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Anaemia in juvenile chronic arthritis: serum inhibition of normal erythropoiesis in vitro

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SUMMARY Serum from patients with juvenile chronic arthritis (JCA) was shown to inhibit colony formation by normal erythropoietic progenitor cells cultured in vitro. The inhibition was proportional to the degree of anaemia and to certain indices of activity of the arthritis and systemic disease. It occurred in a dose dependent manner with increasing serum concentration and was independent of previous blood transfusion or administered drugs. Erythropoietic progenitor cells from the bone marrows of anaemic patients with JCA showed normal requirements for accessory cells (T lymphocytes and macrophages) in culture, and autologous accessory cells were not deficient in providing normal growth requirements.

Juvenile chronic arthritis (JCA) is the term used to describe a heterogeneous group of arthritic disorders of childhood[1] in which anaemia is a common manifestation. The degree of the anaemia is related to the severity and type of the disease.[2] Previous reports have shown iron deficiency,[3] haemolysis,[2] and erythroid aplasia[4] to be causative factors in some patients, but in the majority no specific aetiology is demonstrated, and it is ascribed to the 'anaemia of chronic inflammatory disease'.[5] There has been no investigation of this type of anaemia in children, but in adults with rheumatoid arthritis we have recently described a serum inhibitor of in vitro erythropoiesis.[6]

Techniques now available for in vitro culture of haemopoietic progenitor cells permit detailed investigation of the physiology of normal erythropoiesis and also of the mechanisms of such putative inhibition. The colony forming assay in semisolid medium allows differentiation and proliferation of primitive and more mature erythroid progenitor cells from the bone marrow. Small colonies of the more mature cell type develop after seven days in culture (colony forming unit erythroid, CFU-e), while large multicentric colonies of the more primitive cell (burst forming unit erythroid, BFU-e) develop after 14 days.[7] BFU-e, but not CFU-e, can also be cultured from peripheral blood mononuclear cells.[8] Optimal colony formation requires the presence of accessory cells, monocytes/macrophages, and T lymphocytes or their secreted products during the very early stages of differentiation and proliferation,[9 10] whereas erythropoietin is active primarily on more mature erythroid precursors.

In a number of bone marrow failure states (pure red cell aplasia, aplastic anaemia) clonal assays have implicated inhibition of haemopoietic progenitors in the pathogenesis.[11] Humoral inhibitors and suppressor lymphocytes have been demonstrated, and macrophages have been claimed to inhibit erythropoiesis in the anaemia of chronic infection.[12] Recently, inhibition of BFU-e growth in vitro has been demonstrated in anaemic adult patients with rheumatoid arthritis (RA),[6] and other evidence for such a mechanism has been put forward.[13 14] The object of this study was to examine the effect of serum from patients with JCA on normal erythroid progenitors and to seek to correlate this with the severity of the anaemia and disease activity. An additional study of JCA bone marrow cells in culture was performed to investigate whether progenitor cell-accessory cell interactions were normal and to examine the effect of autologous serum on marrow BFU-e development.

Patients and methods

PATIENTS

Serum was obtained from 31 patients with JCA (Eular-WHO criteria)[1] who were selected by one of

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Table 1 *Clinical data and routine investigations of patients*

