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Local and Systemic Response of Mice to Interferon-α1-Transfected Friend Leukemia Cells

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DBA/2 mice were injected subcutaneously with an interferon (IFN)-α/β-resistant line of Friend erythroleukemia cells (FLC) transfected with the mouse IFN-α1 gene. These tumor cells produced IFN constitutively, and mice had persistently high levels of IFN in the circulation. We examined the IFN-induced host mechanisms responsible for the local inhibition of growth of these IFN-α-transfected FLC and some of the unusual systemic effects of constant interferonemia such as extramedullary hematopoiesis in the liver, an increase in myeloid cells in the spleen, and persistently elevated splenic natural killer (NK) cell activity. In addition, both DBA/2 +/bg and beige mice developed a rapid and specific resistance to intravenous challenge with parental FLC. In previous experiments DBA/2 beige mice could not be protected by exogenous IFN-α/β. The differences in the response of mice to the constitutive production of IFN-α by IFN-α-transfected tumor cells and their response to exogenous IFN is discussed in terms of the effects of IFN on the host and of antitumor therapy. (Am J Pathol 1995, 147:445–460)

There is an increasing number of articles describing the behavior of a variety of experimental murine tumors transfected with numerous cytokines such as interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, macrophage colony-stimulating factor (M-CSF), granulocyte-M-CSF (GM-CSF), tumor necrosis factor (TNF), and interferon (IFN)-γ. The stated purpose of this research is to understand the mechanisms of the antitumor effects of these cytokines, in the hope that cytokine-secreting tumor cells may be therapeutically useful in patients.[2, 3] With the exception of our own reports on the transfection of tumor cells with the murine IFN-α gene,[4–6] there have been few published reports on the behavior of IFN-α[7] or IFN-β-transfected tumor cell lines in mice. As IFN-α has been used in the treatment of some patients with cancer,[8] we have presented in some detail experiments on the local and systemic response of mice to injection of these cells.

We chose initially to transfect the IFN-α/β-resistant metastatic 3CI8 line of Friend leukemia cells (FLC) with the murine IFN-α1 gene,[4] because we have previously shown that exogenous IFN-α/β exerted a clear-cut antitumor effect in mice injected with these tumor cells.[9, 10] These IFN-α1-transfected FLC seem to us a most interesting and unique experimental system for two reasons. First, the FLC of the 3CI8 line are totally resistant to mouse IFN-α/β.[11] We have studied the characteristics of this cell line and shown that despite the presence of high affinity receptors for IFN-α/β,[12] these cells are totally resistant *in vitro* to all the activities of IFN-α/β tested and, furthermore, can even be shown to be resistant within the mouse to the antiviral effect of IFN α/β[13] as well as the enhancing effect of IFN on the expression of H-2 antigens.[14] Thus, we concluded that the antitumor effects of IFN-α/β in FLC-injected mice were mediated by the reaction of the host to the tumor and were not due to the effects of IFN on the tumor cells themselves.[9, 10] Sec-

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ond, IFN-α1-transfected 3Cl8 FLC secrete large amounts of IFN, and persistent interferonemia can be detected over a period of several weeks.[4] We know of no other experimental model in which there is a constitutive production of large amounts of IFN-α over a significant period of time. In virtually all other experimental models in which mice were injected with transfected cytokine producing cells, secreted cytokines have not been detected in the sera with the exception of high-producing TNF-transfected tumor cells,[15, 16] and one report of IFN-γ-transfected tumor cells.[17] In fact, the localization of the secreted cytokine has been considered an advantage by many investigators,[18–21] in order to avoid systemic effects of the cytokines that may prove detrimental to the host. Although these systemic effects observed after the administration of some cytokines have been referred to simply as "toxic," it seems to us that it is of considerable interest to understand the nature of these effects, because they may contribute to our understanding of the physiological role of these cytokines and may well contribute to their therapeutic usefulness in treating local or systemic disease.

In this article we attempt to understand the host mechanisms that are responsible for restricting the local growth of these IFN-α1-transfected IFN-α-resistant FLC injected subcutaneously (s.c.) and we also describe some of the systemic changes associated with the persistent production of IFN-α1, especially in terms of extramedullary hematopoiesis in the liver and an increase in myeloid cells in the spleen. These changes have not been heretofore described in mice treated with IFN. Lastly, we show that s.c. inoculation of these IFN-α1-transfected FLC is accompanied by a very rapid and specific resistance to intravenous challenge with the parental highly metastatic line of FLC.

Materials and Methods

Mice

6- to 7-week old male DBA/2 mice were obtained from Charles River Breeding Laboratories (Italia Calco, Italy). Breeding pairs of DBA/2J-CO-bg^{8J} beige/beige (bg/bg) and heterozygote +/bg mice were obtained through the courtesy of Dr. G. Carlson (Jackson Laboratories, Bar Harbor, ME). A colony was then raised and maintained at Villejuif, France. These mice were shown to be deficient in natural killer (NK) cell activity.[22] Male Swiss nude mice were purchased from Charles River Laboratories (Calco) at 4 weeks of age and kept for 1 week before use. Splenectomy, irradiation, and anti-asialo GM1 treatment of nude mice have been previously described.[23]

Tumor Cells

The IFN-α/β-resistant clone 3Cl8 of FLC passaged *in vitro* was originally obtained from Dr. E. Affabris, Rome.[11] The cells were subsequently passaged *in vivo* by weekly intraperitoneal (i.p.) injection of syngeneic DBA/2 mice. These FLC were highly metastatic for the liver and spleen[24] and exhibited a specific membrane glycoprotein pattern distinct from the original non-metastatic FLC.[25] IFN-α1-producing FLC (clone 11) and transfection control FLC (clone TC-2) were isolated after transfection of *in vivo* passaged FLC as described in detail elsewhere.[4] TC-2 FLC maintained the highly metastatic phenotype typical of *in vivo* passaged FLC even after several *in vitro* passages.[4] IFN-α-Cl 11 FLC secreted IFN α into the culture fluid (approximately 256 to 512 U/ml), whereas no IFN activity was secreted by clone TC-2 FLC. In some experiments we also used the IFN-α/β-resistant cell line[26] of the transplantable murine B-cell lymphoma L1210 transfected with the murine IFN-α1 gene using the same retroviral vector pLMu IFN-α1 TneoL as previously described.[4] These cells also secreted IFN into the culture fluid (256 to 512 U/ml). L1210 cells (H-2^d) are syngeneic for DBA/2 mice. The transfected cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) containing 300 μg/ml of G418 (calculated to give 100% antibiotic activity; Geneticin, GIBCO Grand Island, NY). Mice were injected s.c. in the dorsum after shaving.

IFN Titration

IFN was titered on L929 cells as previously described.[27] IFN titers were expressed as international units.

Antibodies for Cell Population Depletion

The antibodies used to deplete specific cell populations were Ig-purified fractions of ascites of clone GK.1.5 (anti-CD4$^+$), clone T1B-5 105 (anti-CD8$^+$), and clone RB6–8C5 (anti-granulocyte). The procedures concerning injection of antibodies were described previously.[28] The efficacy of cell depletion *in vivo* using the different antibodies was controlled by fluorescence-activated cell sorter (FACS) analysis using specific monoclonal antibodies.

Determination of the Different Spleen Cell Populations

Different populations of cells within the spleen were determined using fluor-labeled antibodies against specific surface antigens. 1×10^6 cells were incubated with the appropriate antibody for 30 minutes at 4°C in HBSS containing 5% FCS and 0.1% sodium azide. Cells were washed three times in HBSS and fixed in 1.0 ml formalin (1% in PBS). The percentage of each respective cell population in individual spleens was determined using an EPICS cytofluorograph (Coulter, Hialeah, FL). The total number of each cell type within individual spleens was approximated by multiplying the percent positive cells by the total number of cells determined by light microscopy for that same spleen. Antibodies used were phycoerythrin (PE)-labeled clone RM4–5 antibody against mouse $CD4^+$ helper T cells (PharMingen, San Diego, CA); PE-labeled clone 53–5.8 antibody against mouse $CD8^+$ cytotoxic T cells (PharMingen); PE-labeled clone RB6–8C5 antibody against mouse Gr-1^+ granulocytes (PharMingen); FITC-labeled anti-mouse F4/80; and FITC-labeled affinity purified anti-mouse Ig antibodies (Biosys, Compiégne, France). Appropriate PE- and FITC-labelled mouse immunoglobulin isotype controls were obtained from PharMingen and used to determine nonspecific fluorescent antibody binding.

