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$Al(OH)_3$-adjuvanted vaccine-induced macrophagic myofasciitis in rats is influenced by the genetic background

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Abstract

Macrophagic myofasciitis (MMF) is a specific histopathologic lesion involved in the persistence for years of aluminum hydroxide [$Al(OH)_3$] at the site of previous intramuscular (i.m.) injection. In order to study mechanisms involved persistence of MMF lesions, we set up an experimental model of MMF-lesion in Sprague–Dawley and Lewis rat, by i.m. injections of 10 μL of an $Al(OH)_3$-adjuvanted vaccine. An evaluation carried out over a 12-month period disclosed significant shrinkage of MMF lesions with time. A radioisotopic study did not show significant aluminium uptake by $Al(OH)_3$-loaded macrophages. A morphometric approach showed that Lewis rats with Th1-biased immunity had significantly smaller lesions than Sprague–Dawley rats with balanced Th1/Th2 immunity. Concluding, our results indicate that genetic determinatives of cytotoxic T-cell responses could interfere with the clearance process and condition the persistence of vaccine-induced MMF-lesions.

Keywords: Inflammatory myopathies; Macrophagic myofasciitis; Experimental model; Aluminium hydroxide; Vaccine; Adverse effects; Histopathology; Morphometry; Th1; Th2; Radioisotopic study; Sprague–Dawley rat; Lewis rat

1. Introduction

Macrophagic myofasciitis (MMF) is a recently described histopathologic lesion, mainly detected in adult patients with chronic fatigue and diffuse myalgias [1,2]. MMF consists of a pathognomonic focal epi-, peri- and endomysial infiltration of large PAS-positive and major histocompatibility complex class 1 antigen (MHC-1)-positive macrophages, intermingled with $CD8^+$T-cells, in the absence of conspicuous muscle fibre damage [1,2]. At electron microscopy, macrophages constantly enclose crystal material in their cytoplasm [1,3], representing aluminium hydroxide [$Al(OH)_3$], an immunologic adjuvant incorporated in vaccines to stimulate Th2 immune responses [2]. Analysis of patients history established that MMF assesses long-term persistence of $Al(OH)_3$ at site of previous intramuscular (i.m.) injection [2,4], time elapsed from last immunization with an $Al(OH)_3$-containing vaccine to muscle biopsy ranging from 3 months to 8 years in our series (median: 53 months) [5].

The low detection rate of MMF among vaccine receivers undergoing deltoid muscle biopsy prompted WHO to propose the working hypothesis that MMF could occur in a predisposed subset of individuals with impaired ability to clear aluminium from muscle [4]. In fact, the main difficulty relies on the lack of firm data about normal residence time of $Al(OH)_3$ in muscle tissue after i.m. injection in normal individuals [2]. Long-term follow-up of vaccinated monkeys indicates that MMF lesions are destinated to vanish, only 2/4 monkeys still displaying macrophagic infiltration 12 months after injection [6]. This experimental result notably differs from that observed in patients, in which the median persistence time of macrophagic infiltrates was

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53 months. This led us to study factors that could interfere with the clearance process.

For this purpose, we induced in rats MMF lesions by i.m. administration of a human-equivalent dose of an $Al(OH)_3$-adjuvanted vaccine. Once phagocytozed by macrophages [7–9], $Al(OH)_3$ promotes cell survival [10], and antigen presenting cell functions [11]. Such vaccine-loaded macrophages strongly express the ferritin receptor (CD71) through which extracellular aluminium can enter cells [12]. Therefore, we first investigated whether exogenous soluble aluminium from another source than vaccine could be captured by $Al(OH)_3$-loaded macrophages and eventually impede solubilization of aluminium from the crystal. Genetic determinatives of cytotoxic T-cell responses could also represent factors interfering with the clearance process. Indeed, while a large number of macrophages remain accumulated at site of $Al(OH)_3$ injection [2], an unknown proportion of aluminium-loaded antigen presenting cells migrate to the regional lymph node [7] where they likely initiate the primary immune response to the vaccine antigen. It is widely accepted that effector $CD8^+$T-cells generated in the lymph node are in charge of clearing out cells bearing the cognate antigen on MHC-1 molecules in peripheral tissues [13]. Consistently, CD8 + T-cells in MMF closely contact MHC-1-expressing macrophages accumulated in muscle and fascia [2] suggesting an effector-target interplay. To test this hypothesis, we compared the size of vaccine-induced lesions in inbred rat strains Sprague–Dawley and Lewis that differ by the intensity of cell-mediated immune responses [14].

