MECHANISM OF REJECTION OF VIRUS PERSISTENTLY INFECTED TUMOR CELLS BY ATHYMIC NUDE MICE*

By NAGAHIRO MINATO, BARRY R. BLOOM, CHARLOTTE JONES, JOHN HOLLAND, and LOLA M. REID‡

From the Departments of Microbiology and Immunology, and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, and from the Department of Biology, University of California at San Diego, La Jolla, California 92093

The congenitally athymic nude mouse has been widely used to study the tumorigenicity of a variety of human and animal neoplasms (1–6). Yet, nude mice appear not to be totally immunodeficient; they do not have a higher incidence than normal mice of chemical carcinogenesis or spontaneous tumors and show infrequent metastases of tumors known to be metastatic in their original host (4, 7–10). Although growth in the nude mouse is a major criterion for tumorigenicity of primary or cultured cells (5, 6), it is increasingly clear that many tumors fail to grow in the nude mouse (4, 11), even though the basis for this discrimination between different tumors remains unclear. The present studies were initiated to develop an appropriate model system for defining both the mechanism of discrimination between different types of tumors, and the possible mechanisms available for tumor rejection in the nude mouse. For these studies, cell lines known to be highly tumorigenic in the nude mouse were modified by rendering them virus persistently infected (P.I.)[1] with a variety of RNA viruses. It will be demonstrated that most such P.I. tumor cells are effectively rejected by the nude mouse. We believe that the studies on the rejection of virus P.I. tumor cells by the nude mouse may provide useful insights into the immunological control of tumor cells having altered surface properties and into the mechanisms of resistance to persistent viral infections as well.

Materials and Methods

Mice. BALB/c nude mice were derived from breeding stock obtained from Dr. G. Sato (University of California, San Diego) and maintained in an isolated colony (4). They were monitored regularly by screens for viruses, and by autopsy of randomly selected experimental and breeder mice. Conventional BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. 5–8-wk-old mice were used in these experiments.

P.I. Cell Lines. BHK21 hamster kidney cells and HeLa human cervical carcinoma cells were grown in vitro in Eagle's minimal essential medium supplemented with 7% heat-inactivated calf serum. P.I. virus cell lines were established by infecting cells with stocks of virus containing large numbers of defective interfering particles, except for BHK-vesicular stomatitis virus (VSV), in which purified defective-interfering (DI) particles and a homologous, cloned temperature-sensitive mutant of VSV was used (12).

* Supported by U.S. Public Health Service grants P30-CA1330, AI07118, AI10702, AI09807, AI19627, and RG1006 from the National Multiple Sclerosis Society.

‡ Recipient of a Sinsheimer Career Development Award.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; DI, defective-interfering; NK, natural killer; P.I., persistently infected; VSV, vesicular stomatitis virus.

Growth of Tumors in Nude Mice. Various virus P.I. or uninfected cell lines from tissue cultures were harvested and inoculated subcutaneously in 0.2 ml in the flank of nude mice. The number of viable cells infected ranged from 10^1 to 2×10^7. Results were scored as follows: (*a*) tumors were relatively localized neoplasms that were soft, well vascularized, rapidly growing, and which had achieved a 2–4-cm diameter by 4-wk postinoculation; (*b*) nodules were restricted, hard lesions which arose in 4–8 wk, stabilized at an ≅5-mm diameter, and persisted in that benign form indefinitely; (*c*) complete rejection was said to have occurred if the inoculation of tumors failed to produce a palpable lesion, either a tumor or a nodule, within 6 mo. All lesions were biopsied, fixed in Bouin's solution, and stained with hematoxylin-eosin or Masson's trichrome stain. Complete autopsies were done on all injected animals to obtain evidence for metastatic lesions. All tumors and nodules were cultured and cultured cells analyzed by karyotyping to confirm the species of origin.

Irradiation Procedures. For studies on the effect of radiation of the animals on tumor growth, BALB/c nude females were irradiated with 600 rads of ^{135}Cesium and maintained thereafter in a Biogard hood (Bio-Rad Laboratories). Greater than 95% of the animals survived 4–5 mo after irradiation.

Test for Virus Production

The presence of virus in tumors or nodules was assessed by cocultivation of a tissue mince with BHK21 cells or HeLa cells at 33°C. Cytopathic effects (CPE) were monitored for 6 d, most positive CPE being found by 3 d. In addition, primary and cultured cells were stained with fluorescent antibody to the virus, after acetone fixation, by an indirect procedure, using goat anti-rabbit fluorescein-conjugated antibody purchased from Meloy Laboratories, Inc., Springfield, Va.

CYTOTOXIC ASSAY (*a*) ^{51}Cr-release method: the assay was performed according to the method of Trinchieri et al. (13). Trypsinized target cells (10^6) were resuspended in 0.5 ml medium and incubated with 100 μCi of $Na_2\,^{51}CrO_4$ (Amersham Corp., Arlington Heights, Ill.), for 1 h at 37°C in 5% CO_2. After three washes, they were seeded into microtiter wells at a density of 10^4 cells/wells, and incubated overnight. The plates were washed three times with warm medium. A 0.2-ml vol of effector-cell dilution or medium alone was added and incubated for 10 h. After the incubation, 0.1 ml of supernate from each well was collected, and the radioactivity (A) was counted in an LKB gamma-counter (LKB Instruments, Inc., Rockville, Md.). To each well, 0.1 ml of 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) was added, the plates were incubated overnight, and 0.1 ml of each supernate was again collected for counting of radioactivity (B). ^{51}Cr release (E) was calculated as:

$$E = \frac{A}{B + A/2} \times 100.$$

The specific ^{51}Cr release (R) was calculated as:

$$R = \frac{E - S}{100 - S} \times 100;$$

where (S) is the percent ^{51}Cr release of target cells in the presence of medium alone.

(*b*) ^{3}H-uridine method: the procedure was essentially the same as described by Fujiwara et al. (14) and McFarland (15) with slight modification. Trypsinized target cells were seeded into microtiter wells at a density of 10^4 cells/well, and incubated overnight. 1 μCi of [5-^{3}H]-uridine (Amersham Corp.) was added to each well and incubated for 3–4 h at 37°C in 5% CO_2. The plates were then washed three times with warm medium. Effector-cell dilutions or medium (0.2 ml) were added to each well, and incubated at 37°C in 5% CO_2 for 10 h. After the incubation, the plates were washed with warm phosphate-buffered saline (PBS), trypsinized, and the cells from each well were collected separately by a semiautomatic cell harvester. The radioactivity was then counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The cytotoxicity (R) was calculated as:

$$R = \frac{\text{cpm (medium alone)} - \text{cpm (effector cells)}}{\text{cpm (medium alone)}} \times 100.$$

SPLEEN CELL SEPARATION PROCEDURES. (*a*) Nylon wool column: spleen cells were passed over nylon wool columns according to the method of Julius et al. (16). 10^8 spleen cells of nude or BALB/c mice in 10% fetal calf serum (FCS)-DME were added to the column (0.6 g nylon wool/10 ml), incubated for 40 min at 37°C, and then eluted with 20 ml of warm medium. Viable cell recovery after the column passage was ≅15% with nude spleen cells, and 20–25% with BALB/c spleen cells. The column-passed spleen cells of BALB/c did not respond to either concanavalin A (Con A) or bacterial lipopolysaccharide and, only after the addition of 2% peritoneal exudate cells, was the Con A response restored. Thus, this cell fraction was functionally depleted of macrophages and B cells.

