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ORIGINAL PAPER

Homeopathic treatments modify inflammation but not behavioral response to influenza antigen challenge in BALB/c mice

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Background: **Influenza affects thousands of people worldwide every year, motivating the development of new therapies. In this work, the effects of two homeopathic preparations (influenza biotherapies and thymulin) were chosen following two different rationales: isotherapy and endo-isotherapy models. The homeopathic effects were evaluated individually considering the inflammatory and behavioral responses against influenza virus antigen were studied in BALB/c mice.**
Methods: **Male adult mice were treated orally and blindly for 21 days with highly diluted influenza virus or with thymulin, and were divided in two sets of experiments. The first series of experiments aimed to describe their behavior, using an open field (OF) device. In the second series, mice were challenged subcutaneously with influenza hemagglutinin antigen (7 μg/200 μl) at day 21. At day 42, behavior and inflammation response were evaluated.**
Results: **No behavioral changes were seen in OF tests at any time point after treatments. Flow cytometry and morphometry revealed significant changes in T and B cell balance after influenza antigen challenge, varying according to treatment.**
Conclusion: **The results show that both homeopathic treatments induced subtle changes in acquired immune anti-viral response regulation. A deeper understanding of the mechanism could elucidate their possible use in influenza epidemiological situations.**
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Keywords: Homeopathy; Influenza virus; Influenza hemagglutinin antigen; Behavior; Thymulin; Immunology

Introduction

Influenza A virus has been responsible for successive pandemics around the world, leading to more deaths than natural disasters, mainly due to the continuous changes in its antigenic structure.[1–5] The continuous impact of influenza virus on the human population has motivated the development of new approaches less sensitive to these variations, including homeopathy,[6,7] with special focus on biotherapies, that are medicines prepared from etiologic agents following homeopathic procedures (ultradilution and succussion).

In previous *in vitro* studies made by our group, intact influenza biotherapy 30DH was able to change cellular

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and biochemical aspects of MDCK and J774G8 cell lines with an increase in TNF-α production by these macrophages cells.[6,12] On the other hand, thymulin, a nine-aminoacid thymic hormone which modulates several endocrine and immunological effects, has also shown important immunomodulation effects on birds and mice challenged with bacteria and protozoa, even when prepared as 5CH homeopathic dilutions,[8–11] but there is no experimental description about thymulin 5CH effects in viral disease. Thus, we could compare two different approaches to influenza induced body changes: (a) classical isotherapy (influenza biotherapy) and (b) homeopathic preparations of an endogenous immune-modulator peptide (endo-isotherapy with thymulin). The motivation to the present study was to compare the putative effects on inflammation and behavior of both homeopathic preparations, in order to reveal some critical aspects of their clinical potential to influenza treatment.

Materials and methods

Animals

Male 21 days old BALB/c mice were kept in *quarantine* in micro-isolators (Techinoplast®) during 7 days, until the beginning of the experiment. After this adaptation, the animals were randomly divided in groups, receiving sterile water and commercial food *ad libitum*. The animals were kept under controlled environmental conditions (temperature: 20–24°C; humidity: 50–65%; light/dark cycle: 12/12 h; air cycle: 75 changes/hour) throughout all the experimental period. Water and food consumption and body weight were checked three times a week. Their handling was done exclusively by authorized people throughout the experimental period.

Ethics

The protocol was approved by the Ethics Committee on Animal Use (CEUA), at the Federal University of Rio de Janeiro, under reference numbers: DFBCICB 037 (Experiment 1) and DFBCICB 040 (Experiment 2). All animal procedures were done according to EU Directive 2010/63/EU for animal experiments.

Randomization

The animals were randomly distributed in boxes and weighed. Before starting the tests, the average of weight of each box was evaluated and did not show statistically significant differences (ANOVA-one way) in any of the two experimental phases.

