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Association of immune parameters with clinical outcome in stage III colon cancer: results of Southwest Oncology Group Protocol 9009

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Abstract Levamisole (LMS), utilized in the adjuvant treatment of patients with stage III colon cancer, is immunomodulatory. To determine whether alterations in immune parameters before, during and after 12 months of 5FU/LMS therapy correlate with disease-free survival, 38 patients enrolled on Southwest Oncology Group (SWOG) protocol 8899 received extensive lymphocyte phenotypic analysis prior to therapy and 3, 6, 12 and 15 months after treatment initiation. The median follow-up of patients is 41 months. Significant increases in the proportion and total number of $CD56^+$ natural killer cells were seen, starting at 3 months and continuing until 15 months ($P < 0.001$). Increases in the total numbers of cells expressing CD25 (interleukin-2 receptor), VLA4 and the combinations of CD4:CD45RA and CD4:CDw29 were not evident during therapy but were seen at 15 months ($P < 0.05$: CD25, CD4:CDw29, CD4:CD45RA; $P < 0.001$: VLA4). Low levels of $CD8^+$ cells prior to treatment initiation and after 3 months of therapy correlated with early relapse within the first year of 5FU/LMS treatment. Patients who have remained disease-free ($n = 22$, median follow-up 45 months) demonstrated increases in the total numbers of $CD8^+$, $CD25^+$, $CD56^+$, $VLA4^+$, CD4:CDw29 and CD4:CD45RA cells, primarily at 15 months. In contrast, patients who relapsed had decreased numbers of $CD8^+$, CD4:CDw29, CD4:CD45RA and $VLA4^+$ cells and minimal increases in $CD56^+$ and $CD25^+$ cells. Statistically significant differences between the late-relapse group and the group remaining disease-free were seen for $CD25^+$, CD4:CD45RA and CD4:CDw29 cells at the 15-month assay time ($P = 0.0276$, $P = 0.0349$, $P = 0.0178$ respectively). In conclusion, multiple alterations in lymphocyte phenotype, with increases in the proportion and total number of cells involved in cell-mediated immune responses, were seen during and especially following completion of therapy with 5FU/LMS. Many of these changes are significantly associated with clinical outcome and may be useful for risk stratification of stage III colon cancer patients following completion of adjuvant therapy.

Key words Levamisole · Natural killer cells · Lymphocyte phenotype · Tumor immunology

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Introduction

Levamisole (LMS) in conjunction with 5FU is effective in reducing the incidence of relapse following surgical resection for patients with stage III (Duke's C) colon carcinoma [4, 11, 15, 25]. LMS is an imidazothiazole

derivative that is used extensively in underdeveloped countries and in veterinary medicine as a broad-spectrum anti-helminthic. The first report citing immunostimulatory effects of LMS was in 1971 when Renoux and Renoux [19] found that it potentiated the protective effect of the *Brucella* vaccine in mice. LMS appears have minimal clinical effectiveness in settings characterized by extensive bulky disease, with its greatest efficacy being apparent when it is administered adjuvantly [2].

In healthy individuals, LMS appears to have minimal effects on the intact immune system [21, 22] though this agent does restore cutaneous delayed hypersensitivity in anergic cancer patients [24]. In addition, patients receiving LMS with 5FU as adjuvant therapy for colon cancer exhibit an increase in the proportion and total number of $CD56^+$ natural killer (NK) cells during the course of therapy [6]. This increase in $CD56^+$ NK cells correlated with clinical outcome in a small series of patients studied. In that study [6], patients also exhibited an increase in the proportion and total number of cells expressing CD25, the interleukin-2 (IL-2) receptor, and in serum levels of soluble IL-2 receptor (sIL-2R). A separate study of patients receiving 5FU/LMS also found that $CD25^+$ cells and sIL-2R increased following therapy [3] and it appears that LMS alone can increase sIL-2R levels [8]. LMS has also been shown to be synergistic with IL-12 in the induction of a Th1-type immune response in vitro [5]. IL-12 is obligatory in the induction of differentiation of naive T cells to interferon-γ and IL-2-producing Th1 cells [7, 12, 14, 23]. Th1 cells are important in the induction and propagation of cell-mediated immune responses against tumors, virally infected cells, and parasitic infections [1]. Taken together, these data suggest that LMS influences cells of the immune system to allow for more effective tumoricidal activity.

