-α, suggesting a relationship with hydrocarbon's adjuvanticity. Whether this is relevant in human vaccination is a difficult issue due to the complex effects of vaccines and the fact that immunotoxicological effects vary depending on species, route, dose, and duration of administration. Nevertheless, the potential of adjuvant hydrocarbon oils to induce autoimmunity has implications in the use of oil adjuvants in human and veterinary vaccines as well as basic research.

Keywords: Adjuvant; Autoimmunity; Pristane

1. Introduction

The weak immunogenicity of many foreign antigens can be overcome through the use of adjuvants [1,2]. Although the precise mechanisms of action are poorly understood, adjuvants such as Incomplete Freund's adjuvant (IFA) or alum have been used for many years in human and veterinary vaccination [3,4]. Adjuvants also are used routinely in immunological research [2]. Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane) is a new adjuvant oil to replace the mineral oil used in IFA and is the main component of a new adjuvant, MF59, used in human vaccines [5,6]. It also is present in TiterMax® (Accurate Chemical & Scientific Co., Westbury, NY), an adjuvant widely used in animal research [7].

A single intraperitoneal (i.p.) injection of pristane (2,6,10,14 tetramethylpentadecane) induces a lupus-like syndrome in virtually any strain of mouse [8–10] as well as plasmacytomas [11] or chronic destructive arthritis in susceptible strains of mice [12] and rats [13]. Pristane is derived from mineral oil, a byproduct of petroleum distillation. IFA consists of a mineral oil Bayol F plus the emulsifier Arlacel A [14]. Although the risk of use in human vaccines is considered minimal [3], i.p. injection of IFA or its oil component Bayol F [15] induce plasmacytomas in BALB/c mice as does pristane [11]. IFA or squalene induces inflammatory arthritis in susceptible mice and rats [14,16], but it was not known whether they could induce a lupus-like autoimmunity like pristane. We have recently reported that a single i.p. administration of IFA or squalene can precipitate lupus-like autoimmunity in non-autoimmune BALB/c mice [17,18]. The induction of lupus-specific autoantibodies by adjuvant oil may have implications for the immunization of both humans and experimental animals.

In this article, we will provide an overview of murine lupus induced by pristane, the induction of autoimmunity by adjuvant oils IFA and squalene, and the current understanding of the risk of autoimmunity with vaccination.

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2. Pristane-induced lupus model in mice

2.1. Induction of autoimmunity by pristane

A single i.p. injection of pristane induces hypergammaglobulinemia and lupus-related autoantibody production in virtually any non-autoimmune immunocompetent strain of mouse [8,19]. Immune-complex deposition in the kidneys is common although histologically apparent glomerulonephritis with proteinuria is less common and is strain and environment dependent [9,20–22].

Following pristane injection, a polyclonal increase of the T-cell independent isotypes IgM and IgG3 occurs along with the production of low affinity IgM anti-ssDNA antibodies, typically peaking at 2 weeks (phase I) [9,19]. Based on production of low affinity IgM class antibodies and the disappearance or dramatic decrease of peritoneal B-1 cells within 2 weeks of pristane injection [23], B-1 cells may play an important role in this phase. Whether B-1 cells die, differentiate, or migrate into other tissues is not known. After about a month, the phase I is followed by a polyclonal increase in T-cell dependent isotypes IgG1, IgG2a, and IgG2b [19]. IgG2a may peak at 4–5 months whereas IgG1 increases gradually. IgG2a/IgG1 ratios (a surrogate marker of Th1/Th2 balance) typically peak at 3–5 months reflecting this course. High affinity IgG lupus-related autoantibodies to nRNP/Sm, Su [8,9], and ribosomal P [10,20] appear as early as 6–8 weeks and the titers rapidly increase between 2–3 months in many mice (Phase II). Other specificities such as antibodies to nuclear factor (NF) 45/90/110 [10,24], myositis-associated autoantibodies to OJ (isoleucyl tRNA synthetase complex) [25], and signal-recognition particle (SRP) (M. Hirakata, unpublished) also were found in some mice during this phase. IgG anti-chromatin and -dsDNA antibodies are absent or at very low levels before 6 months but may increase later on in BALB/c mice (Phase III) [26]. In susceptible strains of mice such as DBA/1, chronic destructive arthritis is seen 4–8 months after treatment and active inflammation resolves leaving bone destruction and deformity [12]. Plasmacytoma develops in susceptible strains of mice such as BALB/cAn or NZB after 6 months [11].

2.2. Genetic factors

As opposed to the susceptibility of only a limited number of strains to pristane-induced arthritis [12] or plasmacytomas [11], lupus-related autoantibodies are induced in virtually any immunocompetent strain regardless MHC or other genetic background [10]. However, the specificity of autoantibodies produced by different strains is strikingly different and appears to depend mainly on non-MHC background genes. MHCs tested include H-2a, b, d, k, q, and s but regardless the MHC, strains with the same background produced the same specificities; BALB/c background mice with H-2b, d, and k all produced anti-nRNP/Sm and -Su but no anti-ribosomal P (M. Satoh, unpublished) whereas B10 mice with H-2b, k, q, and s produced anti-ribosomal P and -NF45/90/110 [10,24] with little anti-nRNP/Sm ([10] and A. Mizutani, unpublished).

2.3. Environmental factors

When specific pathogen free (SPF) and conventional mice were compared, SPF mice had reduced and delayed autoantibody production and hypergammaglobulinemia, suggesting the role of microbial stimuli in this model [19]. The reduced induction of autoantibodies in C3H/HeJ mice, which are resistant to LPS stimulation due to mutant TLR4, is consistent with this observation [27]. However, germfree mice lacking any exogenous live microorganism including normal bacterial flora, produced anti-nRNP/Sm and -Su antibodies at frequencies and levels comparable to SPF mice, indicating that live microorganisms are not necessary in lupus autoantibody production in this model (A. Mizutani et al., submitted). Most strains of mice have endogenous murine leukemia viruses (MuLV) and mouse mammary tumor viruses (MMTV) that may play an important role in autoimmune responses [28]. Pristane reactivates endogenous ecotropic and xenotropic MuLV [29]. When exogenous MMTV free C3HeB/FeJ mice and endogenous ecotropic MuLV free CE/J mice were tested, they produced anti-nRNP/Sm, -Su, chromatin, and -ssDNA antibodies at frequencies comparable to control mice, indicating that these viruses are not essential to pristane-induced lupus [30]. However, other types of endogenous MuLV (xenotropic, polytropic) and endogenous MMTV are still present in germ-free mice and could play a role. Other undetermined environmental factors may also play a role since variability between batches of mice and cages have been observed (M. Satoh, unpublished).

