

Host Humoral and Cellular Immune Mechanisms in the Continued Suppression of Friend Erythroleukemia Metastases after Interferon α/β Treatment In Mice

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Summary

DBA/2 mice were injected intravenously with 2×10^6 3C18 Friend erythroleukemia cells (FLC), a cell line resistant to interferon α/β (IFN- α/β). Although daily administration of mouse IFN- α/β markedly increased the mean survival time, most IFN-treated mice continued to harbor FLC in different organs. To investigate the mechanisms responsible for this persistent suppression of FLC growth in IFN-treated mice, we undertook a series of adoptive transfer experiments with sera and spleen cells. Sera from FLC-injected, IFN-treated mice were very effective in conferring protection on DBA/2 mice even when injected systemically (intravenously) 18–24 h before intravenous challenge with FLC. These sera also exhibited antitumor activity when injected subcutaneously or intraperitoneally together with FLC. The protective factor in serum was shown to be an immunoglobulin. FLC-injected, IFN-treated mice developed antibodies to FLC demonstrable by radioimmunoassay and complement-dependent cytotoxicity. Sera from these mice recognized a specific 65-kD FLC membrane antigen(s) not detectable on membrane extracts from RBL-5 or ESb tumor cells, or on normal spleen cells. FLC-injected, IFN-treated mice also developed a specific cellular response demonstrable by transfer of protection with spleen cells injected intravenously or subcutaneously. Analysis of the responsible spleen cell populations indicated that the effector cells were neither T nor B cells. These results demonstrating the importance of host humoral and cellular immune mechanisms in the persistent suppression of FLC in IFN-treated mice may be relevant to the use of IFN- α/β in patients in whom tumors may regress and tumor cells may then remain latent for extended periods of time.

We have been interested in how IFN inhibits tumor growth and especially how it inhibits the development of tumor metastases. Using an experimental mouse model, we showed that IFN- α/β treatment was very effective in inhibiting the development of Friend erythroleukemia cell (FLC)¹ metastases and in increasing mouse survival time (1, 2). Use of IFN- α/β -resistant lines of FLC indicated that IFN was most likely not acting directly on the tumor cells themselves (3–6), but acted through host mechanisms (1), and our previous experiments emphasized the importance of

an intact immune system in achieving optimal therapeutic effects (7).

In the months after injection of tumor cells, it became apparent that FLC often remained latent in different organs even after IFN treatment had been discontinued. These observations suggested that either the phenotype of tumorigenicity of residual FLC had changed or that IFN-treated mice developed means of restraining FLC tumor growth. As our experimental results showed that FLC recovered from the livers of surviving IFN-treated mice still conserved their tumorigenic and metastatic capacity, we investigated the nature of possible host mechanisms. The results of the experiments presented herein show that FLC-injected, IFN-treated mice

¹ Abbreviations used in this paper: FLC, Friend erythroleukemia cell; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus.

developed specific humoral and cellular immune mechanisms that we believe are responsible for the persistent suppression of FLC growth in these mice.

Materials and Methods

Mice

DBA/2 mice were obtained from the pathogen-free breeding colony of the Institut de Recherches Scientifiques sur le Cancer (Villejuif).

Tumor Cells

The IFN- α/β -resistant clone 3C18 of FLC, obtained from Dr. E. Affabris (3) (Istituto Superiore di Sanità, Rome, Italy), was passaged in our laboratory by weekly intraperitoneal injection of DBA/2 mice. These cells were highly metastatic for the liver and spleen (8–10). The phenotype of IFN resistance of 3C18 FLC was confirmed several times in the course of these experiments by cultivating *in vivo* passaged cells in the presence of IFN- α/β (4). The ESb lymphoma, a spontaneous metastatic variant of a methylcholanthrene-induced T cell lymphoma (11), was obtained from V. Schirrmacher (Heidelberg, FRG) and was passaged intraperitoneally in DBA/2 mice. RBL-5 tumor cells, a Rauscher virus lymphoma line, were originally provided by J.P. Lévy (Paris, France) and passaged intraperitoneally in C57Bl/6 mice.

Quantitation of Tumor Growth

Intravenous. Mice injected intravenously with FLC die with macroscopic tumor invasion of the liver and spleen. The mean day of death of DBA/2 mice injected intravenously with serial 10-fold dilutions of 3C18 FLC is highly reproducible, and the standard error is very small. As can be seen in Fig. 1, the mean day of death for mice injected intravenously with 10^5 FLC was 12.4 ± 0.4 and 17.4 ± 0.6 d for mice injected with 10^2 FLC (i.e., a difference of 1,000-fold in the number of FLC injected can be equated with a ~ 5 -d difference in the mean day of death). Approximately four FLC constituted one LD₅₀ (1).

Subcutaneous. DBA/2 mice were shaved and 24 h later injected in the dorsal shoulder region with 3C18 FLC. The diameter of tumor nodules was measured with callipers.

Intraperitoneal. Mice were killed and the peritoneal cavity was washed with 2 ml of cold RPMI containing 2% FCS. The total number of cells recovered from each mouse was estimated by colony formation in agarose (4).

IFN- α/β and Control Preparations

Mouse IFN- α/β was prepared from suspension cultures of mouse sarcoma C243 cells infected with Newcastle disease virus (NDV). The methods of production, partial purification, and assay have previously been described (12). Control preparations consisted of the supernatant from cultures of C243 cells in which the IFN inducer, NDV, was omitted. IFN and control preparations were then concentrated 50-fold. IFN was assayed by inhibition of cytopathic effect of vesicular stomatitis virus (VSV) on L cells in monolayer cultures (0.2 ml/well) in microplates (Falcon Labware, Oxnard, CA). Units are expressed in mouse reference units. The specific activity of partially purified IFN was $\sim 2 \times 10^7$ U/mg protein.

Titration of Antibodies to FLC in the Sera of FLC-injected, IFN-treated DBA/2 Mice

Radioimmunoassay. 50 μ l of twofold serial dilutions of sera was

added to 3C18 FLC (5×10^4 cells in 50 μ l of PBS containing 1 mg/ml of BSA [PBS-BSA]) in Millititer™ SV 96-well filtration plates (SVLP 5.0 μ m hydrophilic durapore; Millipore Continental Water Systems, Bedford, MA). After 1 h at 4°C, cells were washed four times with 200 μ l/well of PBS-BSA, using a filter apparatus (Millititer™; Millipore Continental Water Systems) and a suction pump. 100 μ l containing $\sim 10^5$ cpm of anti-mouse Ig, ¹²⁵I-labeled species-specific F(ab)₂ fragment (Amersham Corp., Arlington Heights, IL), was added to each well. After 1 h of incubation at 4°C, cells were washed extensively. Dried filters were removed and counted for cell-bound radioactivity in a gamma counter (Beckman Instruments, Inc., Palo Alto, CA). Pooled sera from normal untreated mice were used as a negative control in each test. Triplicate samples for each serum dilution were used. The end-point dilution of a given serum exhibiting an anti-FLC reactivity significantly higher than the corresponding dilution of normal control serum ($p < 0.01$) was estimated as the specific anti-FLC titer.

