Recombinant Human Interleukin-4 Inhibits Growth of Some Human Lung Tumor Cell Lines In Vitro and In Vivo

By Max S. Topp, Michael Koenigsmann, Anthony Mire-Sluis, Dorothea Oberberg, Florian Etelbach, Zofia von Marschall, Michael Notter, Birgit Reufi, Harald Stein, Eckhard Thiel, and Wolfgang E. Berdel

Cytokines play an important role in activating the immune system against malignant cells. One of these cytokines, interleukin-4 (IL-4), has entered clinical phase I trials because of its immunoregulatory potency. In the present study we report that recombinant human (rh) IL-4 has major direct antiproliferative effects on one human lung cancer cell line (CCL 185) in vitro as measured by a human tumor cloning assay (HTCA), tritiated thymidine uptake, and counting cell numbers and marginal activity in a second cell line (HTB 56) in the HTCA. This activity could be abolished by neutralizing antibody against rhIL-4. The biological response of the tumor cells to the cytokine is correlated with expression of receptors for human IL-4 on both the mRNA level and the protein level. The responsive cell line, CCL 185, secretes IL-6 after being incubated with rhIL-4. On the other hand, neutralizing antibodies against IL-6 showed no influence on the growth modulatory efficacy of rhIL-4 in this cell line. Furthermore, CCL 185 does not show detectable production of IL-1, tumor necrosis factor alpha or interferon gamma after incubation with rhIL-4. Thus, the response to rhIL-4 is not mediated through autocrine production of these cytokines triggered by rhIL-4. In a next series of experiments some of the cell lines were xenotransplanted to BALB/c nu/nu mice. Subsequently, the mice were treated for 12 days with two doses of 0.5 mg/m² rhIL-4 or control vehicle subcutaneously per day. Treatment with rhIL-4 yielded a significant inhibition of tumor growth versus control in two of the non-small cell lung cancer cell lines being responsive in vitro (CCL 185, HTB 56). Histology of the tumors in both groups showed no marked infiltration of the tumors with murine hematopoietic and lymphocytic cells consistent with the species specificity of IL-4. In contrast, no tumor growth inhibition was found in the small cell lung cancer cell lines (HTB 119, HTB 120) being nonresponsive in vitro. We conclude that rhIL-4 has direct antiproliferative effects on the growth of some human non-small cell lung cancer cell lines in vitro and in vivo, which together with its regulatory effects on various effector cell populations makes this cytokine an interesting candidate for further investigation in experimental cancer treatment.

INTERLEUKIN-4 (IL-4), a 20-kD glycoprotein, is a cytokine with pleiotropic functions that was first described to enhance DNA synthesis of purified resting mouse B cells. IL-4 is mainly produced by CD4+ T cells, and to a lesser extent by mast cells and human tumor-associated natural killer cells. The functional properties of IL-4 include activation of resting B cells, enhancement of IgE production in activated B lymphocytes, an increase in expression of the low-affinity receptor for IgE (CD 23), being a growth factor for T cells, and their activation to cytolytic T lymphocytes. In contrast to these functions shared by different mammalian species, IL-4 is also a switch factor for IgG1 in murine B cells and increases tumoricidal activity of murine macrophages. Whereas in human B cells the IgG production is altered to IgG1, IgG2, and IgG3, and supernatants of human macrophages cultured with IL-4 can show a decreased activity against melanoma cells. The receptor for IL-4 is not only expressed on hematopoietic cells, but also on endothelial cells. Recent reports indicate that the IL-4 receptor (IL-4R) is also detectable on solid tumors, such as colon and lung cancer. In one report the receptor for IL-4 is coexpressed with epithelial growth factor (EGF) receptors in adenocarcinomas and squamous cell lung carcinomas but lacks expression in the other histologic subtypes of lung carcinoma. However, the functional role of this IL-4R in lung tumors is not yet understood.

This study demonstrates that recombinant human (rh)IL-4 has a direct antiproliferative effect on some lung carcinoma cell lines in vitro and in vivo. This effect is correlated with expression of the IL-4R.