Tests of Splenic NK Cell Activity

Cytotoxic activity of spleen cells was assessed in a radioactive chromium release assay using mouse YAC target cells as described.[29] YAC cells (5×10^6) were radiolabeled by incubation with 200 μCi of ^{51}Cr (2 mlCi/ml; Amersham, Les Ulis, France) for 1 hour at 37°C in 0.5 ml RPMI 1640 plus 5% FCS and then washed extensively. Labeled target cells (10^4) were incubated in round-bottomed microplates (Nunc, Roskilde, Denmark) with freshly harvested spleen cells in lymphocyte-to-target-cell ratios of 100:1, 50:1, 25:1, and 12.5:1 for 4 hours at 37°C. Supernatant (0.1 ml) was collected at the end of the incubation period and counted for γ-radioactivity. The percentage of cytotoxicity was calculated by the following formula: 100 × (cpm released from YAC cells incubated with lymphocytes – cpm released spontaneously from YAC cells/cpm released from YAC cells treated with 100 μl 2 N NCl – cpm released spontaneously from YAC cells).

Dosage of Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)

Murine GM-CSF in the sera was determined using an ELISA kit (Endogen, Boston, MA). As a positive control a known amount of murine GM-CSF was obtained from Immunex (Seattle, WA) and added to the sera to be tested.

Light and Electron Microscopy

Organs were fixed in 4% buffered formalin and embedded in paraffin wax. Sections (5 μm) were cut and routinely stained with hematoxylin and eosin. For ultrastructural examination, small pieces of liver were cut into 1 mm^3 pieces and fixed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer at 4°C for 1 hour. Samples were post-fixed in 1% osmium tetroxide in Millonig's phosphate buffer for 1 hour at 4°C, washed in distilled water, immersed in 1% aqueous uranyl acetate for 1.5 hours at room temperature, dehydrated through ascending grades of ethanol, and embedded in Spurr's resin. Sections were cut on a Reichert OmU3 ultramicrotome. Semithin sections (0.5 to 1.0 μm) were stained with toluidine blue/pyronin Y in borax and examined by light microscopy. Ultrathin sections (60 to 80 nm) were stained in 1% aqueous uranyl acetate and Reynolds lead citrate and examined with a Philips EM300 electron microscope.

Statistical Analyses

Within each experimental group, a one-way analysis of variance was used, and the means were subsequently compared using Fisher's PLSD or Scheffe's F test.

Results

Tumorigenicity of IFN-α1-Transfected Clone 11 FLC Normal and Immunodeficient DBA/2 Mice

As IFN-α1-transfected Cl-11 FLC maintained the resistance to the antiproliferative action of IFN-α/β characteristic of parental IFN-α/β-resistant 3Cl8 FLC, we concluded that the decreased tumorigenicity of these cells was due to the effect of IFN-α1 on the host rather than to an autocrine action of IFN on the tumor cells themselves.[4] It was of interest, therefore, to determine the tumorigenicity of IFN-α1-transfected Cl-11 FLC in immunodeficient mice.

Immunocompetent DBA/2 +/bg mice injected s.c. with control transfected TC-2 FLC developed rapidly growing tumors (Figure 1A) and died with liver and spleen metastases 20 to 35 days after tumor cell injection. In contrast, mice injected with IFN-α1 Cl-11 developed very small tumors (Figure 1A), which exhibited large areas of necrosis at 15 to 30 days. In all experiments there was a marked increase in the survival time and overall survival of mice inoculated with IFN-α1 Cl-11 cells compared with mice inoculated with TC-2 cells. IFN-α1 Cl-11 FLC showed the same decreased tumorigenicity in NK cell-deficient DBA/2 beige (bg/bg) mice as in DBA/2 +/bg mice (Figure 1B). Injection of DBA/2 mice with monoclonal antibodies to deplete either $CD4^+$ or $CD8^+$ lymphocytes or granulocytes did not affect either the tumor growth curve (Figure 1C), the percentage of tumor takes, or the survival time of DBA/2 mice injected s.c. with IFN-α1-producing clone 11 cells (data not shown). IFN-producing Cl-11 cells did form rapidly growing tumors, however, in totally immunosuppressed Swiss SIA nude mice (ie, splenectomized, irradiated, and treated with anti-asialo GM1 antibody), even though there was still a slight difference in the growth curve of this tumor compared with the control TC-2 tumors (Figure 1D). There was no difference in the survival time or overall survival between these two groups of mice (data not shown). These latter results clearly indicated that the *in vivo* rejection of IFN-producing 3Cl8 cells was not due to a loss of the inherent *in vivo* growth capacity of these cells but rather to an IFN-induced host-mediated response in the immunocompetent mouse.

Morphological Examination of TC-2 and IFN-α CL-11 FLC Tumors in DBA/2 Mice

To gain further insight into the possible host mechanisms mediating the decreased tumorigenicity of IFN-α1-producing Cl-11 cells in immunocompetent