Complement-mediated Cytotoxicity

Specific cytotoxic activity of sera was assessed in a chromium release assay. FLC from tumor ascites were radiolabeled by incubation of 5×10^6 FLC with 200 μ Ci of ⁵¹[Cr] sodium chromate (2 mCi/ml; CEA/ORIS, Gif sur Yvette, France) for 1 h at 37°C in 0.5 ml RPMI 1640 plus 5% FCS and then washed extensively. ¹⁰⁴ labeled target cells were incubated in round-bottomed microplates (Nunc, Roskilde, Denmark) with serial twofold dilutions of serum in 0.2 ml total volume at 4°C for 1 h and subsequently with rabbit complement diluted 1:30 for 30 min at 37°C; 0.1 ml of supernatant was collected at the end of the incubation period and counted for gamma radioactivity. The percentage of cytotoxicity was calculated with the following formula: $100 \times [(cpm \text{ released in serum} + C - cpm \text{ released in C}) / (cpm \text{ released in detergent} - cpm \text{ released in C})]$.

Identification by Western Blot of FLC Membrane Proteins Recognized by Antibodies in the Sera of FLC-injected, IFN-treated Mice

Cell membrane fractions were prepared by sucrose gradient as previously described (13, 14). The protein content of cell membrane fractions was assayed by the method of Lowry et al. (15). The NP-40 membrane protein fractions (corresponding to 10^7 cells) were diluted 1:1 with sample buffer (1% SDS, 2% mercaptoethanol, 1:10 electrode buffer, 10% glycerol), boiled for 4 min, and loaded onto a 10% acrylamide-SDS slab gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes for 4 h at 250 mA. Nitrocellulose membranes were saturated by incubation with 2% BSA in PBS for 1 h at room temperature. Strips were incubated overnight at 4°C with different sera diluted 1:100 in PBS containing 2% BSA. After washing with PBS containing 0.2% BSA and 0.1% NP-40, the strips were incubated with 1 μ Ci/ml ¹²⁵I-labeled anti-mouse Ig (Fab fragment), for 2–4 h at room temperature. The nitrocellulose strips were exposed at -80°C for 2–12 h with an intensifying screen.

Separation of Ig Fraction from Sera with Antitumor Activity

Protein A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was prewashed in a 40-mM Hepes buffer solution, pH 8.0. As a control, BSA was coupled to activated Sepharose 4B (7 mg/ml in Hepes 40 mM, pH 8.0). Potential sites of nonspecific binding were blocked with 1 M ethanolamine, pH 8.0, for 2 h

at room temperature. Conditions of elution and purification of IgG by anion exchange chromatography are given in the legend to Table 1.

Preparation of Splenic Cells and Cell Fractionation

Spleen cell suspensions were prepared aseptically in RPMI 1640 and 2% FCS. After sedimentation of clumps of tissue, the cell-rich supernatant was filtered on a loose-meshed nylon gauze, the cell suspension centrifuged, and the cell pellet resuspended. For cell fractionation, pooled spleen cells from several mice were prepared aseptically. T, B, and nul cell subsets were separated by negative panning selection (16). 5×10^7 splenocytes were incubated for 40 min at 4°C over Petri dishes precoated with antibodies (10 µg/ml of Ig-purified fractions from serum or ascites). After agitation, the nonadherent cells were carefully pipetted, washed three times, and counted. Cell aliquots were stained with fluoresceinated antibodies and analyzed by flow cytometry (FacsScan™; Becton Dickinson & Co., Mountain View, CA) for monitoring cell depletion. Antibodies used for depletion were clone 4.221 against Thy-1.2⁺ cells (17), clone LICR-LAU-RLI 172.4 against CD4⁺ T cells (18), clone 3.155 against CD8⁺ T cells (19), and sheep anti-mouse Ig polyclonal antibodies against B cells (Biosys, Compiègne, France). Fluorescein-conjugated antibodies from the following clones were used as monitoring reagents: clone 30-H12, anti-Thy-1.2⁺ (20); clone GK1.5, anti-CD4⁺ (21); clone 53-6.7, anti-CD8⁺ (20); clone MARK-1, anti-rat κ chain (Biosys); and affinity-purified anti-mouse Ig antibodies (Biosys).

Statistical Analysis

Within each experimental group, the one-way variance analysis test was used after verification of homogeneity of the variances by Bartlett's test, and subsequently, the means were compared using Duncan's and/or Tukey's test. When necessary, an inverse transformation of the survival times was performed to homogenize the variances.

Results

Effect of IFN-α/β Treatment on the Survival Time of DBA/2 Mice Injected Intravenously with 3C18 FLC. 10 experiments have been undertaken to determine the effect of IFN-α/β treatment on the evolution of FLC tumors and the survival time of DBA/2 mice injected with 2×10^6 3C18 FLC (equivalent of $\sim 5 \times 10^5$ LD50 [1]). The overall mean survival times were 8.7 ± 0.1 d for 115 untreated or control-treated mice, and 64.5 ± 10.1 d for 95 mice treated with at least 8×10^5 U of IFN-α/β daily for at least 1 mo (Fig. 2). All control FLC-injected mice died with characteristic massive tumor involvement of the liver and spleen (1). Of the 95 IFN-treated mice, 46 died with tumor involvement of the liver and spleen; 32 mice had no gross tumor involvement of the liver and spleen but had tumor involvement of different organs (kidney, ovaries, submaxillary, retroaortic and retroperitoneal lymph nodes, meninges, and vertebral column); and 11 mice were found dead without any detectable gross tumor. The mean day of death for IFN-treated mice dying with tumor in the liver and spleen was 54.0 ± 12.3 d, and was 71.4 ± 18.0 d for mice dying without tumor in these

