Levamisole Effects on Major Histocompatibility Complex and Adhesion Molecule Expression and on Myeloid Cell Adhesion to Human Colon Tumor Cell Lines

Edward S. Kimball, M. Carolyn Fisher*

Background: The drug levamisole has been successfully used in combination with fluorouracil to increase the disease-free interval and survival of patients who have undergone surgical resection of Dukes' stage C colon cancer. Levamisole is thought to affect the host immune response. Several recent studies have examined its effect on the expression of major histocompatibility complex (MHC) class I molecules, but the results have been inconsistent. An equally important requirement for a host cellular immune response is the adhesion of leukocytes to tumor cells. The latter may be required for cell-mediated antitumor cytotoxic responses. Purpose: We evaluated the ability of levamisole to affect the expression of MHC class I molecules and cell-adhesion molecules and determined whether levamisole could affect leukocyte adhesion to tumor cells that had been treated with the drug. Methods: A panel of four human colon tumor cell lines (HT-29, SW-620, HCT-15, and LoVo), A-375 human melanoma cells, and human umbilical vein endothelial cells (HUVEC) were cultured in the presence of levamisole and examined by solid-phase enzyme immunoassay to determine the level of expression of MHC class I, intercellular adhesion molecule 1 (ICAM-1), vascular cell-adhesion molecule 1 (VCAM-1), leukocyte immunoglobulin VLA-4, and lymphocyte-functional antigen (LFA-1) molecules. Adhesion of HL-60 and THP-1 myeloid cells to tumor cells was also evaluated. Tumor necrosis factor (TNF) at 10 ng/mL was used as a positive control for increasing adhesion molecule expression and cell-cell adhesion. The statistical significance of differences in cell surface molecule expression and functional adhesion between treated and control cells were tested by use of analysis of variance and the two-tailed Dunnett's test. Results: Treatment with levamisole (0.1 and 1 ug/mL) caused the levels of MHC class I expression to increase approximately threefold above control levels on HCT-15 and LoVo colon tumor cells (P<.05 in each case) compared with untreated cells, caused minimal increases on HT-29 cells (to 1.5 times control levels), but caused no significant increases on SW-620 colon tumor or A-375 melanoma cells. The HCT-15 and LoVo colon tumor cells had very low basal MHC expression. Levamisole (1 ug/mL) increased VCAM-1 expression on HT-29 and SW-620 colon tumor cells to 4.3 and 2.4 times (P<.05 in each case) control levels, respectively, doubled ICAM-1 expression on HT-29 cells (P<.05), and increased LFA-1 expression on HT-29, LoVo, and A-375 cells to 2.1, 3.2, and 1.8 (P<.05 in each case) times control levels, respectively. TNF (10 ng/mL) was used as a positive control and yielded increased expression of MHC class I molecules on the HT-29, LoVo, SW-620, and HCT-15 cells (2.5, 7.8, 1.9, and 4.8 times control levels, respectively; P<.05 in each case). TNF increased VCAM-1 expression to 4.2 times the vehicle-treated control levels (P<.05) on HT-29 cells and increased ICAM-1 expression on HT-29, LoVo, and SW-620 cells (8.4, 1.8, and 1.9 times vehicle control levels, respectively; P<.05 in each case). THP-1 and HL-60 cells demonstrated increased adhesion to levamisole-treated HT-29 colon tumor cells. HL-60 cells also exhibited increased levamisole-mediated adherence to LoVo and HCT-15 cells. Adherence by THP-1 was significantly improved after levamisole treatment of the HUVEC, SW-620, and A-375 cells (P<.05 in each case). Conclusions: Levamisole can directly affect the expression and function of molecules that are engaged in cell-cell recognition and signaling on the surfaces of some tumor cell lines. However, no consistent pattern between cell-adhesion molecule expression, cell-cell adhesion, or levamisole concentration could be discerned. [J Natl Cancer Inst 1996;88:109-16]

Interactions between T lymphocytes (T cells) and accessory cells depend on antigen presentation by accessory cells through recognition of major histocompatibility complex (MHC) molecules. Cytolytic T cells recognize antigen when presented by MHC class I molecules. Similar recognition requirements for T-cell- and macrophage-mediated tumor cell destruction have also been found to occur (I). Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin supergene family (2,3), is found on lymphocytes and monocytes (4,5) and can be induced on endothelial cells by cytokines, such as interleukin 1 (IL-1), tumor necrosis factor (TNF), and interferon (IFN) (4-6). Recognition of and functional binding to ICAM-1 are requirements for successful presentation of antigens to T cells by accessory cells (7-9).
counter receptors for ICAM-1 on accessory cells are three heterodimeric proteins of the integrin family consisting of a common beta chain (beta 2 = CD18) and three different alpha chains (CD11a, CD11b, and CD11c) (10-13).

Studies (1, 14-16) have indicated that ICAM-1 expression on tumor cells may be important for host immune, cell-mediated cytotoxicity. For example, cytokines such as IFN, TNF, and IL-1 are able to increase ICAM-1 expression on tumor cells in vitro (e.g., HT-29 human colon tumor cells) and are reported to render the cells susceptible to macrophage-mediated killing (14). Corecognition of ICAM-1 and MHC class I has also been observed to be a vital factor of host cell-mediated tumor cytotoxicity (1).

