Perioperative Immunotherapy with Recombinant Interleukin 2 in Patients Undergoing Surgery for Colorectal Cancer

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ABSTRACT

Major surgery impairs the cellular immune response. We have therefore studied the immunological effects of low-dose recombinant interleukin 2 given to patients undergoing surgery for colorectal cancer to determine whether this agent has potential in perioperative adjuvant immunotherapy. Patients were randomly allocated to control (n = 13) or treatment groups (n = 12). Immunological studies of both lymphocyte function and subset number were performed preoperatively and on Days 1, 4, 7, and 10. Treatment with recombinant interleukin 2 prevented the postoperative fall in both natural killer and lymphokine-activated killer cell cytotoxicity, clearly demonstrated in the control group. The treatment group also showed in vivo T-cell activation with an initial lymphopenia followed by a rebound lymphocytosis and upregulation of the subset markers CD25 (interleukin 2 receptor) and CD45RO (T-memory cells). These combined effects may have important consequences in controlling metastatic dissemination of tumor during the vulnerable perioperative period.

INTRODUCTION

At present the treatment of most gastrointestinal malignancies, including colorectal cancer, is unsatisfactory. Surgery is the mainstay of treatment but, even when all macroscopic disease is removed at operation, the majority of patients still die from recurrent disease (1). As surgery alone is inadequate, several groups have used adjuvant chemotherapy and had modest success in Dukes' Stage C colon cancer (2, 3). This treatment, however, is given for a full year after the operation and is associated with some morbidity.

We are interested in the potential of perioperative immunotherapy using the cytokine rIL-2 in improving the outlook for patients with intestinal malignancy. IL-2 is a key molecule in the cellular immune system (4), being necessary for the proliferation and maturation of T-lymphocytes (5) and the development of LAK cells from NK cell precursors (6). LAK cells have the ability to kill adenocarcinoma cells in vitro and, when given in combination with rIL-2, have demonstrated activity against solid tumors in patients (7).

The perioperative period is a logical time for adjuvant immunotherapy for several reasons. (a) The tumor bulk is at its minimum. (b) The cellular antitumor immune responses, which are already depressed in patients with gastrointestinal cancer (8), are decreased further by major surgery (9). (c) Finally, tumor cells are shed into the circulation at operation (10, 11) and, with host immune defenses already attenuated, there is a clear risk of dissemination. This is proven beyond doubt in animal models (12).

As a first step in assessing the use of perioperative immunotherapy with rIL-2, we have performed a randomized study to examine effect of this cytokine on a number of components of the cellular immune system in patients undergoing surgery for colorectal cancer. High-dose rIL-2 is extremely toxic (7) and clearly unsuitable in this vulnerable perioperative period, so we have evaluated a low-dose s.c. regimen. This route of administration produces sustained plasma levels of IL-2 over a 12-h period (13) and has afforded response rates similar to i.v. rIL-2 in patients with malignant melanoma and renal cell carcinoma (14).

MATERIALS AND METHODS

Patients. Twenty-five patients undergoing elective surgery for histologically confirmed colorectal cancer were studied (Table 1). Patients were prospectively randomized into control and treatment groups. The control group proceeded through surgery in the normal manner. The treatment group each received 1.8 x 106 IU/m2 of rIL-2 s.c. (EuroCetus, Harefield, United Kingdom) twice daily for 5 days starting the evening before surgery. Injections were administered at 8 a.m. and 8 p.m. The study was approved by Leeds Eastern District Clinical Research (Ethics) Committee.

Immunological Studies. Baseline assessment of lymphocyte number and function was performed on the preoperative day and repeated on postoperative Days 1, 4, 7, and 10. From each patient a 35-ml sample of peripheral venous blood was drawn between 7 and 8 a.m., before the morning dose of rIL-2. In each instance 5 ml were collected into EDTA and the remaining 30 ml into bottles containing 8 drops of preservative-free heparin.