Patient No	*Disease type*	*Joint count**	*Global disease activity†*	*Haemoglobin‡ (g/dl)*	*Erythrocyte sedimentation rate (mm/1st h)*	*C reactive protein‡ (mg%)*	*IgG (g/l)*	*Percentage BFU-e growth§*	*Percentage CFU-GM growth§*	*Medications‖*	*Previous transfusion*
1	Systemic	5	3	6·9	30	8·4	13·3	30	64	1, 2	
2	Systemic	4	2	8·4	58	14·3	11·2	61	105	1, 4, 5	
3	Systemic	3	4	6·9	88	20	19·5	50	64	1	+
4	Systemic	5	4	9·1	80	3·5	12·8	51	79	1	
5	Systemic	15	4	9·2	65	14	18·1	27	64	1	
6	Systemic	0	4	8·1	74	23	35·1	36	85	1	+
7	Systemic	6	2	6·9	70	12	9·4	59	100	1	+
8	Systemic	9	4	10·4	70	17	11·1	53	79	1, 4	
9	Systemic	8	3	11·5	31	3·2	15·7	56	64	1	
10	Systemic	12	4	10·7	34	16·9	10·7	39	60	1, 3	+
11	Systemic	16	3	11·4	33	NA	13·8	54	75	1	+
12	Systemic	2	2	12·2	115	2·2	1·4	108	88	1, 5	
13	Systemic	8	2	12·1	40	4·5	7·6	98	86	1, 4	
14	Poly RF+	13	3	11·5	47	3·6	20·0	63	69	1, 3	+
15	Poly RF+	10	3	10·8	45	17	13·8	6	81	1, 2, 4	+
16	Poly RF−	3	2	11·4	74	10	18·3	66	64	1, 3	+
17	Poly RF−	2	1	13·6	7	0·5	NA	91	64		+
18	Poly RF−	9	3	10·1	17	0·5	11·6	59	68		
19	Poly RF−	2	1	10·6	30	<0·5	18·8	23	88		
20	Pauci	3	2	11·8	15	<0·5	10·4	101	68		
21	Pauci	1	1	10·6	30	1·3	8·6	54	79		
22	Pauci	1	1	11·5	10	0·5	9·2	79	100		
23	Pauci	2	1	12·3	3	0·4	12·2	96	105		
24	Pauci	2	1	12·2	NA	<0·1	9·8	93	100		
25	Pauci ext	9	3	11·2	19	0·9	15·8	93	100	6	
26	Pauci ext	8	2	9·3	53	5	13·0	61	110	1	
27	Pauci ext	5	2	9·7	48	1·1	19·1	40	75		
28	Pauci ext	5	3	9·4	30	1·1	12·5	82	71	6	
29	Pauci ext	3	1	10·8	45	3·9	24·3	46	100		
30	Pauci ext	5	2	9·6	48	3·1	18·6	66	100		
31	Pauci ext	9	4	9·8	48	4	24·9	38	65		

*Joint count according to the method of the co-operating clinics committee of the American Rheumatism Association.[14]
†Global disease activity=measured summation incorporating joint count, duration of early morning stiffness, and presence of systemic features.
‡Haemoglobin g/dl×10=g/l; C reactive protein mg%×10=mg/l.
§Expressed as a proportion of growth in the presence of control serum.
‖Medications: 1=oral prednisolone, 2=sodium aurothiomalate, 3=D-penicillamine, 4=chloroquine, 5=chlorambucil, 6=auranofin.
NA=not available.

us (ARH) to give a range of disease type and activity and severity of anaemia. The disease type was subgrouped by the form of onset and disease progress into: systemic; polyarticular positive for IgM rheumatoid factor (poly RF+); polyarticular negative for IgM rheumatoid factor (poly RF−); pauciarticular (pauci); and pauciarticular onset with extending joint involvement (pauci ext). Three of these patients with severe anaemia had bone marrow aspirations performed as part of their clinical investigation. In all patients faecal occult blood tests were negative. In three patients with a haemoglobin level below 11·5 g/dl (115 g/l) serum ferritin levels were below 25 ng/ml (μg/l) and, unlike the patients of Koerper *et al*,[3] they did not respond to oral iron therapy. In the three severely anaemic patients on whom bone marrow aspirates were taken there were normal marrow iron stores. No patient had evidence of renal impairment, and other causes for anaemia were excluded. Clinical assessment of disease activity by one of us (ARH) was made with a count of active joints[15] and a global assessment of disease activity in four grades, based on the severity of joint inflammation, duration of morning stiffness, and the presence and severity of systemic symptoms. Measurement of haemoglobin (Hb), erythrocyte sedimentation rate (ESR, Westergren), serum C reactive protein (CRP, radial immune diffusion), immunoglobulin (laser nephelometry), and rheumatoid factor (latex, RAHA) were obtained. Two of the 31 patients were positive for rheumatoid factor. All patients were receiving non-steroidal anti-inflammatory drugs and 19 were also receiving disease modifying drugs: prednisolone (17), sodium aurothiomalate (two), auranofin (two), chloroquine (four), D-penicillamine (three), and chlorambucil (two) (Table 1).