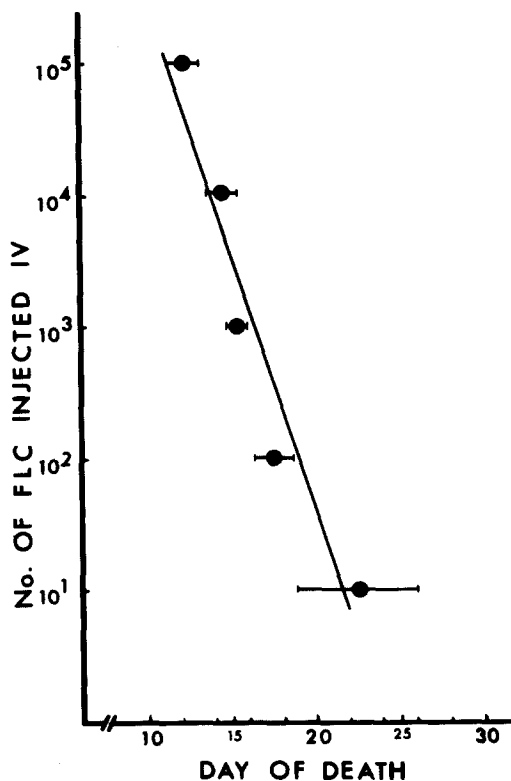


Figure 1. Tumorigenicity of 3C18 FLC injected intravenously in DBA/2 mice. 6-wk-old male DBA/2 mice were injected intravenously with 0.2 ml of 10-fold dilutions of 3C18 FLC. There were seven mice per 10-fold dilution. The slope coefficient of the dose-response (day of death) line of regression was highly significant ($p < 0.001$; $1/y = 9.98 \times 10^{-3} \ln x + 150.10^{-2}$; $r = 0.741$), with no significant deviation from linearity. The means \pm SE are indicated and were estimated after $1/y$ transformation ($y =$ day of death).

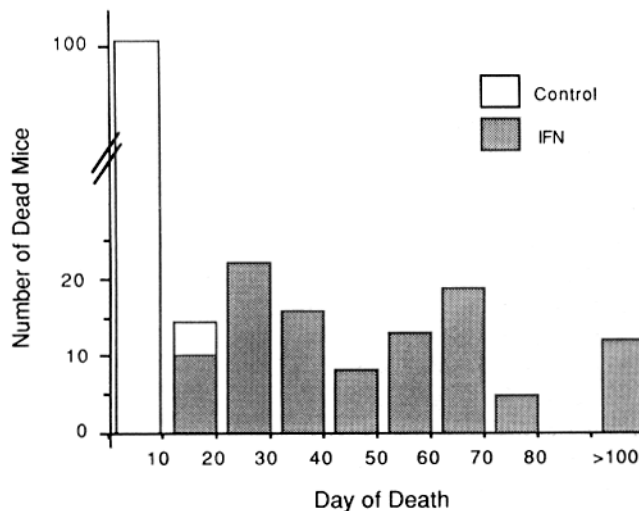


Figure 2. Effect of IFN-α/β treatment on the survival time of DBA/2 mice injected intravenously with 2×10^6 3C18 FLC. In all experiments, IFN treatment was initiated 3 h after tumor injection (1) and continued daily for at least 31 d and never beyond 72 d. Mice were injected intraperitoneally with different IFN-α/β preparations varying between 8×10^5 and 2.4×10^6 U/mouse/d.

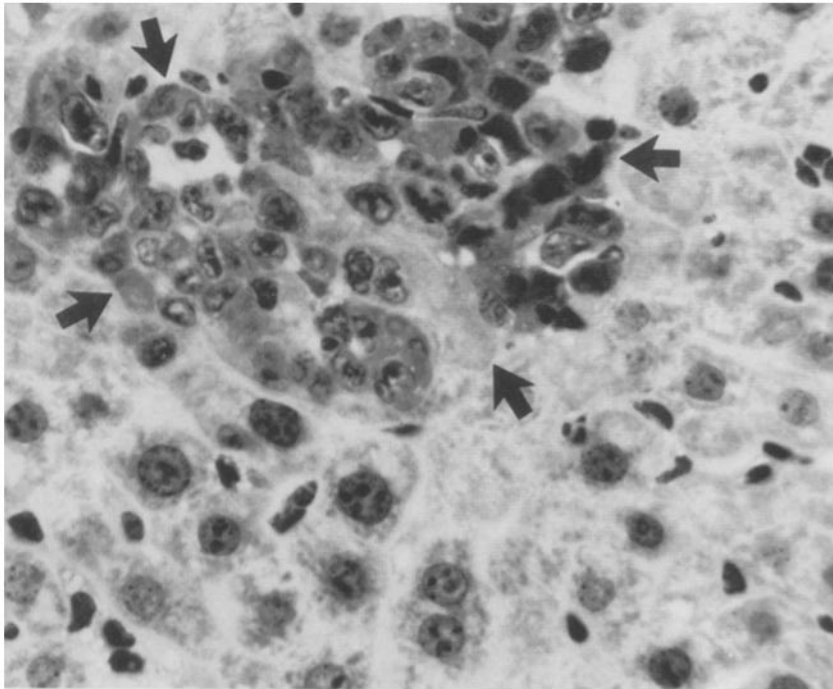


Figure 3. Morphologic appearance of a focus of FLC (delimited by arrows) persisting in the liver of an IFN-treated mouse killed 63 d after intravenous inoculation of 2×10^6 3C18 FLC and treated intraperitoneally daily for 1 mo with 1.6×10^6 U of mouse IFN- α/β . Hematoxylin and eosin ($\times 600$).

organs but with tumor elsewhere or without gross tumor (the difference was not significant). Of 12 mice surviving beyond 100 d (Fig. 2), three mice died at 110, 351, and 491 d with tumor involvement of the liver and spleen, whereas three other mice had no detectable tumor when killed at 150 or 812 d or found dead at 130 d. Six other mice are still alive.

Some FLC-injected IFN-treated mice appeared clinically well, but when killed were found on gross or microscopic examination to harbor tumor cells in some organs. Fig. 3 shows a focus of FLC in the liver of a DBA/2 mouse killed 63 d after intravenous inoculation of 3C18 FLC. No inflammatory host reaction was seen within or in proximity to these tumor foci.

These experimental results and those previously reported (1, 7) suggested that host mechanisms were responsible for continued suppression of tumor growth in various organs. To characterize the nature of these mechanisms, we undertook a series of passive transfer experiments using either sera or spleen cells from surviving FLC-injected, IFN-treated mice. In the experiments to be described, sera or spleen cells were taken from IFN-treated mice without gross evidence of tumor 18–154 d after inoculation of FLC.