While it is clear that LMS induces specific and consistent alterations in various measures of immune function, whether it is these immunomodulatory effects of LMS that account for the beneficial effects seen in patients with stage III colon cancer is uncertain. This study was undertaken to examine changes in immune function in patients receiving 5FU and LMS to attempt to confirm the prior report [6] suggesting that immune parameters might be useful in predicting clinical outcome in these patients.

Materials and methods

SWOG9009 study design

SWOG (Southwest Oncology Group) protocol 9009 was a prospective correlative immunology study in conjunction with SWOG8899 (INT-0089), an adjuvant treatment protocol for patients with stage III and high-risk stage IIB colon cancer. A group of 38 patients with stage III colon cancer, who were randomized to receive standard 5FU/LMS on SWOG8899, were enrolled on the immunology study at various sites across the country between March 1991 and August 1992. Patients received treatment according to Moertel [15] with 5FU at 450 mg/m^2 everyday for 5 days followed by weekly 5FU at 450 mg/m^2 starting at day 29 and continuing for 11 months. LMS was administered at 50 mg orally three times a day for days every other week starting at day 1. Heparinized whole blood samples were obtained from patients for extensive lymphocyte phenotypic analysis by flow cytometry and functional cytotoxicity assays prior to treatment, at 3 months, 6 months, 12 months (completion of 5FU/LMS) and 15 months (3 months after completing therapy) and sent by overnight courier at room temperature to the principal investigator's laboratory for analysis. Samples were drawn on treatment days prior to administration of 5FU and LMS. Therefore all samples were obtained 1 week following 5FU and 2 weeks following LMS administration. All patients gave their written informed consent before participating at the treating institution, in addition to giving consent for the parent study, SWOG8899.

Sample preparation and flow cytometry

Samples were processed and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on a Ficoll/Hypaque gradient. The cells were incubated with fluorscene-isothiocyanate- or phycoerythrin-conjugated murine monoclonal antibodies directed against cell-surface antigens at 4 °C for 30 min. Cells were then washed twice with phosphate-buffered saline (PBS) with 50 mg/dl bovine serum albumin and fixed for 10 min with PBS/1% formaldehyde prior to analysis with an Epics 753 flow cytometer. All samples were run concomitantly with unstained cells to assess viability and autofluorescence and with conjugated isotype-specific controls. Two-color analysis was performed for most markers. Forward and right-angle scatter gatings were set to include resting and activated lymphocytes and eliminate debris and dead cells. Antigen expression, given as a percentage of total cells, includes events recorded above a negative gate established at the upper limit of fluorescence in the absence of primary antibody minus background reactivity observed with the relevant IgG isotype control. The channel number corresponding to mean fluorescence intensity was determined for positive cells using an arbitrary scale from 1 to 256. A minimum of 10 000 cells were analyzed for each experiment.

Antibodies utilized for phenotypic analysis included those directed against CD3 (IOT3, clone ×35, IgG2a; Amac, Westbrook, Me.), CD4 (T4, clone SFCI12T4D11, IgG1, Coulter, Hialeah, Fla.), CD5 (T1, clone SFCI24T6G12, IgG2a; Coulter), CD8 (T8, clone SFCI21Thy2D3, IgG1; Coulter), CD11a (clone 25.3.1, IgG1; Amac), CD16 (leu-11, clone NKP15, IgG1, Becton Dickinson, San Jose, Calif.), CD19 (B4, clone 89B, IgG1; Coulter), CD25 (IL-2R, clone 2R1.2, IgG1; T-cell Diagnostics, Cambridge, Mass.), CDw29d (4B4, IgG1; Coulter), CD45RA (2H4, clone 2H4LDH11LD139, IgG1; Coulter), CD49a (VLA-1, clone TS2/7, IgG1; T-cell Diagnostics), CD49d (VLA-4, clone HP2/1, IgG1; Amac), CD56 (NKH-1, clone N901, IgG1, Coulter), T cell receptor δ chain (TCRδ1, clone 5A6.E9, IgG1; T cell Diagnostics). The CD45RA antibody utilized, Coulter 2H4, has 99% reactivity with $CD45RA^+CD4^+$ cells and only 7.2% reactivity with $CD45RO^+CD4^+$ cells [13]. Isotype controls utilized included IgG1 and IgG2a (Coulter).

