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A lethal myeloproliferative syndrome in mice transplanted with bone marrow cells infected with a retrovirus expressing granulocyte-macrophage colony stimulating factor

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Murine bone marrow cells infected with a novel recombinant retrovirus, MPZen(GM-CSF), were engrafted into lethally irradiated recipients. The transplanted animals developed extremely high circulating levels of GM-CSF (up to 3 × 10^5 units/ml), and greatly elevated peripheral nucleated cell counts (up to 110 × 10^6 per ml). Their haemopoietic tissues contained GM-CSF proviral DNA and produced substantial levels of GM-CSF. The mice died within 4 weeks of transplantation with extensive neutrophil and macrophage infiltration of the spleen, lung, liver and peritoneal cavity and significant infiltration of both heart and skeletal muscle by neutrophils, macrophages and eosinophils. The thymus and lymph nodes were deficient in lymphoid cells. No disease occurred when infected cells from haemopoietic tissues of the primary transplanted animals were injected into normal or sub-lethally irradiated mice. Dysregulated GM-CSF expression by haemopoietic cells thus produces a fatal albeit non-neoplastic myeloproliferative syndrome.

Key words: GM-CSF/leukaemia/retroviral-vectors/transplantation

Introduction

The haemopoietic colony stimulating factors (CSFs) are a family of glycoproteins that are directly responsible *in vitro* for the survival, proliferation, differentiation and functional activation of neutrophils, macrophages, eosinophils, megakaryocytes and, in association with other haemopoietic growth factors, erythroid cells (reviewed in Metcalf, 1984, 1986). Molecular cloning of the four known CSFs has facilitated *in vitro* production of large quantities of these factors and enabled analysis of their action in animals and in man (Kindler *et al.*, 1986; Groopman *et al.*, 1987; Metcalf *et al.*, 1987a,b; Nienhuis *et al.*, 1987; Welte *et al.*, 1987). Intense interest is currently focused on determining the potential of the CSFs for reversing a number of human haematological disorders that have arisen either spontaneously or as a result of treatment of cancers with cytotoxic drugs.

Granulocyte-macrophage CSF (GM-CSF) directly stimulates neutrophils, macrophages and eosinophils and their progenitors *in vitro* (Metcalf, 1984). Moreover, injection of GM-CSF into mice was recently shown to induce a 2-fold increase in blood neutrophils and a substantial increase in the number and functional activity of peritoneal neutrophils, macrophages and eosinophils (Metcalf *et al.*, 1987a), providing evidence that this molecule may act as a regulator for these cell populations *in vivo*. Cells from certain human myeloid leukemias have been shown to contain GM-CSF mRNA (Young and Griffin, 1986; Cheng *et al.*, 1988). In addition, a factor-dependent and non-tumorigenic murine cell line, FDC-P1, became tumorigenic when infected with retroviruses expressing GM-CSF (Lang *et al.*, 1985; Laker *et al.*, 1987). It is therefore pertinent to ask whether dysregulated GM-CSF production in normal haemopoietic cells plays a role in myeloproliferative syndromes and/or leukemia.

Transgenic mice harbouring a murine GM-CSF cDNA have recently been used to investigate the consequences of constitutive GM-CSF production *in vivo* (Lang *et al.*, 1987). GM-CSF was readily detected in the serum of the transgenic mice, but the pattern of expression of the transgene suggested that it was only active in macrophages.

To attempt to assess the effect of constitutive GM-CSF production by a wider range of normal haemopoietic cells *in vivo*, we have utilized an alternative protocol involving the transplantation of lethally-irradiated mice with haemopoietic cells expressing a GM-CSF gene introduced via retroviral infection *in vitro*. To achieve adequate expression of GM-CSF, a new retroviral vector, pZen, was developed. This study documents that the haemopoietic tissues of mice transplanted with marrow cells infected with Zen(GM-CSF) virus produce high levels of GM-CSF, resulting in previously unattainable circulating levels of the factor. Such animals rapidly succumb to a fatal disease associated with a large increase in the numbers of circulating and infiltrating neutrophils, macrophages and eosinophils and a failure of repopulation of lymphoid organs. The implications of these results for differentiation commitment and leukaemogenesis are discussed.

Results

Construction of a new GM-CSF retrovirus

Preliminary experiments utilizing a GM-CSF virus (GMV) (Figure 1, bottom; Lang *et al.*, 1985) showed that the level of GM-CSF synthesis by normal haemopoietic cells infected with this virus was insufficient for autonomous growth. Since transcription of the parental virus [ZipNeoSV(X); Cepko *et al.*, 1984] containing only the *neo*^{r} gene was better in haemopoietic cells than that of GMV or other recombinant viruses carrying an additional gene near the 5′ long terminal repeat (LTR) (Bowtell *et al.*, 1988), we constructed a vector, pZen, which expresses the gene of interest from the site formerly occupied by *neo*^{r} (Figure 1, top). A variant vector, pMPZen, incorporated the enhancer from the LTR of the myeloproliferative sarcoma virus (MPSV) (Bowtell *et al.*, 1987), which is more effective in myeloid cells than that of Moloney virus (Bowtell *et al.*, 1987, 1988). Because the 3′ untranslated region of the GM-CSF cDNA used in

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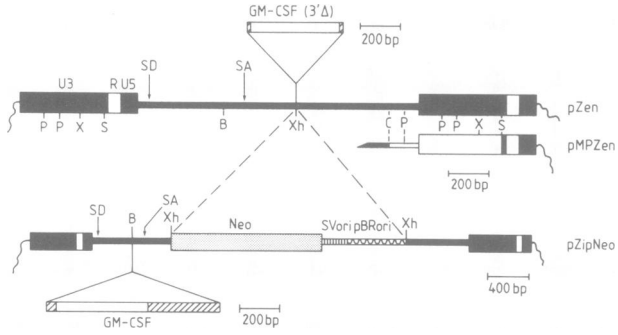

