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Original Paper

Red Blood Cell Polyamines, Anaemia and Tumour Growth in the Rat

V. Quemener,[1] J.Y. Bansard,[1] M. Delamaire,[2] S. Roth,[3] R. Havouis,[1] D. Desury[1] and J.-Ph. Moulinoux[1]

[1]Laboratoire de Biologie Cellulaire, URA CNRS 1529, C.H.U. de Rennes, 2 Avenue du Professeur Léon Bernard, 35043 Rennes Cedex, France; [2]Centre Régional de Transfusion Sanguine, rue Pierre Jean Gineste, 35000 Rennes Cedex, France; and [3]Klinik und Poliklinik für Urologie, Westfälische Wilhems-Universität Münster, Albert-Schweitzer-Strasse 33, 4400 Münster, Germany

In rats with Mat Lylu prostatic carcinoma, significant changes in blood composition and red blood cell (RBC) characteristics were observed. Anaemia, characterised by a decrease in the number of RBC and the reduction of haemoglobin and the iron content in plasma, was correlated with tumour size and the accumulation of spermidine and spermine in the RBC. In large tumours, spermidine levels were increased by 8-fold over normal value. Spleen weight and splenic spermidine concentrations were enhanced in animals with tumours. After splenectomy, the rate of tumour growth decreased by 30%. It is proposed that anaemia in tumour-bearing animals is caused by enhanced RBC lysis, owing to the alteration of the rheological properties of RBC. These may be caused by the alterated surface characteristics due to polyamine accumulation. RBC lysis and high concentrations of polyamines in RBC and spleen appear, not only to favour tumour growth, but also to compromise the immunological defence mechanisms against neoplastic invasion.

Key words: cancer, polyamines, erythrocytes, anaemia, rat, prostatic carcinoma
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INTRODUCTION

IN PATIENTS suffering from cancer, symptoms such as cachexia, abnormalities in blood coagulation, anaemia and immunosuppression are frequently observed. Although unexplained and not directly related to tumour growth, these symptoms are known by the term paraneoplastic syndrome [1]. Paraneoplastic syndrome is not only known in man, but also in experimental animals. Several authors have reported that animals with grafted tumours develop marked anaemia [2, 3]. In the case of Lewis lung carcinoma in mice, anaemia is normocytic, accompanied by a marked reticulocytosis and a decreased RBC survival time, suggesting its haemolytic origin [2]. A microangiopatic origin was discussed to explain the observations but was not demonstrated.

Reasons other than the enhanced destruction of the RBC owing to biochemical changes, or a direct haemolytic effect of tumour cells, have also been discussed [4].

Physiologically, the senescent erythrocytes are recognised and sequestered by reticuloendothelial macrophages before they are phagocytosed. A glycopeptide, detectable in old but not in young erythrocytes, is involved in erythrocyte sequestration as a recognition factor [5], and a senescent cell antigen immunologically related to band 3 protein membrane is necessary for phagocytosis of the old erythrocytes [6]. In addition to antigenic recognition of senescent RBC, mechanical destruction of erythrocytes can occur in the spleen when RBC are devoid of their two important characteristics: extreme pliability and mechanical stability. There is evidence that *in vitro* the structural basis for these physical properties are due to a specific protein–phospholipid complex which constitutes the membrane skeleton. These skeletal structures are in a state of dynamic equilibrium. They can be destabilised by changes in the ionic strength [7].

Polyamines are cationic molecules found in association with skeletal proteins in normal erythrocytes. Increased levels of polyamines are observed in membranes derived from erythrocytes of sickle cell anaemia patients [8]. In this disorder, the erythrocytes are abnormal with a decreased pliability and a short life-span. Polyamines, especially spermine, are known to

Correspondence to V. Quemener.
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decrease membrane pliability and to stabilise the membrane skeleton of human erythrocytes *in vitro* [8].