2.4. Role of cytokines

Cytokines play an essential role in the pathogenesis of autoantibody production and lupus in humans and mice. Recent studies suggest a critical role of Th1 cytokines, in particular IFN-γ, in the pathogenesis of lupus in MRL/*lpr* mice and NZB x NZW (F1) (B/W) mice, although IL-4 may also play an important role in B/W mice [31,32].

IL-6, IL-12, and TNF-α are overproduced in the peritoneal cavity of pristane-treated mice [17,33]. Peritoneal, spleen, and lymph node cells from pristane-treated mice overproduce IL-6, IL-12, TNF-α, IFN-γ in vitro and shifts cytokine balance towards Th1 [17,25,33,34]. Data from our laboratory using cytokine gene deleted mice suggest a critical role of IL-6 in the production of IgG anti-dsDNA and -chromatin antibodies in pristane-induced lupus [26]. IFN-γ appears to play more important role than IL-6 in the induction of anti-nRNP/Sm autoantibodies though the IL-6 may play a role in maintaining levels of these autoantibodies [21].

2.5. Interaction of genetic and environmental factors

SJL/J and other mice with H-2s produce autoantibodies to the nucleolar protein fibrillarin, a specificity found in some patients with scleroderma [35]. However, SJL/J and other H-2s mice produce lupus-specific autoantibodies to cytoplasmic antigen ribosomal P when i.p. injected with pristane [10,20]. These observations indicate that mice with same genetic background can respond to chemicals by producing either scleroderma-related anti-fibrillarin antibodies or lupus-related anti-ribosomal P antibodies, depending on which chemical they are exposed to. This is consistent with observations in humans indicating that monozygotic twins and other relatives of a SLE patient may produce different kinds of autoantibodies or even develop different autoimmune diseases [36].

In B6 mice, a defect in Fas-mediated apoptosis due to mutation in Fas (B6/*lpr*) or Fas-ligand (B6/*gld*) leads to a lupus-like autoimmune syndrome with anti-chromatin antibodies but not anti-Sm or ribosomal P autoantibodies [33]. In contrast, pristane-treatment in B6 mice induces anti-nRNP/Sm, -Su, and -ribosomal P antibodies with little anti-chromatin or ssDNA antibodies [33]. On the CBA background, the *xid* mutation (CBA/N, *xid* mice) is associated with spontaneous production of anti-RNA helicase A autoantibodies along with the production of IL-4 and IL-6 [25]. Pristane treatment shifts cytokine balance toward Th1, inducing IFN-γ and IL-12, and suppresses anti-RHA antibodies. Pristane treatment in immunocompetent CBA/CaJ mice induces anti-nRNP/Sm, -Su, and -chromatin antibodies [25]. Mice with impaired Fas-mediated apoptosis such as B6/*lpr* and B6/*gld* mice, are resistant to pristane-induced lupus [33]. Furthermore, pristane does not enhance the spontaneous production of anti-chromatin/DNA antibodies or nephritis (MRL/*lpr* mice) in these strains whereas lupus in MRL+/+ is accelerated, suggesting that *lpr* or *gld* and pristane are antagonistic in the induction of lupus (A. Mizutani et al., submitted). NZB/W F1 mice also spontaneously produce anti-RHA antibodies. Pristane-treatment shifts the cytokine balance toward Th1, inhibiting anti-RHA production while inducing anti-nRNP/Sm and -Su antibodies [34]. These results suggest that a single genetic factor (*lpr*, *xid*) or environmental factor (pristane) can induce distinctive subsets of autoantibodies in the mice with same genetic background (e.g. B6, CBA), consistent with the possibility that there may be several different pathways to the development of SLE.

3. Induction of lupus-related autoantibodies by adjuvant oils

In our recent reports, the autoimmune responses induced by two adjuvant oils (IFA, squalene) and medicinal mineral oils were compared with those induced by pristane [17,18]. Three-month-old female BALB/cJ mice received a single i.p. injection (0.5 ml) of either pristane, squalene, IFA, medicinal mineral oils (MO-F, MO-HT, MO-S from drug stores and supermarkets) or phosphate buffered saline. Additional age-matched untreated mice were also used as control.

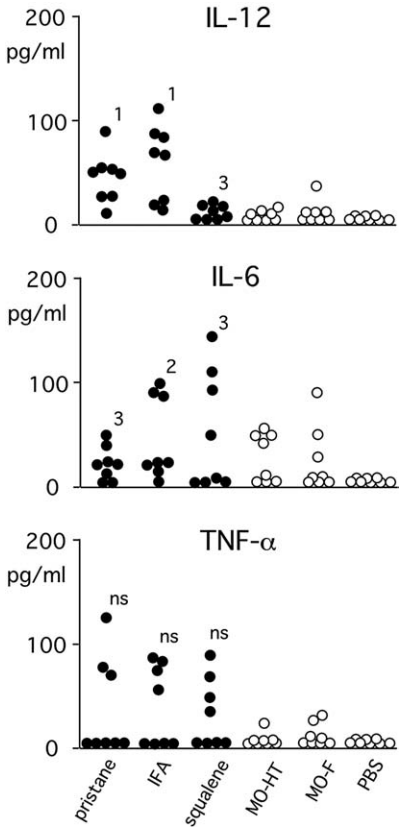

Fig. 1. IL-12, IL-6, and TNFα levels in peritoneal lavage. IL-12, IL-6, and TNFα levels in peritoneal lavage from BALB/cJ mice 2 weeks after treatment with hydrocarbon oils or PBS were measured by ELISA. [1] $P < 0.0005$ vs. PBS group, [2] $P < 0.001$ vs. PBS group, [3] $P < 0.05$ vs. PBS group (Mann–Whitney test) ns, not significant.

3.1. Cytokines

Intraperitoneal injection of adjuvant oils is followed by the influx of inflammatory cells such as macrophages, T-cells, and B-cells and ultimately granuloma formation in peritoneal cavity [37]. In vivo cytokine production measured as cytokine levels in peritoneal lavage fluid indicates that various types of hydrocarbons including adjuvant oils and medicinal mineral oils induce IL-12, IL-6, and TNF-α [17]. However, the hydrocarbons that induce lupus-related anti-nRNP/Sm and -Su antibodies (pristane, IFA, and squalene) are associated with in vivo (peritoneal lavage fluid) and in vitro (culture supernatant) production of these cytokines, in particular IL-12, at early (2 weeks–3 months) time points when autoantibodies develop [17] (Fig. 1).