(*b*) Anti-serum treatment: to remove any T cells present, 10^7 spleen cells in 1 ml of medium were mixed with anti-Thy 1.2 serum (final dilution 1:10) or normal mouse serum (final dilution 1:10) as a control at 4°C for 30 min. They were then incubated for 40 min at 37°C with 0.5 ml of normal rabbit serum as a source of complement (final dilution 1:10) which had been previously absorbed with BALB/c spleen cells. The anti-Thy 1.2 + complement treatment suppressed >90% of the Con A response of BALB/c spleen cells without significant effect on the LPS response. For B-cell depletion, the spleen cells were incubated with rabbit anti-mouse IgM serum (final dilution 1:20) and rabbit serum at the same conditions as above. By this treatment, ≅70% of the LPS response of the BALB/c spleen cells was eliminated.

(*c*) Trypsin treatment: normal nude spleen cells were resuspended at a density of 1×10^7 cells/ml in 0.5% or 2.0% trypsin-PBS at 37°C for 30 min. After treatment, the cells were washed twice. Approximately 70% of the cells were recovered after the trypsin treatment.

Results

Tumorigenicity of BHK21 cells, HeLa Cells, and Virus P.I. Cell Line Derived from Them. Both HeLa and BHK21 cells are highly tumorigenic in BALB/c nude mice, forming soft, well vascularized tumors within 3 wk in 100% of the animals injected with as few as 10–100 cells. As shown in Table I, these same cell lines P.I. with measles, mumps, influenza (NWS Flu), VSV, or rabies virus (data not shown) rarely, if ever, form tumors. In a portion of these animals, a neoplasm is never palpable (all the mice injected with $<10^4$ cells and varying numbers injected with $> 10^4$ cells), and in the remainder, a hard, poorly vascularized nodule forms within 4 wk. Representative mice exhibiting tumors or nodules are illustrated in Fig. 1. In histological studies of the neoplasms (Fig. 1), the benign nodules contrast with the tumors in that they show strong fibrous reaction, encapsulation and a significant mononuclear cell infiltrate.

Nodules forming in response to injection of P.I. cells often persist longer than 6 mo. Virally infected cells persist within the nodule, because virus could be identified by any of three techniques: (*a*) cocultivation; (*b*) by the resistance of the primary explanted cultures to superinfection by the homologous virus; and (*c*) by surface immunofluorescence for surface viral glycoprotein antigens. In all cases, karyotypic analysis showed that the virus-containing cells from the nodules derived from the species of cells inoculated and not from the host.

There could be two explanations for these results: (*a*) an intrinsic deficiency of the P.I. cell lines to grow in vivo relative to the parental HeLa or BHK21 cells; or, (*b*) active thymus-independent mechanisms of rejection by the nude mouse.

A number of studies have minimized the likelihood of the former interpretation. Previous studies have shown that the growth rates in vitro of the virus P.I. cell lines

TABLE I

Tumorigenicity of BHK21, HeLa, and Their Daughter Cell Lines P.I. with Various RNA Viruses

Characteristic examined	BHK21 cells infected with				HeLa cells infected with		
	----	VSV	Mumps	Flu	----	VSV	Measles
I. Tumorigenicity in normal nude mice							
Percent with no palpable lesion	0	20	17	0	0	33	47
Percent with tumor only	100	0	0	0	100	0	0
Percent with nodule only	0	80	83	84	0	67	53
Percent with nodule + subsequent tumor by							
4 wk	0	0	0	16	0	0	0
6 mo*	0	36	33	100	0	0	0
Presence of virus in:							
nodule/tumor/host‡	NA§	+/−/−	+/−/−	+/−/−	NA	+/NA/−	+/NA/−
(number of mice tested)	(18)	(25)	(6)	(19)	(15)	(15)	(15)
II. Tumorigenicity in nude mice irradiated with 600 rads							
Percent with tumor	100	100					
Presence of virus in: tumor/host‡	NA	+/+					
(number of mice tested)	(12)	(12)					

All the mice were injected with cells subcutaneously and examined for neoplasms 4 wk later (except where indicated). A more detailed presentation of the characteristics of the neoplasms and of the properties of the viral infections is presented in Table V.

* The percentage of mice with nodules at 4 wk which by 6 mo showed tumor development associated with the nodule.

‡ Host tissues: spleen, lung, liver, serum, and lymph nodes.

§ NA, not applicable.

are comparable to those of the uninfected cell lines (12). The reduced tumorigenicity of the P.I. cell lines is not a result of viral infection of the hosts because prior acute infection of the mice with live VSV or NWS-flu virus does not alter the tumorigenicity of the P.I. cell lines or of the uninfected parental cell lines. The suppression of the tumorigenicity of the P.I. cell lines is directly correlated with the persistent viral infection because: (*a*) P.I. cells cloned in vitro in the presence of antiviral antibodies are again highly tumorigenic in the nude mice; (*b*) prior injection of the mice with uninfected parental cells or with P.I. cell lines does not alter the tumorigenicity of uninfected or virus P.I. cells inoculated on the contralateral side of the mouse; and (*c*) coinjection of a mixture of BHK21 cells with BHK-VSV cells into mice resulted in tumors associated with or surrounding nodules. Thus, even under conditions in which the growth of the P.I. cells was restricted locally, the growth of the uninfected parental cells was not altered.

