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Effects of Cytokines on *in vitro* Colony Formation of Primary Human Tumour Specimens

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Although under study to alleviate chemotherapy-induced bone marrow toxicity, cytokines can stimulate *in vitro* growth of solid human tumour cell lines. The effects of granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3) on *in vitro* colony formation of primary human tumours was studied in a capillary soft-agar cloning system. Of 108 tumour specimens from 100 patients, 85 specimens were tested against all three factors at concentrations ranging from 0.1 to 1000 ng/ml. 44 of 100 tumours showed adequate growth in controls. 8 out of 43 (19%) specimens were significantly stimulated by GM-CSF, 6 of 40 (15%) by G-CSF and 10 of 44 (23%) by IL-3. Sensitivity to all three cytokines was observed in 4 of 44 (9%) specimens. By light microscopy the appearance of colonies from stimulated specimens was identical to that of controls. Sensitivity to cytokines was independent from sensitivity to epidermal growth factor, transferrin or insulin. Sensitivity to GM-CSF, G-CSF and IL-3 may be aberrantly expressed in a subgroup of solid human tumours.
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INTRODUCTION

GRANULOCYTE–MACROPHAGE colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3) are involved at different levels in the proliferation and maturation of human haematopoietic precursor cells [1, 2]. IL-3 induces formation of a variety of colony types, including mature cells as well as multipotent stem cells [39]. GM-CSF predominantly induces colonies of macrophage and granulocytic lineage and also stimulates eosinophil precursors, while G-CSF more specifically induces proliferation and differentiation of neutrophil colonies [2]. The complementary DNAs to their respective mRNAs have been cloned and recombinant GM-CSF, G-CSF and IL-3 are being used in clinical trials to alleviate chemotherapy-induced bone marrow toxicity [1]. However, these cytokines can also stimulate *in vitro* growth of human solid tumour cell lines [4, 5]. Also, various tumours have been reported to produce colony-stimulating factors which might in turn stimulate their growth by paracrine or autocrine mechanisms [5–10].

We have evaluated the effect of GM-CSF, G-CSF and IL-3 on clonogenic cells from freshly obtained human solid tumour specimens in a capillary soft-agar cloning system.

MATERIAL AND METHODS

Cytokines and growth factors

Lyophilised recombinant human GM-CSF, G-CSF and IL-3 were provided at a specific activity of 5×10^7 U/mg by Behringwerke, Marburg. The compounds were resuspended in phosphate-buffered saline pH 7.4 (PBS)/BSA (2 mg/ml) to stock concentrations of 1, 10, 100, 1000 and 10 000 ng/ml and stored at −20°C. Human epidermal growth factor (EGF) was purchased from Flow. Substantially iron-free transferrin and bovine insulin were obtained from Sigma. Stock concentrations between 10^{-5} and 10^{-11} mol/l were prepared for EGF and transferrin in PBS/BSA. For insulin, stock concentrations of 5×10^{-5} to 5×10^{-11} mol/l were used.

Collection of cells

Tumour specimens (pleural effusions, ascites or surgical biopsy specimens) from 100 patients were obtained by sterile standard techniques as part of routine clinical diagnostic or surgical procedures. Preservative-free heparin (10 U/ml, NOVO) was added to prevent coagulation. Effusions were centrifuged at 150 *g* for 5–7 min and the supernatants discarded. The pellets were suspended in McCoy's 5A medium (GIBCO) containing 5% horse serum (HS), 10% fetal calf serum (FCS) (both GIBCO), 2 mmol/l sodium pyruvate, 2 mmol/l glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin and 35 μg/ml L-serine (Sigma). Solid tumour specimens were minced and passed through steel meshes (100 and 50 μm, Linker, Kassel). Single cell suspensions were prepared by sedimentation and further mechanical dissociation (25 or 27 G needles) when necessary.