Homeopathic solution preparation

The homeopathic solutions used in this study were prepared according to the Brazilian Homeopathic Pharmacopoeia,[13] using a mechanical automatic device (Autic®) for succussion. The protocol was designed to compare two different rationales: the effects of classical isotherapy and those of endogenous molecules isotherapy (endo-isatherapy). The chosen dilutions for both, H3N2 biotherapy and thymulin (decimal or centesimal, respectively), were selected according to previous experimental results.[9,13]

The biotherapies were prepared using purified H3N2 influenza virus sample (A/AICHI/2/68), at 10,240 HAU/25 μl as a starting point, following a standardized protocol established by Siqueira et al.[12] Briefly, we previously detected that the use of water as solvent preserve the virus particle. On the other hand, when ethanol was used as solvent, the virus particles were lysate. In the present work, we compared the efficacy of both medicines, considering the use of both solvents: water (called as intact H3N2) and ethanol (called as inactivated H3N2). Both solvents and protocols are usually used in current homeopathic pharmaceutical practice.

Two variations of biotherapy were prepared: a) the first using 70% alcohol as solvent in all decimal dilution steps, originating the 30DH H3N2 inactivated virus biotherapy; and b) the second using sterile distilled water as solvent from 1st to the 10th or 28th decimal dilution and 70% alcohol in the last two steps, to prepare the 12 and 30DH dilutions, respectively. The use of water as the initial solvent kept the virus particles intact, originating the H3N2 intact virus biotherapy. The control samples corresponded to water, whose preparation followed the same dilutions and procedures except for the addition of influenza virus (water 30DH).

Thymulin 5CH was prepared using a thymulin 4CH matrix (Boiron®), which was prepared from the synthetic zinc-free peptide, whose purity was 98.66%. This matrix was diluted at a centesimal basis (1:100) in 30% alcohol and mechanically succussioned (Autic®) to obtain the 5CH homeopathic dilution, whose estimated concentration is 4×10^{-13} M, a hundred fold less concentrated than the physiological effective level.

All treatments were offered to mice into the drinking water by free access, according to.[8–10] The test solutions were diluted 1:100 in 250 ml of drinking water, in such a way that the final alcohol concentration was 0.3%, low enough not to produce behavioral changes in mice.

Experimental groups and study design

All procedures were done in blind. Thus, medicines flasks were labeled with a code, whose identification was kept under the responsibility of a laboratory staff not involved with any experimental procedure. The codes were broken only after statistical analysis.

Set one: In this experimental set, 62 animals were used and the behavioral parameters and the influence of the starting point of the biotherapy (intact and inactivated H3N2 influenza virus) were evaluated. The experimental groups were distributed as follows (N = 14 mice per group):

Group A – Animals treated with Thymulin 5CH (N = 14).

Group B – Animals treated with water 30DH (N = 14).

Group C – Animals treated with H3N2 inactivated virus 30DH (n = 14).

Group D – Animals treated with H3N2 intact virus 30DH (n = 14).

Group E – Untreated animals (control; n = 6).

Mice were manipulated in two steps: the 'B' (behavior) step and 'I' (inflammation) step. In the 'B' step, the animals were kept under observation for the first 21 days of treatment, when they were submitted to open field test (OF). After, 31 animals (7 in A−D; 3 in E) were euthanatized and part was challenged subcutaneously with 7 μg/200 μl of influenza viral hemagglutinin antigen (A/WISCONSIN/67/2005, NIBSC CODE: 06/120), composing the 'I' (inflammation) step. They were monitored up to day 42, when they were observed again in the OF and submitted to euthanasia too. Spleen, lungs, heart and mediastinal lymph nodes were weighed in a semi-analytical balance (Marte®). Representative fragments were fixed in 10% buffered formalin for conventional histological procedures. The sections were stained by hematoxylin-eosin method and observed at light microscope for histological and histomorphometric analysis.[9]

The OF test was performed for 5 min, in which the number of stools, urine wells, frequency of walking, rearing, grooming and immobility time were registered by a digital camera and analyzed later. To avoid biases, the whole procedure was always done in the morning. After every animal exchange, the OF was cleaned with 20% alcohol, due to the possible contaminant olfactory stimuli.[9]

Set two: Based on the results obtained in the first protocol, described above, two different biotherapy dilutions (12DH and 30DH) were used from the intact H3N2 influenza virus sample (A/AICHI/2/68). Twenty animals were distributed in 4 Groups (A−D), as follows:

Group A − Animals treated with Thymulin 5CH (N = 5).