PERIPHERAL BLOOD STUDIES

Sera from the 31 patients with JCA were screened for inhibitory activity in the BFU-e colony forming assay by one of us (PJP) without knowledge of the clinical data. Group O peripheral blood mononuclear cells from five normal volunteers were prepared by Ficoll-Paque centrifugation, and after incubation in plastic tissue culture plates the non-adherent cell fraction (PB-NAC; <5% macrophages present) was used as the source of erythroid progenitors (BFU-e) in methylcellulose culture at 5×10^5 cells/ml. Serum autologous with the PB-NAC or sera from patients with JCA were added at 10% final volume. Sera from five selected patients were titrated at varying saline dilutions to define the dose-response relationship of the serum inhibitor. BFU-e colonies were counted at 14 days by direct vision using an inverted microscope. Myeloid colonies (CFU-GM), identified by distinct morphology and absence of haemoglobin, were also counted at 14 days. BFU-e and CFU-GM counts were obtained from simultaneous cultures of PB-NAC with autologous or JCA sera; the counts obtained with autologous serum were designated as a standard 100%, and the counts obtained with JCA sera as a proportion of that (percentage growth, Table 1). The mean colony count in control cultures for BFU-e was 50 (range 28–69) and for CFU-GM was 6 (range 3–8) per 5×10^5 PB-NAC.

BONE MARROW STUDIES

Bone marrow (BM) cells obtained from posterior iliac crest aspirates on three anaemic JCA patients were fractionated to provide two progenitor cell populations. After Ficoll-Paque centrifugation a mononuclear cell fraction non-adherent to plastic was prepared (BM-NAC; <5% macrophages present). A proportion of these cells was subjected to T lymphocyte depletion by a single neuraminidase treated sheep erythrocyte rosette technique,[15] and virtually complete macrophage removal was achieved by centrifugation over a sucrose gradient (5–15%, 100 *g* for seven minutes).[17] This second progenitor population was designated as the low density fraction minus T lymphocytes (LDF, T°; <1% macrophages). Coculture experiments to investigate the interaction of progenitor cells with accessory cells (adherent cell-macrophages and T lymphocytes) were performed using autologous bone marrow adherent cells (80–90% peroxidase positive) and autologous peripheral blood T lymphocytes (95% sheep erythrocyte rosette forming cells) as previously described.[6] LDF, T° were cultured at $0\cdot5\times10^5$ cells/ml, and adherent cells added at $0\cdot2-0\cdot8\times10^5$ cells/ml or T lymphocytes added at 10×10^5/ml in 1% methylcellulose cultures. BFU-e growth from both BM-NAC and low density fractions in the presence of autologous or heterologous ABO compatible serum was examined for evidence of inhibition of growth by the autologous serum.

CULTURE DETAILS

All cultures were performed in 1% methylcellulose, Iscove's complete medium, 1% deionised bovine serum albumin (Sigma), 30% fetal calf serum (Sera Lab), 10^{-4} M 2-β-mercaptoethanol, and sheep step III erythropoietin (Connaught) at 2 U/ml. Duplicate samples of all cultures were incubated in 5% CO_2/95% air at 37°C in a fully humidified incubator until 14 days when erythroid bursts were counted.

Test human sera added to culture was not decomplemented before use.