Cell viability and cytotoxicity

Blood samples were obtained from the participating sites 24–72 hours after phlebotomy because of delays inherent in overnight mail delivery and the study design. Cell viability for flow cytometry was more than 98% for all samples, regardless of the time of sample arrival, suggesting that flow-cytometric data were not significantly affected by transport delays. Quality control on samples from a patient treated at the principal investigator's base institution revealed no significant differences in flow cytometry data if heparinized blood was evaluated after 24 h, 48 h or 72 h at room temperature. However, testing for non-MHC-restricted cytotoxicity, performed with a standard ^{51}Cr-release assay utilizing K562 erytholeukemia cells as targets, was markedly affected by transport delays. Samples received within 24 h had higher percentage cyto-

toxicities than samples received after 48 h or 72 h. At all effector:target ratios (10:1, 50:1, and 100:1) there were several samples that arrived after more than 48 h that exhibited 0% cytotoxicity. An amendment to the protocol was therefore instituted, which required that samples be transported on Monday through Thursday only, with next-morning delivery required. Samples received after more than 24 h were discarded. This improved the reliability of cytotoxicity testing and no further samples exhibited zero killing. However, because the accuracy of testing from the initial assay times was compromised, no further analysis of cytotoxicity data was attempted.

Patient follow-up

Patient follow-up data were obtained through the SWOG statistical office and the Eastern Cooperative Oncology Group (ECOG) with information from SWOG8899. The median follow-up for all patients is 41 months (range 5–52 months). The median follow-up for patients remaining free of disease is 45 months; 22 patients have remained with no evidence of disease, 5 relapsed early while on therapy, and 9 relapsed late, following completion of a full year of 5FU/LMS. Two patients are lost to follow-up and are unevaluable with respect to disease status.

Statistical analysis

A paired *t*-test was utilized for comparison of samples at various study time to prestudy values for individual patients. Comparisons of values across groups with different clinical outcome were made through an unpaired *t*-test with Welsh's correction to adjust for unequal variances. Survival curves were plotted by means of Kaplan-Meier estimates [9].

Results

Lymphocyte phenotype

The results of phenotypic analyses for selected antigens tested at each assay time are presented in Table 1. Starting at month 3 and continuing through month 15, there was a significant increase in the proportion and total number of circulating $CD56^+$ NK cells (range $P < 0.05$ to $P < 0.001$). No change was seen in levels of CD16-expressing cells. CD16 identifies the $Fc_\gamma III$ receptor, which is expressed on a subpopulation of NK cells. Other significant changes included an increase in the proportion and total number of CD25(IL-2 receptor)-expressing cells ($P < 0.05$), the proportion and total number of $CD4^+$ helper T-cells coexpressing CD45RA or CDw29d ($P < 0.05$), and the proportion and total number of cells expressing the adhesion protein VLA4 (CD49d, $P < 0.001$). VLA4 and CDw29 are cell-surface-adhesion molecules in the integrin family. CD29 corresponds to the β chain of multiple integrin molecules and defines the β1 integrin family. VLA4 is a β1 integrin formed by the association of α4 and β1. VLA4 binds to VCAM-1 (CD106) and is expressed on lymphocytes and hematopoietic progenitor cells. Expression of both of these antigens can increase variably under conditions of cellular activation. The CD45 antigens represent cell-surface tyrosine phosphatases also widely expressed on hematopoietic cells. CD45RA is expressed on "naive" T cells which are primed to respond to processed antigen [20]. A second isoform of CD45, CD45RO, defines a subset of helper T cells termed "memory" T cells. Antibodies to CD45RO were not utilized as part of the antibody panel in SWOG9009. For CD45RA, CDw29d and VLA4, significant alterations were seen only at the 12-month and/or the 15-month assay and were not seen in earlier samples obtained during adjuvant 5FU/LMS therapy. There was a small increase in the proportion but not in total number of $CD8^+$ cytotoxic T cells at 15 months. Other antigens that did not demonstrate any significant change during therapy included CD3, CD4, CD5, CD11a, CD19, VLA1 (CD49a) and the δ chain of the γδ T cell receptor, which identifies a small subset of circulating and intraepithelial γδ T cells.