Sera from FLC-injected IFN-treated Mice Can Transfer Protection to DBA/2 Mice Injected with FLC. As can be seen from the pooled results of 11 experiments illustrated in Fig. 4 A, a single injection of sera from surviving FLC-injected, IFN-treated mice conferred a marked protection on DBA/2 mice when injected intravenously 18–24 h before an intravenous challenge with FLC. Considering all the results together, the mean day of death was 13.5 ± 0.2 d for 66 untreated mice; 13.1 ± 0.2 d for 41 mice treated with normal sera; and 19.0 ± 0.6 d for 65 mice treated with sera from FLC-injected,

IFN-treated mice ($p < 0.001$). Analysis of each of the 11 experiments showed a highly significant ($p < 0.001$) difference in each experiment between the group of mice injected with sera from FLC-injected, IFN-treated mice and the two control groups of mice.

Kinetic experiments showed that the protective factor was not present in the sera of FLC-injected, IFN-treated mice 7 d after inoculation of the tumor (data not shown) but was present in sera from mice 18–58 d after inoculation of FLC in all 11 experiments. Sera from mice treated with IFN but not injected with FLC did not confer protection on DBA/2 mice (Fig. 4 A). Sera that protected mice against challenge with FLC did not protect DBA/2 mice challenged intravenously with methylcholanthrene-induced ESb lymphoma cells (Fig. 4 A).

Protection was also observed when sera from FLC-injected IFN-treated mice were mixed with FLC and injected subcutaneously (Fig. 5 A) or intraperitoneally (data not shown).

Transfer of Spleen Cells also Confers Protection. As can be seen from the pooled results of seven experiments illustrated in Fig. 4 B, spleen cells from FLC-injected, IFN-treated mice conferred some degree of protection on DBA/2 mice when injected intravenously 18–24 h before challenge with FLC. Considering all the results together, the mean day of death was 13.3 ± 0.2 d for 38 untreated mice; 13.5 ± 0.2 for 38 mice injected with normal mouse spleen cells; and 16.3 ± 0.7 d for 51 mice injected with spleen cells from FLC-injected, IFN-treated mice ($p < 0.001$). Analysis of the results of each individual experiment showed a highly significant difference ($p < 0.001$) in five experiments between the protection induced in mice receiving spleen cells from FLC-injected, IFN-treated mice and the two groups of control mice. No significant

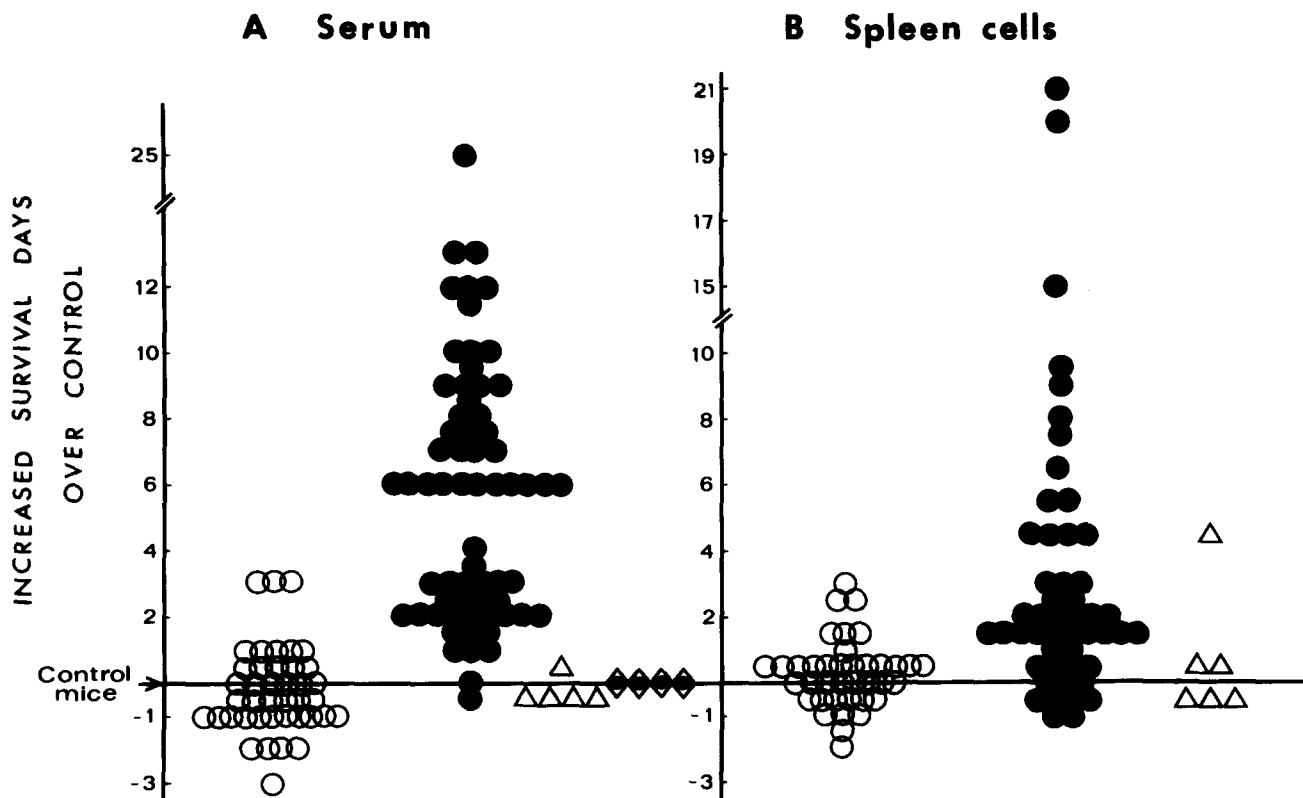


Figure 4. Capacity of sera or spleen cells from surviving FLC-injected, IFN-treated mice to confer protection on DBA/2 mice injected intravenously with 3C18 FLC. In 11 experiments, male or female adult DBA/2 mice were injected intravenously with 2×10^6 3C18 FLC and treated daily for at least 3 wk with mouse IFN- α/β ($\geq 8 \times 10^5$ U/mouse/d). 18–154 d thereafter, surviving mice were killed and sera or spleen cells from mice without gross evidence of tumor were injected intravenously into normal adult male or female DBA/2 mice. In each experiment, other groups of mice were injected with normal DBA/2 mouse sera or spleen cells or left untreated. 18–24 h later, all mice were injected intravenously with 8×10^4 to 1.6×10^5 3C18 FLC ($\sim 2\text{--}4 \times 10^4$ LD₅₀). The mean day of death of five to six untreated mice in each experiment was taken as the baseline (solid horizontal line). The day of death of serum-treated mice or mice injected with spleen cells is plotted in relation to the mean survival time of the untreated mice in each experiment. Each symbol represents, therefore, an individual serum-treated or spleen cell-injected mouse challenged with 3C18 FLC. (A) Individual serum samples or pools of sera from 3–16 FLC-injected, IFN-treated mice were injected (0.2 ml of undiluted sera) into 65 adult DBA/2 mice (●). Sera from normal DBA/2 mice (○) or mice treated with IFN- α/β but not injected with FLC (Δ) were injected into 41 or 5 other DBA/2 mice, respectively. DBA/2 mice receiving sera from FLC-injected, IFN-treated mice but challenged intravenously with 6×10^4 ESb lymphoma cells are indicated (\diamond). The survival of these mice was compared with that of untreated mice injected with ESb cells. (B) Between 1 and 2×10^7 spleen cells from surviving FLC-injected, IFN-treated mice were injected (in 0.2 ml) into 51 normal adult DBA/2 mice (●). Spleen cells from normal mice (○) or mice treated with IFN- α/β but not injected with FLC (Δ) were injected into 38 or 6 other DBA/2 mice, respectively.