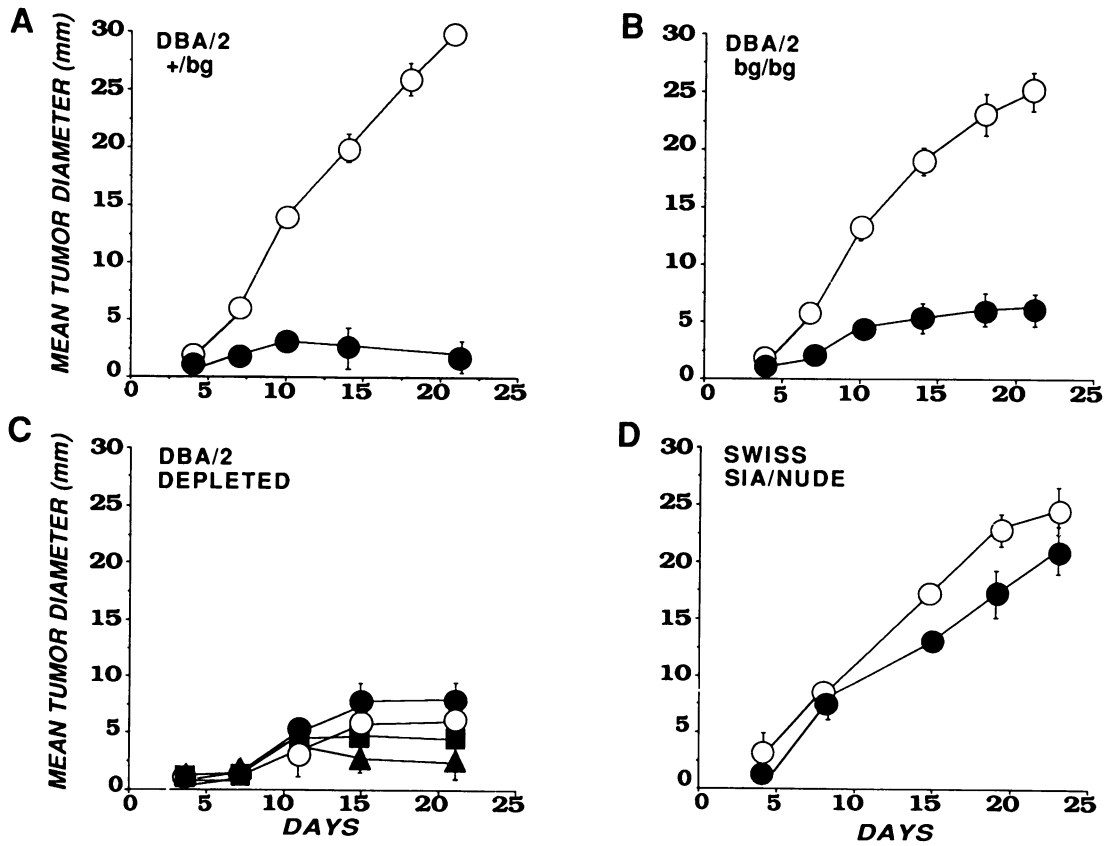

Figure 1. *Tumorigenicity of IFN-α1-transfected Cl-11 FLC and transfection control TC-2 cells injected s.c. in normal and immunodepressed mice.* (A *and* B) *8-week-old male DBA/2 +/bg and bg/bg mice were injected with 2 × 10^6 TC-2* (○) *or IFN-α1 Cl-11 FLC* (●). There were six mice in each group. (C) *6–7-week old male DBA/2 mice were treated on day −2, 0, 5, 9, and 15 (as indicated in Materials and Methods) with anti-granulocyte antibodies* (▲), *anti-CD4 antibodies* (●), *anti-CD8 antibodies* (■), *or kept untreated* (○). *On day 0, all the mice were injected s.c. with 2 × 10^6 IFN-α1 Cl-11 cells. There were eight mice per group.* (D) *Swiss SIA nude mice were injected s.c. with 2 × 10^6 TC-2* (○) or IFN-α1 Cl-11 FLC (●). *There were 10 mice in each group.*

DBA/2 mice, we examined in detail the morphology of s.c. tumors at 1, 4, 7, and 14 days from mice injected with transfection control TC-2 cells or IFN-α1-transfected Cl-11 cells.

Light Microscopy

At day 1, no major differences were observed between clone TC-2 and IFN-α1 Cl-11 FLC. The tumor was initially localized beneath the platysma, and necrotic cells were observed within the central area of the tumors in both groups of mice. On day 4, in mice injected with TC-2 cells, the tumor had infiltrated upward through the muscle layer of the platysma to the level of the hair follicles, and a few macrophages and fibroblasts were detected surrounding the leading edge of the tumor. At this time the pattern of tumor growth in mice injected with TC-2 cells was similar to that of Cl-11 cell-injected mice with the exception that in the latter there was a prominent infiltrate of macrophages, neutrophils and lymphocytes together with fibroblasts at the tumor edge. The presence of macrophages was confirmed by positive Mac-1 antibody staining and nonspecific esterase cryostat preparations. The lymphocytes stained with antibodies to CD3 and CD4, but not with antibody to CD8.

At 7 and 14 days, in mice transplanted with TC-2 cells, the tumor had ulcerated onto the surface of the skin with only a few neutrophils and macrophages around the edges of the tumor (Figure 2, A and B). In mice injected with IFN-α1 Cl-11 cells, the tumors were still at the level of the reticular collagen and there was extensive individual cell necrosis and apoptosis. No vascular abnormalities were observed. At the leading tumor edge, many macrophages and some neutrophils, eosinophils, and lymphocytes were present (Figure 2, C and D). By day 14, in some mice injected with IFN-α1 Cl-11 cells only residual necrotic tumor was evident, surrounded by macrophages, neutrophils, and fibroblasts (not shown). In other mice a small rim of viable tumor cells persisted for several weeks.

Electron Microscopy

At day 4 the TC-2 cell tumors exhibited large sheets of viable tumor cells (Figure 3A) with very occasional necrotic cells, while IFN-α1 cl-11 tumors showed degenerating and necrotic tumor cells with evidence of apoptosis (Figure 3, B and C). These apoptotic cells were characterized by nuclear changes in which there was condensation of the nuclear heterochromatin to a crescentic position at the periphery of the nucleus. In addition, apoptotic bodies were present composed of dense chromatin fragments surrounded by cytoplasm, organelles, and intact plasma membrane (Figure 3, B and C). There was also a prominent inflammatory cell infiltrate consisting of neutrophils, eosinophils, occasional lymphocytes, and large numbers of macrophages (Figure 4). The capillaries showed no abnormalities and in particular no endothelial cell damage.

Morphological Examination of Different Tissues of Mice Bearing IFN-α Cl-11 FLC and TC-2 FLC

As most mice bearing IFN-α Cl-11 s.c. had persistent interferonemia (Table 1), we examined the morphology of different tissues of these mice to determine whether there were any systemic changes that might have been induced after tumor inoculation. We were especially interested in changes in the liver and spleen as 3Cl8 FLC-injected s.c. are highly metastatic to these organs.[30]

Liver: Presence of Extramedullary Hemopoiesis in the Livers of DBA/2 Mice Injected with IFN-α Cl-11 Cells and IFN-α1-Transfected L1210 Cells

Four mice in each group were sacrificed at 7, 13, and 20 days after inoculation of tumor cells as described in Table 1. At 13 and 20 days after subcutaneous tumor inoculation, a striking increase in the number of islands of hemopoietic cells was found in the livers of the Cl-11 treated mice when compared with the livers of mice injected with TC-2 cells or left uninjected (Table 2). This hemopoiesis was of both the erythroid and myeloid series confirmed ultrastructurally by the presence of either promyelocytes or normoblasts in the individual islands (Figure 5, A and B). Viral particles were never observed by electron microscopy in any of the cells in these islands.

Ultrastructural changes were also seen in the hepatocytes of the mice injected with Cl-11 cells with many fat globules present and, in addition, abnormal intracytoplasmic inclusions in the region of the endoplasmic reticulum. These inclusions consisted of a network of small interwoven tubules of ~20 nm in diameter, which were in direct continuity with the endoplasmic reticulum (Figure 6a) and were identical to the tubular aggregates previously described in the hepatocytes of newborn mice treated with IFN-α/β.[31] Similar tubular aggregates were seen within reticulocytes found in the liver sinusoids (Figure 6B). These tubular aggregates were never seen in the livers of untreated mice or mice injected with TC-2 cells.

As a further control to determine that IFN was responsible for the extramedullary hematopoiesis in the liver, we examined the livers of DBA/2 mice injected