Cell Surface Markers. Enumeration of leukocyte subpopulations was performed by flow cytometry using a panel of directly conjugated monoclonal antibodies directed against cell surface antigens. EDTA-stored blood was labeled within 4 h of collection using a whole blood technique. In brief, 100 µl of undiluted whole blood were incubated for 15 min with a mixture of two monoclonal antibodies, each in a 1:10 dilution. The red cells were lysed using FACS lysing solution (Becton Dickinson Immunochemistry Systems, San Jose, CA). The leukocytes were pelleted and washed once in phosphate-buffered saline containing 0.1% azide before being stored at 4°C in the dark until analysis. The following monoclonal antibodies were obtained from DAKO, Ltd. (High Wycombe, United Kingdom): UCHT1 (CD3, total T-cells); MT310 (CD4, helper/inducer T-cells); DK25 (CD8, cytotoxic/ suppressor T-cells); ACT-1 (CD25, p55 subunit IL-2 receptor); UCHL1 (CD45RO, T-cell activation marker); and mouse immunoglobulin G1 isotype controls. Anti-Leu-11c (CD16, NK cells) and anti-Leu-19 (CD56, NK and LAK cells) were obtained from Becton Dickinson Immunochemistry Systems. All antibodies were directly conjugated with either FITC or phycoerythrin.

The cells were analyzed on a Becton Dickinson FACSscan. Lymphocytes were gated by low-angle forward and right-angle light scatter properties. The percentage of cells reactive with each monoclonal antibody was determined by comparison of fluoresceinlabeled cells with cells that had been labeled with isotype controls. Analysis was performed using Lysis 2 software (Becton Dickinson), and results were determined by 4-quadrant analysis having gated for >99% positive cells on isotype control samples.

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The abbreviations used are: rIL-2, recombinant interleukin 2; NK, natural killer; LAK, lymphokine-activated killer; PBMC, peripheral blood mononuclear cells; HBSS, Hank's buffered salt solution; IL-2, interleukin 2; FITC, fluorescein isothiocyanate.
Total leukocyte counts and differential cell counts were performed on a Technicon H1 analyzer in the pathology department at St. James's Hospital.

Lymphocyte Separation. PBMC were separated from heparinized blood by Lymphoprep (Nycomed Pharma, Oslo, Norway) density centrifugation (15); washed twice in HBSS; and resuspended in complete medium consisting of RPMI 1640 (Gibco, Ltd., Uxbridge, United Kingdom) supplemented with 10% fetal calf serum (Imperial Laboratories, Andover, United Kingdom), gentamicin (200 μg/ml), Fungizone (2.5 μg/ml), nonessential amino acids, mercaptoethanol (50 μmol), and sodium pyruvate (5 mmol). The cells were counted in a Neubauer counting chamber and diluted in complete medium to the cell density required for each experiment.

Natural Killer Cell Cytotoxicity Assay. Freshly isolated PBMC were used for determination of NK cell activity using a standard 4-h chromium release cytotoxicity assay with the cell line K562 as target (16). In brief, 5 × 10^5 effector cells (PBMC) were incubated with 3^11 Cr-radio labeled K562 target cells (1 × 10^4) in triplicate wells of round-bottomed microtiter plates in a total reaction volume of 200 μl of complete medium. Serial doubling dilutions of effector cells were performed so that effector:target ratios from 50:1 to 6.25:1 were achieved. After incubation at 37°C in a humidified atmosphere containing 5% CO2 for exactly 4 h, 100 μl of supernatant were removed from each well, and its radioactivity was measured on a Cobra gamma counter (Canberra Packard). Spontaneous and maximal 3^11 Cr release was measured by incubating 1 × 10^4 target cells with complete medium alone and with detergent, respectively. The percentage of cytotoxicity of the target cells at each effector:target ratio was calculated from the standard formula: (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100%.

Lymphokine-activated Killer Cell Assay. For the generation of LAK cells, 15 × 10^6 fresh PBMC were cocultured with 1000 units/ml of recombinant IL-2 (Roche Products, Ltd., Basle, Switzerland) in 10 ml of complete medium. The upstanding tissue culture flasks were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO2 with gentle agitation daily. The cells were harvested, washed once in HBSS, resuspended in complete medium, and counted for use as effectors in the cytotoxicity assay. This was performed in the same way as for the NK assay above but using two NK-resistant cell lines as targets, DAUDI (a reference target for LAK cell activity) and COLO 205 (a colonic adenocarcinoma cell line).

