

ORIGINAL ARTICLE

Blood cells with fetal haemoglobin (F-cells) detected by immunohistochemistry as indicators of solid tumours

M Wolk, J E Martin, R Constantini

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See end of article for
authors' affiliations

Correspondence to:
Dr M Wolk, Central
Laboratories, Israel
Ministry of Health, Ya'akov
Eliav St 9, PO Box 34410,
Jerusalem 91342, Israel;
moshe.wolk@eliav.health.
gov.il

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Aims: Fetal hemoglobin (HbF) is an established serological indicator of cancer. However, its distribution in tumour tissues is rarely investigated. Therefore, HbF was studied immunohistologically in different cancers characterised by high blood HbF concentrations.

Methods: Anti-HbF was immunoaffinity purified and used to study HbF immunohistochemically in the following cancers: germ cell tumour (GCT), trophoblastic disease (TD), lymphoma, myelodysplastic syndrome (MDS), multiple myeloma (MM), and ovarian adenocarcinoma (OA).

Results: In GCT a distinction was made between tumours substantially without HbF positive red blood cells (F-RBC) and those with F-RBC. Those without F-RBC were non-metastatic mature teratomas and dermoid cysts. Those containing F-RBC were mainly embryonal carcinomas and metastatic teratomas. HbF positive myeloid cells (F-MLC), HbF positive normoblasts (F-NBS), and F-RBC were common in the bone marrow and in the lymphoid tissues of lymphoma, MDS, and MM. In TD, normal and nucleated F-RBC were seen in the trophoblastic villi in one case with incomplete molar pregnancy (ICM) but not in other cases of ICM and complete molar pregnancy. However, F-RBC and F-MLC were seen in the decidua of both types of TD. Generally, F-cells were observed either within blood vessels or concentrated in certain areas of the neoplastic tissue.

Conclusions: HbF was evaluated as an inducible marker within different tumour tissue blood cells. The dual distribution of these cells—circulating in the blood or concentrated in areas of the neoplastic tissues—might reflect the two independent serological indicators of HbF: one in whole blood and the other in plasma of patients with cancer.

Fetal haemoglobin (HbF) is the main haemoglobin of the late fetus and the newborn, constituting around 80% of the total haemoglobin.¹ In normal adults, HbF comprises less than 1% of the total haemoglobin.^{2–4} However, increased concentrations of HbF are common in some haemoglobinopathies,⁵ in addition to many neoplasms, including those studied here, namely: germ cell tumour (GCT),^{6,7} trophoblastic disease (TD),^{7,8} lymphoma,^{9,10} multiple myeloma (MM),^{10–12} myelodysplastic syndrome (MDS),^{12–14} and ovarian adenocarcinoma (OA).^{7,12}

"Some early studies claimed that fetal haemoglobin is produced by the tumour cells of germ cells tumours, rather than by the red blood cells residing in the tumour"

Recently we were able to distinguish between whole blood HbF and plasma HbF as two independent indicators of cancer.^{7,12} The origin of the whole blood HbF is the circulating red blood cell (RBC). As such, whole blood HbF is not an oncofetal protein, produced by tumour cells, but an inducible protein in many neoplasms. In contrast, the origin of plasma HbF was not clear, especially with regard to non-haematological solid tumours. For instance, among 23 cases of testicular germ cell tumour,^{7,14} 14 had raised plasma HbF, most of them with normal whole blood HbF. Similarly, in persistent trophoblastic diseases, raised plasma HbF was very common.¹² Some early studies^{6,15} claimed that HbF is produced by the tumour cells of germ cells tumours, rather than by the RBC residing in the tumour. In our preliminary immunohistochemical study,⁷ we found faint staining of tumour cells using haemoglobin A absorbed anti-HbF antibody. However, it was still unclear whether plasma HbF is a classic oncofetal protein, produced by tumour cells, or a

component of haemopoietic cells or RBC, concentrated inside the tissue of certain tumours. In our attempt to solve this problem, and to re-evaluate our preliminary observations,⁷ we prepared a new batch of immunoaffinity purified anti-HbF antibody, and the results obtained using this reagent are presented here.

