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Skin reactivity to thimerosal and phenol-preserved Montenegro antigen in Brazil

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Abstract

A randomized double-blind trial was performed to determine the frequency of positive reactions to the Montenegro antigen (leishmanin) preserved in thimerosal (Merthiolate™) 1:10,000 or phenol 0.4%. The respective products were tested separately in 400 young healthy individuals from a non-endemic area for *Leishmania*ses. Each volunteer received one of the following reagents: merthiolated antigen, phenolated antigen, merthiolated saline, or phenolated saline. The frequency of positive responses to each reagent after the first application was as follows: 0% (phenolated saline), 9.2% (merthiolated saline), 34.6% (antigen in phenolated saline), and 41.1% (antigen in merthiolated saline). After 1 week, volunteers who had tested positive for merthiolated or phenolated antigen were retested with the respective preservative, while negatives were retested with the preservative they had not received during the first test. In all, 331 volunteers who received merthiolated saline during the study, of whom 41 (12.4%) tested positive. Meanwhile, 326 volunteers who received phenolated saline, 4 (1.2%) tested positive. Positive reactions in each group were similar in relation to gross appearance skin reactions. Considering the high frequency of hypersensitivity to thimerosal in the study population, it is recommended that this compound should be replaced as a preservative of the leishmanin antigen. Almost 30% of positive reactions to Montenegro antigen in what is considered a non-endemic region was surprising and will be the object of future studies.

Keywords: *Leishmania*; Skin test; Thimerosal; Phenol

1. Introduction

The Montenegro skin test (MST) (Montenegro, 1926) is considered the most important complementary test in the diagnosis of tegumentary *Leishmania*sis (TL) and is also widely used in epidemiological studies and as

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an indicator of inapparent infection with *Leishmania* (Restrepo Isaza, 1980; Souza et al., 1992; Zijlstra and el-Hassan, 1993; Ben Salah et al., 2005; de Castro et al., 2005). However, standardization of a reagent for universal use has still not been achieved, and various formulations are used in Brazil and elsewhere in the world (Melo et al., 1977; Reed et al., 1986; Alimohammadian et al., 1993; Abramson et al., 1995; Akuffo et al., 1995).

Thimerosal (thiomersal, merthiolate) has been used as a preservative in vaccine and skin test antigens for decades (Marzochi et al., 1998). Marzochi et al. (1998) described a post-vaccinal MST conversion rate of 66% in volunteers who received only placebo (thimerosal 1:10,000 in saline) in a Phase I tegumentary *Leishmania*sis vaccine trial in Brazil, suggesting that the thimerosal present in the vaccine and in the Montenegro reagent received by the vaccinated volunteers could act as a confounder in similar vaccine evaluation studies.

Several studies have shown that thimerosal is allergenic (Forstrom et al., 1980; Seidenari et al., 1989), capable of inducing delayed hypersensitivity as demonstrated by skin tests (Maibach, 1972; Lebrec et al., 1999). Thimerosal present in intramuscular vaccines is associated with the occurrence of adverse effects, and the utilization of such vaccines can also cause hypersensitivity to thimerosal (Cox and Forsyth, 1988; Osawa et al., 1991).

Seeking to evaluate the frequency of delayed hypersensitivity to thimerosal and its possible interference with the MST, this study compared the response to Montenegro antigen conserved in thimerosal or phenol in healthy volunteers from leishmaniases non-endemic areas using a randomized double-blind design.

2. Patients and methods

The study was designed according to Resolution 196/96 of the Brazilian National Health Council and approved by the Brazilian Ministry of the Army. The FIOCRUZ Ethics committee also approved the study. Informed consent was obtained from all the volunteers who presented after an explanatory lecture on the *Leishmania*ses and on the study's objectives and risks. The following data were obtained through individual interviews: place of residence, vaccines received in the previous 2 years, previous skin tests, history of allergies, routine use of products that could contain thimerosal (eye drops, nasal drops) and other medications, and prior knowledge of *Leishmania*ses.

A total of 400 male volunteers with an age range of 18–23 were recruited into the study. The volunteers came from the city of Santa Maria in Rio Grande do Sul, which is not known to be endemic for *Leishmania*sis. Volunteers known to be allergic to thimerosal and/or phenol and those who presented any signs or symptoms of any disease at the time the tests were performed were excluded from the study. The sample size was calculated for a statistic power of 80% to discriminate differences of 15% between the frequency of hypersensitivity to phenol (5% Imperato and Diakité, 1969) and to thimerosal, expected to be around 20% (Program Statcalc-EPIINFO version 6.0).