difference was observed between experimental and control groups in two other experiments.

In four experiments, spleen cells from FLC-injected, IFN-treated mice were mixed with 3C18 FLC in ratios of 75:1 to 133:1 and injected subcutaneously. As seen from the results of an experiment illustrated in Fig. 5 B, a clear-cut inhibition in the development of subcutaneous FLC tumors was observed compared with the two groups of control FLC-injected mice.

Presence of Antibodies to FLC in the Sera of Surviving FLC-injected IFN-treated Mice. We first determined the presence of antibodies to FLC in FLC-injected, IFN-treated mice using a radioimmune binding assay. 19 of 20 IFN-treated mice developed significant antibodies to FLC, whereas untreated FLC-injected DBA/2 mice had not developed antibodies to FLC by the time of death (7–14 d) (Fig. 6).

As shown in Fig. 7, sera from surviving mice exerted a

clear-cut cytotoxicity on FLC target cells in the presence of complement. Sera from untreated mice or mice injected only with IFN were totally devoid of cytotoxic activity. Thus, the presence of complement-fixing antibodies resulted from specific immunization of the host against membrane-bound antigens expressed on FLC cells.

By Western blotting, a pool of sera from FLC-injected, IFN-treated mice exhibited a strong reactivity to specific FLC membrane antigen(s) in the 65-kD region of the gel (Fig. 8) but did not react with cell surface membrane proteins from other mouse tumor cells (i.e., RBL-5 and ESb cells) or normal DBA/2 spleen cells (Fig. 8). Sera from control FLC-injected mice did not detect the 65-kD antigen(s) (Fig. 8).

Evidence that the Anti-FLC Factor in the Sera of FLC-injected IFN-treated Mice Is an Ig. The capacity of these sera to confer protection on DBA/2 mice challenged intravenously with FLC was abrogated by preincubation with 2×10^8 FLC for