MATERIALS AND METHODS

Cells. HTB 53, HTB 56, HTB 119, HTB 120, and CCL 185 are human lung carcinoma cell lines and were purchased from the American Type Culture Collection (Rockville, MD). Allogeneic human lymphokine activated killer (LAK) cells were generated from peripheral blood of healthy young donors and cultivated with 10³ U rhIL-2/mL at a cell concentration of 1 × 10⁶/mL medium. CTLL 16, an IL-2 growth-dependent mouse hybridoma, was a gift from Dr W. Falk (German Cancer Research Center, Heidelberg, Germany). Cell culture techniques have been described recently in detail.

Cytokines and antibodies. RhIL-4 was purchased from Genzyme (origin Escherichia coli; Cambridge, MA), PBH (origin yeast; Hannover, Germany), and R & D Systems (origin E. coli; Minneapolis, MN). For the in vivo experiments rhIL-4 was a gift from Dr D.L. Urdal (Immunex, Seattle, WA). Activity was evaluated by simultaneously comparing antiproliferative effects of the various rhIL-4 batches in the human tumor cloning assay (HTCA) with the cell line CCL 185 and showed no significant differences between the batches used. Neutralizing rabbit polyclonal antibody against human (h)IL-4 was purchased from Genzyme. Sheep hIL-1α antiserum (89/582), sheep hIL-1β antiserum (89/584), and goat hIL-6 antiserum (89/586) were gifts from the National Institute for Biologic-

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Submitted April 12, 1993; accepted July 2, 1993.

Supported by a grant of the Deutsche Forschungsgemeinschaft (DFG Be 822/4-3).

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0006-4971/93/8209-0021$3.00/0

Blood, Vol 82, No 9 (November 1), 1993: pp 2837-2844

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ical Standards and Control (Potters Bar, England). RhIL-4 was obtained from Cetus (Frankfurt, Germany). Ki-67 equivalent MIB 1 antibody was purchased from Dianova (Hamburg, Germany).

HTCA. Cells were tested in the HTCA modification yielding the best clonal growth for the respective cell line. The HTCA in agar containing capillaries (HTCAcap) was performed as previously described\textsuperscript{19,20} with the cell line CCL 185. For evaluation of clonal growth of the cell lines HTB 119 and HTB 120, a methylcellulose assay (HTCAmix)\textsuperscript{21} was used. The cell lines HTB 53 and HTB 56 were tested in a newly developed HTCA using mixtures of methylcellulose and agar (HTCAmix) in the following modification: cells were detached by trypsinisation and washed with their own growth medium, resuspended with 1 mL RPMI 1640 medium (GIBCO, Glasgow, Scotland) plus 10% fetal calf serum (FCS) and counted by trypan blue staining to yield a final concentration of 3 x 10⁶ cells/mL. Viability of the cells >80% was required before cells were taken for an experiment. Methylcellulose solution was produced by boiling 0.5 L distilled water with 21 g methylcellulose (Sigma No M 0512, Deisenhofen, Germany). Five hundred millilitre cold Isevo's modified Dulbecco's medium (double concentrated; GIBCO, Cat No 041-01980M) was added to the methylcellulose after cooling down to 37°C. This mixture was kept in 3.6-ML aliquots at -20°C. Agar was made by boiling 3 g Difco agar (Agar Noble; Difco Lab, Detroit, MI) in 100 mL distilled water for 30 minutes, and consecutively 10 mL of the boiling agar were added to 20 mL RPMI 1640 (37°C). The incubation mixture was made up of 3.6 mL methylcellulose solution, 2.7 mL Hyclone FCS (Logan, UT; Kat. No. A-1111-D), 0.06 mL mercaptoethanol (0.084 x 10⁻³ mol/L mercaptoethanol in distilled water; Sigma, No M-3148, and filtered sterile), 0.3 mL cell suspension, 0.8 mL Isevo's and 1.6 mL Agar/RPMI 1640 mixture. This incubation mixture was vortexed thoroughly and kept in the dark at 37°C for 20 minutes. RhIL-4 or control vehicle was added to Lux dishes (suspension culture dishes, 35 x 10 mm, No 117116; Miles Scientific, Naperville, IL) as a solution of 0.1 mL RPMI 1640 plus 0.1% bovine serum albumin (BSA) and rhIL-4. An aliquot of 1 mL of the incubation mixture was then added to the Lux dishes. This final incubation mixture contained the final cytokine concentrations as indicated in the results, thus tumor cells were exposed to the cytokine for the complete assay period. For all HTCA modifications applied the antibody against hIL-4 tested was incubated for a 1-hour period at room temperature with the cytokine before being added to the assay. The antiserum against hIL-1 and hIL-6 were added simultaneously to the assay with the rhIL-4. The colony formation was evaluated with an inverted microscope before and after an incubation period of 10 days at pH 7.2, 37°C, 5% CO₂, and high humidity.