ICAM-1-mediated, cell-adhesive interactions make up only a part of the spectrum of intercellular molecular contacts. For example, vascular cell adhesion molecule-1 (VCAM-1) interactions with its counter receptor (the leukocyte integrin VLA-4 [very late antigen-4, also called a4b1 integrin]) (11, 17, 18) may be as important as those that depend on ICAM-1. There are reports that mutual recognition of VCAM-1 and VLA-4 between T cells and macrophages is also important in evoking immune responses (11, 18).

The drug levamisole has been successfully used in combination with fluorouracil (5-FU) to increase the disease-free interval and survival of patients who have undergone surgical resection of Dukes’ stage C colon cancer. There is an extensive literature [reviewed in (19)] regarding the ability of levamisole to stimulate the immune system. However, the mechanism by which it operates as an immunomodulator, especially in the context of adjuvant chemotherapy of colon cancer, has not yet been determined. Potential mechanisms that have not been previously examined might involve the expression of adhesion molecules and the functionality of cell–cell adhesion. Several studies (20-22) of MHC class I expression on tumor cells after levamisole treatment have produced conflicting results. Accordingly, in this study, a panel of human colon tumor cell lines (HT-29, SW-620, HCT-15, and LoVo), A-375 human melanoma cells, and primary human umbilical vein endothelial cells (HUVEC) were examined for changes in MHC and adhesion-molecule expression on their cell surfaces as a function of treatment with levamisole or a TNF-positive control (15, 23) as well as for concomitant effects on the ability of monocytic (THP-1) and promyelocytic (HL-60) cells to adhere to them.

Materials and Methods

Materials

Levamisole was obtained from the Janssen Research Foundation (Beerse, Belgium). The source of 2',2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diaminonitro salt (ABTS) was the Pierce Chemical Co. (St. Louis, MO); ABTS was supplied as a solution. Human recombinant TNF was purchased from Biotech International (Camarillo, CA). Antibodies used for enzyme immunoassay (EIA) studies were purchased from the following sources: HLA-ABC. clone W6/32 (Harlan Bioscience, Indianapolis, IN); ICAM-1 (CD54), clone 8H10 (Immunootech, Westbrook, ME); lymphoendothelial antigen (LFA-1) (CD11a), clone 25.3.1 (Immunotech); VCAM-1, clone 1G11 (Immunootech); VLA-4 (CD49d), clone HP2/1 (Immunootech); and goat anti-mouse immunoglobulin G (IgG) Fc fragment-horseradish peroxidase (Jackson Immunoresearch, Media, PA).

Cell Lines

The tumor cell lines used were randomly chosen from a collection of human cell lines available to our laboratory. The HT-29, SW-620, HCT-15, and LoVo colon tumor cell lines were originally obtained from the American Type Culture Collection (ATCC) (Rockville, MD). HCT-15 and SW-620 were derived from patients with Dukes’ stage C colon cancer. The Dokes’ classification was not determined for the HT-29 and LoVo lines. All four cell lines were low expressors of carcinoembryonic antigen. LoVo and HCT-15 originated from epithelial tumors. HT-29 was derived from a microvillus epithelial tumor, and SW-620 originated from a tumor consisting of round, bipolar cells. The A-375 malignant melanoma cell line was also obtained from the ATCC. Moreover, both the THP-1 monocytic leukemic cell line and the HL-60 promyelogenous leukemia cell lines were obtained from the ATCC. HUVECs were obtained as primary cultures from Clonetics, Inc. (San Diego, CA).

Solid-Phase EIA for Tumor Cell-Surface Molecule Expression

HUVECs were maintained in endothelial cell growth medium (Clonetics, Inc., Rockville, MD). Tumor cell lines were maintained in Dulbecco’s minimum essential medium with high glucose supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glucose. Cell culture media and fetal bovine sera were purchased from JRH Biosciences (Lenexa, KS). For solid-phase EIA, all cells were maintained at subconfluent densities and were plated at 15,000 cells per well into 96-well culture plates 18 hours in advance of any treatment. Complete medium was used for all experiments. For induction of cell-surface adhesion molecules, complete medium was replaced with 200 μL fresh complete medium containing TNF (10 ng/mL) as a positive control, levamisole, or complete medium only. All treatments were in quadruplicate. Cultures were incubated for 48 hours, as described by Webb et al. (14), washed with phosphate-buffered saline (PBS), pH 7.2, and then analyzed in situ for cell-surface molecule expression. Shorter culture times were less than optimal for ICAM-1 expression, consistent with published reports (14, 15). Twenty micrograms of monoclonal antibody (diluted 1:5, 1:10, or 1:20 in PBS containing 0.1% bovine serum albumin) was placed in all wells. Plates were incubated at 4°C for 4 hours, washed three times with cold PBS, blocked with 100 μL PBS containing 1% normal goat serum for 30 minutes, and again washed three times with cold PBS. Next, 100 μL of a 1:5000 or 1:10,000 dilution in PBS of purified goat anti-mouse IgG Fc fragment conjugated to horseradish peroxidase was added to each well. Following a 1-hour incubation at 4°C, the wells were washed three times with cold PBS, and 150 μL of ABTS solution was added. Color development was allowed to proceed at 4°C for 18 hours, except for the A-375 and HCT-15 cell lines that required no more than 1 hour of incubation for ICAM-1-specific reactions. Color development was quantitated at 405 nm on a 96-well plate reader (Molecular Devices, Inc., Menlo Park, CA). Color development times of 18 hours for A-375 and HCT-15 cell lines yielded optical density readings greater than or equal to 2. Because of the different basal levels of cell-surface molecule expression found for each cell line, combined data are expressed as the percent of control to simplify comparisons between cell lines for changes in cell-surface molecule expression.

