JCI The Journal of Clinical Investigation

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J Clin Invest. 1983;72(2):707-717. https://doi.org/10.1172/JCI111020.

Research Article

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Role in Nude Mice of Interferon and Natural Killer Cells in Inhibiting the Tumorigenicity of Human Hepatocellular Carcinoma Cells Infected with Hepatitis B Virus

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ABSTRACT The human hepatoma cell line, PLC/PRF/5, which is persistently infected with hepatitis B virus (HBV), has integrated HBV-DNA, secretes HBV surface antigen (HBsAg), and does not grow readily in congenitally athymic (*nu/nu*) mice. The present investigation was undertaken to ascertain whether the low tumorigenicity of this cell line was governed by a host immune response and/or was related to expression of HBsAg. Subcutaneous injection of 4–5 × 10^6 cells into BALB/c nude mice produced localized encapsulated tumors with morphologic features of primary hepatocellular carcinoma in 25% of the animals within 29–40 d. No tumor growth was observed at lower cell inocula. In contrast, SK-HEP-1, an HBV-negative human hepatoma cell line, produced tumors at 1–5 × 10^6 cells inocula in 66% of the animals. Immunosuppression of mice with antilymphocyte serum (ALS) or irradiation increased tumor incidence in mice inoculated with 1 × 10^6 PLC/PRF/5 cells to almost 100% and produced local invasiveness. Immunosuppression also reduced the latency, i.e., time to tumor appearance, and increased mean tumor weight. These results suggest that tumorigenicity was limited by the host immune response.

The nature of the response was delineated by treating nude mice challenged with tumor cells with sheep anti-mouse interferon globulin (anti-IFN). When 2 × 10^6 cells were injected, tumor growth occurred in 75% of anti-IFN-treated mice, whereas controls injected with the same number of cells, but not receiving anti-IFN, failed to develop tumors. The tumors in the anti-IFN-treated mice were highly invasive and the latency period until tumor appearance was reduced to 3–5 d. An inverse correlation was found between susceptibility of the hepatoma cells to natural killer (NK) activity in vitro and resistance to tumor growth in vivo. In vitro cytotoxicity for PLC/PRF/5 cells was eliminated by anti-NK 1.1 and complement, establishing the effector cell as an NK cell. NK cell activity 14 d after inoculation of mice with PLC/PRF/5 cells was augmented against PLC/PRF/5 target cells but not against SK-HEP-1 cells. Treatment of mice with ALS, irradiation, or anti-IFN abolished NK activity against PLC/PRF/5 cells. Co-cultivation of nude mouse spleen cells with PLC/PRF/5 but not with HBsAg or SK-HEP-1 cells induced secretion of murine IFNα. These results suggest that the IFN/NK cell system may play a role in limiting tumorigenicity and invasiveness of HBV-infected human hepatocellular carcinoma cells by a mechanism similar to that found for other cells persistently infected with viruses.

INTRODUCTION

Hepatitis B virus (HBV)[1] infection is a worldwide public health problem. It is estimated that 200 million

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Received for publication 12 July 1982 and in revised form 22 April 1983.

[1] *Abbreviations used in this paper:* AC, sheep antiserum to contaminants in the IFN preparation; ADCC, antibody-dependent cell-mediated cytotoxicity; ALS, rabbit anti-

people are hepatitis B surface antigen (HBsAg) carriers. HBV is transmitted vertically and horizontally (1, 2), and in geographical areas where HBV is endemic, such as the Far East, Southeast Asia, and the Mediterranean Basin, HBsAg carriers represent 5–15% of the general population (2, 3). In recent years, evidence has accumulated that HBV is etiologically associated with primary hepatocellular carcinoma (PHC) (2, 3). Epidemiological studies indicate that in areas where HBV is endemic, 40–90% of patients with hepatocellular carcinoma are HBsAg carriers and that the tumor incidence is 20–200 times higher in carriers than in the general population (2, 3). Our ability to study the pathophysiology, immunopathology, and molecular biology of HBV infection and its relation to PHC has been enhanced recently by the establishment of human hepatoma cell lines that secrete HBsAg (4, 5).

Recently, we and others have characterized one of these human hepatoma cell lines, PLC/PRF/5, which is persistently infected with HBV. As shown by molecular hybridization studies, this cell line contains four to six copies per cell of integrated HBV DNA and secretes 300–500 ng HBsAg/10^6 cells/d in culture. These cells produce no other known HBV protein (such as hepatitis B core antigen [HBcAg] or hepatitis B e antigen [HBeAg]) and do not contain the viral DNA polymerase (4, 6–11).

Like many other virus-infected human tumor cell lines, PLC/PRF/5 cells do not readily grow in congenitally athymic (*nu*/nu) mice (12–14). However, immunosuppression by antilymphocyte serum (ALS) or irradiation (x-irrad) markedly augmented the tumorigenicity of PLC/PRF/5 cells in nude mice. This suggested a strong inverse correlation between tumorigenicity and the host immune response. Since PLC/PRF/5 cells express HBsAg, we investigated further the possibility that rejection of these cells was due primarily to the expression of HBsAg antigens, as has been shown in other systems (14, 15). Our results indicate that splenic lymphocytes from intact nude mice injected with PLC/PRF/5 cells develop cytotoxic activity against these cells in vitro and that the host cell responsible for this activity appears to be a natural killer (NK) cell. SK-HEP-1, a human hepatoma cells line that is HBsAg negative, is resistant to lysis. Because interferon (IFN) has been shown to be a major regulatory factor controlling NK activity (16, 17), we have attempted to assess the role of IFN in vivo and correlate this activity with viral expression and/or tumorigenicity.

mouse lymphocyte serum; anti-HBc, antibodies to HBcAg; anti-HBe, antibodies to HBeAg; anti-HBs, antibodies to HBsAg; anti-IFN, sheep antiserum to mouse IFN; DME, Dulbecco's-modified Eagle's medium; CPE, cytopathic effect; FCS, fetal calf serum; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HeLa-MS, HeLa cells persistently infected with measles virus; IFN, interferon; LPS, bacterial lipopolysaccharide; LSP, liver-specific protein; MEM, minimal essential medium; MLTC, mixed lymphocyte tumor cells cultures; NK, natural killer; PHC, primary hepatocellular carcinoma; poly I:poly C, polyinosinic-polycytidilic acid; VSV, vesicular stomatitis virus; x-irrad, irradiation.