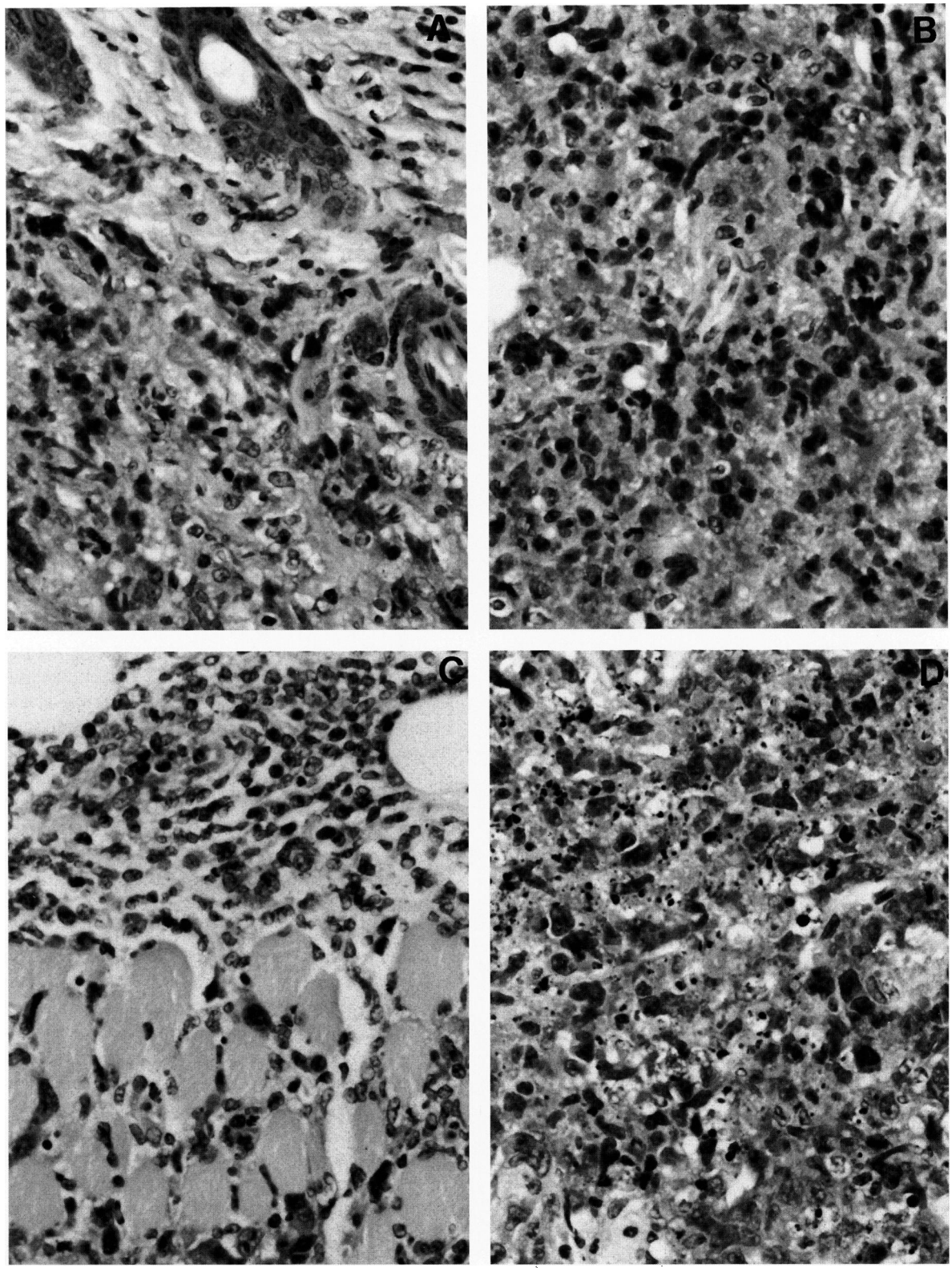

Figure 2. (A *and* B) *DBA/2 mouse 7 days after injection with* 2×10^5 *TC-2 cells.* A *shows invasion of the tumor to the level of the hair follicles with minimal infiltration by neutrophils and macrophages.* ×350. B *shows the tumor mass in which there is no necrosis.* ×350. (C *and* D) *DBA/2 mouse 7 days after injection with* 2×10^5 *IFN-α1 Cl-11 cells* C *shows invading tumor cells at the level of the platysma (lower part of micrograph) surrounded by a conspicuous infiltrate (upper part of micrograph) containing numerous macrophages.* ×350. D *shows a typical region of the tumor with extensive necrosis.* ×350.

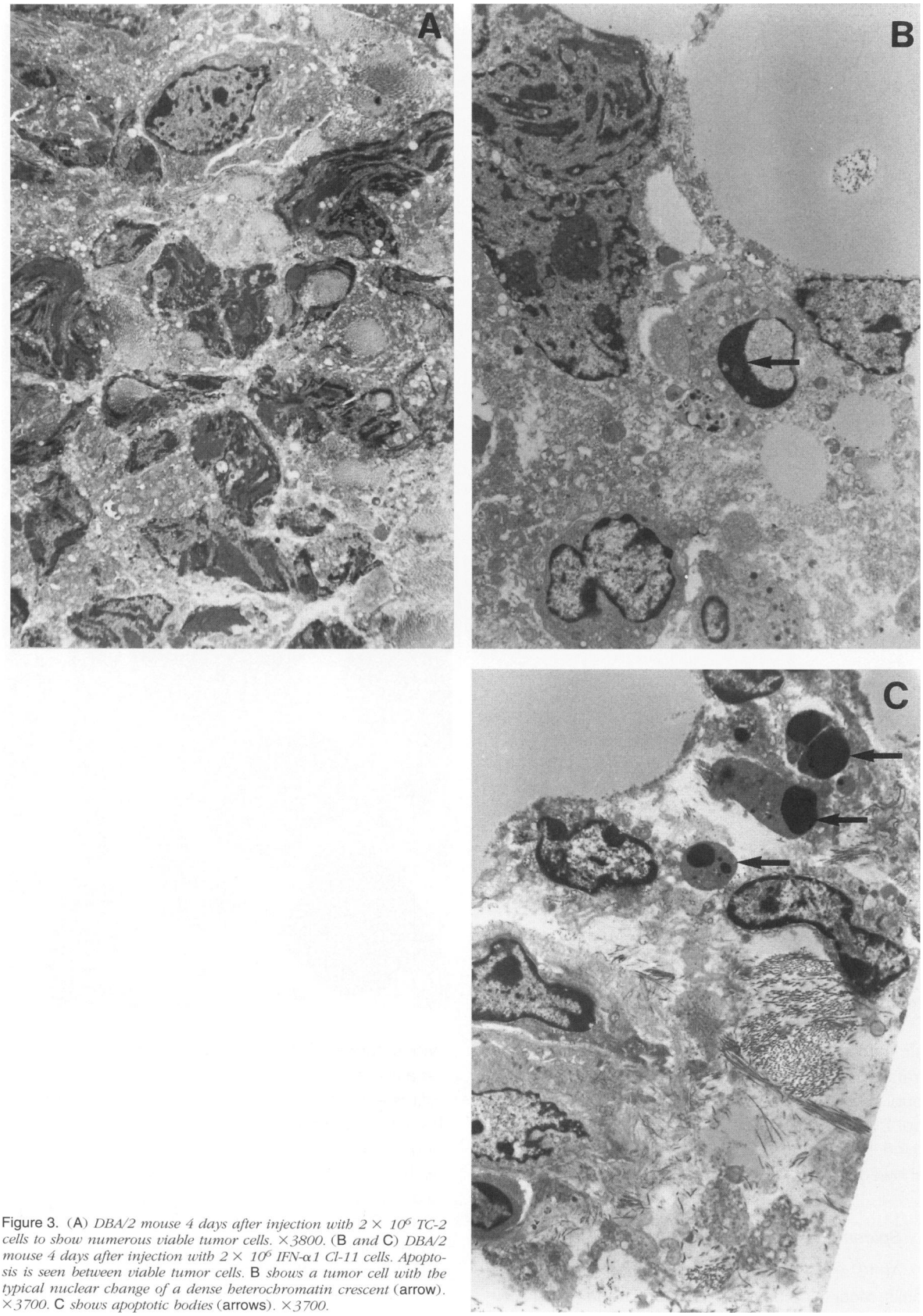

Figure 3. (A) *DBA/2 mouse 4 days after injection with 2 × 10⁶ TC-2 cells to show numerous viable tumor cells. ×3800.* (B *and* C) *DBA/2 mouse 4 days after injection with 2 × 10⁶ IFN-α1 Cl-11 cells. Apoptosis is seen between viable tumor cells.* B *shows a tumor cell with the typical nuclear change of a dense heterochromatin crescent* (arrow). *×3700.* C *shows apoptotic bodies* (arrows). *×3700.*

