**Immunomodulation by SanPharma Fungal Metabolic Products**

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**ABSTRACT**

**Objective:** In the present study, a series of fungal metabolite products from SanPharma (Dohren, Germany) were tested for effects on human peripheral blood leukocytes *in vitro* using standard immunologic methods.

**Background:** Therapeutic strategies used in German biological medicine often include treatment (oral, nasal, rectal, topical, or injection) with fungal or bacterial products, also known as “isopathic remedies,” of which some are limited to metabolic products, whereas others include microbial cell lysates and cell wall fragments as well. The SanPharma products are based on metabolites, and do not contain microbial cell wall compounds.

**Methods:** Activation of natural killer (NK) cells was evaluated by cell surface immunostaining using CD3, CD56, CD69, and CD25 monoclonal antibodies. Production of interferon-γ was evaluated by enzyme-linked immunosorbent assay (ELISA) on supernatants collected after 5 days’ culture of peripheral blood mononuclear cells (PBMC). Direct mitogenic effect was assessed using the lipophilic membrane dye PHK26 (Sigma-Aldrich, St. Louis, MO) in a fluorescence-based proliferation assay, in which fluorescence intensity is reduced upon cell divisions. Cell viability upon exposure to fungal metabolites was assessed using propidium iodide staining and flow cytometry.

**Results:** All fungal metabolite products specifically induced the expression of the CD69 marker on human CD3-negative, CD56–positive NK cells, but not CD3-positive T cells, *in vitro*, as shown by the induction of the CD69 marker on up to 50% of NK cells after 18 hours’ culture with metabolites. Only one of the five metabolite products, Roqueforti, induced cyclooxygenase-2 (COX-2), indicating that nuclear factor–kappaB (NFκB)-mediated signaling may not have been involved in the NK activation by the other four products. The Notatum product reduced baseline levels of COX-2, indicating an anti-inflammatory effect. No evidence of toxic or mitogenic effects was found.

**Conclusions:** The fungal metabolite products from SanPharma specifically activate human NK cells *in vitro*.

**INTRODUCTION**

This study was performed to assess some fundamental issues regarding certain products associated with the therapeutic principles of German biological medicine. These often include the use of microbial and fungal products, also known as isopathic remedies. These remedies are manufactured from microbial cultures, according to nonmainstream principles, and although dilute, are not homeopathic. Despite their extensive use in Europe, and some expansion on the North American natural products market for complementary cancer treatments, the use of such products is often associated with a belief in alternative biologic concepts. To date, no peer-reviewed research has addressed efficacy of these type of product, despite plenty of parallel mainstream cancer research that has pointed to the interesting possibilities of general and tumor-specific immunomodulation using microbial products, in new offspring of vaccine strategies and immune support.

Mainstream cancer treatment is currently embracing new treatments based on findings that certain known microbes are able to trigger immune reactivity through molecular mimicry; that is, similarities between microbial antigens and cancer cell antigens. Many patients with cancer express some degree of anergy; they are unable to respond to recall antigens because of a reprogramming of the T cells that are specific for such antigens. In other words, lymphocytes with specific receptors...
toward certain antigens, presumably including tumor cell antigens, are energized—they receive signals to remain unresponsive. The induction of specific immune reactivity toward tumor cells via novel microbial-based vaccine strategies is receiving substantial interest, and include the current treatment of choice for superficial bladder cancer using Bacillus Calmette-Guerin (BCG) vaccine, as well as several experimental vaccine strategies for breast cancer based on mycobacteria and Staphylococcus. Other experimental models include mycobacterial-based vaccines for non–small-cell lung cancer and mesothelioma and melanoma.

Despite these exciting new turns in modern cancer treatments, the terminology surrounding the microbial-based isopathic remedies used in German biological medicine remains predominantly based on older concepts of microbiology, on research performed before deoxyribonucleic acid (DNA) was discovered and basic aspects of the immune system were established. The most striking difference between these simplified views and modern microbiology is the realization that obscure microbial presences may be contributing factors in a number of diseases, and that microscopic assessment of morphology alone is not sufficient for conclusions regarding such microbial involvement, chemical composition, gene expression, and pathogenicity.

From a chemical perspective, the SanPharma fungal products tested in this study should be viewed as a suspension of fungal metabolites, based on cultures of the fungal species behind each product. Fungal metabolites remain the focus of intense research efforts in both the pharmaceutical and natural products industry. Several classes of molecules of pharmaceutical interest are fungal metabolites, including toxins, antibiotics including novel blockers of viral enzymatic activity, and anti-inflammatory and anti-cancer compounds, including specific inhibitors of NFκB transcriptional activity.