Tritiated thymidine uptake. The effect of rhIL-4 on the spontaneous proliferation of the lung tumor cell lines was measured by using the tritiated thymidine uptake assay. Cells were freshly detached from plastic tissue flasks by a 1-hour incubation at 4°C with 0.6 mmol/L ethylene diamine tetra acetate (EDTA) in phosphate-buffered saline (PBS). Then, cells were washed in their own growth medium with FCS, resuspended with 1 mL RPMI 1640 plus 10% FCS, and counted with trypan blue staining to yield a final concentration of 2.5 x 10⁶ cells/mL. A 0.1-mL aliquot of the cell suspension was added to every well of a 96-well plate (NUNC, Kamstrup, Denmark). Every well was then treated with a 0.1 mL solution of different concentrations of rhIL-4 to yield final concentrations of 10 pg—10 ng/mL or control vehicle. Plates were incubated at 37°C, pH 7.2, in 5% CO₂, and high humidity. After 16 hours cells were pulsed with 0.5 μCi of (³H) thymidine/well (specific activity, 5.0 Ci/mmol; Amersham and Buchler, Braunschweig, Germany) and incubated for a further 8-hour period. The samples were then processed and counted in the LKB Betaplate system (LKB; Pharmacia, Freiburg, Germany). Values are depicted as the means plus/minus SD.

Cell numbers. All human tumor cell lines except the tumor cell line HTB 53 were incubated (initial cell concentration 5 x 10⁷/mL) in 8 mL medium plus 5% FCS with 10 ng/mL rhIL-4 for 5 days at conditions as above (tritiated thymidine uptake assay). At day 5 cells were detached by trypsinization and counted by trypan blue staining.

Analysis of cytokine production. Cells (5 x 10⁵/mL) were cultured with CG medium (Camon Labor Service, Wiesbaden, Germany) plus 1% FCS and 10 ng/mL rhIL-4 at conditions described above (tritiated thymidine uptake assay). The production of other cytokines (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, IL-1, and IL-6) induced by preincubation with rhIL-4 was quantified by measuring the supernatant of cultures at days 2, 4, and 6 with enzyme-linked immunosorbent assay (ELISA) for TNF-α and IFN-γ and bioassaying all mentioned cytokines. Methods have been recently described in detail.\textsuperscript{22,23}

Detection of specific IL-4 binding sites (receptors) by fluorescent-activated cell sorting (FACS) analysis. The expression of the IL-4R on the cell surface was evaluated by flow cytometry using a phycoerythrin-conjugated IL-4 (PE-IL-4; R & D Systems). Cells were detached as mentioned above (tritiated thymidine uptake assay) and washed twice at 500 x 5 minutes with the provided buffer and counted by trypan blue staining to give a final concentration of 4 x 10⁶ cells/mL. Twenty-five microliters of the cell suspension were exposed for 1 hour to 40 μL PE-IL-4 and kept at 4°C in the dark. The cell samples were vortexed thoroughly several times and finally washed twice with the buffer provided. The pellet was resuspended with 100 μL propidium iodide (concentration 2 μg/mL; P4 170, Sigma) in PBS and incubated for 15 minutes at conditions as above, washed twice, and resuspended with 200 μL PBS. The cell samples were then analyzed using a FACS analyzer (Becton Dickin- son, Heidelberg, Germany). Specific binding was determined by 1-hour preincubation with a 100 molar excess amount of nonla- beled rhIL-4 as described by Ohara and Paul.\textsuperscript{24}