2. Material and methods

2.1. Animals and experimental procedures

This study was conducted in accordance with the EC guidelines for animal care [Journal officiel des communautés européennes, L358, December 18, 1986]. We used 5-week-old female rats, including 50 Sprague–Dawley (SD) rats that have a naturally well-balanced immunity, and 40 Lewis (Lw) rats that predominantly develop Th1 immune responses [14]. The animals were kept at constant temperature (22 °C) and pressure (15 Pa) under a 12:12 h day/night cycle with food and water ad libitum. All experiments were carried out under general anesthesia by i.p. injection of 0.1 mL/100 g of 6% phenobarbital.

Since most cases of human MMF are caused by anti-HBV vaccines immunization was performed using a commercially available aluminium hydroxide-adjuvanted vaccine. According to Smil [15], 10 μL was considered as a human equivalent dose of vaccine since average weight of rats was about 120 g at time of injection. Rats received i.m. injection of 10 μL of either Engerix® B20 (vaccinated groups, V) or NaCl 0.9% (controls) into one *tibialis anterior* muscle. Skin was incised then i.m. injection was performed longitudinally with a needle inserted 10 mm deep into muscle.

2.2. Myopathological study

Muscle samples were conventionally processed for light microscopy, and studied as 10 μm cryosections stained by hematoxylin–eosin. Vaccine-induced MMF-like lesions, defining experimental myofasciitis (EMF), were assessed by the presence of at least one infiltrate of basophilic large cohesive macrophages [2]. Macrophages were assessed by immunohistochemical expression of ED-1 antigen [16]. Lymphoid component of EMF lesions was semi-quantitatively evaluated and expressed as lymphocyte score (LS): 0, absent; 1+, mild, 2+, moderate; 3+, marked (more lymphocytes than macrophages). In addition, a quantitative approach was performed at 2 and 12 months by counting the total lymphocyte number observed within MMF lesions. Detection of T-cells in EMF lesion was performed by immunofluorescence using monoclonal antibody to rat CD5 (MCA52R, Serotec, UK) 1/200.

2.3. Radio isotopic study

To assess whether circulating aluminium could be trapped in the MMF lesion, a single dose of 0.4 ng ^{26}Al was injected i.v. in 6 V-SD at day 30 post-injection (fully constituted EMF lesion) and 4 SD controls. Animals were sacrificed 24 and 72 h post-i.v. injection and both *tibialis anterior* muscles were removed. Quantification of the radioisotope was done in muscle tissue by accelerator mass spectroscopy (AMS), as previously described [17]. The $^{26}Al/^{27}Al$ ratio and ^{26}Al concentration were determined in each sample. The ratio of trapped ^{26}Al fractions in right and left *tibialis anterior* muscles ($AbsFr_{r/l}$) was calculated, allowing evaluating aluminium uptake in EMF lesions.

2.4. Comparative clearance of MMF lesions in two different rat strains

Rats were sacrificed at 1, 2, 3, 6 and 12 months post-injection. At each time point, 10 vaccinated (5 V-SD; 5 V-Lw) and 6 controls (3 SD; 3 Lw) had removal of both *tibialis anterior* muscles. Muscles were serially sectioned for pathologic evaluation. EMF lesions were irregular and consisted of central vaguely fusiform bulk, often accompanied by small satellite lesions. Evaluation of EMF lesions was based on size and lymphoid component of lesions. The lymphoid component was evaluated by LS determination (see supra). The size of lesions was assessed using the Cavalieri estimator procedure that allows accurate estimation of an irregularly shaped lesion volume [18]. Briefly, 10–15 equally spaced cross-sections were systematically sampled from an array of serial sections representing 1 mm of muscle, section count starting from the first one hitting the lesion. The volume of lesion (V_L) was calculated