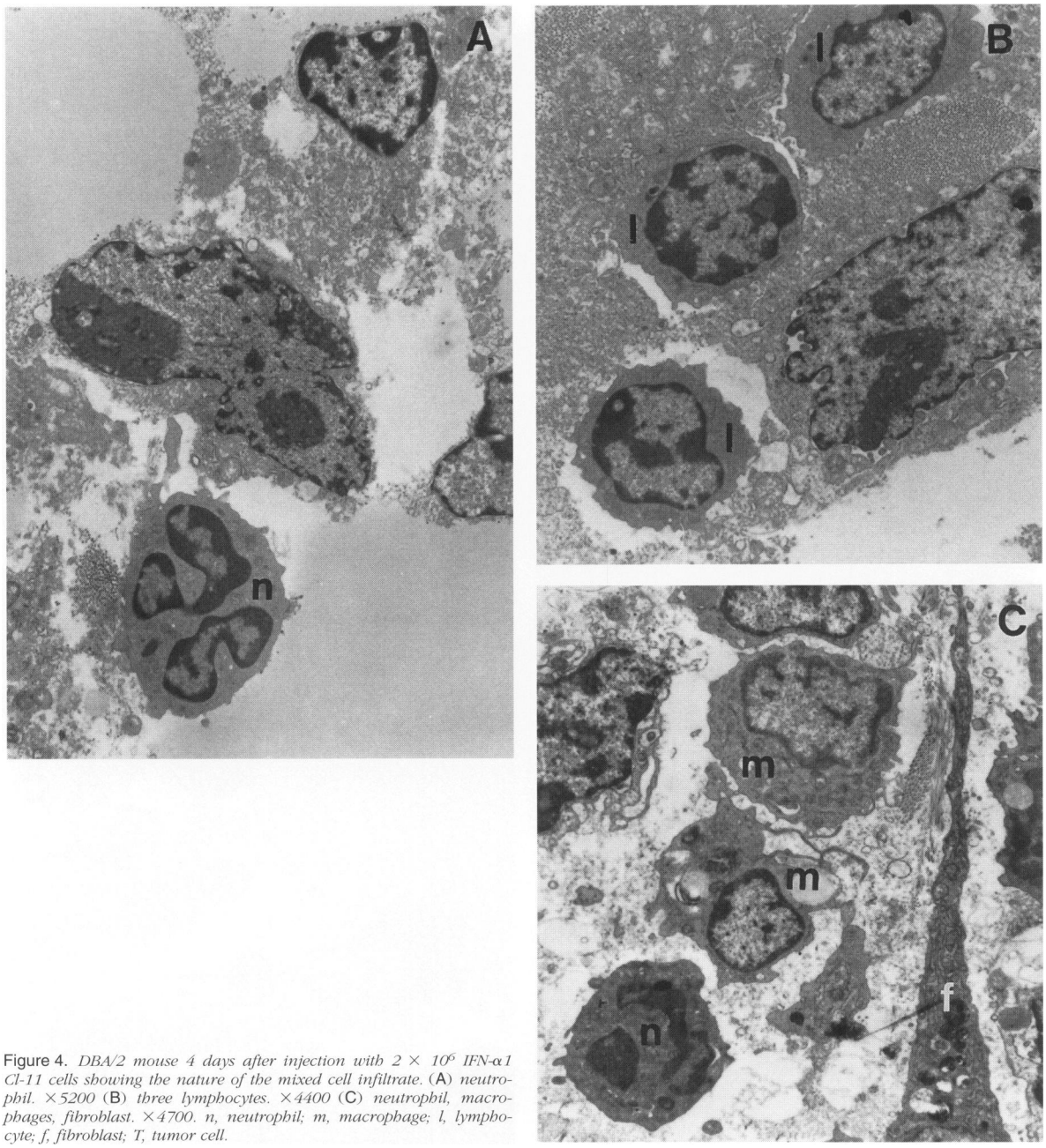

Figure 4. *DBA/2 mouse 4 days after injection with 2 × 10⁶ IFN-α1 Cl-11 cells showing the nature of the mixed cell infiltrate.* (A) *neutrophil. ×5200* (B) *three lymphocytes. ×4400* (C) *neutrophil, macrophages, fibroblast. ×4700. n, neutrophil; m, macrophage; l, lymphocyte; f, fibroblast; T, tumor cell.*

s.c. either with a high IFN producer clone of IFN-α1-transfected B lymphoma L1210 cells (T. Kaido, unpublished observations) or with the transfection control L1210 cells, or left untreated. Only the livers of mice injected with IFN-α1-transfected L1210 cells showed myeloid and erythroid hematopoiesis.

Spleen

At 13 and 20 days after s.c. inoculation of Cl-11 cells, most mice developed splenomegaly. Histologically these spleens showed hyperplasia but no obvious tumor. Therefore, we determined by FACS analysis the different cell populations in the spleens of mice sacrificed in the days following inoculation of tumor cells (as described in Table 1). The most striking changes were in the spleens of mice inoculated with IFN α1 Cl-11 cells. At 7, 13, and 20 days, there was a marked increase in the number of myeloid cells (Table 3) and at 13 and 20 days there was an increase in the number of macrophages ($F4/80^+$ cells). Using FITC-labeled anti-Friend viral gp70 protein we could not detect any FLC by FACS analysis in the spleens of mice bearing IFN-α1 Cl-11 tumors, whereas we

Table 1. *IFN Activity in the Sera of DBA/2 Mice at Different Times After s.c. Injection with IFNα1-Transfected or Control Plasmid-Transfected 3CL8 FLC*

Day after tumor injection	Mouse no.	Tumor cells injected		
		None	TC-2	IFN-α1 CL-11
7	1	<80	<80	<80
	2	<80	<80	640
	3	<80	<80	640
	4	<80	<80	640
13	5	<80	<80	160
	6	<80	<80	640
	7	<80	<80	320
	8	<80	<80	640
20	9	<80	ND	640
	10	<80	ND	3,840
	11	<80	ND	640
	12	<80	ND	<80

DBA/2 mice were injected s.c. with 2×10^6 cells. On the indicated day mice were bled from the retro-orbital plexis and serum was collected and stored at −20°C. IFN activity was determined after all samples had been collected. The values indicate the reciprocal of the IFN titer in the sera. ND, not done.

Table 2. *Myeloid and Erythroid Cell Colonies in the Livers of DBA/2 Mice Injected with IFN-α1-Cl-11 Cells**

Days after tumor cell injection	Mouse no.	Tumor cells		
		Uninjected	TC-2 cells injected	Cl-11 cells injected
7	1	4	7	2
	2	0	3	8
	3	1	8	9
	4	3	4	4
13	5	1	3	70
	6	1	2	76
	7	2	7	53
	8	3	0	29
20	9	1	ND	129
	10	3	ND	384
	11	4	ND	26
	12	13	ND	47

The experimental details are those given in the legend to Table 1, as these are the same mice. A total of 40 fields of liver sections were examined by light microscopy at a magnification of ×10.

*Total number of myeloid and erythroid cell colonies counted in the liver.

could detect viral gp70$^+$ cells in the spleens of TC-2 injected mice after 2 weeks (data not shown).

To determine whether the appearance of myeloid colonies in the liver and the increase in myeloid cells in the spleen were due to the presence of GM-CSF, we tested the sera of normal mice and mice injected with IFN-α1 Cl-11 cells for GM-CSF using a sensitive enzyme-linked immunoadsorbent assay. GM-CSF was not detected in any of these sera (data not shown).

We also tested these same spleens for NK cell activity. A marked increase in NK cell activity was observed only in mice injected with IFN-α1Cl-11 cells, and this activity remained elevated for 3 weeks (Figure 7). An increase in NK cell activity was not observed in DBA/2 bg/bg mice at any time tested after inoculation of Cl-11 cells (data not shown).

Kidney and Lung

We have previously described renal[32] and pulmonary lesions[33] in newborn mice (but not adult mice) treated with potent preparations of IFN-α/β. We examined the kidneys and lungs of DBA/2 mice bearing Cl-11 or TC-2 cell tumors. No abnormalities were detected.

Resistance of Mice Injected s.c. with IFN-α1 Cl-11 FLC to Challenge with Parental 3C18 FLC Injected i.v.

As DBA/2 mice bearing IFN-α1 Cl-11 tumors s.c. have interferonemia, it was of interest to determine their response to an i.v. injection of parental 3Cl8 FLC. As can be seen in Table 4, DBA/2 mice bearing Cl-11 cell tumors (but not TC-2 tumors) showed a marked resistance to i.v. injection of parental 3Cl8 FLC, a resistance that was observed as early as 3 days after inoculation of the IFN-α1 Cl-11 cells (exp. 1). This resistance to parental 3Cl8 FLC injected i.v. was also seen in DBA/2 beige (bg/bg) mice bearing s.c. Cl-11 cell tumors (Table 4, exps. 2 and 3). DBA/2 mice bearing Cl-11 cell tumors were not protected against an i.v. challenge with the unrelated ESb tumor, which also metastasizes to the liver and spleen.[34]

Use of IFN-α1-transfected CL-11 Cells as Therapy in DBA/2 Mice Injected I.V. with Parental Tumorigenic FLC

We have previously shown that a single s.c. injection of IFN-α1-producing FLC increased the survival time of mice even when this treatment was initiated up to 8 days after s.c. implantation of tumorigenic FLC.[4] The results in Table 5 show that treatment with either irradiated or viable IFN-α1-producing Cl-11 FLC initiated after i.v. injection of 3Cl8 FLC markedly increased the survival time of DBA/2 mice.