In a previous study, we showed that when RBC were incubated *in vitro* with radiolabelled polyamines and then submitted to SDS–PAGE electrophoresis, radioactivity was strongly associated with band 3 protein [9]. We have also reported that polyamines are found at abnormally high concentrations in the RBC of tumour-bearing animals [10], and of patients with different types of cancer [11–13]. The clinical use of RBC polyamines as an index of tumour cell proliferation has been clearly established. Undoubtedly, the excessive polyamines which are transported by RBC are of tumour origin [14].

Our aim in this work was to characterise the biochemical changes in the RBC of rats grafted with the Mat Lylu prostatic carcinoma, and to explore the anaemia occurring during tumour growth.

MATERIALS AND METHODS

Chemicals

Usual laboratory chemicals and reagents, as well as dansyl chloride, *o*-phalaldehyde, polyamines and amino acids were obtained from Sigma Co. (St Louis, Missouri, U.S.A.).

Animals and tumours

A total of 107 3-month-old Fisher-Copenhagen rats, weighing 170–220 g, bred in our laboratory, were used in this study. Animals were housed in plastic cages with free access to food and water. Room temperature (21–23°C), humidity (55–65%) and a 12 h light–12 h dark cycle were kept constant. Rats were separated into random groups of controls and tumour grafted for each experiment. The Mat Lylu prostatic carcinoma cells were routinely maintained in culture in RPMI 1640 medium (Eurobio, Les Ullis, France). Cells (2×10^6) were injected subcuteaneously in the flank of the animals. All experiments were repeated at least twice. Tumour volume was determined using a calipers every second day. Animals were sacrificed at different periods of tumour growth, usually 14, 21, 28 days after tumour cell implantation. Blood was collected by retro-orbital bleeding or after decapitation.

Splenectomy

Twenty-four hours before tumour cell grafting, animals were submitted to surgical removal of the spleen. Anaesthesia was done by i.p. injection of 166 mg/kg of Ketalar (Parke Davis Lab., Courbevoie, France). Splenic vascularity was clamped with a surgical suture thread Maxon® 6.0 (Robert & Carrière Lederle, France). Control animals were sham-operated.

Assays methods

Blood. Blood was collected into EDTA sterile vials and analysed for red blood cell (RBC), leucocyte and platelet numbers, haemoglobin, haematocrit, mean cell volume (MCV) and mean cell content of haemoglobin (MCH) by the use of a Coulter T 540 (Coultronics, Margency, France).

Reticulocyte number. A version of the method of Lee and associates [15] was used. Aliquots of the blood samples (5 μl) were incubated for 30 min with Thiazol Orange 1/10 000. Using a Cytoron Absolute fluorimeter equipped with an Argon laser (Orthodiagnostic, Roissy, France), fluorescence of 6×10^4 cells was measured at 488 nm. Data are expressed as percentage of fluorescent cells in the RBC population.

Biochemical analysis. Iron and urea concentrations were determined in plasma by the use of a SMAC Technicon analyser (Bayer Diagnostics, France). Erythropoietin concentrations were measured in serum (blood samples collected in sterile vials with no additive) with the radio-immunologic EPO-Trac® ^{125}I method. Kits were obtained from INSCTAR Corp. (Stillwater, Minnesota, U.S.A.).

ATP and 2,3-DPG concentrations were measured in the RBC pellet according to the published methods [16, 17].

Polyamine determinations. Blood samples were collected in a 0.129 M buffered sodium citrate solution and centrifuged at 1200*g* for 10 min at 4° C. The RBC pellet was washed twice with 4 volumes of 0.14 M NaCl and treated with 10% perchloric acid. The erythrocyte number was determined in an aliquot of the pellet: 1 ml of packed RBC contains 8×10^9 erythrocytes. For polyamine determinations, the perchloric acid extracts were submitted to dansylation and HPLC separation [18].

Spleens were homogenised in 10 volumes of ice-cold 10% perchloric acid, maintained for 1 h at 0°C and centrifuged at 3000*g* for 10 min at 4°C. Polyamines were determined in the perchloric acid extract by the same procedure as the RBC polyamines.

