Effect of sulfurous (thermal) water on T lymphocyte proliferative response
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Salvatore Valittutti, Flora Castellino, and Piero Musiani

We studied the effect of sulfurous water thermal therapy on the phenotype and the proliferative response of peripheral lymphoid cells from ten subjects affected by chronic upper respiratory disease and from six suffering from articular and periarticular disorders. Sulfurous water (S-H2O) therapy did not modify the phenotype and function of peripheral blood mononuclear cells (PBMC) nor did it modify systemic immunologic reactivity. A different result was obtained by analyzing the response to mitogens of peripheral blood mononuclear cells in cell cultures containing graduated amounts of S-H2O. These “in vitro” studies have shown an important dose-dependent inhibitory effect of S-H2O on mitogen induced T lymphocyte proliferation and on [IL2 production. H38 present in S-H2O seems to be the primary component responsible for inhibition. Our results are consistent with a local immunosuppressive role Q/S-H2O, which may explain part of the observed therapeutic effect of inhalation therapy on upper respiratory allergic disorders.

INTRODUCTION
The use of thermal spring water therapy has been a tradition for centuries, not only for its hygienic aspects, but also for its therapeutic properties. Any possible therapeutic benefit resulting from the use of thermal therapy has been based on empirical data that have reported mainly subjective sensations of improved health in subjects complaining of various disorders with often complex and/or heterogeneous etiology. The rare reported data confirming the effectiveness of spa therapy are available in clinical and epidemiologic studies. Considering the difficulty in finding any scientific foundation on which to base the validity of spa therapy, the beneficial effects frequently obtained in patients treated with thermal therapy have been ascribed to the geographic locations of these spas, immersed in nature, and to a reduction or absence of stress more than to an actual therapeutic activity of thermal water. In any case, therapy using sulfurous water (S-H2O) is often prescribed because it is considered clinically valid in treating chronic inflammations of immunoallergic pathogenesis of various organs and systems. In fact the application of S-H2O through inhalation and mud baths is widely used in the treatment of inflammatory diseases of the skin, of the upper respiratory tract, and of the joints with reasonable clinical benefits. It is possible that certain substances present in the water may have a disinfecting, cleaning and liquefying effect on external secretions, but it is also possible that the same substances influence the immune response against a wide range of infectious agents. In order to evaluate the possible immunomodulating activity of S-H30, we studied its effect on the phenotype and on the proliferative response of lymphoid elements of peripheral blood in subjects with chronic diseases who undertook thermal therapy. In addition, we evaluated the proliferative response to mitogens of lymphoid cells in the presence of graduated amounts of S-H30 in order to reproduce “in vitro” conditions similar to those created locally during therapy. Our results suggest a local immunosuppressive role of S-H2O, which appears to be an interesting modulator of immunologic reactivity, and which can be used in the study of the complex interactions between cells of the immune system. Sulfurous water therapy does not appear to modify the phenotype and function of peripheral blood mononuclear cells (PBMC) nor does it seem to modify systemic immunologic reactivity. On the other hand, in vitro studies indicate that S-H2O, and principally the sulfuric acid it contains, have an inhibitory effect on T cell activation and proliferation induced by mitogens. These results suggest, therefore, that thermal therapy with S-H2O can provide beneficial effects in chronic inflammatory disorders with an immunologic pathogenesis by inhibiting the immune response at a local level.