Table 1 Lymphocyte phenotype analysis, SWOG9009. Values indicate median and range (in parentheses). n = number of patient samples available for processing at that time. *WBC* white blood cells × $(\mu l)^{-1}$. Antigen presence is shown as a percentage of all peripheral blood mononuclear cells. Antigens not shown (see text): CD3, CD3:TCRδ, CD5, CD11a, CD19, VLA1. Total = cells × $(\mu l)^{-1}$

Antigen	Prestudy (n = 38)	Month 3 (n = 38)	Month 6 (n = 31)	Month 12 (n = 28)	Month 15 (n = 24)
WBC	7.2 (3.9–12.7)	5.9 (3–11.1)**	5.8 (3.2–9.7)**	6.1 (2.4–10.3)**	6.6 (3.8–8.6)
Total lymph	1711 (792–4745)	1775 (534–4462)	2024 (323–3686)	1713 (420–3780)	1827 (460–3112)
CD4 (%)	42 (6–63)	43 (23–67)	48 (24–67)	49 (23–69)	49 (9–81)
Total CD4	736 (78–2083)	745 (191–2461)	873 (147–2479)	904 (179–2232)	953 (97–1820)
CD4/CD45 (%)	1.3 (0.1–24.1)	1.7 (0–16.2)	1.8 (0.3–50.1)	4.5 (0.8–25.4)*	5.5 (0.8–32.3)
Total CD4/CD45	28 (1–382)	31 (0–271)	35 (1–360)	69 (7–533)	110 (7–639)*
CD4/CD29 (%)	11.4 (0.1–50.1)	11.6 (0.1–32.7)	13.9 (1.3–58.8)	23 (0.5–36.3)*	25.9 (4.6–53.5)*
Total CD4/CD29	165 (1–1442)	224 (2–856)	272 (4–1056)	326 (8–1372)	477 (53–1554)*
CD8 (%)	16 (0–42)	18 (6.5–51)	19 (1.4–51)	16.5 (0–52)	19 (7.9–63)*
Total CD8	288 (0–1524)	312 (109–1913)	349 (237–1718)	271 (0–1784)	343 (63–1967)
CD16 (%)	9.6 (0–34.4)	10.7 (0.8–25.3)	12.5 (0–37.4)	11.4 (2.1–35.8)	10.6 (0.9–31.6)
Total CD16	183 (0–935)	183 (15–706)	215 (0–1211)	212 (24–536)	145 (14–635)
CD25 (%)	0.15 (0–27.5)	0.5 (0–6.7)	0.4 (0–11.4)	0.8 (0–4.9)*	1.2 (0–16.6)*
Total CD25	1.5 (0–431)	8.8 (0–134)	9.4 (0–76)	7.9 (0–103)	20.1 (0–482)*
CD56 (%)	9.5 (0–44.2)	14.8 (0.4–38.2)**	19.3 (1–40.6)**	15.8 (3–41.1)*	13.9 (3.5–45.1)*
Total CD56	187 (0–973)	232 (6–1229)*	247 (23–1381)	284 (34–1418)	259 (55–1404)
VLA4 (%)	45 (0–85)	46 (0–86)	54 (0–94)	67 (23–89)*	70 (14–95)**
Total VLA4	778 (0–2420)	768 (0–3851)	897 (0–3205)	1134 (151–2545)	1229 (159–2966)**

*,** Paired *t*-test in comparison to prestudy: *$P < 0.05$; **$P < 0.001$

Immune parameters analyzed by clinical outcome

Of the 36 patients evaluable for clinical outcome, 22 remain disease-free (no evidence of disease) with a median follow-up of 45 months and 14 have relapsed (median follow-up 41 months). Of the 14, 5 relapsed early while receiving 5FU/LMS; 9 relapsed late, following completion of a full year of 5FU/LMS. Immune parameters were analyzed on the basis of stratification among clinical outcome groups. Results of the phenotypic analysis from the prestudy assay did not differ among patient groups, except for the total of $CD8^+$ cells (Table 2), which reached marginal statistical significance when early-relapse patients were compared to those who relapsed late or remained disease-free ($P = 0.078$). This difference was noted despite the fact that there were only 5 patients in the early-relapse group. Significantly depressed levels of total $CD8^+$ cells in the early-relapse group persisted at the 3-month assay time compared to the group that remained disease-free ($P = 0.0138$) and compared to those who relapsed late or remain disease-free ($P = 0.0165$). When all patients were stratified for prestudy total CD8 levels, progression-free survival for patients with low levels (fewer than 200 cells/μl) was worse than that for patients with high prestudy CD8 levels (above 200 cells/μl, Fig. 1). The number of events is not sufficient to assess statistical significance for this analysis.

