Defective Natural Killer and Phagocytic Activities in Chronic Obstructive Pulmonary Disease Are Restored by Glycophosphopetalical (Inmunoferón)

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We have investigated both modifications in natural (innate) immunity caused by chronic obstructive pulmonary disease (COPD) and the effects of a glycophosphopetalical immunomodulator (Inmunoferón) treatment on COPD-associated immunodefficiencies. In a double-blinded clinical trial, 60 patients with COPD received glycophosphopetalical or placebo during 90 consecutive days at oral doses of 3 g/d. Fifty-six sex- and age-matched healthy control subjects were included as a reference group for immunologic parameters. Peripheral blood natural killer (PBNK) cell cytotoxic activity and phagocytic activity of peripheral monocytes/macrophages (Mo/Ma) and polymorphonuclear (PMN) cells were assessed at baseline and then again at the end of treatments. We found both PBNK activity and phagocytic activity to be significantly decreased in patients with COPD compared with levels in healthy volunteers. The treatment with glycophosphopetalical provoked significant stimulatory effects on PBNK cytotoxic activity. This stimulation was not mediated by an increase in CD3+CD56+ NK cells. Further, glycophosphopetalical significantly increased the percentage of monocytes and PMNs that phagocytize Escherichia coli in vitro, as well as increased phagocytic indices. We conclude that peripheral blood cells of patients with COPD show clear defects in natural immunity that are partially rescued by glycophosphopetalical.

Chronic obstructive pulmonary disease (COPD) is a clinically defined respiratory disease with a high prevalence worldwide (1). Although the intrinsic mechanisms involved in the pathogenesis of COPD are partially unknown, it has been shown that COPD is frequently associated with several immune disturbances. Abnormalities in the distribution and in the function of T-lymphocyte (2–5) and natural killer (NK) cell (6–8) subsets in patients with COPD have been documented in the literature. Alterations in effector functions and cytokine production by monocytes and polymorphonuclear cells (PMNs) have been described in patients with COPD (7–14). The pathophysiology of these immune alterations is complex. Several contributory factors may be involved in this immunologic impairment, including cigarette smoking (5, 15–17), age (18), and relapsing viral and bacterial respiratory infections (1, 7, 10). Of the aforementioned factors, the contribution of smoking to observed immunodeficiencies in COPD has proved the most controversial.

The pathogenic relevance of the impairment of phagocytic cells and NK lymphocytes in patients with COPD is unclear. Although patients with defects in NK (19) or phagocytosis activity (20, 21) are highly susceptible to viral or bacterial infections, the relative contribution of these impairments to the increased prevalence of acute respiratory infections in patients with COPD (1, 10) has yet to be established. Epidemiologic studies have implicated recurrence of acute respiratory infections as one of the major factors associated with the progression of chronic airway obstruction (1, 22). In patients with COPD, superimposed acute respiratory infections result in clinical relapses. The argument for the use of immunomodulators in patients with COPD is well supported by clinical evidence of reduced recurrence rates of acute respiratory infections (23–26).

Glycophosphopetalical is an immunomodulator that has been proven to increase NK and some macrophage activities in human and animal models (18, 27). In animal models, the capacity of glycophosphopetalical to potentiate natural (innate) and specific immunity is related to the induction of endogenous production of interleukin-12 (IL-12) and interferon gamma (IFN-γ) while partially inhibiting the production of tumor necrosis factor-alpha (TNF-α) (28, 29). Glycophosphopetalical potentiates the in vitro immune response against some viruses (30) and viral vaccines (31), demonstrating the potential in vivo immunologically mediated antiviral activity of the drug. Whereas the efficacy of glycophosphopetalical in protecting patients with COPD from acute infectious exacerbations is well established (23–26), the mechanism of its ability to rescue immune alterations in humans remains undefined. Because observed changes in natural immunity in COPD may contribute to increased risk of infectious complications, we investigated the effect of glycophosphopetalical on peripheral blood natural killer (PBNK) activity and monocytes/macrophages (Mo/Ma) and PMN phagocyte activity in a large population of COPD patients in a randomized, placebo-controlled, double-blind clinical trial. We also included sex- and age-matched healthy control subjects as a reference group against which to compare the ability of glycophosphopetalical to reverse immunoalterations seen in patients with COPD.