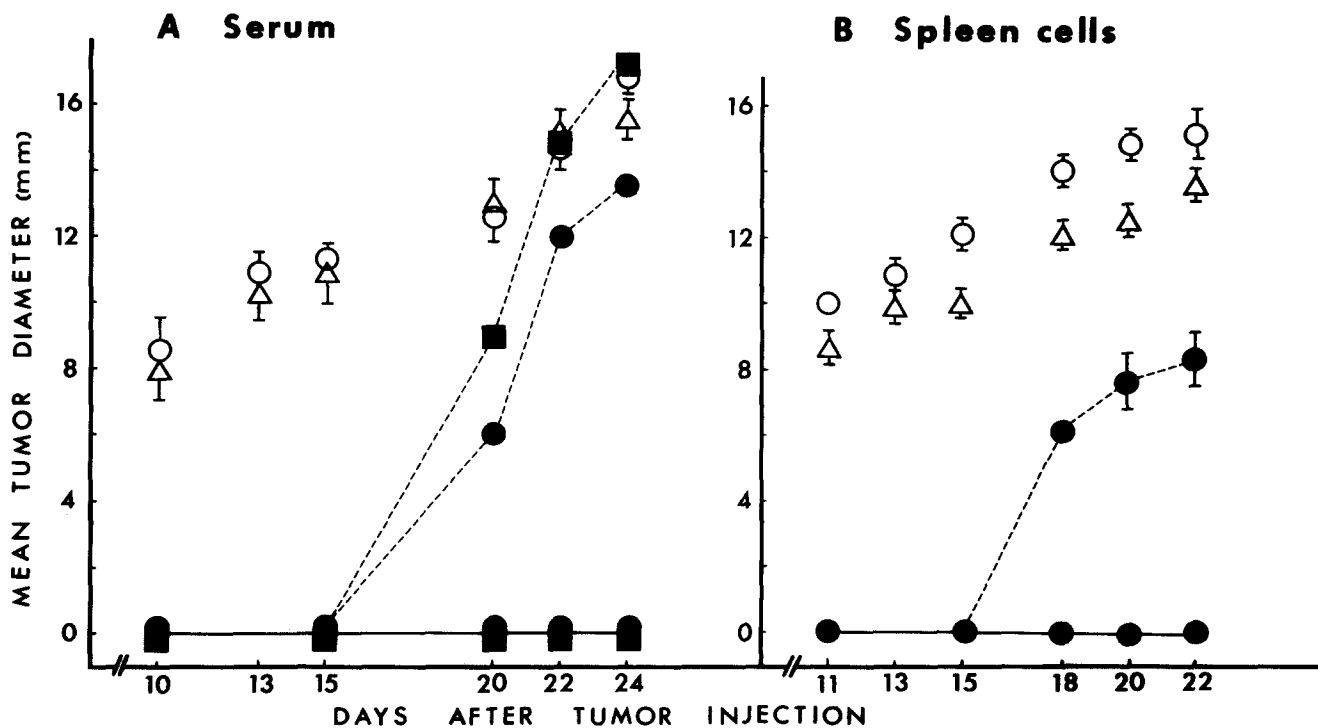


Figure 5. The inhibitory effect of sera or spleen cells from FLC-injected, IFN-treated mice on the growth of 3C18 FLC injected subcutaneously. Sera and spleen cells were obtained from 13 IFN-treated DBA/2 mice without gross evidence of tumor 32 d after intravenous inoculation of 3C18 FLC. (A) The pool of sera used in this experiment had been shown to be highly effective when injected intravenously in protecting mice challenged with FLC intravenously. In this experiment, 1:2 and 1:6 dilutions of sera were mixed in equal volumes with 3C18 FLC for 15 min at room temperature before injection. 5-wk-old female DBA/2 mice were injected subcutaneously with 0.2 ml of the mixture containing 2×10^5 3C18 FLC (i.e., $\sim 2 \times 10^3$ LD₅₀ when injected subcutaneously). There were five mice in the group injected with a 1:4 final dilution of this pool of sera and FLC (●), and five mice in the group injected with a 1:12 final dilution of sera and FLC (■). The 3 of 10 mice in these two groups developing tumors (one mouse at the 1:4 dilution and two at the 1:12 dilution of sera) are indicated (---). In another group of five mice, a 1:4 final dilution of normal mouse sera was mixed with FLC (○), and in a third group of five mice, BSA (100 μg/ml) was mixed with FLC (Δ). All 10 control injected mice developed subcutaneous tumors and died at 34.2 ± 2.9 and 35.9 ± 1.0 d with tumor metastases in the liver and spleen. Only 1 of 5 mice injected with FLC and a 1:4 dilution of protective sera and 2 of 5 mice at a 1:12 dilution of sera developing tumors died with tumor metastases. The remaining seven mice did not develop tumors and were tumor free when killed at 87 d. (B) 2×10^8 spleen cells/ml from FLC-injected, IFN-treated (●) or normal DBA/2 (○) mice were mixed in equal volumes with 1.5×10^6 3C18 FLC/ml and 0.2 ml of the mixture injected subcutaneously into 7-wk-old male DBA/2 mice. Each mouse received 1.5×10^5 3C18 FLC, which was $\sim 1.5 \times 10^3$ LD₅₀. There were eight mice per group. Another group of eight mice (Δ) was injected with FLC mixed with cell culture medium containing 2% FCS. In the group receiving spleen cells and FLC from FLC-injected, IFN-treated mice, four mice developed tumors as indicated (-----), and four mice did not develop subcutaneous tumors. All 16 control FLC-injected mice died with tumor metastases in the liver and spleen (mean day of death for untreated and normal spleen cell treated mice was 29.4 ± 1.3 and 24.9 ± 1.8 d, respectively). In contrast, 4 of 8 of the mice receiving spleen cells from FLC-injected, IFN-treated mice died with visceral metastases (mean day of death was 62.4 ± 8.1 d), and four mice were tumor free when killed 135 d after tumor inoculation. The symbols indicate mean tumor diameter (mm) per group. The SE is shown by the bar when it exceeded the diameter of the symbol.

2 h at 4°C. Thus, the mean day of death of five control FLC-injected mice was 12.1 ± 0.5 d vs. 17.0 ± 1.6 d for five mice pre-injected with sera from FLC-injected, IFN-treated mice ($p < 0.001$). In contrast, when this latter serum was preincubated with FLC, the mean survival time of five recipient mice was reduced to 13.2 ± 0.8 d, a difference that was significant compared with the mean survival time of mice injected with unabsorbed sera. Preincubation of a protective serum with 2×10^8 normal DBA/2 spleen cells under the same conditions did not diminish the protective activity of the serum.

We could show that the protective serum factor was an Ig by incubating serum from FLC-injected, IFN-treated mice with protein A coupled to Sepharose 4B. Thus, as can be

seen in Table 1, incubation with protein A-Sepharose depleted the serum of its capacity to confer protection, whereas the same serum incubated with BSA-Sepharose retained its protective activity. The eluate from the protein A-Sepharose 4B was dialyzed and applied to a Mono Q anion-exchange column. The material from the IgG peak demonstrated a significant antitumor activity (Table 1).

Analysis of the Effector Cells Responsible for Adoptive Protection. In an attempt to characterize the effector cells responsible for protection, we undertook three experiments in which spleen cells from tumor-free FLC-injected, IFN-treated mice were subjected to various depletion procedures, including removal of Ig⁺, or CD4⁺ or CD8⁺ lymphocytes. As shown in Fig. 9, the depletion of Ig⁺, or CD4⁺ or CD8⁺ lym-

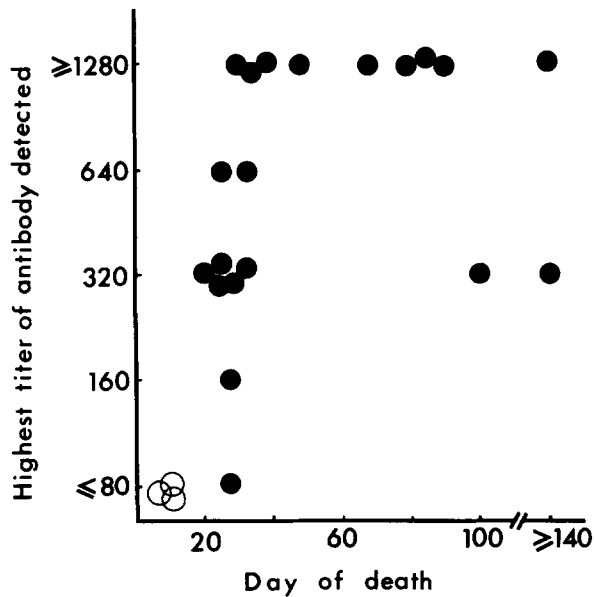


Figure 6. Presence of antibodies to FLC in FLC-injected, IFN-treated mice. 20 7-wk-old female DBA/2 mice were injected intravenously with 10^6 3C18 FLC. 3 h later and daily thereafter, mice were injected intraperitoneally with 10^6 U of IFN- α/β (●) or a control preparation (○). Sera from three control-treated mice were taken 7 d after tumor cell injection. The mean day of death of the six control mice was 7.8 ± 0.4 d. Orbital venous blood was taken every 14 d from tagged individual mice (a minimum of one sample to a maximum of six samples per mouse, depending on the survival time of each mouse). Sera were tested for the presence of antibodies to FLC as described in Materials and Methods. The highest titer of anti-FLC antibodies detected for each mouse (in the course of the total observation period) is plotted against the survival time of the same mouse.