Isolation of total cellular RNA and Northern blot analysis. Total cellular RNA was isolated from cells using the RNAzol B method (WAK-Chemie Medical GmbH, Bad Homburg, Germany). Subse- quently, 15 μg isolated RNA of every cell sample was electrophoresed in a 1% agarose gel containing 2.4% formaldehyde and then transferred by vacuum in 10X standard saline citrate (SSC) to a nylon membrane (Hybond-N membrane; Amersham). The blots were baked for 2 hours at 80°C. After prehybridization for 3 hours at 42°C in 5X Denhardt's solution, 50% formamide, 1% sodium dodecyl sulfate (SDS), 1 mol/L NaCl, 50 mmol/L Piperrazine-N,N'-bis(2-ethanesulfonic acid), 10 mmol/L EDTA, and 100 μg/mL her- ring sperm DNA, the blots were exposed to an overnight hybridiza- tion (using the same solution as for prehybridization with the addition of 0.3 mol/L NaCl) with a cDNA probe (1.9-kb fragment of 4A2 cDNA [5' end to NcoI]) for the hIL-4R, which was a gift from the DNAX Research Institute (Palo Alto, CA). The cDNA probe was previously radiolabeled with [α-³²P]dCTP using a multiprime DNA labeling system (Amersham). The blots were then washed twice for 5 minutes in 2X SSC:0.1% SDS at room temperature and twice for 20 minutes in 0.1X SSC:0.1% SDS at 60°C. Finally, the blots were autoradiographed for 18 hours with a Kodak x/Omatic film (Eastman Kodak, Rochester, NY) at -80°C. The blots were checked for integrity and comparable loading of RNA by reprobing with a β-actin–specific oligonucleotide (Dianova). The human β-actin probe was radiolabeled with [α-³²P]dCTP using a DNA tailing kit (Boehringer-Mannheim, Mannheim, Germany), then prehybridized and hybridized as described above and exposed to a Kodak x/Omatic film for 16 hours.
Animals. Four-week-old female athymic BALB/c nu/nu mice were obtained from The Jackson Laboratory (Ry, Denmark). Mice were kept under specific pathogen-free conditions and fed on an autoclaved standard diet (Altromin) and sterilized water at low pH. The human lung tumor cell lines (numbers as indicated in the figures) were xenotransplanted intracutaneously into the flank as single cell suspensions in 0.1 mL PBS. Mice were randomized to control and treatment groups (10 mice per group) thereafter. Tumor growth was measured with callipers at days as indicated. Mean diameter (length plus width/2) is given as tumor size in millimeters.

Cytokine plasma levels. Plasma levels of rhIL-4 were measured at 10 minutes and 1 hour after the last injection of 2.5 µg rhIL-4 in 0.1 mL PBS plus 0.1% BSA or control vehicle by ELISA purchased from R & D Systems.

Tumor pathology. Tumors were excised from additional animals in each group at days 12, 16, and 49 and processed by paraffin embedding. Tumors were stained by hematoxylin-eosin or with Ki-67 equivalent MIB 1 antibody, a MoAb reacting with a nuclear proliferation-associated antigen, to evaluate the percentage of proliferative cells. For the latter staining we have used a modified APAAP technique as described.25

Statistics. Results were evaluated statistically by the Mann-Whitney test or Kruskal-Wallis test as indicated. P values <.05 were interpreted as indicating significant differences.

RESULTS

Effects of rhIL-4 in vitro. In a first set of experiments using the HTCA we showed that rhIL-4 displayed a significant antiproliferative effect in vitro in two of five lung carcinoma cell lines (CCL 185 and HTB 56) (Table 1). However, strong effects blocking ≥50% of the colony formation were only observed in the CCL 185 cell line (Table 1). The response to rhIL-4 was clearly dose dependent (Table 1) and could be reversed by preincubating rhIL-4 with a neutralizing rabbit polyclonal antibody against hIL-4 in both sensitive cell lines (Fig 1 shows the results with CCL 185 as an example). Additionally, there was some saturation of the growth inhibition by rhIL-4 at concentrations between 1 and 10 ng/mL (Table 1). Furthermore, the tritiated thymidine uptake of the cell line CCL 185 could be substantially suppressed by rhIL-4 (Table 2). It was only marginally influenced by rhIL-4 in the cell lines HTB 56 (10 ng/mL rhIL-4: 94.6% of the controls, P = .03) and HTB 53 (10 ng/mL rhIL-4: 88.6% of the controls, P = .004), but remained uninfluenced by rhIL-4 in the two small cell lung cancer cell lines HTB 119 and HTB 120 (data not shown). Exposure of cell cultures to rhIL-4 for 5 days also showed a significant inhibition of cell growth as measured by counting cell numbers in the cell line CCL 185 (Table 2) but not in the cell lines HTB 56, HTB 119, and HTB 120 (data not shown).