3.2. Hypergammaglobulinemia

Pristane, squalene, and IFA all induced hypergammaglobulinemia of T-cell dependent isotypes IgG1, IgG2a, and IgG2b. However, IgG2a increased less dramatically in

squalene or IFA-treated mice than in pristane-treated mice. IgG1 was increased predominantly in the squalene group [17,18]. The IgG2a/IgG1 ratios increased markedly in pristane-treated mice and slightly in the IFA group, but not in the squalene group [17], suggesting that Th1 cytokine production was less prominent in the IFA and squalene groups than in the pristane group. Although medicinal mineral oils also induced hypergammaglobulinemia, their effects on T-cell dependent isotypes were less significant compared with those of adjuvant oils. T-cell independent isotypes IgM and IgG3 were often dominant in this group, similar to the effects of silicone oil [38].

3.3. Antinuclear and anti-cytoplasmic antibodies

Antinuclear and anti-cytoplasmic antibodies in sera from BALB/cJ mice 3 and 6 months after treatment were examined by indirect immunofluorescence [18]. Representative staining patterns by sera from mice 3 months after IFA or squalene treatment are shown in Fig. 2. Titers of antinuclear antibodies in the pristane or IFA -treated mice were higher than those in the untreated mice at 3 months ($P < 0.01$ and $P < 0.05$, respectively, Mann–Whitney test) [18]. Titers of anti-cytoplasmic antibodies were higher in pristane, squalene, or IFA -treated mice ($P < 0.01$), and to a lesser degree in the MO-F group ($P < 0.05$ vs. untreated group, Mann–Whitney test) compared with the untreated group. Although the frequency of antinuclear antibodies (1:80 or higher) was significantly higher than control only in the pristane group, anti-cytoplasmic antibodies were more frequent in pristane, squalene, IFA, and MO-F treated mice than in controls ($P < 0.01$–0.05 by Fisher exact test) at 3 months (Fig. 3) [18]. Data at 6 months were similar to those at 3 months, however, the anti-cytoplasmic antibodies, which probably reflect autoantibodies to heat shock proteins [39], appeared to be less frequent than those at

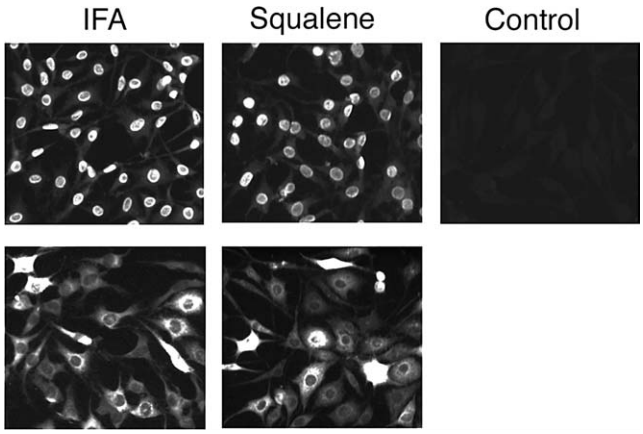

Fig. 2. Antinuclear and anti-cytoplasmic antibodies by immunofluorescence. L929 cells (mouse fibroblast) stained with serum from IFA or squalene-treated BALB/cJ mice 3 months after treatment, showing nuclear (top) and cytoplasmic (bottom) staining. Negative staining by a serum from an untreated mouse is shown (control). Serum dilution 1:40, original magnification 200×.

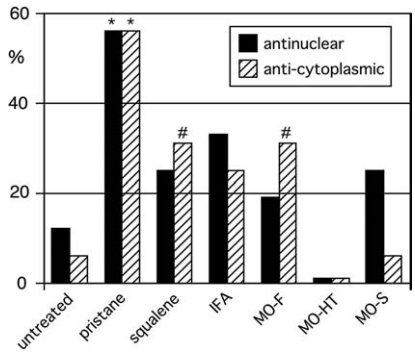

Fig. 3. Frequency of antinuclear and anti-cytoplasmic antibodies by immunofluorescence. Sera from BALB/cJ mice 6 months after treatment were examined at 1:40 dilution and the titer was estimated with titration emulation (ImageTiter, RhiGene Inc.). Frequency of antinuclear and anti-cytoplasmic antibodies (≥1:80) in each group ($n = 12$–24) is shown. * $P < 0.01$, # $P < 0.05$ (one-tailed) vs. PBS group by Mann–Whitney test.

3 months in the squalene or IFA treated group (Fig. 2). This suggests that the production of anti-cytoplasmic antibodies may be an early or less persistent response than other autoantibody responses.

3.4. Anti-nRNP/Sm and -Su antibodies

Autoantibodies in sera from BALB/c mice 6 months after treatment were examined by immunoprecipitation [17] (Figs. 4A and 5). In addition to pristane-treated mice, some IFA or squalene-treated mice, but not medicinal mineral oil treated mice, produced anti-nRNP/Sm and/or anti-Su antibodies [17] (Fig. 4A). Mice treated with pristane produced anti-nRNP/Sm (15/24, 63%) or anti-Su (13/24, 54%), and 19/24 (79%) produced at least one of these specificities ($P < 0.001$ vs. PBS group by Fisher exact test). Although less frequent than seen in the pristane group, 20% of the IFA-treated mice and 25% of squalene-treated mice ($P < 0.05$ and $P < 0.01$ vs. PBS by Fisher exact test, respectively) were either anti-nRNP/Sm or anti-Su positive, in contrast to the absence of these antibodies in mice treated with medicinal mineral oils [17] (Fig. 5). In western blotting using affinity purified U1snRNPs [40], sera from IFA-treated mice reacted with U1-70K protein (Fig. 4B). Other autoantibodies common in human lupus such as anti-ribosomal P, -Ro, or La, were not found [17]. Commercially available medicinal mineral oils (MO-H, MO-F, MO-S) did not induce these autoantibodies although some MOs induced anti-chromatin and -ssDNA antibodies more efficiently than IFA or squalene [18].