METHODS

Mice

BALB/c *nu/nu* mice, originally obtained from Dr. G. Sato (University of California, San Diego), and CBA/N nude mice from Dr. Carl Hansen (National Institutes of Health, Bethesda, MD) were used. Mouse colonies were maintained in isolation within barrier quarters under positive air pressure with HEPA-filtered air. Breeding stocks were kept in laminar flow cage racks (Biogard, Baker Co., Inc., Sanford, ME); experimental animals were kept on open shelves in cages with individual filter bonnets (18). Mice were monitored regularly for virus infections, other pathogens, and disease processes by autopsy of randomly selected animals. 4–6-wk-old-mice were used in all experiments.

Cell lines

PLC/PRF/5, a human hepatoma cell line established by Alexander et al. (4), which synthesizes HBsAg in vitro (4, 6, 7), was obtained from Dr. I. Millman (Fox Chase Cancer Center, Philadelphia, PA). These cells also express several liver-derived proteins (5, 7, 19). SK-HEP-1 (20), another human hepatoma cell line, was a gift from Dr. Jørgen Fogh (Sloan Kettering Institute, Walker Laboratories, New York). This cell line expresses liver-specific protein (LSP) (19) but does not express HBsAg (unpublished observation). Both cell lines were grown as monolayers in minimal essential medium (MEM, Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 10 mM nonessential amino acids, 2 mM L-glutamine, penicillin (100 μg/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml).

YAC-1 cells, derived from a Moloney leukemia virus-induced lymphoma of A/Sn mice (21), were maintained in continuous suspension culture in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FCS, L-glutamine, and antibiotics as above. HeLa-MS, a HeLa cell line persistently infected with measles virus, was obtained from Dr. John Holland (University of California, San Diego) and was grown as monolayers in MEM supplemented with 8% calf serum (14, 22).

Mouse fibroblasts (L929), used for IFN assays, were grown on Dulbecco's-modified Eagle's medium (DME) supplemented with 10% FCS, glutamine, and antibiotics, and human trisomic-21 skin fibroblasts (Detroit 532; American Type Cell Culture Collection, Rockville, MD), used for human IFN assay, were grown on RPMI 1640 medium supplemented with 10% FCS, glutamine, and antibiotics.

Preparation of cell suspensions for injection

Subconfluent cultures were trypsinized with trypsin-EDTA, and cell viability was determined by trypan blue exclusion. The cells were centrifuged, resuspended in serum-free medium, and then injected subcutaneously at different concentrations into the flank region of nude mice (7, 14).

Detection and evaluation of tumor growth

Mice were examined daily for tumor appearance. Mouse weight was monitored at the beginning and end of experiments. Tumors were measured with a caliper at the longest longitudinal and horizontal axis and tumor volume was expressed in cubic millimeters. At the end of each experiment, mice were autopsied; tumor, liver, spleen, lungs, kidneys, lymph nodes, and bone marrow were fixed in 10% buffered formaldehyde and processed as described previously (7). Results were evaluated by three independent investigators. Tumor invasiveness was defined as macroscopic penetration into the thoracic or abdominal cavities and/or microscopic invasiveness from the innoculation site through the fibrous capsule surrounding the tumor and into the surrounding muscle, fat, or associated subcutaneous tissue.

Radioimmunoassay (RIA) for detection of HBV markers in nude mouse serum

Blood was collected by retrobulbar puncture or exsanguination of mice and was allowed to clot (7). Solid-phase RIA for detection of various HBV markers in mouse serum were performed with commercially available kits (Abbott Laboratories, North Chicago, IL) (7). Mouse serum was assayed for HBsAg and HBeAg and for antibodies to HBsAg (anti-HBs), HBcAg (anti-HBc), and HBeAg (anti-HBe).

Immunosuppression procedures

Antilymphocyte serum (ALS). Rabbit anti-mouse lymphocyte serum (M. A. Bio-Products, Walkerville, MA) was tested for lysis of BALB/c nude mouse spleen cells. Batches of ALS that caused 50% specific lysis of spleen cells at 1:1,000 dilution with complement were used. Mice were injected intraperitoneally with 0.1 ml of ALS 24 h before and after inoculation of PLC/PRF/5 cells and twice weekly thereafter, until the end of the experiments. No mortality occurred in mice treated with ALS alone.

X-irrad. Nude mice were irradiated with 600 rad from a ^{137}cesium source (Atomic Energy, Ottawa, Canada) 10 d before injection of PLC/PRF/5 cells (15).