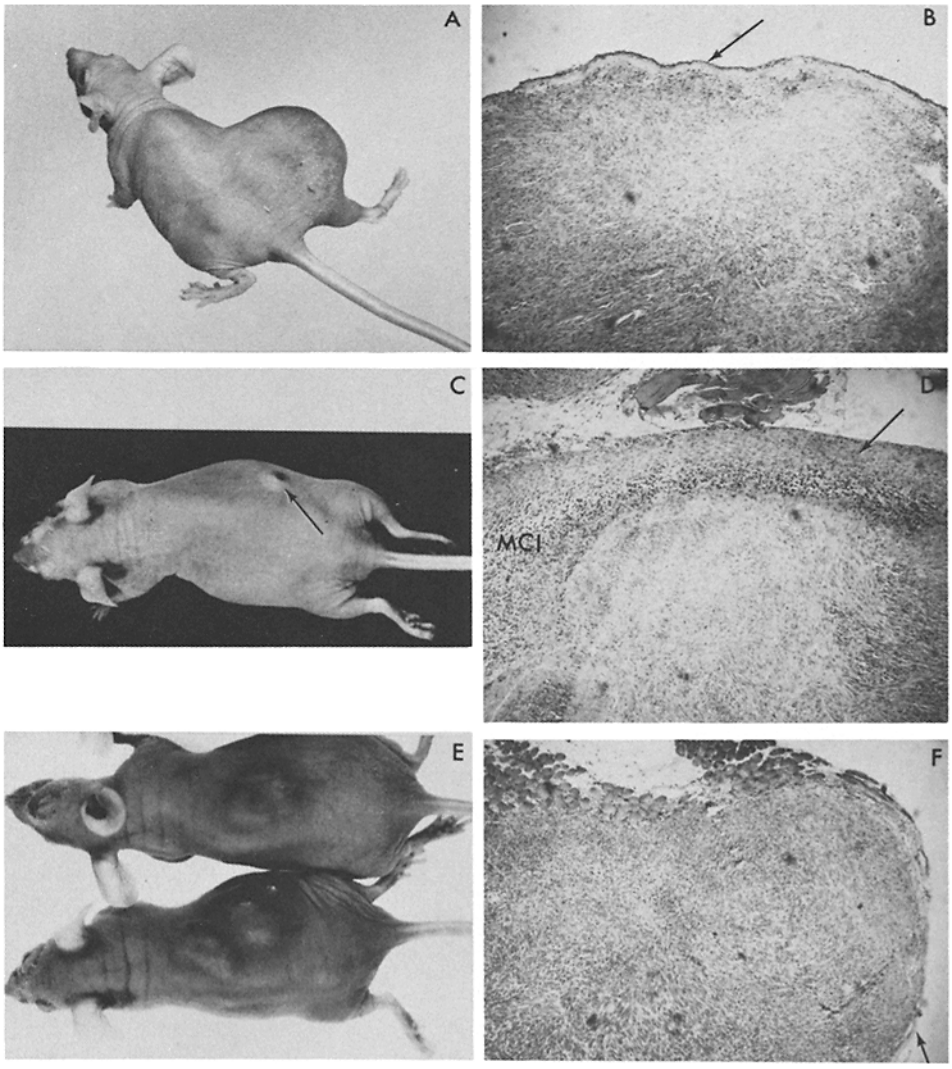

FIG. 1. Neoplasms in BALB/c nude mice injected with BHK21 or BHK-VSV cells. A. The mouse was inoculated with 10^6 BHK21 cells subcutaneously. The tumor was initially palpable by 10 d, and the animal was moribund with the tumor by 6 wk. B. A section of a tumor produced in a nude mouse injected with 10^6 BHK21 cells. Note the thin but distinct fibrous capsule (arrow) infiltrated with a small population of mononuclear cells. The 8-μm paraffin section was stained with haematoxylin and eosin. Magnification: × 130. C. The mouse was inoculated with 10^6 BHK-VSV cells subcutaneously. The small neoplasm noted on the right side of the mouse (arrow) developed by the 4th wk after inoculation and persisted in this benign form for >6 mo. D. Section from a nodule produced in a nude mouse injected with 10^6 BHK-NWS flu cells. Similar results were obtained from tumors from injections with other virus P.I. cell lines. Note the thick fibrous capsule (arrow) and the mononuclear infiltrate (MCI) surrounding the BHK-flu cells. The 8-μm paraffin section was stained with haematoxylin and eosin. Magnification: × 260. E. The mice were irradiated with 600 rads of ^{135}Cesium and subsequently injected with 10^6 BHK-VSV cells 12 d after irradiation. The tumors were palpable within 2 wk, and the mice were moribund with the tumors by 6 wk. F. Section of a tumor produced in an irradiated nude mouse injected with 10^6 BHK-VSV cells. The capsule surrounding the tumor was thin (arrow) and could be readily seen only with Masson's trichrome stain. Note the deficiency in the mononuclear cell infiltrate in the capsule. The 8-μm paraffin section was stained with Masson's trichrome. Magnification: × 130.

Curing of the P.I. Cell lines in Vivo. In some of the mice inoculated with virus P.I. tumor cells and maintained for >2 mo, incipient tumors were detected forming adjacent to the nodules. Primary cultures of the nodules and the tumors indicated that the former contained virally infected BHK21 or HeLa cells and that the latter contained cells with no virus infection as detectable by cocultivation, superinfection of explants or immunofluorescence. The tumors thus appeared to result from in vivo selection against the infected cells and the escape of cells that were cured of the viral infection. The fact that cells, cured of a persistent viral infection, regain tumorigenicity comparable to that of the uninfected parental cells corroborates the finding above that the growth restriction is critically dependent on the persistent viral infection.

Effect of Radiation of the Hosts on the Tumorigenicity of the P.I. Cell Lines. The possibility of an active rejection mechanism capable of discriminating between virally infected and uninfected tumor cells was strengthened when it was observed that whole body irradiation of nude mice with 600 rads of ^{135}Cesium or ^{160}Cobalt rendered the mice unable to suppress the growth of the virus P.I. cell lines (Table I). Mice, injected with 10^6 BHK21-VSV cells or with BHK21 cells 12 d after irradiation, developed tumors within 3 wk. The mice were moribund with the tumors by 5–6 wk after the inoculation. In animals inoculated on the day of irradiation, tumors developed more slowly and were not palpable until 5–6 wk after inoculation. Almost no signs of cellular infiltration or encapsulation were seen in histological specimens of virus P.I. tumors from irradiated nude mice (Fig. 1). Examination of primary cultures of the tumors derived from the BHK-VSV cells indicated that all neoplasms contained cells infected with viruses, and infectious particles were found in the spleen, kidney, liver and serum of the hosts. Karyotypically, the cultured cells were those of donor not host origin.

Preferential In Vitro Cytotoxic Activity of Normal Spleen Cells against Xenogeneic Tumor Cell Lines Persistently Infected with Viruses. The mechanism of rejection of the P.I. tumor cells by the nude mice was investigated by studying the in vitro cytotoxic activity of spleen cells derived from normal nude mice against P.I. or uninfected tumor cell lines. Spleen cells from normal nude mice 6–8 wk old were preincubated in tissue culture plates for 2 h to remove adherent cells, and mixed with prelabeled target cells. Two assays for cytotoxicity were used, a ^{51}Cr-release method and a ^{3}H-uridine-labeling assay, both carried out for 10 h. The latter was used because some P.I.-cell lines were difficult to label with ^{51}Cr. Spontaneous cytotoxicity assayed simultaneously by the two methods on HeLa and HeLa-measles cells is shown in Fig. 2, indicating marked cytotoxicity of the viral infected cells, and little or no cytolysis of the uninfected parental cells. A comparable or slightly greater degree of sensitivity was found in the ^{3}H-uridine assay. The ^{3}H-uridine assay was chosen for routine use. As shown in Fig. 3, this phenomenon was general, in that BHK-mumps, BHK-VSV, and HeLa-VSV, as well as HeLa-measles were all killed to various degrees by spleen cells from normal nude mice, although uninfected parental cells were not effectively killed. HeLa measles was consistently the most susceptible to spontaneous cytotoxicity among the virus P.I. lines, paralleling the in vivo observation that HeLa-measles was invariably rejected in nude mice whereas other virus P.I. cell lines were sometimes cured of their virus infection and regained tumorigenicity.