MATERIALS AND METHODS

Subjects

The programme of this research, including studies on archival and stored clinical materials, was approved by the East London and the City Health Authority research ethics committee. Patients' histological specimens were selected from the collection of the department of histopathology, Royal London Hospital, UK. They included the following diseases: GCT (26 cases), TD (14 cases), lymphoma (seven cases), MDS (seven cases), myeloma (seven cases), hairy cell leukaemia (10 cases), and OA (six cases). In parallel with the study of pathological tissues we tested normal tissues of the same organs.

Abbreviations: AFP, α fetoprotein; CM, complete molar pregnancy; EC, embryonal carcinoma; F-MLC, fetal haemoglobin positive myeloid cells; F-NBS, fetal haemoglobin positive normoblasts; F-RBC, fetal haemoglobin positive red blood cells; GCT, germ cell tumour; hCG, human chorionic gonadotrophin; HbF, fetal haemoglobin; HbFSRBC, fetal haemoglobin coated sheep red blood cells; ICM, incomplete molar pregnancy; MDS, myelodysplastic syndrome; MM, multiple myeloma; MMT, metastatic mature teratoma; NMT, non-metastatic mature teratoma; NSS, normal sheep serum; OA, ovarian adenocarcinoma; OD, optical density; PBS, phosphate buffered saline; RBC, red blood cells; TD, trophoblastic disease; YS, yolk sac

Table 1 Affinity purification of sheep anti-HbF serum

Purification fraction	Anti-HbF titre (A)	OD280 (protein concentration; B)	Ratio of anti-HbF to protein (A/B)
Original anti-HbF serum	1000	35	28.5
Eluate I	86	2.0	43
Eluate II	160	0.70	228.5
Eluate II (control; NSS)	0	0.73	0

Purification product in eluate II = $228.5/28.5$ (eightfold). The recovery of anti-HbF in eluate II = $160 \times 100/1000 = 16\%$.
HbF, fetal haemoglobin; NSS, normal sheep serum.

Affinity purification of anti-HbF antibody

The HbF antiserum was prepared in sheep and was rendered specific for HbF by absorbing it to HbA.¹⁶ The same antiserum has been used in the past in serological studies of HbF in patients with cancer.^{4 7 12 16 17} This antibody was further purified by absorbing it to HbF coated sheep red blood cells (HbFSRBC) and selectively eluting the anti-HbF. The HbFSRBC were prepared using glutaraldehyde as the coupling agent, as described previously.¹⁶ The affinity purification was performed by the following steps:

- (1) Extraction of the immunoglobulin by precipitation with 40% saturated ammonium sulfate.
- (2) Dialysis $\times 3$ against phosphate buffered saline (PBS; pH 7.5).
- (3) Absorption 1 : 1 with HbFSRBC for four hours at room temperature.
- (4) Disposal of the supernatant containing the unabsorbed protein, followed by washing the HbFSRBC with two volumes of PBS.
- (5) Elution of the anti-HbF from the HbFSRBC was performed batchwise by two steps.
 - (a) Shaking with a 1 : 1 volume of 0.2M glycine/HCl (pH 2.7) for five minutes then saving the supernatant by spinning for five minutes at $1010 \times g$ at 4°C . The supernatant, designated eluate I, was immediately adjusted to pH 7.5 by the addition of 1M Na_2HPO_4 .
 - (b) The HbFSRBC from (a) were eluted again by shaking them with 1.3 volumes of 0.2M glycine/HCl (pH 2.7) for 15 minutes. The supernatant, designated eluate II, was saved and its pH adjusted as in (a).
- (6) Eluates I and II were dialysed $\times 3$ against PBS then diluted 1 : 1 with glycerine for storage at 4°C .

The efficiency of purification was estimated by comparing the ratios of antibody titre to protein concentration, before and after purification. The anti-HbF titre was measured by the graded haemagglutination system,¹⁶ and the protein concentration was inferred from the optical density (OD) at 280 m μ .