Anti-IFN globulin. Sheep anti-mouse IFN serum (globulin fraction) was prepared as previously described (23, 24). The globulin at a dilution of 1:1,000, completely neutralized 200 U of mouse IFNα. Sheep antiserum to the contaminants in the IFN preparation (AC), which lacked anti-IFN activity, was used as a control. Both the anti-IFN serum and the AC serum were diluted 1:3 with phosphate-buffered saline (PBS) and absorbed with a one-third volume of packed BALB/c spleen cells and erythrocytes for 30 min at 4°C. Mice were injected intravenously with 0.1 ml of anti-IFN or AC serum concomitantly with the inoculation of tumor cells. Mice received a second injection of anti-IFN or AC serum 7 d later. There was no mortality in groups treated with anti-IFN or with AC serum alone.

Preparation of effector cells

Spleens from tumor-bearing or untreated nude mice were passed through a fine cellector sieve (Bellco Glass, Inc., Vineland, NJ), washed three times with PBS and resuspended in MEM plus 10% FCS at a concentration of 1×10^7 cells/ml. Spleen cells nonadherent to nylon wool were obtained according to the method of Julius et al. (25). Briefly, 2×10^8 spleen cells in RPMI 1640 medium with 10% FCS were added to a column of 0.6 g nylon wool, incubated for 45 min at 37°C, and eluted with 80 ml prewarmed RPMI 1640 plus 10% FCS. Recovery of viable cells after this procedure was ~15%. The eluted spleen cells, which were subsequently used for cytotoxicity assays, did not respond to concanavalin A (Con A) or bacterial lipopolysaccharide (LPS). Thus, this cell fraction was functionally depleted of macrophages and B cells.

Treatment of monoclonal anti-Thy 1.2

Monoclonal anti-Thy 1.2 antibody was purchased from New England Nuclear, Boston, MA. Spleen cells (5×10^7 cells/ml) were incubated with monoclonal anti-Thy 1.2 (final dilution 1:1,000) and rabbit low toxicity complement (Low Tox, Cederlane, Ontario, Canada; final dilution 1:12) for 45 min at 37°C in 5% CO_2.

Treatment with alloantiserum NK 1.1

Polyclonal mouse anti-NK 1.1 (26) was obtained from Dr. S. Pollack (Seattle, WA). 3×10^7 spleen cells were incubated with anti-NK 1.1 (final dilution 1:30) and rabbit complement (final dilution 1:30) for 45 min at 37°C. Treatment of nude mouse spleen cells with this antiserum reduced the splenic cytotoxicity against YAC-1 cells by 50%. Rabbit complement alone had no effect.

Natural cytotoxicity assay

Cytotoxic activity of mouse spleen cells against various target cells was performed as described by Minato et al. (15). Briefly, adherent target cells (PLC/PRF/5 or SK-HEP-1) were trypsinized, washed once in complete medium, and labeled with 100 μCi of ^{51}Cr (sodium salt, sp act 300–650 mCi/mmol, Amersham Corp., Arlington Heights, IL) for 1 h at 37°C in 5% CO_2 with periodic gentle agitation. Cells were washed three times, seeded in flat-bottomed microtiter wells at a density of 2×10^4 cells/well and incubated overnight. YAC-1 cells were labeled as described above and added to U-bottomed microtiter plates at a concentration of 1×10^4 cells/well. Effector spleen cell suspensions from tumor-bearing or untreated mice were added at ratios of 100:1, 50:1, and 25:1, centrifuged (100 *g* for 2 min), and incubated for 8 h for hepatoma cell lines or 4 h for YAC-1 cells. After incubation, the plates were centrifuged and 0.1 ml of the supernatant fraction was harvested from each well for determination of ^{51}Cr release. For maximal release, 0.1 ml of 2 N HCl was added to each well and specific release was calculated as previously described (15).

Spontaneous ^{51}Cr release was 20.2±0.9% (n = 23) for PLC/PRF/5 cells and 17.3±1.2% (n = 5) for SK-HEP-1 cells after 8-h incubation, and 20.8±4.1% (n = 5) for YAC-1 cells after 4-h incubation. In some experiments, cytotoxic activity of effector cells was determined by linear regression analysis of percent cytotoxicity against three effector/target ratios and was expressed as lytic units (LU) per 10^7 spleen cells (27). 1 LU was defined as the number of effector cells required to produce 20% specific lysis of 2×10^4 hepatoma cells.

IFN induction by mixed lymphocyte-tumor cell cultures (MLTC)

1×10^6 human hepatoma cells were cultured overnight in 24-well Linbro tissue culture plates (Flow Laboratories,

Inc., McLean, VA). The medium, was removed and 1–2 × 10^8 normal BALB/c *nu/nu* spleen cells in 1 ml of DME plus 10% FCS were added to the monolayers of hepatoma cells. After incubation for 24–36 h at 37°C in 5% CO_2, culture supernatants were collected, centrifuged, and stored at −70°C until use. 10–100 μg of purified HBsAg (a gift from Dr. L. R. Overby and Dr. I. K. Mushahwar, Abbott Laboratories) were added to 1 × 10^8 spleen cells prepared as above.

IFN titrations

Mouse IFN titers were measured by the microtiter method by determining 50% plaque reduction of encephalomyocarditis (EMC) virus on L929 cell monolayers. In these assays, one unit was approximately equal to one National Institutes of Health mouse IFN standard reference unit. Human IFN was measured by the inhibition of 50% of vesicular stomatitis virus (VSV) cytopathic effect (CPE) on monolayers of human trisomic-21 skin fibroblasts (Detroit 532). One unit in our assay system corresponded to 0.6 reference units. IFN titers were expressed as the reciprocal dilution that produced 50% plaque reduction or inhibition of CPE.