Group B − Animals treated with H3N2 intact virus 30DH (N = 5).

Group C − Animals treated with H3N2 intact virus 12DH (N = 5).

Group D − Animals treated with water 30DH (N = 5).

After 21 days of treatment, all animals were challenged subcutaneously with the influenza viral hemagglutinin antigen (A/WISCONSIN/67/2005, NIBSC CODE: 05/236) at the concentration of 7 μg/200 μl and monitored for another 21 days, when they were submitted to euthanasia and autopsied. The peritoneal washing fluid was harvested and a pool of samples from each experimental group was prepared in order to reach sufficient number of suspended cells to be analyzed in the flow cytometer, according to cell population balance. Each spleen was weighed and submitted to fixation in 8% paraformaldehyde for subsequent immunohistochemical analysis.

Flow cytometry

The peritoneal washing fluid was harvested using 5 ml of cold RPMI medium, centrifuged (2000 rpm, 5 min) and re-suspended in 1 ml of hemolytic buffer (GIBCO®) for two minutes. The viability test was made by counting cells in a Neubauer chamber (HAUSSER-SCIENTIFIC), using 0.1% of Trypan blue dye to verify viability. Thus, cells were incubated with CD16/CD32 anti-mouse antibody (1:100) diluted in 1% PBS-BSA for 30 min at 4°C, to block Fc *gamma* II and III.

Aliquots were divided into three tubes: tube 1) incubated only with 1% PBS-BSA (negative control, unmarked cells); tube 2) containing 1% anti-CD23 FITC, anti-CD5 PerCP, anti-CD19 APC and anti-CD11b PE antibody solution (combo 1); tube 3) containing 1% anti-CD4 PE, anti-CD8 AF 405, anti-CD19 PE Cy5.5 and anti-CD25 AF488 antibody solution (combo 2). The antibodies were incubated for 30 min at 4°C. After further washing in PBS, the cells were re-suspended, fixed in 1% paraformaldehyde and kept at 4°C. The counting was done using ATTUNE Acoustic Focusing Cytometer® (APPLIED BIOSYSTEM) within 48 h. All antibodies were supplied by INVITROGEN®.

The granularity and cell size were selected to determine the lymphocyte and phagocyte peritoneal sub-populations. From the lymphocyte gate, the B2 cells were determined by the simultaneous expression of CD19 and CD23, and the B1 cells were determined by the simultaneous expression of CD19 and CD11b. The quantity of CD5 positive (B1 a) and CD5 negative (B1 b) cells was calculated from the latter sub-population. From the phagocyte gate, double CD19−CD11b positive cells were labeled as 'B1-derived phagocytes' (BDP) and CD11b positive−CD19 negative cells were labeled as 'mature phagocyte'.[14]

The analysis of T cells subsets was made from the lymphocyte gate. The CD19 negative cells were delimited in a specific gate and CD4 and CD8 positive populations were determined.[14] An additional counting was done from each of these sub-populations to determine the percentage of T regulatory CD25 positive cells.

The Flow Jo 8.7 software was used for data analysis. Ten thousand events were considered by the system for each sample. The compensation was made after a single marker set of samples. The balance between cell subpopulations was represented graphically.