Tumour cloning assay

The capillary human tumour cloning assay was used [11]. Cells from fresh human specimens were seeded at a median density of 5.2×10^4 per capillary (range 4.0×10^4 to 6.3×10^4) in 100 μl glass capillaries. Various concentrations of the cytokines were added for continuous exposure experiments. Six capillary tubes were used for each data point. Each experiment included one set of controls with PBS/BSA (2 mg/ml) and a set of controls with ammonium monovanadate 1 mmol/l (Merck) to assure the presence of a good single-cell suspension (positive

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control) [12]. Capillary tubes were incubated at 37°C for 14–21 days and the agar transferred to microscope slides. Colonies (cell aggregates ≥ 50 μm) were manually counted in an inverted microscope. *In vitro* colony formation was considered adequate when the PBS/BSA control had a mean of 3 or more colonies per capillary and the positive control showed 30% or less colony formation compared with the PBS/BSA control. An increase in tumour colony formation was considered significant if the number of colonies compared with the control was increased 1.5 fold or more. At least two independent investigators evaluated colony morphology.

Histology

For further histological examination, colonies were fixed in 2.5% glutaraldehyde (Merck) and embedded in paraffin. Serial sections were stained with haematoxylin and eosin.

Statistical analysis

Data were calculated as means and standard deviations of four to six evaluable determinations per data point. Survival was calculated by expressing the average number of tumour colony-forming units from cells exposed to recombinant cytokines or growth factors as a percentage of the average number of untreated controls [13]. We used Fisher's exact test.

RESULTS

108 freshly obtained tumour biopsies and effusions from 100 patients with malignant diseases were cultured. 4 specimens with negative histology did not form colonies in control capillaries and were not stimulated by GM-CSF, G-CSF or IL-3. These specimens were excluded from further analysis. 4 specimens were inevaluable because of fungal or bacterial contamination. Table 1 summarises the tumour types for the remaining 100 specimens. Growth in control capillaries was observed in 44 specimens. The median number of colonies formed per capillary was 9.3 (range 3.0–162.0). 6 specimens were excluded because of too many cell clumps and 50 specimens did not grow in control capillaries. Each specimen was incubated with cytokines at final concentrations ranging from 0.1 to 100 ng/ml. In addition, 27 evaluable specimens were incubated with cytokines at 1000 ng/ml.

GM-CSF significantly stimulated colony formation in 8 out of 43 (19%) specimens; 12 specimens were sensitive at the highest concentration tested ($P = 0.003$) (Table 2). Sensitivity of each of these specimens to GM-CSF was concentration-dependent. G-CSF stimulated 6 out of 40 specimens (18%) ($P < 0.02$). Again, sensitivity was concentration-dependent.

Table 1. Tumour characteristics

Type	No. growing*		Source: solid/effusion
Renal	9/20	(45%)	19/1
Hepatobiliary	4/11	(36%)	11/0
Melanoma	8/11	(73%)	11/0
Colorectal	6/15	(40%)	13/2
Ovary	5/6	(83%)	0/6
Breast	3/3	(100%)	0/3
Sarcoma	2/6	(33%)	6/0
Other	7/28	(25%)	11/17
Total	44/100	(44%)	71/29

*In control capillaries without cytokines.

Table 2. Effect of recombinant human GM-CSF, G-CSF and IL-3 on colony formation in soft agar by human solid tumours

	Concentration of cytokine (ng/ml)				
	0.1	1	10	100	1000
GM-CSF	1/43*	2/43	5/43	7/43	2/20
G-CSF	0/40	2/40	4/40	5/40	1/20
IL-3	3/44	3/44	4/44	9/44	2/20

*No. specimens with stimulation/no. specimens with growth in control capillaries.