Fig. 1. Schematic representation of retroviruses expressing GM-CSF. The vectors all utilize a pBR322 backbone for replication in *E. coli*. The U3, R and U5 elements of the LTR are indicated. For the pZipNeo (Cepko *et al.*, 1984) and pZen vectors, both LTRs derive from Moloney leukemia virus, but most of the U3 region of the 3′ LTR in pMPZen is derived from the myeloproliferative sarcoma virus (see Materials and methods). The derivation of pZipNeo(GM-CSF), GMV, has been described by Lang *et al.* (1985). The coding regions of the GM-CSF cDNA inserts are shown open, untranslated regions hatched. Arrows labelled SD and SA indicate splice donor and acceptor sites used to generate the subgenomic RNA encoding the gene 3′ to SA. SVori indicates the SV40 origin of replication and pBRori the pBR322 origin of replication. Restriction endonuclease sites are abbreviated as follows: P, *Pvu*II (not all sites outside LTR shown); X, *Xba*I; S, *Sac*I; B, *Bam*HI; Xh, *Xho*I.

GMV contained sequences subsequently shown to confer mRNA instability (Shaw and Kamen, 1986), a smaller fragment lacking these sequences was used for the Zen(GM-CSF) viruses (Figure 1). To obviate the need for helper virus, virus-producing lines were generated by transfecting retroviral plasmid DNA into the $\psi 2$ packaging line (Mann *et al.*, 1983).

Autonomous growth of foetal liver cells infected with MPZen(GM-CSF) virus

To assess the efficacy of the Zen-based vectors for GM-CSF expression, foetal liver cells were infected by co-cultivation with irradiated Zen(GM-CSF) virus-producing Ψ2 cells or, as a control, with the parental Ψ2 line, in medium containing pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) as an exogenous source of CSFs. When the non-adherent foetal liver cells were washed and plated in medium lacking PWM-SCM, all cells in the mock-infected control cultures died within 10 days. Those exposed to the MPZen(GM-CSF) virus, however, continued to slowly proliferate (doubling time 4 days) for 5−6 weeks and remained viable for a further 12 weeks, when the cells were frozen down. Most of the cells proliferating in all of these cultures were of uniform size, round, adherent and, on staining, resembled macrophages. They all contained high levels of proviral DNA and expressed GM-CSF viral RNA (data not shown). Conditioned medium (prepared after 17 weeks of culture) stimulated FDC-P1 but not 32Dcl.23 cell proliferation, indicating that the cells were secreting GM-CSF. When the cells ($0.5-1 \times 10^6$) from three separate experiments were injected into non-irradiated syngeneic mice, they failed to provoke tumours. Moreover, no proviral DNA could be detected by Southern blot analysis of haemopoietic tissues of these mice 7 weeks after transplantation, their white cell counts were normal and the serum contained no detectable GM-CSF. These results demonstrated that GM-CSF production by infected macrophages could promote their long-term proliferation and survival *in vitro*, but did not render them tumorigenic.

Table I. Analysis of spleen colonies

Colony[a]	Viral DNA[d]	Infected mice[b] Total CFC		Control mice[c] Total CFC	
		−SCM	+SCM	−SCM	+SCM
1	0	0	0	0	0
2	~6	125	275	0	2975
3	3	0	0	0	0
4	2	0	0	0	0
5	1	0	0	0	0
6	~6	0	25	0	0
7	1	2100	2175	0	25
8	0.3	175	800	0	0
9	0.8	125	325	0	0
10	1	925	3525	0	75

[a]Spleen colonies dissected 13 days after transplantation of irradiated mice with ~10^5 bone marrow cells co-cultured with MPZen(GM-CSF) virus-producing $\psi 2$ fibroblasts or, as a control, with the parental $\psi 2$ line, which produces no virus. A portion (50−80%) of each colony was screened by Southern blot analysis for proviral DNA, while the remaining cells were stained or cultured.
[b]5/5 colonies were taken from one mouse, 7/7 from another. Two colonies have been excluded because insufficient DNA was obtained for viral analysis.
[c]10/15 colonies were taken from one mouse.
[b]Viral DNA was detected by Southern blot analysis of *Xba*I digests using a GM-CSF cDNA probe. The average number of inserts per cell was estimated by comparison with the intensity of bands derived from the endogenous GM-CSF gene.

GM-CSF virus infection of progenitor cells (CFC) and multipotential cells (CFU-S)

To ascertain whether the level of GM-CSF expression by the Zen viruses was sufficient for autonomous growth of progenitor cells, foetal liver or bone marrow cells were cultured in semi-solid agar medium after co-cultivation. No colonies grew in cultures of mock-infected cells in the absence of PWM-SCM. In contrast, cultures exposed to Zen(GM-CSF) or MPZen(GM-CSF) virus produced 25−100% as many colonies as those growing with PWM-SCM. Colony formation was linear with cell number cultured (from 600−10^5 cells), suggesting that proliferation of CFC was autonomous. As expected of day 7 cultures stimulated by GM-CSF, the colonies growing in the absence of PWM-SCM were composed of neutrophils, neutrophils and macrophages, macrophages and, occasionally, eosinophils. Colonies of erythroid cells were also observed when infected foetal liver cells were cultured with erythroprotein, but not in control cultures of uninfected cells. Twenty-four individual neutrophil and/or macrophage colonies grown from bone marrow in the absence of PWM-SCM were picked individually into 1 ml liquid medium and maintained with weekly medium changes. After 3 weeks, seven of the cultures contained increased numbers of cells (up to 4×10^4), all macrophages, which persisted for a further 9 weeks and the medium contained 600−2500 units of GM-CSF.