Functional Adhesion Bioassay

The adherence of THP-1 monocytic and HL-60 promyelocytic cells as effector cells to a monolayer of target cells was measured. The effector cells were labeled with the fluorescent dye 5-carboxyfluorescin diacetate (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Target cells were plated in 48-well tissue culture dishes at densities designed to reach confluence by 48 hours. This varied according to the growth rate of the cell line used. During the 48-hour incubation, the cells were grown in control medium, medium containing TNF (10 ng/mL), or levamisole (1 μg/mL). The cells were then washed twice with PBS, and 5 x 10^5 freshly labeled effector cells in 100 μL PBS were added and incubated at 37°C for 30 minutes. After being washed with warm PBS (37°C), adherent cells were quantitated by lysing the culture with 200 μL 1 N NaOH to yield a homogeneous solution for measuring fluorescence with a fluorescent plate reader (Millipore Corp., Bedford, MA). A standard curve of graded numbers of fluorescent effector cells was run with each experiment to calculate the numbers of adherent cells.

Materials and Methods

Levamisole was obtained from the Janssen Research Foundation (Beerse, Belgium). The source of 2',2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diaminonitro salt (ABTS) was the Pierce Chemical Co. (St. Louis, MO); ABTS was supplied as a solution. Human recombinant TNF was purchased from Biotech International (Camarillo, CA). Antibodies used for enzyme immunoassay (EIA) studies were purchased from the following sources: HLA-ABC, clone W6/32 (Harlan Bioscience, Indianapolis, IN); ICAM-1 (CD54), clone 8H10 (Immunootech, Westbrook, ME); lymphoendothelial antigen (LFA-1) (CD11a), clone 25.3.1 (Immunotech); VCAM-1, clone 1G11 (Immunootech); VLA-4 (CD49d), clone HP2/1 (Immunootech); and goat anti-mouse immunoglobulin G (IgG) Fc fragment-horseradish peroxidase (Jackson Immunoresearch, Media, PA).

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Journal of the National Cancer Institute, Vol. 88, No. 2, January 17, 1996
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Statistics

Each cell line was tested separately for the effect of levamisole on functional cell adhesion or on cell-surface molecule expression as measured by solid-phase EIA. A range of levamisole doses was tested in each experiment and was compared with results obtained from cells treated with vehicle only. TNF (10 ng/mL) as a positive control group was compared against the vehicle control group. In all experiments, levamisole doses overlapped with doses used in previous experiments but may also have included doses not previously tested.

EIA data were normalized to untreated controls. Non-specific binding was subtracted from all wells, and the ratio of optical density in experimental wells to untreated control wells (E/C) was taken. Mean non-specific bindings ± standard deviation for the experiments reported herein were as follows: A-375, 0.23 ± 0.04; HCT-15, 0.24 ± 0.07; HT-29, 0.29 ± 0.16; LoVo, 0.31 ± 0.08; and SW-620, 0.35 ± 0.03. All functional adhesion data were compared with those obtained with vehicle-treated controls.

A one-factor analysis-of-variance layout with n, treatment groups and one control group was used for both the surface molecule expression (EIA studies) and the functional adhesion studies. Hypotheses tested within cell lines were of the form:

$$\mu_i - \mu_j \leq 0 \text{ versus alternative: } \mu_i - \mu_j > 0,$$

where $$\mu_i, i = 1 \ldots n, \text{ and } \mu_j, \text{ respectively, denote the population means for } n, \text{-treated groups and the population mean for the control group. The type I error rate of this family of hypotheses was controlled at the } 5\% \text{ level through use of the two-tailed Dunnett's test.}$$

Optical density measurements in EIA studies were re-expressed as a ratio of experimental optical density/control (untreated) optical density to allow comparisons between cell lines with different basal levels of cell-surface molecules. Normality of these data within each cell line was verified through use of the one-tailed Shapiro–Wilks test.

Results

MHC Class I Expression on Tumor Cell Lines

Comparisons from a series of experiments with levamisole are shown in Fig. 1. The four colon tumor cell lines tested expressed low levels of MHC class I, and TNF treatments increased expression 1.5- to 7.5-fold. MHC class I increases on LoVo, HCT-15, and HT-29 cell lines were dependent on levamisole concentration. When incubated with levamisole (0.01-1 μg/mL), MHC class I expression in LoVo and HCT-15 was increased to as much as 3.5 and four times vehicle-treated control levels; $$P<.05$$. HT-29 cells had a weak response to levamisole (1.5 ± 0.13 times control levels; $$P<.05$$) but only with 0.01 μg/mL levamisole. Other levamisole concentrations had no significant effect. In addition, this cell line had low resting-state MHC surface expression. SW-620 did not show increased MHC expression in response to increasing levamisole concentrations; in fact, a decrease occurred with 0.01 μg/mL levamisole. A-375 malignant melanoma failed to respond to levamisole or TNF with increased MHC class I expression (data not shown).

