Reduced in vitro erythroid progenitor cell growth in bronchial cancer

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SUMMARY Peripheral blood and bone marrow were studied in 21 men with disseminated untreated bronchial cancer in an attempt to define abnormalities of erythropoiesis associated with the development of anaemia. Haemoglobin concentration at or below 13 g/dl was present in 13 cases. Marrow morphology was normal in all cases except one, in which small numbers of tumour cells were found. Clonal assay of erythroid progenitors showed a significant decrease in the number of BFU-E ($p = 0.03$) and CFU-E ($p = 0.01$) compared with cultures from normal marrow (12 subjects). The growth of granulocyte and macrophage progenitors (GM-CFCs) was similar in patients with bronchial cancer and normal subjects. When normal marrow was incubated in the presence of serum from bronchial cancer patients, no inhibitory factors could be detected either for BFU-E or CFU-E growth.

In all patients circulating T8 numbers were significantly raised ($p = 0.0002$). Consequently, the median T4:T8 ratio in blood was 1.2, and this was significantly lower than the ratio of 1.7 found in 20 normal subjects ($p = 0.036$). In 18 patients the bone marrow T4:T8 ratio of 1.1 was significantly lower than the ratio of 2.9 found in seven normal subjects ($p = 0.04$). Total blood white cell counts, neutrophils, and monocyte numbers were also increased ($p = 0.0001$; $p = 0.0001$; $p = 0.002$).

There is, as yet, no satisfactory explanation for anaemia in chronic disease. Ineffective erythropoiesis does not seem to be responsible for the mild anaemias seen in Hodgkin's disease,¹ myelomatosis,² and rheumatoid arthritis,³ and this distinguishes the condition from iron deficiency anaemia. Red cell life span is not severely shortened, but the erythropoietic response to the onset of anaemia seems to be blunted.³ Some workers have attributed this to a failure of erythropoietin production,⁴⁻⁶ although this remains controversial.⁷⁻⁹ Circulating inactivators of erythropoietin have been implicated in the anaemia of chronic renal failure.¹⁰ This could explain why sera from patients with bronchial cancer and rheumatoid arthritis were reported to inhibit in vitro erythropoiesis,^{11,12} although it seems increasingly likely that failure of erythroid cell production, in vivo and in vitro, is caused by abnormalities in other cell populations which regulate erythroid progenitor cells. Thus monocytes support the in vitro proliferation and differentiation of erythroid colony forming cells in the presence of sera from non-anaemic, but not from

anaemic, donors with rheumatoid arthritis.¹² Macrophages from anaemic patients with fungal infection inhibit the proliferation of erythroid colony forming cells, whereas they stimulate their development after the infection has been eliminated,¹³ and inhibitors of erythropoiesis present in chronic renal failure sera act indirectly by preventing T cells, monocytes, and macrophages from elaborating factors which support in vitro growth of erythroid colony forming cells.¹⁴ Furthermore, quantitative and qualitative changes in T cell subsets have been noted in the anaemia of acquired immune deficiency syndrome (AIDS),^{15,16} myelodysplastic syndromes,¹⁷ and aplastic anaemia,¹⁸ and, in the latter two conditions, are associated with moderate to severe depression of numbers of erythroid colony forming cells in the marrow.

We investigated the associations between progenitor cell numbers and serum inhibitory factors, neutrophils, monocytes and T cell subsets in the anaemia of lung cancer.

Material and methods

Marrow aspirates and blood samples were taken from

21 newly diagnosed male patients with disseminated bronchial cancer (age range 51–78 years). These comprised 17 small cell carcinomas, one adenocarcinoma, and three squamous cell carcinomas. Normal marrow was obtained from male patients of similar age (50–79 years) undergoing cardiac surgery or total hip replacement. Normal blood was taken from age matched male controls. A pool of AB serum was prepared from healthy donors. Fully informed consent was obtained from all subjects.

SERA

Both the control pooled AB serum preparation and individual bronchial cancer sera were heat inactivated at 56°C for 30 minutes and were subsequently filtered (0.45 µm) and stored at –20°C. On thawing, sera were refiltered (0.45 µm) to remove aggregates before being added to culture.