Table 2 Stratification by clinical outcome – total CD8 *LR* late relapse, *NED* no evidence of disease

Clinical group	$CD8^+$ cells $(\mu l)^{-1}$					
	Prestudy			3 month		
	Mean	± SD	*n*	Mean	± SD	*n*
Early relapse	175	183*	5	245	85**	5
Late relapse	413	238	9	314	211	9
NED	357	263	22	464	331	21
LR and NED	373	253	31	419	305	30
Total *n*			36			35

* $P = 0.078$ versus LR and NED

** $P = 0.0138$ versus NED; $P = 0.0165$ versus LR and NED

Different patterns in the changes from baseline for several antigenic markers were noted between patients who remain without disease and those who relapsed (Fig. 2). Differences in the percentage change in CD8 were most marked early in therapy at the 3-month assay time. The principal contribution to this was from patients experiencing an early relapse during 5FU/LMS therapy. At 12 and 15 months, CD8 levels were higher than baseline for patients remaining disease-free and depressed for patients who eventually relapsed. Similar findings were noted for $CD56^+$ levels, with an increase above baseline in the disease-free group of 100% and 43% at 12 and 15 months, compared to a reduction or a minor (8%) increase in the group suffering relapse.

A marked difference in percentage change from baseline was seen at 15 months for CD25. CD25 expression rose above baseline in both groups but the magnitude of the increase was much greater in patients remaining disease-free (5330%) than in those who relapsed (275%). A similar pattern for the percentage change from baseline at 15 months was noted for CD4:CD45RA cells and CD4:CDw29 cells, where value below baseline were observed in patients who had relapsed, and dramatic (at least 200%) increases were seen in patients remaining disease-free.

The majority of absolute differences between groups with different clinical outcomes, in the total number of cells expressing a specific phenotypic marker, occurred at 15 months, 3 months after completion of adjuvant 5FU/LMS, at which point several statistically significant differences are seen (Table 3). Comparisons at this assay time are limited to patients who relapsed late, for whom samples were submitted, and those which remained disease-free. Early-relapse patients were withdrawn from the study protocol and samples were not obtained from these patients at 15 months. Results for CD8, CD56 and VLA4 revealed a non-statistically significant trend toward higher levels in patients remaining disease-free. Significant differences between groups were noted for CD25-expressing, CD4:CDw29

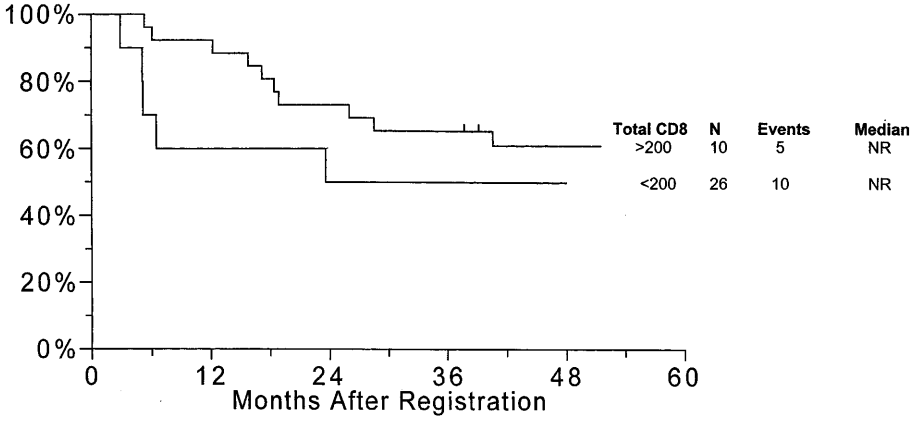

Fig. 1 Progression-free survival stratified for total prestudy CD8 [cells × $(\mu l)^{-1}$]. Curves represent Kaplan-Meier estimates. *NR* median survival not yet reached