Polyinosinic-polycytidilic acid

Polyinosinic-polycytidilic acid (poly I:poly C, Sigma Chemical Co., St. Louis, MO) was injected intravenously (0.1 mg/mouse) 24 h before the natural cytotoxicity assay.

RESULTS

Augmentation of tumorigenicity of PLC/PRF/5 cells in immunosuppressed mice. When PLC/PRF/5 human hepatoma cells are injected subcutaneously into athmic nude mice, a small white nodule appears at the injection site within 3–5 d. This nodule may regress or form a tumor depending on the number of cells injected. The minimal number of PLC/PRF/5 cells required to produce tumors was 4–5 × 10^6 cells in BALB/c *nu/nu* mice and 3 × 10^6 cells in CBA/n *nu/nu* mice. 25% of untreated BALB/c *nu/nu* mice developed tumors within 29–40 d and in CBA/n *nu/nu* mice tumors appeared between 30 and 34 d after cell injection (Table I). In untreated nude mice,

TABLE I

Effects of Immunosuppression on Tumorigenicity of PLC/PRF/5 Cells in BALB/c and CBA/n Nude Mice

Mouse strain	Treatment	No. of injected cells × 10^6	Latency	Tumor frequency: No. mice with neoplasm / No. mice injected	P*	Invasiveness‡ +/−
			d			
BALB/c *nu/nu*	None	0.5–3§	—	0/10	—	−
		4–5§	29–40	7/28	—	−
		6–10	12–22	29/36	—	−
	ALS‖	0.001–2§	—	0/21		−
		3	16–19	4/6		−
		4–5	11–21	15/16	<0.02	+
		6–10	11–21	23/26		+
	X-irrad¶	5	11–21	9/9	<0.02	+
	Anti-IFN**	2	3–5	3/4		+
		5	3	5/5	<0.05	+
		10	3	4/4		+
CBA/n	None	2§	—	0/5		−
		3§	30–34	5/6		−
		5–10	12–14	6/7		−
	ALS	3–10	12–16	10/11		+

* *P* values assessed in accordance with the chi-square test. The data from the different treatment groups were compared with the untreated controls for one inoculum dosage, 4–5 × 10^6 cells.

‡ Invasiveness was established as macroscopic and/or microscopic tumor cell invasion through the fibrous capsule and into the thoracic and/or abdominal cavities (Results).

§ Mice were maintained for up to 60 d to determine whether tumors developed. All other mice were killed on day 21, except for the anti-IFN-treated group.

‖ Mice were injected intraperitoneally with 0.1 ml of ALS, 24 h before and after PLC/PRF/5 cell inoculation and twice weekly thereafter.

¶ Mice were irradiated with 600 rad 10 d before tumor cell injection.

** Mice were injected with 0.1 ml of anti-IFN intravenously concomitantly with the inoculation of PLC/PRF/5 cells, and 7 d later.

tumors by microscopic evaluation were localized, well encapsulated, and well vascularized. Treatment of BALB/c *nu/nu* mice with ALS or x-irrad resulted in augmented tumorigenicity of PLC/PRF/5 cells, as measured by a shorter latency period, increased tumor weights, and increased frequency of tumor development from 25 to 97–100% (Tables I and II). In contrast to untreated mice, tumors in immunosuppressed animals were invasive, with macroscopically evident penetration into the abdominal and thoracic cavities in 80% of the mice, and invasiveness at microscopic levels evident in all immunosuppressed mice. No metastases were detected by histopathological examination of several sections of paraffin-embedded material from lung, liver, kidneys, bone, or lymph nodes. Since these tissues were not cultured to maximize the chance of finding microscopic, metastatic lesions, definitive conclusions with respect to the presence or absence of metastases were not made. Similar results were obtained with CBA/n mice treated with ALS.

For comparison, BALB/c *nu/nu* mice were injected subcutaneously with 1–5 × 10^6 human hepatoma SK-HEP-1 cells that do not express HBsAg. Tumors appeared within 19–25 d in 8/12 animals.

Effect of treatment with anti-IFN globulin. Injection of sheep anti-mouse IFN globulin resulted in a greater augmentation of tumorigenicity of PLC/PRF/5 cells in BALB/c nude mice as compared with x-irrad or ALS treatment (Tables I and II). The number of cells required to produce tumors was reduced from 4–5 × 10^6 to 1 × 10^6 and tumor frequency was increased to virtually 100%. The latency period was shortened considerably, as tumors were palpable and visible within 3–5 d (Table I). Mean tumor weight in anti-mouse IFN-treated animals at 14 d after cell inoculation reached the same levels as tumors in ALS-treated or irradiated mice after 21 d. In untreated mice or mice treated with AC serum, there were no palpable or visible tumors by day 14 following injection of 2–5 × 10^6 tumor cells. However, tumor cells could be identified microscopically at the injection site. In contrast, in anti-IFN-treated mice, mean tumor volumes by day 14 were 1,341, 2,196, and 5,783 mm^3 in mice injected with 2, 5, and 10 × 10^6 PLC/PRF/5 cells, respectively. Tumors in these mice were extremely invasive locally, but no metastases were observed macroscopically or microscopically.

TABLE II
Effects of Immunosuppression on the Tumor Weight in BALB/c Nu/Nu Mice

No. of mice	Treatment	Days after inoculation	Mean tumor weight
			mg
7	None	21	18 (0–32)
9	X-irrad	21	157 (78–394)
15	ALS	21	326 (91–1435)
5	Anti-IFN	14	370 (167–580)

Adult BALB/c nude mice were injected with 4–5 × 10^6 PLC/PRF/5 cells. For treatment protocols see legend, Table I.