Characteristics of the Effector Cells Responsible for the Spontaneous Killing of Virus P.I. Cell Lines. A variety of selective enrichment and depletion procedures was applied to spleen cells from nude mice to characterize the cytotoxic effector cell in the HeLa-

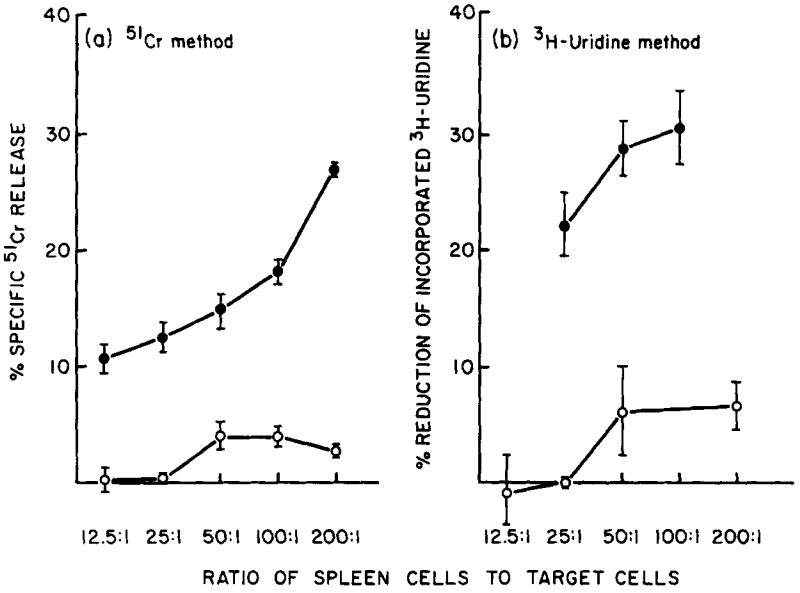

FIG. 2. The spontaneous cytotoxicity of the normal nude mouse spleen cells against HeLa and HeLa-measles. HeLa and HeLa-measles cells were labeled with $Na_2{}^{51}CrO_4$ (a) or with ^{3}H-uridine (b) as described in Materials and Methods. Spleen cells from normal nude mice, that had been preincubated to eliminate adherent cells, were added to these target cells at varying lymphocyte/target cell ratios. After a 10-h incubation, culture supernates (^{51}Cr method) or the labeled cells (^{3}H-uridine method) were harvested, and the cytotoxicity was calculated as described in Materials and Methods. The means of triplicate cultures ±SE are shown. (○): HeLa target, (●): HeLa-measles target.

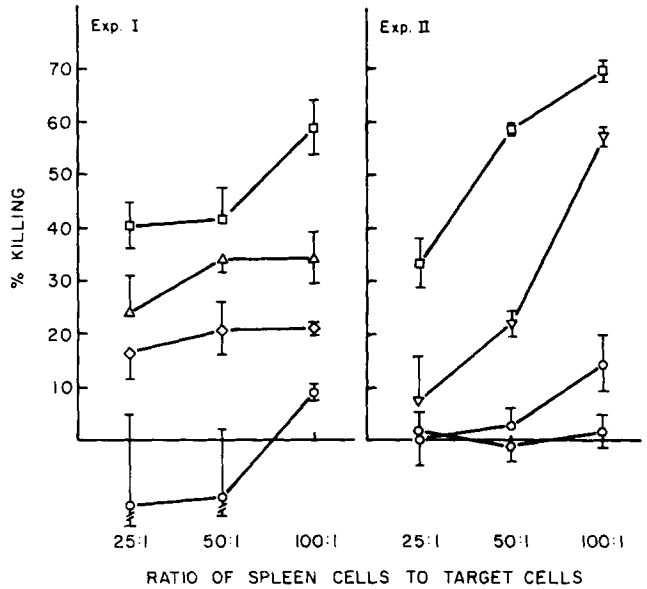

FIG. 3. Spontaneous cytotoxicity of normal nude mouse spleen cells against various uninfected or virus P.I. cells lines. The cytotoxicity was assayed using the ^{3}H-uridine method. The means and standard errors of triplicate cultures are shown. (○): HeLa target, (□): HeLa-measles target, (△): HeLa-VSV target, ((◯)): BHK target, (◇): BHK-mumps target, (▽): BHK-VSV target.

TABLE II

*Partial Characterization of the Effector Cells in Nude Mice Responsible for the Spontaneous Cytotoxicity against HeLa-Measles Cells**

Exp. No.	Treatment	Cytotoxicity	% Response
		%	
I	None	44.0± 6.4	100
	Elimination of glass adherent cells	54.3± 9.1	124
II	None	41.3± 4.4	100
	Nylon column passed	48.5± 3.5	117
	Trypsin (0.5%, 30 min)	46.7± 2.2	113
	Trypsin (2.0%, 30 min)	32.7± 2.5	79
	Anti-Ig + C′	44.5± 4.4	108
	Anti-Thy 1.2 + C′	24.6± 3.5	60
III	NMS + C′	51.3± 1.2	100
	Anti-Thy 1.2 + C′	33.5± 7.3	65
IV	NMS + C′ (× 2)‡	44.8± 2.1	100
	Anti-Thy 1.2 + C′ (× 2)	31.0± 4.0	69

* Spleen cells from normal nude mice were variously treated as described in Materials and Methods, and their spontaneous cytotoxicities against HeLa-measles target cells were assayed by ^{3}H-uridine assay. In exp. I and II, the cell numbers were adjusted after various treatments to the same numbers, and in exp. III and IV, the cells were resuspended to the original volume without the adjustment of cell numbers after the treatments. In all experiments, A/T ratio was 100:1, and the means ± SE of the triplicate wells are shown.

‡ The treatment with antiserum and complement was repeated twice.

measles system. As shown in Table II, the cytotoxic activity was enriched by elimination of glass-adherent cells and by passage through nylon wool columns, suggesting that the effector cell is neither a macrophage nor a B cell. Similarly, the cytotoxic activity was unaffected by treatment of the spleen cells with anti-Ig + complement. The cytotoxic activity was resistant to trypsin treatment. Spleen cells still showed significant activity even after treatment with 2% trypsin for 30 min.

Because cytotoxic activity was found in athymic nude mouse spleen cells and was higher than that in conventional mice of the same background, i.e., BALB/c (data not shown), it seemed improbable that conventional T cells were responsible for the cytotoxicity. Recently, however, it has been reported that some spleen cells express weak Thy 1 antigen even in the nude mouse (17). Consequently, we examined the effect of anti-Thy 1.2 + complement treatment on the killing by nude spleen cells. As shown in Table II, after treatment with anti-Thy 1.2 + C, under conditions in which a >90% reduction of T-cell activity of conventional mouse spleen was found (as assessed by Con A stimulation), treated nude mouse spleen cells still retained significant spontaneous cytotoxicity of HeLa-measles targets, although the activity was consistently reduced by 30–35%. These results indicate that the cells cytotoxic to the virus P.I. cell lines are not conventional B cells, T cells, or macrophages. From the above characteristics and the finding that the cytotoxic activity is detected in unprimed, normal animals, the effector cell in this system would appear to be the so-called natural killer (NK) cell. However, when such cells were retreated with anti-Thy 1.2 + complement, a consistent partial reduction in activity (30–40%) was noted.

