4-1-1992

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JOURNAL OF VIROLOGY, Apr. 1992, p. 2532–2535
0022-538X/92/042532-04$02.00/0

Vol. 66, No. 4

Generation of "Natural Killer Cell-Escape" Variants of Pichinde Virus during Acute and Persistent Infections

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Received 12 November 1991/Accepted 7 January 1992

Pichinde virus (PV) strain AN 3739 was determined to be sensitive to natural killer (NK) cells in vivo by enhanced replication in NK cell-depleted mice. An NK-sensitive subclone (PV-NK^{s1}) was serially passed in mice whose NK cells had previously been activated by an interferon inducer, and two plaque isolates were shown to be resistant to NK cells but not to interferon. Inoculation of severe-combined-immunodeficient mice with PV-NK^{s1} led to a persistent infection resulting in an NK-resistant viral population. This is the first demonstration of the isolation of viral "NK-escape" variants, as defined by the ability of the virus to replicate in vivo.

Viruses evolve under the selective pressure of host defense mechanisms, resulting in immunological escape variants or even in the formation of new viral species (6). The antigenic drift of influenza viruses and changes in neutralizing antibody epitopes during persistent lentivirus infections are examples of selection by antiviral antibodies (9, 13). A cytotoxic T-cell-resistant variant of lymphocytic choriomeningitis virus (LCMV) capable of escaping surveillance during persistent infection has also been demonstrated (10). These antibody- and cytotoxic-T-cell-escape variants arise from mutations in their neutralization antibody-binding sites and in immunodominant T-cell epitope sequence, respectively.

Natural killer (NK) cells play roles in the natural resistance to a variety of viral infections, and their activities may remain elevated for long periods of time during persistent infections (2–3, 4, 15–17). The only persistent infection adequately examined for the role of NK cells has been persistent LCMV infection of mice. Depletion of the NK cells in these persistently infected mice with antibodies did not enhance LCMV synthesis, nor did it enhance LCMV synthesis in acutely infected mice (1, 4). Thus, LCMV, which normally causes persistent infection in mice under conditions of elevated NK cell responses, appears to be an NK-resistant virus.

While screening a variety of viruses for sensitivity to NK cells in vivo, by using enhanced replication in NK cell-depleted mice as an indicator of NK sensitivity, we found that Pichinde virus (PV) fell into the category of being NK sensitive (17). PV is an arenavirus distantly related to LCMV, but, unlike LCMV, it does not normally establish persistent infection in mice. We speculated that, given its similarity to the NK-resistant LCMV, it might be possible to select NK-resistant variants of PV by passage in mice in the presence of NK cells. Here we report the generation of such variants.

Isolation of NK-cell-resistant variants of PV. NK-resistant variants of PV were established from an NK-sensitive plaque-purified subclone (referred to henceforth as PV-NK^{s1}) of the AN 3739 strain of PV (17). C3H/HeSnJ mice (Jackson Laboratory, Bar Harbor, Maine), 4 to 8 weeks of age, were inoculated intraperitoneally (i.p.) with 100 μg of the interferon (IFN)-inducing poly(I)-poly(C) (Sigma Chemical Co., St. Louis, Mo.) in order to activate NK cells. Two days later the mice were challenged with 2×10^6 PFU of PV-NK^{s1} diluted in Eagle's minimal essential medium supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal bovine serum (MEM). After 3 days, the mice were sacrificed, and spleen homogenates were prepared and used to inoculate monolayers of L-929 cells. Isolated plaques were collected and dispersed by sonic oscillation, and a virus stock was grown in L-929 cells maintained in MEM. To examine the sensitivity of the virus to NK cells in vivo, groups of four to six C57BL/6 male mice which were 4 to 8 weeks old (Jackson Laboratory) were injected with 100 μl of a previously determined optimal dilution of an ascites preparation of the immunoglobulin G 2a PK 136 monoclonal antibody (MAb) to NK 1.1 (7) in MEM, in order to selectively deplete NK-cell activity (17). Control mice received 100 μl of MEM instead of the MAb. Similar results were obtained whether the MAb was administered i.p. or intravenously. The mice were then inoculated i.p. with the virus. Three days after inoculation of the virus, PFU titers in the spleens of individual mice were examined by plaque assays with Vero cells. NK cell activity was monitored by using spleen cells from each individual mouse in a microcytotoxicity assay with YAC-1 cells (14). Comparisons between groups were done by using the Student *t* test.