Lymphocyte Proliferation Assay. Freshly isolated PBMC were stimulated using the T-cell mitogen concanavalin A (Sigma Chemicals, Ltd., Poole, England) as previously described (17). Lymphocytes at a concentration of 10^6 cells/ml were cocultured with 20 μg/ml of concanavalin A in a final volume of 200 μl in flat-bottomed microtiter plates for 40 h at 37°C in a humidified atmosphere containing 5% CO2. Lymphocytes without mitogen were simultaneously cultured and used to give background activity. Lymphocyte proliferation was determined by adding 1 μCi of tritiated thymidine (Amersham, Little Chalfont, United Kingdom) to each well for the final 18 h of culture. Cells were harvested on a Dynatech 2000 cell harvester, and the [3H]thymidine uptake of cells was measured on a Philips beta counter. Results are expressed as cpm × 10^3 of stimulated lymphocytes.

Effects of Perioperative Blood Transfusion. Perioperative allogenic blood transfusion requirements were documented on all patients. Results were then reanalyzed within each group with respect to the need of transfusion.

Statistical Analysis. Statistical analysis was performed between groups using the t test for unpaired data and within a group using a paired t test. Results are expressed as the mean ± SE with a probability value of <0.05 regarded as statistically significant.

RESULTS

Patient Outcome. All patients receiving rIL-2 treatment tolerated the injections well. There was a significant increase in maximum postoperative pyrexia from 37.4 ± 0.4°C (maximum temperature, mean ± SD) in the control group to 38.6 ± 0.7°C in the treatment group (P < 0.05), but this was limited to the treatment period. No other treatment-related side effects were noted.

Lymphocyte Phenotype Analysis. Before the operation both groups had identical numbers and subpopulations of lymphocytes. In each there was an early postoperative lymphopenia, more marked in the treatment group (Table 2). By Day 7, however, the total lymphocyte numbers in the control arm had returned to preoperative levels, whereas those in the treatment arm showed a highly significant rebound lymphocytosis (P < 0.01).

In the control group, the percentage expression of the lymphocyte subset markers did not change over the postoperative period. By contrast, treatment with rIL-2 had profound effects on a number of the subpopulations measured (Table 3). The percentage of T-cell (CD3) expression fell significantly during the early postoperative period before returning to preoperative levels by Day 4. This decrease was due to a specific fall in T-helper cells (CD4) and, hence, the helper:cytotoxic ratio (CD4:CD8) also fell significantly (Fig. 1). The T-cytotoxic cell (CD8) expression was unaltered by treatment as were the null cell markers (CD16 and CD56). Expression of the activation marker CD25 (IL-2 receptor subunit) fell on the first postoperative day, but this simply mirrors the decrease in circulating CD4 cells, on which this marker is mostly expressed. However, as shown in Fig. 2, CD25 median fluorescence (demonstrating IL-2 receptor density on circulating cells) was significantly raised throughout the treatment period to more than double preoperative values. The other activation marker CD45RO (T-memory cells) also rose, but only during the posttreatment lymphocytosis (Table 3). Changes in activation marker expression differed between subsets, and CD25 positivity varied specifically within the CD4+ helper T-cell subset, whereas changes in CD45RO expression were identifiable on both CD4+ and CD8+. No changes were observed within the null cell population.

Lymphocyte Function Analysis. After the operation, NK cell activity of PBMC was significantly reduced in the control patients. Those receiving rIL-2, however, showed no such fall in

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Preoperative</th>
<th>1</th>
<th>4</th>
<th>7</th>
<th>10</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td>1.18 ± 0.15*</td>
<td>0.85 ± 0.12</td>
<td>0.78 ± 0.11*</td>
<td>1.02 ± 0.15</td>
<td>0.92 ± 0.15</td>
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<tr>
<td>rIL-2</td>
<td>1.23 ± 0.21</td>
<td>0.56 ± 0.06*</td>
<td>0.60 ± 0.14*</td>
<td>2.36 ± 0.36*</td>
<td>1.70 ± 0.17*</td>
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* Mean ± SEM of lymphocyte count × 10^3. Significance calculated using paired t test.