Immunohistochemistry and histomorphometry

The spleen samples embedded in paraffin were cut in 5 micron sections and mounted on sylane treated slides for immunohistochemistry procedures. The antigen retrieval was done by moist heat to 80°C for 20 min in an electric pan (PANASONIC®) immersed in citrate buffer (DAKO). The endogenous peroxidase activity was blocked by incubation with 5% H_2O_2 in methanol for 15 min at room temperature. The sections were treated with normal horse serum 2.5% (VECTOR) for twenty minutes at room temperature to block nonspecific adsorption sites. The respective primary antibodies (Table 1) were incubated overnight, at 4°C, in a humid dark chamber. On the following day, the sections were washed in PBS and treated with conjugated polymer with secondary antibody anti-mouse IgG (VECTOR), for 30 min, at room temperature. The revelation of peroxidase activity was done by DAB (DAKO). The sections were mounted after to be counterstained with Harris hematoxylin (HE). The negative control was done simultaneously by adding pure DAKO® antibody diluents instead of antibody.

Table 1 Markers used in the immunohistochemistry

Marker	*Cell*	*Supplier*	*Molecular target*	*Clone*	*Origin species/target*	*Dilution*
Anti-CD3	T cells	Serotec	CD 3	CD312	Rat−mouse	1:80
Anti-CD11b	Migrating phagocytes	Serotec	CD11b	M1/70.15	Rat−mouse	10%
Anti-CD45R	B Lymphocytes (specifically)	Serotec	Surface protein (LCA)	RA3-6B2	Rat−mouse	0.5%

Table 2 Bodyweight (g) and relative weight of spleen of mice at the day of necropsy, from different groups (mg). Pathogenetic protocol (P set). Values represent the mean and standard deviation of each group

Weight	*Control*	*Water 30DH*	*Inactivated H3N2 30DH*	*Intact H3N2 30DH*	*Thymulin 5CH*
Body (g)	26.55 ± 7.79	26.25 ± 6.41	18.52 ± 4.70	23.56 ± 6.22	28.45 ± 3.14
Relative weight (mg)	5.85 ± 0.71	6.62 ± 2.48	8.94 ± 2.33	5.38 ± 1.10	4.66 ± 0.052

The slides of heart, lung and mediastinal lymph nodes were analyzed qualitatively. The diameter of follicles and germinal centers were measured in pixels and the ratio between both diameters was considered for statistical analysis, in order to evaluate the spleen proliferative activity. The slides stained by immunohistochemistry were assessed for the distribution of B and T lymphocytes and by the delimitation of the positivity area (in pixels) of each marker, in relation to the total follicle area. All morphometric analysis were done using images captured from 4× objective and the *Image Tool 3.0* software (UTHSCSA, University of Texas Health Science Center; San Antonio, Texas), except the number of CD11b+ cells by follicle, that was counted by visual analysis.

Statistical analysis

All results were analyzed using ANOVA/Tuckey Krammer or Kruskal−Wallis/Dunn, according to the degree of homogeneity of the samples, previously assessed by the Bartlett test. The balance between different cell populations counted by flow cytometry was analyzed by the X^2 test. The difference was considered statistical significant when $p \leq 0.05$.

Results

In the behavior protocol, no significant results were seen after the OF test analysis, considering both times: 21 and 42 days. There was no statistically significant difference between the relative weights of organs (Table 2), or in spleen histomorphometry (*data not shown*) after the first 21 days, before antigen inoculation. However, after the viral hemagglutinin antigen inoculation, the animals presented an increase in the relative weight of spleen as well as bodyweight loss. These aspects may suggest the use of intact H3N2 virus as a starting point of the biotherapy preparation (Set one, Group D) may cause a pathogenetic effect (Figures 1 and 2).