2.1. Reagents

Antigens were prepared by the Reference Center for Diagnostic Reagents (BIOMANGUINHOS-FIOCRUZ-RJ) using the PH8 strain of *Leishmania amazonensis* (IFLA/BR/1967/PH8) containing 40 μg of proteic nitrogen/ml (Melo et al., 1977). Identical batches of antigen suspension were preserved in 0.85% NaCl solution with either thimerosal 1:10,000 or 0.4% phenol. The same preservative solutions were prepared separately without the antigens. The four formulations were stored identically and coded by an observer who was independent of the production and test application process.

2.2. Testing

The order of application of the reagents was obtained through randomization in blocks of four, with the volunteers allocated to treatment at the moment in which they arrived at the testing site. The volunteers were allocated in four groups according to the reagent received at the initial test (Table 1): group I ($n = 102$), merthiolated antigen; group II ($n = 101$), phenolated antigen; group III ($n = 97$), merthiolated saline; group IV ($n = 100$), phenolated saline.

Each allocated volunteer initially received a single intradermal application of 0.1 ml of one of the reagents on the anterior left forearm. All volunteers remained under observation for 45 min after the application. Readings were performed 48 h later as proposed by Sokal (1975) and were considered positive with an induration $\geq$5 mm measured at the largest diameter. Any local or systemic responses to the tests were also recorded.

2.3. Retesting

One week after the first test the volunteers were retested on the opposite forearm, based on the allocation and the result of the first test. Individuals from groups I and II, when positive, received the homologous diluent solution for the antigen initially administered to them,

while the negatives received the heterologous diluent solution. Positive individuals from groups III and IV were retested with 0.1 ml of 0.85% saline solution, while negatives received phenolated or merthiolated saline, respectively.

2.4. Analysis and interpretation of results

The results were analyzed using EpiInfo version 6.0 and SPSS for Windows (Statistical Package for Social Sciences). p-values <0.05 were considered significant. To study the frequency of positive reactions to the antigen and preservatives, the following assumptions were considered: (1) that no volunteer presented current or prior *Leishmania* infection and (2) that there was no *Leishmania* transmission in the study area.

3. Results

The groups were homogeneous in relation to: mean age, frequency of individuals vaccinated in the previous two years, and frequency of allergic antecedents. The majority of the volunteers who were initially tested (340/400; 85%) were re-tested. One hundred and sixty nine of the volunteers (169/400; 34.7%) came from the city of Santa Maria, while the rest came from other non-endemic areas in the State of Rio Grande do Sul. Minorities of volunteers reported using thimerosal before (13.3%), or had previously received other skin tests (8.7%), all negative. The majority of volunteers who reported previous intra-muscular vaccination (295/296) had received tetanus toxoid (TT) 15–90 days prior to the beginning of the study.

Table 1 shows the response to skin tests in the four groups studied. Frequency of positive responses to each reagent following the first application was: 0% (phenolated saline), 9.2% (merthiolated saline), 34.6% (antigen in phenolated saline), and 41.1% (antigen in merthiolated saline). Of the 87 volunteers who presented positive skin tests, 83 in were re-tested, which includes 41 positive to merthiolated antigen in group I, 33 positive to phenolated antigen in group II and 9 positive to merthiolated saline in group III. Of the 41 volunteers who were re-tested with merthiolated saline, 13 (31.7%) were positive. Of the 33 volunteers who were re-tested with phenolated saline, only 1 (3.0%) was positive. Of the 9 volunteers in group III who were retested with plain saline, none were found positive.

Two hundred fifty seven of the 313 volunteers who did not react were re-tested; 125/257 received merthiolated saline where 14.4% of them tested positive. The remaining volunteers (132/257) received phenolated saline, only a minority (2.4%) tested positive ($\chi^2 = 11.80$, $p < 0.001$). All these negative volunteers received each reagent only once, to avoid double application of any product in the same volunteer.

Table 1
Responses to skin tests in the four groups studied

Groups[a]	Results initial test	n	Volunteers retested (n)	Reagent used in retest	Results of retest	n
I ($n = 102$)	Positive	42	41	Merthiolated saline	Positive	13
					Negative	28
	Negative	60	50	Phenolated saline	Positive	3
					Negative	47
II ($n = 101$)	Positive	36	33	Phenolated saline	Positive	1
					Negative	32
	Negative	65	51	Merthiolated saline	Positive	5
					Negative	46
III ($n = 97$)	Positive	9	9	Plain saline	Positive	0
					Negative	9
	Negative	88	75	Phenolated saline	Positive	0
					Negative	75
IV ($n = 100$)	Positive	0		–		
	Negative	100	81	Merthiolated saline	Positive	14
					Negative	67
Total		400	340			

[a] I: merthiolated antigen; II: phenolated antigen; III: merthiolated saline; IV: phenolated saline.

