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# Mechanisms of Experimental Cancer Cachexia

## Local Involvement of IL-1 in Colon-26 Tumor

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**Abstract.** In the colon-26 (C-26) tumor model, the cytokine IL-6 is an important factor involved in experimental cancer cachexia. Recent in vitro data indicated that IL-1 plays a role in the interaction between host macrophages and C-26 cells that express IL-1R, resulting in the amplification of tumor IL-6 production. To investigate the role of IL-1 on the development of C-26 cachexia in vivo, the effect of specific blockade of the action of IL-1 with reagents against IL-1R was evaluated. Both IL-1R antagonist (IL-1RA) and the mAb 35F5 directed against IL-1R type I, prevented binding of radioactive IL-1, and inhibited IL-1-induced IL-6 synthesis by the C-26 cell line. Whereas a systemic administration of these reagents did not reverse weight loss in C-26-bearing mice, intratumoral injections of IL-1RA significantly reduced cachexia. Furthermore, body composition analysis confirmed that this treatment improved lean tissue and fat, as well as hypoglycemia and serum IL-6 level. The fact that the treatment did not change the tumor burden suggests that it affected the host directly. These results support the hypothesis that, at the microenvironment of the C-26 tumor, IL-1 is involved in the cachexia endured by the host. *Journal of Immunology,* 1993, 150: 2341.

Neoplastic diseases are frequently associated with metabolic changes collectively known as cancer cachexia. These abnormalities include wasting of both fat and muscle tissues, anorexia, asthenia, hypoglycemia, and anemia (1, 2). Cachexia long has been recognized as an important cause of death in cancer patients (3), and patients who exhibit weight loss have a reduced response to chemotherapy (4). Understanding the mechanisms that lead to cachexia therefore is important.

TNF has been suggested as a mediator of cancer cachexia because it suppresses key metabolic enzymes and induces anorexia and weight loss in animals (5–8). Recently, however, an experimental cachexia model has been identified that appears to involve another cytokine. The model uses a cell line derived from C-26,[2] which retains the transplantability of the original tumor in syngeneic mice, and fulfills the criteria of early onset wasting without apparent anorexia. It also involves a relatively small tumor burden (9). In at least this model, IL-6 appears to have a more significant role than TNF in mediating the myriad parameters of cachexia (9). In culture, the C-26.IVX cell line expresses high affinity IL-1R type I, and fM concentrations of IL-1 but not TNF, upregulates IL-6 production (10). In addition, significant potentiation of tumor IL-6 secretion can be seen when the line is co-cultured with syngeneic mononuclear phagocytes. In this cellular interaction, the mAb 35F5, directed against murine IL-1R type I, blocks IL-6 synthesis (10).

IL-1RA is a naturally occurring protein (11), which is useful in blocking several IL-1-mediated pathologies in vivo (12). Accordingly, utilizing IL-1R blocking reagents, the role of IL-1 in the development of C-26-mediated cachexia was investigated.

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[2] Abbreviations used in this paper: C-26, colon-26 adenocarcinoma; IL-1RA, IL-1R antagonist; (CD)$F_1$, BALB/c × DBA/2$F_1$.

## Materials and Methods

### Mice

Virus-free, male BALB/C × DBA/2 (CD)$F_1$, mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Mice were housed under conventional conditions and used at 10 to 12 wk of age.

### Reagents

Human rIL-1β was a gift from Dr. Y. Hirai (Otsuka Pharmaceutical, Tokushima, Japan). [$^{125}$I]IL-1α (sp.act. 2000 Ci/mmol.) was from Amersham (Arlington Heights, IL). Purified IL-1RA was obtained after the cloning, expression, and purification procedures described previously (11, 13). The sterile material had a purity of greater than 99% by SDS-PAGE, and endotoxin content was determined to be less than 0.5 ng/mg of protein. For in vivo experiments, IL-1RA was diluted in sterile Dulbecco's $Ca^{++}$- and $Mg^{++}$-free PBS (GIBCO, Grand Island, NY). This diluent also served as a vehicle for control injections. The rat mAb 35F5 (14) (IgG1) was obtained from Dr. R. Chizzonite (Hoffman-LaRoche, Nutley, NJ). Purified rat IgG was obtained from Sigma (St. Louis, MO).

### Assays

The IL-1 radioreceptor assay on C-26.IVX (obtained without trypsinization) and on EL-4.6.1 cells was performed as previously described (10). The presence of IL-6 in serum and culture-conditioned medium was performed by utilizing the B-9 cell line assay (10) and ELISA (Endogen, Boston, MA). The addition of mAb against murine IL-6 and a murine IL-6R completely abrogated IL-6-dependent proliferation by test samples.