The time course of anti-nRNP/Sm autoantibody production in IFA vs. pristane-treated mice was compared using ELISA [41] (Fig. 6). Two mice started to produce anti-nRNP/Sm antibodies 2 months after IFA treatment in this group. The time of onset and levels were similar to those in pristane-treated mice [8,9]. The titers of anti-nRNP/Sm antibodies induced by IFA or squalene were as high as 1.28×10^5 by ELISA, comparable to at least some pristane-induced antibodies [17]. Two squalene-treated mice started to pro-

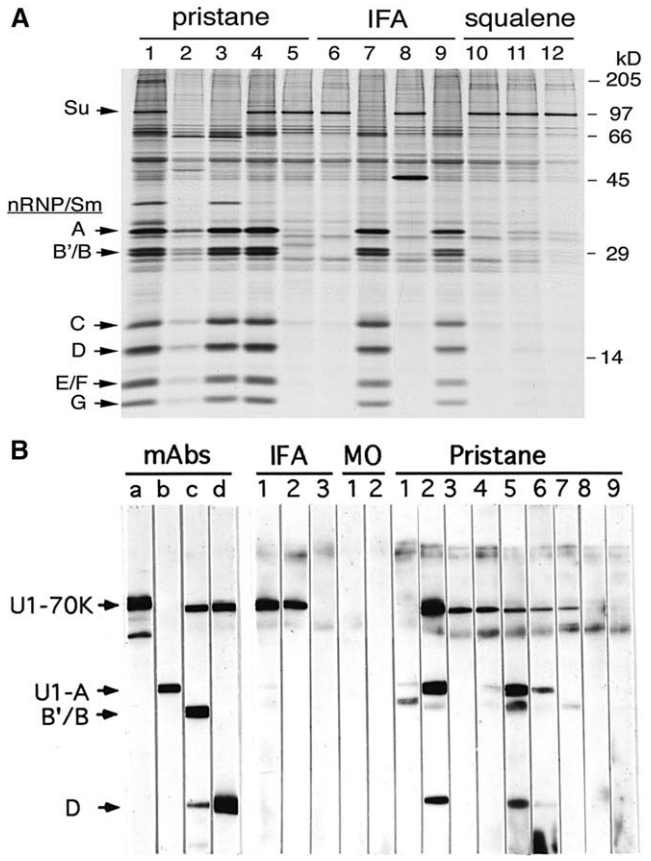

Fig. 4. Autoantibodies to nRNP/Sm and -Su in sera from IFA-treated mice. (A) Immunoprecipitation. ^{35}S-labeled K562 cell extract was immunoprecipitated with sera from BALB/cJ mice 6 months after pristane (lanes 1–5), IFA (lanes 6–9) or squalene (lanes 10–12) treatment. Lanes 1–4, 7, and 9, anti-nRNP/Sm positive sera (proteins A, B′/B, C, D, E/F, and G); lanes 1, 4–6, 8, 10–12, anti-Su positive (Su). (B) Western blotting. Purified U1 snRNPs were separated by SDS-polyacrylamide gel electrophoresis and subjected to western blot analysis using sera (1:2000 dilution) from mice 6 months after treatment with IFA (lanes 1 and 2, anti-nRNP/Sm positive; lane 3, negative), MO (MO-HT, both negative), or pristane (lanes 1–7, anti-nRNP/Sm positive; lanes 8 and 9, negative). Left panel, immunoblots using mAbs: a, 2.73 (anti-U1-70K); b, 9A9, anti-U1A; c, Y2, anti-Sm B′/B + D; d, 2G7, anti-Sm D.

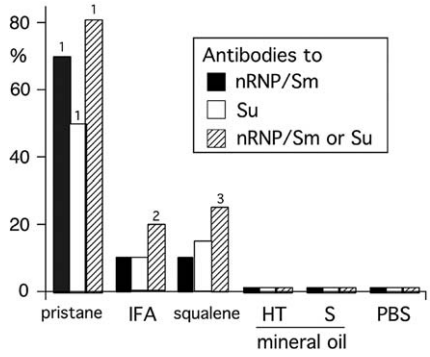

Fig. 5. Frequency of anti-nRNP/Sm and -Su antibodies by immunoprecipitation. Sera from BALB/c mice 6 months after treatment were tested by immunoprecipitation. Frequency of anti-nRNP/Sm and -Su antibodies are summarized. n = 20–28/group [1] $P < 0.001$, [2] $P < 0.05$, [3] $P < 0.01$ vs. PBS group (Fisher exact test).

duce anti-nRNP/Sm antibodies at 3 and 6 months, respectively (not shown). None of MO or PBS treated mice had anti-nRNP/Sm antibodies, in agreement with immunoprecipitation and western blotting data.

It should be emphasized that the anti-nRNP/Sm and -Su autoantibodies described in the present study are not low affinity polyreactive natural autoantibodies, which typically are detectable only by ELISA [42]. They are mainly IgG, are not detected in pristane-treated nude mice [43] and have titers comparable to those in MRL/*lpr* mice or SLE patients [17,37]. They immunoprecipitate a highly restricted group of proteins from crude cell extracts (Fig. 4A) and are reactive with particular antigens on western blotting (Fig. 4B).

3.5. IgG subclasses of anti-nRNP/Sm autoantibodies

Since IFN-γ plays a critical role in anti-nRNP/Sm antibody production [21], IgG subclasses of anti-nRNP/Sm antibodies were examined using ELISAs (Fig. 7) to see whether the IFN-γ dependent isotype IgG2a is dominant. IgG subclasses were tested in 7 sets (3 and 6 months after treatment) of sera from pristane-treated mice, two sets of IFA treated sera, and two sets of squalene treated mouse sera. All sera from the pristane-treated mice had IgG2a-predominant anti-nRNP/Sm antibodies. One set of IFA treated sera (C) also had IgG2a-predominant anti-nRNP/Sm antibodies at 3 (C-3M) and 6 months (not shown). IgG2b was dominant in the other mouse (D), especially at 6 months (D-6M). Squalene induced IgG2a anti-nRNP/Sm antibodies in both mice (E and F). However, mouse F developed high levels of IgG1 anti-nRNP/Sm antibodies between 3 and 6 months, consistent with the increased total level of the IgG1 in squalene treated mice [17,18]. Thus, although anti-nRNP/Sm antibodies usually were mainly IgG2a, other isotypes such as IgG1 and IgG2b also were produced, especially in IFA or squalene treated mice, consistent with less intense skewing towards Th1 in IFA and squalene-treated mice than pristane-treated mice.

The predominant increase in IgG1 [17,18] and the switching of anti-nRNP/Sm antibodies from IgG2a to IgG1 as the anti-nRNP/Sm antibody response develops, as seen in a squalene-treated mouse (Fig. 7 mouse F-3M, 6M), may reflect IL-6 overproduction (Fig. 1) during this period [17].