Previous works published with homeopathic pathogenetic trials detected high incidence of pathogenetic effects

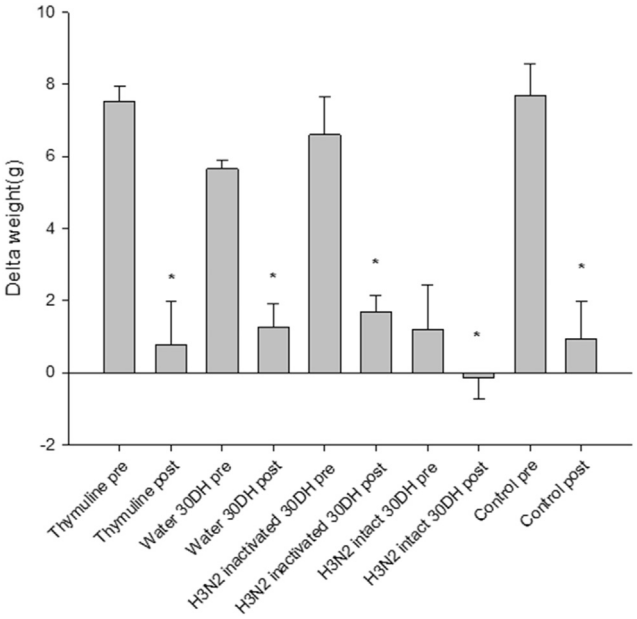

Figure 1 Comparison of weight gain ('delta') of mice from set I, during 21 days before (pre) and 21 days after antigen administration (post). Weights were measured in grams. *$p \leq 0.01$, Student, '*t* test.

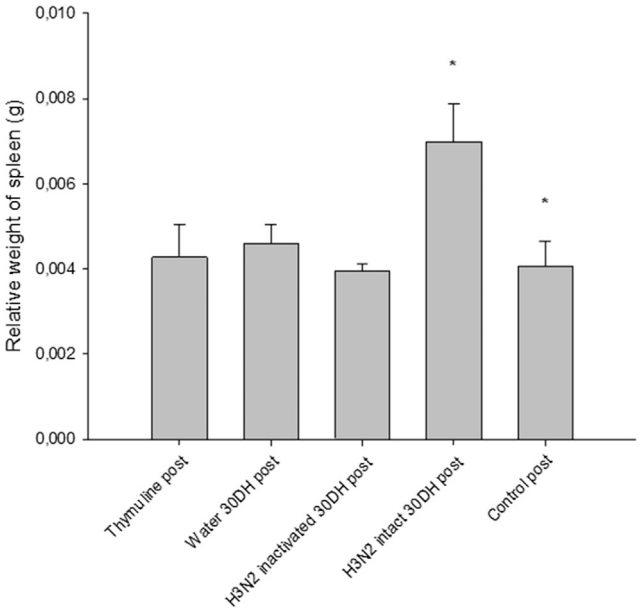

Figure 2 Spleen relative weight of mice from set I, 21 days after antigen administration. Weights measured in grams. *$p \leq 0.05$, ANOVA, Tuckey Krammer, in relation to all experimental groups, including control.

Table 3 Spleen Germinal Center/Follicle area ratio (pixels), measured by histomorphometry after 21 days from antigen inoculation in mice. Set I. ANOVA. Values represent the mean and standard deviation of each group

Area (pixels)	*Control*	*Water 30DH*	*Inactivated H3N2 30DH*	*Intact H3N2 30DH*	*Thymulin 5CH*
Follicle	131,665 ± 67,453	186,351 ± 106,833	164,531 ± 87,581	198,205 ± 103,053	189,540 ± 105,474
Germinal center	36,957 ± 52,764	57,637 ± 47,581	44,725 ± 41,081	56,676 ± 54,664	55,443 ± 40,740
Ratio	0.2827	0.3092	0.2718	0.2859	0.2925

Table 4 Number of CD11b+ cells per spleen follicle and positive area of CD45RA+ cells (B lymphocytes) and CD3+ cells (T lymphocytes) in relation to the total microscopic spleen follicle area in different groups (set 2). ANOVA