in this arbitrarily selected length of muscle tissue. If h is the distance between each two sections (1,2,3,…n) and S_L the surface of the lesion on each of these sections measured by image analysis (KS 400.3.0, Zeiss, Germany), $V_L = h\Sigma S_L$ (15). The largest lesion surface measured in serial cross sections correlated well with the calculated V_L ($P < 0.0001$). Maximal areas were for the purpose of non-linear regression because it permitted the inclusion of an additional time-point. SPSS® 11.0 and CurveExpert® software packages (GraphPad, San Diego, CA) were used for morphometric analysis.

2.5. Statistics

Results were expressed as mean ± SD. Statistical analyses were achieved with Kruskal–Wallis non-parametric ANOVA and Mann–Whitney tests, using GraphPad InStat® 3.05 software. A P-value < 0.05 was considered significant.

3. Results

3.1. I.m. injection of $Al(OH)_3$-adjuvanted vaccine causes macrophagic myofasciitis lesions

A single i.m. vaccine injection of 10 μL was sufficient to induce an MMF-like inflammatory lesion at the injection site (Fig. 1). One month post-injection, the lesion was focal or multifocal and consisted of accumulations of large basophilic ED-1-positive macrophages, without multi-nucleated giant cell formation, in peri- or endomysium.

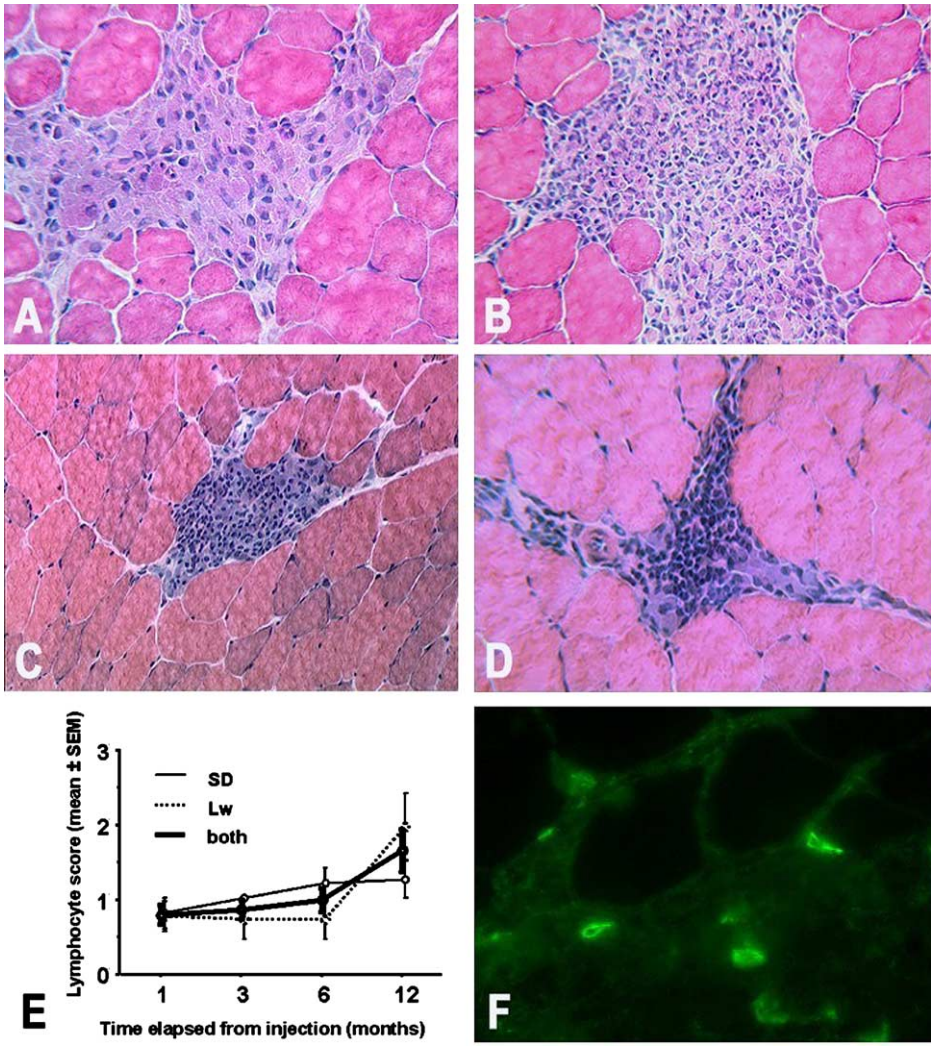

Fig. 1. Macrophagic myofasciitis (MMF) lesions in rat: myopathological study of injected *tibialis anterior* muscle (10 μm frozen sections). (A–D): MMF lesions at 3 months (A), 6 months (B), and 12 months post-injection (C, D): focal infiltrates of large cohesive macrophages within muscle tissue, with scattered lymphocytes (hematoxylin–eosin; A: ×400; B: ×250). MMF lesions at 12 months post-injection (C, D) showed conspicuous enrichment in lymphocytes (hematoxylin–eosin; ×400). (E): Semi-quantitative evaluation of lymphoid component within MMF lesions (lymphocyte score): progressive T-cell enrichment of MMF lesions between month 1 and 12 ($P < 0.05$). (F): Immunofluorescence: CD5-positive T-cells in MMF lesion intermingled with macrophages (×630).