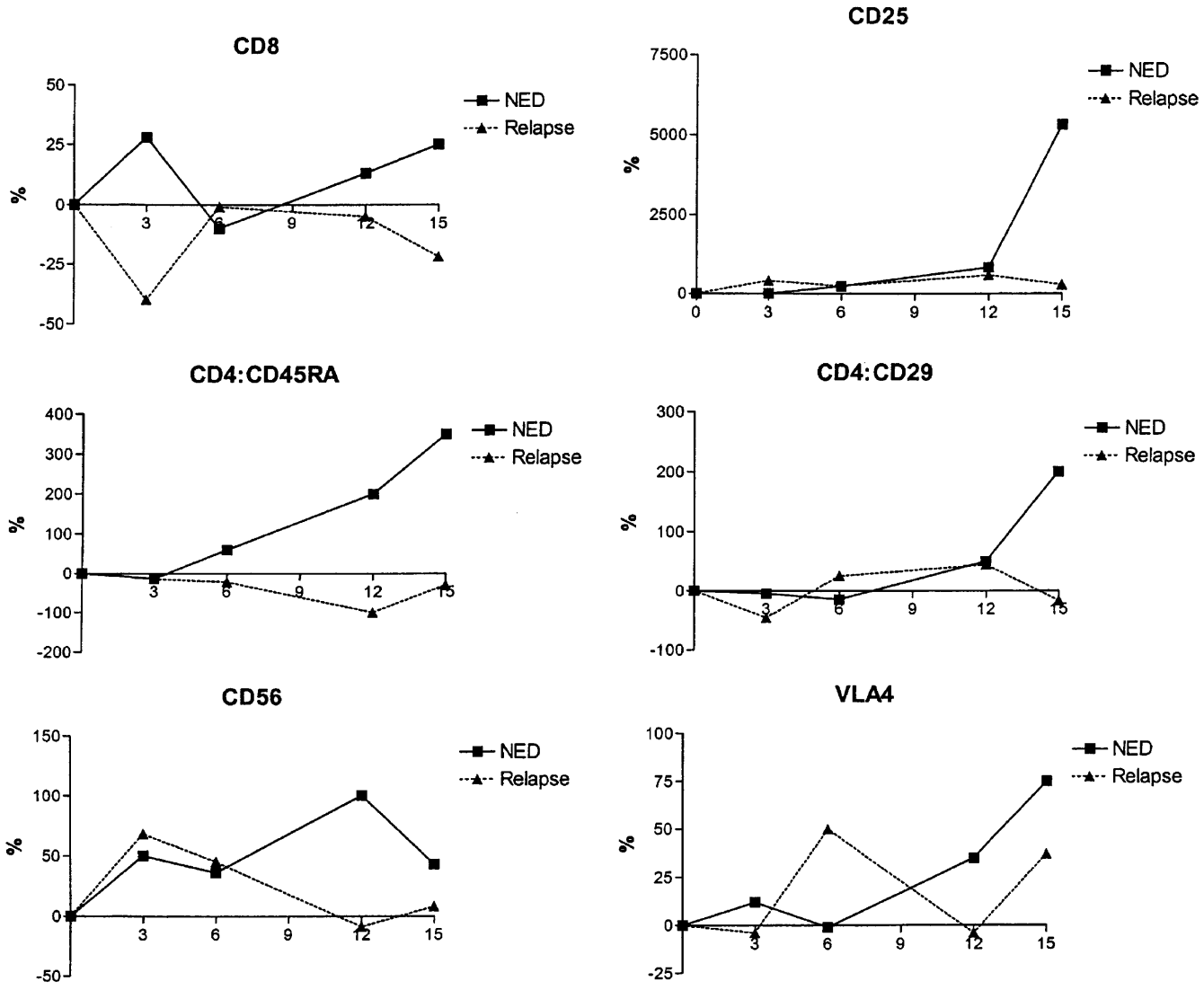

Fig. 2 Percentage change from baseline in the total number of cells expressing a particular antigen, over time in months. 0 = prestudy assay time. ■ Median values for patients remaining free of disease (*NED*). ▲ Median values for patients experiencing relapse

and CD4:CD45RA cells ($P = 0.0276$, $P = 0.0349$, $P = 0.0178$ respectively).

Discussion

This study confirms that specific and consistent immune changes occur during adjuvant therapy with 5FU and levamisole and supports the contention that this combination of medications is immunomodulatory. Increases in $CD56^+$ NK cells, activated T cells and specific T cell subsets indicate activation of cell-mediated components of the immune response and are concordant with prior reports of cellular immune activation with 5FU/LMS and with LMS alone [3, 5, 8, 24]. While LMS alone exhibits immunostimulatory activity, the relative contribution of 5FU toward this activity, whether positive or negative, is uncertain and not resolved on the basis of this study.

Whether the immunological actions of LMS alone or in the combination 5FU/LMS contribute to anticarcinogenic activity is not known. It has been speculated that LMS may make cells more sensitive to 5FU-dependent cytotoxicity through inhibition of tumor cell tyrosine phosphatases, though it is known that it does not directly modulate thymidylate synthase inhibition [10]. However, the correlations seen in this study between immune changes and clinical outcome support pilot data from an earlier study [6] and suggest that immune alterations play some role in the activity of this adjuvant regimen. Most of the differences between the group remaining disease-free and the group experiencing a late relapse are seen at the 15-month assay time. Higher levels of $CD25^+$-activated T cells at this point are consistent with other studies demonstrating increases in CD25 or sIL-2R following LMS administration [3, 6, 8]. Alterations in CD4:CD45RA (naïve) and CD4:CDw29 T cell subsets, and their correlation with clinical outcome, are a novel finding of this study. These subsets

