Immunomodulation with low dose levamisole in patients with colonic polyps

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Abstract

Background: Levamisole (LMS) has immunomodulatory activity, stimulates the immune system of healthy, normal volunteers and has been proposed previously as a colon cancer preventive agent. *Methods:* Patients with a history of colonic polyps who are at increased risk of colon cancer received LMS in a placebo-controlled, double-blinded clinical trail with crossover design. Primary endpoints were immunologic and included flow cytometry of peripheral blood mononuclear cells (PBMCs), measurement of interferon-γ copy number (IGCN) in PBMCs, and an ex vivo serum immune assay. *Results:* No differences were seen in the expression of multiple antigens by flow cytometry pre- and post-LMS. The IGCN partitioned subjects into two distinct groups defined by a γ-distribution which had a differential response to LMS. Those with low basal IGCN had a lower percentage of CD25 expressing PBMCs and responded to low dose LMS by producing more PBMC-derived interferon-γ and increasing the expression of CD25 and two NK cell markers, CD16 and CD56. In contrast, subjects with a high basal IGCN responded to low dose LMS with a reduction in PBMC-derived interferon-γ and a decrease in the expression of CD25. *Conclusions:* In aggregate, these responses suggest that LMS may act as an immunostimulatory agent for one group, those with low basal IGCN, and as an immunosuppressive agent for the other. LMS may not be an optimal agent for most patients with colonic polyps and should be avoided in patients with normal immune function. IGCN may be useful as an immunologic surrogate endpoint biomarker in future cancer prevention trials with immunomodulatory agents.
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1. Introduction

Levamisole (LMS) is an imidazothiazole anti-helminthic which is effective in reducing the risk of relapse when given adjuvantly to patients with surgically-resected stage III colon cancer [1,2], though it is no longer utilized clinically in this setting. It is thought to act, in part, though stimulation of the cellular immune system. In patients, LMS has been shown to increase the number of circulating natural killer (NK) cells [3], the expression of membrane CD25 (IL2 receptor) [4] and serum levels of soluble IL2 receptor [5]. In vitro, LMS modulates NK cell-mediated tumor lysis [6], tumor cell MHC class I expression [7,8] and IL12-dependent Th1 immune responses [9,10].

The role of LMS-mediated immunomodulation in vivo was examined in a dose escalation trial in normal volunteers [10]. Significant increases in the proportion of peripheral blood mononuclear cells (PBMCs) expressing the NK antigen CD16, especially the CD16/CD56(+) subpopulation, was noted. Only small effects on Th1 cellular immune function and serum cytokine levels were seen. Low dosages of LMS, 50 mg/day – 3 times/week, were equally efficacious in stimulating the immune system as clinically utilized dosages which are up to three-fold higher. In contrast to the standard dosages, minimal toxicity was seen at the low, 50 mg/day, dose.

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The appeal among the lay public of utilizing an immunostimulatory agent for cancer prevention is well documented [11]. There is, however, a paucity of scientifically-controlled data to validate this approach. In order to address this, we enrolled patients with a history of colonic polyps onto a placebo-controlled clinical trial with low dose LMS. Patients with colonic polyps are at significantly increased risk for the development of colon cancer, the third leading cause of cancer death for men and women in the United States [12]. While the primary mechanisms involved in colonic polyp formation are not immunologic, there is some evidence that immune function in patients with polyps is not entirely normal [13,14]. Endpoints in this study were immunologic including both general measures of immune activity as well as specific assays of cellular immune function. We report for the first time that low dose LMS is an effective immunostimulant only for patients with evidence of impaired basal cellular immune function and describe a novel assay which partitions patients into two groups with differential responses to LMS.

2. Patients and experimental methods

2.1. Clinical trial design and sample acquisition

Eighteen patients with a history of colonic polyps were identified through the University of California, Irvine (UCI), Division of Gastroenterology and the Chao Family Comprehensive Cancer Center. Each patient signed written informed consent for this study which was approved by the UCI Institutional Review Board. Levamisole (LMS) and an identically-appearing inert placebo tablet were obtained from Johnson & Johnson. Patients were randomized to receive either LMS at 50 mg/day – 3 times/week or placebo on the same schedule, every other week for 12 weeks. After 12 weeks, patients were crossed over to the other arm of the study and treated for an additional 12 weeks. The overall duration of participation in the trial was 24 weeks. Randomization was performed according to a table of random numbers and the key was retained by the study pharmacist. Patients, the treating physicians, and laboratory personnel performing immunologic assays were blinded as to when the patient was receiving active drug versus placebo.

Heparinized blood and serum samples for laboratory analyses were obtained at study initiation, after the first 12 weeks (at crossover), and after 24 weeks (at completion of the study). Any toxicity notations were also made at these timepoints.

2.2. Flow cytometry

Heparinized blood was diluted 1:1 with Hanks' buffered salt solution and peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on a Ficoll/hypaque gradient (Organon Technika, NC). Following washes cells were incubated for 30 min at 4 °C with FITC- or PE-conjugated monoclonal antibodies directed against the following antigens: CD4, CD8, CD16, CD25, CDw29 (co-stain with CD4), CD45RA (co-stain with CD4 to identify naïve helper T-cells), CD45RO (co-stain with CD4 to identify memory helper T-cells), CD56, CD95 (Fas) and CD178 (Fas ligand). After additional washes and fixation with 1% formaldehyde, cells were analyzed by flow cytometry. Controls included unstained cells to assess viability and autofluorescence and cells incubated with conjugated isotype-specific controls.

2.3. Interferon-γ copy number (IGCN) in PBMCs

PBMCs were obtained as described above and RNA was isolated with a TRIzol reagent from 10×10^6 cells. cDNA was synthesized in a 20 μl reaction containing 5 μg of total RNA, 200 UMmuLV reverse transcriptase and 500 ng of oligo(dT)20 primers (Promega, Madison, WI). PCR products were analyzed by CytoXpress Quantitative PCR system (BioSource International, Camarillo, CA) with spectrophotometric readouts with a Biorad 96-well Elisa plate reader at 450 nm. The CytoXpress system is based on biotin-labeled primer incorporation into PCR products, followed by hybridization to sequence-specific capture oligos in microplate wells. The captured sequences are detected and quantified in enzyme-streptavidin/substrate reaction and the optical density measured. Primers were utilized to provide a quantitative assessment of the number of copies of interferon-γ mRNA in a defined amount of total PBMC RNA.

2.4. Statistics

Flow cytometric data and IGCN values were tested for significance with a two-tailed Wilcoxon signed rank (paired, non-parametric) test with significance defined as $p < 0.05$ (actual values reported). For comparison across groups, an unpaired *t*-test with Welch's correction for unequal variances was utilized. Correlations between different data sets were examined with the Spearman's ''*r*'' correlation test. The entire IGCN data set was examined for ''goodness to fit'' to a γ-distribution model by χ^2 and plotted as observed quantiles versus fitted γ-quantiles. A further description of the statistical analysis describing the populations defined by IGCN is included under results.

3. Results

3.1. Flow cytometry

Results of flow cytometry for the expression of multiple antigens on PBMCs are shown in Table 1. The expression of CD4, multiple subsets of CD4 cells including CD4/CDw29, CD4/CD45RA (naïve), and CD4/CD45RO (memory) and