Table 2
Association between response to skin tests and the study variables

Variables*	Initial test ($n=400$)			Retest ($n=340$)		
	Positive	Negative	p	Positive	Negative	p
Prior vaccination						
Yes	65	231	1.000	30	223	0.230
No	22	82		6	81	
History of allergies						
Yes	20	59	0.446	8	59	0.238
No	67	254		28	245	
Prior use of medications						
Yes	8	46	0.217	4	41	1.000
No	267	79		32	263	
Prior use of topical thimerosal						
Yes	77	269	0.745	32	260	0.800
No	10	43		4	44	

*The volunteers were asked about receiving vaccination before entering the study, about previous history of allergies, use of medication and use of any product containing thimerosal (eyedrops, etc.). **There was no statistically significant association with the study variables, based on Fischer's exact tests.

Adding the tests and retests, 331 volunteers received merthiolated saline during the study, of whom 41 (12.4%) were positive, while 326 volunteers received phenolated saline, of whom 4 (1.2%) were positive. The frequency of positive reactions to saline containing thimerosal among those who received it once was significantly less than among those who received it twice (14.4% versus 31.7%; $\chi^2 = 6.22$, $p < 0.05$, the same was true for phenolated saline (2.4% versus 3.0%, $\chi^2 = 4.98$ $p < 0.05$).

Local reactions to the tests could not be differentiated based on the reagents received and were identical to a classical positive reaction to the Montenegro skin test. The local reactions to the mertiolated saline injected were identical in appearance those of the mertiolated Montenegro antigen. In other words, with the naked eye, as it is performed the reading of a standard skin test, it could not be discriminated a positive result to the test with the antigen or with the mertiolated saline alone. The diameter of indurations obtained with the first test for merthiolated saline varied from 3 to 24 mm and from 4 to 24 mm for the retest. The four volunteers with positive reactions to phenolated saline presented indurations of 4, 6, 6, and 18 mm. The volunteer who presented an induration of 18 mm reacted positively to the initial test with phenolated antigen (15 mm) and had injured the site of the second test before the 48 h reading was performed.

Table 3
Number of volunteers with local or systemic reactions to skin tests in the four groups

Local/systemic reactions	Groups[a]				Total
	I	II	III	IV	
Local edema/erythema					
Initial test	5	2	3	0	10
Retest	0	1	0	4	5
Local pruritus					
Initial test	29[b]	29[b]	17	1	76
Retest	3	3	0	5	11
Fever, blisters, urticaria, local pain					
Initial test	0	0	0	0	0
Retest	0	0	0	0	0

[a] I: merthiolated antigen; II: phenolated antigen; III: merthiolated saline; IV: phenolated saline.
[b] The frequency of local pruritus was statistically associated with the injection of merthiolated or phenolated antigen and not with the injections of the saline solutions ($p < 0.001$, χ^2 test).

Frequency of positive reactions to the tests was not associated with a history of allergies or allergic diseases in the volunteers, utilization of medications, topical use of thimerosal, or having received vaccines prior to the study (Table 2). The size of indurations following the tests and retests was also no different comparing previously vaccinated to non-vaccinated individuals (Wilcoxon, $p > 0.05$).

Side reactions to the tests are described in Table 3. The appearance of edema/erythema was associated with receiving thimerosal (Pearson $\chi^2 = 3.75$, $p < 0.05$), while the appearance of pruritus was associated with receiving the *Leishmania* antigen, whether merthiolated or phenolated (Pearson $\chi^2 = 33.27$, $p < 0.0001$). All of the local reactions to retests (five cases of edema and nine of local pruritus) occurred in volunteers who received merthiolated saline (Table 3).

4. Discussion

The possibility that thimerosal may induce a false-positive reaction to the Montenegro skin test emerged from the work by Marzochi et al. (1998), who observed that responses to intradermal injection of thimerosal were identical to those induced by the application of merthiolated *Leishmania* antigen. Based on this observation, the study was conducted in a non-endemic area for *Leishmania*ses, so that only the allergic components of thimerosal and phenol would be analyzed. The current results confirm the study by Marzochi et al. (1998), demonstrating the sensitizing capacity of thimerosal in some 12% of the volunteers tested (Table 1), as well as: (1) the morphological similarity between the intradermal reactions to thimerosal and the Montenegro antigen and (2) the association between receiving thimerosal and a higher frequency of side effects to the tests (Table 3). Sensitization to thimerosal was significantly more frequent than to phenol (Table 1), which showed sensitization in only four individuals.