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METHODS

Inclusion and Exclusion Criteria

Subjects of the study were individuals of both sexes between the ages of 40 and 80 with a diagnosis of COPD as defined by the American Thoracic Society guidelines (32) and a Karnofsky score greater than 70. Inclusion criteria. Diagnosis of COPD was defined by the following spirometry parameters: FEV₁/FVC < 70% and FEV₁ between 35 and 70% with no evidence of a significant reversible component as evidenced by a less than 15% (200 ml) change from baseline after bronchodilator challenge. At least two episodes of acute respiratory exacerbations during the previous year were required for inclusion into the study. Respiratory exacerbations were defined as having at least one of the following: worsening productive cough, increasing production of purulent sputum, increasing dyspnea, or pneumonia. Further, subjects must have had a history of smoking at least one pack a day for 20 yr and have quit smoking at least 6 mo before study inclusion.

Exclusion criteria. Subjects unable or unlikely to comply with the study protocol (e.g., drug or alcohol dependence) were excluded. Female subjects were excluded in the case of pregnancy, breast feeding, or refusal to use a standard birth control method. Subjects with previously identified allergies to Immunoferon (glycophosphopeptical) or one of its components were excluded.

Subjects with congenital or acquired immunodeficiencies or autoimmune disease were also excluded. In addition, subjects with the following diagnoses were excluded: malabsorption syndrome, hypercalcemia, bronchiectasis, active pulmonary tuberculosis, pulmonary or extrapulmonary neoplasm, cystic fibrosis, restrictive pulmonary fibrosis, cardiac insufficiency greater than or equal to class III; advanced renal insufficiency (serum creatinine > 4 mg/dl) or liver disease with a Child-Pugh score of 7 to 15 (Grade B or C).

Subjects who in the 3 mo before the study received immunosuppressants, immunomodulators, cimetidine, or other medications considered to modify the immune response except inhaled corticosteroids (maximum dose of 800 μg of beclomethasone or equivalent dosing of other inhaled corticosteroids) and subjects who received systemic corticosteroid therapy within 2 wk before entrance into the study were excluded. Further, any subjects who experienced an acute respiratory exacerbation treated with antibiotics within the month before the study and those with a clinical destabilization within 15 d of the study were not included.

Subjects

In a double-blind clinical trial 60 patients with COPD as defined previously who were randomized to receive glycophosphopeptical (n = 30) or an indistinguishable placebo (n = 30). Subject characteristics are shown in Table 1. Before study entry, all recruited subjects received extensive information about the study, were given the opportunity to ask questions, and signed a witnessed informed consent. An age- and sex-matched control group of 56 healthy, nonsmoking donors with a mean age of 61 ± 8 yr was used for the immunologic studies. The study was approved by the Research and Ethics Committee of Alcalá University.

The study began in September 1998, before the onset of winter in Spain. Peripheral venous blood was collected from all patients with COPD and healthy volunteers to assess baseline immunologic parameters. Sixty patients with COPD received either glycophosphopeptical or placebo during 90 consecutive days during the winter months. Peripheral blood for final measurements of immunologic parameters was collected again within 6 d of terminating treatment. The total number of subjects reported for any given experiment reflects the number of subjects available for the given analysis. Four patients in the placebo group were lost to follow-up. No deaths occurred during the study period. Any discrepancy between the enrolled patients and final subject number reflects either patient dropout or insufficient sample to perform the experiment.

Treatments

Glycophosphopeptical (AM3 Immunoferon; I. F. Cantabria, Madrid, Spain) is an orally given immunomodulator with a low toxicity profile that is a polysaccharide/protein compound purified from Candida utilis. Previous dose-finding and kinetic studies with glycophosphopeptical demonstrated that 3 g/d was the optimal dose for maximal immunostimulation without side effects (25). Six capsules per day (two 300-mg capsules three times a day) of either glycophosphopeptical or placebo were therefore administered orally during 90 consecutive days.