3.6. Anti-ssDNA and anti-chromatin antibodies

Levels of IgG anti-ssDNA and anti-chromatin antibodies were tested (ELISA) at 3 and 6 months after treatment [18]. Squalene or IFA did not induce IgG anti-ssDNA antibodies, in contrast to the significant induction by pristane or mineral oils MO-F or MO-S ($P < 0.01$–0.05, vs. untreated, squalene, or IFA-treated group). Although IFA induced IgG anti-chromatin antibodies, the levels were low (~1–10 units com-

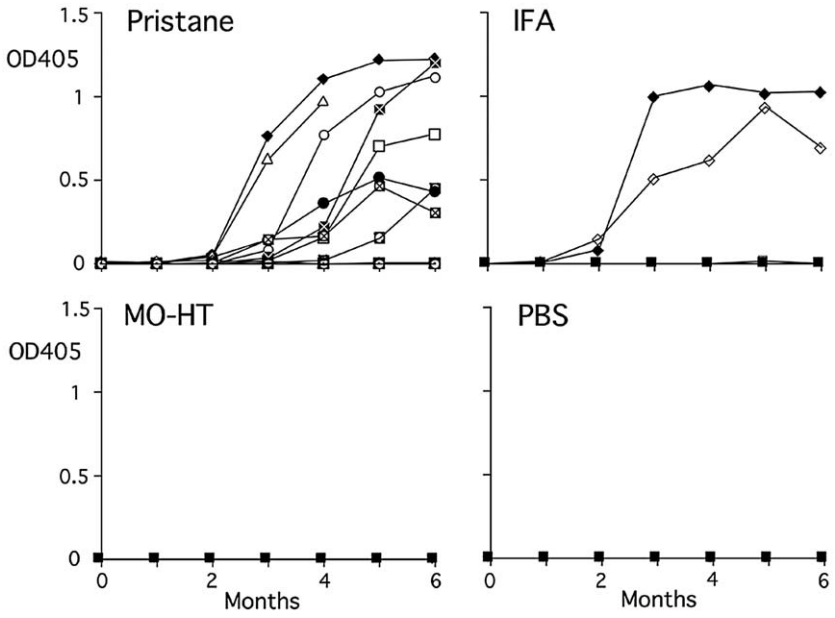

Fig. 6. Time course of anti-nRNP/Sm autoantibody production. Sera were collected monthly from mice treated with MO-HT, IFA, pristane, or PBS and tested (1:500 dilution) for IgG anti-nRNP/Sm antibodies by ELISA. Levels of these antibodies in sera from individual mice are shown as a function of time. n = 12/group.

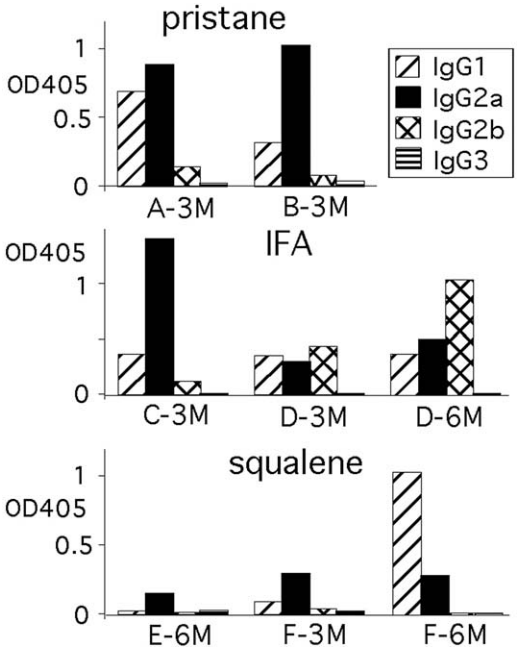

Fig. 7. IgG subclasses of anti-nRNP/Sm antibodies. IgG subclasses of anti-nRNP/Sm antibodies in sera of six mice (A–F) 3 and 6 months after treatment with pristane, IFA, or squalene were determined (ELISA). IgG2a predominated in many cases.

pared with hundreds to thousands units by MRL/lpr or NZB/W F1 mouse sera) and squalene did not induce significant levels of anti-chromatin antibodies [18]. These data suggest that different types of hydrocarbons may induce different types of autoantibodies.

Immune responses induced by pristane, adjuvant oils, medicinal mineral oils, and silicon oil are summarized in Table 1.

4. Mechanisms of action of hydrocarbon oil adjuvants

Although the precise mechanisms of action are poorly understood, adjuvants have been used for many years in human and veterinary vaccination [3,4,44]. Adjuvants also play an essential role in basic research while being called "immunologist's dirty little secret", for without them most investigations of experimental autoimmunization, would be impossible [2,45].

Several recent articles have revisited the mode of action of adjuvants in an effort to reclassify them based on their potential mechanisms of action [44,46–48]. Cox and Coulter [44] classified the modes of action of adjuvants as; (1) immunomodulation (modification of the cytokine network), (2) presentation (preserve conformational integrity and present this to immune effector cells), (3) CTL induction, (4) targeting (delivery of an immunogen to immune effector cells), and (5) depot generation. IFA and squalene tested in the present study are particulate adjuvants, which exist as microscopic particles and owe at least some of their adjuvant activity to this property. These water-in oil emulsions mainly act via immunomodulation and depot effects [44].

The effects of IFA are somewhat confusing and appear to have different effects depending on the systems used. Immunization with an autoantigen in IFA can induce tolerance while the same antigen in CFA induces autoimmune disease [49]. While IFA, pristane, squalene, or hexadecane itself induces chronic arthritis [12,50], they prevent CFA-induced adjuvant arthritis [51]. Injections of antigens with IFA were thought to be prone to induce tolerance because IFA serves as excellent antigen depot for months, providing the first signal without bacterial products that stimulate signal 2. However, other researchers believe that there is abundant signal 2 in

Table 1
Immunological effects of hydrocarbons

	Polyclonal		Autoantibodies		
	IgM, IgG3	IgG1, IgG2a, IgG2b	IgM anti-ssDNA	IgG anti-nRNP/Sm, Su	IgG anti-chromatin/DNA
Pristane	+	++	+	+++	+
Squalene	+	+	+	+	–
IFA	+	+	+	+	+
Medicinal mineral oil	++	+–	+	–	+–/+
Silicone oil	++	+–	+	–	+–

lymphoid system and propose that the dose and duration of antigen exposure determine the fate of immune response [52]. This issue is controversial and different type of Th response, Th1 by CFA vs. Th2 by IFA, may be more critical in the induction of immune responses [1]. Nevertheless, IFA is an effective vaccine adjuvant by itself [3] and induces autoimmune chronic arthritis [16,50] and lupus-related autoantibodies as shown in this paper.

A recent study using squalene-based adjuvant MF59 emphasized an importance of macrophage trafficking and apoptosis and suggested that dendritic cells acquire antigen and adjuvant by uptake of the apoptotic macrophages [53]. Another study showed that adjuvant enhanced survival of bone marrow-derived macrophages and even induced DNA synthesis [54]. Recent advances in understanding the biological functions and ligands of toll-like receptors (TLR) revealed that many agents considered as adjuvants induce cytokine production via interactions with TLRs [55]; monophosphoryl lipid A (MLA) and other lipid A mimetics via TLR4, poly I:C via TLR3, and CpG DNA via TLR9. Stimulation of different TLRs lead to dendritic cell maturation and induction of distinct Th responses [55].