Statistical analysis

The non-parametric Mann & Whitney ranking test (for unpaired values), linear correlation tests and multiple correspondence analysis were used for statistical evaluation of the data.

RESULTS

Blood cell characteristics in animals with tumour

A significant reduction of the RBC counts and of haemoglobin (Hb) concentrations, and an enhancement of the leucocyte counts were observed in animals grafted with tumours. Consequently, the haematocrit was reduced (Table 1). These changes were accentuated during tumour growth. Platelet counts were not significantly affected (Table 1). A significant inverse correlation was found ($P < 0.0001$) between the RBC counts and the tumour volume. Similar correlation was observed between tumour volume and Hb levels or haematocrit. The leucocyte counts increased with the size of the tumour ($P < 0.0001$).

RBC characteristics

The mean RBC volume (MCV) and the haemoglobin content per cell (MCH) were significantly increased 20 days after tumour grafting (tumour volume greater than 40 cm^3) (Table 1). In contrast, the ATP and 2,3-DPG concentrations in RBC were slightly but not significantly enhanced in cancerous rats (Table 2).

The reticulocyte counts were significantly increased in animals with large but not in those with small tumours (Table 3). There was a correlation between reticulocyte counts and tumour volume ($P < 0.0001$). With severe anaemia (animals with tumours larger than 60 cm^3; Table 1), the reticulocyte concentration reached more than 10^6 cells/mm^3 (34% of the RBC count).

Spermidine and spermine concentrations increased with increases in the tumour volume ($P < 0.0001$). For large tumours, mean spermidine levels were 8-fold the normal value (Table 3), but individual animals showed 12-fold increases.

Table 1. Blood cell counts in rats during Mat Lylu carcinoma growth. Animals (n = 43) *were grafted with tumour cells and blood cell counts (red blood cells, RBC; erythrocytic mean cell volume, MCV; mean cell hemoglobin, MCH) were measured in animals with tumours of different volumes. Controls* (n = 15) *were healthy rats. Data are means* (SEM)

	Controls ($n = 15$)	Mat Lylu grafted Day 14* ($n = 15$)	Day 21 ($n = 14$)	Day 28 ($n = 14$)
Tumour volume (cm^3)	—	14 (2)	44 (2)	76 (4)
RBC ($10^6/mm^3$)	8.1 (0.1)	7.3 (0.2)†	5.6 (0.3)§	5.1 (0.3)§
Leucocytes ($10^3/mm^3$)	8.0 (0.8)	12 (1)†	32 (4)§	48 (3)§
Platelets ($10^3/mm^3$)	594 (83)	553 (83)	530 (61)	401 (48)
Haemoglobin (g/dl)	13.7 (0.2)	12.4 (0.3)‡	10.1 (0.4)§	9.2 (0.4)§
Haematocrit (%)	41.8 (0.6)	37.5 (0.9)‡	31 (1)§	29 (1)§
MCV (fl)	51.3 (0.3)	51.4 (0.3)	55.2 (0.8)‡	56.4 (0.9)§
MCH (pg)	16.9 (0.1)	17 (0.2)	18.4 (0.7)†	18.1 (0.3)†

* Day on which animals were sacrificed following tumour inoculation.
Significantly different from controls with †$P < 0.01$, ‡$P < 0.001$, §$P < 0.0001$.

Table 2. Red blood cell ATP and 2,3-DPG concentrations in rats grafted with the Mat Lylu carcinoma. Animals were sacrificed with a mean tumour volume of 32 ± 4 cm^3, *20–22 days after grafting* (n = 10), *controls* (n = 9). *Data are expressed in mg per g of haemoglobin (Hb). Mean values (SEM)*

	ATP (mg/g Hb)	2,3-DPG (mg/g Hb)
Controls	1.9 (0.2)	5.2 (0.2)
Mat Lylu	2.5 (0.3)	5.6 (0.15)

Table 3. Polyamine levels in red blood cell and spleen of rats grafted with the Mat Lylu carcinoma. Animals were sacrificed at two different periods of tumour growth. Data are reticulocyte count (% of RBC), spleen weight and polyamines (PA) levels in red blood cells (RBC) and spleen. Mean values (SEM)