Of interest to therapeutic approaches to chronic inflammatory diseases is the finding that some fungal metabolites possess potent anti-inflammatory activity, and that some are able to inhibit an inflammatory response without causing generalized immunosuppression. This prompted the authors to examine whether this type of situation could be found for the SanPharma fungal metabolite products (Table 1).

This study was performed to assess some fundamental issues regarding the SanPharma fungal products, and evaluate whether any effects could be identified using standard immunologic methods. The test panel included cell viability, induction of proliferation, natural killer (NK) cell activation, and production of interferon-γ. No evidence of toxic or mitogenic effects was found. It was found that all fungal metabolite products tested possessed the ability to specifically activate human NK cells in vitro.

**MATERIALS AND METHODS**

**Fungal remedies**

The following products were obtained from the SanPharma distributor BioResource Inc. (Cotati, CA): Mucor, Notatum, Quentans, Roqueforti, Aspergillus, and Kombination, in 10-mL bottles of “4X” (i.e., 10⁴) dilute in a base of purified water, 0.09 g sodium chloride, and 0.01 g potassium sorbate. Initially, all products were added to human cell cultures in serial dilutions from 10-fold down to 10,000-fold dilution. Subsequent experiments used only 1:10 dilutions, as further dilutions did not produce significant effects.

**Buffers, serum, and reagents**

The following were obtained from Sigma-Aldrich (St. Louis, MO): Dulbecco’s phosphate-buffered saline without calcium and magnesium (#D8537), RPMI 1640 medium without phenol red (#R7509), Histopaque-1077 (#H8889), fetal calf serum (FCS) (#F6178), phytohemagglutinin (PHA) (#AL-4144), human recombinant interleukin-2 (hu rIL-2) (#IL-7908), and the PKH26 red fluorescent cell linker mini-kit (Sigma-Aldrich #MINI26). The author’s in-house immuno-

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**Table 1. List of Product Names, Fungal Species, and Natural Occurrence, Regarding the Tested Fungal Metabolites**

<table>
<thead>
<tr>
<th>Product name*</th>
<th>Species name</th>
<th>Phylum</th>
<th>Natural occurrence</th>
<th>OSHA comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucor</td>
<td>Mucor racemosus</td>
<td>Zygomycota</td>
<td>Mold (soil/fruit/juices/dust/manure)</td>
<td>Allergen, irritant, hypersensitivity pneumonitis, dermatitis</td>
</tr>
<tr>
<td>Notatum</td>
<td>Penicillium notatum, syn. Chrysogenum</td>
<td>Ascomycota</td>
<td>Mold</td>
<td>Allergen, irritant, hypersensitivity pneumonitis, dermatitis</td>
</tr>
<tr>
<td>Quentans</td>
<td>Penicillium frequentans, syn. glabrum</td>
<td>Ascomycota</td>
<td>Blue-green mold (bread, cheese, fruits, nuts)</td>
<td>Used for production of blue cheese, proteases and ketones</td>
</tr>
<tr>
<td>Roqueforti</td>
<td>Penicillium roquefortii</td>
<td>Ascomycota</td>
<td>Mold (soil, decaying plant matter)</td>
<td>Produces aflatoxin</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Aspergillus niger, Mucor racemosus, Mucor niger</td>
<td>Ascomycota</td>
<td>“Black mold”</td>
<td></td>
</tr>
</tbody>
</table>

*All products from BioResource Inc., Cotati, CA, distributor for SanPharma. OSHA, Occupational Safety and Health Administration.
fluorescence (IF) buffer was prepared using PBS with 0.01 g/mL bovine serum albumin and 0.05% sodium azide. The following reagents and monoclonal antibodies were obtained from Becton-Dickinson (San Jose, CA): FACS lysing solution (10x), CD45-FITC, CD14-FITC, CD3-PerCP, CD56-PE, CD56-FITC, COX-1-FITC/COX-2-PE, and isotype control antibodies.

Cells

Peripheral venous blood from healthy human volunteers was obtained after informed consent by venopuncture into sodium heparin-coated vacutainer vials. Immediately upon blood draw, human peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation, in which 5 mL of whole blood was layered onto 5 mL of Histopaque-1077, and centrifuged at 400 g for 25 minutes. The PBMC-rich interface was harvested, washed twice, and used in the assays described in the following.