ICAM-1 Expression on Human Tumor Cell Lines

HT-29 expression of ICAM-1 was increased by 10 ng/mL TNF (Table 1) as reported by Webb et al. (14). When HT-29 cells were cultured with levamisole, increased ICAM-1 expression was observed for cells treated with 0.1 and 1 μg/mL. Smaller increases were observed for cells treated with 0.01 and 0.001 μg/mL, whereas concentrations lower than 0.001 μg/mL were without effect (data not shown). Maximum average ICAM-1 levels after treatment with 1 μg/mL levamisole were 2 ± 0.18 times ($$P<.05$$) control levels. In contrast to HT-29, the SW-620, LoVo, and HCT-15 cell lines failed to respond to levamisole. SW-620 did not respond to TNF, results obtained with LoVo were nonsignificant, and HCT-15 cells failed to respond to TNF. HCT-15 cells, unlike the other three cell lines tested, have extremely high constitutive levels of ICAM-1 expression.

There was no increase in ICAM-1 expression on primary HUVEC or A-375 melanoma cells that had been cultured with levamisole, although both cell lines responded to TNF (2.5- and

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Fig. 1. Increased major histocompatibility complex (MHC) class I expression on four human colon tumor cell lines in response to levamisole and tumor necrosis factor (TNF). Cells were cultured for 48 hours with the indicated concentration of levamisole or 10 ng/mL TNF and then analyzed in situ for MHC class I molecule with the use of a solid-phase enzyme immunoassay technique. Lower concentrations of levamisole (not shown) were inactive on these cells. All treatments were run in quadruplicate. Data shown are normalized to control and are expressed as the ratio of optical densities, where $$E/C = (\text{experimental optical density/vehicle-treated control optical density})$$. Results shown are mean $$E/C$$ ± standard error. Base-line optical densities at 405 nm for vehicle-treated cells and numbers of experiments were as follows: HT-29 (0.09 ± 0.03; n = 4), SW-620 (0.52 ± 0.16; n = 3), LoVo (0.02 ± 0.01; n = 4), and HCT-15 (0.07 ± 0.01; n = 4).$$P<.05$$ versus control, analysis of variance and Dunnett’s two-tailed test.

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Table 1. Effect of levamisole on cell-surface expression of adhesion molecules

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Antibodies to</th>
<th>10 ng/mL tumor necrosis factor</th>
<th>1 µg/mL levamisole</th>
<th>0.1 µg/mL levamisole</th>
<th>Base-line optical density†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>ICAM-1</td>
<td>8.40 ± 0.941</td>
<td>2.00 ± 0.18‡</td>
<td>1.56 ± 0.18‡</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>4.17 ± 0.841</td>
<td>4.29 ± 0.74‡</td>
<td>3.50 ± 0.66‡</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
<td>1.08 ± 0.38</td>
<td>1.09 ± 0.20</td>
<td>1.54 ± 0.40</td>
<td>0.34 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>LFA-1</td>
<td>1.60 ± 0.24</td>
<td>2.10 ± 0.29‡</td>
<td>1.53 ± 0.28</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>SW-620</td>
<td>ICAM-1</td>
<td>1.88 ± 0.16‡</td>
<td>0.94 ± 0.05</td>
<td>1.00 ± 0.06</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>1.74 ± 0.51</td>
<td>2.37 ± 0.70‡</td>
<td>2.39 ± 0.72‡</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
<td>1.56 ± 0.55</td>
<td>1.13 ± 0.09</td>
<td>1.40 ± 0.17</td>
<td>0.58 ± 0.11</td>
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<tr>
<td></td>
<td>LFA-1</td>
<td>1.28 ± 0.20</td>
<td>1.21 ± 0.11</td>
<td>1.04 ± 0.08</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>LoVo</td>
<td>ICAM-1</td>
<td>1.76 ± 0.03</td>
<td>0.84 ± 0.23</td>
<td>0.90 ± 0.25</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>1.35 ± 0.39</td>
<td>1.71 ± 0.29</td>
<td>1.44 ± 0.25</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
<td>0.87 ± 0.09</td>
<td>1.13 ± 0.08</td>
<td>1.18 ± 0.08</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>LFA-1</td>
<td>4.51 ± 1.89‡</td>
<td>3.23 ± 1.04‡</td>
<td>1.51 ± 0.87</td>
<td>0.15 ± 0.14</td>
</tr>
<tr>
<td>HCT-15</td>
<td>ICAM-1</td>
<td>0.82 ± 0.07</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.05</td>
<td>1.40 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>0.65 ± 0.13</td>
<td>1.80 ± 0.70</td>
<td>1.25 ± 0.70</td>
<td>0.45 ± 0.16</td>
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<td>VLA-4</td>
<td>2.29 ± 0.86</td>
<td>1.93 ± 0.34</td>
<td>0.65 ± 0.13</td>
<td>0.30 ± 0.05</td>
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<td>LFA-1</td>
<td>2.54 ± 1.26</td>
<td>2.69 ± 1.03</td>
<td>1.51 ± 0.37</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td>A-375</td>
<td>ICAM-1</td>
<td>2.90 ± 0.12‡</td>
<td>1.20 ± 0.15</td>
<td>1.10 ± 0.15</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>1.40 ± 0.25</td>
<td>1.38 ± 0.21</td>
<td>1.37 ± 0.02</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
<td>0.94 ± 0.06</td>
<td>1.11 ± 0.05</td>
<td>1.05 ± 0.03</td>
<td>1.47 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>LFA-1</td>
<td>1.18 ± 0.32‡</td>
<td>1.83 ± 0.37‡</td>
<td>1.73 ± 0.46</td>
<td>0.10 ± 0.04</td>
</tr>
</tbody>
</table>