BONE MARROW

Marrow samples were collected in 10 ml Eagle's medium (Flow Laboratories) containing 2% heat inactivated, filter sterilised fetal calf serum and 15 U/ml preservative free heparin at 4°C. The cells were immediately centrifuged at 500 g for 10 minutes, the supernatant discarded, and the pellet resuspended in 5 ml fetal calf serum. Bone marrow granules were broken down by aspiration through a 25 gauge needle. Nucleated cells were separated by centrifugation on Ficoll-Hypaque (Pharmacia) at 400 g for 30 minutes, the interface layer nucleated cells recovered and washed twice with Hepes-buffered Eagle's medium at pH 7.4.

ENUMERATION OF CELL POPULATIONS IN UNFRACTIONATED BLOOD AND MARROW

Blood populations were enumerated on a Hematrak differential cell counter (Becton Dickinson) and marrow populations by visual differential counts on Wright-Giemsa stained films.

ENUMERATION OF BLOOD AND MARROW OKT4, OKT8, AND MO2 POPULATIONS IN MONONUCLEAR CELL FRACTIONS

Cell aliquots (2×10^6 cells) were incubated for 30 minutes at 4°C with 5 µl of monoclonal antibody OKT4, OKT8 (Ortho Diagnostics), or MO2 (Coulter Clone Ltd). After two washes in phosphate buffered saline (PBS), 2% bovine serum albumin (BSA, Sigma Diagnostics), and 0.02% sodium azide cells were further incubated with 5 µl of fluorescein isothiocyanate-labelled rabbit antimouse Ig (Dako Ltd; F.313), or fluorescein isothiocyanate-labelled goat antimouse Ig (Hybritech), for 30 minutes at 4°C. After two further washes in PBS + BSA + azide cells were fixed in 2% paraformaldehyde.

Surface fluorescence was excited at 488 nm and emission measured at 520–540 nm. Each preparation was counted at 500 cells/second, and a total of 10^4 cells were counted using a FACS III (Becton Dickinson) with gates set to encompass all mononuclear cells.

CELL CULTURE

Erythroid colony forming cells

Interface mononuclear cells from the Ficoll procedure were cultured by a modification of the technique described by Iscove *et al.*,¹⁹ in which 2×10^5 nucleated cells/ml were cultured in α medium (Flow) containing 0.8% methylcellulose (Dow, A4M Premium), 2.1 g/l bicarbonate (Wellcome), 10^{-4} M β -mercaptoethanol (BDH), 10^{-7} M sodium selenite (BDH), 10 mg/l vitamin E (Eastman), 10 mg/l ribonucleosides and deoxyribonucleosides, 100% saturated human transferrin (Behringwerke, 10–20 µM), antibiotic and antimycotic solution (Gibco), and 1.0 U/ml anaemic sheep erythropoietin (Step III, Connaught). Pooled normal human AB serum (33% v/v) was added to obtain the final culture mix. Triplicate cultures were incubated at 37°C in air with 5% carbon dioxide in a fully humidified incubator. Colony forming units-erythroid (CFU-E) were scored after seven days and burst forming units-erythroid (BFU-E) after 14 days.

Granulocyte-macrophage colony forming cells (GM-CFCs)

Interface mononuclear cells were cultured, as previously described²⁰ in duplicate in 35 mm Petri dishes at a concentration of 2×10^5 cells/ml of α medium (Gibco Europe Ltd) containing 15% v/v of newborn calf serum (special Bobby calf serum, Gibco Europe Ltd) and 0.3% of agar, and supplemented with 10% v/v dialysed human placental conditioned medium (HPCM) as a source of GM-CSF. Human placental conditioned medium was prepared after the method of Burgess.²¹ GM-CFCs were scored after seven and 14 days of culture at 37°C in air with 5% carbon dioxide in a humidified incubator.

TESTS FOR SERA INHIBITORY ACTIVITY

Erythroid colony forming cells from group O rhesus positive donor marrows were cultured under limiting serum conditions (25% pooled, control AB serum). Individual BC sera and some normal control (non-anaemic) sera were added at 10% of final culture volume, bringing the final serum concentration to 35%.