TABLE III

Inhibition by Various Virus P.I. Cell Lines of Spontaneous Cytotoxicity against HeLa-Measles

Exp. no.	Percent cytotoxicity against HeLa-measles in the presence of various unlabeled target cells*					
	None	HeLa	HeLa-measles	HeLa-VSV	BHK-mumps	BHK-VSV
I	61.8 ± 0.7 (0.7%)§	60.6 ± 1.5 (1.9%)	20.7 ± 2.8 (67.5%)	n.t.‡	n.t.	62.3 ± 2.6 (0.8%)
II	64.2 ± 1.2	n.t.	51.0 ± 0.2 (20.6%)	63.1 ± 1.2 (1.7%)	66.6 ± 1.2 (−2.8%)	58.2 ± 1.1 (9.3%)

* HeLa-measles cells were labeled with ^{3}H-uridine for 3 h. After three washes, medium or various kinds of unlabeled cells were added to the labeled HeLa-measles, and then spleen cells from normal nude mice were added to an attacker to target cell ratio of 100:1. In exp. I, the ratio of unlabeled cells to labeled HeLa-measles was 4:1, and in exp. II, 10:1. The means triplicate culture ± SE are shown.

‡ Not tested.

§ Percent inhibition of the cytotoxicity is shown in parenthesis.

The partial inhibition of cytotoxicity in this system by high concentrations of anti-Thy 1 + complement is also consistent with recent reports of Herberman et al. (18) using a leukemia target system.

Specificity of Natural Killer Cells in the Cytotoxicity of Virus P.I. Cells. As shown in Fig. 3, NK cells from normal nude spleens had no apparent specificity for individual viruses or cells, other than distinguishing between P.I. and uninfected targets. This observation, however, does not necessarily indicate that NK-cell killing is totally nonspecific. The degree of susceptibility to killing of various P.I. targets always had the same rank order. For example, the order of greatest sensitivity to killing was invariably HeLa-measles > BHK-mumps = BHK-VSV > BHK-VSV-P. To probe the question of specificity in more detail, we used cold target inhibition of killing of virus P.I.+cell lines. HeLa-measles were labeled with ^{3}H-uridine for 3 h, and just before the addition of the effector cells, unlabeled target cells of various types were added to the labeled HeLa-measles cells. The results shown in Table III indicated that the killing of labeled HeLa-measles was blocked by the addition of unlabeled HeLa-measles, but not by cold uninfected HeLa cells. Other virus P.I. cell lines, including HeLa-VSV, BHK-mumps, and BHK-VSV similarly failed to block the killing of HeLa-measles, although they were susceptible to NK killing.

These results suggest, but do not prove, that the killing of the virus P.I. cell lines by NK cells may involve virus-specific recognition, although the whole NK-cell population has cytotoxic activity for a variety of virus P.I. cells.

In Vivo Induction of NK Activity in Nude Mice by the Injection of Virus P.I. Cell Lines. In all experiments described above, spleen cells used for the cytotoxic assay were derived from normal nude mice. We have subsequently examined the cytotoxic activity of spleen cells of mice that had been inoculated with either virus P.I. or uninfected cell lines to ascertain whether any alteration of cytotoxic activity was found in spleens of mice exposed to these cell lines in vivo. BHK, BHK-mumps, or BHK-VSV cell lines were injected subcutaneously into age-matched (6 wk) nude mice, and after various intervals, the cytotoxic activity of the spleen cells was assayed using HeLa-measles as targets. The spontaneous cytotoxic activity of age-matched normal nude mice was assayed simultaneously as control. The results presented in Table IV indicate that a significant increase in the cytotoxic activity over controls was observed in nude mice

TABLE IV

*Kinetics of the Augmentation of NK Activity In Vivo by the Inoculation of Virus PI Cell Lines in Nude Mice**

Exp. No.	Days after inoculation	A/T ratio	Percent cytotoxicity of spleen cells of nude mice			
			None	BHK	BHK-VSV	BHK-mumps
I	2	100:1	24.3	21.1	43.1	27.1
		50:1	0.2	1.0	12.7	20.0
II	5	100:1	37.3	28.5	55.6	NT‡
		50:1	16.8	13.1	46.4	
III	15	100:1	52.8	20.3	63.7	55.9
		50:1	32.8	9.8	53.8	46.7
IV	35	100:1	62.0	NT	NT	65.9
		25:1	22.7			57.4

* BHK or virus PI BHK cell lines (10^6 cells) were injected subcutaneously into nude mice. After various intervals, the cytotoxic activity of the spleen cells was assayed using HeLa-measles as a target. Spleens of two mice were pooled in each experiment. Each time, the NK activity of the normal nude spleen cells (two mice pooled) was also assayed as a control. Assay time was 10 h. Mean cytotoxicity of the triplicate cultures is shown.

‡ NT, not tested.

previously inoculated with virus P.I. cell lines. The augmentation of cytotoxic activity in P.I. cell lines was detected as early as 2 d after inoculation and remained at high levels for at least 35 d. The spleen cells from mice receiving P.I. cell lines, however, still failed to exert any significant cytotoxicity on uninfected HeLa cells. In addition, no augmented cytotoxic activity was observed with spleen cells from nude mice inoculated with uninfected cells; in fact, there was a significant and reproducible depression of NK activity in these mice compared to normal control mice.

Clearly, in vivo induction of NK activity was not specific for inducing virus or carrier cell type, because spleen cells from nude mice injected with either BHK-mumps or BHK-VSV showed augmented cytotoxic activity on HeLa-measles targets. Nevertheless, these spleen cells had little cytotoxic activity for uninfected parental cells, suggesting that the induced killer cells derived from the same population as those in untreated nude mice, and retained a similar spectrum of specificity or activity on different targets.

Selection of Variants: Development of the BHK-VSV Producer (BHK-VSV-P) Cell Line. Because the data suggested that P.I. tumor cells are restricted in the tumorigenicity in nude mice by a host immunological response, we sought, through serial transplantation of the nodules, to obtain variants which would fail to activate a host response and/or would be resistant to that response. BHK-VSV nodules persisted for months, and through a number of passages in nude mice. One serially transplanted nodule formed a slowly growing tumor which shed virus in vivo and in culture. Reinjection of the cultures into nude mice resulted in the growth of tumors containing viruses. This virus-producing line (BHK-VSV-P) has been serially passaged six times in nude mice with intervening isolation and characterization of primary cell cultures between each passage. The characteristics of the BHK-VSV-P line are summarized in Table V. Throughout the passage-culture series, the variant cell line has produced

TABLE V

Comparison of the In Vitro and In Vivo Properties of BHK21, BHK-VSV, and BHK-VSV-P

Characteristic examined	BHK21	BHK-VSV	BHK-VSV-P
Tumorigenicity in nude mice:			
Number of mice tested	18	25	10
Neoplasm which forms	Tumor (100%)	Nodule (80%)	Tumor (100%)
Minimum no. of cells for neoplasm formation	10	10^4	NT
Invasiveness	Invasive	No invasiveness	Extensively invasive
Metastases	−	−	+; Lungs by lymphatic route
Mononuclear cell infiltration into neoplasm	+/−	+ + +	+ + +
Infectious virus levels in vivo (serum, spleen, kidney, and liver)	NA	10^1–10^5 PFU/ml	10^6–10^9 PFU/ml
DI particles in neoplasm	NA	+	+
Virus recovered	NA	Small plaque, ts	Small plaque, ts
VSV neutralizing serum antibody titers	NA	<1/10	<1/10
Percent neoplastic cells with viral antigen	NA	99%	99%
Percent neoplastic cells resistant to VSV superinfection	NA	99%	99%

NT, not tested; NA, not applicable; PFU, plaque-forming unit.