5. Use of hydrocarbon oil adjuvant in vaccines

Squalene-based MF59 and alum are currently the only adjuvants used in FDA-approved commercial vaccines [5]. IFA is an effective and relatively safe adjuvant and was used extensively in the past for influenza, poliomyelitis, and other vaccines [3,4]. The side effects appeared to be minimal and only local side effects such as cysts or abscess formation were confirmed. Association with autoimmune diseases or cancer has not been confirmed epidemiologically [3,56]. However, the risk/benefit ratio was still considered high and IFA is not currently used for commercial vaccines in healthy individuals [4]. Nevertheless, IFA still has been actively used in development of vaccines for patients with aggressive diseases such as HIV, melanoma, human papillomavirus positive gynecological neoplasia, and multiple sclerosis [57]. Squalene is a component of MF59 and other new adjuvants that have been used as adjuvants in influenza, HBV, HSV, HIV, and CMV vaccines [4,6]. Both of these have been widely used in veterinary vaccines as well.

6. Vaccination and autoimmunity

Regarding the effects of vaccination on autoimmunity, there are two aspects to be considered: the induction de novo of autoimmune disease, and the exacerbation of existing autoimmune disorders [58].

6.1. Vaccine-induced autoimmunity in animals

Various types of autoimmune diseases such as autoimmune hemolytic anemia, immune thrombocytopenia, and the immune-mediated polyarthritis syndrome, which usually occurs between 30 and 45 days after vaccination, have been reported in veterinary vaccination [59]. However, only a few studies had an appropriate control or yielded statistically significant results. There are few studies specifically examining autoimmune responses after vaccination. In one, development of autoantibodies to laminin and fibronectin in the dog following vaccination, possibly due to contamination of bovine serum proteins in a vaccine preparation, has been described [60].

6.2. Vaccine-induced autoimmunity in humans

Local side effects of oil adjuvants include reactions at injection site characterized by acute or subacute tissue damage, or later granulomatous reaction. Diverse autoimmune diseases such as autoimmune type I diabetes, multiple sclerosis, and Guillain-Barre syndrome following vaccination have been reported, though this has generated much controversy [61–63].

Older et al. [64] reviewed cases of post-immunization systemic rheumatic diseases, including SLE, rheumatoid arthritis (RA), dermatomyositis, polyarteritis nodosa, and Reiter's syndrome. The common characteristics found were (1) the development of symptoms approximately 1–3 weeks of secondary immunization when the boosted immune response is at its peak, (2) except for the limited association of tetanus toxoid and hepatitis B with RA, the vaccine type and class (live or killed) bear no correlation with the resultant rheumatic disease, (3) involvement of multiple or combination vaccines as well as single agent vaccines are involved [64]. There are other case reports and retrospective studies on RA, SLE and other rheumatic disorders that developed after hepatitis B vaccination [65,66]. Although, no evidence supporting

the association of hepatitis B vaccination and SLE was found epidemiologically in one study [67], additional studies will be required.

A rare long-term mortality follow-up study of army recruits who received influenza virus vaccine with IFA showed no evidence of increased collagen or allergic diseases [56]. Despite numerous case reports on vaccination induced autoimmunity, most epidemiological studies failed to confirm the association and the risk appears to be extremely low or non-existent [63]. Nevertheless, the possibility that certain vaccines can induce or exacerbate autoimmunity, at least in a few susceptible patients, has not been completely ruled out [62].

6.3. Vaccination in patients with lupus

As in vaccination of healthy individuals, both microbial products and adjuvants may have effects on autoimmune diseases. Human and murine lupus are heterogeneous syndromes affected by both genetic and environmental factors. In animal models, microbial products such as LPS accelerate lupus in NZB/W F1 and MRL/*lpr* mice [68]. The adjuvant oil pristane accelerates lupus in NZB/W F1 [34], MRL+/+ (A. Mizutani et al., submitted), and BXSB (H. Yoshida et al., unpublished) mice, but not in MRL/*lpr* mice, suggesting that it can trigger lupus in genetically susceptible host or exacerbate established lupus.

Immunization of SLE patients with pneumococcal polysaccharide, influenza, recombinant hepatitis B, Haemophilus influenza type B (HIB), tetanus toxoid, and other vaccines has been well tolerated in general [64,69]. In a few controlled studies on flare of lupus after vaccination in human, they concluded that there is no difference in flare-up between vaccinated group vs. control [70,71]. One study on poliomyelitis vaccine claimed that lupus flare was more common in the vaccinated group [72], however, this may reflect an unusually low frequency of flare in the control group (no flares in 37 controls for 3-month period) in this study. Some studied the effects of vaccination on activity of SLE based on short-term (1–3 months) observation [73,74]. Although there is no information regarding the duration of acceptable observation period, 1–3 months may not be long enough for the purpose, considering that it takes 2–6 months for adjuvant oils to induce lupus autoantibodies in mice [8,9,34] and that the oil-induced granulomatous inflammation can last for years.

6.4. Autoimmunity induced by vaccines and adjuvant oils

An important factor to consider in vaccine-induced autoimmunity is the fact that vaccines contain a microbial component (or other type of antigens) and adjuvant [75]. Differentiating adverse reactions caused by these two factors is often difficult, or it can even be a result of the combination of both. Nevertheless, the microbial components are generally considered responsible for adverse reactions and minimum attention has been paid to the potential effects of the adjuvant component. Molecular mimicry of a microbial antigen in a vaccine and a host tissue self-antigen is often considered important [61]. Immune complexes also may be formed following vaccination [61,76], deposit in vascular endothelium and induce vasculitis. Induction of cytokines or shifting cytokine balance may also play an important role. Predisposing environmental factors such as the dose and time of vaccination, the age, concurring infections or latent ongoing autoimmune disease, are also likely to be critical factors [62]. Like idiopathic autoimmune diseases, genetic factors also may be important for certain syndrome.

Although adjuvant effects are not usually considered of primary importance for vaccine-induced autoimmunity, it has been pointed out that the adjuvants, not the microbial components of the vaccine, might be responsible for autoimmune phenomena [61]. Adjuvant induces polyclonal activation of B-cells and increased production of natural autoantibodies or preexisting autoantibodies [77]. However, the lupus autoantibodies reported here are clearly not the low affinity natural autoantibodies suggested by this mechanism (see Section 3.5). Even in the absence of antigens, IFA or squalene can induce autoimmunity in animals, as illustrated by the development of autoimmune hepatitis in mice injected with IFA [78] or induction of chronic autoimmune arthritis by IFA or squalene in rodents [14,16].