To determine if haemopoietic mutlipotential cells could be infected with the Zen(GM-CSF) viruses, bone marrow cells from mice previously treated with 5-fluorouracil (5-FU) were infected and transplanted into lethally-irradiated recipients in order to generate spleen colonies. Animals were

sacrificed 12–14 days later, spleens removed and sequential individual spleen colonies dissected. With the Zen(GM-CSF) virus, 35% of the colonies were positive for proviral DNA (eight of 23 analysed). With the MPZen(GM-CSF) virus, viral DNA was detected in 9 of 10 colonies and (except in two cases) the level averaged ≥1 copy per cell (see Table I).

All the virus-bearing spleen colonies were derived from multipotential cells, since they contained variable proportions of erythroblasts, neutrophils (myeloblasts, myelocytes and metamyelocytes), monocytes, macrophages, undifferentiated blasts, and occasionally, eosinophils and megakaryocytes. Bioassays of media conditioned by each of these colonies confirmed that they secreted GM-CSF. Moreover, with one exception, cells from all viral DNA-positive spleen colonies that contained progenitor cells (CFC) yielded significant numbers of autonomous colonies when cultured in semi-solid agar cultures (Table I). These cultures also contained numerous single macrophages. These studies indicated that the Zen-based viruses could efficiently express GM-CSF in the progeny of infected multipotential and progenitor cells.

Abnormalities in animals transplanted with MPZen(GM-CSF) virus-infected cells

To determine the effect of unregulated GM-CSF production *in vivo*, we undertook a systematic analysis of a group of lethally-irradiated mice transplanted with 10^6 post-5-FU bone marrow cells which had been co-cultivated with the MPZen(GM-CSF) virus-producing line. Control animals were injected with 10^6 mock-infected cells co-cultivated with the parental (non-producer) $\psi 2$ fibroblast line. All recipients of virus-infected cells became severely ill 2–3 weeks after transplantation and none survived longer than 4 weeks. Their major abnormalities were shortness of breath, partial paralysis of the hind limbs and, in some cases, opacity of the cornea. Autopsy revealed patchy consolidation of the lungs (11/11) and in some instances, spleen enlargement (7/11), mesenteric lymph node enlargement (6/11), white spots on the liver lobes (6/11) and nodules on the abdominal side of the diaphragm (1/11). The seven control animals autopsied at the same time as the sick mice appeared normal and the remaining control animals were healthy 6 months after transplantation.

Excessive GM-CSF production in animals transplanted with MPZen(GM-CSF) virus-infected cells

High levels of GM-CSF were detected in the sera of all animals transplanted with virus-infected cells (Table II). No GM-CSF was detectable in the sera of control animals. Furthermore, none of the sera contained detectable G-CSF or IL-3, and both the control and virus-bearing animals contained similar levels of erythropoietin (~0.6 units/ml).

In all cases, media conditioned for 7 and 14 days by the bone marrow, spleen and peritoneal cells of mice injected with virus-infected cells contained GM-CSF (but not G-CSF or IL-3) (Table III). These cultures also contained variable numbers of macrophages which persisted without any evidence of extensive mitotic activity until terminated at 4–12 weeks. No CSF activity was detected in conditioned media prepared from control animals.

Southern blot analysis of tissues (Figure 2A) indicated varying levels of proviral DNA. High levels (on average, 1–2 copies/cell) of the expected 2.5 kb *Xba*I fragment were apparent in the spleen, bone marrow, peritoneal cells and lungs. The low and variable level of proviral DNA in the mesenteric lymph node and thymus was probably due, in the former, to infiltration by neutrophils and/or macrophages and, in the latter, to contamination by the adjacent (infiltrated) apical lymph nodes (see below). Northern blot analysis of total RNA extracted from the various tissues showed that the level of the expected full length (2.5 kb) and spliced (2.0 kb) viral RNAs was proportional to their viral DNA content (compare, e.g. Figure 2B and C).

Table II. GM-CSF activity in serum of transplanted animals

Virus	Cells transplanted ($\times 10^{-6}$)	Day of autopsy	No. of animals	GM-CSF[a] (U/ml $\times 10^{-3}$)
+	1.0	14	4	310 ± 170
−	1.1	15	3	<0.18
+	1.1	20–22	2	54 ± 6
−	1.1	20–22	4	<1.1

[a]Mean bone marrow units ± SD.

Table III. GM-CSF activity in medium conditioned by haemopoietic tissues from animals transplanted with MPZen(GM-CSF) virus-infected bone marrow cells

Cells transplanted ($\times 10^{-6}$)	Day of autopsy	Tissue	GM-CSF Activity/10^6 cells (U/ml $\times 10^{-3}$)[a]
1.0	14	BM	38, 109, 18, 19
		Spleen	4, 4, 2, 1
		PC	14, 18, 15, 17
1.1	20–22	BM	18, 29, 74, 326
		Spleen	2, 1, 1, 3, 1
		PC	33, 19, 2, 56, 2

[a]Activity expressed per ml of medium conditioned for 7 days by 10^6 cells from bone marrow (BM), spleen or peritoneal cavity (PC) of individual mice.