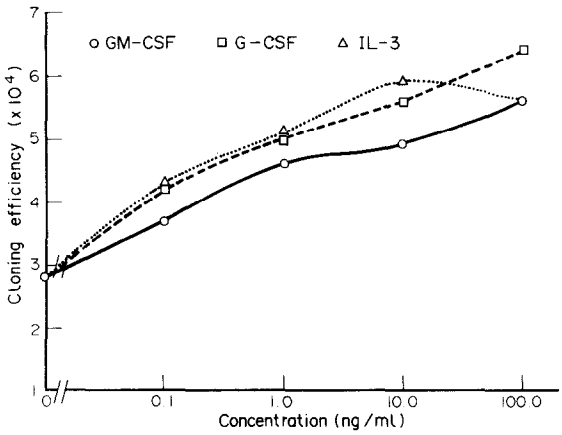


Fig. 1. Concentration–response curve of oesophageal cancer specimen. Cloning efficiency = number of colonies divided by number of cells seeded per capillary.

IL-3 increased colony formation in 10/44 specimens (23%) with 9 specimens being sensitive at 100 ng/ml ($P < 0.001$). In 2 of these specimens concentration-dependency was questionable. 1 specimen was a melanoma with exceptionally good spontaneous growth (50.2 [S.D. 9] colonies per capillary) showing stimulation of 237% survival at 0.1 ng/ml. It is tempting to speculate that exhaustion of available nutrients may have caused the lack of significant further growth stimulation: 1 ng/ml, 232%; 10 ng/ml, 267% and 100 ng/ml, 229% colony formation compared with control. The second specimen, from a colorectal tumour, showed adequate spontaneous growth (13.2 (S.D. 3.1) colonies per capillary) and had 142–162% colony survival in 0.1–100 ng/ml IL-3. With higher cytokine concentrations, more tumours were sensitivity tested with maximum recruitment of colonies being observed at 100 ng/ml. Mean maximum stimulations were: GM-CSF, 187 (40); G-CSF, 177 (34); and IL-3, 181 (41) percent survival. There was no evidence for sensitivity to cytokines of any particular tumour type.

Figure 1 shows the concentration-dependence of an oesophageal cancer specimen to GM-CSF, G-CSF and IL-3. Fig. 2 is an example of a biphasic concentration response.

There was no evidence that colonies in stimulated specimens were comprised of haematopoietic cells (Fig. 3). Colonies grown in the presence of cytokines were indistinguishable from those grown without cytokines. Consistent with reports by other investigators, nuclear and cytoplasmic heterogeneity was observed [14, 15]. Also, occasional multinucleated cells were noted.

Of 27 tumour specimens tested, there was no relation between sensitivities to cytokines and EGF, transferrin or insulin (Table 3).