The different in vivo-passaged, plaque-purified viruses were examined for sensitivity to NK cells compared with NK-sensitive PV-NK^{s1} and then subjected to additional passages in poly(I)-poly(C)-treated C3H/HeSnJ mice. Two NK-resistant variants of PV (PV-NK^{r1} and PV-NK^{r2}) were identified after 3 consecutive passages in vivo. Table 1 shows that splenocytes recovered from mice inoculated with the NK-sensitive PV-NK^{s1} and with either of the variants mediated significant killing of YAC-1 cells. Cytotoxicity was reduced by 80 to 100% by treatment of the mice with MAb to NK 1.1. As shown in Table 2, PV-NK^{s1} consistently replicated to higher titers in the spleens of NK cell-depleted mice than in control mice, as would be predicted for an NK-sensitive virus. In contrast, plaque-purified PV-NK^{r1} and PV-NK^{r2} replicated to high titers in both groups of mice,

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TABLE 1. NK cell-mediated cytotoxicity in control virus-infected mice and in mice depleted of NK cells by using anti-NK 1.1 MAb[a]

Virus	Expt	Results for:	
		Control[b] mice	Mice given anti-NK 1.1[c]
PV-NK^{s1}	1	31 ± 6.9	5.2 ± 3.3
	2	32 ± 1.9	1.8 ± 0.3
	3	35 ± 2.5	2.3 ± 1.7
	4	41 ± 5.0	5.4 ± 1.0
	5	68 ± 9.1	9.1 ± 1.4
	6	28 ± 2.9	2.9 ± 1.4
	7	24 ± 15	−13 ± 0.8
PV-NK^{r1}	1	26 ± 3.5	−0.2 ± 0.5
	2	38 ± 4.3	1.7 ± 0.8
	3	35 ± 5.3	0.0 ± 0.9
	4	45 ± 4.0	5.2 ± 0.6
	5	71 ± 7.9	6.8 ± 3.0
PV-NK^{r2}	1	56 ± 3.1	0.8 ± 0.8
	2	36 ± 5.5	0.6 ± 0.7
	3	56 ± 6.6	5.0 ± 2.4
PV-NK^{scid}	1	39 ± 6.9	4.1 ± 1.0
	2	35 ± 12.3	−12 ± 2.6

[a] Results represent means of the percent specific cytotoxicity against YAC-1 cells in 4-h microcytotoxicity assays ± standard deviations for four to six mice tested separately, using effector/target ratios of 100:1.
[b] 100 μl of MEM injected i.p. or intravenously.
[c] 100 μl of MAb NK 1.1 injected i.p. or intravenously.

regardless of whether the NK cells had been eliminated by treatment of the animals with MAb to NK 1.1. On the average, an increase in the titers of PV-NK^{s1} of 0.8 (6.3-fold) ± 0.3 $\log_{10}$ PFU per spleen was found in the MAb anti-NK 1.1-treated mice. In contrast, depletion of NK cells resulted in insignificant increases in the titers of both PV-NK^{r1} and PV-NK^{r2} (0.1 ± 0.3 and 0.03 ± 0.1 $\log_{10}$ PFU per spleen, respectively). Note that the virus titers in mice inoculated with the NK-resistant variants approached the virus titers found in the anti-NK 1.1-treated mice inoculated with the NK-sensitive PV variant. This is of significance, because a 20- to 40-fold-lower inoculum of the NK-resistant variants was used (Table 1), further indicating that these viruses replicate very efficiently in both NK cell-depleted and in control mice. In addition, we have recently shown that PV-NK^{r1} replicates to similar titers in untreated or NK cell-depleted mice with severe combined immunodeficiency (SCID) (16). PV-NK^{s1}, on the other hand, replicates to significantly higher titers in NK cell-depleted SCID mice (16).

Both the NK-sensitive PV-NK^{s1} and the NK-resistant variant PV-NK^{r1} establish asymptomatic, persistent infection in SCID mice (16). We asked whether an NK-resistant virus could be recovered from SCID mice persistently infected with PV-NK^{s1}. Homozygous SCID mice from the BALB/c CB17 background, bred in microisolator cages in the Department of Animal Medicine at the University of Massachusetts Medical Center, were inoculated i.p. with 2 × 10^6 PFU of PV-NK^{s1} in MEM. At 53 days postinfection the mice were bled, and virus titers were determined in a plaque assay with Vero cells. A virus stock was grown in L-929 cells. The sensitivity to NK cells of this non-plaque-purified virus isolate (PV-NK^{scid}) was examined as described above. Table 1 shows the results from two independent experiments. PV-NK^{scid} replicated to similar levels in control C57BL/6 mice and in mice treated with MAb to NK 1.1. Note that in the latter group of mice, NK cell activity was completely abrogated by the treatment with the MAb (Table 1). The above results are a clear indication that NK-resistant variants of an NK-sensitive virus may arise by in vivo selection during persistent infection.