In an attempt to quantify how many clones of infected cells were present in the transplanted mice, *Eco*RI-digested DNA from the various tissues was also analysed (data not shown). Since MPZen(GM-CSF) proviral DNA is not cut by this enzyme, proviruses inserted at different chromosomal locations are released as fragments of different size. No distinct fragments containing viral sequences were detected in most of the tissues which had yielded high levels of the 2.5 kb *Xba*I proviral fragment. The haemopoietic organs of these mice were thus constituted from multiple clones. In three mice, specific virus-bearing fragments were detected above a heterogeneous background, indicating a dominant contribution by a restricted number of clones. In one animal, DNA from the bone marrow, spleen and peritoneal cells displayed an identical size pattern, so cells in these three tissues were all probably the progeny of one or a very few cells.

Haematological parameters in animals with excessive GM-CSF production

Animals transplanted with MPZen(GM-CSF) virus-infected cells differed markedly from controls in the number and relative proportions of haemopoietic cell types. Elevated numbers of cells were apparent in the spleen, peripheral

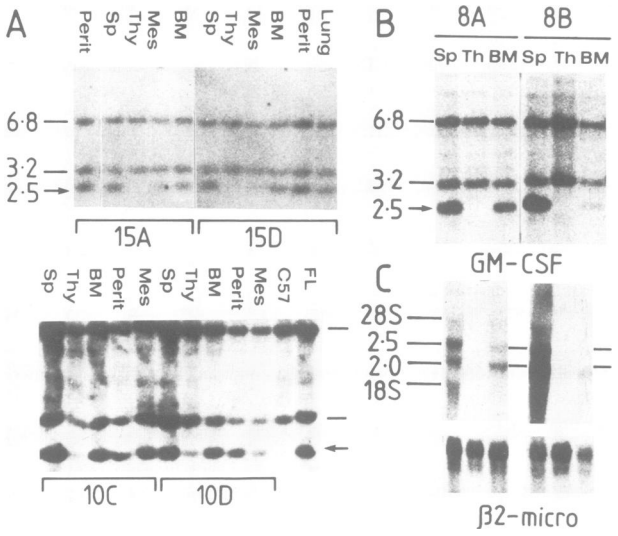

Fig. 2. Location and expression of MPZen(GM-CSF) provirus in tissues from irradiated mice transplanted with virus-infected bone marrow cells. (**A**) Southern blot analysis with a GM-CSF cDNA probe of *Xba*I digested DNA from tissues taken 14 (mouse 15A, 15D) and 21 days (mouse 10C, 10D) after transplantation with 10^6 virus-infected marrow cells. *Xba*I cuts within each LTR and releases GM-CSF provirus as a 2.5 kb fragment. Tissues analysed were spleen (Sp), peritoneal cells (Perit), thymus (Thy), mesenteric lymph node (Mes), bone marrow (BM) and lung. C57BL/6/J WEHI DNA (C57) and MPZen(GM-CSF) virus-infected foetal liver cells (FL) served as negative and positive controls, respectively. (**B**) Southern blot analysis with a GM-CSF cDNA probe of *Xba*I digested DNA from the spleen, thymus and bone marrow of two mice killed 13 days after transplantation with 10^5 bone marrow cells. (**C**) Northern blot analysis of total RNA (4 μg) isolated from the same tissues as analysed in (B). A GM-CSF cDNA probe was used first and then, as a control for the amount of RNA loaded, a β2-microglobulin probe.

blood and peritoneal cavity (Table IV). This increase was largely due to the presence of excess neutrophils, monocytes and macrophages (Figure 3). While the bone marrow had reduced cellularity (Table IV), its neutrophil content was marginally increased (Figure 3). Eosinophils were increased in all four tissues (Figure 3). Decreased numbers of erythroblasts and lymphocytes were observed in the bone marrow, but increased numbers of these cells were present in the spleen.

Overall, there were 15 times as many neutrophils, 7 times as many monocytes and macrophages, 9 times as many eosinophils and twice as many erythroblasts in the bone marrow, spleen, peripheral blood and peritoneal cavity of the virus-bearing mice as compared to the controls, while the numbers of lymphocytes, blasts and mast cells were comparable. The total body content of neutrophils, monocytes and macrophages was in fact much greater than these data suggest, as many non-haemopoietic tissues (see below) had large accumulations of these cell types. Moreover, since the thymus was atrophic and the lymph nodes were infiltrated (see below), the mice may actually have been deficient in lymphocytes.

The expanded numbers of neutrophils and macrophages were not paralled by an increase in progenitor cells. The number of these cells (GM-CFC) in the femoral bone marrow of recipients of infected cells was reduced compared with controls, but the total number of GM-CFC in the spleen was variable (see Table IV). No GM-CFC were detected

Table IV. Cellularity and number of GM-CFC in haemopoietic tissues of transplanted mice

Cells transplanted ($\times 10^{-6}$)	Day of autopsy	Virus	Bone marrow	Spleen	Peripheral blood	Peritoneum
			Cellularity ($\times 10^{-6}$)[a]			
1.0	14–15	+	5 ± 3	700 ± 180	110 ± 14	17 ± 3
		−	10 ± 2	210 ± 4	0.7 ± 0.3	2 ± 0.3
1.1	20–22	+	2 ± 1	520 ± 52	89 ± 73	35 ± 9
		−	11 ± 6	190 ± 25	2 ± 1	2 ± 0.8
			Number of GM-CFC ($\times 10^{-3}$)[a]			
1.0	14–15	+	0.4 ± 0.4	56 ± 23	ND	0
		−	2 ± 1	70 ± 30	ND	0
1.1	20–22	+	1 ± 0.8	72 ± 24	ND	0
		−	5 ± 3	24 ± 22	ND	0

[a]Mean data (±SD) from same animals shown in Table II.
ND=Not Determined.