The study of a homogeneous population allowed for comparable groups, so it was not necessary to perform more than one simultaneous application in the same individual. This decreases the risks of increasing positive reactions to the test based on a dose effect, both of antigens and of different preservatives, which could occur if volunteers received various applications. Retesting was done on the contralateral forearm from the initial test.

Given the possibilities of sensitization by merthiolate or phenol (Nascimento et al., 1993; Satti et al., 2002; De Luca et al., 2003), we chose not to repeat application of the antigen with either thimerosal or phenol. The purpose of retesting with only diluent was to distinguish between individuals that had reacted only to the *Leishmania* antigen (those who were negative on retesting) and double positive individuals (those who reacted to two consecutive tests). However, the positive reaction to thimerosal was significantly greater among those who received it twice, suggesting that the thimerosal present in Montenegro antigen can obscures the test's sensitizing capacity.

Although various authors reported sensitization to thimerosal associated with receiving vaccines, our data do not show this association. The small number of non-vaccinated individuals in the four groups may make the analysis difficult. Neither was it possible to associate delayed hypersensitivity to thimerosal with a history of allergies or use of medications by volunteers (Table 2), and it was not possible to demonstrate cross-reactivity between hypersensitivity to thimerosal and other products, as described previously (Goncalo et al., 1996) (e.g. piroxicam).

The development of hypersensitivity reactions is associated with different exposure routes to allergens and is regulated by different immune mechanisms. Although 86% of the volunteers used topical thimerosal, none reported signs or symptoms of current or past allergy to the product, even though 12.4% of them showed post-test delayed hypersensitivity, which was unfamiliar to them. The intradermal antigen inoculation route is associated with the development of delayed hypersensitivity, while the topical use of subcutaneous inoculations usually triggers reactions of the immediate type, which are more easily perceived by the patient.

The high frequency of delayed hypersensitivity to thimerosal, the similarity between the reaction induced by this compound and that elicited by the MST, the association between receiving thimerosal and the higher frequency of side effects to thimerosal (Table 3) indicate that this compound should be replaced as the preservative for the Montenegro antigen and other antigens applied in intradermal tests. The low sensitivity induced by phenolated saline in our study (1.2%) and the rare occurrence of side effects associated with this reagent suggest that it could be used to replace thimerosal until the development of new antigens free of non-specific components.

It was necessary to get consistent and evaluable results about the safety and the allergic potential of the preservatives used in the leishmanin and subsidiates the design of other field evaluations to verify if the non-specific reactions found to the preservatives occurs in other regions and countries.

Considering the absence of infection or disease from *Leishmania* among the volunteers and the absence of transmission of this parasite in the study area, the

response in the group that received the antigen should be the same as that which received only the corresponding diluent. However, from the 41 positive volunteers re-tested with mertiolated saline (Table 1), 28 were negative to re-testing, suggesting that they were skin-test positive, without sensitization by the preservative used. Studies are under way to elucidate the reason for the increased frequency of true positives to the Montenegro antigens with or without thimerosal in an area which is presumed to be non-endemic and without any association with allergy to the preservatives.

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References

Abramson, M.A., Dietze, R., Frucht, D.M., Schwantz, R., Kenney, R.T., 1995. Comparison of new and old world leishmanins in an endemic region of Brazil. Clin. Infect Dis. 20, 1292–1297.

Akuffo, H., Darce, M., Maasho, K., Berhan, T.Y., 1995. In vivo evaluation of immune responses in leishmaniasis: the use of cross-species leishmanin preparations for skin testing. Am. J. Trop. Med. Hyg. 53, 16–22.

Alimohammadian, M.H., Kivanjah, M., Pak, F., Gaznavia, A., Kharazmi, A., 1993. Evaluation of the efficacy of Iran leishmanin and comparison with leishmanins from Wellcome (UK) and Roma (Italy) in cured cutaneous leishmaniasis patients [see comments]. Trans. R. Soc. Trop. Med Hyg. 87, 550–551.

Ben Salah, A., Louzir, H., Chlif, S., Mokni, M., Zaatour, A., Raouene, M., Ismail, R.B., Dellagi, K., 2005. The predictive validity of naturally acquired delayed-type hypersensitivity to leishmanin in resistance to Leishmania major-associated cutaneous leishmaniasis. J. Infect. Dis. 192, 1981–1987.