Tumors or nodules were excised from the mice, minced thoroughly, and cultured in MEM supplemented with 7% heat-inactivated calf serum. Tests for viral antigen expression and for resistance to homologous VSV Indiana and challenge infection were performed on primary cultures of tumors or nodules by the procedures described in Materials and Methods.

large, soft, rapidly growing, invasive tumors which are metastatic by the lymphatic route to the lung, liver, lymph nodes, and other organs. Histologically, however, these tumors are invariably surrounded with a large mononuclear cell infiltrate, suggesting the existence of a host response. Primary cultures of these tumor cells released large amounts of mature infectious virus and defective-interfering (DI) particles. The BHK-VSV cultures expressed very small amounts of VSV surface antigens, whereas BHK-VSV-P cells express surface antigens in amounts comparable to lytically infected cell cultures. Several findings indicate that the BHK-VSV-P cell line contains a mutant virus (J. Holland, and C. Jones. Unpublished observations). The cells infected with VSV-P are capable of yielding larger than usual amounts of virus and DI particles. Oligonucleotide maps of the VSV-P virus show significant differences from those of wild type VSV. Moreover, purified VSV-P virus, and DI particles isolated from BHK-VSV-P tumor have initiated new virus P.I. cell lines of BHK21 cells which produce virally infected tumors, rather than nodules, when injected into nude mice. Among the virus P.I. cell lines tested, BHK-VSV-P is unique in its ability to metastasize as well as to remain P.I. and form tumors in the nude mouse. As shown in Fig. 4 b, BHK-VSV-P was found to be less susceptible to the spontaneous cytotoxicity of normal nude spleen cells than any other P.I. cell lines tested, although it was slightly more susceptible than uninfected BHK cells. Conversely, as shown in Fig. 4 a, spleen cells of mice injected with BHK-VSV-P showed extraordinarily

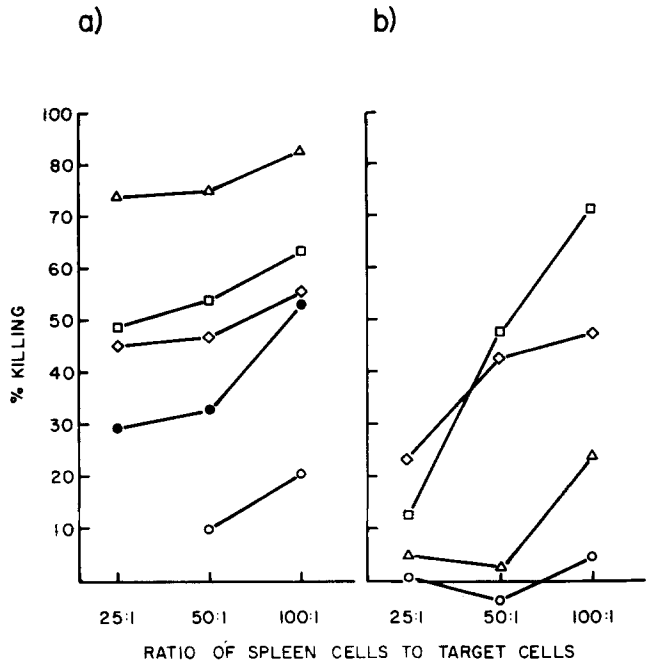

FIG. 4. (a) The effect of injection of BHK21 cells or various BHK P.I. cell lines on the spontaneous cytotoxic activity of nude mouse spleen cells. 10^6 cells of BHK21, BHK-mumps, BHK-VSV, or BHK-VSV-P were injected subcutaneously into nude mice. 2 wk later, two to three spleens of each group were pooled and their spontaneous cytotoxic activities on HeLa-measles were assayed using ^{3}H-uridine method. The cytotoxic activity of the age-matched, normal nude mice spleen cells was also assayed as a control. Each value represents the mean value of triplicate culture. (●), normal mice; (○), BHK-injected mice; (◇), BHK-mumps-injected mice; (□), BHK-VSV-injected mice; (△), BHK-VSV-P-injected mice. (b) The comparison of the susceptibility of BHK-VSV-P to spontaneous cytotoxic activity of normal nude mouse spleen cells with those of BHK21, BHK-mumps, and BHK-VSV. Each point represents the mean value of triplicate cultures. (○), BHK target; (◇), BHK-mumps target; (□), BHK-VSV target; (△), BHK-VSV-P target.

augmented cytotoxicity on HeLa-measles targets. These results indicate that BHK-VSV-P is the best inducer of NK activity on a variety of infected targets, a finding that correlates well with the strong cellular reaction at the tumor site in vivo. It is likely that its tumorigenicity is related to the fact that BHK-VSV-P is relatively resistant to the cytolytic activity of NK cells. In any case, in this exceptional variant cell line, a dissociation was observed between susceptibility to spontaneous cytotoxic activity in vitro and ability to augment the cytotoxic activity in vivo.

Discussion

A number of viruses are known to persist for long times in animals and man, some of which may be responsible for chronic diseases, particularly slow neurologic diseases (19, 20). The persistent viral infection represents a paradoxical situation in that viruses manage to survive in a host which has generated appropriate immune responses to protect it from the acute phase of viral infection. It has been amply demonstrated that various cultured cells, such as HeLa and BHK21, can be P.I. by enveloped RNA viruses, e.g., VSV, measles, mumps, influenza, and rabies (12, 21, 22). Although the viral mechanisms leading to persistent infection in cultured cells

have been extensively studied, particularly in defining the roles of viral defective interfering particles and conditional lethal mutants (21, 23), the nature of the host immune responses to such P.I. cells remains largely unexplored.

In these and previous studies (22, 24, 25) we observed that virus P.I. cell lines expressed little tumorigenic potential when injected into athymic nude mice. In contrast, the uninfected parental cells, even as few as 10–100 cells, formed rapidly growing, well-vascularized tumors. The P.I. daughter cell lines failed to grow or formed benign nodules at all seeding densities between 10^4 and 10^7 cells. Histologically, these nodules showed a marked host reaction to the virally infected cells. The reduced tumorigenic potential is strictly correlated with the presence of the persistent viral infection, because cells cured in vivo or in vitro of their viral infection are again highly tumorigenic in the nude mice. Further, coinjection of P.I. cells with uninfected parental cells results in the restriction of growth only of the virally infected cells. Evidence supporting an active host response as responsible for the restriction of growth of P.I. cells derives from the findings that marked mononuclear cell response appeared at the sites of inoculation and that irradiated nude mice failed to reject the P.I. cell lines.

An analysis of the in vitro cytotoxic activity of spleen cells from nude mice for virus P.I. cell lines indicated: (*a*) P.I. cell lines, but not uninfected parental cell lines, were susceptible to spontaneous cytotoxic activity measured by two assays: (*b*) in vivo inoculation of P.I. cell lines, but not uninfected parental cells, induced an enhanced spontaneous cytotoxic activity for P.I. target cells in vitro; (*c*) the induction of cytotoxicity was not specific for the inducing virus or the cell used for the induction, suggesting that the in vivo induction was mediated by some nonspecific mediators.