As a control for the immunohistochemical staining we repeated purification steps 1–6 on normal, unimmunised sheep serum (NSS).

Immunohistochemical staining

We used the peroxidase labelled avidin–biotin method.¹⁸ Formalin fixed, paraffin wax embedded cross sections were cut at 3 μm , dewaxed, then quenched with 3% H_2O_2 in methanol. The incubation steps were as follows: (1) blocking with normal rabbit serum, diluted 1/10, for 20 minutes; (2) primary antibody (anti-HbF; eluate II), diluted 1/20, for 60 minutes; (3) secondary antibody (affinity purified biotin labelled rabbit anti-sheep IgG; Zymed Laboratories, San Francisco, California, USA), for 10 minutes; (4) streptavidin–biotin complex (Dako A/S, Glostrup, Denmark), for 10

minutes; and (5) chromagen (DAB solution; Biogenex, San Ramon, California, USA), for four minutes. Steps 2 to 4 were each followed by washing $\times 2$ for two minutes with Tris buffered saline (pH 7.2). The sections were counterstained with Gill's haematoxylin for 30 seconds, blue differentiated, dehydrated, and mounted. Each section had parallel control staining using the affinity purified NSS immunoglobulin as the primary antibody in step 2.

RESULTS

Affinity purification

The degree of purity was measured by dividing the antibody titre by the OD at 280 m μ . After purification, the purity of the antiserum had increased eightfold (table 1).

Immunohistochemistry of tumours

To confirm the specificity of our purified anti-HbF we checked it in parallel with the control unimmunised antibody (NSS) on normal placenta. In the blood vessels of the trophoblastic villi of the normal placenta, approximately half the non-nucleated RBC and all the nucleated RBC were positively stained for HbF (fig 1). The control NSS, checked in parallel with the anti-HbF antibody, showed no traces of staining after the application of the chromagen.

Germ cells tumours

Tumours from 26 patients were studied (table 2) immunohistochemically for HbF. In some of the tumours the immunohistochemical results of conventional tumour markers hCG (human chorionic gonadotrophin) and AFP (α fetoprotein), were available from the archive reports, and these were compared with our HbF results. In embryonal carcinoma (EC), including sections of testis and retroperitoneal lymph nodes, high numbers of HbF positive RBC (F-RBC) were seen in three of four patients. The F-RBC were congested in "blood lakes" surrounding tumour cells (fig 2A).

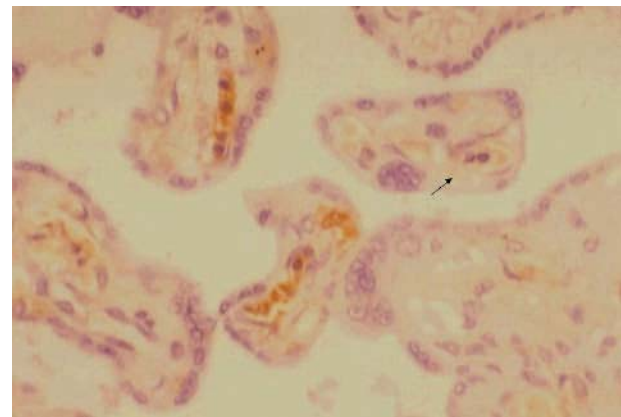


Figure 1 Immunostaining (orange cytoplasmic staining) of nucleated and non-nucleated red blood cells in the microvilli of normal placenta. Arrow, unstained normal RBC.

In two cases of metastatic mature teratoma (MMT), similar high numbers of F-RBC were seen in tumours of the brain. These cells were also congested in "blood lakes" and large haemorrhagic spaces, and comprised 30% of all RBC (fig 2B). In the blood vessels of these sections, 5% of the RBC were F-RBC. In two other cases of MMT, including one lymph node, and one testis, no F-RBC were seen. Five cases of non-metastatic mature teratoma (NMT) of the testis were all negative for F-RBC. Similarly, in nine cases of ovarian teratoma represented by dermoid cysts, no F-RBC were observed. However, in one case of ovarian dermoid cyst, high numbers of congested F-RBC were seen, resembling the other positive cases. A similar positive picture of F-RBC was seen in the germinoma component of an ovarian mixed yolk sac (YS) and germinoma tumour. The YS component was also HbF negative in another case of mixed YS and MMT.