Table 3 Differences among clinical outcome groups at 15-month assay time: total antigen expression. *P* values for comparison between patient groups

Patient group and antigen	No. of cells × (μl)$^{-1}$			Change from baseline (%)	*P*
	Mean	± SD	Median		
Late relapse (n = 4)					
CD 8	243	230	145	−22	
CD4:CD45RA	42	56	20	−30	
CD4:CDW29	235	225	159	−17	
CD56	294	323	163	+8	
CD25	5.7	57	5.0	+275	
VLA4	960	881	605	+37	
NED (n = 14)					
CD 8	565	499	419	+25	NS
CD4:CD45RA	191	182	131	+350	0.0178
CD4:CDW29	623	388	544	+200	0.0349
CD56	87	333	289	+43	NS
CD25	91	128	38	+5330	0.0276
VLA4	1509	652	1313	+75	NS

have not been examined previously in this clinical group, though specific effects of LMS on T cell subsets have been seen in vitro [5].

It is interesting that immunological changes are seen primarily at 12 and 15 months. If immunostimulation contributes to the effectiveness of 5FU/LMS, this may explain the relative lack of activity of 5FU/LMS when administered for a more limited 6-month course [16, 17]. A recently reported prospective trial suggested that 5FU/LMS for 6 months is slightly less successful than 5FU/LMS for 12 months and the combination of 5FU/lecovorin/LMS for 6 or 12 months [18]. Detailed immunological testing has not been performed on patients receiving the three-drug combination or on patients receiving 5FU/leucovorin alone. The delayed immunological response suggests that prolonged exposure to LMS may be necessary and that this agent may prime cells for subsequent activation. It is also tempting to speculate that immunological responsiveness may be suppressed during 5FU administration and that sequential, rather than concurrent use of 5FU and LMS may be more clinically efficacious.

There are several limitations to this study. It is a small pilot study of only 38 patients and, because of the relative lack of usefulness of cytotoxicity testing, immunological analysis was principally static rather than functional. In addition, the analysis of clinical outcome was retrospective rather than prospective and, because it would be clinically inappropriate, there was neither an untreated control group nor a group receiving 5FU alone. Despite these limitations, the results are significant in that they support prior in vitro and in vivo investigations and suggest that immune testing may be useful in stratifying patients for risk. Identification of patients, following surgery and prior to adjuvant therapy, who will relapse early is currently not possible. This study suggests that total CD8 may be a predictive marker for early relapse, at least for patients receiving 5FU/LMS. Low prestudy CD8 may identify a population for which the standard 5FU/LMS is not an appropriate adjuvant treatment regimen. Immune parameters may also be useful in risk-stratifying patients following adjuvant 5FU/LMS. Nearly 40% of patients with stage III disease will relapse, regardless of the adjuvant regimen utilized. To advance the treatment of patients with stage III colon cancer further, it is imperative that prognostic markers be developed that will identify patients who need additional or more aggressive adjuvant therapy. This study suggests that comparison of immune parameters obtained at the completion of therapy to those obtained in the same individual prior to adjuvant therapy initiation may be useful in this regard, at least for patients receiving LMS-containing regimens. The ultimate utility of such immune testing will await additional larger, prospective studies with both static and functional immunological correlates.

