Investigation of Cytokine Expression in Human Leukocyte Cultures with Two Immune-Modulatory Homeopathic Preparations

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ABSTRACT

Background: The efficacy of homeopathic medicines for maintaining human health and treating disease has been extensively examined in clinical trials. However, there is a paucity of preclinical evaluations of the effects of homeopathic medicinal preparations on cellular signaling pathways relevant to the applications of these preparations.

Materials and methods: In this study, the immune-modulatory effects of Phase 6 (for the stimulation of the nonspecific defense system) and Flu Terminator® (for influenza and viral diseases) (Be Well Homeopathics Inc. Miami, FL), two homeopathic preparations developed for the purpose, were evaluated in normal human leukocyte cultures in vitro.

Results: Both Phase 6 and Flu Terminator stimulated the production of pro- and anti-inflammatory cytokines by human leukocytes, although higher doses often produced a weaker response than lower doses. The carrier solvent (20% ethanol) failed to elicit any cytokine synthesis.

Conclusions: The results of the in vitro studies suggested that ultralow concentrations of ingredients in Phase 6 and Flu Terminator were capable of eliciting a human immune response.

INTRODUCTION

Homeopathy stands out as an experimental discipline, as is evidenced by the vast amount of homeopathic data collected over more than 2 centuries. However, homeopathy remains controversial from the perspective of Western medicine because of the apparent inadequacy or inconsistency of data to support use of the ultralow concentrations in which homeopathic medications are given. Consequently, the prevailing attitude does not consider the small amount of ingredients used in homeopathy as being capable of inducing significant changes in classic pharmacologic models.1

Homeopathic research has evolved over the past 20 years with the advent of comprehensive biomedical research methodologies that make subtle effects more amenable to detection (i.e., clinical trials, observational studies, statistical evaluations, computerized storage programs, and instrumental or laboratory testing). Some innovative models have been used to contribute to the debate about the efficacy of homeopathic medications, including demonstration of the efficacy of ultrahigh dilutions of IgE on basophil degranulation, inhibition of basophil degranulation by ultrahigh dilutions of histamine, and other models that incorporate the measurement of cell viability and cytokine induction, which may be diagnostic laboratory techniques for determining the immunologic potency of various homeopathic preparations and may help clarify the clinical benefit of therapies with these substances.2–5

For the past 10 years, homeopathic research has ventured into the evaluation of combination formulas used to treat a...
A variety of clinical conditions.\textsuperscript{6–9} In many respects, this is a considerable challenge, given the controversies surrounding research on single-component homeopathic preparations. However, by focusing on well-established endpoints and using conventional research methodologies, it is possible to elicit meaningful data from well-designed preclinical studies. The field of allergy and immunology has been viewed as a bridge into homeopathy since both disciplines share the concept that modulations in biologic systems are sensitive to very low concentrations of natural or endogenous substances.\textsuperscript{1} Therefore, appropriate models for evaluating multicomponent homeopathic preparations and determining their effects on immune function or mediators of immunity are essential. Traumeel\textsuperscript{®} S (Heel GmbH, Baden-Baden, Germany), an extensively evaluated commercial homeopathic preparation with a combination of ingredients, has been shown to modulate the secretion of interleukin (IL)-1\textbeta, tumor necrosis factor (TNF)-\textalpha and IL-8 in immune cells.\textsuperscript{10} Thus, modulation of mediators of immunity may represent an opportunity to assess appropriate homeopathic mixtures using \textit{in vitro} models. In the present study we attempted to further validate this hypothesis by exploring the effects of 2 multicomponent homeopathic formulas, Flu Terminator\textsuperscript{®} and Phase 6, on a panel of pro- and anti-inflammatory cytokines in human leukocyte cultures.