Trophoblastic disease

Table 3 summarises the results for trophoblastic disease. Sections from nine patients with complete molar pregnancy (CM) and five patients with incomplete molar pregnancy (ICM) were analysed. No differences between the two types of TD were noted, except in one case of ICM where normal and nucleated F-RBC were seen in chorionic villi blood

vessels, similar to normal placenta. In all other cases, no F-RBC were seen in the chorion. In most cases, there were young or mature forms of fetal haemoglobin positive myeloid cells (F-MLC) or fetal haemoglobin positive normoblasts (F-NBS), clustered or dispersed in inflammatory parts of the decidua (fig 2C, D) and the F-MLC were usually associated with F-RBC. In five cases, unusually high numbers of F-RBC were also seen in haemorrhagic spaces of the decidua, as was also seen in the GCTs (fig 2A).

Lymphoma

Lymph nodes from six patients were examined and in three of these samples 20% of the RBC in the haemorrhagic regions were F-RBC (fig 2E). Bone marrow samples were examined in four patients and all of these samples had small clusters (up to four cells), mainly of F-MLC, at different stages (fig 2F), occasionally with some F-NBS and F-RBC. The lymphocytes were all negative for HbF. In one spleen sample examined we also saw such clusters of F-cells with no HbF staining of the lymphocytes.

Myelodysplastic syndromes

In five of the seven cases we saw many large clusters of F-RBC, together with F-NBS, in the bone marrow (fig 3A).