Proliferation

Freshly purified PBMC were stained with PKH26 according to the manufacturer’s protocol. In brief, cells were resuspended in the provided Diluent C at a concentration of 20 x 10^6/mL, and added to an equal volume of freshly prepared working concentration of PKH26 in the provided diluent. The cells were mixed by pipetting for 30 seconds, and gently agitated by rotation for the remaining 4.5 minutes of the incubation with PKH26. FCS was added, incubated for 1 minute under gentle agitation, after which 10 mL of RPMI with 10% FCS was added. Cells were washed four times in RPMI, resuspended at 10^6 cells/mL in RPMI with 10% FCS, 1% L-glutamine, and 1% penicillin/Streptomycin. Cells were distributed in sterile U-bottom 96-well microplates (NUNC, Denmark) at 200 μL/well. Ten (10) microliters of fungal metabolite products were added in triplicate. Negative controls consisted of untreated wells in triplicate. Positive controls were treated with either PHA (2 μg/mL) or human recombinant IL-2 (range, 50–500 U/mL). Culture plates were incubated at 37°C, 5% CO₂, for 5 days, removed from the incubator, transferred to flow cytometry vials in 200 μL RPMI, and acquired by flow cytometry. Analysis of proliferation was conducted using the ModFit 2.0 software, in which the amount of cells remaining in the parent population was compared to cells that had divided and thus lost fluorescence intensity, as PKH26 is shared between daughter cells.

Induction of the CD69 NK-cell–activation marker

Freshly isolated PBMC were resuspended in RPMI 1640, and aliquoted into U-bottom NUNC microwell plates at 10^6/mL. Negative control wells were left untreated. Fungal metabolites were added at 1:10 final dilutions in quadruplicate for each product. Plates were incubated at 37°C, 5% CO₂ overnight (18 hours). The following morning, the plates were washed, and cells resuspended in IF buffer. For each set of wells, all wells were stained with CD3 and CD56, one well was used for staining with an isotype control antibody, and the remaining three wells were stained with CD69. Monoclonal antibodies were added, incubated for 10 minutes at room temperature, washed twice, fixed in 1% formalin, and acquired by flow cytometry. Analysis of CD69 fluorescence intensity on CD3-negative, CD56-positive NK cells versus CD3-positive T cells was performed using CellQuest Pro (Becton-Dickinson, San Jose, CA) and FlowJo (Tree Star, Ashland, OR).

Induction of cyclooxygenase-2 (COX-2) expression

Freshly drawn whole blood was aliquoted in 200 μL aliquots into U-bottom NUNC microwell plates. Negative control wells were left untreated. Positive control wells were treated with bacterial lipopolysaccharide LPS at a concentration of 0.2 μg/mL. The plates were incubated at 37°C, 5% CO₂, for 5 hours to allow the induction of COX-2 enzyme in neutrophils and monocytes. Samples were washed in saline containing sodium azide, and permeabilization of leukocytes with simultaneous lysis of erythrocytes was performed in a two-step procedure, as described by Ruitenbergen and Waters. In brief, 165 μL of FACS lysing solution was added to each sample, and incubated for 10 minutes in the dark at room temperature. The cells were pelleted by centrifugation, the FACS lysing solution was removed, and FACS lysing solution containing saponin was added to all wells. The plates were incubated at 10 minutes in the dark at room temperature, centrifuged, supernatant removed, and cells washed in IF buffer. After the wash, 50 μL IF buffer was added to each well, and a COX-1FITC/COX-2PE monoclonal antibody cocktail was added. The samples were incubated for 30 minutes at room temperature in the dark, washed once, and fixed in formalin. Analysis of the amount of intracellular COX-2 enzyme present in neutrophils and monocytes was determined using the CellQuest Pro software.

Microbial testing

Testing was performed to assess whether live fungal organisms were present in any of the fungal metabolite products, using standard quality control methods based on the 3M microbiology system. In brief, 1 mL of fungal metabolite product was plated onto a 3M yeast and mold film, and the film was sealed according to the instructions from the manufacturer. As a negative control, 1 mL of distilled water was plated in parallel. Films were incubated for 5 days and visually inspected for colony formation.

RESULTS

The SanPharma fungal metabolites activate human NK cells in vitro

All fungal metabolic products induce activation of human CD3−, CD56+ NK cells, as seen by the upregulation of the
NK cell activation marker CD69 (Fig. 1). In contrast, no up-regulation of CD69 was seen on cells within the CD3\(^+\) T lymphocyte subset.

The data also were evaluated for any changes in the expression of CD25 (IL-2 receptor) on CD3\(^-\)CD56\(^+\) NK cells, CD3\(^+\)CD56\(^+\) T cells, and CD3\(^+\)CD56\(^-\) T cells. No changes were observed in fluorescence intensities of untreated versus treated CD3\(^+\)CD56\(^-\) cells (data not shown). Furthermore, no induction of interferon-\(\gamma\) was found.