	Water 30DH	*Thymulin 5CH*	*Intact H3N2 12 DH*	*Intact H3N2 30DH*
CD11b+	1.420 ± 0.773	1.860 ± 0.282	0.817 ± 0.573	1.850 ± 1.084
CD45RA+ ratio	0.54 ± 0.10	0.43 ± 0.14	0.48 ± 0.17	0.48 ± 0.13
CD3+ ratio	0.35 ± 0.13	0.43 ± 0.12	0.51 ± 0.10	0.43 ± 0.07

when different homeopathic medicines were evaluated.[15] Additionally, when we evaluated the occurrence of respiratory symptoms in Brazilian children who received homeopathic medicines (homeopathic complex and InfluBio) in comparison to placebo group, we detected the occurrence of respiratory symptoms in these later than in those who received the homeopathic medicines, suggesting a pathogenetic effect triggered by these medicines.[16]

The germinal center/follicle area ratio of spleen did not present significant differences among groups (Table 3), and no significant differences were seen regarding to CD3+ (T cell) and CD45+ cell (B lymphocytes) area in proportion to total follicle area, indicating that treatments were not able to switch humoral to cellular immune response in a systemic level (Table 4).

In the flow cytometry (Figures 3 and 4), when the balance between different cell sub-populations was analyzed using a contingency table and the X^2 test, the lymphocyte/macrophage ratio in mice treated with both thymulin 5CH and intact biotherapic 30DH was equally balanced. A predominance of macrophage number in relation to lymphocytes was seen in the other groups, without statistical significance (Table 5 A and B). Table 5 (A and B) also shows that thymulin 5CH treated group presents increase in the T/B cell proportion, similar to that found in the intact biotherapic 12DH group. On the contrary, mice treated with intact biotherapic 30DH presented decrease in the T/B cell ratio in relation to water 30DH. The ratio (or balance) between both cell types was calculated as [percentage of T cells/percentage of B cells] and compared between groups by X^2 test. Concerning the CD4/CD8 T cells ratio, no statistical significance was seen among groups.

Discussion

In the present work, a new attempt to register 'proving' effects[17,15] in experimental animals was presented, without significant results, considering the behavioral data obtained from the OF test, body and organ weights and histomorphometry. Similar difficulties were also shared by Coelho et al.,[18] who were not able to demonstrate pathogenetic effects when rats were treated with *Dolichos*

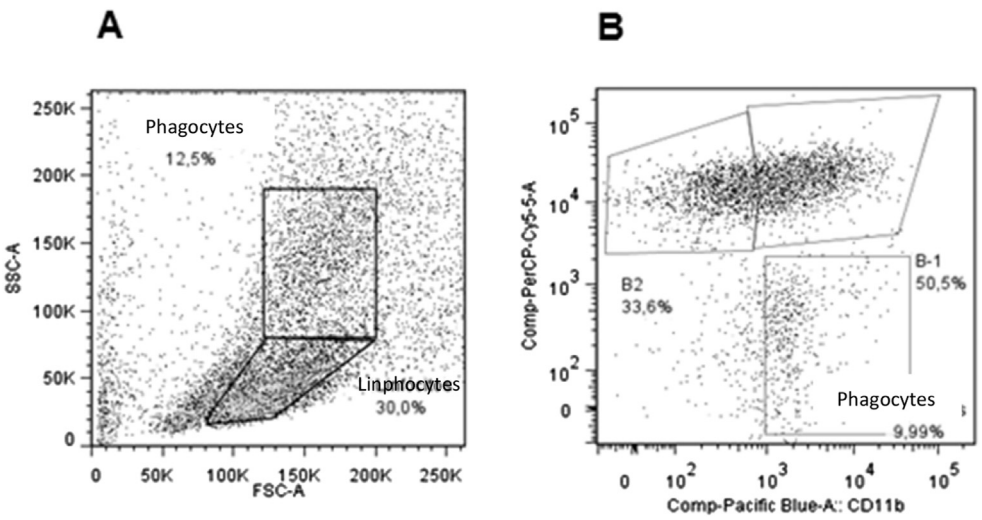

Figure 3 Illustrative scattering diagram representing the percentage, distribution and selection of B cells populations in flow cytometry of peritoneal washing fluid harvested from a control mouse. (A) Lymphocyte and phagocyte gates identified on the basis of cell size (y axis) and lumpiness (x axis); (B) from the lymphocyte gate, a secondary diagram was extracted representing B1 (CD19+ CD23−), B2 (CD19+ CD23+) cells and phagocytes (CD19− CD11b+). Graphs obtained from Flow Jo 8.7 software.