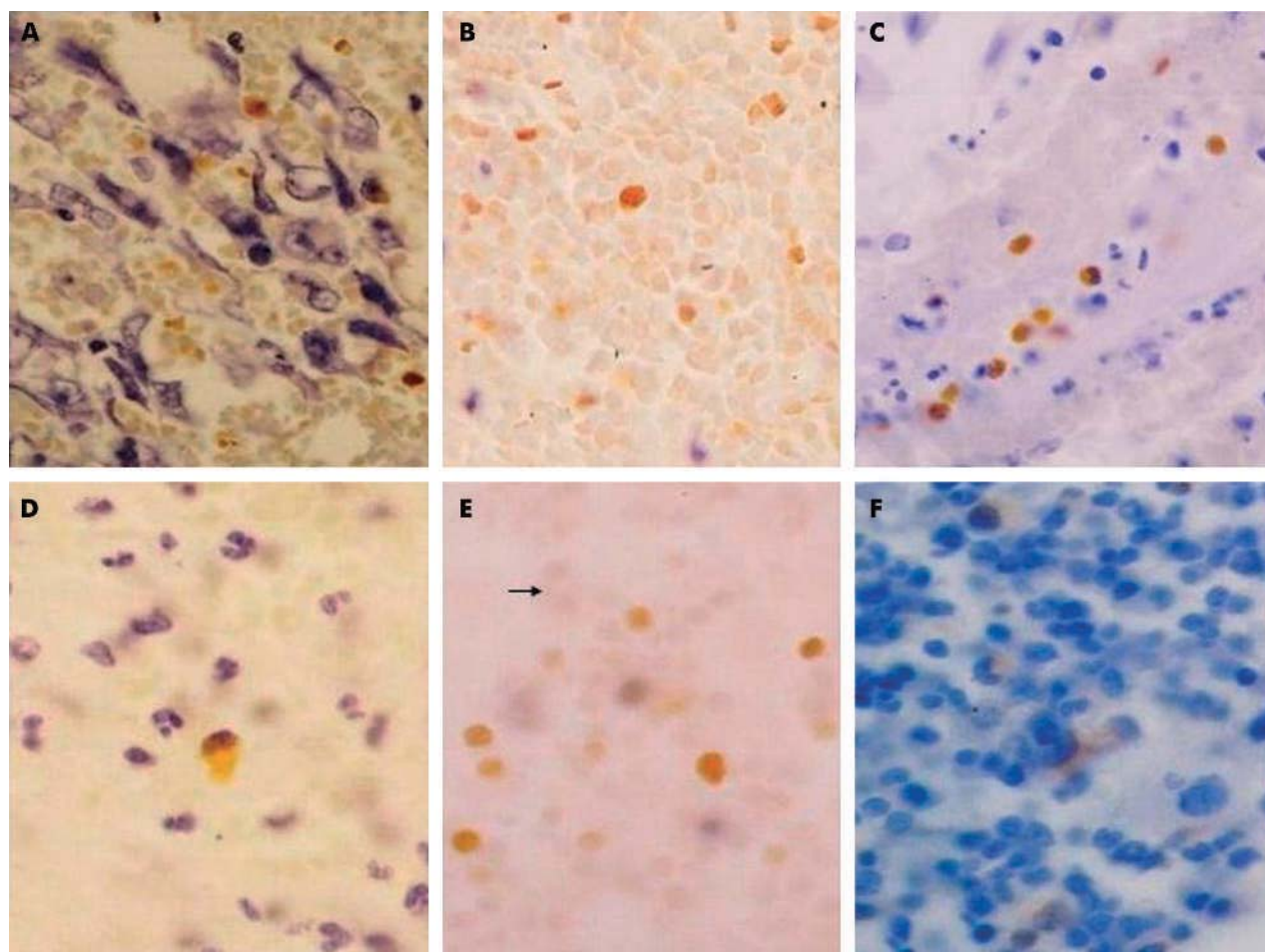


Figure 2 (A) Immunostained (orange) fetal haemoglobin positive red blood cells (F-RBC) in "blood lakes" surrounding tumour cells in testicular embryonal carcinoma tissue. (B) Immunostained (pale to strong orange) F-RBC in large haemorrhagic space of embryonal carcinoma in brain. (C) Immunostaining (orange) of clustered fetal haemoglobin positive myeloid cells (F-MLC) and F-RBC in the decidua of incomplete molar pregnancy (ICM). (D) Immunostaining (orange) in one dispersed F-MLC in the decidua of ICM. (E) Immunostained (pale to strong orange) F-RBC in the haemorrhagic region of a lymph node from a patient with non-Hodgkin lymphoma. Arrow, unstained normal RBC. (F) Immunostained (orange) F-MLC in the bone marrow of a patient with non-Hodgkin lymphoma.

Table 2 Distribution of F-RBC in germ cell tumours

No	Sex	Organ	Tumour	F-RBC distribution	Comment
1	Male	Testis	EC	Congested (30%) F-RBC in blood lakes surrounding the tumour cells (fig 2A)	hCG and AFP negative
2	Male	Testis	EC	Positive as in 1. Also 2–5% F-RBC in blood vessels	No F-RBC in the counterpart normal testis
3	Male	Retroperitoneal lymph node	EC	Positive as in 2	
4	Male	Testis	EC	None	hCG and AFP positive
5	Male	Brain	MMT	Congested (30%) F-RBC in blood lakes and haemorrhagic zones (fig 2B) in blood vessels 5%	hCG positive AFP negative
6	Female	Brain	MMT	Positive as in 5	
7	Male	Retroperitoneal lymph node	MMT	None	
8	Male	Testis	MMT	none	
9	Female	Ovary	Mixed germinoma and YS	In germinoma positive as in 1. In YS none	AFP positive in YS. hCG negative
10–14	Male	Testis	NMT	None	
15	Male	Brain	Mixed seminoma and mature teratoma	None	
16	Female	Lymph node	Mixed YS and mature teratoma	None	AFP positive in YS
17	Female	Ovary	Dermoid cyst	Positive as in 2	
18–26	Female	Ovary	Dermoid cyst	None	

AFT, α fetoprotein; EC, embryonal carcinoma; F-RBC, fetal haemoglobin positive red blood cells; hCG, human chorionic gonadotrophin; MMT, metastatic mature teratoma; NMT, non-metastatic mature teratoma; TD, trophoblastic disease; YS, yolk sac.