The possibility that cytokine shifts toward Th2 might be responsible for the mysterious Gulf war veterans' syndrome has been considered [79]. Some studies focused on possible immunotoxicological effects of squalene adjuvants and described the presence of antibodies to squalene among individuals who received anthrax vaccine, but this is quite controversial [80].

7. Mechanisms of adjuvant oil-induced lupus autoantibodies in our model

Both IFA and medicinal mineral oils are heterogeneous mixtures of various hydrocarbons [18]. There are several possible explanations for the induction of autoantibodies to nRNP/Sm and -Su by IFA but not mineral oils [17]. It is possible that the emulsifier added to IFA, Arlacel A, enhances the immune response [50] and contributes to the induction of autoimmunity. However, the induction of the same set of autoantibodies by highly purified pristane, squalene [17], and hexadecane (Y. Kuroda, manuscript in preparation), which contain no Arlacel A, argues strongly against this possibility. Another possibility is that the amount of pristane in the oil is the critical factor. IFA contains 9–67-fold more pristane than the medicinal oils, but total pristane content of the former was only 0.17% [18]. Although it is possible that trace amounts of pristane are sufficient to induce lupus-related autoantibodies, a more likely explanation is that there are many components in mineral oil that can induce lupus-related autoantibodies. Induction of lupus-related autoanti-

bodies by at least three pure hydrocarbon oils, pristane, squalene, and hexadecane (Y. Kuroda, manuscript in preparation) supports this possibility [17,18].

What biochemical characteristic is associated with the ability to induce IgG anti-nRNP/Sm and -Su autoantibodies is an important question. It is possible that the ability to induce lupus autoantibodies correlates with adjuvanticity of the oil. Intermediate length hydrocarbons (C15–C20) are shown to have high adjuvant activity [14]. IFA consists mainly of more inflammatory C15–C25 hydrocarbons compared with C25–C35 hydrocarbons in MOs [18]. The long average chain length of MOs may explain the absence of anti-nRNP/Sm and Su autoantibodies in mice treated with MOs compared with pristane or IFA. However, another active adjuvant hydrocarbon, squalene, is a C30 hydrocarbon [6] yet retains the capacity to promote autoimmunity. Therefore, it is likely that other physical characteristics such as viscosity, density, carbon type (i.e. cyclo-, iso-, or straight carbon), or sulfur content [81] could influence different biological activities. The identification of factors influencing the potency of different mineral oil preparations in the induction of lupus autoantibodies may provide important clues to identify safe adjuvants.

A single i.p. injection of hydrocarbon oils has a prolonged effect on cytokine production by peritoneal antigen-presenting cells (APCs) [17]. In particular, production of IL-6, IL-12, and TNF-α, at early time points (up to 2–3 months after injection) in vivo (peritoneal lavage) and in vitro (culture supernatant) appears to be associated with the ability of oils to induce autoantibodies to nRNP/Sm and -Su [17]. The importance of IL-6, IL-12, and IFN-γ in pristane-induced autoantibodies was confirmed from experiments using cytokine knockout mice [21,22,26]. Cytokine transgenic mice that over-express IL-4, IL-6, or IFN-γ, all spontaneously produce antinuclear antibodies [82]. Induction of ANA also has been reported in patients treated with interferon-α or anti-TNF-α antibodies [83]. These data suggest that a simple overproduction of particular cytokine or shifting cytokine balance may be enough to induce some type of autoimmunity.

Although most studies on lupus focus on T-cell or B-cell abnormalities, APC abnormalities also have been reported [84] and primary macrophage abnormalities can induce a lupus-like syndrome [85]. APCs play a critical role in polarization of Th1 vs. Th2 immune response via production of cytokines such as IL-6, IL-10, IL-12, and TNF-α, and other mechanisms [86]. Among them, the levels of IL-12 p70 produced by APCs are of major importance [86]. Spontaneous proliferation of macrophages from mice treated with pristane and other hydrocarbon oils and survival for prolonged periods in vitro without stimulation (M. Satoh et al., unpublished) may be an important feature for autoimmunity. Aberrant APC functions such as impaired phagocytosis, apoptosis, proliferation, or overproduction of IL-12 and other cytokines by activated APCs stimulated by oil adjuvant, may be a primary pathogenic mechanism in hydrocarbon oil-induced lupus.

Recent studies indicated that many agents considered as adjuvants, are ligands of TLRs and induce cytokine production via TLRs [55]. In pristane-induced lupus, autoantibody production was diminished in TLR4 mutant C3H/HeJ mice [27]. However, it is not known whether the immunological effects of pristane, IFA or squalene directly involve TLRs. Further studies on the role of TLRs in adjuvant oil-induced autoimmunity are under way in our laboratory.

Injection of adjuvant oil induces inflammation and apoptosis in tissues [53]. Therefore, what is happening in hydrocarbon oil injected mice can be similar to immunizing animals with apoptotic cells [87]. Selection of a highly restricted set of antigens as targets of autoimmunity due to non-specific inflammation is another question that needs to be addressed. Induction of anti-nRNP/Sm and -ribosomal P, specificities found in pristane-treated mice, by immunization of whole apoptotic cells has been reported [87]. Modification of certain self-antigens [88] that occur during adjuvant-induced inflammation and apoptosis, either as direct or indirect effects of hydrocarbons, along with genetic factors may determine the autoantibody specificity.

8. Human exposure to mineral hydrocarbons

Humans are exposed to various types of hydrocarbons by various routes including, oral (dietary, medicine), inhalation (air pollution, oil mist in work environment), and cutaneous (cosmetics, contact with mineral oil in work environment) [81,89,90]. Excellent reviews on dietary exposure to mineral hydrocarbons from food-use applications are available [91,92]. Food contamination can be via a direct route such as coatings of food products (cheese, fruits, vegetables), grains de-dusting, confectionary, chewing-gum base, and baking applications (divider oils, pan-release oils). Mineral hydrocarbons can also contaminate food indirectly via corrugated cartons, polystyrene, adhesives, and food-grade lubricants. The sum of these is estimated 1–2 mg/kg/day (20–50 g/year) [92]. In addition to hydrocarbons found in foods, many individual takes pure hydrocarbon oil (medicinal mineral oil) available at drug stores and supermarkets as an intestinal lubricant [93]. The standard dose is as much as 15–30 g/day. It is well known that ingested hydrocarbons are absorbed and cause lipogranulomas (follicular lipidosis) seen in the liver, spleen, and lymph nodes of healthy individuals in industrial countries [94]. In addition to dietary exposure, oil mist in the work place, diesel exhaust, gasoline, jet fuel or other petroleum products, are also common. In particular, health concerns related to inhalation and skin exposure to jet fuels are drawing considerable attention recently [90].