	Controls ($n = 23$)	Mat Lylu ($n = 10$) Day 14*	Mat Lylu ($n = 14$) Day 21–24
Tumour volume (cm^3)		9.3 (0.9)	60 (5)
Reticulocytes (%)	2.5 (0.2)	2.5 (0.15)	20 (2)§
Spleen weight (mg)	421 (23)	439 (19)	806 (61)§
PA in RBC (nmol/8 × 10^9)			
Spermidine	30 (2)	31.5 (3)	251 (21)§
Spermine	1.9 (0.3)	1.7 (0.2)	14 (2)§
PA in spleen (nmol/g)			
Putrescine	18 (2)	24 (1)	99.5 (8)§
Spermidine	687 (66)	827 (38)	996 (61)†
Spermine	354 (40)	405 (23)	192 (16)‡

*Day on which animals were sacrificed following tumour inoculation. Significantly different from controls with †$P < 0.01$, ‡$P < 0.001$, §$P < 0.0001$.

The accumulation of spermidine and spermine in the RBC was correlated with a decrease in RBC counts ($P < 0.0001$) (Fig. 1), a decrease in Hb ($P < 0.0001$) and an increase of the number of reticulocytes ($P < 0.0001$).

Plasma erythropoietin, iron and urea concentrations

Plasma erythropoietin levels were slightly but not significantly increased in rats with tumours (Table 4). A significant decrease in plasma iron levels was observed in animals with tumours (Table 5), confirming the anaemia. The decrease in plasma iron concentrations was correlated with an increase in tumour volume ($P = 0.0001$). In contrast, urea was accumulated in the plasma of the Mat Lylu grafted rats, but the

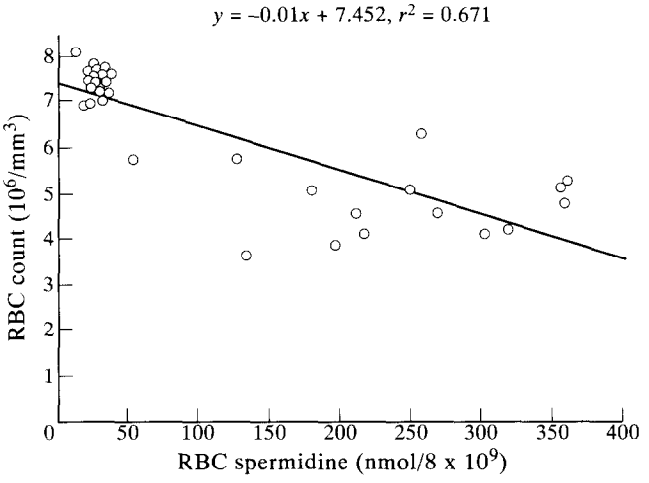

Figure 1. Correlation between RBC counts and RBC spermidine concentrations during Mat Lylu carcinoma growth. Animals were sacrificed at different periods of tumour growth. Correlation with $P < 0.0001$.

Table 4. Serum erythropoietin concentrations in rats during Mat Lylu carcinoma growth. Animals (n = 15) *were grafted with tumour cells and erythropoietin (mU/ml) was determined at different periods of tumour growth. Controls* (n = 14) *were healthy rats. Mean values (SEM)*

	Tumour volume (cm^3)	EPO (mU/ml)
Controls ($n = 14$)	0	35 (1)
Mat Lylu ($n = 6$)	2.6 (0.3)	36 (2)
($n = 5$)	25 (4)	34 (7)
($n = 4$)	72 (4)	43 (6)

Table 5. Plasma iron and urea concentrations in Mat Lylu grafted rats. Animals grafted with tumour cells (n = 14) *were sacrificed with a mean tumour volume of* 51 ± 6 cm^3, 21–28 *days after implantation. Controls were identically treated* (n = 15). *Mean values (SEM)*

	Iron (μmol/l)	Urea (mmol/l)
Controls	29 (2)	6.8 (0.2)
Mat Lylu	15 (1)*	15 (3)†

Significantly different with $*P < 0.0001$, $\dagger P < 0.05$.

plasma urea concentration was not correlated with tumour size (Table 5).