STATISTICAL ANALYSES

Non-parametric statistical analyses were applied to the data to minimise the effect of outlying values. Significance was demonstrated by the Mann-Whitney test and correlations by Spearman rank techniques.²²

Results

HAEMATOLOGICAL DATA ON PATIENTS WITH BRONCHIAL CANCER (TABLE 1)

Taking the bronchial cancer group as a whole, haemoglobin concentrations, serum iron, and total iron binding capacity were reduced, and serum ferritin increased compared with normal control values. Of 21 patients with bronchial cancer, 13 were anaemic (haemoglobin of 13 g/dl or less). Per cent transferrin saturation, per cent erythroblasts, and dyserythropoiesis were within the normal range.

WHITE BLOOD CELL, LYMPHOCYTE, AND MONOCYTE POPULATIONS IN BLOOD AND MARROW OF PATIENTS WITH BRONCHIAL CANCER

Myeloid and monocyte cell numbers were significantly increased in patients' blood but lymphocyte numbers were unchanged (table 2). Total circulating T4 numbers were significantly increased ($p = 0.046$) but less so than T8 lymphocytes, which caused a reduction in the T4:T8 ratio (table 3).

In the Ficoll interface fraction prepared from marrow of patients with bronchial cancer per cent T4 values were unchanged and per cent T8 cells showed a significant increase (at the 5% level of probability), which would explain the significantly depressed T4:T8 ratio (table 3). The proportion of monocytes in marrow of patients with bronchial cancer (as measured by MO2 staining) was unchanged.

PROGENITOR CELL NUMBERS IN MARROW OF PATIENTS WITH BRONCHIAL CANCER

Both CFU-E and BFU-E numbers were significantly reduced compared with numbers in cultures of normal marrow (table 4), although GM-CFC numbers were unchanged. When patients with bronchial cancer were separated into anaemic and non-anaemic groups, CFU-E numbers were reduced only ($p = 0.009$) in non-anaemic patients. BFU-E numbers in the separated anaemic and non-anaemic groups were not significantly different from normal values. There was no correlation between haemoglobin concentration and CFU-E and BFU-E numbers in patients with bronchial cancer ($r = -0.053$, $+0.150$ respectively; $p > 0.1$).

SERUM INHIBITORY ACTIVITY

The figure illustrates the effect of adding 10% of 19 individual bronchial cancer sera to erythroid cultures of normal marrow containing 25% control pooled AB serum. A batch of 10 bronchial cancer sera were tested in one experiment and a batch of another nine in a second experiment. Colony growth in cultures containing 25% AB serum + 10% bronchial cancer serum was compared with growth in cultures containing 25% and 35% AB serum. Five bronchial cancer sera increased CFU-E numbers and seven sera increased BFU-E numbers to a greater degree than 35% pooled AB serum and were, therefore, "strongly supportive" of erythroid colony forming cell growth. One serum reduced BFU-E numbers to a level below

Table 1 Haematological data on 21 patients with bronchial cancer

Patients	Haemoglobin (g/dl)	Serum ferritin ($\mu\text{mol/l}$)	Serum iron ($\mu\text{mol/l}$)	Total iron binding capacity ($\mu\text{mol/l}$)	Transferrin saturation (%)	Erythroblasts (%)	Dyserythropoiesis (%)
Mean (SD)	12 (2)	531 (564)	9 (3)	45 (13)	22 (7)	15 (6)	1 (1)
Range	7-15	20-2093	4-14	23-66	11-35	7-35	0-4
Normal mean (n = 20)	14.4 (1.4)	93 (98)	19 (8)	67 (11)	27 (12)		
Range	13-18	15-300	12-30	45-70	13-49	2-20	0-10
p value	>0.001	0.01	>0.01	>0.01	>0.1		

Table 2 Enumeration of cell populations in unseparated blood of patients studied

	Patients with bronchial cancer (n = 21)			Normal donors (n = 20)			p value
	Median	Mean	Range	Median	Mean	Range	
White cell count ($10^{-9}/\text{l}$)	9.9	12.3	7.4-40.8	6.4	6.5	3.9-8.9	<0.0001
Total polymorphs ($\times 10^{-9}/\text{l}$)	6.6	9.1	4.3-37	3.7	3.8	1.8-6.1	0.0001
Total lymphocytes ($\times 10^{-9}/\text{l}$)	2.0	2.3	0.7-4.3	2.1	2.0	1.3-3.3	0.80
Total monocytes ($\times 10^{-9}/\text{l}$)	0.59	0.68	0.2-1.6	0.31	0.35	0.08-0.67	0.002

