Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice

(natural killer cells/tumor rejection)

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ABSTRACT Baby hamster kidney or HeLa cells form tumors in 100% of athymic nude mice. When such cells are persistently infected (PI) with RNA viruses, such as mumps or measles virus, the tumor cells either fail to grow or form circumscribed benign nodules. Neither the parental nor the virus PI tumor cells form invasive or metastatic lesions in nude mice. Previous studies have indicated a correlation between the susceptibility of virus-PI tumor cells in vitro and the cytolytic activity of natural killer (NK) cells and their failure to grow in vivo. Because interferon (IF) is the principal regulatory molecule governing the differentiation of NK cells, it was possible to test the relevance of the IF-NK cell system in vivo to restriction of tumor growth by treatment of nude mice with anti-IF globulin. This treatment was shown to reduce both IF production and NK activity in spleen cells. Both parental and virus-PI tumor cells grew and formed larger tumors in nude mice treated with anti-IF globulin than in control nude mice. The viral-PI tumor cells and the uninfected parental cells formed tumors in treated mice that were highly invasive and often metastatic. Some human tumor types have been notoriously difficult to establish as tumor lines in nude mice (e.g., primary human prostatic carcinomas). When transplanted into nude mice treated either with anti-IF globulin or anti-lymphocyte serum, two prostatic carcinomas grew and produced neoplasms with local invasiveness and some metastases. The results are consistent with the view that interferon may be important in restricting the growth, invasiveness, and metastases of tumor cells by acting indirectly through components of the immune system, such as NK cells.

Although it is clear that rejection of the virus-PI tumor cell lines in vivo is an active process of the host (10, 11) and can be abrogated by irradiation (10), it remains to be established whether the NK mechanisms effective in vitro has relevance to restriction of tumor growth in vivo. Because interferon (IF) appears to be the major regulatory factor controlling the generation of NK cells (12–15), we have attempted to assess the role of the IF-NK cell mechanisms in vivo by studying the effect of treating nude mice with high-titer antisera prepared against mouse IF on the growth of both virus-PI tumor lines and primary human prostatic tumor cells.

MATERIAL AND METHODS

Mice. Five- to 8-wk-old BALB/c nu/nu mice and the tumor cell lines BHK21 and HeLa, uninfected or PI with measles virus or mumps virus, were maintained as described (10, 11, 16). All cell lines were repeatedly monitored for mycoplasma contamination by using the Hoechst stain and were found to be consistently negative.

Primary Human Prostatic Tumors. Surgical specimens of human prostatic tumors were minced, and 0.2 ml was injected subcutaneously into adult male BALB/c nude mice treated with 20-mpg pellets of dihydrotestosterone. All mice were observed for up to 6 mo for the development of tumors.

Autopsy Procedures. All mice were autopsied and tissues were prepared as described (10, 11, 16).

Anti-Lymphocyte Serum. Rabbit anti-mouse lymphocyte serum (ALS) was purchased from Microbiological Associates, Bethesda, MD. Mice were injected intraperitoneally (i.p.) twice weekly with 0.1 ml of a 1:2 dilution.

Anti-IF Globulin. Sheep anti-mouse IF serum (globulin fraction) was prepared as described (17). The globulin at a dilution of 1:1000 completely neutralized 200 units of mouse leukocyte IF induced by HeLa-Ms cells. The antiserum diluted 1:3 with phosphate-buffered saline was absorbed with one-third vol of packed BALB/c spleen cells and erythrocytes for 30 min at 4°C, and 0.1 ml of the absorbed globulin was injected intravenously (i.v.) into the nude mice at the same time as the subcutaneous (s.c.) inoculation of tumor cells. Normal sheep serum was absorbed as described above and was used as a control in one experiment. In some experiments a sheep antiserum to contaminants in the IF preparation (unpublished data), which lacked anti-IF activity, was used. It was absorbed as described for the antiserum to IF.

Abbreviations: ALS, rabbit anti-mouse lymphocyte serum; IF, interferon; i.p., intraperitoneally; i.v., intravenously; NK, natural killer cells; PI, persistently infected; s.c., subcutaneous; BPH, benign prostatic hypertrophy; BHK cells, baby hamster kidney cells; VSV, vesicular stomatitis virus.