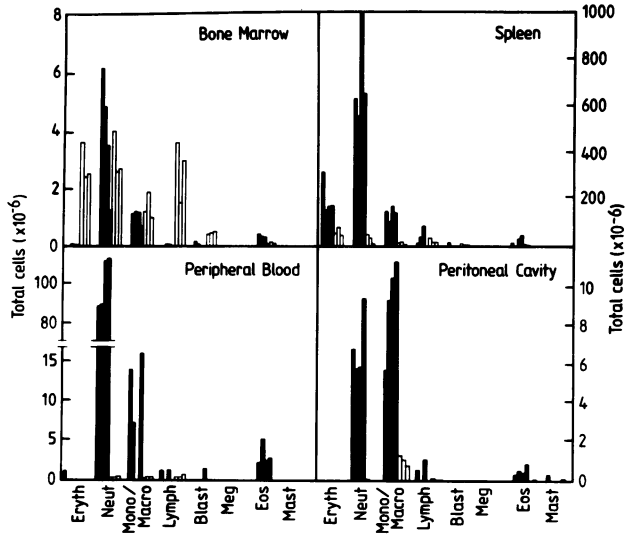

Fig. 3. Total numbers of erythroblasts (Eryth); neutrophilic myelocytes, metamyelocytes, polymorphs (Neut); monocytes, macrophages (Mono/Macro); lymphocytes (Lymph); blast cells; megakaryocytes (Meg); eosinophilic myelocytes, metamyelocytes, polymorphs (Eos) and mast cells in the bone marrow, spleen, peripheral blood and peritoneal cavity of animals transplanted 14–15 days previously with 10^6 post-5-FU treated bone marrow cells. Each column represents an individual mouse, open columns are control mice transplanted with mock-infected cells, black columns are recipients of cells co-cultivated with ψ2 cells producing MPZen(GM-CSF) virus.

in peritoneal cell populations. Cultures lacking PWM-SCM contained apparently factor-independent GM-CFC in numbers ranging up to 76% of those in control cultures to which PWM-SCM has been added. No CSF-independent CFC were detected in control animals.

Pathological changes in tissues of transplanted animals

Histological analysis of animals injected with MPZen(GM-CSF) virus-infected cells revealed extensive invasion of many organs by neutrophils and macrophages. All stages of maturation were apparent amongst the infiltrating cells and