Although the risk of vaccination with hydrocarbon adjuvant in humans appears to be low, at least with the small quantities of oil administered in vaccines [56], accidental or deliberate human inoculation of mineral oil is associated with severe local and systemic effects [95]. It has been suggested that individuals injected with mineral or paraffin

oil for cosmetic purposes are at increased risk of developing systemic autoimmunity [96,97], an idea consistent with the present data in mice. A possible association between hydrocarbon exposure and glomerulonephritis and Goodpasture's syndrome also has been suggested [89].

Humans are heavily exposed daily to various types of hydrocarbons, many of which may have adjuvant activity and the ability to induce lupus-related autoantibodies. Evaluating hydrocarbon exposure in each individual is not easy but an appropriate epidemiological study may help to define the pathological significance of hydrocarbon exposure.

9. Significance of adjuvant oil-induced autoimmunity

Although less than the frequency observed in pristane-treated mice, the induction of lupus-related autoantibodies to nRNP/Sm and -Su in ~25% of IFA or squalene-treated mice is significant by itself, considering the fact that these substances are used as adjuvants in human and veterinary medicine [4,6], as well as in basic immunology research [2].

Induction of autoimmunity by squalene is of particular interest since it is an endogenous lipid abundant in serum and a normal precursor of cholesterol and steroid hormones [6]. Squalene has been used as a dietary supplement and is found in cosmetics in Asian countries despite reported cases of lipoid pneumonia due to its aspiration [98]. Although generally considered to be inert, endogenous lipids such as squalene or intestinal adipose tissue have strong adjuvant effects in the rat arthritis model [99]. The present study clearly showed that the endogenous lipid squalene could induce lupus autoantibodies in normal mice. Recent studies suggest that various endogenous products such as heat shock proteins can stimulate APCs via TLRs and can work as adjuvants [55]. How and under what conditions such endogenous adjuvants cause inflammation or immune stimulation needs to be addressed in future studies.

Induction of autoimmunity by adjuvants also has implications in basic research since adjuvants are essential for experimental immunization [2]. There are reports of the induction of autoantibodies to lupus antigens in animals immunized with peptides or proteins emulsified in Freund's adjuvants [100]. A common feature is the "spreading" of autoimmunity to additional epitopes not carried by the immunizing peptide. An idiotype–anti-idiotype mechanism has been postulated as the mechanism of anti-nRNP/Sm antibody production in another model of lupus in which mice are immunized with monoclonal antibodies in CFA/IFA [101]. The induction of autoantibodies by adjuvant oil itself (IFA or squalene) points out the need for caution in interpreting studies in which antigens emulsified in adjuvant oil induce the lupus-related autoantibodies such as snRNPs or chromatin.

The detection of autoantibodies suggestive of a systemic autoimmune reaction has been successful with very few compounds only, most often in non-conventional strains of mice [102]. Hydrocarbon-induced autoimmunity is the only model in which disease specific high affinity autoantibodies such as anti-Sm and -ribosomal P are induced in a wide variety of standard strains in the absence of immunizing antigens. This model should be helpful to study the mechanisms of autoimmunity and the role of environmental chemicals in lupus.

10. Immunotoxicity testing of vaccines for autoimmunity

In addition to the fact that vaccines contain microbial products and adjuvant, the main challenge in establishing a predictive safety assessment comes from the fact that vaccines act through a highly complex, multistage mechanism in which the vaccine by itself is not the final triggering component [58]. Vaccine induced antibodies or activated T-cells are the actual effectors. Considering this multi-level interaction between the organism and the vaccine, five distinct categories of toxicological effects can be identified and appropriate investigations need to be designed accordingly. (1) Direct toxicity of vaccine components, (2) toxicity linked to the pharmacodynamic activity, (3) the adverse response related to the activation of pre-existing biological processes, (4) toxicity of contaminants and impurities, (5) adverse reactions due to the interaction between the various vaccine components [58]. In the past, induction of autoimmunity by vaccines has been hypothesized to relate mainly or exclusively to the microbial component. However, our data suggest that the direct or indirect effects of adjuvant need to be carefully evaluated in vaccine development.

Animal models remain the best option for mimicking the human situation in toxicological studies, however, a critical challenge is the identification of the "relevant" animal models. The species specificity of the immune function is frequently mentioned as being a major obstacle to safety assessment of vaccines in animals before proceeding to trials in man [58]. Responses in animals vary according to genetic influence and extrapolation to humans is often difficult. IFA induces adjuvant arthritis in rodents but no association of IFA and arthritis was confirmed in humans. Interestingly, the species specificity of mercuric chloride's effects on the immune system illustrates the difficulty in extrapolating animal data to human [103].

Differences in the effects of vaccine components depending on the route and dose are additional factors. Aminocarb is known to have different effects with the strongest effects via i.p. injection. In contrast, mercuric chloride can induce antinucleolar autoantibodies in susceptible strains of mice when given either subcutaneously, i.p., or orally [104]. Another limitation in animals is the difficulty evaluating long-term chronic effects. Some chemicals may have long-term effects over many years, which may be difficult to evaluate in mice over 2 years.

It has been pointed out that rare but serious adverse reactions of vaccination are difficult to detect due to a lack of

statistical power [105]. Even if vaccination in humans is associated with lupus, it is not clear how long it will take and what genetic predisposing factors might be involved. A long-term observation of a large number of subjects like the one performed in army recruits who received influenza vaccine containing IFA [56], will be ideal but practically not easy.

The Environmental Disease Study Group of the American College of Rheumatology (ACR) has suggested an orderly and staged process based on current paradigms in toxicology, epidemiology, and epistemology [106]; Stage 1, proposing the association, Stage 2, testing the association, Stage 3, defining the disorder, and Stage 4, refining the disorder. Despite the numerous reports of vaccine-induced autoimmunity, evaluation of adverse reactions is a highly complex process. A combination of basic animal research and careful clinical and epidemiological studies should offer us a better understanding of the truth in vaccine-induced autoimmunity.

11. Summary and conclusion

Our data suggest that an i.p. injection of adjuvant hydrocarbon oils, a chronic, non-specific inflammatory stimulus, can trigger the production of a highly restricted subset of autoantibodies usually associated only with lupus. This may help in understanding the significance of human exposure to hydrocarbons and it also suggests that caution must be used when adjuvants are used in research studies focusing on autoimmunity. It will be of interest to examine in the future how the unremitting, non-specific, inflammation induced by hydrocarbon oils, generates such a highly restricted subset of autoantibodies associated with lupus. The answer to this question may provide insight as to why the same subset of autoantibodies is produced by lupus patients.

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