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Somatoform and neurocognitive syndromes after HPV immunization are not associated to cell-mediated hypersensitivity to aluminum

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ABSTRACT

Vaccines against human papilloma virus (HPV) have been demonstrated to be very effective to prevent infection-related neoplasms. However, several reports describing heterogeneous post-vaccination phenomena have been published in last few years. The spectrum of these disorders includes both immune-mediated neurological diseases and neuropsychiatric functional disorders. Some researchers speculated about a genetic predisposition, but others hypothesized a role of adjuvants, including some metals and, particularly, aluminum. Here, we tested sixteen young girls developing somatoform and neurocognitive syndromes after the HPV immunization, through MELISA® test, detecting cell-mediated hypersensitivity to several metals. We found no association between these neurocognitive disorders and the results provided by this test; importantly, no patients showed hypersensitivity to aluminum, which is the inorganic adjuvant included in HPV vaccines. Thus, if aluminum played a role in the pathophysiology of musculoskeletal and neurocognitive disturbances occurring in some young girls after HPV immunization, that should recognize other mechanisms than the activation of aluminum-specific lymphocytes.

1. Introduction

Vaccines have been recognized as the most important tool to prevent many infectious diseases associated to high rates of lethality and/or irreversible clinical consequences until the recent past (Siegrist, 2013). Recently, some vaccines have been showed to be able to protect also against the occurrence of specific cancers, by preventing the viral infections involved in the cell transformation process. The first example was provided by vaccines against hepatitis B virus (HBV), which markedly reduced the incidence of liver cancer, in addition to preventing its chronic infection. This vaccine is currently prepared by the DNA recombinant technology: basically, it was developed by inserting the HBsAg genes into yeast cells and the recombinant antigens were added to a formulation including aluminum phosphate or aluminum hydroxide, as adjuvants. Similarly, vaccines against human papillomavirus (HPV) were produced in order to prevent this sexually transmitted infection promoting the occurrence of cervical cancer in women (Shiller and Lowy, 2014). Indeed, HPV-related genital skin and mucosal lesions have a self-limited course in most cases, but the persistence of the genome of some HPV strains has been showed to be significantly associated to the development of cancer: several non-structural proteins of high-risk HPV serotypes (such as HPV-16 and -18) can interfere with replication mechanisms of infected cells leading to cancer promotion (Malik et al., 2014). Even if a 9-valent HPV-vaccine (Gardasil-9®) has been recently licensed, the Italian vaccination program provided the following HPV vaccines until 2015 (Table 1): i) a quadrivalent vaccine, covering serotypes 6, 11, 16 and 18 (Gardasil®); ii) a bivalent vaccine directed against high-risk serotypes 16 and 18 (Cervarix®). Neither vaccine contains any preservative and differences are mainly related to the cell system used to produce the antigenic proteins included in the vaccine: Gardasil® is produced in cells of *Saccharomyces cerevisiae*, whereas Cervarix® is obtained through L1-recombinant baculovirus-infected insect (*Spodoptera frugiperda* SF9, *Trichoplusia ni* Hi 5) cells. Moreover, the former contains aluminum phosphate (225 μg) as an adjuvant and the latter uses AS04 adjuvant system, which comprises an aluminum salt (aluminum hydroxide, 500 μg) and 50 μg of the immunostimulatory molecule 3-O-desacyl-4′-monophosphoryl lipid A (MPL). This molecule is a detoxified form of lipopolysaccharide (LPS)

Abbreviations: HPV, human papilloma virus; HBV, hepatitis B virus; MPL, 3-O-desacyl-4′-monophosphoryl lipid A; LPS, lipopolysaccharide; Ni, nickel; Al, aluminum; Hg, inorganic mercury; Thim, thimerosal; MeHg, methyl-mercury; ASIA, Autoimmune/inflammatory Syndrome Induced by Adjuvants; CRPS, complex regional pain syndrome; POTS, postural orthostatic tachycardia syndrome; CFS, chronic fatigue syndrome; $Al(OH)_3$, aluminum hydrate; MMF, macrophagic myofasciitis; MACD, MMF-Associated Cognitive Dysfunction; AD, Alzhaimer's Disease

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Table 1
General features of human papillomavirus (HPV) vaccines.