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Lymphoprep Nyegaard and Co., Oslo, Norway) density gradient centrifugation, washed three times in NaCl 0.9% buffer, and suspended in RPMI 1640 (Whitaker Bioproducts, Walkerville, MI) containing 10% fetal bovine serum (Biochrom KG, Berlin). After counting, cells were resuspended at 1 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% heat-activated fetal bovine serum, l-glutamine (2 mM Flow Laboratories, Irvine, UK), Hepes (0.5% Flow Lab) and 1% penicillin-streptomycin (Difco Laboratories, Detroit, MI); this will be referred to as complete medium. Cell viability was checked by trypan blue exclusion.

Cytotoxicity Assays

Cytotoxicity was quantitated by a ⁵¹Cr-specific release assay using K-562 target cells, as previously described (26). Target cells were labeled with Na₂⁵¹CrO₄, washed twice, and resuspended in culture medium. Target cells (5 × 10⁶ cells/ml) were mixed with effector cells at different effector to target (E/T) ratios (50:1, 25:1, 12:1, and 6:1) in triplicate round-bottom microwell plates (Soria-Greiner, Madrid, Spain). Controls included target cells incubated either with complete medium (spontaneous ⁵¹Cr release) or with 1 N HCl (maximal ⁵¹Cr release). Plates were incubated for 4 h at 37°C in a 5% CO₂ humid atmosphere. After incubation, 0.1 ml from each well was collected and counted in a gamma counter. All cultures were made in triplicate and mean counts per minute (cpm) used for all calculations. The percentages of specific lysis were calculated as follows: (A–S/M–S) × 100, where A = mean cpm of test samples, S = mean cpm of spontaneous ⁵¹Cr release, and T = mean cpm of maximal ⁵¹Cr release. Spontaneous ⁵¹Cr release was always lower than 15% of maximal release counts.

Immunophenotypic Studies

PBMCs (5 × 10⁶ in 50 μl of phosphate-buffered saline [PBS] with 2% human albumin) were incubated with combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), and phycoerythrin-cya-

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TABLE 1. CHARACTERISTICS OF PATIENTS WITH COPD RANDOMIZED TO PLACEBO OR GLYCOPHOSPHOPEPTICAL

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>Placebo (n = 30)</th>
<th>Glycophosphopeptical (n = 30)</th>
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</thead>
<tbody>
<tr>
<td>Male, N</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62 ± 9</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>FEV₁, L (%)</td>
<td>1.27 ± 0.4</td>
<td>1.38 ± 0.3</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>50.1 ± 13.3%</td>
<td>54.6 ± 8.5%</td>
</tr>
<tr>
<td>Treated with inhaled corticosteroids</td>
<td>52.5 ± 8.6%</td>
<td>52.7 ± 7.9%</td>
</tr>
<tr>
<td>Mean inhaled corticosteroid dosage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking history, pack-years</td>
<td>760 ± 219 mg/d</td>
<td>850 ± 207 mg/d</td>
</tr>
<tr>
<td>Years since quit smoking</td>
<td>(maximum to minimum)</td>
<td>11.9 ± 13 (1–40 yr)</td>
</tr>
</tbody>
</table>
nine-5 conjugate (tricolor)-labeled monoclonal antibodies (MoAbs) against CD3, CD14, CD15, CD16, and CD56, for 30 min at 4°C as previously described (33, 34). Isotype-matched irrelevant FITC-, PE-, and tricolor-labeled MoAbs served as a control for each experiment. After washing cells three times in PBS, individual cell fluorescence was measured and registered with a FACSScan (Becton-Dickinson, San Jose CA). Flow cytometric analysis was completed with the help of Cell Quest software (Becton-Dickinson).