References

1. Abbas AK, Williams ME, Burstein HJ, Chang T-L, Bossu P, Lichtman AH (1991) Activation and functions of CD4$^+$ T cell subsets. Immunol Rev 123:5–22
2. Brabander M de, Cree J de, Vandebroek J, Verhaegen H, Janssen PAJ (1992) Levamisole in the treatment of cancer: anything new? Anticancer Res 12:177–188
3. Brabander M de, Vandebroek J, Wassenaar H, DeCree J, Braisier A, Demoen B, DeRidder R, Jagers E, Roels V, Vogels O (1995) Immunological alterations induced by adjuvant treatment of postoperative colon carcinoma Duke's B or C with levamisole in combination with 5-FU. Anticancer Res 15:2271–2277
4. Chrisp P, McTavish D (1991) Levamisole/fluorouracil: a review of their pharmacology and adjuvant therapeutic use in colorectal cancer. Drugs Aging 4:317–337
5. Holcombe RF, Stewart RM (1997) Augmentation of IL12-dependent Th1 cytokine production by levamisole. Allergy Clin Immunol Int 9:119–120
6. Holcombe RF, Stewart RM, Betzing KW, Kannan K (1994) Alteration in lymphocyte phenotype associated with administration of adjuvant levamisole and 5-fluorouracil. Cancer Immunol Immunother 34:394–398
7. Hsieh C-S, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM (1993) Development of Th1 CD4$^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 260:547–549
8. Janik J, Kopp WC, Smith JW, Longo DL, Alvord G, Sharfman WH, Fenton RG, Sznol M, Steis RG, Creekmore SP, Ewel CH, Hursey J, Urba WJ (1993) Dose-related immunological effects of levamisole in patients with cancer. J Clin Oncol 11:125–135
9. Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Statist Assoc 53:457–481
10. Kovach JS, Svingen PA, Schaid DJ (1992) Levamisole potentiation of fluorouracil antiproliferative activity mimicked by orthovanadate, an inhibitor of tyrosine phosphatase. J Natl Cancer Inst 84:515–519
11. Laurie JA, Moertel CG, Fleming TR, Wiegand HS, Leigh JE, Rubin J, McCormack GW, Gerstner JB, Krook JE, Malliard J, Twito DI, Morton RF, Tschetter LK, Barlow JF (1989) Surgical adjuvant therapy of large-bowel carcinoma: an evaluation of levamisole and the combination of levamisole and fluorouracil. J Clin Oncol 7:1447–1456
12. Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S (1993) Natural killer cell stimulatory factor (interleukin 12 [IL12]) induces T helper type 1 [Th1]-specific immune responses and inhibits the development of IL4 producing Th cells. J Exp Med 177:1199–1204

13. Marimoto, Chikao (1995) CD45 cluster report. In: Schlossman SF, et al (eds) Leucocyte typing. V. Oxford University Press, New York, pp 386–389
14. McKnight AJ, Zimmer GJ, Fogelman I, Wolf SF, Abbas A (1994) Effects of IL12 on helper T cell dependent immune responses in vivo. J Immunol 152:2172–2179
15. Moertel CG, Fleming TR, McDonald JS, Haller DG, Laurie JA, Goodman PJ, Ungerleider JS, Emerson WA, Tormey DC, Glick JH, Veeder MH, Mailliard JA (1990) Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 322:352–358
16. O'Connell MJ, Kahn MJ, Erlichman C, Goldberg RM, Shepherd LE (1996) Optimal duration of fluorouracil plus levamisole colon cancer surgical adjuvant therapy. J Clin Oncol 10:2887
17. O'Connell MG, Laurie JA, Shepherd LE (1996) A prospective evaluation of chemotherapy duration and regimen as surgical adjuvant treatment for high-risk colon cancer: a collaborative trial of North Central Cancer Treatment Group and the National Cancer Institute of Canada Clinical Trials Group. Proc Am Soc Clin Oncol 15:209a
18. O'Connell MJ, Laurie JA, Kahn M, Fitzgibbons RJ, Erlichman C, Shepherd L, Moertel CG, Kocha WI, Pazdur R, Wiand S, Rubin J, Vukov AM, Donohue JH, Krook JE, Figueredo A (1998) Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. J Clin Oncol 16:295–300
19. Renoux G, Renoux M (1971) Effet immunostimulant d'un imidothiazole dans l'immunisation des souris contre l'infection par Brucella abortus. CR Acad Sci Ser D Sci Nat 272:349–350
20. Rudd CE, Morimoto C, Wong LL, Schlossman SF (1987) The subdivision of the T4 (CD4) subset on the basis of the differential expression of L-C/T200 antigens. J Exp Med 166:1758–1773
21. Schiller JH, Lindstom M, Witt PL, Hank JA, Mahvi D, Wagner RJ, Sondel P, Borden EC (1991) Immunological effects of levamisole in vitro. J Immunol 10:297–306
22. Spreafico F (1980) Use of levamisole in cancer patients Drugs 19:105–116
23. Trinchieri G (1993) Interleukin-12 and its role in the generation of Th1 cells. Immunol Today 14:335–338
24. Tripodi D, Parks LC, Brugmans J (1973) Drug-induced restoration of cutaneous delayed hypersensitivity in anergic patients with cancer. N Engl J Med 289:354–357
25. Windle R, Bell PRF, Shaw D (1987) Five year results of a randomized trial of adjuvant 5-fluorouracil and levamisole in colorectal cancer. Br J Surg 74:569–572