*Solid-phase in situ enzyme immunoassay data expressed as (optical density [OD] experimental/OD vehicle-treated control) ± SE. Results are taken from at least three separate experiments run in quadruplicate. ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular cell-adhesion molecule-1; VLA-4 = leukocyte antigen; and LFA-1 = lymphocyte-functional antigen.

†Base-line optical density defined as mean optical density ± standard error at 405 nm for vehicle-treated controls.
‡P<.05 versus control, analysis of variance and two-tailed Dunnett's test. Concentrations ^0.1 µg/mL were less effective or ineffective.

1.9-fold increases, respectively; data not shown). A-375 cells have high constitutive levels of ICAM-1 expression in our studies, and this finding is consistent with published data (14) for the A-375 cell line.

Other Adhesion Molecules Expressed on Tumor Cells

Data from a series of experiments are shown in Table 1. Levamisole at 1.0 and 0.1 µg/mL caused significant increases in VCAM-1 expression on HT-29 and SW-620 cells (maximum changes were 4.3 ± 0.74 and 2.37 ± 0.70 times vehicle-treated controls; P<.05 in all cases) but had no effect on LoVo, HCT-15, or A-375 cell expression of this molecule. TNF had a stimulatory effect on HT-29 but no effect on the other cell lines.

Levamisole (1 µg/mL) significantly increased LFA-1 expression on HT-29, LoVo, and A-375 cells to 2.1 ± 0.29, 3.23 ± 0.10, and 1.83 ± 0.37 times vehicle-treated control levels, respectively (P<.05 in each case). LFA-1 was increased more than twofold on HCT-15, but the increase was not statistically significant. The results for low base-line LFA-1 expression on HT-29 and A-375 are consistent with published reports (14). Neither levamisole nor TNF had significant (P>.05 in all cases) effects on VLA-4 expression on any of the cell lines tested.

Functional Adhesion Studies

In these studies, the binding of HL-60 human promyelocytic cells and THP-1 human monocytic leukemia cells to levamisole-treated target cells was studied (Table 2).

When compared with vehicle-treated HT-29, adhesion of THP-1 cells to HT-29 cells treated with 1 µg/mL levamisole was nearly doubled (P<.05), going from 5.1% ± 0.3% adherence for vehicle-treated cells to 9.8% ± 1.2% adherence after levamisole treatment, with similar increases for HL-60 adhesion (P<.05). Adhesion to HT-29 cells was not affected by levamisole concentrations lower than 0.001 µg/mL (data not shown). Significant (P<.05) increases in HL-60 adherence (3% ± 0.2% for vehicle-treated cells increasing to a maximum of 9.5% ± 1.3%) occurred in LoVo cells cultured with 0.1 µg/mL levamisole. Changes in percent adhesion by THP-1 (18.1% ± 0.8% for vehicle-treated cells to a maximum of 23.2% ± 4%) were nonsignificant (P>.05). Treatment of SW-620 cells with levamisole (1 µg/mL) caused an increase in adhesion by THP-1 cells from 18.6% ± 0.8% to 28.0% ± 2.5% (P<.05) after treatment. Adhesion was improved to 35.8% ± 4% (P<.05) for the TNF-positive control. Smaller increases in adhesion to SW-620 cells by HL-60 cells occurred after levamisole and TNF treatments of SW-620 cells.

There were no improvements in the adherence of THP-1 cells to HCT-15 cells following levamisole treatment of HCT-15. Adherence of HL-60 cells to HCT-15 cells increased after culture with 0.01 µg/mL levamisole, but not at higher concentrations, and therefore may be of no importance.