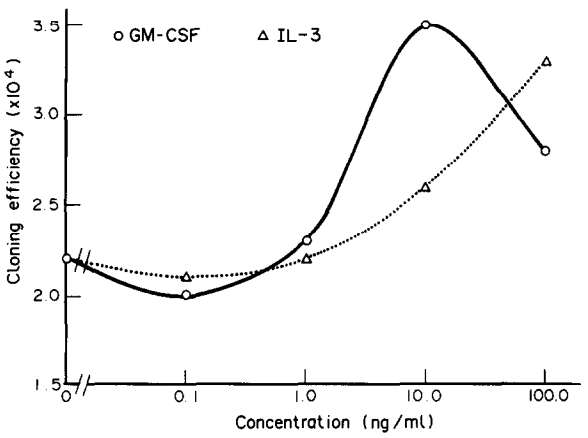


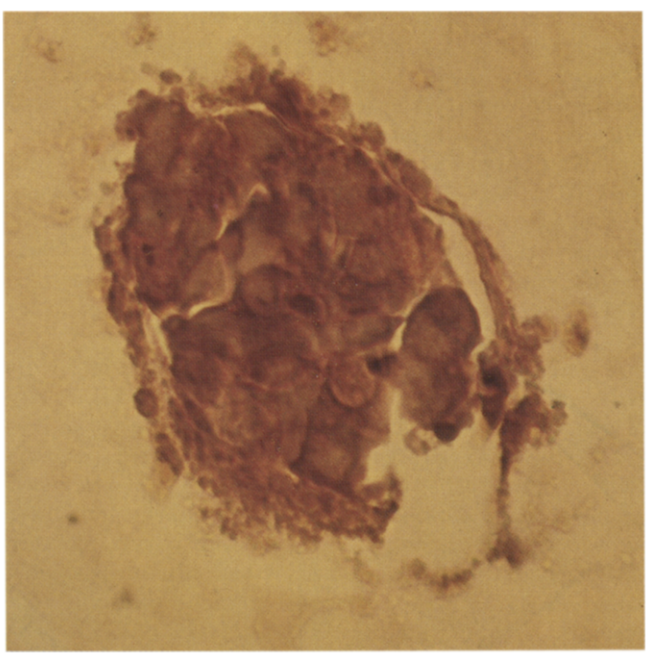
Fig. 2. Concentration–response curve of cholangiocellular carcinoma. Single cells were continuously incubated with GM-CSF, G-CSF, and IL-3.

Sensitivity to cytokines was observed significantly less frequently than sensitivity to EGF (GM-CSF, $P = 0.03$; IL-3, $P = 0.01$; and G-CSF, $P = 0.001$). Also, no significant cross-sensitivity was observed with only 2 specimens being sensitive to all factors studied.

DISCUSSION

Clonogenic cells from a subgroup of human solid tumours exhibited increased growth in the presence of one or more cytokines. Recruitment of cells into active growth was concentration-dependent, with some specimens showing a biphasic response. These observations may indicate susceptibility of the original tumour *in vivo* to the respective cytokine. None of the specimens without growth in control capillaries showed stimulation, which is of interest since other growth factors for solid tumours induce colony formation even in specimens without "autonomous" growth in controls [16, 17] and suggests that the cytokines tested may not be able to induce proliferation of tumour stem cells by themselves but require additional factors. No inhibition of colony formation was observed.

Growth of normal and neoplastic cells can be stimulated by a variety of polypeptide factors *in vitro* and *in vivo* [18]. Most of the experimental evidence for polypeptide-mediated growth stimulation comes from studies of human cancer cell lines. EGF, insulin, transferrin and transforming growth factor alpha stimulate clonogenic growth of primary human tumour specimens in soft agar [16, 17, 19–21]. While haematopoietic growth factors were first considered to be specific mitogens for bone marrow precursor cells, there is now evidence for stimulatory effects on human solid tumour cell lines [4, 5, 22, 23]. However,



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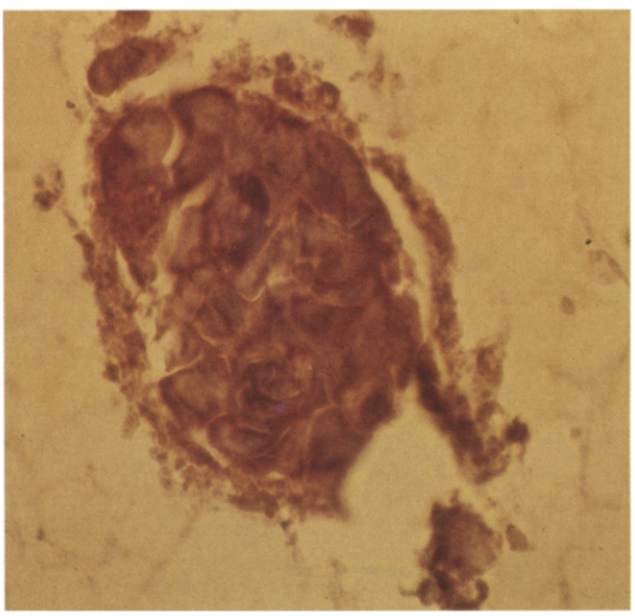