**COX-2 in human peripheral blood monocytes or neutrophils**

As the possibility existed that the SanPharma products could contain some proinflammatory cell wall components, despite the claim that they only contain metabolites, the authors assessed whether the products induced the production of COX-2 enzyme in human neutrophils and monocytes. As a positive control, bacterial lipopolysaccharide (LPS) was used. It is known that LPS induces a strong expression of COX-2 in monocytes, and a weaker induction in neutrophils. It was found that neither Mucor, Quentans, nor Aspergillus induced COX-2 (Fig. 2). In contrast, Roqueforti induced COX-2. Notatum reduced baseline levels of COX-2 expression.

**None of the fungal metabolic products induces mitogenesis**

The ability of the fungal metabolites to induce mitosis was evaluated using human PBMC in 5-day cultures with and without fungal metabolites, PHA, and IL-2. This was to assess for a possible superantigenic effect, similar to certain lectins. There was no induction of cell proliferation after 5-day exposure to any of the fungal metabolites (Table 2). There was no interference between fungal metabolites and the proliferative responses to either PHA (see Table 2) or IL-2 (data not shown).

**FIG. 1.** Expression of the CD69 cell-surface antigen on CD3\(^-\)CD56\(^+\) natural killer (NK) cells versus CD3\(^+\) T cells after 18 hours in culture in the presence of fungal metabolites. Untreated cells show the baseline expression of CD69. A. The mean fluorescence intensity for CD69 expression on each cell subset is shown as mean ± standard deviation of triplicate samples. Comparison between treated and untreated samples was highly significant for Quentans and Mucor (distributed by BioResource, Cotati, CA, for SanPharma, Dohren, Germany) \((p < 0.001)\), and only marginally significant for all other product \((p < 0.2)\). The data shown are representative of three similar experiments performed on peripheral blood mononuclear cells (PBMCs) from different donors. The level of CD69 expression on untreated NK cells reflects the proportion of circulating activated NK cells in a donor at the time of sampling, and typically was between 5\% and 15\% in samples from healthy donors. B. Flow cytometry dot plots showing the raw data for CD69 versus CD56 fluorescence intensity on CD3\(^-\)CD56\(^+\) PBMCs are shown for representative samples among the triplicates for untreated samples as well as samples treated with fungal metabolite products. The induction of CD69 on NK cells is specific for the CD56\(^{dim}\) subset, and is not seen on the CD56\(^{bright}\) subset.
No evidence of short-term toxicity by fungal metabolites to human white blood cells in culture

The viability of PBMC upon 24-hour exposure to fungal metabolites in culture was assessed by flow cytometry. There was no difference in cell viability between untreated and treated cultures (see Table 2).

Other assessments

Microbiologic testing showed that the products contain no live fungal organisms (data not shown).

DISCUSSION

The data presented here provide a first step toward evaluating by standard immunologic methods the effects of certain microbial-based products; that is, the so-called isopathic remedies, frequently used in German biological medicine. It was found that all the fungal metabolite products tested were able to activate NK cells, but different mechanisms may be involved in the activation by different products. The assay used for this project focused on the well-characterized rapid and transient upregulation of the CD69 cell surface marker on NK cells upon activation.39,40 On other cell types, such as circulating T-regulatory cells, there is a debate about the significance of CD69 expression, and some evidence

<table>
<thead>
<tr>
<th>Table 2. Proliferation and Viability</th>
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<tr>
<td>Treatment</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>Mucor</td>
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<tr>
<td>Notatum</td>
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<tr>
<td>Quentans</td>
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<td>Kombination</td>
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*aAll products from BioResource Inc., Cotati, CA, distributor for SanPharma, Dohren, Germany.
points toward CD69 as a regulatory molecule on T cells.\textsuperscript{41} However, on activated NK cells, the expression is an indication of recent activation. Therefore, the authors analyzed in parallel two cell subsets: CD3$^-$CD56$^+$ NK cells, and the CD3$^+$ T-cell subset. It is clear that the exposure to fungal metabolites used in this study triggered CD69 expression only on the CD3$^-$CD56$^+$ NK cell subset, and not on CD3$^+$ T cells. No induction of the CD25 marker was seen on either NK or T cells. The phenotypic changes indicate that the activation appears to be specific for NK cells, but does not explain underlying mechanisms of action, nor does it lead to a cytotoxic activity and less toward cell division.\textsuperscript{42}

One of the molecular mechanisms that leads to NK cell activation includes NF\textsubscript{κ}B, a signaling pathway that also leads to induction of COX-2. Many NK-activating natural products have some proinflammatory effects, containing compounds that induce COX-2. It was found that with the exception of the Roquefortii product, the fungal metabolite products did not induce COX-2 in human monocytes or neutrophils. The Notatum product reduced baseline levels of COX-2, indicating an anti-inflammatory effect in this product. The statistically significant level of NK cell activation, in the absence of other proinflammatory action, is promising.