We have inferred that the cytotoxicity against the P.I. cell lines is mediated by non-B-, non-T-, nonadherent lymphocytes with characteristics previously described for NK cells on the basis of the following observations: (*a*) cytotoxicity exists in normal unprimed spleen cells in both nude and conventional mice, and is, in fact, greater in the former than the latter; (*b*) the cytotoxic cells are relatively resistant to treatment with anti-Thy 1.2 + C under conditions in which in vitro T-cell activity is abrogated, are totally resistant to anti-Ig + complement, and are nonadherent either to plastic dishes or to nylon wool columns; (*c*), cytotoxic activity is present in normal unstimulated spleen, but is markedly augmented by inoculation of nude mice with various P.I. cell lines; and (*d*) uninfected tumor cells are not susceptible to and unable to induce cytotoxicity in vivo in nude mice. In fact, they frequently depressed the level of spontaneous cytotoxic activity. All these observations correlate with the characteristics of NK cells described in a variety of tumor systems (10, 26–30). Although spontaneous cytotoxic activity of NK cells in mouse spleen or human peripheral blood for most target cells is usually relatively low, it is clear that these cells are susceptible to modulation and enhancement by a variety of agents in vivo, such as the adjuvants BCG (31) and *Corynebacterium parvum* (32), and more recently, interferon or interferon inducers (33–35).

The mode of recognition of tumor cells or virus-infected cells by NK cells remains an intriguing problem. At the first level, it would appear that their acitivity is quite nonspecific, in that normal spleen cells are capable of killing tumor cell lines P.I. with any of three different viruses, correlating well with the ability of nude mice to reject those cell lines in vivo. However, cold target competition experiments described here

suggest rather specific recognition by NK cells, in that blocking of the killing of one virus-infected cell, e.g., HeLa-measles, could be achieved only by unlabeled HeLa-measles, and not by uninfected HeLa or other P.I. cells. Similar observations suggesting rather specific recognition of NK cells have been reported by Welch et al. (36) in the LCM system. Two explanations could be forwarded to explain these apparently divergent specificities. It could be argued that NK cells are a homogeneous population that recognize some unknown structure(s) or cell-surface alteration(s) common to all susceptible cell lines, tumor cells, or virus-infected cells, and, as such, are nonclonal and nonspecific in the immunological sense, but selective in terms of the cells with which they can interact. Alternatively, the NK population could be a heterogeneous one, containing a wide variety of specificities capable of distinguishing between individual viruses and target cells, and these specificities would be expressed clonally. Stimulation of any one subpopulation, however, would lead to the production of a nonspecific stimulatory factor, such as interferon, that could cause the polyclonal expansion or activation of other specificities, resulting in the apparent broad specificity observed in vitro. Although our present results seem to support the latter possibility, they cannot be regarded as conclusive. The previous observation of Herberman et al. (18) and our own data indicating that NK cells express low levels of Thy 1.2, and data of Cantor that NK cells and T cells express Ly 5 determinants[2] are most consistent with the possibility that NK cells represent T-cell precursors. If so, it might be predicted that they would have specific receptors, but would not be restricted in their cytotoxicity by the major histocompatibility complex because they have not been processed by the thymus in the nude mouse (37). The present system of virus P.I cells may prove useful for analyzing the fine specificity requirements for recognition and for evaluating whether specificity is clonally distributed, but clearly more cell-virus combinations must be studied to resolve the question of specificity.

If recognition is specific, it is unclear whether the inducing stimulus is, in fact, virus-infected cells, released virus and/or DI particles. In this regard, it is of some interest that a subline of the BHK cells P.I. with VSV (BHK-VSV-P) which was selected for its ability to withstand the rejection process in nude mice, was found to be the most potent inducer of NK activity in vivo, although the cells were relatively resistant to the NK activity of normal nude spleen cells and formed tumors in nude mice. Compared with other virus P.I. cell lines, BHK-VSV-P sheds large amounts of virus both in vivo and in vitro (J. Holland and C. Jones, unpublished observation). It is our speculation that the large number of virus particles released from BHK-VSV-P may be responsible for the nonspecific augmentation of NK activity through the induction of interferon. Indeed, preliminary experiments show significant interferon titers in the serum of mice inoculated with BHK-VSV-P.

It is not completely clear whether the dissociation between NK-cell susceptibility and NK-cell induction in vivo observed in the BHK-VSV-P subline reflects changes in the properties of the cell, or of the virus following the selection of the subline in vivo. Preliminary results indicate that BHK cells P.I. with virus isolated from the BHK-VSV-P subline behave precisely as the BHK-VSV-P subline, suggesting that the invasiveness of the tumor in vivo and its relative resistance to NK activity in vitro may be primarily regulated by the mutant virus itself.

---

[2] Cantor, H., M. Kasai, F. W. Shen, J. C. Leclerc, and L. Glimcher. Immunogenetic analysis of natural killer activity in the mouse. Immunol. Revs. In press.

In summary, the results indicate that persistent viral infection produces alterations in tumor cells rendering them capable of recognition and rejection by the nude mouse. Although, in general, there was excellent correlation between the capability of the nude mice to reject the P.I. tumors in vivo and the ability of NK cells to lyse the P.I. target cells in vitro, it is important to recognize that variants exist in which these two properties can be dissociated. The present studies lend support to the thesis that NK cells may play an important role, not only against tumor cells (38), but in resistance to viral infections, particularly persistent infections.

Our results may also have relevance for defining the requirement for invasiveness and metastasis of tumors, and in explaining difficulties in heterografting certain types of human tumors, e.g. prostatic tumors, into the nude mouse. (4, 11, 25). At the very least, a number of previous studies attempting to correlate in vitro cellular phenotypes of transformed cells with malignancy as defined by tumorigenicity in the nude mouse (5, 6) require reevaluation, because some cells thought to be nontumorigenic may in fact be malignant but immunologically rejected by the nude mouse.

Summary

Cell lines known to be tumorigenic in the nude mouse were modified by rendering them persistently infected (P.I.) with a variety of RNA viruses, including measles, mumps, vesicular stomatitis virus, and influenza. Although as few as 100 HeLa or BHK cells produced tumors in 100% of nude mice, as many as 2×10^7 of the same cells P.I. with viruses failed to produce tumors. An active host response responsible for restricting the growth of the P.I. cells was suggested by the findings of marked mononuclear cell infiltrates at the inoculation sites and the inability of irradiated nude mice to reject them.

An analysis of the in vitro cytotoxic activity of spleen cells from normal nude mice indicated that: (*a*) P.I. cell lines, but not uninfected cell lines, were susceptible to spontaneous cytotoxicity; (*b*) in vivo inoculation of P.I. lines induced an enhanced cytotoxic activity for P.I. targets in vitro, and this induction was not specific either for inducing virus or cell line; and (*c*) the effector cell had the characteristics of natural killer (NK) cells. Although the specificity of recognition of the various P.I. cell lines remains unclear, cold competition experiments indicated that blocking the killing of one P.I. cell line, e.g. HeLa-measles, could be achieved only by unlabeled homologous cells, i.e. HeLa-measles, and not by uninfected cells or other P.I. lines.

A variant subline of BHK cells P.I. with VSV was selected for its ability to withstand the rejection process in nude mice. These cells formed metastatic and invasive tumors in nude mice. Although they were the most potent inducers in vivo of NK cell activity against various P.I. targets, they were the most resistant of the P.I. lines to NK cell cytotoxicity in vitro. In this system there was a good correlation between tumor rejection in vivo and susceptibility to NK cells in vitro. The present results suggest that NK cells may play a significant role in both rejection of tumor cells, and in resistance to viruses, particularly persistent infections.