Type of HPV vaccine	Gardasil®	Cervarix®
Targeted HPV genotypes	Quadrivalent Human Papillomavirus (Types 6, 11, 16, 18) Recombinant Vaccine	Bivalent Human Papillomavirus (Types 16, 18) Recombinant Vaccine
Vaccine antigenic composition	Each dose contains 20 μg of HPV 6 L1 protein, 40 μg of HPV 11 L1 protein, 40 μg of HPV 16 L1 protein, and 20 μg of HPV 18 L1 protein	Each dose contains 20 μg of HPV 16 L1 protein, and 20 μg of HPV 18 L1 protein
Vaccine adjuvants	Each dose of the vaccine contains ***225*** μg ***of aluminum*** [***as amorphous aluminum hydroxyphosphate sulfate*** (***AAHS***) ***adjuvant***], 9.56 mg of sodium chloride, 0.78 mg of L-histidine, 50 μg of polysorbate 80, 35 μg of sodium borate, and water for injection	Each dose of the vaccine contains ***500*** μg ***of aluminum*** (***hydroxide salt***), 50 μg of 3-O-desacyl-4′ monophosphoryl lipid A, 4.4 mg of sodium chloride, 0.624 mg of Sodium dihydrogen phosphate dihydrate, and water for injection
Route of administration	Intramuscular injection	Intramuscular injection
Vaccine schedule	• 9–26 years: 0.5 ml dose at 0,2 and 6 months	• 9–14 years: two 0.5 ml doses each of 0.5 ml. The second dose given between 5 and 13 months after the first dose. • From 15 years and above: three 0.5 ml doses at 0, 1, 6 months

deriving from Salmonella Minnesota (R595), which was demonstrated to be able to activate the innate immune system, mainly binding TLR4 (Bryan, 2007).

Like most drugs, some adverse events have been reported after vaccinations. Most side effects of vaccinations are local reactions and systemic adverse events after immunization are usually febrile, mild and self-limiting illnesses; severe and/or lethal events have been reported at a very low frequency (around 1 case out of 1–2 million vaccinations) and were often related to allergy (Chen, 1999) However, some concerns among people grew up in last few years about the possibility that some vaccines, including those against HPV, may trigger autoimmune and neurological/neuropsychiatric diseases (Poddighe et al., 2014). Actually, available studies showed no increased global incidence of several immune-mediated disorders after HPV immunization, but in the current post-licensure medical literature a lot of clinical reports describing post-vaccination phenomena and/or immune-mediated reactions can be found (Wraith et al., 2003; Grimaldi-Bensouda et al., 2014). Very recently, we described a case series of 18 young girls developing severe somatoform, neurocognitive and dysautonomic syndromes after they received HPV immunization (Palmieri et al., 2017a). Some scientists proposed that some post-vaccination phenomena might be triggered by the adjuvants included in the vaccine preparation and metals, particularly aluminum, have been claimed as potential noxious agents of vaccines (Shoenfeld and Agmon-Levin, 2011; Tomljenovic and Shaw, 2012; Shaw and Tomljenovic, 2013). Here, we describe the results of tests devoted to evidence cell-mediated hypersensitivity to metals.

2. Material and methods

2.1. Patients

In 2015, eighteen young girls (aged 12–24 years; mean age: 15.2 years) were evaluated in our “second opinion medical network” because of a variable pattern of somatoform, neurological and vasomotor symptoms, as described in Table 2. After an appropriate diagnostic work-up, including blood tests, neuro-radiologic investigations, electro-physiological procedures and neurological evaluations, the patients were diagnosed as being affected with neuropathy with autonomic dysfunction. All the girls had undergone HPV immunization before the onset of symptoms, which appeared within 20 days after the vaccine dose. None of them complained neurocognitive and/or neuropsychiatric symptoms previously. In order to provide further insights on potential pathological mechanisms, 16 out of eighteen aforementioned patients accepted to receive MELISA® blood test, which is addressed to evidence the cell-mediated immunological reactivity to specific metals. All the patients received the MELISA® blood test in the period between April and June 2016, although they underwent the vaccination at a variable time (2010–2014).