Some microbial compounds act as superantigens; that is, strong activators of immune cells by a nonspecific cross-linking of certain types of T cell antigen receptors. This potent and rather nondiscriminatory immune activation is interesting as a general immune-supportive strategy, but would warrant severe caution in a number of clinical situations. The authors tested the ability of the metabolites to induce proliferation in PBMC, using an assay that would be able to detect a proliferative response in even a minor subset of PBMC. No evidence was found for a superantigenic effect of the SanPharma fungal metabolite products, as none of the products were able to elicit a proliferative response among peripheral blood lymphocytes.

Based on the data that the SanPharma fungal metabolites are able to specifically activate human NK cells, but do so without mitogenic, inflammatory, or toxic effects, a new rationale is provided for their use in the integrated cancer clinic. Interestingly, several types of novel adjunct cancer therapies are based on vaccine strategies involving microbial products. Some of the experimental strategies include the use of artificially fused microbial and tumor cell antigens. The possibility that some microbial metabolites could adhere to the surface of tumor cells and assist in eliciting a tumor-specific immune response are interesting but speculative at this point.

In terms of mainstream methods of adjunct cancer treatment, the best studied microbiologic products today include the \textit{Staphylococcus} enterotoxins, and the best studied mycoplasma-based vaccine is the Bacillus Calmette-Guerin (BCG), based on attenuated \textit{Myocobacterium bovis} (Table 3). The recent experimentation with reintroduction of certain genes deleted during initial attenuation, has demonstrated that certain secreted low-molecular-weight antigens enhance the protective effects against tuberculosis and increase T-cell activation;\textsuperscript{43} therefore, it can be speculated that these low-molecular-weight antigens also may improve the therapeutic efficacy against bladder cancers. Once the discussion of microbial metabolites is moved from bacterial to fungal cultures, a different dimension is entered. On one hand, fungal toxins are well studied; on the other hand, fungal secondary metabolites represent a vast, relatively uncharted terrain of biosynthetic possibilities. Fungal metabolic products span a wide range of clinical applications, including many antibiotics starting with penicillin, the platin chemotherapeutic drugs, and selective immunosuppressive drugs such as cyclosporine, given after organ transplants to prevent rejection of the transplanted organ. Much pharmacologic and biotechnologic processing research is focused on the discovery of novel fungal metabolites.\textsuperscript{44}

**CONCLUSIONS**

Several different mechanisms of action of the fungal metabolites are possible. The most obvious and likely explanation is that they contain specific immunostimulatory metabolites. In light of clinical use on the fungal metabolites in the integrated cancer clinic, further research should focus on a more detailed evaluation of NK activation, as well as anti-inflammatory effects and possible effects on dendritic cells.

### Table 3. Examples of Microbial-Based Treatments in Mainstream Cancer Treatment and Experimental Strategies

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Organism</th>
<th>Cancer</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td>\textit{Staphylococcus aureus} (enterotoxins)</td>
<td>Breast, liver, lung, melanoma</td>
<td>Experimental models (murine)</td>
</tr>
<tr>
<td>Mycoplasmal</td>
<td>\textit{Mycobacterium tuberculosis}</td>
<td>—</td>
<td>T-cell activation (in vitro)</td>
</tr>
<tr>
<td>Fungal</td>
<td>Experimental \textit{Candida} vaccines</td>
<td>Superficial bladder cancer</td>
<td>Cancer treatment (human)</td>
</tr>
</tbody>
</table>

BCG, Bacillus Calmette-Guerin.
NK ACTIVATION BY FUNGAL METABOLITES

ACKNOWLEDGMENTS

This study was sponsored by BioResource, Inc. (Cotati, CA), the North American distributor of SanPharma products, who wished to assure that biologic effects could be demonstrated and quantified by the use of the products they distribute, using standard immunologic methods. The work was performed at NIS Labs, a private immunology research laboratory specializing in assessment of natural products on immune activation.

REFERENCES

31. Rey-Ladino J, Koochesfahani KM, Zaharik ML, et al. A live and inactivated Chlamydia trachomatis mouse pneumonitis strain induces the maturation of dendritic cells that are phe-

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