Expression of HBV proteins. HBsAg was undetectable in the serum of untreated mice 14 and 21 d after injection of 5–10 × 10^6 cells (Table III). However, at these times, tumors were very small or absent. Elevated serum HBsAg levels were detected in ALS- or anti-IFN-treated mice and correlated with the presence of large tumors. The possibility that anti-IFN or other forms of immunosuppression might enhance the emergence of clone(s) of PLC/PRF/5 cells expressing HBeAg was examined.

Experiments were performed to maximize for expression of HBeAg. Tumors from anti-IFN, ALS-treated, or untreated mice were transplanted directly to conventional BALB/c mice, C57BL/6 (*beige/beige*) mice and to ALS-treated BALB/c *nu/nu* mice. The rate of tumor take was 50–70% in BALB/c *nu/nu* mice injected with tumor cells from untreated or ALS-treated mice. However, tumors did not grow in BALB/c nude mice injected with tumor cells from anti-IFN-treated mice. All tumors were rejected after injection of BALB/c or C57BL/6 (*beige/beige*) mice. Low levels of antibodies to HBsAg (three times greater than control values) were detected in 17% of injected mice in the different groups (data not shown). However, anti-HBe and anti-HBc were not detected in serum up to 3 mo after direct transplantation of tumors in all animals tested, regardless of mouse strains (data not shown). Moreover, HBcAg was undetectable by immunofluorescence or immunoperoxidase techniques in tumor tissues of immunosuppressed nude mice.[2]

Natural cytotoxicity of spleen cells from tumor-bearing mice. Spleen cells from BALB/c *nu/nu* mice given high doses of tumor cells (10^7) induced significant lysis of ^{51}Cr-labeled PLC/PRF/5 target cells as compared with spleen cells from untreated mice (Table IV). Cytotoxic activity was highest 14 d after tumor cell injection, causing 37.7% specific lysis in contrast to HBV-negative SK-HEP-1 target cells that were resistant to lysis by the same spleen cells. These cells produced tumors in untreated BALB/c *nu/nu* mice and at low inoculum dosages of 10^6 cells. Augmented activity was also observed against YAC-1 cells, standard NK targets (Table IV), suggesting that the effector cells were NK cells. To exclude a possible antibody-

[2] Gerber, M. A. Personal communication.

TABLE III

RIA for HBsAg and HBeAg in Serum of PLC/PRF/5-injected BALB/c Nu/Nu Mice

HBV marker	Treatment	Days after tumor cell inoculation*	No. of mice	^{125}I (cpm)		P
				Threshold value‡	Serum levels	
				mean±SE		
HBsAg	Normal mice	---	10	727±62	346±46	---
	None	14	5	727±62	390±50	NS
	Anti-IFN	14	3	727±62	10,309±821	<0.01
	None	21	2	727±62	370±35	NS
	ALS	21	10	727±62	9,844±894	<0.01
HBeAg	Normal mice	---	5	647±72	308±28	---
	None	14	5	647±72	349±35	NS
	Anti-IFN	14	21	647±72	368±52	NS
	None	21	2	1,634±172	778±115	NS
	ALS	21	11	1,634±172	831±95	NS

All ALS- and anti-IFN-treated mice had palpable tumors at the time of bleeding. Untreated mice had no palpable tumor at day 14 or at day 21.

* Adult BALB/c *nu/nu* mice were injected with 5–10 × 10^6 PLC/PRF/5 cells.

‡ Threshold values represent the cutoff point, which was 2.1 times counts per minute of the mean negative control obtained by testing normal nude mouse serum. All data above this value represent a positive result.

dependent, cell-mediated cytotoxicity (ADCC) response, we added monoclonal antibodies against HBsAg (28) to mouse spleen cells and saw no increase in cytotoxic activity against PLC/PRF/5 cells (data not shown), although they are lytic in the presence of complement (28). As shown in Fig. 1, a minor peak of increased spleen cell cytotoxicity in tumor-bearing mice was noted on day 3, reappeared on day 10, remained at a constant level for 4 d (days 12–15) and then decreased rapidly. After filtration of spleen cells through nylon wool, 70% of the cytotoxic activity against the same target cells observed on day 14 was retained. By 22 d after tumor cell injection, spleen cell cytotoxic activity was reduced to control levels, concomitant with growth of large tumors. As shown in Fig. 2, anti-IFN treatment caused a marked reduction of cytotoxic activity against PLC/PRF/5 target cells. Similar results were obtained in mice immunosuppressed by ALS or x-irrad and injected with PLC/PRF/5 cells (Table V).

The phenotype of the predominant effector cell in this system was established as NK 1.1, Thy 1.2 using appropriate antisera and complement. Specific killing of PLC/PRF/5 cells was reduced by 60% (two exper-

TABLE IV

Cytotoxic Activity of Spleen Cells from Tumor-bearing Mice against Various Target Cells

Injected tumor cells	Days after injection	Target cells: percent specific lysis*		
		PLC/PRF/5	SK/HEP-1	YAC-1
None	—	10±1.2	6.5±0.7	24.7±2.8
PLC/PRF/5	3	13.4±1.1	NT	30.2±2.9
PLC/PRF/5	7	6.6±0.5	0	NT
PLC/PRF/5	14	37.0±3.9	9.6±1.0	51.2±4.5

* Data presented as mean±SE of triplicate experiments of percent specific ^{51}Cr release. The above experiment is representative of data obtained from six independent experiments. Adult BALB/c *nu/nu* mice were injected subcutaneously with 1 × 10^7 PLC/PRF/5 cells. Age-matched BALB/c *nu/nu* mice were used as controls. Cytotoxicity data are represented at an effector-to-target cell ratio 100:1. Incubation period was 8 h for hepatoma target cells and 4 h for YAC-1 targets. NT, not tested.