Discussion

There are three major points of interest in this article, which we will discuss separately.

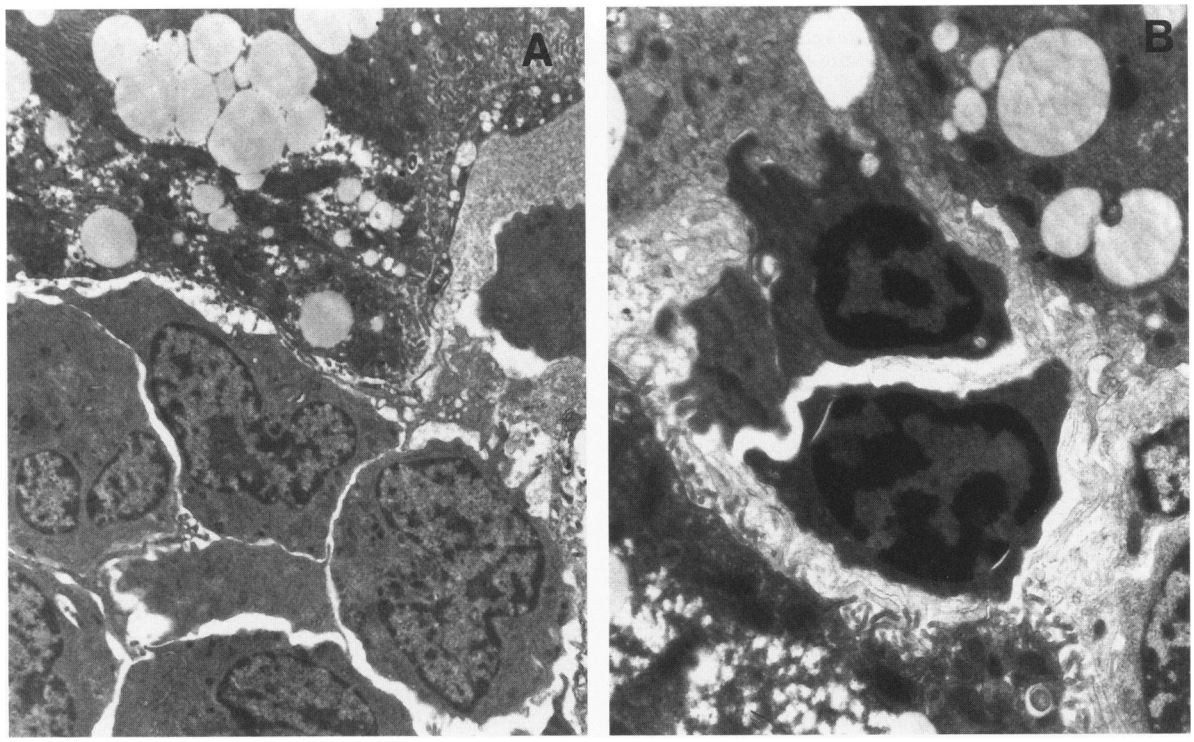

Figure 5. *Electronmicrographs from the liver of a DBA/2 mouse inoculated with IFN-α1 Cl-11 cells at day 13 (see legend to Table 1).* (**A**) *Liver sinusoid containing a group of promyelocytes. Numerous lipid droplets are seen in the hepatocyte. ×4200.* (**B**) *Liver sinusoid containing normoblasts characterized by the dense nuclear heterochromatin and sparse cytoplasmic organelles. Lipid droplets are present in the surrounding hepatocytes. ×8300.*

The Nature of the Local Response to Implantation of IFN-α1-Transfected FLC

The 3Cl8 clone of FLC transfected with the mouse IFN-α1 gene was selected for resistance to IFN-α/β and was previously shown to be totally resistant to IFN-α/β *in vitro*[11, 12] and *in vivo*.[13, 14] To our knowledge, no other tumor cell line has been so extensively characterized as to its resistance to the cytokine used to transfect. In most other studies using cytokine-transfected tumor cells, only resistance to the *in vitro* antiproliferative effects of the cytokine, such as TNF,[16,18,19] IL-7,[35] and M-CSF[36] has been investigated. In some systems it has been shown that the cytokine-transfected cells were resistant to the antiproliferative effects (eg, IFN-γ) but that the expression of H-2 antigens was increased.[17, 37] Thus, it is difficult in such instances to determine whether the decreased tumorigenicity of the cytokine-transfected tumor cell was due to direct effects of the cytokine on the tumorigenic phenotype (direct antiproliferative effects, effects on the expression of cell surface antigens, etc.) or due to host-mediated effects. We conclude that in our system the decreased tumorigenicity was due to the effects of secreted IFN-α on the host rather than to direct effects of IFN-α on the tumor cells themselves.

We have previously shown that exogenous IFN-α/β induced an ischemic necrosis of well established 3Cl8 FLC tumors implanted s.c..[30] This necrosis was considered to be due to IFN-induced damage to the endothelium of the tumor blood vessels.[38] Here, we have investigated the kinetics of the early events following the implantation of the IFN-α-transfected FLC. Although initially (days 1 to 3) there was no difference between control and IFN-α1-transfected tumor cell growth, the IFN-α1-transfected tumors subsequently failed to grow. There was extensive individual tumor cell necrosis and apoptosis (Figures 2 and 3), but no vascular abnormalities were noted. Although we have noted apoptosis in another model with IFN-α1-transfected TS/A tumor cells[5] and it has been reported that IFNs can induce apoptosis *in vitro*,[39] apoptosis is often observed in degenerating tumors regardless of the immediate cause.[40–42]

Although there was a prominent host cell infiltrate of the IFN-α1 Cl-11 tumors composed of macrophages, neutrophils, lymphocytes, and fibroblasts, no one cell type predominated. This heterogeneous host cell response has been reported for other cytokine-transfected tumor cells. Important macrophage infiltrates have been reported for IL-2, IL-4, TNF, IFN-γ, and