Treatment with 1 µg/mL levamisole modestly increased the adhesion of THP-1 cells to A-375 cells (from 29.9% for vehicle-treated cells to 36.5% ± 0.7 for cells treated with 1 µg/mL levamisole; P<.05). TNF (10 ng/mL) treatment of A-375 cells caused increased adhesion by THP-1 cells, increasing from 29.9% (vehicle-treated cells) to 48.2% adherence (P<.05).
Table 2. Adhesion to levamisole-treated tumor cells and human umbilical vein endothelial cells (HUVEC)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HT-29 % adhesion ± standard error</th>
<th>HCT-15 % adhesion ± standard error</th>
<th>SW-620 % adhesion ± standard error</th>
<th>LoVo % adhesion ± standard error</th>
<th>A-375 % adhesion ± standard error</th>
<th>HUVEC % adhesion ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.0 ± 0.6</td>
<td>39.2 ± 1.8</td>
<td>14.8 ± 0.6</td>
<td>3.0 ± 0.18</td>
<td>ND</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>0.001 μg/mL LMS</td>
<td>7.6 ± 0.4†</td>
<td>46.0 ± 3.4</td>
<td>22.2 ± 2.2†</td>
<td>5.4 ± 0.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.01 μg/mL LMS</td>
<td>9.7 ± 1.0†</td>
<td>47.6 ± 2.0†</td>
<td>20.3 ± 1.4†</td>
<td>8.1 ± 0.8†</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.1 μg/mL LMS</td>
<td>8.1 ± 1.3†</td>
<td>38.8 ± 7.4</td>
<td>17.2 ± 0.9</td>
<td>9.5 ± 1.3†</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1 μg/mL LMS</td>
<td>7.5 ± 1.2†</td>
<td>40.2 ± 1.3</td>
<td>18.2 ± 1.8</td>
<td>8.0 ± 1.6†</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10 ng/mL TNF</td>
<td>7.6 ± 1.6†</td>
<td>38.7 ± 1.3</td>
<td>18.0 ± 1.5</td>
<td>5.9 ± 1.2†</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data are from two to six experiments performed in quadruplicate. LMS = levamisole; TNF = tumor necrosis factor; ND = not done.

1P<.05; vehicle-treated effector cells adhering to drug-treated targets versus vehicle-treated targets, analysis of variance and two-tailed Dunnett’s test.

Table 2. Adhesion to levamisole-treated tumor cells and human umbilical vein endothelial cells (HUVEC)*

While ICAM-1 levels on HUVEC were not affected by levamisole, there was increased adhesion by THP-1 cells to levamisole-treated HUVEC (from 46.6% [vehicle-treated cells] to 57.3% adhesion [P<.05]). This result suggests that other adhesion molecules have been affected by levamisole treatment of HUVEC. Adherence by HL-60 cells increased by approximately 4.5-fold to 18.5% ± 2.8% after TNF treatment of HUVEC, but there were no increases for HUVEC cultured with levamisole.

Blocking experiments (Fig. 2) were performed to ascertain the relative contributions of ICAM-1 and VCAM-1 to adhesion between HT-29 and THP-1 cells. Adhesion to TNF-treated cells appeared to be partly dependent on ICAM-1 (45% inhibition) but not on VCAM-1. Adhesion to vehicle-treated HT-29 cells could be inhibited 58% and 53% by antibodies to ICAM-1 or VCAM-1, respectively, while adhesion to levamisole-treated HT-29 cells was inhibited 27% and 33% by antibodies to ICAM-1 and VCAM-1, respectively. The fact that this relative percentage is decreased from vehicle-treated control cells implies that there may have been other adhesion molecules affected by levamisole treatment that were not examined in this study. When these antibodies were combined with antibodies to their respective counter receptors, inhibition was not greatly changed (32% for ICAM-1 + LFA-1 antibodies and 35% for VCAM-1 + VLA-4 antibodies).

![Fig. 2. THP-1 cell adhesion to levamisole-treated HT-29 colon tumor cells is partially blocked by antibodies to ICAM-1 and VCAM-1. HT-29 cells were stimulated with either 1 μg/mL levamisole (LMS) or 10 ng/mL tumor necrosis factor (TNF) or were left unstimulated (Veh) as indicated. The cell-adhesion mixtures contained, in addition to the effector cells (THP-1) and target cells (HT-29), 20 μg/mL of the indicated antibody or antibodies. Results are from one experiment performed in duplicate.](https://academic.oup.com/jnci/article-abstract/88/2/109/867215)
Table 3 summarizes cell-adhesion results in the context of changes in adhesion-molecule expression on tumor cell lines treated with levamisole.

**Discussion**

Our results demonstrate that levamisole can elevate the expression of MHC class I, ICAM-1, VCAM-1, and LFA-1 on certain tumor cell lines. Adhesion by THP-1 and HL-60 cells was increased to those tumor cell lines that demonstrated increased ICAM-1, VCAM-1, and/or LFA-1 as a function of levamisole treatment (Table 3); however, except for HT-29 cells, no clear concentration-dependent response for cell-cell adhesion could be discerned. Although adhesion of THP-1 cells to levamisole-treated HT-29 cells appeared to be related to increased ICAM-1, VCAM-1, and LFA-1 levels, no similar general relationships could be discerned for the other cell lines. Concentration-related responses were observed for the effect of levamisole on MHC class I expression on HCT-15 and LoVo cells, for VCAM-1 on SW-620 cells, for LFA-1 expression on LoVo and HCT-15 cells, and for ICAM-1 on HT-29 cells, but no consistent overall relationship between concentration-dependent responses could be discerned for effects on surface-molecule expression and cell adhesion among all of the cell lines tested. The fact that a relationship could not be found between levamisole doses and the various phenomena examined in this study suggests that 1) there may be different levels of cell-associated receptors or response elements affected by levamisole in the various tumor cell lines tested; 2) other, possibly more relevant, surface molecules were not studied; 3) there may be diversity in the expression of these molecules on tumor cells; and 4) the increased adhesion observed with some cell lines may reflect changes in adhesion-molecule configuration and avidity rather than changes in numbers.