2.2. MELISA® test

The MELISA® test is performed by using lymphocytes isolated from anticoagulated blood through Ficoll-Paque, as described elsewhere (Stejskal et al., 1994; Valentine-Thon and Schiwara, 2003). Briefly, after resuspension to a concentration of 1×10^6 lymphocytes/ml, 1 ml is pipetted into a 24-well cell culture plate pre-coated with metal solutions in 2 to 3 concentrations. Three negative controls (only lymphocytes in medium) and one positive control (lymphocytes in Medium containing Poke Weed Mitogen) were included in each test. After a 5-day period of incubation, a defined amount of cell suspension from each well is transferred to a new 24-well plate where the cells are pulsed with methyl-3H-thymidine and, thus, radioactivity is measured, according to a specific protocol, in order to calculate the Stimulation index (SI = cpm in test well/cpm in negative control wells), expressing the proliferative response of lymphocytes. A positive reaction was defined as SI value ≥ 3, whereas SI value between 2 and 3 indicates possible sensibilization and SI value < 2 is considered negative (Frigerio et al., 2011).

Blood samples obtained from our patients were sent to a licensed laboratory within 24 h (Medical Center SRL, 21,018 Sesto Calende [VA], Italy), where the test was performed. Patients' lymphocytes were tested for the following substances: nickel (Ni), alum (Al), inorganic mercury (Hg), thimerosal (Thim) and methyl-mercury (MeHg).

3. Results

Raw data of MELISA® tests for all patients are displayed in Table 3. Among 16 young girls receiving MELISA® test, only 6 patients showed a clearly positive lympho-proliferative response to any of the tested substances, according to validated cut-off values (SI $>$ 3): only nickel ($n = 4$), only inorganic mercury ($n = 1$) and both nickel and inorganic mercury ($n = 1$). Of these girls, 4 were immunized with Gardasil® and 2 received Cervarix®.

In addition to these six clearly positive patients, some mildly positive (SI comprised between 2 and 3) reactions were observed too: nickel ($n = 2$), inorganic mercury ($n = 3$), methyl-mercury ($n = 2$). Four of these 7 mildly positive results were observed in 4 patients showing clear positivity for another substance. On the other side, eight patients showed no high or mild positivity to any tested substance and they were vaccinated through both Cervarix® ($n = 4$) and Gardasil® ($n = 4$). Overall, no patient showed a lympho-proliferative reaction for aluminum or thimerosal.

4. Discussion

Available studies showed no overall increase of autoimmune diseases after vaccinations, except few specific cases (Wraith et al., 2003). As regards HPV vaccines specifically, post-marketing studies evidenced no increased incidence of autoimmune and/or neurological diseases

Table 2
Main clinical features of patients.

Patients	HPV vaccine	Main core symptoms	Autoimmunity
# 1	Cervarix®	Fatigue, myalgia, arthralgia	–
# 2	Gardasil®	Low-grade fever, memory loss, fatigue	–
# 3	Gardasil®	Myalgia, arthralgia, fatigue	–
# 4	Cervarix®	Muscle weakness, skin rashes, sleep disturbances, memory loss	Diabetes mellitus type I
# 5	Gardasil®	Low-grade fever, skin rashes, cognitive impairment	–
# 6	Cervarix®	Myalgia, arthralgia, memory loss, sleep disturbance	–
# 7	Gardasil®	Muscle weakness, memory loss, dry mouth	Diabetes mellitus type I
# 8	Gardasil®	Headache, arthralgia, muscle weakness	–
# 9	Cervarix®	Muscle weakness, skin rashes, cognitive impairment	–
# 10	Cervarix®	Muscle weakness, dizziness, syncope	–
#11	Gardasil®	Arthralgia, insomnia, syncope, muscle weakness	Raynaud syndrome
# 12	Gardasil®	Arthralgia, muscle weakness, sleep disturbance, headache	Autoimmune thyroiditis
# 13	Gardasil®	Nausea, asthenia, fever, myalgia	–
# 14	Gardasil®	Arthralgia, sleep disturbances, fatigue	Autoimmune thrombocytopenia
# 15	Cervarix®	Arthralgia, skin rashes	IgA nephropathy
# 16	Cervarix®	Fever, fatigue, sleep disturbances	–

Table 3
Results of MELISA test.