Cox, N.H., Forsyth, A., 1988. Thiomersal allergy and vaccination reactions. Contact Dermatitis 18, 229–233.

de Castro, E.A., Luz, E., Telles, F.Q., Pandey, A., Biseto, A., Dinaiski, M., Sbalqueiro, I., Soccol, V.T., 2005. Eco-epidemiological survey of Leishmania (Viannia) braziliensis American cutaneous and mucocutaneous leishmaniasis in Ribeira Valley River, Parana State. Brazil. Acta Trop. 93, 141–149.

De Luca, P.M., Mayrink, W., Santiago, M.A., Nogueira, R., Conceicao-Silva, F., Melo, G., Mendonca, S.C., 2003. Randomized, double-blind, placebo-controlled study on the immunogenicity of the leishmanin skin test. Trans. R. Soc. Trop. Med. Hyg. 97, 709–712.

Forstrom, L., Hannuksela, M., Kousa, M., Lehmuskallio, E., 1980. Merthiolate hypersensitivity and vaccination. Contact Dermatitis 6, 241–245.

Goncalo, M., Figueiredo, A., Goncalo, S., 1996. Hypersensitivity to thimerosal: the sensitizing moiety. Contact Dermatitis 34, 201–203.

Lebrec, H., Bachot, N., Gaspard, I., Kerdine, S., Guinnepain, M.T., Laurent, J., Pallardy, M., 1999. Mechanisms of drug-induced allergic contact dermatitis. Cell. Biol. Toxicol. 15, 57–62.

Maibach, H.I., 1972. False-positive intradermal skin tests and thimerosal. JAMA 220, 126.

Marzochi, K.B., Marzochi, M.A., Silva, A.F., Grativol, N., Duarte, R., Confort, E.M., Modabber, F., 1998. Phase 1 study of an inactivated vaccine against American tegumentary leishmaniasis in normal volunteers in Brazil. Mem. Inst. Oswaldo Cruz. 93, 205–212.

Melo, M.N., Mayrink, W., da Costa, C.A., Magalhaes, P.A., Dias, M., Williams, P., Araujo, F.G., Coelho, M.V., Batista, S.M., 1977. Standardization of the Montenegro antigen. Rev. Inst. Med. Trop. Sao Paulo 19, 161–164.

Montenegro, J., 1926. A cutis reacção na leishmaniose. Ann. Fac. Med. Univ. São Paulo 1, 323–330.

Nascimento, M.D., Alcantara-Neves, N.M., Muniz, M.E., Nunes, S.F., Paranhos, M., de Carvalho, L.C., 1993. Induction and modulation of the immune response to Leishmania by Montenegro's skin test. Trans. R. Soc. Trop. Med. Hyg. 87, 91–93.

Osawa, J., Kitamura, K., Ikezawa, Z., Nakajima, H., 1991. A probable role for vaccines containing thimerosal in thimerosal hypersensitivity. Contact Dermatitis 24, 178–182.

Reed, S.G., Badaro, R., Masur, H., Carvalho, E.M., Lorenco, R., Lisboa, A., Teixeira, R., Johnson Jr., W.D., Jones, T.C., 1986. Selection of a skin test antigen for American visceral leishmaniasis. Am. J. Trop. Med. Hyg. 35, 79–85.

Restrepo Isaza, M., 1980. The Montenegro test in the epidemiology of South American leishmaniasis. Bol. Oficina Sanit Panam 89, 130–138.

Satti, I., el Hassan, A., Khalil el, T.A., Akuffo, H., 2002. The effect of repeated leishmanin skin testing on the immune responses to Leishmania antigen in healthy volunteers. Trans. R. Soc. Trop. Med. Hyg. 96, 565–567.

Seidenari, S., Manzini, B.M., Modenese, M., Danese, P., 1989. Contact sensitization to thimerosal in healthy subjects. G. Ital. Dermatol. Venereol. 124, 335–339.

Sokal, J.E., 1975. Editorial: measurement of delayed skin-test responses. N. Engl. J. Med. 293, 501–502.

Souza, W.J., Sabroza, P.C., Santos, C.S., de Sousa, E., Henrique, M.F., Coutinho, S.G., 1992. Montenegro skin tests for American cutaneous leishmaniasis carried out on school children in Rio de Janeiro Brazil: an indicator of transmission risk. Acta Trop. 52, 111–119.

Zijlstra, E.E., el-Hassan, A.M., 1993. Leishmanin and tuberculin sensitivity in leishmaniasis in the Sudan, with special reference to kala-azar. Trans. R. Soc. Trop. Med. Hyg. 87, 425–427.