The expression of MHC class I protein was increased on two colon tumor cell lines (HCT-15 and LoVo) to approximately four times the level expressed by vehicle-treated controls and by a smaller amount on HT-29 cells following in vitro levamisole treatment. The functional significance of the observed increases is not clear, since these cell lines had low basal levels of MHC class I expression and the net increases were correspondingly small. These data showing increased MHC expression for some cell lines treated with levamisole conflict with those of other published reports. Goodrich et al. (20) examined CaCo-2 human colon tumor and MDA MB-435 human breast carcinoma cell lines and observed that the expression of MHC class I proteins was decreased after in vitro culture with levamisole. In that study, however, there was no quantitation of MHC expression and the concentration of levamisole (10 μg/mL) used may not easily be achieved in patients (19,24). However, in the present study, considerably lower concentrations of levamisole were used. Colon tumor cell lines that had low MHC class I expression responded with increases in MHC class I, consistent with small increases seen for WiDr human colorectal cancer cells (22) that express moderate amounts of MHC, while high MHC class I expression by SW-620 cells was generally unaffected by levamisole treatment. The apparent decrease in MHC class I expression observed on SW-620 cells treated with low-dose (0.001 μg/mL) levamisole is not clearly understood, but it may be a property of cells that have high MHC surface expression. Bell-shaped concentration responses (e.g., primary antibody responses) have been observed in the past to occur with levamisole (25).

In another report, Schiller et al. (21) found no effect for levamisole on three other human colon tumor cell lines (SKCO-1, Colo 205, and LS-174T) for expression of ICAM-1 or MHC class II. In contrast, Janik et al. (26) reported that levamisole administration following resection of primary tumors caused increases in MHC class I expression in all patients given a dose of 5 mg/kg levamisole or higher. Most recently, Abdalla et al. (22) reported that 5-FU increased the level of MHC class I protein expressed on WiDr colon tumor cells; this was slightly further enhanced by levamisole treatment. MHC class I steady-state messenger RNA (mRNA) was increased by 5-FU and was increased further by levamisole. Levamisole alone increased MHC mRNA transcription, but the effect of levamisole on MHC cell-surface expression in the absence of 5-FU was not evaluated. Our results extend the findings of Abdalla et al. (22) and Janik et al. (26).

Reports (1,27) suggest that increases in MHC class I expression by tumor cells help render those cells susceptible to cytolytic T-cell- or to LAK-mediated cytotoxicity, especially if this is accompanied by high cell-adhesion molecule expression. The increases in MHC class I expression mediated by levamisole, although small in terms of net increases, occurred in three of four colon tumor cell lines tested; in some instances, in-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>LFA-1</th>
<th>VLA-4</th>
<th>MHC-I</th>
<th>Adhesion by</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NC</td>
<td>↑ (NS)</td>
<td>HL-60</td>
</tr>
<tr>
<td>SW-620</td>
<td>NC</td>
<td>↑</td>
<td>NC</td>
<td>NC</td>
<td>NC+</td>
<td>HL-60</td>
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<td>NC</td>
<td>↑ (NS)</td>
<td>↑</td>
<td>NC+</td>
<td>↑</td>
<td>THP-1</td>
</tr>
<tr>
<td>HCT-15</td>
<td>NC+</td>
<td>↑ (NS)</td>
<td>↑ (NS)</td>
<td>↑ (NS)</td>
<td>↑</td>
<td>THP-1</td>
</tr>
<tr>
<td>A-375</td>
<td>NC+</td>
<td>NC</td>
<td>↑</td>
<td>NC</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Surface-expression results derived from solid-phase enzyme immunoassay (ELA) data. ND = not done. NC = no change. NS = not statistically significant (P>0.05), ANOVA and two-tailed Dunnett's test.

+High base-line optical density (OD) (>0.4) for solid-phase ELA on vehicle-treated cells. ↑ = increased surface expression ≥1.5 times that of base-line OD. Statistically significant (P<0.05), analysis of variance (ANOVA) and two-tailed Dunnett's test, unless indicated otherwise. ↑ = increased adhesion, statistically significant (P<0.05), ANOVA and two-tailed Dunnett's test.
creases in MHC class I expression were accompanied by increases in cell adhesion. The functional significance of these changes in net MHC expression is not clear. Definitive studies to address this aspect of immune-mediated tumor cell killing as a potential result of levamisole treatment have not been performed, and the effect of levamisole on MHC class I expression in vivo is not well established. However, it is worth noting these observations in the context of the reported therapeutic benefit provided by levamisole in the adjuvant treatment of Dukes' stage C colon cancer (28,29).