Patient	Vaccine	NC [cpm]	PC [SI]	Ni [SI]	Al [SI]	Hg [SI]	Thim [SI]	MeHg [SI]
#1	Cervarix	535	493,3	**9,2**	1,6	***2,4***	1,2	1,3
#2	Gardasil	489	492,3	**22,7**	0,8	0,9	1	0,6
#3	Gardasil	1093	152,8	0,6	0,5	0,7	0,7	0,9
#4	Cervarix	1093	246,8	1,1	0,5	1,5	0,7	0,5
#5	Gardasil	587	343,7	***2,4***	0,9	***2,9***	1	1,1
#6	Cervarix	538	338,3	1,7	1,4	0,8	1	0,6
#7	Gardasil	437	256,1	1,4	1,1	0,7	0,1	0,9
#8	Gardasil	1018	237,3	***2,4***	0,6	**6,6**	0,7	1,4
#9	Cervarix	1248	127	1,1	0,8	***2,4***	0,5	1,2
#10	Cervarix	1686	145,8	**9**	0,5	**5,9**	0,5	1,8
#11	Gardasil	423	527,4	**4,4**	1,3	1,5	1,1	2
#12	Gardasil	636	194,6	**5,7**	0,3	1,4	0,8	***2,9***
#13	Gardasil	661	264,3	1,5	0,8	0,9	0,7	0,6
#14	Gardasil	533	317	1,2	1,1	0,9	0,8	0,5
#15	Cervarix	1213	215,7	0,7	0,6	0,8	0,9	0,6
#16	Cervarix	450	270,7	0,8	0,8	0,6	1	0,5

(Grimaldi-Bensouda et al., 2014). However, several reports describing heterogeneous post-vaccination phenomena have been published last few years: although the spectrum of these disorders includes some immune-mediated neurological diseases, actually most are represented by not well-defined syndromes characterized with general and not specific musculoskeletal, neurological and psychiatric clinical manifestations (Poddighe et al., 2014; Pellegrino et al., 2015; Karussis and Petrou, 2014).

In our case series (see Table 2), an "acute" phase dominated by low-grade fever, muscle and joint pains, non-specific skin rashes, headache and sensorial disturbances have been often described. During the following weeks or months, those symptoms subsided to lead to persistent disturbances resembling complex regional pain syndrome (CRPS), postural orthostatic tachycardia syndrome (POTS), chronic fatigue syndrome (CFS), fibromyalgia or other somatoform disorders (Palmieri et al., 2017a; Palmieri et al., 2017b). According to some authors, most of these clinical manifestations might be consistent with an autonomic dysfunction and, particularly, Kinoshita et al. hypothesized a small fiber neuropathy triggered by HPV immunization in susceptible people (Martínez-Lavín, 2015; Kinoshita et al., 2014). Very recently, some authors coined the term HPV vaccination associated neuro-immunopathetic syndrome (HANS) to refer to the ensemble of clinical conditions, allegedly occurring after the administration of HPV vaccine to some subjects. Indeed, this entity includes symptoms referring to four clinical domains: (i) autonomic and neuroendocrine; (ii) cognitive and emotional; (iii) environmental hypersensitivity and pain perception; (iv) musculoskeletal. This experimental animal model first supported the hypothesis that there might be some hypersensitive subjects to HPV vaccine because of unclear predisposing factors and/or environmental concomitant events causing a damage of the blood-brain barrier probably (Aratani et al., 2016; Nishioka et al., 2014).

Among the etiological theories attempting to explain those post-vaccination phenomena, the potential role of some metals (in particular, aluminum) included in the adjuvant complex of some vaccines has been considered. Aluminum has potential immunologic and neurotoxic effect. Studies demonstrated that aluminum could affect both innate and adaptive immune response, through the activation of NLRP3 inflammasone pathway, the up-regulation of antigen-presenting function and also the antigen protection. Moreover, due to its lipophilic nature, aluminum can bind cell membrane phospholipids, promoting some oxidative damage, which could provide some danger signals to the immune system. Finally, aluminum is an adjuvant and an antigen at the same time, as there is evidence that exposure to this metal can create an immunological memory causing delayed hypersensitivity (Kashiwagi et al., 2014; Lauren et al., 2016; Ruwona et al., 2016; He et al., 2015).