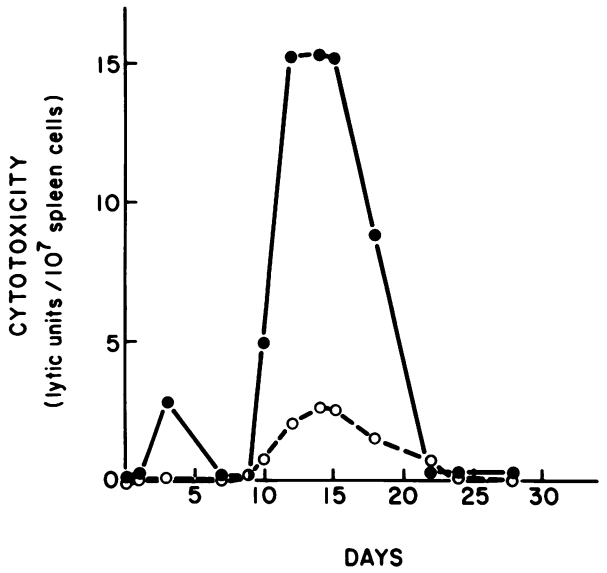

FIGURE 1 Development of NK activity in BALB/c *nu/nu* mice injected with PLC/PRF/5 cells against ^{51}Cr-labeled PLC/PRF/5 target cells. Adult BALB/c *nu/nu* mice were injected subcutaneously with 1×10^7 PLC/PRF/5 cells. Age-matched mice were used as controls. Results are the mean of three individual experiments using spleens from three separate animals in each experiment. Cytotoxic activity is expressed in lytic units as calculated from three different effector/target ratios: 100:1, 50:1, 25:1 (Methods). ●, mice injected with PLC/PRF/5 cells; O, age-matched normal control mice.

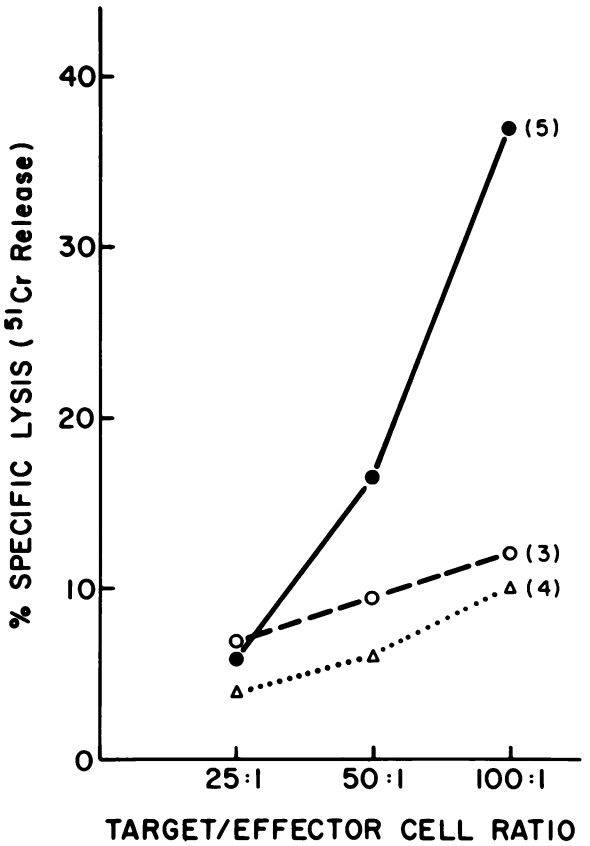

FIGURE 2 Influence of anti-IFN serum injected on NK cell activity against PLC/PRF/5 target cells. Adult BALB/c *nu/nu* mice were injected subcutaneously with 1×10^7 PLC/PRF/5 cells and assayed for splenic cytotoxicity against ^{51}Cr-labeled homologous target cells 14 d later. Data are presented as mean percentage of ^{51}Cr-specific release from triplicate experiments after 8 h of incubation. ●, mice injected subcutaneously with PLC/PRF/5 cells; Δ, mice injected with PLC/PRF/5 cells and treated with 0.1 ml anti-IFN serum once every 7 d as described in Methods; O, age-matched normal BALB/c *nu/nu* control mice; ( ), number of mice tested.

iments) after treatment with anti-NK 1.1 serum and complement and by 40% using monoclonal anti-Thy 1.1 serum and complement (four experiments). We refer to the effector cell in this system as an NK cell, probably of the NK_T subset (29). Mice injected with 10^6 HeLa-Ms cells or with 100 μg poly I:poly C, both of which are known to augment NK activity, showed enhanced splenic cytotoxic activity against PLC/PRF/5 and YAC-1 cells (Table V).

Regulation of effector cells by IFN. On the basis of previous studies of persistently infected tumor cells (15, 24), it seemed possible that IFN was responsible for the augmentation of NK activity seen against both PLC/PRF/5 and YAC-1 tumor cells. Consequently, mouse IFN was measured in supernatants of PLC/PRF/5 cells co-cultured with mouse spleen cells and elevated titres were found but not with SK-HEP-1 cells or purified HBsAg co-cultured with mouse spleen cells (Table VI). These results correlate well with the selectivity of cytotoxic activity for PLC/PRF/5 targets. Interestingly, supernatants from SK-HEP-1 cultures, but not PLC/PRF/5, contained detectable levels of human IFN (Table VI).