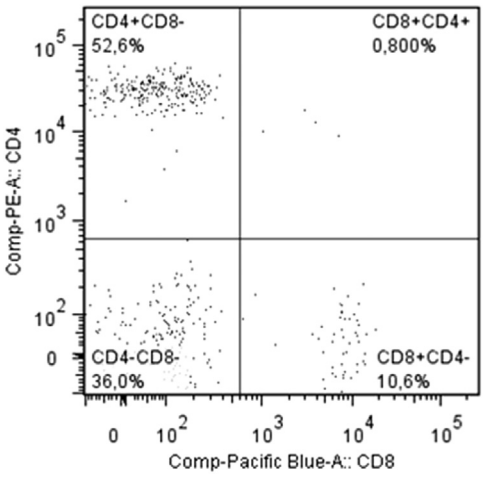

Figure 4 Illustrative scattering diagram representing the distribution and selection of T cell sub-populations in flow cytometry of peritoneal washing fluid harvested from a control mouse. The diagram was extracted from the lymphocyte gate. The predominant population was CD4+ cells. Graphs obtained from Flow Jo 8.7 software.

pruriens homeopathic preparations. However, in the present experiments, after the viral hemagglutinin antigen inoculation, the animals presented alterations in the relative weight of spleen and weight lost, which could be attributed to a pathogenetic effect. Indeed, few experimental studies about proving effects in animals have been done to give enough substantial knowledge about this phenomenon.[19] No significant results were observed with inactivated biotherapic, what can be explained by Roberto Costa's theory. According to him, it is important to maintain the microorganisms active or alive in the first dynamizations, in order to preserve the infectious power of viral particles.[20,21] Roberto Costa developed this kind of biotherapy using living infectious microorganisms as etiological agents, and called this preparation as 'living nosodes', registering better clinical responses from these uses.

After viral antigen inoculation, reduction in the body weight could be seen in those treated with intact biotherapic 30DH, maybe as a mimesis of viral infection, as previously demonstrated by Lin.[22] Since no statistically significant difference was detected in food consumption, the meaning of this observation could be related to putative changes in animal metabolism, involving the release of several cytokines, as seen in natural viral infections. Similar effects were already registered after treatment of mice with homeopathic solutions of *Euphorbia tirucalli* Lineu.[23] On the other hand, the data obtained from the histomorphometry analysis revealed increase in germinal center area when animals were inoculated with viral antigen, indicating the efficiency of this model to induce specific immune response, although no difference was seen after treatments, including the T and B lymphocytes population ratio, identified by immune-histochemistry.

The flow cytometry analysis revealed that thymulin 5CH treatment was able to induce an increase in the T/B cell ratio, similar to that found in the intact biotherapic 12DH group. The treatment of mice with intact biotherapic 30DH, instead, leads to decrease in the T/B cell ratio, indicating that different potencies of the same medicine can modulate the immune system by diverse ways. Considering our results, summarized in Table 5 (A and B), we can suggest H3N2 intact 30DH would be more efficient to promote humoral response against viral antigens; however, thymulin 5CH and H3N2 intact 30DH were able to induce a switch toward non-B CD19-cells, in which population T lymphocytes and NK cells can be found. This kind of response is more efficient against viral infection, as expected to thymulin 5CH, which was able to change the balance of lymphocyte sub-types toward T cells.[8–10] Our results show that the intact biotherapic 12DH caused the same change, but stronger. However, as we mentioned above, the concentration of thymulin in potency 5CH is 100 times shorter than the physiological, i.e., it is not possible to detect any physiological effect.