* Versus preoperative values, P < 0.05.

* Versus preoperative values, P < 0.01.
Table 3  Effect of treatment with rIL-2 on the percentage expression of a number of lymphocyte subset markers, in patients undergoing surgery for colorectal cancer

<table>
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<tr>
<th>Postoperative days</th>
<th>Treatment group</th>
<th>Preoperative</th>
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<td></td>
<td>CD3</td>
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<td>64.2 ± 2.6*</td>
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<td>58.5 ± 3.7*</td>
<td>65.5 ± 2.7</td>
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<td></td>
<td>rIL-2</td>
<td>67.4 ± 2.6</td>
<td>58.7 ± 3.3*</td>
<td>65.1 ± 3.5</td>
<td>73.0 ± 2.0*</td>
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<td>CD4</td>
<td>Control</td>
<td>38.2 ± 2.2</td>
<td>35.5 ± 2.5</td>
<td>37.4 ± 2.7</td>
<td>40.8 ± 2.8</td>
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<td></td>
<td>rIL-2</td>
<td>40.3 ± 2.4</td>
<td>26.8 ± 1.9*</td>
<td>38.4 ± 4.2</td>
<td>45.8 ± 2.4</td>
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<td>CD8</td>
<td>Control</td>
<td>27.3 ± 2.6</td>
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<td></td>
<td></td>
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<td>Control</td>
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<td></td>
<td>rIL-2</td>
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<td>6.7 ± 0.9*</td>
<td>12.2 ± 3.9</td>
<td>26.6 ± 3.8c</td>
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<td>CD45RO</td>
<td>Control</td>
<td>32.0 ± 2.6</td>
<td>30.9 ± 2.4</td>
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<td>33.0 ± 2.6</td>
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<tr>
<td></td>
<td></td>
<td>rIL-2</td>
<td>31.4 ± 3.2</td>
<td>27.5 ± 3.2</td>
<td>34.2 ± 4.5</td>
<td>44.7 ± 3.8c</td>
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* Mean ± SEM of percentage expression, statistical analysis performed using paired t test.

a Versus preoperative values, * P < 0.05.
c Versus preoperative values, * P < 0.01.

Fig. 1. Effects of perioperative rIL-2 on the CD4:CD8 ratio of peripheral blood lymphocytes in patients undergoing surgery for colorectal cancer. Points, mean; bars, SEM. **, P < 0.01 versus baseline value, *, P < 0.05 between the two groups. Heavy bar indicates the treatment period.

Fig. 2. Effect of perioperative rIL-2 IL-2 receptor (CD25) density on peripheral blood lymphocytes in patients undergoing surgery for colorectal cancer. Results are given for the median channel number from the flow cytometric analysis of lymphocytes labeled with the CD25-FITC monoclonal antibody. **, P < 0.01; ***, P < 0.01 versus baseline values. ***, P < 0.01 between the two groups. Heavy bar indicates the treatment period. Points, mean; bars, SEM.

activity. This was the case regardless of the effector:target ratio; the 50:1 ratio is shown in Fig. 3.

The ability to generate LAK cells from PBMC is similarly reduced by surgery in the control group. By contrast, treatment with rIL-2 enhanced LAK activity against both the target cell lines, with that for Daudi shown in Fig. 4. The results for Colo 205 are identical (data not shown). Endogenous LAK activity was not observed in any patient in the control and only occasionally in the treatment group (data not shown).

Basal lymphocyte proliferation (without concanavalin A) was the same in both groups (data not shown). Maximal lymphocyte proliferation is shown in Table 4; there was no perioperative change within the control group, but the treatment group showed a marked fall on Day 1 before returning to preoperative values by Day 4.

Effects of Blood Transfusion. The transfusion requirement was not significantly different between the two groups, with 7 of 13 patients in the control group requiring 2.6 ± 1.5 units per patient transfused compared with 9 of 12 in the treatment group requiring 3.2 ± 1.8 units per patient transfused. There were no differences between the two groups with respect to any of the parameters measured, although the actual numbers are very small.