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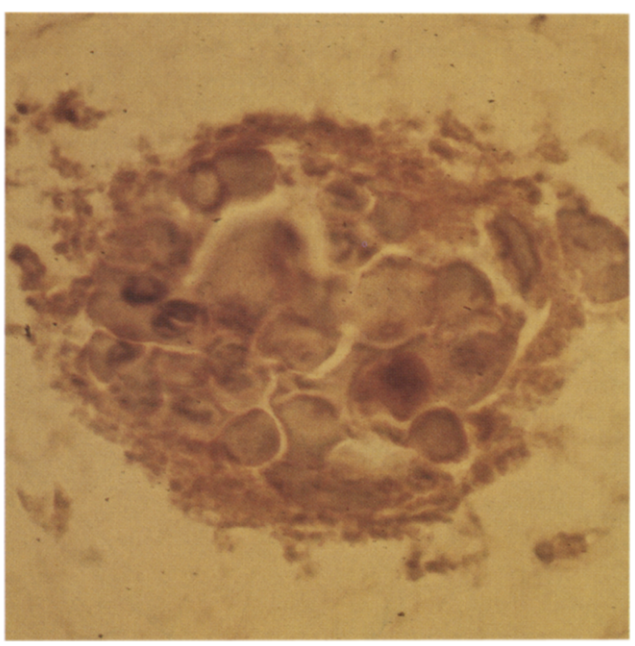

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Fig. 3. Individual colonies from melanoma specimen stimulated by cytokines (100 ng/ml). a = control, b = GM-CSF and c = IL-3.

Table 3. Response of primary human tumour specimens to cytokines and other growth factors

Tumour Type	Sensitivity to: GM-CSF	G-CSF	IL-3	EGF	Transferrin	Insulin
Melanoma	+	ND	+	+	+	+
Ovary	+	+	+	+	+	+
Hepatobiliary	+	ND	+	−	−	−
Colorectal	+	−	+	+		+
Liver	−	ND	−	+	+	−
Liver	−	−	−	−	+	+
Kidney	−	−	−	+	−	−
Kidney	+	−	−	−	−	−
Kidney	−	−	−	+	−	−
Kidney	+	−	−	+	−	−
Melanoma	−	−	−	+	−	+
Ovary	−	−	−	+	+	+
Ovary	−	−	−	+	+	+
Sarcoma	−	−	−	+	+	+
UPS	−	ND	+	+	−	−
UPS	−	−	−	+	−	+
UPS	−	−	−	+	−	−
UPS	−	−	−	−	−	−
Melanoma	−	−	−	−	−	−
Colorectal	−	−	−	−	−	−
Colorectal	−	−	−	−	−	−
Colorectal	−	−	−	−	−	−
Kidney	−	−	−	−	−	−
Kidney	−	−	−	−	−	−
Kidney	−	−	−	−	−	−
Kidney	−	−	−	−	−	−
Pancreas	−	−	−	−	−	−

*GM-CSF, G-CSF and IL-3, 0.1 ng/ml–100 ng/ml; EGF, transferrin 10^{-12}–10^{-6} mol/l and insulin 5×10^{-12} to 5×10^{-6} mol/l. ND = not done. UPS = unknown primary site.

Munker *et al.* did not find any stimulatory effects of GM-CSF [24]. A variety of human tumour cell lines synthesise and secrete cytokines, and have been used as a source for purification of certain cytokines [25].

Our data support the notion that the biological activity of cytokines is not restricted to haematopoietic cells. As has been suggested by others, growth stimulation of tumour cells might render otherwise dormant cells sensitive to subsequent chemotherapy [4, 26, 27]. The lack of cytotoxic effects of GM-CSF in our studies contrasts with findings in a smaller series of specimens reported by Salmon and Liu [28]. Possible explanations include differences in the recombinant material used as well as in the experimental systems.