Flow Cytometry Studies on Phagocytic Activity
Each assay was performed in duplicate with 100 μl of cold heparinized whole blood according to methods previously described using Phagotest (ORPEGEN Pharma, Heidelberg, Germany) (35). In brief, after the addition of 20 × 10⁹ FITC-labeled opsonized Escherichia coli (Becton Dickinson) the samples were incubated for 10 min at 37°C. To suppress the fluorescence of bacteria attached to the outside of the cells, 100 μl of ice-cold quenching solution (trypan blue) was added to the samples before washing and pelleting (2 times at 250 g × 5 min). Samples were then treated with FACS lysing solution (Becton Dickinson) that lyses red cells while fixing leukocytes. Negative control samples were treated as above with the exception of the initial incubation step, which included cells alone or with E. coli incubated on ice; and cells alone incubated at 37°C. Staining with propidium iodide allowed for identification of leukocytes. Fluorescence was analyzed with a FACSScan (Becton-Dickinson, San Jose CA) both the percentage of monocytes and PMN phagocytes with phagocytosed bacteria and the mean phagocytic activity per phagocytic cell (mean channel intensity) were determined. The total phagocytic activity (phagocytic indices) of the population was determined by multiplying the percentage of phagocytic cells by the mean phagocytic capacity per cell.

Statistical Analysis
Differences between groups were compared for normally distributed data by two-tailed Student’s t test. Comparisons of pretreatment and posttreatment immunologic parameters within groups were completed by paired t-test. Significance was set at p ≤ 0.05.

RESULTS
Decreased Circulating NK Activity in Patients with COPD Is Rescued by Glycophosphopeptical Treatment
The NK activity of PBMCs from patients with COPD was investigated before randomization (baseline) and then again after the end of glycophosphopeptical treatment. As shown in Table 2, patients with COPD showed significantly lower baseline PBNK cell activity at all E/T ratios than did age-matched healthy control subjects. Further, at baseline values there were no significant differences between the PBNK activity in glycophosphopeptical or placebo treatment groups of COPD patients (Table 2). At follow-up we show no significant change in PBNK cell activity in the placebo group (Figure 1A). In contrast, glycophosphopeptical treatment significantly increased PBNK activity from baseline at all E/T ratios than did age-matched healthy control subjects. Further, at baseline values there were no significant differences between the PBNK activity in glyco-

TABLE 2. PBNK CELL ACTIVITY SEEN IN HEALTHY PEOPLE AND IN PATIENTS WITH COPD

<table>
<thead>
<tr>
<th>Groups</th>
<th>Effector:Target Cell Ratio</th>
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<tbody>
<tr>
<td></td>
<td>6:1</td>
</tr>
<tr>
<td>Healthy people, n = 50*</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Overall patients with COPD, n = 56</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Placebo, n = 26</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Glycophosphopeptical, n = 30</td>
<td>8 ± 3</td>
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</table>

* n = number of patients studied.
† Statistically significant differences between healthy control subjects and patients with COPD (p < 0.001).

Figure 1. Effects of glycophosphopeptical on PBNK cytotoxic activity against K-562 target cells in patients with COPD. Specific lysis was determined in each subject by triplicate cytotoxicity assays performed at different E/T ratios at baseline and after completing a 90-day course of treatment with placebo (n = 26; Panel A) or glycophosphopeptical (n = 30; Panel B). *Indicates the existence of significant differences between baseline levels and posttreatment levels of cytotoxic activity (p < 0.05; paired samples Student’s t test).

Glycophosphopeptical Treatment Rescues Percentage of Circulating Phagocytic Cells and Phagocytic Activity Per Cell in Patients with COPD
Tables 3 through 5 show the percentage of phagocytic cells, mean phagocytic activity per cell, and phagocytic indices for each cell type as measured in patients with COPD and healthy control subjects at baseline and follow-up. Patients with COPD had significantly depressed phagocytic activities of monocytes and PMNs compared with healthy volunteers (Table 3). Whereas the three baseline parameters of phagocytic activity of both monocytes and PMNs in the COPD group were significantly depressed with respect to that of healthy donors, glycophosphopeptical treatment, but not placebo, normalized these responses at posttreatment follow-up.