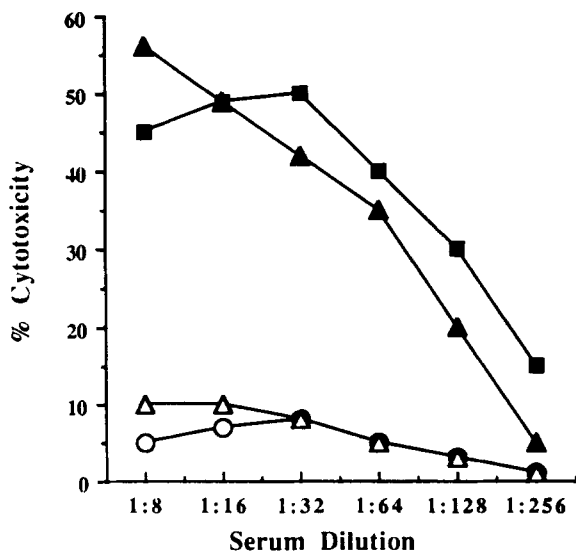


Figure 7. Cytotoxicity of serum from FLC-injected, IFN-treated DBA/2 mice for target 3C18 FLC. 7-wk-old female DBA/2 mice were injected intravenously with 2×10^6 3C18 FLC and treated daily with 1.6–3.2 $\times 10^6$ U of IFN- α/β . 28 d later, blood was taken from a surviving mouse (▲). 34 d later, a pool of sera from 15 other FLC-injected, IFN-treated mice without any visible tumor was also tested for cytotoxicity (■). Blood was also taken from a normal mouse (○) and an IFN-treated mouse not injected with FLC (Δ). Each point represents the mean of triplicate wells. The SE was $<5\%$.

Figure 8. Western blot analysis of sera from FLC-injected, IFN-treated DBA/2 mice. Plasma membrane fractions were extracted from 3C18 FLC, RBL-5 cells, ESb lymphoma cells, and spleen cells from untreated DBA/2 mice. Electrophoresis and Western blot analysis were performed as described in Materials and Methods. Pooled sera from untreated or control preparation-treated DBA/2 mice injected intravenously with 3C18 FLC were used as a negative control (taken 7–10 d after tumor cell injection). Sera from FLC-injected, IFN-treated mice were taken from a pool of 13 mice 32 d after tumor cell inoculation. None of the mice had visible tumor. Molecular weight markers were phosphorylase B (97,400); BSA (66,000); OVA (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); and lysozyme (14,400).

phocytes did not modify the protective capacity of the remaining spleen cells as determined either by the delay in the appearance of subcutaneous tumors or by the lower incidence of mice with tumors (Fig. 9 A), or by a decrease in the size of the tumors (Fig. 9 B). In the same experiments, spleen cells doubly depleted of $CD4^+$ and $CD8^+$ lymphocytes or $CD4^+$ and Ig^+ cells, or $CD8^+$ and Ig^+ lymphocytes still exerted an anti-FLC activity (data not shown). In another experiment, sequential depletion of Ig^+ , $CD4^+$, and $CD8^+$ lymphocytes did not modify the protective capacity of the remaining spleen cells (data not shown). These results indicated that the effector cells were neither B nor T lymphocytes.

Discussion

IFN- α/β has proven very effective in inhibiting the development of visceral metastases and in increasing survival time even after the intravenous injection of large numbers of FLC. About 10–15% of treated mice were considered cured (Fig. 2) (1, 2). The pattern of tumor growth in IFN-treated mice was totally different from that observed in control mice. Whereas the latter died rapidly with massive tumor involvement of the liver and spleen, IFN-treated mice died months later, some with tumor in the liver and spleen, and others with tumor in the kidneys, meninges, vertebral column, and lymph nodes. Many mice appeared clinically well for long periods but when killed were found to harbor nests of tumor cells in different organs. Thus, in most mice IFN therapy resulted in a suppression of tumor growth rather than an eradi-

Table 1. Abrogation of the Protective Capacity of Sera from FLC-injected, IFN-treated Mice by Passage on Protein A-Sepharose 4B

Group	Pretreatment of sera	No. of mice*	Mean day of death (\pm SE)	
A	Control	6	14.7 \pm 0.7	} NS s
	Protein A-Sepharose 4B	6	14.0 \pm 0.3	
	Sera from FLC-injected, IFN-treated mice†	6	23.8 \pm 1.6	
B	BSA-Sepharose 4B	6	23.8 \pm 1.6	}
	Buffer	5	12.8 \pm 0.2	
	Eluate from protein A-Sepharose 4B; Mono Q ANION exchange column	5	14.2 \pm 0.4	

(A) 0.75 ml of sera was mixed on a mechanical stirrer with 0.5 ml of protein A-Sepharose 4B gel or with BSA linked to Sepharose 4B for 1 h at 4°C. The gels were centrifuged at 2,500 rpm for 15 min and the supernatants tested for anti-FLC activity in mice.

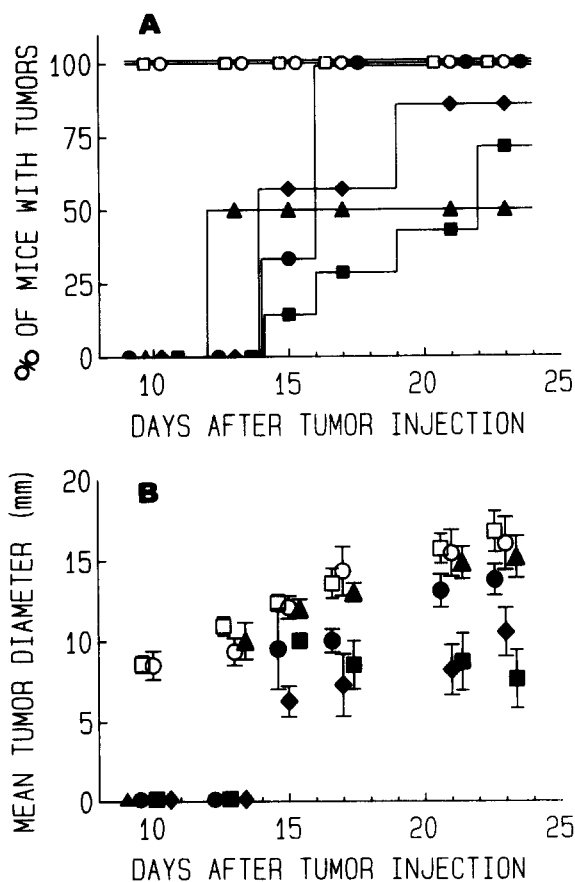
(B) The bound fraction of the serum was eluted from the protein A-Sepharose 4B gel with 0.1 M sodium citrate, pH 4.0, dialyzed (20 mM Tris-HCl, pH 7.7), and applied to a Mono Q (anion exchange) column (Pharmacia Fine Chemicals). Proteins were eluted with a linear NaCl gradient (0–0.5 M). Igs eluted \sim 0.2 M NaCl. 2 ml of the concentrated material from the IgG peak (2 mg) was injected intravenously into mice to determine anti-FLC activity.

* 7-wk-old male DBA/2 mice were injected intravenously with 0.2 ml of material to be tested. 1 h later, mice were injected intravenously with 10^5 3C18 FLC.