MATERIALS AND METHODS
Sixteen patients (nine males and seven females), ten affected by different chronic or recurring upper respiratory inflammatory diseases and six with articular and periarticular diseases, were randomly selected for this study. In addition there were eight healthy young people as voluntary controls. Peripheral blood samples were drawn to obtain PBMC. Cells and Culture Conditions Peripheral blood mononuclear cells were isolated on a Ficoll-Hypaque density gradient. Cells were washed, resuspended, and cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum at a cell concentration of 1 >< 10^6 cells/mL in the presence of PHA (0.3 rig/mL) (Wellcome Laboratories, Beckenham_UK), or of anti-CD3 monoclonal antibody (MoAb) (OKT3: Ortho Diagnostic, Raritan, NJ) (2.5 ng/ mL). In the experiments of mixed lymphocyte reaction (MLR). PBMC were cultured in RPMI 1640 medium supplemented with 10% fetal calf
serum at a cell concentration of 1 X 10⁶ cell/mL in the presence of lethally irradiated (5 ≥ 105 RADS) heterologous 5 ≥ 105/mL PBMC. **Monoclonal Antibodies Anti-CD25 MoAb (II-IT4-4H3)’ was a gift from Dr. E. L. Reinherz (Dana Farber Cancer Institute, Boston, MA). Anti-CD3 (OKT3), AntiCD2 (OKT1), Anti-CD4 (OKT4), and Anti-CD8 (OKT8) were purchased from Ortho Diagnostics (Raritan, NJ), and Anti-CD19 (Anti-LEU 12) and Anti-M5 (AntileEU M5) from Becton and Dickinson (Mountain View, CA). Immunofluorescence Staining Cells (1 ≥ 10⁵) were incubated with human AB serum for 20 minutes and subsequently stained for 30 minutes at 4 °C with the appropriate MoAb or irrelevant isotype-identical control antibody. A second incubation was performed on washed cells for 30 minutes at 4 °C with fluorescein-conjugated (Fab’2) goat anti-mouse Ig antibody (Cappel Laboratories, Cochranville, MD). The cells were then washed twice and analyzed on a Spectrum III cytofluorograph (Ortho Instruments, Westwood, MA). Washing medium contained 10% human AB serum. **Cytokines** Purified human recombinant IL1-alpha (specific activity 1 to 2 ≥ 10⁵ it U/mg protein) was generously donated by Dainippon Pharmaceutical Co (Osaka, Japan). One unit of IL1 was defined as the amount sufficient to give half maximal response on mouse thymocyte costimulator assay compared with our laboratory standard. Purified human recombinant (rec) IL2 preparation was obtained from Genzyme Corporation (Boston, MA). The IL2 activity was evaluated as the ability to support the proliferation of the IL2-dependent murine T cell line (CT-L-L) BD. 2.118.803 The IL2 titer was calculated by using a probit analysis computer program to define the reciprocal titer of the test sample that gave 50% of maximal cpm of our laboratory standard which was calibrated against the Biological Response Modifiers Program reference reagent human IL2 (Jurkat). (Lot ISDP-841, containing 500 reference units/mL). Recombinant IFN-gamma was kindly provided by Dr. M. Bruda (Roche, Nutley, NJ). The antiviral activity of the IFN-gamma preparation was determined before each test by inhibition of the cytopathic effect on WISH cells. An international laboratory standard of recombinant human IFN-gamma was always included in order to express the titers in international units (IU/mL). The titers of the preparations of recombinant IFN-gamma used here were between 6 and 10 ≥ 107 IU/mL. All recombinant reagents were found to contain less than 0.05 ng/mg protein of lipopolysaccharide by the Limulus Amebocyte Test (M. A. Bioproducts Wolkersville, MD). **Proliferation Assays** Peripheral blood mononuclear cells were incubated at 37 °C for 72 hours (for mitogenic assay) or 144 hours (for MLR). Cultures were established in microtiter plates (microtest II, Falcon 3040) in triplicate and consisted of 0.2 mL complete culture medium containing 2 ≥ 105 cells/well. Six hours before termination of the cultures 0.5 mCi of 3H-thymidine (2 Ci/mmol, Amersham International, Amersham, UK) was added. At the end of incubation period the cells were harvested on a microcell harvester (Titertek, Flow Laboratories, Irvine, Scotland) and the SH-thymidine incorporated into DNA was measured by counting the samples in a liquid scintillation counter. **Production of C all are Supernatants from PBMC C and Macrophages** Macrophages (Mo) were obtained from PBMC by 2-hour adherence on plastic dishes. Adherent cells were removed by a 10-minute exposure to 0.02% ethylene diaminetetraacetic acid in Ca²⁺ free Hanks’ balanced salt solution. Macrophages (2 ≥ 105/mL) were cultured for 24 hours in the presence of LPS (10 ag/mL) and the supernatant was collected. IL2-containing supernatants were prepared from 36 hours PBMC culture (2 ≥ 10⁶ cell/mL) carried out in the presence of P1.1A. IL1 and IL2 activities were evaluated as reported above. **Statistical Analysis** Group comparison for determination of significance was done using Student's two sample t test with P<.05 as the significance threshold. **RESULTS** Peripheral blood mononuclear cells were obtained from eight normal subjects and from 16 patients who were subjected to thermal treatment. Ten of the patients were treated with a 14-day cycle of inhalatory therapy for upper respiratory disorders and six with applications of mud therapy for articular and periarticular diseases. The peripheral blood samples were drawn before and after the therapeutic cycle. The phenotypic studies done on PBMC did not show a significant difference in the expression of the various surface antigens (Table 1), suggesting that inhalatory or mud therapy does not induce alterations in the lymphoid subsets. Similarly, the proliferative response to mitogens of patient PBMC was not affected by the S-H2O therapy. A different result was obtained by analyzing the response to mitogens of lymphoid elements in cell cultures containing graduated amounts of isotonic sulfurous water or physiologic solution. As shown in Figure 1 there was an inhibition of lymphocyte proliferation depending on the percentage of S-H2O present in the culture medium. Controls that used 5% and 10% physiologic solution showed no significant inhibition of T cell proliferative response, suggesting that the inhibitory effect exerted by isotonic S-H2O was not due to an alteration of culture conditions such as reduction of serum protein content or modification of mineral
Similar levels of proliferative activity inhibition were found in mitogen-stimulated PBMC from healthy subjects and from patients before and after therapy. The inhibitory activity of S-H2O was more evident when a weaker and relatively more physiologic stimulus was applied, such as the antibody anti-CD3 compared with that exerted by PHA. In fact, the resulting inhibition in anti-CD3 MoAb-stimulated cultures was 73%, and 85% with the respective presence of 5% and 10% of S-H2O. The inhibition obtained in cultures stimulated with PHA did not exceed 24% (with 5% of S-H2O) and 35% (with 10% of S-H10). It is important to note that the inhibition was proportionate to the amount of S-HZO added to the cultures.