Received for publication 4 October 1978.

References

1. Pantelouris, E. M. 1968. Absence of Thymus in a Mouse Mutant. Nature (*Lond.*). **217**:370.
2. Rygaard, J. 1973. Thymus and Self-Immunobiology of the Mouse Mutant Nude. John Wiley and Sons, New York.

3. Fogh, J., and B. Giovanella. 1978. The Nude Mouse in Experimental and Clinical Research. Academic Press, Inc., New York.
4. Reid, L., and S. Shin. 1978. Transplantation of heterologous endocrine tumor cells in nude mice. *In* The Nude Mouse in Experimental and Clinical Research. J. Fogh and B. Giovanella, editors. Academic Press, Inc., New York. 313.
5. Freedman, V. H., and S. Shin. 1974. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell. **3**:355.
6. Stiles, C. D., W. D. Desmond, L. M. Chuman, G. Sato, and M. H. Saier, Jr. 1976. Relationship of cell growth behavior in vitro to tumorigenicity in athymic nude mice. *Cancer Res.* **35**:3300.
7. Stutman, O. 1978. Spontaneous, viral and chemically induced tumors in the nude mouse. *In* The Nude Mouse in Experimental and Clinical Research. J. Fogh and B. Giovanella, editors. Academic Press, Inc., New York. 411.
8. Koene, R., J. Gerlag, J. Hagemann, and P. Wijcleved. 1974. Rejection of skin grafts in the nude mouse. *Nature* (*Lond.*). **251**:69.
9. Maguire, H., Jr., H. C. Outzen, R. P. Custer, and R. T. Prehn. 1976. Invastion and metastasis of a xenogeneic tumor in nude mice. *J. Natl. Cancer Inst.* **57**:439.
10. Herberman, R. B. 1978. Natural cell mediated cytotoxicity in nude mice. *In* The Nude Mouse in Experimental and Clinical Research. Academic Press, Inc., New York. 135.
11. Reid, L., I. Lear, F. Merk, J. Albert, and J. Geller. 1978. Androgen-dependent human prostatic carcinoma tumor line. *Cancer Res.* **19**:151.
12. Holland, J. J., L. P. Villarreal, R. M. Welsh, M. B. A. Oldstone, D. Kohne, R. Lazzarini, and E. Scolnick. 1976. Long-term persistent vesicular stomatitis virus and rabies infection of cells *in vitro*. *J. Gen. Virol.* **33**:193.
13. Trinchieri, G., and D. Santoli. 1978. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J. Exp. Med.* **147**:1314.
14. Fujiwara, H., T. Hamauka, K. Teshima, H. Aoki, and M. Kitagawa. 1976. Preferential generation of killer or helper T-lymphocyte activity directed to the tumor-associated transplantation antigens. *Immunology.* **31**:239.
15. McFarland, H. A. 1974. *In vitro* studies of cell-mediated immunity in an acute viral infection. *J. Immunol.* **113**:173.
16. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.
17. Loor, A., and G. E. Roelants. 1974. High frequency of T lineage lymphocytes in nude mouse spleen. *Nature* (*Lond.*). **251**:229.
18. Herberman, R. B., M. E. Nunn, and H. T. Holden. 1978. Low density of Thy 1 antigen of mouse effector cells mediating natural cytotoxicity against tumor cells. *J. Immnol.* **121**:304.
19. Horta-Barbosa, L., D. A. Fuccillo, W. T. London, J. T. Jabbour, W. Zeman, and J. L. Sever. 1969. Isolation of measles virus from brain cell cultures of two patients with subacute sclerosing panencephalitis. *Proc. Soc. Exp. Biol. Med.* **132**:272.
20. Maugh, T. H. 1977. Multiple sclerosis: two or more viruses may be involved. *Science* (*Wash. D. C.*). **195**:768.
21. Holland, J. J., and L. P. Villarreal. 1974. Persistent noncytocidal vesicular stomatitis virus infections mediated by defective T particles that suppress virion transcriptase. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2956.
22. Holland, J. J., B. L. Semler, C. Jones, J. Perrault, L. Reid, and L. Roux. 1978. Role of DI, virus mutation and host response in persistent infections by enveloped RNA viruses. *ICN-UCLA Symp. Mol. Cell. Biol.* **11**:57.
23. Preble, O. T., and J. S. Younger. 1975. Temperature-sensitive viruses and the etiology of chronic and inapparent infections. *J. Infect. Dis.* **131**:467.

24. Reid, L., C. Jones, and J. Holland. 1979. Virus carrier state suppresses tumorigenicity of tumor cells in athymic (nude) mice. *J. Gen. Virol.* In press.
25. Reid, L., J. Holland, C. Jones, B. Wolf, G. Niwayama, R. Williams, N. O. Kaplan, and G. Sato. 1978. Some of the variables affecting the success of transplantation of human tumors into athymic nude mice. *In* Symposium on the use of (athymic) nude mice in cancer research. G. Fisher, editor. New York. 123.
26. Herberman, R. B., M. E. Nunn, and D. H. Laurin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer* **16:**216.
27. Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to the genotype. *Eur. J. Immunol.* **5:**112.
28. Kiessling, R. E., E. Klein, H. Pross, and H. Wigzell. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5:**117.
29. Herberman, R. B., M. E. Nunn, T. H. Holden, and D. H. Laurin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer.* **1:**230.
30. Becker, S., and E. Klein. 1976. Decreased "natural killer" effect in tumor-bearing mice and its relation to the immunity against oncorna virus-determined cell surface antigens. *Eur. J. Immunol.* **6:**892.
31. Wolfe, S. A., D. E. Tracey, and C. S. Henney. 1976. Induction of "natural killer" cells by BCG. *Nature (Lond.).* **252:**584.
32. Ojo, E., O. Haller, and H. Wigzell. 1978. *Corynebacterium parvum*-induced peritoneal exudate cells with cytolytic ability for tumor cells are nonphagocytic cells with characteristics of all being natural killer, NK, cells. *Int. J. Cancer.* **21:**444.
33. Gidlund, M., A. Orn, H. Wigzell, A. Senik, and I. Gressor. 1978. Enhanced NK activity in mice infected with interferon and interferon inducers. *Nature (Lond.).* **273:**759.
34. Santoli, D., G. Trinchiari, and H. Koprowsky. 1978. Cell-mediated cytotoxicity against virus-infected target cells in humans. II. Interferon induction and activity of natural killer cells. *J. Immunol.* **121:**532.
35. Herberman, R. B., M. E. Nunn, H. T. Holden, S. Staal, and J. Y. Djeu. 1977. Augmentation of natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic target cells. *Int. J. Cancer.* **19:**555.
36. Welsh, R. M., and R. M. Zinkernagel. 1977. Heterospecific cytotoxic cell activity induced during the first three days of acute lymphocytic choriomeningitis virus infection in mice. *Nature (Lond.).* **268:**646.
37. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J. Exp. Med.* **147:**882.
38. Höller, O., M. Hansson, B. Kiessling, and H. Wigzell. 1977. Role of nonconventional natural killer cells in resistance against syngeneic tumor cells *in vivo*. *Nature (Lond.).* **270:**609.