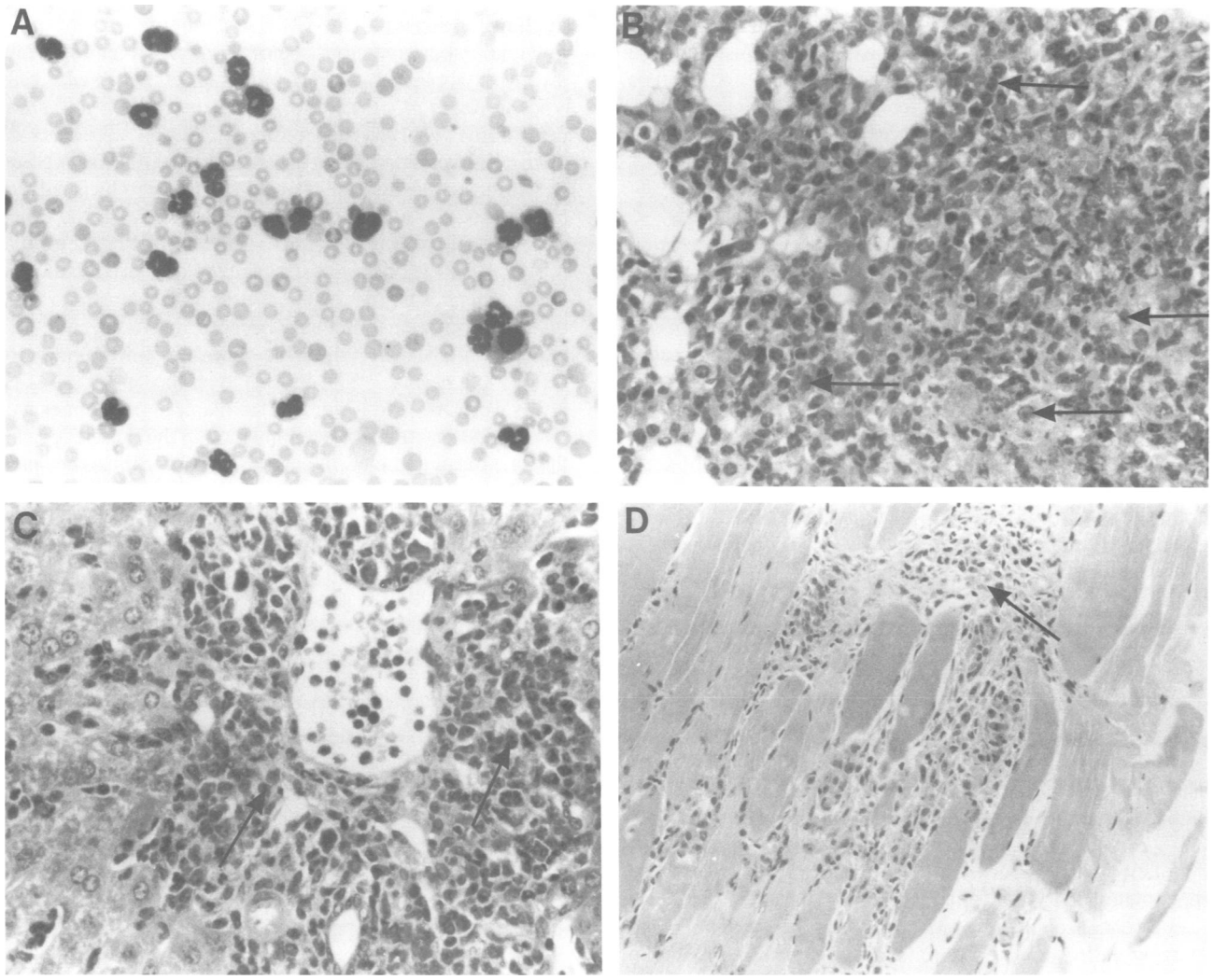

Fig. 4. Photomicrographs of (**a**) peripheral blood smear showing numerous neutrophils, (**b**) section of lung with infiltrating neutrophils and macrophages (arrowed), (**c**) section of liver showing periportal infiltrations of neutrophils (arrowed), (**d**) section of skeletal muscle with focal collection of infiltrating cells (arrowed). All photomicrographs of tissue from animals transplanted 21 days previously with 10^6 post-5-FU treated bone marrow cells co-cultivated with $\psi 2$ cells producing MPZen(GM-CSF) virus.

mitotic activity was prominent. Of the non-haemopoietic organs, those most affected were lung and liver. The lung exhibited focal accumulations of granulocytes and macrophages (Figure 4B), sometimes replacing all the alveolar tissue in a lobe. In other areas of the lung, the alveolar walls were thickened because of infiltration by granulocytes and macrophages. In both the solid infiltrates and the alveolar walls, granulocytes were the dominant population. Infiltration of the lung was not associated with necrosis or visible microorganisms, the pleural membranes were not thickened and no emphysema was apparent.

The livers uniformly exhibited extensive periportal accumulations of infiltrating cells (Figure 4C) together with large numbers of dispersed cells located in the hepatic sinusoids. Some periportal accumulations were composed wholly of macrophages, while others contained a high proportion of granulocytes and some eosinophils. The dominant cells in the dispersed sinusoidal populations were granulocytes.

Both the heart and skeletal muscle tissue commonly showed infiltration (both focal and dispersed) by macrophages, granulocytes and eosinophils (Figure 4D) with evidence of some phagocytosis of fragments of muscle cell cytoplasm by macrophages.

The eyes of some mice exhibited small numbers of infiltrating cells, mainly macrophages, and in some cases there appeared to be partial destruction of the photoreceptor layer with detachment of the retina.

The gonads did not exhibit infiltration but were commonly surrounded by collars of a mixed population of macrophages and granulocytes. No infiltrates were observed in salivary gland, pancreas, skin, kidney or gut.

Sections of the femur often revealed large focal aggregates of either macrophages or granulocytes and, even though other areas of the marrow showed superficially normal haemopoiesis, erythroid and lymphoid cells were subnormal in frequency.

The thymus was invariably devoid of lymphoid cells in the cortex and contained only small numbers of lymphoid cells in the medulla, in sharp contrast to the essentially normal thymus seen in control mice. The apical lymph node adjacent to the thymus and the mesenteric lymph node were

similarly depleted of lymphoid cells. No infiltration by granulocytes or macrophages was observed in the thymus. In some lymph nodes, there was extensive invasion of the hilar region by granulocytes and macrophages, and nodules of macrophages exhibiting mitotic activity were sometimes apparent in what normally would have been lymphoid regions of the organ.

Transplantation of infected haemopoietic cells into secondary recipients

Bone marrow, spleen or peritoneal cells from primary recipients failed to produce tumours when transplanted (either intravenously, intraperitoneally or subcutaneously) into sub-lethally irradiated (4.5 Gy) or unirradiated secondary recipients. All animals (24) injected with bone marrow, spleen or peritoneal cells remained healthy when examined 8–44 weeks after transplantation and, of six animals assayed, none had elevated white cell counts or serum GM-CSF levels. These experiments also indicated that, as expected, no detectable replication-competent GM-CSF virus was present in the primary animals. This conclusion was confirmed by culturing FDC-P1 cells in the presence of serum or irradiated spleen cells from primary (nine animals) or secondary (four animals) recipients, no factor-independent cells containing MPZen(GM-CSF) viral DNA were detected after removal of growth factor.

Discussion

The data presented in this paper establish that constitutive, high level expression of the haemopoietic growth factor GM-CSF in an adult haemopoietic population, drives this population to behave in a highly abnormal manner when repopulating an irradiated recipient.

In essence, recipients of MPZen(GM-CSF) virus-infected marrow cells showed the following set of abnormalities: extremely high serum GM-CSF levels; massive overgrowth of granulocyte and macrophage populations in the blood and spleen with extensive infiltration of the lung and liver; limited repopulation of lymphoid organs; decreased numbers of marrow erythroid and lymphoid cells, compensated in the case of erythroid populations by rises in spleen erythropoiesis; increased spleen cellularity; autonomous progenitor cell growth and grossly elevated levels of GM-CSF in media conditioned by haemopoietic tissues.

The population injected into the irradiated mice was a mixture of infected and uninfected cells at various stages of differentiation, ranging from multipotential stem cells to mature macrophages. Furthermore, as shown by the Southern blot analysis, repopulation in many animals was unlikely to have been by the progeny of a single cell. Therefore several mechanisms could be proposed to account for the abnormal overgrowth of granulocytes, macrophages and eosinophils in these mice and their deficiency in lymphoid cells. These are not mutually exclusive and include disturbed differentiation commitment patterns in multipotential cells, increased proliferative rate of progenitors, an increased life span of infected mature cells (e.g. macrophages), and infiltration by these cells of some lymphoid organs displacing lymphoid differentiation. Excessive production by the mature cells of GM-CSF and possibly other factors may in turn affect the proliferation and differentiation of other cells.