Sensitivity of the PV variants to NK cells in vitro. Although we found that the PV variants were greatly divergent in their sensitivities to NK cells in vivo, we were unable to consistently and unequivocally demonstrate a difference in their sensitivities to NK cells as assessed by in vitro microcytotoxicity assays with monolayers of L-929 cells incubated with the different viruses for various periods of time and exposed to either endogenous or activated NK cells (data not shown). An analogous phenomenon has been found with murine cytomegalovirus. Whereas this virus is extremely sensitive to NK cells in vivo, murine cytomegalovirus-infected cells appear to be less sensitive than uninfected cells to lysis by activated NK cells in short in vitro cytotoxicity assays (3).

IFN is capable of protecting target cells from NK cell-mediated lysis, and we have reported that infection of fibroblasts with the NK-sensitive murine cytomegalovirus blocks the ability of IFN to protect targets from NK cells; in contrast, the NK-resistant LCMV is a poor inhibitor of IFN-mediated protection (3). We therefore tested whether PV infection of L-929 cells inhibited IFN-mediated protection. Cells were infected with PV-NK^{s1} or PV-NK^{r1} at a multiplicity of infection of about 0.1, incubated for 48 h, and then exposed overnight to 10,000 U of beta IFN (IFN-β). They then were exposed to PV-NK^{s1}-induced C3H/HeSnJ mouse NK cells. IFN nearly completely protected uninfected L-929 cells from NK cell-mediated lysis (33% for

TABLE 2. Replication of NK-sensitive and NK-resistant variants of PV in control and NK cell-depleted mice[a]

Virus	Expt	Results for:		P value[d]
		Control[b] mice	Mice given anti-NK 1.1[c]	
PV-NK^{s1}	1	<2.1	2.9 ± 0.5	<0.005
	2	2.5 ± 0.5	3.1 ± 0.4	<0.05
	3	3.4 ± 0.7	4.7 ± 0.2	<0.005
	4	3.1 ± 0.4	3.6 ± 0.4	<0.05
	5	3.7 ± 0.3	4.5 ± 0.2	<0.0025
	6	3.5 ± 0.1	4.2 ± 0.0	<0.0005
	7	3.5 ± 0.3	4.5 ± 0.2	<0.001
PV-NK^{r1}	1	3.6 ± 0.6	3.6 ± 0.3	>0.9
	2	3.6 ± 0.2	3.5 ± 0.8	<0.8
	3	3.6 ± 0.5	4.0 ± 0.7	<0.25
	4	3.8 ± 3.5	3.5 ± 0.3	<0.05
	5	4.4 ± 0.4	4.9 ± 0.3	<0.1
PV-NK^{r2}	1	3.3 ± 0.2	3.2 ± 0.7	<0.8
	2	2.6 ± 0.5	<2.7 ± 0.7	<0.9
	3	3.5 ± 0.3	3.4 ± 0.4	>0.9
PV-NK^{scid}	1	5.0 ± 0.3	4.6 ± 0.4	<0.25
	2	5.4 ± 0.7	5.5 ± 0.2	<0.9

[a] See Table 1, footnote *a*. Results were determined by the Student *t* test and are given as $\log_{10}$ PFU per spleen.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] Inocula: PV-NK^{s1}, 1 × 10^6 to 3 × 10^6 PFU per mouse; PV-NK^{r1} and PV-NK^{r2}, 5 × 10^4 PFU per mouse; PV-NK^{scid}, 1 × 10^6 PFU per mouse.

TABLE 3. Sensitivity of PV variants to IFN

Treatment	Amt of virus (log_{10} PFU/ml)	
	PV-NK^{s1}	PV-NK^{r1}
Medium	5.1	5.0
IFN-α		
12 U	4.4	4.7
120 U	4.1	4.0
1,200 U	3.3	2.6
IFN-β		
12 U	5.3	5.3
120 U	4.1	4.0
1,200 U	3.7	3.2
IFN-γ		
0.5 U	5.2	4.5
5 U	5.0	5.0
50 U	4.3	3.5
IFN-β (120 U) + IFN-γ (5 U)	3.8	3.3
IFN-β (1,200 U) + IFN-γ (50 U)	3.0	2.8

control and 3.7% for IFN-treated mice; effector/target ratio = 100:1) but protected virus-infected cells by only about 50% (with PV-NK^{s1}, 53% for control and 26% for IFN-treated mice; with PV-NK^{r1}, 50% for control and 26% for IFN-treated mice). Thus, even though PV infections caused some impairment of the IFN-mediated protection, no differences in the degree of IFN-mediated protection were noted between the NK-sensitive and NK-resistant variants on this cell line.