† 7-wk-old male DBA/2 mice were injected intravenously with 2×10^6 3C18 FLC and treated daily with 2.4×10^6 U of IFN- α/β . 22 d later, sera from 13 mice without gross evidence of tumor were pooled.

^s $p < 0.001$.

^{||} $p < 0.01$.



of tumor, and FLC persisted often in a latent state even after IFN treatment had been discontinued.

Although IFN treatment of some human and mouse tumor cells in culture has resulted in a reversion of the transformed phenotype to a normal phenotype (22–24), we found no evidence that IFN treatment of mice resulted in a similar reversion of the tumorigenic phenotype of FLC. Thus, FLC recovered from the liver of IFN-treated mice conserved the same capacity to form colonies in agarose and to induce tumors as the original FLC suspension (data not shown). On the other hand, having shown that an intact immune system was

Figure 9. Inhibitory effect of depleted and undepleted populations of splenic lymphocytes from FLC-injected, IFN-treated mice on the growth of 3C18 FLC tumors injected subcutaneously in DBA/2 mice. The donor spleen cells were taken from 9–10-wk-old male DBA/2 mice injected intravenously with 2×10^6 3C18 FLC and treated daily intraperitoneally with 2×10^6 U of IFN- α/β . 29 d later, six mice without any visible tumor were killed, and the spleen cells were depleted of Ig⁺, CD4⁺, or CD8⁺ lymphocytes by panning on antibody-coated plastic dishes. Monitoring of depleted cell populations by flow cytometry showed <2% of contaminating cells (see Methods and Materials). The undepleted and depleted spleen cell populations were mixed with FLC in a ratio of 50:1 and injected subcutaneously. There were six or seven mice per group. The symbols refer to mice injected with FLC and BSA (□); FLC and normal spleen cells (O); and FLC and total spleen cells from FLC-injected, IFN-treated mice (●). This total cell population was then depleted by panning of CD8⁺ lymphocytes (▲); CD4⁺ lymphocytes (◆); and Ig⁺ lymphocytes (■). (A) The percent of mice in each group developing subcutaneous tumors. (B) The mean tumor diameter \pm SE for the mice with tumors.

essential in achieving optimal therapeutic effects of IFN early after inoculation of FLC (7), we postulated that the immune system was involved in the continued suppression of FLC tumor in the months after tumor inoculation. The fact that FLC remained quiescent in different organs indicated that these host mechanisms probably operated at a systemic level. To gain some insight into the possible effector mechanisms, we undertook a series of adoptive transfer experiments using either sera or spleen cells from FLC-injected, IFN-treated mice. The potency of the anti-FLC effect of the serum from these mice was evident when one considers that: (a) this effect was demonstrable not only through close local contact of serum with FLC in a Winn-type assay (Fig. 5 A), but also by systemic administration of serum 18–24 h before the intravenous injection of FLC (Fig. 4 A); and (b) a single injection of serum resulted in a mean increase of survival time of 6 d (Fig. 4 A), which, extrapolating from the titration curves of FLC in normal mice (Fig. 1), was the equivalent of a 1,000-fold decrease in the initial tumor load.

Several arguments indicate that the protective factor in the serum of FLC-injected, IFN-treated mice is an Ig. First, FLC-injected, IFN-treated mice develop antibodies to FLC demonstrable by radioimmunoassay (Fig. 6), complement-dependent cytotoxicity (Fig. 7), and immunoblotting (Fig. 8). Second, the protective factor is tumor cell specific in that sera from mice injected with IFN but not injected with FLC are ineffective in transferring protection and that sera protective against FLC do not protect mice challenged with an unrelated tumor (ESb) (Fig. 4 A). Third, and most importantly, the protective factor can be absorbed out by preincubation with FLC; it binds to protein A-Sepharose and can be recovered in the IgG fraction after elution from the protein A-Sepharose gel column (Table 1).

We can only speculate at present as to the nature of the relevant FLC antigens that trigger the humoral response in IFN-treated mice. The immunoblots indicated that sera from 3C18 FLC-injected, IFN-treated mice reacted with a 65-kD cell surface protein(s) from FLC, not present on RBL-5 and ESb cells or on normal spleen cells (Fig. 8). The reactivity of these sera with a protein(s) in the 65-kD region of the gel could reflect the presence of antibodies to gp70-related Friend leukemia virus proteins that are highly expressed on the cell membrane of 3C18 FLC (25). However, these sera also reacted with membrane preparations from a Friend leukemia virus nonproducer cell line (745) (data not shown) that does not express the gp70 antigen (25), suggesting that these mice may be reacting to tumor-specific antigens common to FLC lines.

Spleen cells from FLC-injected, IFN-treated mice also mediated protection against FLC when transferred both systemically (Fig. 4 B) or locally in Winn assays together with FLC (Fig. 5 B). Analysis of the cell separation experiments aimed at identifying the effector cells showed that neither the depletion of T lymphocytes nor B lymphocytes reduced the protective potential of spleen cells from FLC-injected, IFN-treated mice (Fig. 9). It was therefore deduced that T and B lymphocytes did not directly participate in tumor suppression, at least at the effector stage of the process, and that null cells were most probably responsible for passive transfer of protection.

One of the major questions posed by our experiments concerns the role of IFN in the promotion of an immune response to FLC. IFN might enhance the humoral immune response itself or the effector mechanisms. There are a few examples of the enhancing effect of IFN- α/β on the primary antibody response of mice to sheep erythrocytes (26) and to rabies virus vaccine (27), but we are unaware of any published reports on the effects of IFN on the antibody response to tumor antigens. On the other hand, IFN might also affect the effector stage of the response by enhancing the expression of Fc γ receptors on lymphocytes (28). A third possibility would be that by exerting an antitumor effect, IFN treatment permitted the mouse to survive long enough to develop an immune response.

The experimental results presented herein on the mechanisms operative in the IFN- α/β -induced continued suppression of FLC tumor growth may be relevant to the use of IFN- α in patients with different forms of cancer. Thus, although IFN- α has induced remissions and tumor regression in some patients, tumor cells often remain latent for extended periods of time without being eradicated (29), suggesting that host mechanisms, humoral and/or cellular, maintain a continued suppression of tumor growth. It is also of interest that IFN- α appears to inhibit some tumors in which defined tumor antigens have been described, i.e., melanoma and renal cell carcinoma (30–32). It would be of interest to know whether IFN treatment affects the antibody response of these patients to tumor antigens, and if so, whether there is any correlation between a beneficial response to IFN therapy and the antibody response. The efficacy of IFN- α therapy may be related not only to the multiple effects of IFN on the tumor or the host, but also to the immunogenicity of a given tumor and the capacity of antibody or effector cells to reach the tumor.

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