\begin{table}[h]
\centering
\caption{ Constituents of Flu Terminator and Phase 6\textsuperscript{a}}
\begin{tabular}{ll}
\hline
Flu Terminator\textsuperscript{®} & \\
\hline
\textbf{Gelsemium} & 3x \\
Zincum glaconium & 3x \\
Zincum metallicum & 3x \\
Aconitum napellus & 4x \\
Eupatorium perfoliatum & 4x \\
Phosphorus & 5x \\
Asclepias vincetoxicum & 6x \\
Influenzinum & 12x \\
Anas barbariae hepatis & \\
and cordis extractum & 200K\textsuperscript{b} \\
\hline
Phase 6 & \\
Taraxacum officinale & 1x\textsuperscript{c} \\
Galium aparine & 1x \\
Thuja occidentalis & 3x \\
Aloe vera & 3x, 6x, 12x, 30x \\
Phytolacca decandra & 3x, 11x, 19x, 27x, Euphorbium officinarum 6x \\
Zincum metallicum & 6x \\
Arsenicum album & 6x, 14x, 22x, 30x \\
Viscum album & 8x, 16x, 24x \\
Conium maculatum & 30x \\
Histaminum hydrochloricum & 200x \\
Formicum acidum & 200x \\
\hline
\end{tabular}
\end{table}

\textit{Note:} Plant-based substances are indicated with italics.
\textsuperscript{a}Both products are formulated and manufactured by Be Well Homeopathics Inc, Miami, FL.
\textsuperscript{b}K, Korsakovian dilution, which uses the same container for each serial dilution. The potency numbers that follow the homeopathic remedies are indicative of the number of dilutions and successions from the original mother tincture.
\textsuperscript{c}Dilution of common dandelion.

\section*{MATERIALS AND METHODS}

\subsection*{Preparations and reagents}

Phase 6 and Flu Terminator are homeopathic preparations that were obtained from the manufacturer, Roswell Park Memorial Institute (RPMI) cell culture medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and antibiotics were purchased from Invitrogen, Inc., Corporation, Carlsbad, CA. Enzyme-linked immunosorbent assay (ELISA) reagent kits for human-specific cytokines (IL-1, IL-4, IL-10, and interferon [IFN]-\textgamma and TNF-\textalpha) were purchased from BD Biosciences, San Diego, CA. The constituent ingredients of Flu Terminator and Phase 6 are listed in Table 1.

\subsection*{Isolation of human leukocytes and incubation with Phase 6 and Flu Terminator}

Leukocytes were isolated from human blood samples collected from healthy volunteers using Histopaque 1066 (Sigma Chemical Co., St. Louis, MO) gradient centrifugation.\textsuperscript{9} Cells were washed with PBS and 10\textsuperscript{6} cells/mL were incubated for 24 hours with varying doses of Phase 6 or Flu Terminator (0–200 \textmu L/mL) in RPMI medium supplemented with 10% FBS and antibiotics at 37\textdegree C in a 5% humidified CO\textsubscript{2} incubator. Human leukocytes treated with 0–200 \textmu L of 20% ethanol (the carrier) served as controls in order to determine the carrier effects of Phase 6 and Flu Terminator.

\subsection*{Analysis of cytokine synthesis}

After 24 hours of drug incubation, cell-culture medium was removed by centrifugation at 400 g for 10 min. Cytokines (IL-1, IL-4, IL-10, IFN-\gamma, and TNF-\textalpha) synthesized and released into the medium by leukocytes were estimated by ELISA, using reagent kits from BD Biosciences. Briefly, 100 \textmu L of medium was used for a multiwell ELISA proto-
col according to the manufacturer’s recommendations, and the synthesis of cytokines (in concentrations of pg/mL) by the leukocytes was quantified. The experiment was repeated thrice for estimation of standard deviation values, and the estimates of cytokine concentrations were analyzed statistically through analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Phase 6 induced the production of TNF-α (Fig. 1) and IFN-γ (Fig. 2) at doses of Flu Terminator and Phase 6 ranging from 10 µL to 100 µL, with a decrease in production at 200 µL (p < 0.01). However IL-1, IL-4, and IL-10 were not induced by Phase 6. The cells treated with increasing quantities of 20% ethanol failed to show any cytokine production, indicating that the effect of the two homeopathic preparations was not due to the solvent carrier.

Flu Terminator induced production of IL-10 and TNF-α (Figs. 3 and 4), apparently in a dose-dependent manner (p < 0.01). However, Flu Terminator failed to induce synthesis of IL-4 and IFN-γ. The cells treated with increasing quantities of 20% ethanol failed to show any cytokine production, indicating that the effect was not due to the solvent carrier.