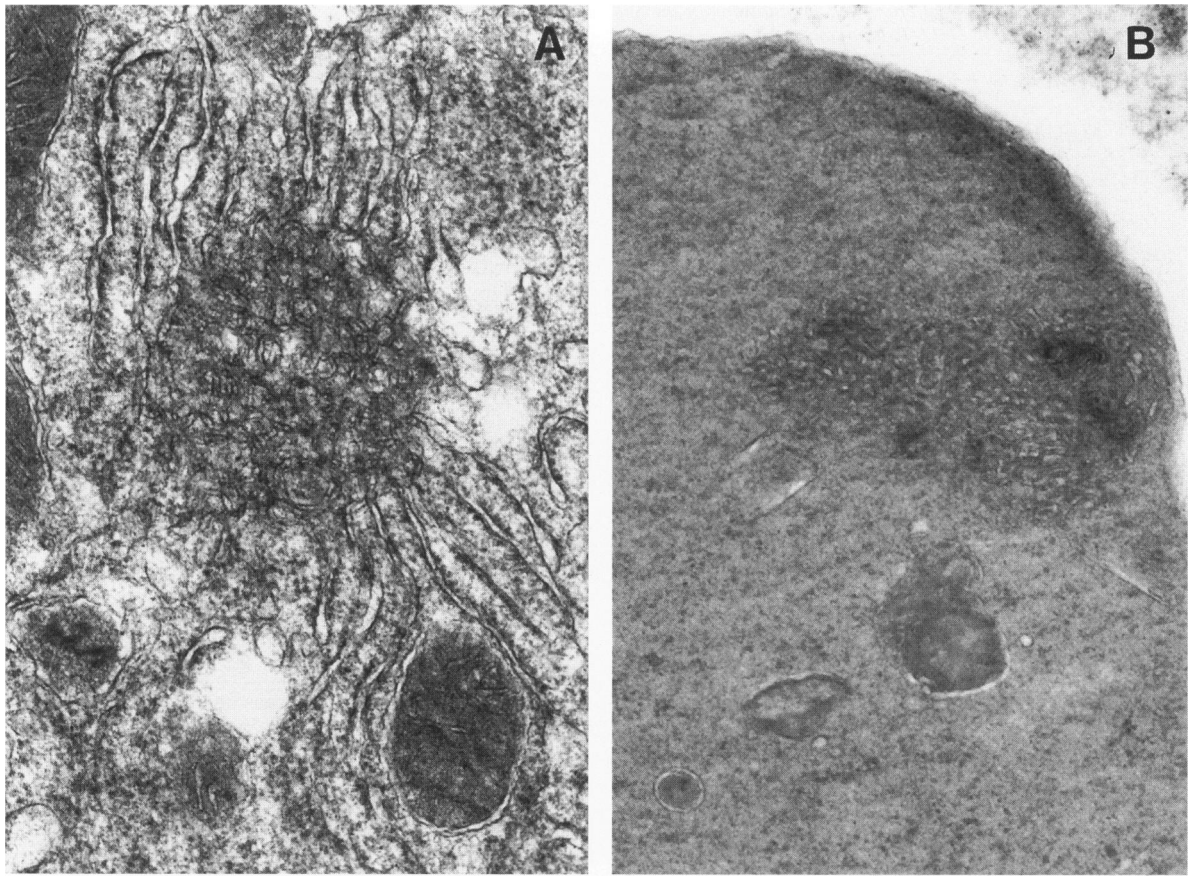

Figure 6. *DBA/2 mouse inoculated with IFNα1 Cl-11 cells at day 13.* (**A**) *Electronmicrograph showing a tubular aggregate composed of a network of small tubules continuous with the granular endoplasmic reticulum within a hepatocyte. ×42,000.* (**B**) *A tubular aggregate is seen within a reticulocyte. ×42,000.*

G-CSF-transfected tumor cells[17,18,43–45]; neutrophils and CD4$^+$ and CD8$^+$ lymphocytes for IL-2-transfected tumor cells[46]; and eosinophils for IL-4- and IL-5-transfected tumor cells.[43,47,48] The general impression is, however, that with few exceptions[46] most investigators have been unwilling to attribute the observed tumor necrosis to any particular host cell. In accord with these conclusions the attempts to deplete certain host cell populations with specific antibodies or to use immunosuppressed mice have often met with uncertain and sometimes conflicting results. For example, TNF-transfected tumor cells still failed to grow in allogenic nude mice in one study,[16] whereas in another study TNF-transfected cells grew in CD4$^+$ and CD8$^+$ lymphocyte-depleted mice.[19] Likewise, the role of T lymphocytes in IL-2 transfected tumor cells implanted s.c. was evaluated differently in three studies.[49–51] In our experiments, the decreased tumorigenicity of IFN-α1-transfected FLC could not be attributed solely to NK cells, CD4$^+$ or CD8$^+$ lymphocytes, or granulocytes (Figure 1, A–C), although the tumor cells did grow in totally immunodepressed SIA nude mice (Figure 1D). We conclude from our studies that the decreased tumorigenicity of IFN-α1 Cl-11 cells is due to the action of the secreted IFN on host tissues, and we suspect that multiple host cell types and mechanisms are involved.

The Systemic Response of Mice to Implantation of IFN-α1-Transfected FLC

Although administration of large amounts of IFN-α/β to suckling mice can induce liver necrosis and death,[52] a progressive glomerulonephritis,[32] or pulmonary cysts,[33] we have never observed any marked systemic changes in adult mice treated daily with the same potent preparations of IFN. However, the half-life of IFN-α/β in the circulation after i.v. or i.p. injection is measured in minutes or a few hours, respectively,[53] and we have never detected IFN-α/β in the blood 24 hours after inoculation of large amounts of IFN. In con-

Table 3. *Distribution of Different Cell Populations in the Spleens of DBA/2 Mice Injected s.c. with TC-2 or Cl-11 Tumor cells*

Marker	Tumor	Percent positive cells/spleen			Approximate total number of positive cells/spleen ($\times 10^{-7}$)		
		7 days	13 days	20 days	7 days	13 days	20 days
$CD4^+$	None	15.8 ± 2.0	15.7 ± 2.5	13.3 ± 2.7	2.8	2.5	2.7
	TC-2	16.1 ± 0.4	12.2 ± 0.7	ND	3.4	2.2	ND
	Cl-11	15.3 ± 1.1	10.1 ± 0.5	8.5 ± 0.9	2.8	2.9	2.9
$CD8^+$	None	6.2 ± 1.2	6.5 ± 0.6	5.2 ± 0.3	1.1	1.0	1.0
	TC-2	6.2 ± 0.4	5.5 ± 0.3	ND	1.3	1.0	ND
	Cl-11	6.1 ± 0.5	4.8 ± 0.4	4.7 ± 0.7	1.1	1.4	1.6
IgG^+ (B cells)	None	63.7 ± 1.9	58.9 ± 1.1	63.6 ± 1.2	11.5	9.4	13.0
	TC-2	61.4 ± 1.0	51.0 ± 2.0	ND	12.9	9.2	ND
	Cl-11	68.0 ± 0.6	46.9 ± 1.8	40.1 ± 0.9	11.2	14.0	14.0
Gr-1^+ (myeloid)	None	11.6 ± 4.3	6.4 ± 0.3	7.9 ± 1.2	2.1	1.0	1.6
	TC-2	16.1 ± 2.2	8.9 ± 0.5	ND	3.4	1.6	ND
	Cl-11	*32.3* ± 1.4	*21.5* ± 2.1	*26.2* ± 4.7	*5.8*	*6.2*	*8.9*
F4–80^+ (macrophages)	None	16.0 ± 1.1	10.1 ± 0.9	14.4 ± 0.5	3.0	1.6	2.3
	TC-2	20.6 ± 1.0	9.3 ± 1.1	ND	4.3	1.7	ND
	Cl-11	15.7 ± 0.6	*18.8* ± 2.2	*18.3* ± 0.9	2.8	*5.4*	*6.2*
Total cells per spleen ($\times 10^{-8}$)	None				1.8	1.6	2.0
	TC-2				2.1	1.8	ND
	Cl-11				1.8	2.9	3.4

The numbers represent the mean ± SEM of four spleens as described for Table 1. The techniques have been described in Materials and Methods. ND, not done. The numbers in italics refer to significant differences.

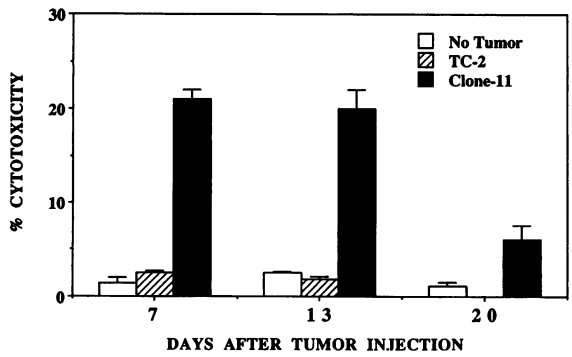

Figure 7. *Natural killer cell activity in the spleens of DBA/2 mice after s.c. injection with TC-2 and Cl-11 cells. The % cytotoxicity is the mean ± SEM of the same four spleens as described for Tables 1 and 2. Spleen cell: Target cell ratios of 100:1, 50:1, 25:1, and 12.5:1 gave comparable results. For convenience, only the results of the ratio of 100:1 are presented. The techniques have been described in the Materials and Methods.*

trast, in mice injected s.c. with the IFN-α1-transfected Cl-11 cells, significant amounts of IFN can be detected in the blood at any given time (see ref. 4 and Table 1). Thus, the mouse is exposed continuously to high blood levels of IFN. We know of no other experimental model in which high levels of IFN are produced constitutively *in vivo*. IFN-α has been detected in the blood of patients with a variety of autoimmune diseases[54] including severe AIDS,[55] and in the blood of macaque monkeys infected with simian immunodeficiency virus (M. Tovey, unpublished results), but in virtually all other instances IFN can be detected for only a very short period of time after viral infection.