Myofiber alterations were minimal, and consisted of rounded atrophic fibers smothered by endomysial infiltrates (Fig. 1A–D), and focal myopathic changes along the needle path. Lymphocyte infiltrates, hardly detected at 1 month post-injection, became more conspicuous with time (Fig. 1C–E). Lymphocytes expressed the rat T-cell marker CD5 (Fig. 1F). They were intermingled with macrophages and occasionally formed perivascular cuffs.

3.2. Exogenous soluble aluminium is not trapped by $Al(OH)_3$-loaded macrophages

Accelerator mass spectroscopy showed that after a single dose of 0.4 ng ^{26}Al injected i.v. ^{26}Al uptake by *tibialis anterior* muscle was similar in MMF-bearing V-SD rats and in controls injected with NaCl (NS) (Table 1). Moreover, at the individual level, ^{26}Al uptake by the vaccine-injected muscle was similar to that of the contra-lateral muscle (Table 1). This result did not support the hypothesis of a significant circulating aluminium uptake in MMF lesions.

3.3. Macrophagic infiltrates shrink with time

Stereotyped lesions were observed 1 month (5/5), 2 months (5/5), 3 months (4/5), 6 months (5/5) and 12 months (5/5) after injection in SD rats (Fig. 1A–D). All lesions exceeded 1 mm in length at serial sectioning. Progressive shrinkage of lesions occurred from month 1 to 12, as assessed by both calculation of V_L and measurement of the largest lesion cross section (Fig. 2A). The mean lesion volume dramatically decreased from month 1 to 6 (shrinkage by 43%) and decreased at a lower rate up to month 12. The largest lesion of cross section estimated at four time points allowed us to draw a curve responding to the following formula: $y = 0.97 + 0.795/x$, according to Motulsky and Christopopoulos [19] (Fig. 2A). Shrinkage of lesions was associated with progressive T-cell enrichment, the lymphocyte score increasing from month 1 to 12, the difference being significant between month 1 and 12 ($P < 0.05$) (Fig. 1E). Moreover, the total number of lymphocyte within MMF lesions was found significantly higher at month 12 than at month 2 (mean ± SD per section: 18.8 ± 6.8 at month 2, 42.6 ± 9.5 at month 12).