STATISTICAL ANALYSIS

The relation between percentage BFU-e growth and

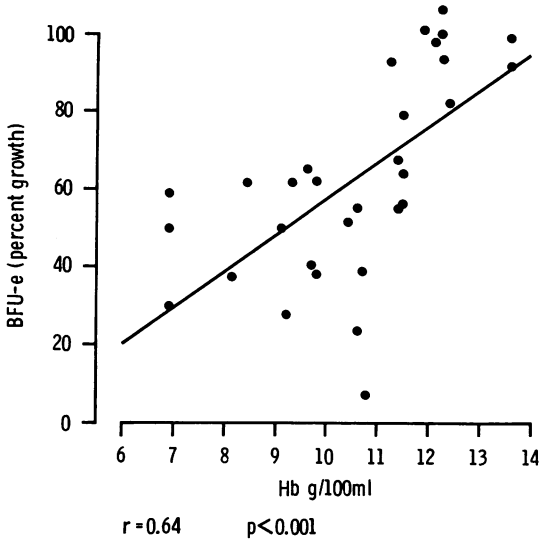

Fig. 1a

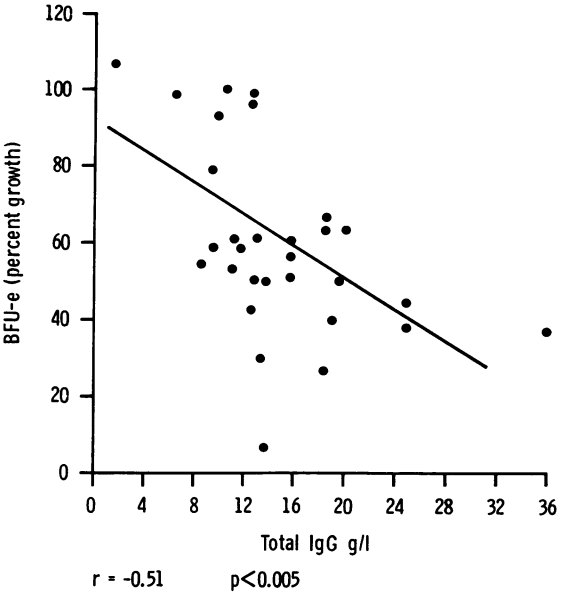

Fig. 1c

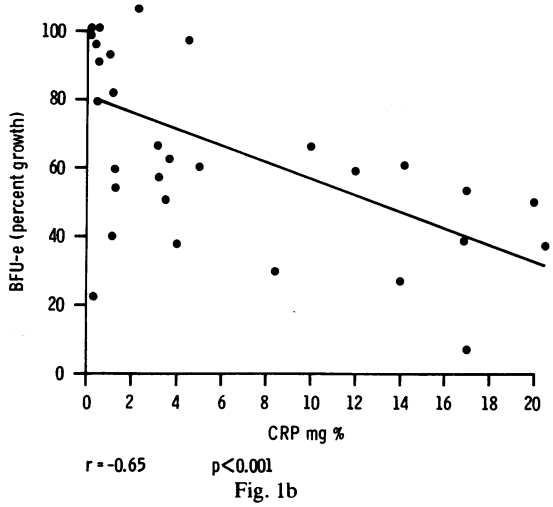

Fig. 1b

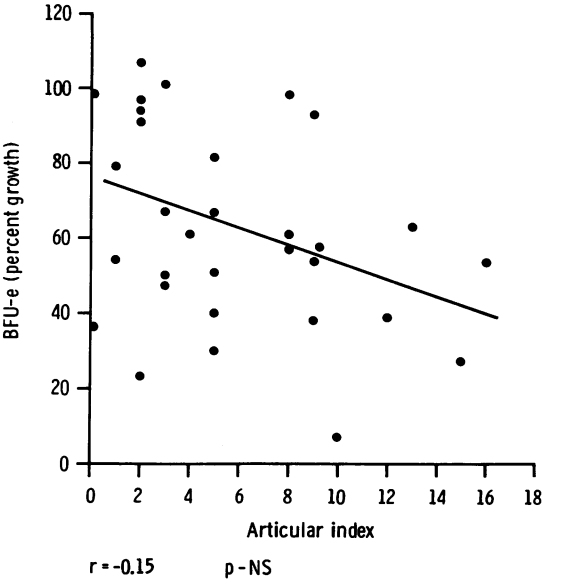

Fig. 1d

FIG. 1 *Percentage erythroid bursts v (a) haemoglobin (n=31), (b) CRP (n=30), (c) IgG (n=30), (d) articular index (n=31). (Hb g/100 ml×10=g/l; CRP mg%×10=mg/l.)*

the clinical and laboratory variable indices of disease activity was assessed for each patient by the linear regression method. In addition, using the GLIM computer program[18] 14 explanatory variables were investigated in various combinations to try to find a function that best fitted the observed percentage growth. The GLIM computer program is a generalised multiple regression method which was chosen to allow the various possible combinations of these variables to be considered in a multivariate way; to examine each one separately when the others were also involved could be seriously misleading. Vari-

Table 2 *Relation between disease severity, BFU-e, and CFU-GM growth in the presence of serum from 31 patients with JCA*

Disease activity	*Mean percentage growth of BFU-e**	*Mean percentage growth of CFU-GM*
Mild (grade 1, n=7)	73 (29)†	77 (19)
Moderate (grade 2, n=8)	73 (23)	90 (17)
Severe (grade 3, n=8)	55 (30)	74 (12)
Very severe (grade 4, n=8)	42 (10)	72 (11)

*The reduction in BFU-e growth is significant to the level p=0·009 (GLIM).
†Values are given as mean (SD).

ables of a yes/no type were coded as 0 or 1, and the three missing values (Table 1) were replaced by averages of the appropriate variables to get a complete data set.