Table 3 Enumeration of T lymphocyte and monocyte subsets in mononuclear fractions prepared from normal and bronchial cancer blood and marrow

		Patients with bronchial cancer			Normal donors			p value
		Median	Mean	Range	Median	Mean	Range	
Blood from: 21 patients with bronchial cancer and 20 normal donors	%T4	37	38	20-60	41	39	19-56	0.48
	%T8	29	30	15-46	24	23	13-41	0.026
	Total T4 ($\times 10^{-9}/l$)	1.0	1.1	0.6-2.1	0.80	0.79	0.3-1.3	0.046
	Total T8 ($\times 10^{-9}/l$)	0.80	0.88	0.4-2.5	0.36	0.47	0.2-0.9	0.0002
	T4:T8 ratio	1.2	1.4	0.8-2.6	1.7	1.9	0.5-3.5	0.036
Marrow from: 18 patients with bronchial cancer and seven normal donors	%T4	17	18	3-33	16	16	8-25	0.63
	%T8	15	16	3-36	6.0	9.0	2-27	0.046
	T4:T8	1.1	1.7	0.3-10	2.9	3.5	0.4-7.4	0.04
	%MO2*	5.0	6.1	0-15	11	13	2.4-38	0.25

*Monocytes estimated by MO2 staining.

Table 4 Progenitor cell growth in cultures of bronchial cancer marrow

		Progenitors per 10 ⁵ mononuclear cells						
Progenitor type		Patients with bronchial cancer			Normal donors			p value
		Median	Mean	Range	Median	Mean	Range	
Day 7	CFU-E	42 (n = 21)	63	0–230	157 (n = 12)	165	15–485	0.01
	BFU-E	7 (n = 21)	17	0–107	26 (n = 14)	29	0–65	0.03
	GM-CFC	76 (n = 8)	83	15–214	102 (n = 15)	117	62–184	0.2
Day 14	GM-CFC	27 (n = 8)	31	12–67	63 (n = 15)	59	8–118	0.1

that observed in cultures supplemented with 25% AB serum—that is, was inhibitory to erythroid colony forming cell growth. Most bronchial cancer sera were “weakly supportive” and increased erythroid colony forming cell numbers, but to a lesser extent than 35% pooled AB serum.

Discussion

In this paper we have reported measurements of marrow and blood functions that might be related to erythropoietic failure in patients with bronchial cancer. Decreased serum iron concentration and total iron binding capacity and increased serum ferritin are all characteristic of the anaemia of chronic disease,^{3 11 23 24} as is a rise in blood neutrophils.^{24 25} Neutrophils contain lactoferrin,²⁶ which, if released in large quantities during neutrophilia, might both depress serum iron and restrict iron supply to maturing erythroid cells.^{24 25} It seems unlikely that these mechanisms could have been responsible for the anaemia in our patients, as they would have caused iron deficient erythropoiesis with obvious ineffective red cell formation, yet ineffective erythropoiesis does not seem to be a contributory factor in anaemia in

chronic disease.^{1 3} In any case there was no correlation between blood neutrophils and serum iron concentration ($r = -0.461$; $0.05 < p < 0.1$) in our patients.

Monocytes were also increased in the blood of patients with bronchial cancer, but, as for neutrophils, their numbers were not related to the degree of anaemia, and monocyte numbers in the marrows of these patients were similar to normal. The changes in lymphocyte subsets in our patients also deserve comment. The decreased T4:T8 ratio is largely the result of increases in T8 cells in both blood and marrow. In marrow the T4:T8 ratio showed a negative correlation with the number of blood neutrophils ($r = -0.525$; $p < 0.05$), but there was no obvious explanation for this.