Receptor expression. The non-small cell lung cancer cell lines CCL 185, HTB 53, and HTB 56 show hIL-4 R transcripts detectable in 15 µg cellular RNA by Northern blot analysis. In contrast, the small cell lung cancer cell lines HTB 119 and HTB 120 show no transcripts for the hIL-4 R (Fig 2). LAK cells were used as positive controls and the mouse hybridoma CTLL 16 cell line14 as a negative control (Fig 2). Cellular RNA was also hybridized with a human β-actin–specific oligonucleotide to confirm RNA integrity and to compare RNA loading in single lanes with the exception of CTLL 16 (Fig 2).

Furthermore, we have used FACS analysis to evaluate whether the antiproliferative response was correlated with specific binding of rhIL-4 to hIL-4 binding sites on the cell surface. As negative controls14 we have used the mouse hybridoma cell line CTLL 16 (Fig 3A), for positive control staining we have used LAK cells (data not shown). FACS analysis showed a high percentage of CCL 185 cells specifically binding hIL-4 (Fig 3B). Marginal expression of hIL-4 binding sites was also detected on the cell surface of the small cell lung cancer cell line HTB 53 and HTB 56 (data not shown). However, HTB 53 did not show clear expression of hIL-4 binding sites in this analysis (Fig 3D). Interestingly, CCL 185, the only cell line with unequivocal responsiveness to IL-4 in all three in vitro assay systems, showed the strongest expression of binding sites. Finally, the hIL-4–nonresponsive cell lines HTB 119 and HTB 120 showed no expression of hIL-4 binding sites (data not shown).

Production of cytokines. Another question of particular interest was whether the antiproliferative response to rhIL-4

Table 1. Effects of rhIL-4 on Colony Formation (HTCA) of Human Lung Carcinoma Cell Lines In Vitro

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Controls (%)</th>
<th>10 pg</th>
<th>100 pg</th>
<th>1 ng</th>
<th>10 ng</th>
<th>No.</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL 185*</td>
<td>63.4 ± 7.1</td>
<td>100 ± 18.0</td>
<td>88.0 ± 17.1</td>
<td>65.6 ± 26.6</td>
<td>50.1 ± 26.7</td>
<td>45.1 ± 30.3</td>
<td>18 0.0001</td>
</tr>
<tr>
<td>HTB 53†</td>
<td>75.0 ± 13.0</td>
<td>100 ± 14.0</td>
<td>94.7 ± 11.7</td>
<td>101.1 ± 16.5</td>
<td>109.9 ± 6.7</td>
<td>94.1 ± 15.3</td>
<td>6 0.2156</td>
</tr>
<tr>
<td>HTB 56†</td>
<td>109.5 ± 11.6</td>
<td>100 ± 9.4</td>
<td>94.9 ± 10.4</td>
<td>104.2 ± 16.3</td>
<td>74.3 ± 12.7</td>
<td>71.7 ± 11.0</td>
<td>12 0.002</td>
</tr>
<tr>
<td>HTB 119†</td>
<td>34.7 ± 1.3</td>
<td>100 ± 28.1</td>
<td>98.8 ± 25.9</td>
<td>93.9 ± 18.8</td>
<td>106.1 ± 39.0</td>
<td>95.1 ± 29.5</td>
<td>12 0.8983</td>
</tr>
<tr>
<td>HTB 120†</td>
<td>28.7 ± 3.6</td>
<td>100 ± 12.5</td>
<td>109.9 ± 31.4</td>
<td>89.9 ± 29.6</td>
<td>115.0 ± 31.6</td>
<td>107.0 ± 16.9</td>
<td>6 0.1551</td>
</tr>
</tbody>
</table>

Controls are shown as mean ± SD of absolute numbers of colonies/capillary or dish. Values are given for each experiment and represent means of sixfold assays. No. represents numbers of capillaries or dishes counted at each single condition (0, 10 pg, 100 pg, 1 ng, 10 ng/mL of rhIL-4). P values determined by Kruskal-Wallis test.

* Colonies measured by HTCAcap.
† Colonies measured by HTCAmix.
‡ Colonies measured by HTCAMc.
...directed by rhIL-4–enhanced production of other cytokines as measured in the cell supernatants or by an induced extracellular (public) autocrine loop for IL-6.