At baseline the patients with COPD in the glycophosphopeptical and placebo treatment groups did not significantly
differ in phagocytic activity of peripheral blood monocytes (Table 4) or PMNs (Table 5). Glycophosphopeptical treatment significantly increased both the percentage of phagocytic cells in PB monocytes (Table 4) and PMNs (Table 5) and the phagocytic indices of monocytes (Table 4) and PMNs (Table 5). Although the mean phagocytic capacity per individual cell trended upward after glycophosphopeptical treatment in both monocytes (Table 4) and PMNs (Table 5), the increases did not reach statistical significance. Patients with COPD in the placebo-treated group did not demonstrate any significant changes at follow-up in any of the three parameters of the PB monocyte (Table 4) or PMN (Table 5) phagocytic activity.

To investigate whether the differences in active phagocytic cell number reflected differences in monocyte or PMN phagocytes, phenotypic analysis was done for CD14 and CD15 in healthy and COPD patients (glycophosphopeptical and placebo groups). There were no significant differences in the percentage of monocytes (CD14bright CD15dim) or PMN phagocytes (CD14dim CD15bright) within any of the study groups either at baseline or at follow-up (data not shown). Further, glycophosphopeptical or placebo treatment did not significantly alter the percentage of PMNs or monocytes (data not shown).

**DISCUSSION**

The presence and relevance of immunoalterations in COPD have been repeatedly challenged. This study did not attempt to define whether immunoalterations were caused directly by COPD pathophysiology. However, this report clearly demonstrates that patients with COPD have decreased PBNK cytotoxic activity and impaired monocytes and PMN phagocytic activities when compared with age-matched healthy control subjects. Although alterations in NK cell activity in COPD have been correlated with tobacco consumption (8, 15, 36), no prior study has been done that consistently demonstrates a direct effect of COPD in the absence of active smoking. Because we excluded active smokers from this study, the defective PBNK activity seen in patients with COPD cannot be explained by active tobacco use alone. Much of the controversial data about COPD-mediated alterations in innate and specific immunity can likely be accounted for by the fact that the onset of COPD and its clinical evolution are influenced by multiple factors such as genetics, tobacco, infectious exacerbations, seasonal variations, and patient age. Treatment with glycophosphopeptical restores PBNK activity to the level of healthy control subjects despite the multifactorial origin of declines in PBNK activity among patients with COPD.

Together with NK cells, the Mo/Ma system as well as PMNs play a pivotal role in innate immunity. Functional alterations in Mo/Ma and PMNs have been described in many human infectious diseases, but also in patients with increased predisposition for developing respiratory infections such as COPD (1, 7, 10, 11, 18, 37–39). In this study we have demonstrated that patients with COPD show defects in phagocytic functions of Mo (Table 4) and PMNs (Table 5).

A novel finding of this study is that glycophosphopeptical treatment returns monocyte phagocytic activity in patients with COPD to the level seen in healthy control subjects (Table 4). Although it has been previously demonstrated that glycophosphopeptical treatment increases PMN phagocytic activity in COPD patients treated with placebo or glycophosphopeptical (p < 0.05; Student’s t-test).
TABLE 4. EFFECT OF GLYCOPHOSPHOPEPTICAL OR PLACEBO ON PHAGOCYTIC ACTIVITIES OF PERIPHERAL BLOOD Mo AMONG PATIENTS WITH COPD

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups of Treatment</th>
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<tbody>
<tr>
<td></td>
<td>Healthy Volunteers</td>
</tr>
<tr>
<td></td>
<td>(n = 30)*</td>
</tr>
<tr>
<td>% cells positive for phagocytosis</td>
<td>Baseline</td>
</tr>
<tr>
<td>Mean phagocytic activity per cell</td>
<td>81 ± 14</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>301 ± 94</td>
</tr>
</tbody>
</table>

* = number of patients assessed.
† Significant increase from baseline; p < 0.001.
‡ (%)= the percentages of increases or decreases (negative numbers) with regard to baseline values.
§ Mean phagocytic activity per cell was obtained as the difference between the fluorescence of monocytes incubated with E. coli-FITC at 37°C and the background fluorescence of negative control monocytes incubated with E. coli-FITC on ice.

The phagocytic index is the product of the percentage of cells positive for phagocytic activity by the mean phagocytic activity per cell.