A reduced T4:T8 ratio has been reported in anaemias associated with AIDS^{15 16} and myelodysplasia,¹⁷ and an increased marrow T8 lymphocyte burden has been reported to be responsible for the suppression of erythropoiesis in aplastic anaemia.¹⁸ Lymphocyte function has been shown to be changed both in bronchial cancer^{27 28} and rheumatoid arthritis,²⁹ although blood T lymphocytes from patients with rheumatoid arthritis stimu-

Erythroid colony forming cells in bronchial cancer

Figure One of two similar experiments. Effect of adding 10% bronchial cancer serum or 10% pooled normal AB serum to cultures of normal marrow already containing 25% AB serum. Ten bronchial cancer sera (batch 1) were tested in one experiment and another nine bronchial cancer sera (batch 2) were tested in second separate experiment. ●—CFU-E; ○—BFU-E numbers/10⁵ mononuclear cells; ----, colony numbers in cultures supplemented with 25% and 35% AB serum.

lated in vitro erythropoiesis to the same degree as did T lymphocytes from normal donors.^{12 30} In our patients there were no correlations between lymphocyte subset numbers and haemoglobin concentration, so the importance of the changes in these populations to the aetiology of anaemia in bronchial cancer remains unclear.

The perturbations in neutrophil, monocyte, and lymphocyte populations did not correlate with the reduction in erythroid progenitors in bronchial cancer marrows. It seemed plausible that the increased demand for mature neutrophils in patients with bronchial cancer might be met by increasing GM-CFCs at the expense of erythroid colony forming cell production.²⁵ GM-CFC numbers, however, were not raised in our patients, and the excess blood neutrophils are presumably released from storage pools. The presence of normal numbers of GM-CFCs suggests that the multipotential stem cell compartment is unaffected. Erythropoietic impairment must, then, occur either shortly before the formation of erythroid colony forming cells or soon enough afterwards to prevent the appearance of colonies in vitro and

increased erythroblast numbers in vivo.

It is tempting to conclude that impaired growth of erythroid colony forming cells underlies the anaemia in bronchial cancer. Erythroid colony forming cell suppression, however, was most noticeable in the non-anaemic group of patients; anaemic patients showed only an insignificant trend towards fewer erythroid colony forming cells. Furthermore, four of 13 anaemic patients possessed large numbers of CFU-E, and there was no correlation between erythroid colony forming cells and haemoglobin concentrations in patients with bronchial cancer. Consequently, it seems unlikely that the anaemia in all such patients could arise from poor erythroid colony forming cell growth. Tumour cell infiltration into the marrow was noted in only one patient and does not seem to have been responsible for reduced numbers of erythroid colony forming cells.

By showing significant suppression of erythroid colony forming cell growth, our data differ from published findings on mixed cancers⁶ and rheumatoid arthritis,^{12 30} in which erythroid colony forming cell numbers in normal and diseased donors were similar.

There are some methodological differences between these earlier studies and the work we report here, and these could be critical to the results.

Although none of the irregularities in blood and marrow cell populations apparently correlates with haemoglobin concentration or erythroid colony-forming cell numbers in patients with bronchial cancer, a qualitative rather than a quantitative change in neutrophils, monocytes, or lymphocytes may be responsible for impaired erythropoiesis.¹²⁻¹⁴ Indeed, marrow adherent cells from anaemic patients with chronic disease have been shown to inhibit *in vitro* erythropoiesis.^{13,31} Any inhibitory factor would have to act over a short range as we have been unable to corroborate a previous report¹¹ that inhibitors of erythropoiesis circulate in bronchial cancer sera. Only one of our bronchial cancer sera proved inhibitory when added to normal marrow cultures containing 25% AB serum; most "weakly supported" erythroid colony formation. Our observation that several bronchial cancer sera "strongly supported" erythroid colony forming cell proliferation is consistent with our earlier report⁹ that some patients with bronchial cancer do respond to their anaemia by increasing production of erythropoietin (and perhaps other positive regulators of erythropoiesis as well).

To summarise, considerable perturbations occur in mature haemopoietic populations and in the erythroid progenitor compartments in patients with bronchial cancer, although the associations between these phenomena are obscure and their role, if any, in the onset of anaemia remains undefined. Anaemia in bronchial cancer may result from the interplay of several of these population imbalances or, alternatively, the changes reported here may be epiphenomena associated with a more fundamental haemopoietic defect.

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