Spleen weight and polyamine concentration

As shown on Table 3, the spleen weight increased during tumour growth, doubling in animals with large tumours, 21–24 days after implantation. The splenic putrescine and spermidine concentrations were greatly enhanced and spermine concentrations were significantly lower in animals with a large tumour (Table 3). Because of the differences in the weight, the total spermine content was not altered in the spleen.

Effects of splenectomy

It was found, in two independent experiments, that when animals were submitted to a total splenectomy 24 h before tumour grafting, tumour growth was slowed significantly (Fig. 2). Twenty-one days after grafting, the tumour volume was reduced by more than 30%. In one experiment, splenectomy preceded grafting by 6 days. In this case, tumour growth was also significantly reduced (data not shown). After splenectomy, the RBC counts in rats with tumours were slightly enhanced but not restored to control values (data not shown).

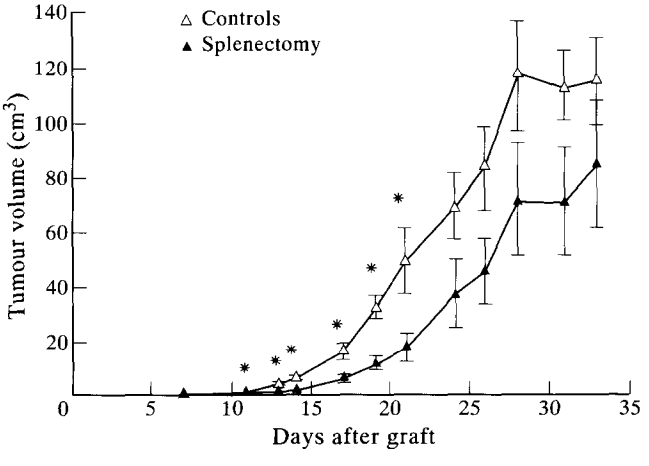

Figure 2. Influence of splenectomy on the Mat Lylu carcinoma growth. Twenty-four hours before tumour cell graft, animals were submitted to surgical removal of the spleen. Data are tumour volume measurements in splenectomised animals compared to sham operated controls: mean ± S.D. $*P < 0.05$.

DISCUSSION

Rats grafted with the Mat Lylu prostatic carcinoma show abnormal blood composition 14 days after tumour cell implantation. The changes are accentuated during tumour growth. In agreement with observations on mouse tumour models [19, 20], we observed, in the tumour-bearing rats, that leucocyte counts were enhanced and RBC counts were reduced. As a result of these changes, the haematocrit decreased during tumour growth. Anaemia, characterised by hemoglobin and RBC reductions, was progressive with the growth of the tumour and was associated with a reduction of plasma iron concentrations similar to the effect with acute haemolysis. An increase of the spleen weight, combined with hyperleucocytosis and hyper-reticulocytosis, was also noticed. These observations are indicative of an anaemia of haemolytic origin. This type of anaemia has already been reported for other tumours [2, 21]. Concomitant with the development of the anaemia, we found increasing levels of polyamines in the RBC, reaching 8-fold the normal value in large tumours. The increase in RBC spermidine and spermine was correlated the tumour size. The mean RBC volume was also significantly enhanced during tumour growth, indicating that the anaemia was macrocytic. In contrast, ATP and 2,3-DPG concentrations were slightly but not significantly modified in the erythrocytes, indicating normal function. Fifteen days after tumour grafting, the number of circulating reticulocytes was dramatically enhanced indicating that the bone-marrow regeneration process was not affected. Surprisingly, plasma erythropoeitin was not significantly changed, although it is well known that it is greatly enhanced in cases of severe anaemia

RBC from animals with tumours exhibit an enhanced uptake of extracellular polyamines [9]. The excessive polyamines in RBC are of tumour origin [14]. The fact that the RBC flux is greater in tumour than in normal tissues [22] and that tumour cells have the capacity to lyse erythrocytes through a tumour haemolytic factor [4] may explain the anaemia, but it does not explain the reduction of tumour growth after splenectomy.