The results reported in this article also demonstrate that levamisole has the ability to directly affect the expression and function of some molecules that are engaged in cell–cell recognition and signaling on the surfaces of some tumor cell lines. Two of the four colon tumor cell lines tested exhibited increased cell adhesion with THP-1 monocytic cells, as did HUVEC. This may reflect an important feature of lymphocyte and antigen-presenting cell interactions resulting from levamisole treatment and may constitute an important aspect of the mechanism of action of levamisole as an immune regulator. Recently, Holcombe et al. (30) reported that levamisole increased the expression of CD56, a marker for natural killer (NK) cell activation. NK cells may recognize tumor targets via ICAM-1 and LFA-1. A report (31) suggests that this recognition may result from intracellular signaling by activated ICAM-1 and LFA-1 and not necessarily only from direct recognition and binding to these molecules.

Treatment of myeloid effector cells with levamisole may also affect adhesion to target cells. Preliminary studies showed that adhesion of THP-1 cells to levamisole-treated HT-29 cells was increased from 9.8% to 11.2% when the THP-1 cells were treated with 1 μg/mL levamisole (data not shown). In the case of levamisole-treated SW-620 cells, adhesion was increased from 28% ± 2.5% to 44.8% ± 7.1% (P<.05) with levamisole-treated THP-1 cells. This result requires further examination. In addition, attempts were made to determine if levamisole-mediated increases in cell adhesion translated into improved cell-mediated cytotoxicity. With THP-1 cells, none of the colon tumor cell lines that showed increased adhesion to THP-1 after levamisole treatment were rendered more sensitive to THP-1-mediated cytotoxicity (Kimball ES: unpublished results). This finding suggests that if the increased adhesion-molecule expression has functional significance, it may not relate to monocyte-mediated cytotoxicity, but may still relate to antigen presentation and/or T-cell-mediated cytotoxicity.

The alterations in cell-surface molecules studied in this article may only partly explain levamisole-mediated cell–cell interactions as a mechanism of action. The diversity and lack of congruence between in vitro responses reported by us and others (20-22,26,30) for levamisole-treated colon tumor cell lines reflect the fact that the single mechanism of action may be ascribed to levamisole in the treatment of colon cancer. Increased cell adhesion via levamisole treatments provides a potential mechanism for some of its reported activities as an immune modulator, while increased effector cell recognition of tumor cells via MHC and adhesion molecules provides another hypothetical mechanism for its use as a cancer therapeutic agent.

References

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Telomerase Activity in Human Breast Tumors

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Background: The activity of the ribonucleoprotein enzyme telomerase is not detected in normal somatic cells; thus, with each cell division, the ends of chromosomes consisting of the telomeric repeats TTAGGG progressively erode. The current model gaining support is that telomerase activity in germline and immortal cells maintains telomere length and thus compensates for the "end-replication problem." Purpose: Our objective was to determine when telomerase activity is reactivated in the progression to malignant breast cancer and if knowledge of telomerase activity may be an indicator for the diagnosis and potential treatment of breast cancer. Methods: Using a polymerase chain reaction-based telomerase activity assay, we examined telomerase activity in 140 breast cancer specimens (from 140 patients), four phyllodes tumors (from four patients), 38 noncancerous lesions (20 fibroadenomas, 17 fibrocystic diseases, one gynecomastia; from 38 patients), and 55 adjacent noncancerous mammary tissues (from 55 of the 140 breast cancer patients). In addition, 33 fine-needle-aspirated breast samples (from 33 patients) were analyzed. Results: Among surgically resected samples, telomerase activity was detected in 130 (93%) of 140 breast cancers. Telomerase activity was detected in 68% of stage I primary breast cancers, in 73% of cancers smaller than 20 mm, and in 81% of axillary lymph node-negative cancers. Moreover, the activity was detected in more than 95% of advanced stage tumors but in only two (4%) of 55 adjacent noncancerous tissues. While telomerase activity was not detected in any of 17 specimens of fibrocystic disease, surprisingly low levels of telomerase activity were detected in nine (45%) of 20 fibroadenomas. Among samples obtained by fine-needle aspiration, 14 (100%) of 14 patients whose fine-needle-aspirated specimen contained telomerase activity and who subsequently underwent surgery were confirmed to have breast cancer. Multivariate analysis of 125 specimens from patients for whom data were available on age at surgery, stage of disease, tumor size, lymph node status, tumor histology, and menopausal status indicated that stage classification exhibited the strongest association with telomerase activity (for stage I versus stages II-IV: odds ratio = 1.0 versus 73.4; 95% confidence interval = 2.0-959.0; P = .02). Conclusion: Telomerase activity was detected in more than 95% of advanced stage breast cancers. It was absent in 19%-32% of less advanced cancers. Since a determination of any association between telomerase activity and patient survival is not possible at the present time, it remains to be determined whether lack of telomerase activity predicts for favorable outcome. [J Natl Cancer Inst 1996;88:116-22]

Notes

We thank Dr. Charles Bowden for his insightful comments and valuable advice and Fran Stewart for her assistance with statistical analyses.

Manuscript received June 21, 1995; revised October 11, 1995; accepted October 20, 1995.

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See "Notes" section following "References."