Multiple myeloma

In the bone marrow of four of seven patients we saw many large clusters containing a mixed population of F-MLC, F-NBS, and F-RBC. At the periphery of the bone marrow, there were only F-RBC (fig 3B). In all the samples examined the plasma cells were negative for HbF.

Hairy cell leukaemia

Bone marrow samples from 10 patients were examined and F-RBC were seen in only one. In one spleen sample examined, 15% of the cells in the blood vessels were F-RBC and in its pulp there were clusters of F-RBC together with F-NBS (fig 3C).

Ovary adenocarcinoma

Ovarian tumours from six patients were tested. When the usual dilution of the 1/20 anti-HbF antibody was used no positive results were seen, but when the antibody was less dilute (1/5 dilution), F-RBC were detected in two of the six patients. These F-RBC were seen in the blood vessels (1–2%) and dispersed in regions of loose connective or fat tissue.

Controls

In parallel with each pathological organ (testis, ovary, brain, lymph nodes, spleen, and bone marrow), at least three samples of normal organs were examined by the same immunochemical techniques for F-cells. All these controls were HbF negative.

DISCUSSION

Our affinity purified anti-HbF antibody did not detect HbF in the tumour cells of GCTs. This is in contradiction to previous work^{6,7,15} demonstrating immunostaining of HbF in tumour cells. With regard to our previous preliminary work,⁷ we were able to prove that the positive staining of tumour cells was non-specific, because affinity purification of the same anti-HbF antibody obliterated that staining. We found that the HbF in our GCT samples was present in RBC (F-RBC) rather than tumour cells. Although the number of specimens was small, it seems that in GCT F-RBC are indicators of aggressive rather than less aggressive tumours (table 2). Most cases of EC and two MMT cases (table 2) contained F-RBC. Like MMT, EC was previously reported as a metastatic disease in approximately 66% of patients.¹⁹ Similarly, in dysgerminoma (table 2), previously reported as a metastatic disease,²⁰ there were many F-RBC. In contrast,

Table 3 The distribution of fetal haemoglobin positive cells in trophoblastic diseases

No	Disease	In inflammatory decidua	In haemorrhagic decidua	In chorion
1	CM	Randomly dispersed single F-RBC and F-MLC	(–)	None
2	CM	Clustered and dispersed F-MLC and F-RBC (fig 2C)	2–3% F-RBC	None
3	CM	Randomly dispersed single F-RBC and F-MLC	(–)	None
4	CM	Large clusters of up to 20 F-MLC and F-RBC	8% F-RBC	None
5	CM	None	0.3% F-RBC	None
6	CM	None	1% F-RBC	None
7	CM	Randomly dispersed single F-RBC and F-MLC	(–)	None
8	CM	None	(–)	None
9	CM	None	(–)	None
10	ICM	Clusters of F-MLC mainly with only few F-RBC	(–)	None
11	ICM	Clusters of F-MLC mainly with only few F-RBC	(–)	None
12	ICM	Clusters of F-MLC mainly with only few F-RBC	2% F-RBC	None
13	ICM	Randomly dispersed single F-MLC (fig 2D)	2% F-RBC	None
14	ICM	Decidua not present		F-RBC mature and nucleated

(–), No haemorrhagic decidua present; CM, complete molar pregnancy; F-MLC, fetal haemoglobin positive myeloid cells; F-RBC, fetal haemoglobin positive red blood cells; ICM, incomplete molar pregnancy.