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Persistent infection of HeLa or BHK cells with any of several RNA-enveloped viruses [e.g., mumps, measles, vesicular stomatitis virus (VSV)] dramatically reduces their tumorigenicity in nude mice (10, 11). In most of the mice, no neoplasms were palpable. However, with some of the cell cultures, most commonly BHK cells infected with mumps virus or VSV, benign nodules formed that stabilized at approximately 0.5 cm in diameter and persisted at that size for months (Fig. 1, Table 1). Histopathology of these nodules (Fig. 2B) revealed tumors surrounded by a thick fibrous capsule infiltrated extensively by large numbers of lymphocytes, macrophages, and some neutrophils. Invasiveness or evidence of metastases were never observed.

Tumorigenicity of Virus-PI and Uninfected Tumor Cells in Nude Mice Treated with Anti-IF Globulin. To explore the role of the IF-NK cell system in vivo in rejecting the virus-PI tumors, we attempted to inhibit the recruitment of NK cells by neutralizing endogenous IF through use of high-titered anti-IF globulin. All nude mice treated with anti-IF globulin showed rapidly growing tumors irrespective of whether mice were challenged with virus-PI or uninfected tumor cells (Table 1, Fig. 1). Although both HeLa and BHK cells formed tumors in 100% of both control and anti-IF-treated groups, the average tumor weights were significantly greater in the anti-IF-treated mice (7 times greater with BHK cells and 3 times greater with HeLa cells) than in control mice. In the control groups, none of the mice injected with measles-infected HeLa cells and half the mice injected with mumps-infected BHK cells formed palpable neoplasms, whereas in the anti-IF-treated groups, 100% of the mice receiving either of these tumor cell lines formed palpable neoplasms. Furthermore, all of the tumors in control mice were encapsulated and failed to invade the surrounding tissues. In contrast, the histopathology of the neoplasms in the anti-IF-treated group uniformly revealed (i) absence of a stromal capsule (Fig. 2C), (ii) absence of significant host cell infiltrate (Fig. 2 C and D), (iii) invasiveness into surrounding tissues (Fig. 2 C and D), and (iv) increased incidence of metastases (Fig. 2 E and F). The route of spread appeared to be by way of the lymphatics for those lesions found. These results indicate that endogenous IF has a critical role in restriction of the growth and spread of these xenogeneic tumor cells.

Effects of Anti-IF Globulin Treatment in Vivo on NK Activity of Nude Mice Spleen Cells in Vitro. Two basic mechanisms could underlie the action of endogenous IF in mediating resis-

![Fig. 1. Influence of anti-IF serum on the tumorigenicity of HeLa and its daughter cell line PI with mumps. All mice received 10⁶ tumor cells s.c. Mice 1–4 were injected with HeLa cells and 5–8 were injected with mumps-infected HeLa cells. Mice 1, 2, 5, and 6 received phosphate-buffered saline, and mice 3, 4, 7, and 8 received anti-IF globulin.](image)

### RESULTS

**Tumorigenicity of Virus-PI and Uninfected HeLa and BHK Cells in Nude Mice.** HeLa and BHK2.1 cells formed rapidly growing neoplasms in athymic nude mice (Fig. 1, Table 1) (13). By 4 wk after inoculation of 10⁶–10⁷ HeLa or BHK cells into nude mice, tumors developed with average diameters greater than 2 cm in all animals. The histopathologic study of these tumors (Fig. 2A) indicated well-circumscribed neoplasms with moderate host cell infiltration. The tumors were well encapsulated by a fibrous capsule and were rarely invasive into the surrounding tissue or the muscle of the body wall. Tumor metastases were rarely seen.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Virus infection</th>
<th>Duration, weeks</th>
<th>Treatment*</th>
<th>Frequency of neoplasia</th>
<th>Neoplasm weight, g</th>
<th>% mice with</th>
<th>Invasive tumor</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK</td>
<td></td>
<td>2</td>
<td>PBS</td>
<td>8/8</td>
<td>0.36 ± 0.16</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BHK</td>
<td>Mumps</td>
<td></td>
<td>AIF</td>
<td>8/8</td>
<td>2.49 ± 0.99</td>
<td>100</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td>3</td>
<td>PBS</td>
<td>7/16</td>
<td>0.26 ± 0.17</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Mumps</td>
<td>3</td>
<td>AIF</td>
<td>3/6</td>
<td>0.18 ± 0.1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Measles</td>
<td>3</td>
<td>AIF</td>
<td>3/4</td>
<td>0.65 ± 0.48</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Mumps</td>
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<td>AIF</td>
<td>18/18</td>
<td>3.8 ± 1.7</td>
<td>100</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