Table 1
Accelerator mass spectroscopy: uptake of ^{26}Al by MMF tissue

	$AbsFr_{r/l}$	
24 h post-i.v. injection		
Controls ($n=2$)	0.84 ± 0.08	
Vaccinated ($n=3$)	1.30 ± 0.48	NS
72 h post-i.v. injection		
Controls ($n=2$)	0.69 ± 0.2	
Vaccinated ($n=3$)	1.04 ± 0.28	NS

$AbsFr_{r/l}$: ratio of ^{26}Al fractions in right and left *tibialis anterior* muscles.

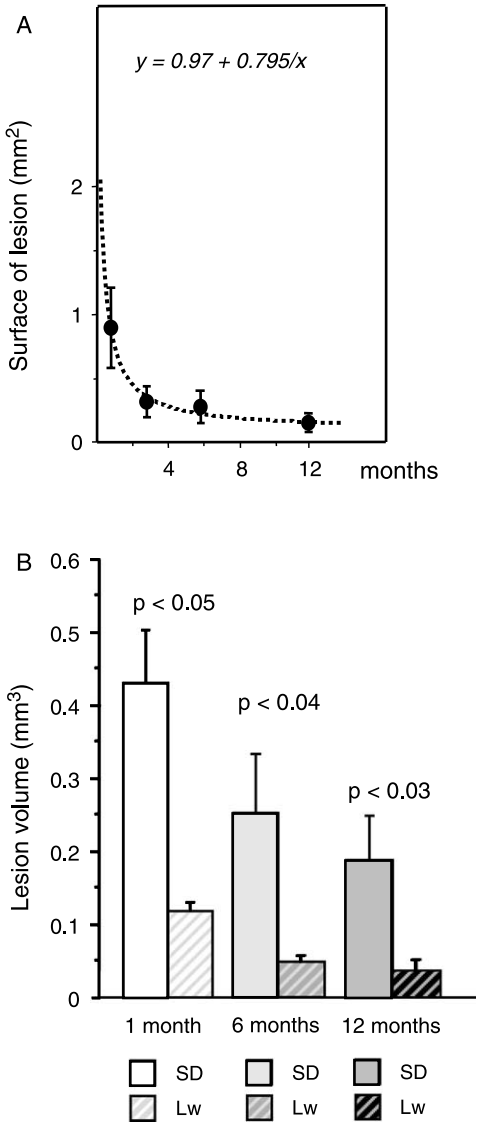

Fig. 2. (A): Modelling of MMF-lesion decrease in SD-rats extrapolated from the largest lesion of cross section estimated at four time points: curve responding to the formula $y = 0.97 + 0.795/x$. (B): Variation of MMF lesions volume (Cavalieri estimator procedure) according to time elapsed from injection (1, 6 and 12 months): difference between SD and Lw rats.

3.4. The clearance process depends on genetic background

As compared to V-SD rats, V-Lw rats showed a similar rate of detection of muscle lesions after serial sections (month 1 post-vaccination, 5/5; month 2, 5/5; month 3, 4/5; month 6, 4/5; month 12, 4/5). In contrast, volume of lesions was significantly lower in V-Lw than in V-SD, as observed in all animals and at all time points: month 1 ($P < 0.05$), month 6 ($P < 0.04$) and month 12 ($P < 0.03$) (Fig. 2B). The lymphocyte score increase from month 6 to month 12, and was milder at month 12 in V-SD than in V-Lw rats (Fig. 1E).

4. Discussion

In this study, we showed that a single 10 μL i.m. injection of $Al(OH)_3$-adjuvanted vaccine to rats was sufficient to induce focal macrophage accumulation forming an MMF-like lesion, undergoing progressive shrinkage and T-cell enrichment, still detectable 12 months after injection, the clearance of which depending on the genetic background.