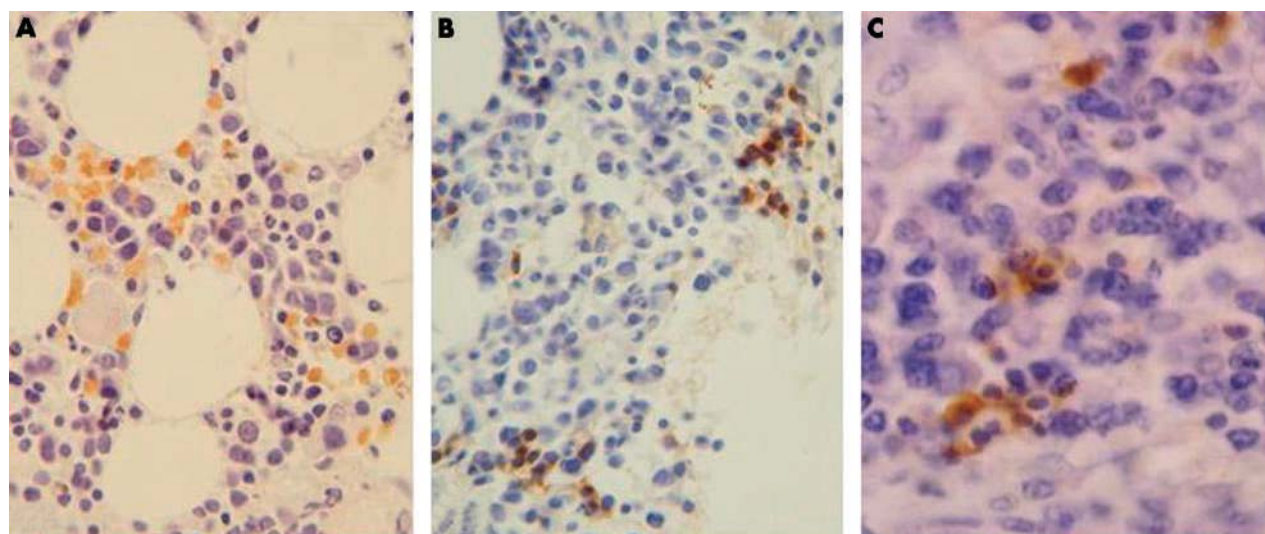


Figure 3 (A) Immunostained (orange) clustered fetal haemoglobin positive red blood cells (F-RBC) and fetal haemoglobin positive normoblasts (F-NBC) in the bone marrow of a patient with myelodysplastic syndrome. (B) Immunostained (orange) clustered fetal haemoglobin positive myeloid cells, F-NBC, and F-RBC in the bone marrow of a patient with multiple myeloma. (C) Immunostained (orange) F-RBC and F-NBC in the spleen of a patient with hairy cell leukaemia.

in the less aggressive disease NMT (table 2), and in dermoid cyst (table 2), known to be a benign tumour,²¹ there were essentially no F-RBC. The extravascular congestion of F-RBC is probably the source of plasma HbF in patients with GCT.⁷ The large blood vessels seen in all of these tumours do not contain F-RBC or have fewer numbers of F-RBC than is seen in the extravascular regions of HbF positive GCTs. HbF, although not produced by the tumour cells, might be a potential tumour indicator, complementary to the conventional markers of GCT, such as hCG and AFP. For instance, in table 2 we see that in one case (number 1) with a high number of F-RBC neither hCG nor AFP was detected, whereas in another case (number 9), F-RBC were congested in the germinoma compartment but AFP was detected only in the YS compartment.

“The extravascular congestion of fetal haemoglobin positive red blood cells is probably the source of plasma fetal haemoglobin in patients with germ cell tumours”

In TD (table 3) the extravascular F-cells are also probably the source of the plasma HbF found in this disease.⁷ In TD, we found in addition to F-RBC, young and mature F-MLC in clusters or single cells in the inflammatory decidua (fig 2C, D), which were only a minority of the total MLC population of that zone. The presence of F-MLC in TD is interesting, and should be investigated further to reveal the relevance and specificity of these cells in these tumours. However, we were unable to evaluate the difference between CM and ICM. The only case with F-RBC in the chorionic villi may be related to an advanced stage of ICM, although more cases would need to be studied to prove this assumption.