DISCUSSION

A wide variety of human tumors grow in athymic nude mice, and these animals have been used to assess tumorigenicity of various cell lines as models for experimental chemo- and immunotherapy. Therefore, a comparative investigation of the tumorigenicity of PLC/PRF/5 and SK-HEP-1 cells in these mice was performed. Preliminary observations indicated that athymic nude mice actively suppress growth and tumor formation of PLC/PRF/5 cells (7). In contrast to SK-HEP-1 cells, which were highly malignant in BALB/c *nu/nu* as were other neoplastic cell lines (14, 15, 18), PLC/PRF/5 cells required higher cell injec-

TABLE V
Effects of Various Treatments on Spleen Cell Cytotoxic Activity

Treatment of mice*	Specific ^{51}Cr release from target cells‡	
	PLC/PRF/5	YAC-1
	%	
None	11.2±0.3	24.7±2.8
1×10^7 PLC/PRF/5 cells s.c.	37.6±3.2	51.2±4.5
ALS alone	8.7±1.2	NT
ALS and 1×10^7 PLC/PRF/5 cells s.c.	7.3±0.8	NT
X-irrad	8.8±1.1	NT
X-irrad and 1×10^7 PLC/PRF/5 cells s.c.	7.6±1.4	NT
Poly I: poly C§	75.9±6.8	65.1±5.7
1×10^6 HeLA MS cells s.c.	27.1±3.0	NT

* Adult BALB/c *nu/nu* mice were injected with PLC/PRF/5 or HeLa-MS and killed 14 d after tumor cell injection. Age-matched BALB/c *nu/nu* mice were used as controls.
‡ Data are presented as mean±SE of triplicate experiments of specific ^{51}Cr release at an effector (spleen cells)-to-target cell ratio of 100:1.
§ Mice were injected with 0.1 mg poly I:poly C i.p. 24 h before killing.
NT, not tested.

tion dosages and demonstrated a longer latency until tumor formation. Even with higher cell dosages, tumors were small, were surrounded by a thick fibrous capsule, were infiltrated by numerous host lymphoid cells and occasionally regressed. Only after immunosuppression of nude mice by x-irrad, ALS, or most dramatically by anti-mouse-IFN serum, the tumorigenic potential of PLC/PRF/5 cells was enhanced: the tumor latency was shortened, the minimum inoculum dose was decreased, tumor weight and invasive potential was increased, and there was an absence of a capsule formation or host cell invasion. Similarly, a general assessment of residual host responses to PLC/PRF/5 cells was afforded by studies using CBA/n nude mice. These mice, known to be deficient in both T cell and B cell responses (30, 31), served as more permissive hosts for PLC/PRF/5 cells, even when the mice were not further compromised by ALS treatment. However, local invasiveness of PLC/PRF/5 cells in CBA/n nude mice still required ALS treatment. Therefore, it seems that tumorigenicity of PLC/PRF/5 cells is controlled by multiple host defense mechanisms, including humoral responses and cell-mediated cytotoxicity of non-T cell origin.

TABLE VI
IFN Titers in Mixed Lymphocyte Tumor Cell Culture Supernatants

IFN inducer	Human IFN	Mouse IFN
	IU/ml	
PLC/PRF/5 cells	0	40
SK/HEP-1 cells	80	0
Hepatitis B surface antigen	0	0

1×10^6 human hepatoma cells were cultured for 24 h in 24-well culture plates. Spleen cells from normal BALB/c nude mice at a ratio of 100:1 were added. 24 h later, supernatants were collected and frozen at −70°C until assayed. Purified HBsAg at a final concentration of 100 μg/ml was incubated with 1×10^8 spleen cells for 24 h. Mouse IFN was not detected in medium alone. Human or mouse IFN titers were measured as described in the Methods section.

Previous reports have shown that neoplastic cells persistently infected with RNA-enveloped viruses are rejected by nude mice by mechanisms that are radiosensitive (14, 15). It was shown subsequently that normal nude mice exhibit splenic cytotoxicity against virus-infected tumor cells in vitro but not against the uninfected cells, and that the cell responsible for this cytotoxicity appears to be an NK cell regulated by IFN (15, 17). The IFN-regulated NK response in athymic nude mice was abolished by injection of anti-IFN serum resulting in the augmentation of the tumorigenic potential of virus persistently infected tumor cells (24). In the present studies, in vitro analysis of the cytotoxic activity of spleen cells from normal or PLC/PRF/5-inoculated nude mice indicated that PLC/PRF/5 cells, but not SK-HEP-1 cells, are susceptible to lysis by spleen cells from PLC/PRF/5-injected mice (Table IV). Two peaks of augmented splenic cytolytic activity were seen, a minor one on day 3 and a major one by 12–14 d following inocu-

lation. Both peaks were abolished by immunosuppression by x-irrad, ALS, or anti-IFN serum.

Evidence for the role of murine IFN in restriction of growth of PLC/PRF/5 tumor cells was derived from both in vitro and in vivo studies. Splenic cytotoxic activity correlated with the ability of PLC/PRF/5 cells to induce mouse IFN but not human IFN when co-cultivated with normal mouse spleen cells (Table VI). Since the species specificity for the action of IFN is well established (32), and since there was no correlation with the secretion of human IFN by SK-HEP-1 cells and susceptibility to lysis, these results suggest that the restriction of growth of PLC/PRF/5 cells in nude mice resulted from augmentation of a host response regulated by mouse IFN.