The most striking effect observed in mice bearing IFN-α1-transfected Cl-11 tumors with persistent interferonemia was the development of extramedullary erythroid and myeloid hematopoiesis in the liver (Table 2, Figure 5, A and B). Extramedullary hematopoiesis was not observed in the livers of mice inoculated with the transfection control FLC or left untreated (Table 2). Likewise, FACS analysis of the spleens of mice with IFN-α1 Cl-11 tumors, but not TC-2 tumors, showed a marked increase in the number and percent of myeloid cells (Table 3). The finding that similar islands of extramedullary hematopoiesis were also observed in the livers of mice bearing another IFN-α1-transfected high IFN-secreting tumor cell (the B-cell lymphoma L1210) but were not observed in mice with control transfected L1210 cell tumors strengthens our conclusion that IFN-α was responsible for inducing this hematopoiesis. We are not aware of any report of extramedullary hematopoiesis in IFN-treated patients and we have not observed these changes in IFN-treated adult mice. However, as stressed above, the effects of constant exposure to persistently high levels of IFN in IFN-α1 Cl-11 tumor-bearing mice are probably quite different from those observed after single or repeated injections of IFN.

Table 4. *Challenge of DBA/2 Mice Bearing IFN-α$_1$-Transfected Tumors with Parental FLC*

Experiment	Cells injected s.c.	Days before challenge	Tumor cell challenge	Mean day of death (±S.E.) Normal DBA/2 +/bg mice	Mean day of death (±S.E.) Mutant DBA/2 bg/bg mice
1	None	3	3CI8 FLC	14.8 ± 0.3	Not tested
	TC-2	3	3CI8 FLC	17.0 ± 1.5 *	
	CI-11	3	3CI8 FLC	72.0 ± 9.0	
	None	3	ESb	8.0 ± 0.0	
	TC-2	3	ESb	8.0 ± 0.0 NS	
	CI-11	3	ESb	9.5 ± 0.6	
2	None	3	3CI8 FLC	15.0 ± 0.4	12.0 ± 0.0
	TC-2	3	3CI8 FLC	18.3 ± 2.3 †	Not tested †
	CI-11	3	3CI8 FLC	34.3 ± 5.6	24.0 ± 4.9
	None	3	ESb	8.5 ± 0.3	8.0 ± 0.0
	TC-2	3	ESb	8.7 ± 0.3 NS	Not tested NS
	CI-11	3	ESb	11.2 ± 0.7	8.0 ± 0.0
3	None	10	3CI8 FLC	10.0 ± 0.7	8.0 ± 0.0
	TC-2	10	3CI8 FLC	16.3 ± 1.6 †	Not tested †
	CI-11	10	3CI8 FLC	35.8 ± 5.9	27.0 ± 4.9

Exp. 1: Male, 6–8 week old DBA/2 +/+ mice were injected s.c. with 2 × 10^6 TC-2 control transfected 3CI8 FLC, CI-11 IFN-α^1-transfected 3CI8 FLC, or left uninjected. Three days later mice were injected i.v. with either 1 × 10^5 parental 3CI8 FLC or 2 × 10^4 ESb lymphoma cells. (n = 4). Exp. 2: Male, 10-week-old DBA/2 +/bg or bg/bg mice were inoculated s.c. and challenged i.v. as described in exp. 1. (n = 4 to 6). Exp. 3: Female, 10-week-old DBA/2 +/bg or bg/bg mice were inoculated s.c. as described in exp. 1 and then challenged 10 days later i.v. with 1 × 10^5 parental 3CI8 FLC. (n = 4). NS = not significant.

*$P < 0.001$, †$P < 0.01$.

Table 5. *Therapy of FLC-injected DBA/2 Mice with either Irradiated or Viable IFN-α$_1$-Producing CI-11 FLC*

Experiment (No. of 3CI-8 FLC injected i.v.)	Treatment	No. of dead mice/ Total mice	Mean day of death ± SE
1 (5 × 10^2)	None	10/10	18.8 ± 0.8
	Irradiated CI-11 FLC	2/10	*130,133* ***
	Viable CI-11 FLC	10/10	*87.9 ± 9.4*
2 (5 × 10^3)	None	10/10	15.0 ± 0.2 *
	Irradiated TC-2 cells	10/10	20.3 ± 0.9
	Irradiated CI-11 FLC	8/10	*48.5 ± 16.9* ***
	Viable CI-11 FLC	8/10	*59.2 ± 16.4*

Male 7–8-week old DBA/2 mice were injected i.v. with parental 3CI-8 FLC as indicated. Three hours after tumor injection, mice were injected with either irradiated FLC (20,000 rads; 10 daily s.c. injections of 2 × 10^6 cells) or viable cells (3 weekly s.c. injections of 2 × 10^4 CI-11 FLC). Other mice were left untreated. The experiment was terminated at 5 months. Numbers in italics refer to the groups showing a significant difference compared with controls.

*$P < 0.05$.

***$P < 0.001$.

At present, we have no explanation for this increase in extramedullary hematopoiesis in mice bearing IFN α1-secreting tumors. GM-CSF was not detected in any of the IFN-containing sera of mice injected with IFN-α1 CI-11 cells, but it is possible that GM-CSF was present in physiologically active amounts in the liver and spleen of these mice, or that other hematopoiesis-stimulating cytokines were involved. Extramedullary hematopoiesis in mice persists normally for a few weeks after birth,[56] and we have shown that even after active hematopoiesis ceases, the microenvironment of the liver conserves the capacity to induce erythroid differentiation.[57] It may be that these findings are related to the presence of extramedullary hematopoiesis in adult mice constantly exposed to IFN for several weeks. It is also possible that extramedullary hematopoiesis was secondary to an IFN-induced bone marrow suppression.

Further evidence that the secreted IFN was acting systemically stemmed from 1) the presence of characteristic IFN-induced tubular aggregates[31] in the endoplasmic reticulum of hepatocytes and reticulocytes, and 2) the persistently elevated levels of spleen NK cell activity (Fig. 7). This latter point may be of some therapeutic interest because the enhanced NK cell activity in mice injected with IFN or IFN inducers is of short duration and it is difficult to maintain persistently high levels of NK cell activity.[58]

Resistance of Mice with IFN-α1 Cl-11 FLC Tumors to Challenge with Parental 3Cl8 FLC Injected i.v. and Use of IFN-α1-Transfected Cells as Therapy

One of the important questions in considering the use of cytokine-producing tumor cells in therapy is whether there is any therapeutic advantage over cytokine administration alone or tumor cell therapy alone.[59] Our results show one such advantage, in that DBA/2 mice bearing IFN-α1-Cl 11 tumors develop an early specific resistance to i.v. challenge with parental FLC and that DBA/2 *beige* mice injected with IFN α1-Cl 11 cells were also protected (Table 4). This finding is of interest because we have never been able to protect DBA/2 beige mice treated with potent IFN preparations against an i.v. challenge of FLC.[34] Our results do not permit us to determine whether the difference in the results between these two systems is quantitative (ie, the difference between constant levels of blood IFN compared with repeated injections of IFN) or qualitative (ie, IFN secreted by and in association with immunogenic tumor cells compared with IFN therapy alone).

We have previously shown that a single or repeated s.c. injections of IFN-producing cells in mice with established metastatic tumors results in a definite antitumor effect.[4] We showed herein (Table 5) that repeated s.c. injection of irradiated or viable IFN-α1-producing cells increased mouse survival time even when treatment was initiated after i.v. inoculation of metastatic FLC. The finding that IFN-α1 gene therapy was effective in a highly metastatic tumor system (the FLC tumor) supports the hope that this approach may be of use in the treatment of patients with small residual metastatic foci.

In summary, our results have underlined a number of differences in the response of mice to IFN-α-transfected FLC and to exogenous IFN. Differences were observed in the local host response to the tumor, in the systemic response of the mouse to IFN, and in the resistance induced to challenge with parental tumor cells. Although cytokine-secreting tumor cells may still be at an early stage of development, this approach may add to our understanding of how cytokines act and may suggest better ways of using cytokines in therapy.

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