DISCUSSION

The perioperative period is a critical time for patients with colorectal cancer. Such patients are already in a state of relative immunosuppression (8), when they are exposed to a further immunological assault from surgical resection itself (18-21) and, hence, this is an appropriate time for immunotherapy. Previous studies from this laboratory examined the use of recombinant α-interferon in the perioperative period in patients with gastrointestinal cancer (9). The results were disappointing and showed that, although NK activity was enhanced, there were no other changes in cellular immunity likely to have clinical benefit. These studies, however, led us to investigate the use of rIL-2, which has subsequently become available.
This study shows that perioperative low-dose rIL-2 is safe when given in the perioperative period with, in particular, no evidence of the capillary leak syndrome which characterizes IL-2 toxicity (7). By contrast with the lack of clinical effects, the immunological consequences were profound. We observed in vivo lymphocyte activation, with upregulation of CD25 expression (part of the IL-2 receptor) and an increase in the number of cells expressing the activation marker CD45RO (T-memory cells) (22). The other striking feature was the abrogation of the effects of surgery on NK activity and stimulation of the ability of NK cells to become LAK cells. Normally both of these components of the immune system are profoundly depressed by the operation (9), as seen in the control group. Since these cells are reputed to have an antitumor surveillance role within the circulation, perioperative enhancement of their activity has potential benefit for the patient.

In this study we have measured both the number and function of the null cells (NK and LAK) using cell surface markers and cytotoxicity assays, respectively. CD16 labels NK cells, whereas CD56 is reported as being more specific for the lymphokine-activated phenotype (23). Comparing results from the subset analysis with the functional assay it is clear that the postoperative fall in NK activity in the control group is due to cellular anergy, since the percentage of CD16+ cells remains unchanged. The situation with LAK cells is more complicated because phenotypic analysis using CD56 was performed prior to 3-day culture with IL-2. However, the fact that the percentage of CD56+ cells before culture remained unchanged suggests that, as with the NK cells, cellular anergy is responsible for the LAK cell functional deficit in the control group. We are currently examining the expression of the CD56 marker in lymphocytes being cultured with IL-2. Endogenous LAK cell activity, demonstrated occasionally in the rIL-2-treated group, may yet prove to be significant.

One prominent feature of our results is the previously reported postoperative lymphopenia (7, 24), due specifically to a fall in the number of T (CD3+) lymphocytes. The fall was noted in all patients, but the pattern of T-cell change was markedly different between the two groups. The CD4+/CD8+ ratio was unchanged in the control group, in contrast to the highly significant fall demonstrated by those receiving rIL-2. The specific fall in circulating CD4+ cells is reputedly due to the sequestration of these highly activated cells into peripheral sites (25). Again, this change may be beneficial as it may direct activated lymphocytes to the tumor and lymph nodes. The fall in the ability of lymphocytes in the treatment group to proliferate is also consistent with the sequestration hypothesis, since it implies that the cells in peripheral blood are already near maximally stimulated, although it may in fact reflect a significant reduction in immune capability.

Despite compelling evidence from animal models (26), the benefit of perioperative immunotherapy in patients with malignant disease may not have been established. In respect to colon cancer, it is now suggested that many patients have occult hepatic metastases (27), in spite of having had an apparently curative resection. The benefit from adjuvant therapy with levamisole, a nonspecific immunomodulator, and 5-fluorouracil, amounting to a survival advantage of 30%, is seen in those patients with Dukes' Stage C colon cancer (2), but it is not clear whether it is those patients with occult metastases who gain. Clearly, a short period of treatment with rIL-2 in the perioperative period may be able to deal with cells liberated around the time of surgery, but it is unrealistic to expect major benefit in patients with established metastases. Clinical studies will have to compare standard adjuvant therapy (2) with rIL-2 used both alone and in combination with other biological response modifiers, in an attempt to optimize immunological enhancement during the perioperative period.

In conclusion, we have demonstrated that, although perioperative low-dose rIL-2 has profound effects on the cellular antitumor-immune system, it can be safely given during this vulnerable period. On the basis of these data a clinical trial can be
justified to examine the effect of perioperative rIL-2 on survival after surgical treatment for colorectal cancer.

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