* Significant increase from baseline; p < 0.05.

Glycophosphopeptical enhances the capacity of purified monocytes to phagocyte Candida albicans (25), the sensitivity of this finding was limited by the microscopic technique. In contrast, our flow cytometry method allowed the quantification of the phagocytic activity of a high number of individual cells (n = 10,000), several folds larger than that determined by microscopic observation (n = 300), thus improving the accuracy of the quantification of phagocytic activity. Because of the enhanced sensitivity of this method, we were able to demonstrate that the stimulatory effect of glycophosphopeptical upon the phagocytic activity of monocytes and PMNs is mainly due to an increase in the percentage of cells with phagocytic capacity, although it also reflects an increase in phagocytic capacity per cell. These data, together with the high degree of monocyte activation reported by other investigators (18, 27), and the high degree of monocyte stimulation observed herein (Table 4), clearly suggest the Mo/Ma system is the primary target for the immunologic actions displayed by glycophosphopeptical.

The decline in PMN phagocytic activity in patients with COPD is well established (6, 11, 38). Furthermore, similar studies of flow cytometry have demonstrated comparable findings of diminished phagocytosis and killing of nonencapsulated Haemophilus influenzae by PMNs (39). Our finding that glycophosphopeptical increases the phagocytic activity of PMNs (Table 5) is biologically important because PMNs far outnumber monocytes in peripheral blood and thus are the most frequently found phagocytic cells in the human body.

In conclusion, our use of a large, ex-smoker patient population allows us to show clear deficiencies in natural immune mechanisms such as PBNK cytotoxic activity and phagocytosis of peripheral blood monocytes and PMNs in COPD that cannot be accounted for by active smoking alone. These data in this selected population of COPD patients with a history of a high incidence of recurrent acute respiratory exacerbations (probable infectious origin) further support the possible clinical relevance of the demonstrated COPD-related alterations in natural immunity as a pathway to the development of infectious complications. Further, these alterations are fully rescued by glycophosphopeptical treatment. Given the importance of innate immunity against microbial invasion, it is likely that the defects in NK and PMN/monocyte phagocyte activity are, at least in part, responsible for the high rate of infectious exacerbations seen in patients with COPD. Antimicrobial management of patients with COPD is becoming increasingly difficult with the continued emergence of antibiotic resistance and the presence of multiple strains of H. influenzae with different antimicrobial susceptibility profiles (40) in the sputum of patients with COPD. Thus the use of immunomodulators

TABLE 5. EFFECT OF GLYCOPHOSPHOPEPTICAL OR PLACEBO ON PHAGOCYTIC ACTIVITIES OF PERIPHERAL BLOOD PMNs AMONG PATIENTS WITH COPD

<table>
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<th>Parameters</th>
<th>Groups of Treatment</th>
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<tr>
<td></td>
<td>Healthy Volunteers</td>
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<tr>
<td></td>
<td>(n = 30)*</td>
</tr>
<tr>
<td>% cells positive for phagocytosis</td>
<td>Baseline</td>
</tr>
<tr>
<td>Mean phagocytic activity per cell</td>
<td>720 ± 330</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>64,766 ± 34,410</td>
</tr>
</tbody>
</table>

* = number of patients assessed.
† Significant increase from baseline; p < 0.001.
‡ (%)= the percentages of increases or decreases (negative numbers) with regard to baseline values.
§ Mean phagocytic activity per cell was obtained as the difference between the fluorescence of PMNs incubated with E. coli-FITC at 37°C and the background fluorescence of negative control PMNs incubated with E. coli-FITC on ice.

† The phagocytic index is the product of the percentage of cells positive for phagocytic activity by the mean phagocytic activity per cell.
‡ Significant increase from baseline; p < 0.03.
offers a great advantage in the successful management of these patients. The next step is to test the clinical efficacy of the immunostimulant effects of glycoprophospeptical on NK and PMN/monocyte phagocytic activity in patients with COPD. We are currently enrolling patients in a multicenter double-blind clinical trial in order to evaluate the relationship between immune parameters and rates of superimposed respiratory infections in patients with COPD.

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