Results

The clinical data and routine investigations are summarised in Table 1. Nineteen of the 31 patients were female. The mean age was 10 years (SD 6, range 2–31), and only two patients were aged over 16 years at the time of study.[6 17] The mean disease duration was 5·3 years (SD 4·8, range 0·4–22).

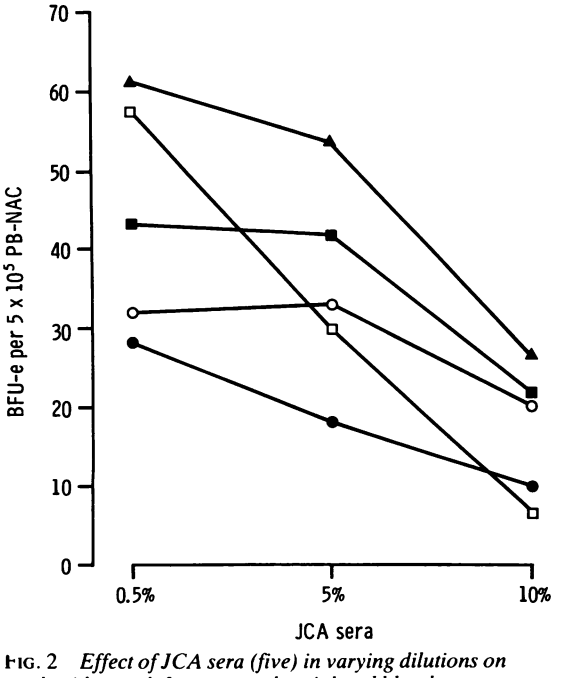

FIG. 2 *Effect of JCA sera (five) in varying dilutions on erythroid growth from normal peripheral blood precursors.*

EFFECT ON NORMAL PERIPHERAL BLOOD BFU-e GROWTH OF SERUM FROM PATIENTS WITH JCA

The addition of JCA serum to normal peripheral blood cultures reduced BFU-e growth, and the reduction occurred in cultures of mononuclear cells from each of the five donors. Plotting the results from all the experiments showed that the degree of inhibition corresponded significantly with the patients' haemoglobin level and with the CRP and total IgG levels (Figs 1a, b, and c). No correlation was found with the joint count alone (Fig. 1d), but the reduction in growth did significantly correlate with the overall assessment of disease activity (Table 2). The percentage growth for each serum sample is recorded in Table 1, and the individual relationships between percentage growth and Hb, CRP, IgG, and joint count are illustrated in Fig. 1.

Further analysis of the variables listed in Table 1 by the GLIM program showed that the observed percentage BFU-e growth was best predicted by five factors: joint count (p=0·020), haemoglobin (p=0·029), IgG (p=0·020), sodium aurothiomalate

Table 3 *Reproducibility of inhibition of BFU-e growth* in the presence of patient serum (10%) using mononuclear cells from separate donors*

Patient sera	*Percentage BFU-e growth*	
	Exp. 1	*Exp. 2*
1	30	25
2	61	50
3	50	62
7	59	62
11	54	38
14	60	50
18	23	35
24	93	105
27	40	50
31	38	36

*Expressed as a proportion of growth in the presence of control serum.

(p=0·008), and auranofin (p=0·033). If haemoglobin was excluded then C reactive protein replaced it for the best fit. The p values represent the probability associated with the introduction of the given variable where the others are already included. The best linear function of the five variables gave an estimate of percentage growth that had a correlation of 0·84 with the observed value. The predictive value of sodium aurothiomalate and auranofin was found from only four patients, but the GLIM program took into account the low number of observations, and we believe this observation reflected the more severe disease activity of these patients.

The relation between percentage BFU-e growth and treatment with 'disease modifying drugs', prednisolone, and blood transfusion was analysed for each patient by the GLIM program and no significant correlation found.

Addition of increasing concentrations of known inhibitory serum to normal peripheral blood cultures produced increasing inhibition of BFU-e development (Fig. 2). This dose-response relationship is that of an inhibitor and would not be the result of the failure of normal serum stimulators of BFU-e.

The reproducibility of inhibition was tested by comparing the effect of sera from 10 of the 31 patients on mononuclear cells from separate donors, and a comparable degree of inhibition was found (Table 3).