Very high numbers of extravascular F-RBC were also found in the lymph nodes of lymphoma, in accord with the high concentration of plasma HbF in patients with lymphoma.^{7 12} The presence of such extravascular F-RBC in lymphoma and in GCT has not been reported previously. No evidence was found for their regeneration within the tumour because there were no precursors of F-cells. Further studies should be conducted to evaluate the role of these F-RBC in tumour development. However, they may have been attracted from

the circulation by the tumour cells, which would have decreased the concentration of HbF in the whole blood, eventually leading to increased plasma HbF. This assumption is supported by the results of the concomitant measurement of whole blood and plasma HbF concentrations—in general, they were inversely related.^{7 12} The detection of such changes between the concentrations of whole blood and plasma HbF might be exploited for diagnostic purposes. However, to verify such a proposed dynamic, a follow up study for HbF in whole blood versus plasma must be performed. Our immunohistochemical findings of F-cells in two spleen samples, one from lymphoma, the other from hairy cell leukaemia, are very similar to previous findings.⁹ In both of these studies there was evidence for fetal haemopoiesis in the spleen of lymphoproliferative and myeloproliferative disease, in addition to the fetal spleen.⁹ Fetal haemopoiesis does not occur in the spleen of healthy adults, so that the spleen is probably the source of the raised whole blood HbF concentrations previously found in patients with hairy cell leukaemia.¹² In MDS, the dense clusters of F-cells may also be the source of extremely high plasma HbF found in such patients.¹² The large clusters of F-cells containing haemopoietic cells (NSB or young MLC) found in the spleen and bone marrow of the patients with haematological malignancies are apparently germinal centres. However, they are not the source of malignancy because the malignancy arose from lymphoid or plasma cells. Thus, in those cases (as in germ cell tumours), HbF is not a direct oncofetal antigen produced by the tumour cells, but an inducible antigen. The mechanism of this induction in adults is not yet clear, and is the subject of a current investigation.²² For instance, it was shown in vitro that some tumour cell lines, including GCT (teratocarcinoma), induced HbF production in precursor erythroid cells.²³ Later on it was found that particular growth factors, including c-kit and interleukin 3, stimulated HbF production in such erythroid cells.²² One source (among others) of c-kit is lymphoma cells.²²

The presence of F-MLC in the bone marrow of haematological malignancies and in the decidua of TD was surprising because myelocytes do not normally produce haemoglobin. However, this is not the first finding of HbF synthesis in transformed myelocytic cells, because the

Take home messages

- Fetal haemoglobin (HbF) was evaluated as an inducible marker within different tumour tissue blood cells
- The dual distribution of these cells—circulating in the blood or concentrated in areas of the neoplastic tissues—might reflect the two independent serological indicators of HbF: one in whole blood and the other in plasma of patients with cancer

expression of HbF (γ and α globins) genes has been reported in myelocytic leukaemic cells.^{24–25} In other studies, myelocytic leukaemic cells grown in culture differentiate spontaneously,^{26–27} or by chemical induction,²⁸ to erythroid cells producing HbF.

The concentration of HbF in F-RBC from OA was lower than that found in the other tumours. It is probable that such poorly detectable F-cells also exist in other tumours. To investigate this question we need to increase the specific activity of our affinity purified anti-HbF antibody.

“The presence of fetal haemoglobin positive myelocytes in the bone marrow of haematological malignancies and in the decidua of trophoblastic disease was surprising because myelocytes do not normally produce haemoglobin”

In conclusion, we have provided immunohistochemical evidence of the source of HbF in both plasma and whole blood, which can be used as an indicator of cancer, and have proved the independence of these two sources.

This adds to our understanding of the importance of serologically high concentrations of HbF in plasma or in whole blood and raises intriguing possibilities of this mechanism of tumour inducible gene expression as a model of other paraneoplastic phenomena.

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Authors' affiliations

M Wolk, Department of Morbid Anatomy and Histopathology, The Royal London Hospital, Central Laboratory, Israel Ministry of Health, Jerusalem 91342, Israel

J E Martin, Pathology Group, Institute of Cell and Molecular Sciences, The Royal London Hospital, London E1 1BB, UK

R Constantin, Department of Pathology, Shaare-Zedek Medical Centre, Jerusalem 91301, Israel

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