Several in vivo studies also point to the relevance of murine IFN to restriction of PLC/PRF/5 cell growth in nude mice. The splenic cytolytic activity of nude mice against PLC/PRF/5 cells was significantly augmented by injection of the nonspecific IFN inducer, poly I:poly C. Even more dramatically, treatment of nude mice with high titer antiserum to mouse IFN resulted in rapid growth and invasiveness of PLC/PRF/5 cells. Anti-mouse-IFN treatment was more effective than the other immunosuppressive treatments. Anti-IFN-treated mice developed visible tumors within 3–5 d, whereas none of the other mice showed tumors. Furthermore, tumor frequency was increased to 100%, required inoculum dosages were decreased and tumor weight was increased. Most striking was the increase in invasive potential of PLC/PRF/5 cells after anti-IFN treatment. In these respects, the restriction of growth of this DNA virus-infected tumor cell line by host responses in nude mice parallels the IFN-NK cell-dependent resistance to RNA virus persistently infected tumor cell lines (14, 15, 17, 24).

From various experiments, we infer that the effector cell in this system is an NK cell. Cytotoxicity against PLC/PRF/5 cells was diminished by treatment with NK 1.1 and complement and with anti-Thy 1 plus complement, which would be the characteristic phenotype of activated NK_T cells (29). ADCC seemed improbable, since killing of PLC/PRF/5 could not be achieved in vitro by addition of monoclonal or polyclonal anti-HBs sera to normal spleen cells in vitro (data not shown).

In analyzing the activation of IFN-regulated mechanisms, it is unclear whether HBsAg expression on the PLC/PRF/5 cell membrane or secretion of HBsAg (a glycoprotein) into the medium is responsible for the immunologic response restricting growth of these cells. Cytoplasmic HBsAg was previously demonstrated in 90% of the PLC/PRF/5 cells in culture and in >50% of tumor cells in BALB/c nude mice (7, 33). However, in one study, HBsAg was present on the plasma membrane of only 15% of the cells in culture at any given time (19). Casali et al. (34) have reported that viral glycoproteins can directly stimulate cell-mediated cytotoxicity independent of IFN regulation. This was not tested in the present study for HBsAg. Nevertheless, purified HBsAg failed to stimulate secretion of mouse IFN in vitro under the conditions studied.

Recently, Dienstag et al. (35) demonstrated enhanced cytotoxic activity of human peripheral blood mononuclear cells against PLC/PRF/5 cells in culture in patients with chronic HBV infection. This response could be blocked by competing with other human hepatoma cells that did not express HbsAg. Thus, the cytotoxicity was considered nonspecific for expression of HBsAg. In contrast, Chisari et al (19), using a similar experimental design and a comparable group of patients, did not find evidence of enhanced cytotoxic activity of peripheral lymphocytes against PLC/PRF/5 cells. Nevertheless, both studies are consistent with our observations that HBsAg did not induce IFN release in spleen cell cultures and, therefore, is not responsible for IFN-induced augmentation of effector cells. Thus, the determinant(s) on the HBV-infected hepatoma cell line responsible for induction of murine IFN secretion remains unclear.

Although PLC/PRF/5 cells contain the complete HBV genome (6), the immunosuppressive protocols did not induce expression of other viral proteins or Dane particles (data not shown). Attempts to detect HBeAg in the serum of PLC/PRF/5 tumor-bearing mice were not successful. Similarly, HBcAg was not demonstrable in tumors of x-irrad or ALS-treated mice. Antibodies to HBeAg were not found consistently in the serum of tumor-bearing athymic nude mice, normal mice, or beige mice inoculated with PLC/PRF/5 cells or in serum from immunosuppressed nude mice inoculated with PLC/PRF/5 cells. However, in a few animals, borderline levels of anti-HBc or anti-HBe were observed. In contrast, in all immunosuppressed animals, a high concentration of HBsAg was present in the serum of tumor-bearing nude mice and correlated with increased tumor weight.

The present findings suggest that the HBV-infected hepatoma cell line, PCL/PRF/5, is restricted in its growth in nude mice by a mechanism(s) bearing many similarities to that causing restriction of growth of RNA virus persistently infected tumor cells (14, 15, 17, 24). This system may therefore prove useful in probing the immunologic mechanisms of defense against human hepatomas with or without HBV infection. These observations may also be utilized to study the effects of chemotherapy and immunotherapy on human hepatoma, using PLC/PRF/5 as the experimental model. In view of the recent findings that human IFN, with or without adjuvant chemotherapy, may inhibit the replication of HBV (36), these effects

could be compared to the possible effect of IFN on hepatocellular carcinoma and on the state of HBV DNA integration.

A new study indicating that our findings may be relevant to man has been published recently by Chin et al. (37) who observed that peripheral blood mononuclear cells of convalescent hepatitis B patients exhibited cytotoxic activity against the PLC/PRF/5 cell line but not against an HBV-negative hepatoma line, Mahlavu. The effector cells had the characteristics of NK cells and could be augmented by exposure to IFN in vitro.

ACKNOWLEDGMENTS

The authors thank Dr. L. R. Overby and Dr. I. K. Mushawar, Division of Experimental Biology, Abbott Laboratories, N. Chicago, IL, for providing purified HBsAg and reagents for HBV marker determinations, and Mrs. E. Hurston for her technical assistance. We are also indebted to Dr. Ion Gresser, Villejuif, France, for kindly providing the anti-mouse IFN globulin and to Dr. Sylvia Pollack (Seattle) for generously making available the NK 1.1 antiserum.

This research was supported, in part, by National Institutes of Health grants (AM17609, AM17702, AM02666, and AM20309), American Cancer Society grant BC-439, the Sara Chait Memorial Foundation, the Gail Zuckerman Foundation, and the Israel Cancer Research Fund.

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