The reduced proliferative response of lymphocytes to mitogens could be due to a direct toxic effect of S-H10 on cells. This possibility was subsequently excluded because cell death, verified at various intervals, was very similar in cell cultures carried out with or without S-I-I2O.

We also observed (Fig 2) that the percentage of inhibition caused by S-I-I20 decreased if the addition of the water to the culture medium was delayed, leading to a reduction of more than 2/3 when the S-H10 was added at 32 hours from the application of the stimulus. A subsequent step in our research consisted in the evaluation of the release of interleukins in the cultures with S-HZO. The production of IL-2 was inhibited by 51 ± 6% and 69 ± 8% in the presence of 5% or 10% of S-H2O, respectively (five experiments). The production of IL I by adherent cells stimulated with LPS sustained no reduction upon addition of 5% and 10% of S-H2O (five experiments).

The results of these experiments were supported by data obtained after the addition of various interleukins to the culture media. The addition of IL 1 rec in various concentrations in our culture systems was unable to remove the inhibition. Conversely, as shown in Figure 3 exogenous IL 2 rec (5 U/mL) almost completely removed the inhibition of the proliferative response of lymphocytes stimulated with anti-CD3. A complete removal of the inhibition was obtained when IFN gamma rec (100 U/mL) and IL 2 rec (5 U/mL) were simultaneously added to the cell culture. IF gamma alone reduced the inhibition by over 60%. The removal of the inhibition of T lymphoid proliferation by IL 2 was not surprising, considering that cells stimulated in the presence of S-H2O expressed IL 2-receptor (CD 25) levels similar to those of stimulated control cells.

Sulfurous water was also effective in inhibiting MLR. In a typical experiment out of six, the 3H-thymidine incorporation after six days of culture was 34.340 ± 1980 SD cpm/well, whereas in the presence of 5% and 10% S-H2O the cpm/well were 26.210 ± 1400 SD and 12.480 ± 920 SD, respectively. If S-I-I20 was added after the third day of MLR culture no inhibition was observed.

Finally we directed our attention to the composition of the thermal water used in our experiments to evaluate the role of its various organic and mineral components on T-cell proliferative response. Our attention was focused on the high concentration of I-I-ZS present in the S-H3O we used. In fact, the very low concentration of other mineral components was considered unable to modify remarkably T cell culture conditions. We subtracted the H15 through acidification of S-H20 to pH 1 and standing at laboratory temperature for one hour because sulfuric acid is not soluble in such a low pH solution, and would therefore be released into the atmosphere. The water treated in this manner showed a significantly lower inhibitory activity compared with sulfurous spring water (Fig 4). The inhibitory activity exerted by S-H2O after this treatment could be due to residue HZS that was not completely subtracted by acidification. In order to evaluate

**Table 1. Phenotypes of Peripheral Blood Mononuclear Cells from 8 Normal Subjects and from 16 Patients Before and After Thermal Therapy**

<table>
<thead>
<tr>
<th>Phenotypes of Cells</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>T Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD2</td>
<td>73 ± 6*</td>
<td>74 ± 9</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>71 ± 8</td>
<td>72 ± 7</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>43 ± 9</td>
<td>44 ± 9</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>29 ± 6</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>B Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD19</td>
<td>11 ± 3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>Monocytes</td>
<td>12 ± 2</td>
<td>13 ± 3</td>
</tr>
</tbody>
</table>

* Results are expressed as % (mean ± SD) of positive cells.