### Measurement of cachexia markers

Mice were inoculated with 0.5 × $10^6$ C-26.IVX cells s.c. to the right flank as described (9, 10). Treatments were performed as indicated in the tables. Mice were weighed between 9 a.m. and 11 a.m. three times per week. The length and width of their tumors were measured by using an engineering caliper, and estimation of tumor weight was calculated, as described, for the same tumor (15). Significant weight loss in C-26-bearing mice occurred between 12 and 14 days after tumor inoculation. Host weight was calculated by subtracting tumor weight (obtained by resection) from total weight. Blood was obtained by cardiac puncture (approximately 0.8 ml), and serum was harvested after the clotting of blood at room temperature for 1 h. Serum was kept frozen (−45°C) until analysis. Measurements of serum glucose were performed using an Ektachem DT-60 analyzer (Eastman Kodak Co., Rochester, NY). Dry weight was determined (after removal of the tumor, blood, and right epididymal fat) by oven drying for 3 days at 85°C.

Table I
*IL-1RA and anti-IL-1R type I mAb block binding of radioactive IL-1 to C-26.IVX cells*

| Addition to Radioreceptor Assay[a] | [$^{125}$I]IL-1α Bound (cpm)[b] | |
|---|---|---|
| | C-26.IVX | EL-4.6.1 |
| Medium | 2237 ± 10 | 3483 ± 25 |
| Human IL-1β (100 ng/ml) | 400 ± 10 | 491 ± 5 |
| Rat IgG (2 μg/ml) | 2257 ± 80 | 3338 ± 210 |
| Anti-IL-1R (2 μg/ml) | 495 ± 5 | 457 ± 35 |
| IL-1RA (30 ng/ml) | 437 ± 20 | 400 ± 30 |

[a] Radioreceptor assay was performed as described in *Materials and Methods*.
[b] Results are expressed as cpm bound ± SD.

### Statistical analysis

Results are presented as mean ± SD. Differences in cachexia markers were calculated using computerized analysis of variance.

## Results and Discussion

First we determined whether reagents capable of recognizing IL-1R would block binding of radioactive IL-1 to the C-26.IVX cell line. As shown in Table I, the mAb 35F5 and IL-1RA completely blocked binding of [$^{125}$I]IL-1α to both the C-26 cell line and to EL-4.6.1 cells in a radioreceptor assay. In a dose-dependent manner, both the mAb and the IL-1RA inhibited IL-1 induced IL-6 production by the tumor line (Table II). On a molar basis, IL-1RA was approximately 240-fold more active than the 35F5 mAb in inhibiting IL-6 synthesis. Also in the table, IL-1RA at millimolar concentration had no agonist activity in inducing IL-6 production by the tumor line.

Next we examined the role of IL-1 in C-26 mediated cachexia. Mice were inoculated with the tumor line and treated with the mAb and IL-1RA, as indicated in Table III. No significant or sustained improvement in tumor-mediated weight loss could be seen when these reagents were administered systemically. Of note, the amount of IL-1RA used in this experiment exceeded the amount used to protect mice from graft-vs-host disease mortality (16) and to block IL-1 induction of IL-6 in vivo (17). The amount of 35F5 mAb used was greater than that shown to increase mortality of irradiated mice (18) and to attenuate turpentine-induced weight loss (19). Together with our previously published data (9), these results suggest that IL-1 does not act in concert with IL-6 in the circulation of C-26 bearing mice as they were losing weight. Nevertheless, the possibility still exists that the amount of blocking reagents used was insufficient to reach the tumor and effectively exert their action. Thus, we attempted to prevent cachexia by the direct administration of IL-1RA to the tumor. As shown in Tables IV and V, intratumoral injections of IL-1RA significantly improved the deleterious systemic effects of the tumor. Typical cachectic parameters, including

Table II
*IL-1RA and anti-IL-1R type I mAb inhibit IL-1-induced IL-6 production by C-26.IVX cells*[a]

| Addition to Culture | | | IL-6 Production | | |
|---|---|---|---|---|---|
| | | | Bioassay | | |
| IL-1 | IL-1RA | Anti-IL-1R | 24 h | 48 h | ELISA 48 h |
| – | – | – | 3 | 35 | 30 |
| + | – | – | 330 | 1100 | 1120 |
| + | 100 | – | 6 | 50 | 50 |
| + | 30 | – | 10 | 87 | NT[b] |
| + | 10 | – | 24 | 210 | 260 |
| + | 3 | – | 90 | 860 | NT |
| + | 1 | – | 154 | 1290 | NT |
| – | 100 | – | 4 | 31 | NT |
| + | – | 3 | 6 | 40 | 80 |
| + | – | 1 | 6 | 40 | NT |
| + | – | 0.3 | NT | 150 | 250 |
| + | – | 0.1 | NT | 500 | NT |
| – | – | 3 | NT | 30 | NT |