Enumeration of myeloid colonies (CFU-GM) failed to show a reduction in numbers by patient serum that correlated with either the haemoglobin level (Table 1) or with disease activity (Table 2).

Table 4 *Effect of autologous and control serum (10%) on BFU-e growth from patient bone marrow cells ($0·5\times10^5$ cells/ml)*

Patient	*Human serum:nil*	*Control*	*Autologous†*
*BM-NAC**			
1	54	57	44
2	35	41	27
3	28	34	19

*LDF, T°+0·4×10⁵ Mø,+10×10⁵ T cells**

Patient	*Human serum:control*	*Autologous†*
1	30	5
2	36	14
3	32	15

*BM-NAC=non-adherent bone marrow cells; LDF, T°=non-adherent, low density fraction bone marrow cells depleted of T lymphocytes and macrophages (Mø).
†Student's *t* paired analysis for BFU-e growth (n=6) in control *v* autologous serum showed significantly better growth in control serum (p<0·001).

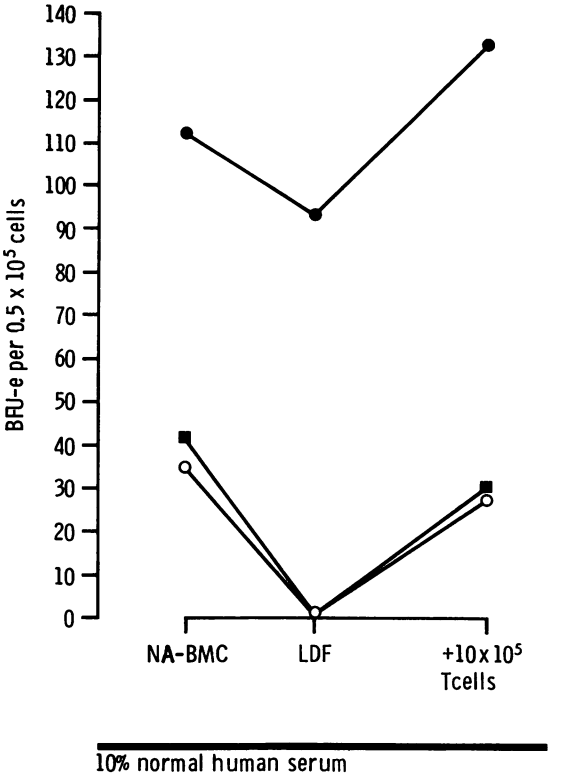

Fig. 3a

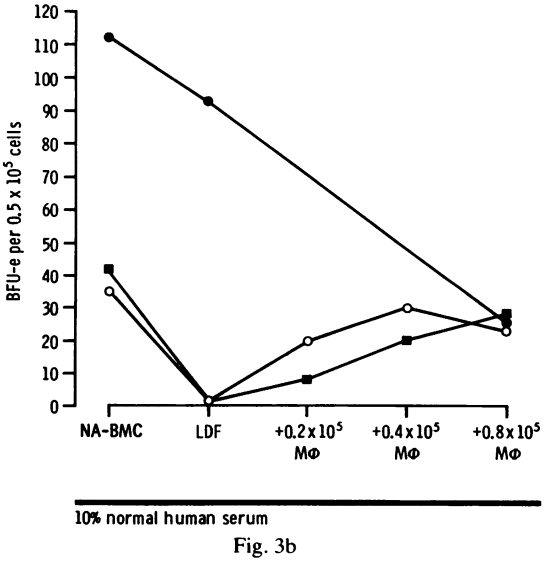

Fig. 3b

FIG. 3 *Effect of (a) T cell readdition and (b) macrophage (Mø) readdition on marrow erythroid burst formation. LDF marrow T cell and macrophage depleted.*

EFFECT ON AUTOLOGOUS MARROW BFU-c GROWTH OF SERUM FROM PATIENTS WITH JCA

In the three anaemic JCA patients from whom bone marrow aspirates were taken the influence of autologous serum on BFU-e growth was compared with that of heterologous ABO matched normal serum. BFU-e numbers both from the BM-NAC and LDF cell pools were reduced by autologous serum. Pooling the results from all six cultures showed the reduction in growth to be significant (Table 4).