To date a number of adverse side effects have been noted in clinical trials with GM-CSF, including fever, bone pain, myalgia, skin reactions, elevation of liver enzymes, thrombocytopaenia and pericarditis (Lieschke *et al.*, 1989; Vadhan-Raj *et al.*, 1988). At doses exceeding 15 μg/kg/day, pericarditis was a dose-limiting toxicity (Lieschke *et al.*, 1988). The experiments in the transplanted mice also predict that high concentrations of GM-CSF produce adverse reactions, and suggest possible mechanisms for the effects observed in clinical trials, e.g. overproduction of macrophages resulting in excess cytokine production.

The changes in the virus-bearing mice were far greater than had previously been observed in transgenic mice expressing GM-CSF under the control of a Moloney virus LTF (Lang *et al.*, 1987). In the latter, the serum GM-CSF levels were 100-fold lower and the major abnormalities were confined to macrophage accumulation in the muscle, eye and the peritoneal and pleural cavities. Apart from some spleen enlargement, no changes to haemopoietic tissues were observed. The more extreme abnormalities seen in the virus-bearing mice may result from the transplantation protocol used, a broader range of haemopoietic cells expressing the exogenous GM-CSF gene, the higher levels of GM-CSF expression achieved or a combination of these factors. While the reasons for the higher levels of expression are still under investigation, data obtained to date suggest that increased transcription from the MPSV LTR (Bowtell *et al.*, 1987, 1988) and the more efficient translation of GM-CSF coding sequences (T.T.Gonda, G.R.Johnson and N.Gough, unpublished) may be important.

In view of the increasing evidence for abnormal transcription of CSF genes in myeloid leukaemic populations in man (Young and Griffin, 1985; Estrov *et al.*, 1986; Cheng *et al.*, 1988), it is pertinent to ask whether the abnormal myelopoiesis in MPZen(GM-CSF) virus-bearing mice can be regarded as a myeloid leukaemia. The fundamental definition of a cancer population is one in which progressive proliferation is incapable of restraint by the body. The recipients of Zen(GM-CSF) virus-infected cells develop a fatal disease with abnormally high and progressively increasing populations of mitotically-active granulocytes, monocyte-macrophages and eosinophils. The histological appearance of the liver and lung is identical to that seen in murine myeloid leukaemia. While the disease was not clonal, clonality is not an obligate feature of cancer. Clonality in spontaneously arising malignancies may simply reflect the fact that the probability of escape from growth control is low and usually involves more than one genetic change. A more compelling argument against classifying the disease as leukaemia is that haemopoietic cells from primary recipients or foetal liver derived cell lines have so far (44 weeks) failed to generate transplanted leukaemias in normal or sub-lethally irradiated secondary recipients.

Since these progressively proliferating populations are apparently not leukaemic, a very intriguing intermediate state between normality and neoplasia has been generated by the constitutive autocrine expression of a specific haemopoietic regulatory gene. Such a population provides a valuable experimental model for known myeloproliferative disorders that predispose to myeloid leukaemia, e.g. juvenile chronic myelogenous leukemia or refractory anaemia with excess blasts. This model system also lends itself to further manipulation by retroviral insertion of other haemopoietic

growth factor genes or oncogenes in an attempt to generate fully leukaemic cells.

Materials and methods

Production of Zen(GM-CSF) retroviruses

The plasmid pZen (Hariharan *et al.*, 1988) was derived from pZipNeoSV(X) (Cepko *et al.*, 1984) by removal of the *Xho*I fragment containing the *neo*^{r} gene and adjacent sequences, followed by religation. In the variant plasmid pMPZen, the 3′ *Cla*I−*Sac*I fragment was exchanged for that of the myeloproliferative sarcoma virus (MPSV), as described previously (Bowtell *et al.*, 1987). The GM-CSF cDNA, pGM3′Δ1.11 (Gough *et al.*, 1987), was inserted into the *Xho*I cloning site of both vectors. Fibroblast lines secreting Zen(GM-CSF) and MPZen(GM-CSF) virus free of helper virus were produced by calcium phosphate transfection of the ψ2 packaging line (Mann *et al.*, 1983) with a mixture of retroviral and pSV2Neo plasmid DNA (Southern and Berg, 1982) at a ratio of 20:1 (w/w) followed by selection in G418 (400 μg/ml). Individual colonies were then picked and expanded for assay.

The relative viral titres of ψ2 clones were determined by comparing their ability to convert FDC-P1 cells to factor-independent growth. FDC-P1 cells (1×10^5) were co-cultivated for 2 days with individual ψ2 clones (2×10^5 cells) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS) and maximal concentrations of pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) (Metcalf and Johnson, 1978), then harvested and washed (3×) in phosphate buffered saline. To minimize the effect of cross-feeding of uninfected cells, the cells were subsequently cultured at a range of cell densities (50−1250 cells) in 1 ml semi-solid agar cultures in the presence or absence of PWM-SCM: ψ2 clones with the highest viral titre were judged to be those producing the highest proportion of autonomously-growing FDC-P1 clones at the lowest number of FDC-P1 cells cultured. The best MPZen(GM-CSF)-secreting ψ2 clone, M5.5, produced 18 factor-independent colonies at 100 cells/ml as against 31 colonies in the presence of CSF. This clone was negative for helper virus secretion by the standard XC plaque assay (Rowe *et al.*, 1970) and RNA prepared from FDC-P1 cells infected with MPZen(GM-CSF) virus lacked any detectable helper virus transcripts.