[a] C-26 IVX ($2 \times 10^5$/well) cells were cultured with IL-1 (10 pg/ml) and with increasing amounts of IL-1 RA or anti IL-1 R type I mAb (indicated in the table as nanograms per milliliter and micrograms per milliliter, respectively). At 24 and 48 h, culture supernatants were collected and subjected to IL-6 bioassay (in units per milliliter) or ELISA (in picograms per milliliter). Variation of triplicate determinations did not exceed 10%.
[b] Not tested.

Table III
*Systemic administration of IL-1 RA and anti IL-1 R type I mAb does not improve weight loss*[a]

| Tumor Bearing | Treatment | No. | Host weight (g) on Day[b] | | | Tumor weight (g)[b] Day 16 |
|---|---|---|---|---|---|---|
| | | | 10 | 13 | 16 | |
| + | PBS | 6 | 25.0 ± 0.5 | 20.6 ± 1.0 | 19.0 ± 0.8 | 1.2 ± 0.2 |
| + | IL-1RA | 6 | 25.5 ± 0.7 | 23.0 ± 0.9[c] | 21.0 ± 1.0 | 1.1 ± 0.3 |
| + | Anti IL-1R (type 1) | 6 | 25.9 ± 0.6 | 20.1 ± 0.8 | 19.1 ± 0.9 | 1.4 ± 0.5 |
| + | Rat IgG | 5 | 25.6 ± 0.3 | 20.4 ± 0.5 | 18.9 ± 0.5 | 1.3 ± 0.3 |
| – | PBS | 5 | 25.3 ± 0.3 | 25.3 ± 0.5 | 25.4 ± 0.5 | |

[a] CD2F1 male mice were injected with C-26.IVX cells (day 0). On day 7 mice were randomized to receive PBS (0.25 ml, s.c. daily), IL-1RA (10 mg/kg, s.c. daily), anti-IL-1R type I mAb and polyclonal rat IgG (12.5 mg/kg, i.p. on days 7, 10, and 13).
[b] Results are expressed as weight of the host (total weight – estimated tumor weight) for days 10 and 13 and the weight of the host (following resection of the tumor) on day 16 ± SD.
[c] $p < 0.025$ from PBS-injected tumor-bearing mice.

Table IV
*Inhibition of Cachexia by Intratumoral Administration of IL-1RA is Dose Dependent*[a]

| Tumor Bearing | Treatment | Initial Weight (g) | Final Body Weight (g) | % Weight Loss | Tumor Weight (g) | Serum Glucose (mg/dl) |
|---|---|---|---|---|---|---|
| – | PBS | 25.1 ± 0.9 | 25.8 ± 0.4 | – | – | 126 ± 3 |
| + | PBS | 25.1 ± 1.3 | 20.2 ± 0.3 | 19.5 | 1.1 ± 0.3 | 32 ± 6 |
| + | IL-1RA (20) | 24.5 ± 0.9 | 20.2 ± 0.8 | 17.5 | 1.1 ± 0.5 | NT[b] |
| + | IL-1RA (50) | 24.6 ± 0.8 | 22.6 ± 1.2[c] | 8.2 | 1.0 ± 0.4 | 64 ± 17[d] |
| + | IL-1RA (100) | 25.2 ± 1.5 | 23.7 ± 1.7[d] | 5.9 | 1.1 ± 0.4 | 72 ± 14[d] |

[a] $CD2F_1$ male mice were injected with C26.IVX cells (day 0). On day 7 mice were randomized. On day 9, 11, 13, and 14 tumors were injected with 0.1 ml PBS or with increasing amounts (shown in parentheses in micrograms) of IL-1RA. Mice were killed on day 15 and final body weight was determined after resection of tumor. Results are expressed as mean ± SD of five mice per group.
[b] Not tested.
[c] Probability value of at least 0.03 from the group which received intratumoral injection of PBS.
[d] Probability value of at least 0.01 from the group which received intratumoral injection of PBS.

weight loss (both fat and muscle tissue), hypoglycemia, and serum IL-6 levels, were significantly improved by the treatment. Of note, the intratumoral administration of the 35F5 mAb or rat IgG at 100 μg/tumor/injection, under the identical conditions described in Table IV, failed to improve cachexia in C-26 bearing mice (not shown). This observation can be explained by the fact that the IL-1RA is at least two orders of magnitude more potent than the mAb in blocking IL-1 activities on the C-26 line in culture (Table II). It is also conceivable that the significantly lower m.w. of the IL-1RA—as compared with the mAb—allowed it to diffuse more easily and thus affect more cells when injected into the tumor.