The increased volume and the high polyamine concentrations in the RBC of tumour-bearing animals could be an explanation for the haemolytic origin of the anaemia. In agreement with this hypothesis, it has been reported that polyamines can crosslink endogenous erythrocyte membrane proteins by forming weak bonds with adjacent negatively charged lipids or protein moieties and lead to rheologically abnormal RBC with a decreased lifespan [7, 8]. These observations support the idea that RBC could be trapped and destroyed by the splenic macrophages because of their abnormal rheological and biochemical characteristics.

Concerning the biochemical characteristics involved in recognition of RBC by reticuloendothelial macrophages, it should be emphasised that when RBC were incubated with ^{14}C spermidine *in vitro*, radiolabelling was mainly detected in band 3 protein after SDS-PAGE electrophoresis [9]. SDS-PAGE analysis of RBC membranes revealed that band 3 proteins were compacted [9] and ^{14}C spermidine binding to band 3 protein of erythrocyte ghosts from 3LL grafted mice was enhanced compared with that of ghosts from erythrocytes of normal mice. Since band 3 proteins are involved in the senescent cell antigen recognition [6], one may suppose that polyamine binding to this structure could influence the macrophagic destruction of RBC. These observations lead to the

idea that high concentrations of polyamines could explain, in part, the shorter RBC lifespan in animals with tumours [2], and be responsible for the establishment of the anaemia during tumour growth.

Some observations support the concept that the changes in red blood cell counts are not just a secondary phenomenon owing to the presence of a tumour. By avoiding the influence of the spleen on RBC destruction, tumour growth was reduced. When RBC are lysed in the spleen, or by other aforementioned mechanisms, erythrocyte polyamines and haemoglobin are released. The tumour cells may re-utilise the released polyamines favouring their growth [23]. Putrescine and spermidine concentrations were significantly higher in the spleen of animals with tumours compared with those of normal rats. The spleen is a reservoir of lymphocytes, natural killer cells and macrophages. All these cells are involved in immunological defence against neoplastic invasion. It has recently been reported by Thomas and associates that polyamines inhibit mitogen-induced Ca^{2+} influx in splenic T cells, specifically $CD4^+$ cells [24]. We have reported that NK cytotoxicity is dramatically decreased in the spleen of mice grafted with the 3LL carcinoma, but completely restored to normal values when the mice are deprived of polyamines [19]. Haemoglobin binds nitric oxide which may be released by activated macrophages. Since the cytostatic activity of macrophages is inhibited in the presence of erythrocytes [25], it is likely that, in haemolytic conditions, haemoglobin could reduce the efficiency of macrophages. Tumour cells are reported to have transferrin receptors on their surface [26]. They can use iron as a growth factor [27, 28]. The major function of RBC is oxygen transport. A deficient oxygenation of tissues is an important factor of tumour growth [29]. All these observations argue in favour of the hypothesis that anaemia, RBC lysis and high concentrations of spermidine in the RBC and the spleen not only favour tumour growth locally but also reduce immunological defence against neoplastic invasion [30].

Our conclusion is that in rats grafted with the Mat Lylu prostatic carcinoma, haemolytic anaemia occurs concomitantly with an increase in RBC and spleen polyamine concentrations. This situation creates a vicious cycle, favouring tumour development. A logical consequence of these conclusions is the use of systematic polyamine deprivation as a treatment for cancer. Since polyamine deprivation normalises tissue and RBC polyamine concentrations, it prevents anaemia, normalises leucocyte counts [18, 31] and restores NK cell activity [19], and it prevents tumour growth in animals [32]. In patients with prostatic cancer, RBC polyamine levels are significantly enhanced [13] and paraneoplastic syndroms such anaemia and immunosuppression frequently observed. Our objective now is to begin clinical trials of polyamine deprivation in patients with prostatic cancer.

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