Analysis of viral DNA and RNA

RNA and DNA were isolated from guanidine−HCl lysates as described previously (Bowtell *et al.*, 1987). Southern blot analysis was performed as described by Cory *et al.* (1983) and Northern blot analysis as described by Gonda *et al.* (1982). The GM-CSF probe was the insert from cDNA clone pGM5′Δ7 (Gough *et al.*, 1985), while that for β2-microglobulin was from pBRcB4 (Parnes and Seidman, 1982). The probes were labeled with [α-^{32}P]dATP using a random hexamer priming kit (Bresatec Limited, South Australia).

Mice

Mice used were 2−3 month old C57BL/6J WEHI and 12 day CBA/CaH/WEHI foetal mice (day of plug discovery = day 0 gestation) from stocks maintained at The Walter and Eliza Hall Institute of Medical Research. For transplantation studies, animals were subjected to whole body irradiation (9.0−9.5 Gy, γ rays generated by ^{60}Co at 0.39 Gy/min at 1.5 m) and transplanted intravenously with the required number of cells within 3 h of irradiation.

Infection of normal haemopoietic cells

Detailed conditions for the infection of normal haemopoietic cells have been described previously (Bowtell *et al.*, 1987). Briefly, 12−13 day foetal liver cells (2×10^5) were co-cultivated with irradiated (35 Gy) virus-producing Ψ2 cells (1×10^5) for 2 days in 5 ml of DMEM and 20% FCS. Normal bone marrow (1×10^6) or day 4 post 5-FU-treated bone marrow cells (minimum of 2×10^6 cells, two femur equivalents) from C57BL/6J WEHI mice were co-cultivated for 5 days in 10 ml of DMEM and 20% FCS with virus-producing ψ2 cells plated 24-h previously at 5×10^4 cells/10 cm dish. To minimize selection of different haemopoietic cell types, all co-cultivations were performed in the presence of optimal concentrations of PWM-SCM, a source of both IL-3 and GM-CSF (Metcalf and Johnson, 1978; Cutler *et al.*, 1985b). Prior to assay all haemopoietic cells were washed (3×) in factor-free medium.

CFU-S and CFC assays

At 12−14 days after transplantation with $0.25-1 \times 10^5$ syngeneic cells, irradiated animals were sacrificed, individual spleen colonies (derived from spleen colony-forming units, CFU-S) dissected from non-confluent spleens and single cell suspensions prepared as described previously (Lala and Johnson, 1978).

The number of CFC (neutrophil, neutrophil-macrophage, macrophage or eosinophil) in different haemopoietic tissues was determined by culturing foetal liver ($0.1-2.0 \times 10^4$ cells /ml), bone marrow (at 1.0, 2.0 and 4.0 $\times 10^4$ cells/ml), spleen (0.5, 1.0 and 2.0×10^5 cells/ml) or peritoneal cells (at 0.2, 0.5 and 1.0×10^4 cells/ml) in triplicate 1 ml agar-medium cultures (Johnson, 1980). Where required, cultures were maximally stimulated by the addition of 0.1 ml PWM-SCM. After incubation for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air, colonies (>50 cells) were counted using an Olympus SZ dissection microscope and the cultures fixed for 1−4 h using 1 ml 2.5% glutaraldehyde. The intact cultures were floated onto glass slides, dried, then stained with Luxol Fast Blue/haematoxylin (Johnson and Metcalf, 1980) for typing of colonies.

Erythroid colonies derived from CFU-E or from BFU-E were scored after 2 or 7 days incubation of 12 day foetal liver cells using 1.0 ml methylcellulose cultures as described previously (Johnson and Barker, 1985). Where required erythropoietin (1.6 units) prepared from human urine (Cutler *et al.*, 1985a) was added to the cultures.

Haemopoietic growth factor assays

GM-CSF and interleukin-3 (IL-3) were assayed using FDC-P1 cells (responsive to GM-CSF and IL-3) and 32Dcl.23 cells (responsive to IL-3) as described previously (Metcalf, 1985). Assays for CSF levels were also performed in parallel using agar cultures of 50 000 C57BL/6/J WEHI bone marrow cells as described previously (Metcalf, 1984). Colonies containing >50 cells were scored after 7 days incubation and morphology of colony cells determined after fixation of intact cultures with 2.5% glutaraldehyde and staining with Luxol Fast Blue/haematoxylin (Johnson and Metcalf, 1980). The number of units of GM-CSF activity was calculated by assigning 50 units to the amount added to cultures of normal C57BL/6 bone marrow cells resulting in 50% of maximum colony numbers. Granulocyte colony stimulating factor (G-CSF) was assayed by its ability to induce differentiation in colonies of the murine myelomonocytic cell line WEHI-3B D$^+$ (Nicola *et al.*, 1988). Erythropoietin (Epo) was assayed in methylcellulose cultures of 12 day CBE/CaH/WEHI foetal liver cells (Cutler *et al.*, 1985a).

Conditioned media

Cell suspensions were prepared from spleen, femoral bone marrow or peritoneum and a 30 μl aliquot (representing 0.6, 1.5 and 1.2% respectively, of the total organ population), was incubated at 37°C in 2 ml of DMEM containing 10% FCS. After 7 days, 1 ml of medium was harvested and replaced by 1 ml of fresh medium. After a further 7 days of incubation, another 1 ml of conditioned medium was collected and both samples were assayed for GM-CSF, IL-3 and G-CSF as described above. If cell survival were evident, the cultures were continued with medium changes weekly for a total period of 12 weeks.

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