**Effects of rhIL-4 in vivo.** Subsequently, we have evaluated putative tumor growth retardation by rhIL-4 in xenografts of some of the cell lines in nu/nu BALB/c mice in vivo. CCL 185 was inoculated intracutaneously at a cell load of $1 \times 10^7$, HTB 56 at $1 \times 10^6$, and HTB 119 at $4 \times 10^6$ per mouse. Starting on day 1 (CCL 185) or on day 5 (HTB 56) after tumor transplantation the animals were treated subcutaneously distant from the tumors twice daily for 12 days with 0.5 mg/m² rhIL-4. There was a significant inhibition of the growth of the CCL 185 tumors in the rhIL-4–treated mice (maximal significance with $P$ value at day 40 was .0019, Mann-Whitney test) as shown in Fig 4A. Three of 10 xenotransplanted tumors in the rhIL-4–treated group showed only minimal growth (tumor size on day 3 was 4 mm; on day 49, 6 mm) throughout the experiment. In addition, there was a significant retardation of tumor growth also in the HTB 56 xenografts (Fig 4B). We were not able to establish tumor growth of HTB 53, thus no in vivo experiments were performed with this cell line. In contrast to the cell lines responsive to rhIL-4, there was no in vivo growth modulation of the small cell lung cancer cell lines being nonresponsive to rhIL-4 in vitro. Tumor growth of HTB 119 xenografts is shown in Fig 4C as example. The in vivo experiments were reproduced at least twice with comparable results. All animals tolerated the treatment with rhIL-4 in PBS plus 0.1% BSA or vehicle without any visible toxicity or death.

**Pathology of the tumors and plasma levels of rhIL-4.** Histologic examination of the tumors showed no major infiltration with hematopoietic or lymphocytic murine cells in both groups (slides not shown). An equal percentage of tumor cells in both groups demonstrated proliferative activity (MIB 1 staining not shown). Plasma levels of rhIL-4 (10 minutes after rhIL-4, 4.2 ng/mL; 1 hour after rhIL-4, 3.6 ng/mL; controls, negative) were clearly exceeding the required amount for a substantial growth inhibition of CCL 185.

**DISCUSSION**

The data demonstrate that rhIL-4 displays major antiproliferative effects against one of five human lung cancer cell lines in vitro (Tables 1 and 2) and against two of these cell lines in vivo (Fig 4). The remaining cell lines tested were essentially nonresponsive in vitro and in vivo (Table 1, Fig 4).

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**Table 2. The Effect of rhIL-4 on the Growth of the Human Lung Carcinoma Cell Line CCL 185 In Vitro in Two Different Assay Systems**

<table>
<thead>
<tr>
<th>Assay System</th>
<th>Control</th>
<th>10 ng of rhIL-4/mL (% Controls)</th>
<th>No. of Assays</th>
<th>Mann-Whitney Test (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritiated thymidine uptake</td>
<td>3106 ± 241.4*</td>
<td>973.5 ± 93.3* (82.3)</td>
<td>6</td>
<td>.0039</td>
</tr>
<tr>
<td>Cell numbers</td>
<td>34.0 × 10⁶ ± 10.5*</td>
<td>21.8 × 10⁶ ± 3.41 (64.1)</td>
<td>4</td>
<td>.0433</td>
</tr>
</tbody>
</table>

* Means ± SD of counts per minute.
† Values of cell numbers are expressed as mean ± SD.

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For details on assay systems used, see Materials and Methods section.
responsive cell line (HTB 56) showed marginal hIL-4 receptor staining in FACS analysis (Fig 3C). Thus, there is no complete correlation between expression of hIL-4R message and sensitivity of the respective cell line to the growth-inhibitory effects of IL-4 or the presence of binding sites on the cell surface in this investigation. Among different other reasons for this observation, posttranscriptional modulation of receptor expression as well as loss of functionality of the receptor molecule might be discussed.

The activity of the cytokine could be abolished by preincubation with neutralizing anti-hIL-4 antibodies (Fig 1). There was a good dose-efficacy relation for serial antibody dilutions in completely reverting the growth-inhibitory effects of rhIL-4. These experiments were performed to relate clearly the growth-inhibitory activity of the material tested to the IL-4 molecule and to exclude nonspecific effects such as those possibly induced by the presence of minute amounts of endotoxin, although the materials tested were specified as being free of endotoxin. Additionally, using excess neutralizing amounts of the anti-IL-4 antibody and the antibody alone without the cytokine rules out extracellular (public) autocrine downregulation of the tumor cell growth by IL-4 itself, because the antibody was without any effect on the tumor cell growth (Fig 1). Furthermore, the antiproliferative activity observed was not mediated by rhIL-4-enhanced extracellular (public) autocrine loops of IL-6 (Table 3) or production of other possible secondary cytokines such as TNF-α, IFN-γ, and IL-1.