* Each mouse received 0.1 ml of either phosphate-buffered saline (PBS), normal sheep serum (NSS), anti-IF globulin (AIF), or anti-IF contaminant serum (AIF^c) i.v. at the same time as the inoculation of 10⁶ tumor cells s.c. The mice were sacrificed either 2 or 3 wk after inoculation and were autopsied. Portions of the lymphoid tissues, of the neoplasm, and of any metastatic tumors were prepared for histopathological observations.

† Average ± SD.
Fig. 2. Influence of anti-mouse IF serum on the histopathology of neoplasms that formed in nude mice injected with BHK or mumps-infected cells. (A) BHK-cell tumor in a nude mouse injected also with phosphate-buffered saline. A thick capsule (arrowheads) demarcates the perimeter of the 2-cm tumor. (x130.) (B) A 4-mm diameter nodule of mumps-infected BHK cells. It is encapsulated (arrowheads) and shows a significant infiltration of host cells. (x80.) (C) Tumor of mumps-infected BHK cells in a nude mouse injected with anti-IF serum. Capsule formation and host cell infiltration at the perimeter of the tumor (arrowheads) are absent, and invasiveness into the s.c. fat tissue is seen. (x170.) (D) Tumor of mumps-infected BHK cells in nude mouse injected with anti-IF globulin. The PI tumor cells have invaded through the muscle of the body wall into the peritoneum. (x80.) (E) Metastatic tumor in the kidney of a nude mouse injected with mumps-infected BHK cells and with anti-IF serum. (x80.) (F) Section of lung indicating several metastatic lesions of mumps-infected BHK cells in the lymphatics surrounding a vein. (x170.) (G) Tumor residues found in the s.c. tissue of nude mice implanted 6 mo previously with prostatic adenocarcinoma cells. The tumor has regressed leaving a residue of cells undergoing squamous metaplasia and keratin formation, surrounded by a stromal capsule and infiltrated by host cells. (x80.) (H) Human prostatic carcinoma (R340) grown in nude mice treated with anti-IF serum. The tumor is solid, invasive, and well-vascularized, with little evidence of host-cell infiltration. (x170.)

Tnance to these tumor cells in nude mice: (i) the IF produced could act directly on the tumor cells, inhibiting their growth; or (ii) IF could act indirectly by potentiating host effector mechanisms, most likely through the NK cell system. Although IF is known to be largely species-specific, and the tumors used here were xenogeneic, we examined the direct effect of 1000 units/ml of mouse IF on the in vitro growth of measles-infected HeLa cells and found no significant difference in their cell growth over a 6-day period (data not shown).

Subsequently, we examined the effect of anti-IF globulin injection in vivo on the NK cell activity of nude mouse spleen cells 3 days after tumor inoculation. NK cell activity was sig-
Table 2. Influence of anti-IF serum on the transplantability of primary human prostatic tumors in BALB/c nude mice

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Surgical specimens</th>
<th>Treatment of mice</th>
<th>Frequency of tumors, (^*) first passage</th>
<th>Transplantability of tumors (^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH</td>
<td>66</td>
<td>None</td>
<td>5/198</td>
<td>0/66</td>
</tr>
<tr>
<td>Adenocarcinoma of prostate</td>
<td>62</td>
<td>None</td>
<td>0/142</td>
<td>0/62</td>
</tr>
<tr>
<td>Adenocarcinoma of prostate</td>
<td>2</td>
<td>AIF</td>
<td>4/4</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ALS</td>
<td>5/5</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None</td>
<td>0/6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Surgical specimens of human prostatic tissues of either benign prostatic hyperplasia or adenocarcinoma were minced for single-cell suspensions and injected into normal male BALB/c nude mice s.c. A portion of the mice also were injected with a single dose (0.1 ml) of anti-IF serum (AIF) i.v. at the same time or with ALS (0.1 ml) twice a week i.p. The mice were observed for 6 mo for tumor formation in the first passage. Tumors appearing in the nude mice were removed and transplanted into normal nude mice for establishment of transplantable tumor cell lines.