The intratumoral administration of IL-1RA resulted in a

Table V
*Reduction in cachetic parameters by intratumoral administration of IL-1RA*[a]

| Parameter | Group 1 | Group 2 | Probability Value | Group 3 |
|---|---|---|---|---|
| Tumor Inoculation | + | + | | – |
| Treatment | PBS | IL-1RA | | PBS |
| Initial weight (g) | 25.7 ± 1.0 | 26.0 ± 1.5 | NS[b] | 26.6 ± 0.8 |
| Final weight (g) | 21.5 ± 1.4 | 24.6 ± 1.2 | 0.005 | 27.7 ± 0.8 |
| Host weight (g) | 20.3 ± 1.4 | 23.4 ± 1.2 | 0.004 | 27.7 ± 0.8 |
| Tumor weight (g) | 1.19 ± 0.05 | 1.13 ± 0.12 | NS | |
| Epididymal fat (mg) | 102 ± 57 | 204 ± 74 | 0.03 | 310 ± 102 |
| Dry weight (g) | 6.5 ± 0.8 | 8.9 ± 1.5 | 0.03 | 10.2 ± 1.1 |
| Serum glucose (mg/dl) | 57 ± 12 | 105 ± 12 | 0.0004 | 153 ± 21 |
| Serum IL-6 (U/ml) | 186 ± 24 | 101 ± 33 | 0.03 | 13 ± 3 |

[a] $CD2F_1$ male mice were injected with C-26.IVX cells (day 0) and were randomized on day 7. On days 9, 11, 13, and 14 tumors were injected with PBS (0.2 ml, group 1) or IL-1RA (100 μg, group 2) and were killed on day 16. Mice in group 3 were non-tumor-bearing age-matched controls. Cachetic markers were quantified as described in *Materials and Methods*. Results are expressed as mean ± SD of six mice/group.
[b] Not significant.

relatively modest reduction (~40%) of serum IL-6, at a time when the protection of the host's weight was more pronounced (Table V). IL-1RA is a competitive antagonist to the action of IL-1 on the C-26 cell line (Table II). Its effect, therefore, is not permanent and may not be sufficient to inhibit IL-6 production by the additional tumor cells generated between the last administration of IL-1RA and the completion of the experiment. Alternatively, our still incomplete understanding of the regulation of IL-6R in the C-26 model, and the fact that serum IL-6 level may represent the unused portion of the cytokine in vivo, make it difficult to evaluate accurately the importance of serum IL-6 level. In addition, we cannot exclude the possibility that the level of IL-6 in the serum is of limited importance, as was recently demonstrated by May et al. (20)

To the best of our knowledge, the results presented here are the first to suggest that events occurring in the tumor mass contribute significantly to wasting, and that IL-1 plays an important role locally at the level of the tumor to initiate and/or exacerbate the wasting syndrome. IL-1 is a potent stimulator of IL-6 synthesis by the C-26 tumor line (10). The results shown here, although supporting the stimulator role of IL-1 in vivo, do not exclude the possibility that the cytokine may also amplify the production of a still undetermined factor/mediator that participates in C-26 cachexia, in addition to IL-6. These factors may also affect the physiology of tumor infiltrating macrophages present in C-26 tumors (10).

IL-6 is a multifunctional cytokine. In addition to its involvement in C-26-associated weight loss (9), it has been implicated in several unrelated chronic inflammatory conditions (21). Thus, an important question in the IL-6 field is how this pleiotropic cytokine contributes to such a wide variety of diseases and whether its involvement in vivo requires other factors that cooperate or synergize with its action.

The fact that intratumoral injection of IL-1RA did not influence tumor burden is important. A previous study attempted to assess the effect of IL-1 on the development of cachexia in the MCG-101 tumor model (22). In that study, however, it was difficult to document a direct effect of IL-1 on wasting because the antibody used inhibited the tumor, thus implicating a role for the cytokine in promoting tumor growth.

The observation that systemically administered IL-1RA and 35F5 mAb could not affect cachexia is not surprising. Many conventional drugs and biologics, including cytokines and mAb, only minimally affect solid tumors, and several physiologic factors have been identified as responsible for the poor delivery of macromolecules to solid tumors (23). Identifying these factors in the C-26 model may further the design of therapeutics that inhibit cachexia.

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