Sensitivity of the PV variants to IFN. Because the selection of the NK-resistant variants of PV was accomplished by using conditions under which high levels of IFN were generated, and because NK cells themselves are capable of synthesizing IFN, we examined whether the PV variants differed in the ability to replicate in vitro in the presence of IFN. To this end, 5×10^5 L-929 cells in MEM were plated on 6-well plates (no. 3406; Costar, Cambridge, Mass.) and incubated for 24 h with different amounts of mouse IFN-α, IFN-β (Lee Biomolecular, San Diego, Calif.), or IFN-γ (Amgen Biologicals, Thousand Oaks, Calif.). The cells were then challenged with 10^5 PFU of PV variant PV-NK^{s1} or PV-NK^{r1}, and cell-free supernatants were harvested 24 h later. The virus titers in the supernatants were determined in plaque assays with Vero cells. As shown in Table 3, which shows representative results for a typical experiment, no significant differences were found in the sensitivities of either of the PV variants to IFN. Additional experiments demonstrated that PV-NK^{scid} and PV-NK^{s1} did not differ significantly from each other in their sensitivities to IFN. We also examined whether a difference existed in the levels of interferon induced in mice inoculated with either of the PV variants. After 3 days of infection, sera from individual C57BL/6 or C3H/HeSnJ mice were tested for IFN by limiting dilution on monolayers of L-929 cells in microtiter wells, followed by challenge with vesicular stomatitis virus, as described previously (1). Data are expressed as the reciprocal of the twofold dilution which showed a 50% inhibition in cytopathic effect per milliliter of serum. As can be seen from Table 4, both PV-NK^{r1} and PV-NK^{scid} induced slightly higher levels of IFN in both C57BL/6 and C3H/HeSnJ mice than did PV-NK^{s1}. This latter observation, however, may only be a reflection of the fact that, as discussed earlier, the NK-resistant variants of PV appear to replicate much more efficiently in vivo than the NK-sensitive variant of PV (Table 2). Nevertheless, taken together the above observations suggest that the greater ability of the NK-resistant variants of PV to replicate in vivo in normal mice is not due to an increased resistance of the viruses to the growth-inhibitory (antiviral) effects of IFN but rather to their increased resistance to the control exerted by the NK cells

Discussion. Correlations between sensitivities to NK cells in vitro and in vivo have been demonstrated with virus-infected tumor cells. Adenovirus-transformed cells with high-level sensitivity to NK cells grow poorly as tumors in athymic nude mice (5). BHK cells persistently infected with vesicular stomatitis virus grow poorly in nude mice, correlating with high-level sensitivity to NK cells in vitro (8, 11, 12). When a vesicular stomatitis virus variant isolated from resistant tumor cells was used to establish a new persistent infection, these tumor cells remained resistant to NK cells in vitro and in vivo.

Our study with PV is the first demonstration of NK-escape variants of a virus—as defined by the ability of the virus to replicate in vivo. Of note is that we did not observe a correlation between NK sensitivity in vivo and sensitivity to NK cell-mediated lysis in vitro. This is not surprising, as we have not seen this correlation with other viruses, such as with the highly NK-sensitive murine cytomegalovirus (3). The sensitivity of virus-infected cells to NK cells in vivo is likely to vary with the cell type infected and to be strongly influenced by cytokines, such as IFN, which can modulate the sensitivity of target cells to lysis (3). We also did not observe a difference in the relative abilities of PV-NK^{s1} and PV-NK^{r1} to inhibit IFN-mediated protection of L-929 target cells in vitro. These studies were, however, done with one cell line, and it is possible that the natural targets of infection in vivo would respond differently in terms of sensitivities to cytokines and to NK cells.

These studies provide direct evidence that a dynamic selection of viral variants which differ in their sensitivities to NK cells occurs in vivo. An NK-resistant variant of a virus should have a selective advantage under conditions of virus-induced NK cell activation, which occurs at high levels in acute infections (14). These studies also indicate that the selective pressure of a continuous NK cell response during a persistent infection may select for NK resistance, as has apparently occurred in nature with LCMV. A molecular analysis of these variants should help in determining the molecular basis for NK sensitivity and resistance.

TABLE 4. Induction of IFN in vivo by variants of PV

Expt	Mouse strain	Log_2 of IFN/ml after induction by[a]:		
		PV-NK^{s1}	PV-NK^{r1}	PV-NK^{scid}
1	C57BL/6	8.3	8.8	10.3
	C3H/HeSnJ	7.8	9.3	10.3
2	C57BL/6	10.0	11.8	11.8
3	C3H/HeSnJ	9.3	13.3	14.8

[a] Results are the mean IFN levels in sera from two animals tested separately. Mice were inoculated with virus as described in Table 2, footnote *d*.

This work was supported by USPHS research grants AI17672 and CA34461.

We thank Beryl Edney for preparation of the manuscript.

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