† Shown as the number of mice with neoplasms/number of mice injected.

‡ Shown as the number of mice with established transplanted tumors/number of mice that received transplants.

Significantly augmented in the nude mice injected with measles-infected HeLa cells compared with normal mice (11). In contrast, in comparable nude mice injected with the anti-IF globulin, no increase in NK cell activity was observed. Similar results were found in IF production in response to coculture with measles-infected cells in vitro. Spleen cells from mice infected with measles-infected HeLa cells produced higher titers of IF in vitro (1600 units/ml) than those from normal mice (500 units/ml), whereas those from comparable mice injected with anti-IF produced no more IF than normal mice (400 units/ml) did. No augmentation of NK cell activity by the virus-PI tumor cell inoculation could be observed in anti-IF-treated mice until 9 days after treatment (data not shown). However, by 21 days, when anti-IF-treated mice had observable neoplasms, the anti-IF-treated mice showed elevated NK cell activity comparable to that seen in nude mice injected with measles-infected HeLa cells alone. These results clearly indicate that a single treatment with anti-IF globulin prevents the induction of NK cell activity and IF production in vivo early after challenge with virus-PI tumor cells, although it does not affect the background levels of preexisting NK and IF-producing cells.

Successful Transplantation of Primary Prostatic Tumors in Anti-IF-Treated Nude Mice. Human prostatic tumors are one of the several human tumors that fail to grow in nude mice. In earlier studies (16, 18) it was found that of 66 surgical specimens of benign prostatic hyperplasia (BPH) transplanted into 198 nude mice, only 2 specimens formed slowly growing neoplasms that could be successfully passaged only twice in nude mice (Table 2). Similarly, of 63 surgical specimens of prostatic carcinoma or adenocarcinoma transplanted into 145 nude mice, none developed into a growing neoplasm. The histopathology of the residues (Fig. 2G) from transplants of either BPH or prostatic carcinomas showed thick stromal capsules, host cellular infiltrates, keratin formation, and squamous metaplasia of the tumors. No invasiveness or evidence of metastases was observed in any of the mice examined.

In the present experiment, surgical specimens of human prostatic adenocarcinomas from two patients were transplanted into BALB/c nude mice treated either with anti-mouse IF, ALS, or normal rabbit or sheep serum. The surgical specimens grew in all of the mice treated with either anti-IF globulin or ALS (Table 2). Both specimens have given rise to transplantable tumor lines that can be maintained in immunosuppressed nude mice. When treatment was interrupted, regression of neoplasms invariably ensued. Paraffin sections (Fig. 2H) from the anti-IF or ALS-treated mice showed a histologic pattern identical to that described for the original prostatic tumor in situ. The tumors were relatively well circumscribed in most cases, and little or no host response was seen. In some of the mice, particularly those treated with anti-IF, the prostatic tumors invaded the peritoneal and pleural cavities.

Discussion

A large body of evidence indicates that (i) NK cells can discriminate between virus-infected cells and uninfected cells in vitro (11, 14, 19–21), (ii) nude mice possess T cell independent defense mechanisms against virus infections and tumor cell growth (5, 11, 14, 19–22), and (iii) there is a general correlation between the level of NK cell activity in vitro and rejection of virus-PI tumor cells in vivo in restricting tumor growth.

The results presented here indicate clearly that treatment of nude mice with anti-mouse IF globulin or serum resulted in rapid growth of three different virus-PI tumor cells. Most striking was the observation that 100% of anti-IF-treated mice developed visible tumors with HeLa-Ms cells in 3 wk, whereas none of the control mice showed any sign of a neoplasm. There was a significant increase in tumor weights and in the tumor’s invasiveness to the surrounding tissues in anti-IF-treated mice, even in the mice injected with HeLa or BHK cells.

Two obvious hypotheses to explain the effects of anti-IF serum can be considered. IF is well-known to have direct inhibitory effects in vitro on the growth of a number of tumor cells and some normal cells (23–25); thus, IF may act directly in vivo to restrict tumor growth. Alternatively, IF may act indirectly by regulating cells of the host, particularly NK cells (12–15). It is well established that both the antiviral and growth-inhibitory effects of IF are rather strictly species-specific (26). The tumor cells used in these studies were either of human or hamster origin and, thus, it was unlikely that mouse IF could act directly on such cells. This was, indeed, confirmed directly in vitro using HeLa-Ms cells.