![Figure 1. Effect of S-H2O on mitogen-stimulated lymphocyte proliferative response. Values from a typical experiment, performed in triplicate (mean ± SD), of eight with similar results.](image-url)
better the role of HZS in the inhibition of T cell proliferation, we conducted experiments using cell cultures enriched with chemically produced H157. As demonstrated in Figure 4, in the cultures containing quantities of HQS similar to those found in spring S-H20 (HZS was titrated according to the procedure described in"), there was an inhibition of lymphocyte proliferation similar to that caused by sulfurous water.

**DISCUSSION**

Thermal sulfurous water therapy has long been prescribed for the treatment of various disorders. Upper respiratory ailments in particular seem to benefit from inhalation therapy. It is possible that in addition to the mucolytic and trophic effect on the respiratory mucosa, S-H20 may have a direct effect on the pathogenetic mechanisms of inflammatory disease.

It is well known that treatment with S-H20 or its vapor gives positive results, especially when there is direct contact of S-H20 with the affected area of the body. In agreement with these clinical observations, our evaluation of the immunologic reactivity of subjects treated with S-H20 (mud baths, inhalation, and irrigation) has demonstrated that the most important variables of systemic immunologic reactivity do not undergo modifications after treatment. In contrast, "in vitro" studies have shown an important dosependent inhibitory effect of S-H20 on the proliferative response of T lymphocytes to mitogens.

A relevant inhibition was observed in cells stimulated with antiCD3 MoAb a mitogen that acts by binding the CD3-T cell receptor complex, thereby exerting a more specific stimulus than that caused by phytoimmunotions. It has been reported recently that human CD4+ helper lymphocytes can be divided into two functional subsets on the basis of antibodies that bind to restricted leukocyte common antigen (CD45) epitopes." It has also been proposed that these subsets represent naive (CD45 RA+) and memory (CD45RO+ or UCHL 1+) T cells. It appears that these phenotypically different subsets differ functionally in activation requirements and lymphokine secretion. Memory cells respond much better than naive cells to activation by agonist MoAb that binds to CD3 receptors. Activation of memory cells leads to secretion of large amounts of IFN gamma while activation of naive cells results in little or no secretion. In contrast, naive and memory cells produce comparable amounts of IL2. The inhibition exerted by S-H20 in our cell cultures appears more evident in anti-CD3 activated cells rather than in those stimulated by PHA. This inhibition could therefore exert itself principally on the T memory subset. Further, the experiments involving the addition of interleukins to cell cultures may be interpreted in this way. In our system IFN gamma seems to play a fundamental role since the addition of this lymphokine reduces inhibition by 60%. Furthermore, S-H20 modulates negatively the production of IL2 and probably IFN gamma, a lymphokine produced mainly by T memory cells. The inhibitory activity of S-H20 seems to exert itself in a specific moment of the activation process leading to T cell proliferation and to manifest itself through a diminished production.
and release of interleukins. In fact the delayed introduction of S-H20 into cell cultures (approximately 30 hours from the stimulus) had a poor effect on lymphocyte proliferation. Sulfurous water does not inhibit the function of accessory cells and in particular their production and secretion of IL1.

Our results suggest that, in our system, S-H30 acts selectively on T cells more specifically on T memory cells. It should be noted that H25 present in S-H20 seems to be the primary component responsible for the inhibitory activity. In fact, cell cultures containing H28 show an inhibitory effect directly correlated to its concentration. In conclusion, part of the therapeutic activity, exerted at the local level by application of S-H20 or its vapor in patients with various chronic inflammatory disorders, may result from the reduction of interleukin production and secretion by T lymphocytes. Inhibition of interleukin production by T memory lymphocytes whose relatively greater frequency has been associated with perseverance of several disorders attributed to altered immunologic reaction seems relevant.

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