Table 5 (A, B) – Number and percentage of sub-type cells present in macrophages (gate M; A) and lymphocytes (gate L; B) gates obtained by flow cytometry analysis from a pool of peritoneal washing fluid samples harvested from five mice per group (set 2). Data represent the absolute number of cells present in each gate and its corresponding percentage of each sub-type. The ratio between both cell types in the different groups was compared by X^2 test, $p \leq 0.01$ in relation to water 30DH. BDP = B1 derived phagocyte

A	*Cells/μl [%]*			
	Group	*Gate MTotal*	*Mature phagocytesCD11b+ CD19–*	*BDP CD11b+ CD18+*
	Thymulin 5CH	457.86	86.45%	3.11%
	H3N2 intact 30DH	446.27	74.13%	1.93%
	H3N2 intact 12DH	696.6	95.56%	2.58%
	Water 30DH	1176.12	96.49%	2.38%

B	*Group*	*Gate LTotal*	*B1 total*	*B1a/B1b*	*B2*	*NON-B CD19-cells*	*CD4*	*CD8*
	Thymulin	501.8	46.75%	B1a: 4.22% B1b: 8.85%	0.031%	50.09%*	2.52%	1.97%
	H3N2 intact 30DH	471.67	69.65%*	B1a: 3.24% B1b: 43%	0.072%	27.6%	1.23%	0.85%
	H3N2 intact 12DH	146.69	54.52%	B1a: 3.17% B1b: 62.36%	0.53%	59.58%	1.12%	2.35%
	Water 30DH	210.85	58.95%	B1a: 5.23% B1b: 33.64%	0.89%	38.08%	0.38%	2.39%

The asterisk represents the statistical significance difference ($p \leq 0.01$) in relation to water 30DH.

Thymulin secreted by thymic epithelial cells is a very important T lymphocyte maturation zinc-dependent peptide, when used in physiological concentrations.[18,24] It is known that the cellular immune response mediated by T lymphocytes with the participation of phagocytes (innate immune response) is a typical anti-virus immune response, by direct cytotoxicity against infected cells or by secreting cytokines that activate macrophages to destroy intracellular agents.[19,25] This is an important parameter to evaluate viral immune response, commonly used in vaccine efficacy evaluation.[26–29]

Taking the data together, the treatments of mice with different protocols show different specific outputs, according to which the use of thymulin 5CH and H3N2 intact 30DH improved the balance of T and B lymphocytes in the sense of better cellular immunity, that is more adequate to virus infection. On the other hand, the use of H3N2 intact 12DH improved the B cells balance (mature B2 cells and their precursors, B1 cells), switching the immune response to a humoral pattern against soluble viral antigens. These differences invite to postulate the usefulness of lower dilutions of H3N2 isotherapy as a prophylactic tool, and the usefulness of higher dilutions as a therapeutic agent to treat the infected host. In fact, our previous work, done with homeopathic medicines (homeopathic complex and InfluBio), both in 30DH, minimized the number of flu and acute respiratory infection symptomatic episodes in children, in comparison to placebo group,[16] corroborating the present results detected in Balb-c mice.

Conclusion

The results highlight the studied homeopathic solutions did not interfere in the animals' behavior; however, they induced modulation changes in the balance of acquired immune anti-viral response. The comparison between intact and inactivated H3N2 influenza virus indicated the importance of maintaining the microorganisms active in the first homeopathic dilutions, in order to preserve the infectious power of viral particles as long as possible. Both homeopathic medicines, H3N2 intact biotherapy and thymulin, were able to modulate the animal's immune response, triggering two different mechanisms according to the potency: a specific switch to humoral immune response against influenza virus antigens, with prophylactic potential, and a specific switch to cellular immune response, that could be efficient after the virus infection.

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