There was some diversity in the response patterns when the different in vitro assays (HTCA, tritiated thymidine uptake, cell numbers) are compared. However, these tests...
Table 3. Effect of rhIL-4 on Inducing Production of IL-6 in the Cell Line CCL 185 (A), and the Influence of Antisera Against IL-6 on the Effect of rhIL-4 on Colony Formation of the Cell Line CCL 185 in the HTCAcap (B)

<table>
<thead>
<tr>
<th></th>
<th>2 Days</th>
<th>4 Days</th>
<th>6 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6*</td>
<td>Control</td>
<td>Nil</td>
<td>30 pg/mL</td>
</tr>
<tr>
<td>+rhlL-4 (10 ng/mL)</td>
<td>12 pg/mL</td>
<td>60 pg/mL</td>
<td>320 pg/mL</td>
</tr>
<tr>
<td>B</td>
<td>Nil</td>
<td>Anti-IL-6†</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52.9 ± 8.9 (100%)§</td>
<td>51.7 ± 14.8 (97.7%)§</td>
<td></td>
</tr>
<tr>
<td>+10 ng/mL rhlL-4</td>
<td>25.5 ± 11.1 (50.1%)§</td>
<td>29.1 ± 6.2 (55.0%)§</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparison of control versus antisera only values yielded P value of .7483, and comparison of 10 ng/mL rhlL-4 only versus antisera with rhlL-4 values yielded P value of .5676 using the Mann-Whitney test.

* Measured with a bioassay.
† Antibody concentration was given to yield neutralizing capacity for 10.0 ng rhIL-6/mL.
§ Numbers of colonies ± SD.
§ Numbers of assays were 6 at each single concentration, numbers in parentheses indicate percent of control.

measure different aspects of antiproliferative activity and failure to demonstrate exact correlations have been reported before. Furthermore, HTCA has been shown to detect reliably modulation of clonal growth of tumor cells by cytokines, and finally, there was an excellent correlation between the in vitro (HTCA) and in vivo results of this study.

Because hIL-4 does not cross-react with the murine IL-4R it is quite unlikely that the therapeutic effect observed in vivo (Fig 4) is caused by an activated murine effector cell population. Furthermore, histologic examination of the tumors in the mice treated with and without rhIL-4 showed no infiltration with murine hematopoietic or lymphocytic cells. This is different from other recent reports demonstrating a mixed cell infiltration of transplanted tumors in which the tumor cells were transfected with the murine IL-4 gene. In these experiments the antitumor effects of IL-4 were mediated mainly by an eosinophilic effector cell population. We interpret tumor growth retardation in our in vivo experiments by rhIL-4 as being induced by a direct antiproliferative effect.

Independently from us, investigators in other laboratories have recently reported that rhIL-4 has an antiproliferative effect on freshly isolated non-Hodgkin’s B-lymphoma cells as well as on solid tumor cell lines derived from human renal, colon, and breast carcinomas. However, those groups of investigators have not shown in vivo effects of the cytokine in the cell types tested. Together, these results indicate that functional hIL-4R may be widely distributed in malignancies of different histologic origin and could be exploited for negative growth regulation of some tumor types by IL-4. However, these and our results must be interpreted with due caution because cell lines can acquire altered properties such as changes in growth requirements, and thus, before final conclusions can be drawn, more experiments are required using fresh tumor specimens.

We conclude that rhIL-4, which has recently entered clinical phase I trials, has direct antiproliferative effects on the growth of some human lung tumors in vitro and in vivo that together with its regulatory effects on various effector cell populations makes this cytokine an interesting candidate for further investigation in experimental cancer treatment.
IL-4 AND LUNG TUMORS

ACKNOWLEDGMENT

The authors thank Dr A. Stiglic and Dr N.-C. Juhr for providing the animal facilities at the Zentrale Tierlaboratorien of the Freie Universität Berlin.

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