To establish whether sensitivity to cytokines in the capillary cloning system was non-specific, specimens were also exposed to known growth factors for solid tumours. Sensitivity to cytokines was largely independent from sensitivity to growth factors such as EGF, transferrin or insulin. Sensitivity to all six growth factors was observed in only 2 specimens and was restricted to individual factors in all other specimens. Since all known actions of cytokines are mediated via membrane receptors, it may be postulated that a subset of solid tumours express surface receptors for haematopoietic growth factors. Attempts to characterise these receptors directly are under way but may be difficult to interpret due to low receptor density and dilution by non-malignant cells.

Stimulation of primary tumour specimens *in vitro* by GM-CSF, G-CSF or IL-3 is of interest since these cytokines are undergoing clinical evaluation to assess their potential for controlling chemotherapy-induced bone marrow toxicity [26, 29, 30]. Extrapolations from our *in vitro* results to treatment will have to take into account clinically relevant concentrations. In our study, stimulation of clonogenic growth was observed in some specimens at 10 ng/ml. How clinically observed serum concentrations reflect cytokine concentrations in the tumour cell will require further research. Our data indicate a concentration-dependent pattern of target cell specificity for cytokines. Clonogenic cells from primary solid human tumours appear to be 10–100 fold less sensitive to cytokines than bone marrow precursors.

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In vivo Interleukin-2 Activated Sheep Lung Lymph Lymphocytes Increase Ovine Vascular Endothelial Permeability by Non-lytic Mechanisms

Daniel E. Bechard, R. Paul Fairman, Daniel B. Hinshaw, Alpha A. Fowler and Frederick L. Glauser

Therapeutic doses of recombinant interleukin-2 (rIL-2) often result in systemic toxicity consistent with increased vascular permeability. rIL-2 activated lymphocytes (IALs) may produce endothelial dysfunction and have cytolytic potential. However, much of the data on IAL cytotoxicity comes from the use of *in vitro* activated IALs. Alternatively, rIL-2 may enhance permeability directly or via release of various cytokines by host effector cells. The cytotoxicity of *in vivo* activated lung lymph lymphocytes has been studied in an ovine model of rIL-2 toxicity. The *in vivo* IALs had no significant endothelial cytolysis at effector to target ratios of 100:1. However, the *in vivo* IALs increased endothelial monolayer permeability to albumin, dependent on the concentration of IALs. rIL-2 induced no endothelial cytolysis or permeability alterations at doses of 10^5 and 2×10^5 U/ml, respectively. These findings suggest that the acute endothelial dysfunction characteristic of the vascular leak syndrome is not due to rIL-2 directly, but is mediated by *in vivo* IALs via non-cytolytic mechanisms and/or the release of secondary cytokines in response to rIL-2.
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INTRODUCTION

ADOPTIVE IMMUNOTHERAPY, the systemic infusion of recombinant interleukin-2 (rIL-2) alone or in combination with *in vitro* expanded rIL-2 activated lymphocytes (IALs) (also called lymphokine activated killer [LAK] cells), is used to treat various animal and human tumours [1, 2]. Tumoricidal activity depends upon the dose and duration of treatment of rIL-2, as well as on the number of *in vitro* IALs infused [3]. Host toxicity (fever, weight gain, malaise, hypotension, azotaemia and respiratory distress) is common. In man, a "vascular leak syndrome" often develops which limits the dose and duration of therapy [4]. The development of increased systemic and pulmonary vascular permeability during rIL-2 adoptive immunotherapy has been substantiated in several animal models [5–8].

Whether the rIL-2, the *in vivo* or *in vitro* IALs, the release of secondary cytokines or the activation of other host cells is responsible for this vascular leak syndrome is unclear. Initially, high doses of rIL-2 were felt to be the cause, although the exact mechanism(s) remain unclear and the available data are contradictory. rIL-2 infusion into isolated, perfused lungs increases microvascular permeability in some [6] but not all models [9]. rIL-2 has also been reported to have no direct