Previous work has indicated that NK cell activity in conventional or nude mice is augmented by the inoculation of tumor cells or virus-PI cell lines (11, 27). In contrast to results with conventional mice, in which the augmentation is evanescent, elevated NK cell activity following virus-PI tumor cell challenge in vivo persists at least 1 mo in nude mice (11). Both virus-PI cell lines, but not the uninfected tumor cells, induced IF production in vitro when cocultivated with normal spleen cells, but the levels of IF produced were dependent on the genetic background of the mice used (14). With the exception of a variant of VSV-infected BHK cells, serum levels of IF in nude or conventional mice challenged with virus-PI tumor cells could not be detected.
In the present experiments, it was shown that anti-IF treatment of mice completely blocked the augmentation of NK cell activity in vivo by virus-PI tumor cells, although pre-existing NK cell activity was unaffected by this treatment. Analysis of the time course of the effects of anti-IF globulin showed that complete blocking of NK induction produced by a single dose lasted approximately 9 days, and a gradual increase of NK cell activity was observed thereafter, probably due to continued stimulation of IF by the virus-PI tumor cells in vivo. The fact that anti-IF serum produced such dramatic effects in vivo, even though serum levels of IF were too low to be detected, suggests either that very low levels of IF are capable of stimulating differentiation of NK cells or, more likely, that the critical events in restriction of tumor growth and invasiveness occur locally. At least two precedents for an indirect action of IF in vivo have been reported (28, 29) with either IF resistant variants of tumor cells or a virus-cell system in vitro that is resistant to the antiviral effects of IF. In both cases, IF was without direct effect in vitro yet provided protection in vivo.

Perhaps the most surprising observation in the present study was that a significant proportion of the anti-IF-treated mice developed remote metastases of the tumors. In a study of over 1000 normal nude mice metastases were rarely seen, although as few as 10–100 highly tumorigenic cells such as HeLa and BHK formed progressive tumors. Yet, in the anti-IF-treated animals challenged with BHK, HeLa, or the virus-PI cell lines, approximately 30% of the animals developed metastases, and all treated mice showed evidence of invasiveness of adjacent tissues by the tumor cells. These results are consistent with recent reports that a high incidence of tumors and metastases following challenge of syngeneic mice with B16 melanoma cells (30, 31) occurred in homozygous beige mice, which are known to be selectively deficient in NK cell function (32). Taken together, these results strongly suggest that the IF-NK cell system may be importantly involved not only in the restriction of local growth and invasiveness of primary tumors but also in preventing metastases in vivo.

The question remained whether this mechanism could explain the failure of certain primary human cell types to grow in athymic nude mice. Human prostatic tumors have been notoriously difficult to establish in nude mice. Of 62 surgical specimens of prostatic carcinoma transplanted into a total of 142 mice, none were successfully established as transplantable tumor cell lines. In the present experiments, two primary prostatic tumors grew in all nude mice treated either with anti-IF globulin or with ALS. Both specimens have been established as lines in nude mice but continue to require immunosuppression for maintenance. The histopathology of these tumors grown in the treated mice parallels that described for the virus-PI cell lines in that there was increased invasive growth, less encapsulation, and an increased inflammatory response.

A variety of recent data (33, 34) and the present experiments suggest that the IF–NK cell mechanism may be relevant not only for restriction of tumor growth in nude mice but also for growth or metastases of autochthonous tumors in man or both. In one study of familial melanoma (34), evidence suggested that a deficiency in the IF–NK cell mechanism may be a predisposing factor to disease.

Note Added in Proof. It has recently been observed (S. Habu, H. Fukui, K. Shimamura, M. Kasai, Y. Nagai, K. Okumura, and N. Tamokiz, personal communication) that antisera to asialo-GM₁ can block NK activity in vivo and in vitro. Treatment of nude mice with this antisera enhanced the incidence and size of transplanted human and murine tumors. These studies provide independent evidence supporting a role for NK cells in the resistance to tumor growth in nude mice.