EFFECT OF AUTOLOGOUS ACCESSORY CELLS ON BFU-e GROWTH FROM PATIENT BONE MARROW

Cultures of bone marrow cells from three anaemic JCA patients gave optimum BFU-e derived colonies in the partially fractionated samples (BM-NAC), with marked reduction in colony counts in two of three samples after accessory cell depletion (LDF, T°). Reconstitution with autologous adherent cells or T lymphocytes restored colony forming capacity (Figs 3a and b) apart from in one patient where adherent cell readdition resulted in macrophage colony overgrowth. Excessive numbers of macrophages have been described as inhibiting BFU-e growth.[19 20]

Discussion

Hypoplasia of the erythroid marrow is not a feature of the anaemia of chronic disorders.[21] Unlike pure red cell aplasia the haemoglobin is maintained, albeit at a low level, and generally there are no transfusion requirements. In JCA, however, as in RA, the erythroid marrow does fail to expand in response to the anaemia with no appropriate increase in erythroblast numbers. In RA recent in vivo ferrokinetic data support the concept of an inadequate erythropoietic response to anaemia,[22] and in addition, increased ineffective erythropoiesis due to death of haem containing progenitors within the marrow has been described both in vivo[23] and in vitro.[24] Inhibition of erythroid maturation could be responsible and occur at any point from the level of the primitive progenitor cell to the fully differentiated erythrocyte.

The in vitro formation of erythroid bursts from primitive BFU-e represents considerable maturation and proliferation which may be blocked at any stage, resulting in a diminution of colony numbers. Our results demonstrate serum inhibition of in vitro erythropoiesis and would be in keeping with failure of the erythroid marrow to expand and the development of the anaemia seen clincally.

Bone marrow cultures from our patients confirm a greater inhibition of BFU-e growth by autologous serum than with normal heterologous serum. The bone marrow cultures also show that colony forming capacity of the BM-NAC is retained, that it is reduced by the depletion of accessory cells, and stimulated by their readdition (Figs 3a and b). This is in agreement with our findings in RA that autologous accessory cells were not deficient in providing growth factors.[6]

Inhibition of erythropoiesis corresponded with the severity of the anaemia and also with the disease activity, implying that it is produced as an integral part of the inflammatory process. Several mediators of the inflammatory responses have the capacity to inhibit cellular proliferation, and of these prostaglandins[25] and interferons[26] may directly affect in vitro haemopoiesis. Although there is no evidence for inhibition of erythropoiesis by prostaglandins of the E series,[25] a prostaglandin F2/α-glycoprotein complex has been described as inhibitory,[27] and further information relating this finding to human inflammatory conditions is awaited.

Interferons (IFN) α, β, and γ have been shown to inhibit colony formation by human erythroid and myeloid progenitors,[26] the greatest effect being on primitive erythroid progenitors.[28] IFNγ, an in vivo product of activated T lymphocytes, has been suggested as the mediator responsible for T cell mediated inhibition of haemopoiesis in some cases of aplastic anaemia.[29] In JCA, IFNγ could not be detected in serum even during acute exacerbation.[30] This evidence, together with the lack of a T lymphocyte suppressive effect in our marrow coculture studies, suggests that an inhibitory role for INFγ is unlikely.

An additional group of non-interferon inhibitors of cell proliferation released by antigen and mitogen activated lymphocytes has been described.[31] Recent studies have shown a relation between these factors and natural killer (NK) cell activity;[32 33] NK cells have been reported to inhibit in vitro erythroid colony formation.[34] These lymphotoxins show chemical and immunological similarities to immunoglobulin,[35] and it is of interest that the serum inhibitor of in vitro erythropoiesis reported by Dainiak *et al* separated with the immunoglobulin fraction.[13]

Previous investigation of the anaemia of chronic disorders has suggested that labile iron is unavailable for erythropoiesis.[36] This process is related to inflammation[37] and interleukin 1 may be the mediator stimulating macrophages to trap iron.[21] The patients with JCA and with the greatest degree of serum inhibition were those with active systemic disease, many of whose features are also thought to be mediated by interleukin 1.[38] Interleukin 1,

however, is highly unlikely to inhibit in vitro erythropoiesis by making iron unavailable, as excess iron is present in the culture medium and inhibition occurs when there are only low numbers of macrophages present in the cultures.

Clarification of the precise mechanisms of serum inhibition of erythropoiesis in JCA will require detailed studies using highly purified progenitor cell and accessory cell populations to elucidate the target cell and fractionation of the serum to permit physicochemical identification of the factor(s) responsible.

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