As assessed by PAS-staining and electron microscopy, $Al(OH)_3$ was not found in extracellular location in humans or animals with MMF. This is discrepant with the classical view based on in vitro dissolution experiments that $Al(OH)_3$ injected into muscle forms extracellular deposits, that are efficiently solubilized by alpha-hydroxycarboxylic acids present in interstitial fluid (citric acid, lactic acid, and malic acid) [20,21]. In line with this old concept, the most recent review on elimination of aluminium adjuvants states that aluminium may be progressively released in a soluble form from $Al(OH)_3$ extracellular crystals, redistributed to different tissues, and gradually secreted into urine but does not take into consideration that a proportion of injected $Al(OH)_3$ is phagocytosed by macrophages [22]. The present study suggests that intracellular location of $Al(OH)_3$ is associated with long-term persistence of the compound at site of injection. Our experiments conducted in rats provided results similar to those found in monkeys [6] and could not substantiate rapid clearance of $Al(OH)_3$-induced muscle lesions.

Although, the lesion persistence endpoint was not reached in a majority of cases, lesion size reduction was significant after one month and lesions appeared markedly shrunk at later stages. This contrasts with the human situation where plump MMF lesions may be observed many years after immunization, suggesting implication of environmental or individual factors of impaired clearance of $Al(OH)_3$ from muscle. On the one hand, the present study did not support a major role of additional sources of exogenous aluminium, since i.v. administration of ^{26}Al was not associated with significant uptake by MMF-lesions. It must be emphasized, however, that this result observed after a single shot experiment does not preclude progressive uptake of soluble aluminium occurring at a low rate in the setting of a chronic intake of alimentary, cosmetic or drug-derived aluminium.

On the other hand, our study supports the hypothesis that the persistence time of MMF lesions depends on genetic factors influencing the immune responses. Indeed, shrinkage of MMF lesions was associated with progressive T-cell enrichment and differed markedly according to the rat strain. Lewis and Sprague–Dawley rats differed in their reaction to the vaccine as Lw rats with Th1-biased immunity had significantly smaller MMF lesions, than SD rats with balanced Th1/Th2 immunity. The striking difference between lesions size in the two rat strains could not be attributed to alimentary aluminium since the two rat strains had identical food and beverage regimens. This observation substantiates the view that interindividual differences may exist in clearance of aluminium from muscle, and that such differences may relate to the immune system, as previously proposed in humans [23]. It seems plausible that specific effector cytotoxic T-cell responses elicited by antigens presented by $Al(OH)_3$-loaded antigen presenting cells in lymph nodes may destroy $Al(OH)_3$-loaded macrophages, re-exposing $Al(OH)_3$ crystals to solubilizing acids of the interstitial fluid leading to progressive elimination of MMF lesions. Interestingly, human MMF is mainly observed in middle aged or elderly individuals, a time when immunosenescence occurs, i.e. when Th1 responses decline in favour of Th2 responses [24]. Aluminium hydroxide is a strong Th2 adjuvant [25], and it seems likely that its long-lasting persistence in immune cells of individuals with weak Th1 responses increases the imbalance of immunity in favour of Th2 function and may contribute to the emergence of a Th2 disease, such as chronic fatigue syndrome (CFS) [26]. Accordingly, MMF patients usually complain of fatigue, myalgias, and mild cognitive alterations, the symptom complex meeting international criteria for CFS in most cases [27]. A case-control study controlled by two French governmental agencies and conducted on patients identified before recognition of the role of vaccines, confirmed that chronic fatigue, associated or not with myalgias, is more frequent and more severe in patients with MMF than in diseased controls without MMF at deltoid muscle biopsy [28]. In addition, chronic low-level stimulation of the immune system, a situation generally thought to underlie CFS [29–32], was reported in MMF patients [33].

Concluding, the present paper provides experimental evidence that $Al(OH)_3$ persists over long periods of time in macrophages at site of previous i.m. injection. Consequently, the short post-exposure period during which symptoms may be ascribed to a drug adverse reaction is likely inappropriate in the setting of vaccines adjuvanted with this compound. The present study also indicates that genetically determined factors involving immune responses could condition the persistence of vaccine-induced MMF-lesions. At present, it would be judicious to dissect more deeply the mechanisms involved in the clearance of MMF lesions and to evaluate whether MMF patients display distinctive immunologic features.

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