Although a growing body of clinical research supports the existence of biologic effects of homeopathic treatments, the principles governing these interventions remain elusive despite abundant theories. The principles of homeopathy will not achieve mainstream medical acceptance without homeopathic preparations being subjected to stringent evidence-based research using conventional methods. However, it is equally essential to recognize that bias by some scientists who dismiss outright the effects of homeopathic preparations as incredible or owing to placebo because the dilutions are so great is not an evidence-based approach to their efficacy.

Both Phase 6 and Flu Terminator are mixtures of highly diluted extracts and minerals that range from 1/10 of the starting material to high dilutions with concentrations beyond (smaller than) Avogadro’s number ($6.23 \times 10^{23}$), which corresponds roughly to a $24\times$ dilution. Both preparations are recommended for general immune defense and for the treatment of viral illnesses, respectively. They were therefore appropriate preparations for evaluating their effects on mediators of immunity, which in our study consisted of pro- and anti-inflammatory cytokines.

The results obtained in our study were reproducible and statistically significant. While it is not possible to draw a specific conclusion other than to note the presence of a biologic effect, several points are noteworthy. In the case of Phase 6, two important proinflammatory cytokines associated with the T-helper 1 (Th1) pathway showed a “U”-shaped response pattern, and not in the dose–response manner typical for a pharmaceutical agent. None of the cytokines associated with the Th2 pathway that were studied were elevated. This is an important observation, suggesting that the effects of the two homeopathic preparations that we studied are not random. Rather they seem to reflect a selective effect on specific biologic pathways in the activation of proinflammatory cytokines.

In the case Flu Terminator, the concentrations of TNF-α, a proinflammatory cytokine, and IL-10, an anti-inflammatory cytokine, were both elevated again, with a “U”-shaped response pattern. While this may appear contradictory, it is important to be aware that the Th1 and Th2 pathways of immune response act in a complementary manner in response to various infections, thus providing a mechanism for immune modulation. The data in our study in no way address the timing of these responses, but do suggest that Flu Terminator may play a role in activation and modulation of the immune response associated with infection.

In either case, skepticism associated with the possibility that ultralow concentrations of homeopathic medications can modulate the expression of these cytokines, irrespective of the theoretical mechanism by which they do this, must be reconsidered. Examination of principles of immunology may provide some support for these observations. Studies of the effects of ultralow concentrations of various sub-
stances have clearly demonstrated reproducible biologic effects in vitro and in vivo.\textsuperscript{2–10,12,13} The concentrations of cytokines required to induce effects in vivo have been found to be in the femtomolar range (10\textsuperscript{-15} M), clearly within the range of homeopathic preparations.\textsuperscript{14–16} Such concentrations are at the lower limits of detection by standard laboratory assays, and whether these preparations can demonstrate biologic effects unequivocally without inferring a concentration (theoretical determination) is therefore unclear. However, there are examples of concentrations of cytokines that demonstrate biologic effects clearly, which have been indirectly determined. These include the inhibition of basophil deregulation by ultralow concentrations of histamine, with confirmation of receptor specificity, and other effects on immune function in vitro of other bioactive compounds at ultralow concentrations.\textsuperscript{4,5} It is also noteworthy that antigens that have immunostimulatory effects may elicit these effects at femtomolar or even subfemtomolar concentrations.\textsuperscript{17–19} Clearly, at least in the case of immune function, it does appear that cells in vitro normally respond to signals associated with ultralow concentrations of bioactive molecules, thus lending some support to the principles of homeopathy.

**CONCLUSIONS**

The impact on cytokine expression of Phase 6 and Flu Terminator in our study lends additional support to the growing body of evidence that dilutions of extracts and minerals do affect biologic systems. Furthermore, such studies also support the possibility that homeopathic agents, as in the case of pharmaceuticals, may affect specific molecular targets, thus defining potential medical applications for these agents. The controversy over the efficacy of low concentrations of homeopathic agents will, however, probably remain until more adequate techniques become available to assess molecular interactions at such concentrations; only then will more reliable models for evaluating effects of homeopathic dilutions be established and expand our knowledge of the physical and biological basis of these interactions.

**REFERENCES**

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