The main result of the present study is no evidence of any specific *in vitro* lymphocyte proliferation upon the stimulation of patients' lymphocytes with specific substances, namely nickel (Ni), alum (Al), inorganic mercury (Hg), thimerosal (Thim) and methyl-mercury (MeHg). Indeed, less than half girls affected with post-HPV vaccination phenomena showed some clear metal hypersensitivity to nickel ($n = 4$), inorganic mercury ($n = 1$) and both nickel and inorganic mercury ($n = 1$), regardless the type of HPV vaccine. However, as regards the perspective of a potential cell-mediated effect of aluminum, the most striking result is that no girls showed an immunological memory against aluminum, according to the results provided by MELISA® test. These findings do not exclude that aluminum might play a role in this post-vaccination phenomena, but do not support that cell-mediate hypersensitivity to aluminum is a fundamental pathological mechanism.

However, aluminum is considered a toxin at multiple levels, as an endocrine disruptor, a pro-oxidant element and a neurotoxin (Nayak, 2002). Indeed, CNS resulted to be the most sensitive target of aluminum and could lead to learning, memory, concentration, psychomotor and behavior impairments. Recent studies revealed that aluminum adjuvant micro-particles and, especially, nanoparticles are able to cross the blood-brain barrier and, thus, could have the potentiality to induce inflammatory and/or direct neurotoxic effects on CNS structures (Park et al., 2016a; Çabuş et al., 2015; Crépeaux et al., 2017). Although aluminum chronic intoxication is known to occur in humans, it is found exclusively in individuals suffering from chronic kidney diseases or in those exposed to high levels of aluminum because of occupational risk (Russo et al., 1992). Interestingly, aluminum was detected also in

neurofibrillary tangles in the hippocampus of patients with Alzheimer's Disease (AD): here, epidemiological studies identified high consumption of aluminum from drinking water as a risk factor for AD (Kandimalla et al., 2016). Actually, in infants there is little risk for aluminum toxicity following immunizations administered according to international recommendations (Glanz et al., 2015).

However, the mode of administration of very small amounts of alum, namely the intra-muscular injection, in addition to its inclusion in specific adjuvant complexes and/or to an undefined genetic individual predisposition, might be claimed to explain the fact that only a few people develop post-vaccination syndromes, despite the regular contact with alum contained in foods or other products.

For instance, in 1998 macrophagic myofasciitis (MMF) was recognized as a rare condition characterized by specific pathological alterations at deltoid muscle biopsy and was demonstrated to be probably related to the persistence of vaccine-derived aluminum hydroxide nanoparticles within macrophages at the site of previous intramuscular injections (Gherardi and Authier, 2012). In addition to local alterations, MMF patients showed a high rate of fatigue, pain syndromes and neuropsychological impairment, leading to the definition of a specific clinical entity, namely MMF-Associated Cognitive Dysfunction (MACD) (Passeri et al., 2011). Although the pathophysiology of neurocognitive impairment in MMF is unclear, aluminum was hypothesized to play a role in MACD pathophysiology and other cognitive dysfunctions (Van Der Gucht et al., 2015). In some animal models, the absorption of aluminum from intra-muscular injection of aluminum hydroxide resulted to be significant: it was demonstrated to entry in bloodstream with potential distribution to the whole body, including the brain. Aluminum hydroxide can form micro- and nano-sized aggregates being captured by cells upon phagocytosis, and recent experimental studies showed that these nano-sized particles can be transported by monocytes/macrophages in lymph nodes, blood and distant organs. Those can enter the brain by a MCP1-driven mechanism; moreover, aluminum can increase endothelial adhesion of circulating leukocytes and could lead to an abnormal release of cytokines inducing synapto-dendritic injury and, thus, affecting inter-neuronal communication and axoplasmic flow. Therefore, some authors raised the hypothesis that MACD could be, at least in part, due to aluminum nano-sized particle neurotoxicity, regardless the activation of specific adaptive immunity against aluminum, as our data demonstrated (Crépeaux et al., 2017; Park et al., 2016b; Couette et al., 2009).

In conclusion, this study showed that a cell-mediated and specific activation of adaptive immune system against aluminum to explain the development of neuropsychiatric symptoms after HPV vaccination is unlikely. Therefore, if there was really a causal effect between HPV vaccine and neuropsychiatric syndromes developed by few girls, the hypothetical role played by aluminum in the pathophysiology, could recognize other mechanisms than the activation of specific lymphocytes to aluminum and metal in general.

Transparency document

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