0022-1767/83/1302-0965$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 130, No. 2, February 1983
Printed in U.S.A.

5-HALO-6-PHENYL PYRIMIDINONES: NEW MOLECULES WITH CANCER THERAPEUTIC POTENTIAL AND INTERFERON-INDUCING CAPACITY ARE STRONG INDUCERS OF MURINE NATURAL KILLER CELLS[1]

EVA LOTZOVÁ,[2] CHERYLYN A. SAVARY, AND DALE A. STRINGFELLOW

From the Department of Clinical Immunology and Biological Therapy, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77030; and the Department of Cancer and Virus Research, The Upjohn Company, Kalamazoo, MI 49001

We tested the effect of three different interferon- (IFN) inducing pyrimidinone molecules with cancer therapeutic potential on natural killer (NK) cells. Peritoneal exudate (PE) cells were selected for these studies because their NK cell cytotoxicity is very low. Augmentation of PE-NK cell cytotoxicity was observed in eight different strains of mice after i.p. administration of 250 mg/kg of each of the pyrimidinones. NK cell induction occurred as early as 6 hr after pyrimidinone administration and peaked 2 to 4 days after treatment; at that time, cytotoxicity was as high as 60 to 90%. Augmentation of NK cell activity was independent of IFN serum levels induced by pyrimidinones and murine H-2 haplotype, and did not exhibit any histocompatibility or species-specific restriction, because it was expressed to syngeneic, allogeneic, and xenogeneic tumor target cells. Characterization studies demonstrated that the cytolytic cells were not macrophages, T cells, or B cells and exhibited NK cell characteristics. NK cell tumor-binding and tumor-killing studies demonstrated that NK cell augmentation was accomplished via both activation and recruitment of NK cells. If one considers NK cells as an important part of tumor immunity (as indicated by murine studies), pyrimidinone molecules may be effective anticancer agents.

Although natural killer (NK)[3] cell-mediated cytotoxicity was originally demonstrated *in vitro*, the relevance of this lymphocyte subpopulation in antineoplastic activity *in vivo* has become increasingly evident (1–5). The latter ability is reflected by lower tumor incidence, greater tumor resistance, and more rapid clearance of radioactively labeled tumors by mice with high NK cell activities and, antithetically, by failure of mice with low or depressed NK cell activities and/or with congenital NK cell deficiencies to display these functions (6–9). In addition to their involvement in resistance to the establishment of primary malignancies, NK cells have been demonstrated to play a role in resistance against metastatic tumor growth in mice (10, 11). By inference from murine data, it is reasonable to postulate a possible role of NK cells in the containment of human malignancies. This possibility, together with the observation that some of the patients with malignant disease exhibit low levels of NK cell cytotoxicity (12–15) and the patients with Chediak-Higashi syndrome (with selective NK cell deficiency) are prone to lymphoproliferative diseases (16), initiated an interest in factors with NK cell augmenting properties. Relatively recently, various natural and synthetic agents were reported to possess such properties; these are represented by bacteria, such as Bacille Calmette-Guerin and *Corynebacterium parvum* (the latter bacteria however, can also cause NK cell suppression under certain conditions; References 17 and 18), various viruses, tumor cells, interferon (IFN), and a variety of chemicals, e.g., poly I:C, tilorone, statolon, and pyran copolymer (2, 19–22). Interestingly, most of these NK cell augmenting agents are also IFN inducers, the observation suggesting that IFN may be of primary importance in the regulation of NK cell-mediated cytotoxicity.

In this study, we investigated the effect of three new IFN-inducing molecules, pyrimidinones, on murine peritoneal exudate (PE) NK cells. PE cells were selected for these studies because they exhibit either no or negligible levels of NK cell activity; thus it was of interest to determine whether PE-NK cells could be induced. The following pyrimidinones were used in our studies: 2-amino-5-bromo-6-phenyl-4 pyrimidinol (ABPP), 2-amino-5-bromo-6 meta fluoro phenyl-4(3H) pyrimidinol (ABmFPP), and 2-amino-5-iodo-6-phenyl-4 pyrimidinol (AIPP). These agents are of clinical interest, because they exhibit antiviral and antineoplastic properties (23, 24) in addition to IFN-inducing potential, in animals. Moreover, ABPP is being clinically investigated for its cancer therapeutic potential, in Phase I study at our Institute.

MATERIALS AND METHODS

Mice. Sixteen to 19-wk-old female A/He, C57BL/6 (abbreviated B6), BALB/c, DBA/2, C3H/Anf, CBA, AKR, and (C57BL/6 × DBA/2) F_1 (abbreviated B6D2F_1) mice were purchased from Cumberland View Farms (Clinton, TN). Experimental mice were maintained in our animal facilities for at least 1 wk after arrival to allow recovery from shipping trauma.

Pyrimidinones. ABPP, AIPP, or ABmFPP were suspended in saline and were injected in the dose of 250 mg/kg into each mouse i.p. at various times before NK cell test (see *Results*). Because of the poor solubility of pyrimidinones, these agents were ground and vigorously mixed by Vortex to achieve a uniform suspension.

IFN assay. IFN levels were determined in the sera of untreated and ABPP, AIPP, and ABmFPP-treated mice (single injection of 250 mg/kg) 3 to 48 hr after injection. Each group consisted of five mice. A standard vesicular stomatitis virus plaque reduction assay was performed on murine L929 cells (25). To standardize the results, the international murine standard (G002-904-511) was included, which had a mean titer of 6200 U/ml in comparison to its accepted titer of 6800 U/ml.

Preparation of effector cells. PE cells were harvested by massage of the peritoneal cavity after injection of 3 ml of saline and aspiration of exudate

Received for publication June 14, 1982.
Accepted for publication October 1, 1982.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by Grants CA 31394 and CA 21062 from the National Cancer Institute.
[2] Reprint requests and all correspondence should be sent to Dr. Lotzová, Department of Clinical Immunology and Biological Therapy, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030.
[3] Abbreviations used in this paper: NK, natural killer; IFN, interferon; PE, peritoneal exudate; ABPP, 2-amino-5-bromo-6-phenyl-4 pyrimidinol; ABmFPP, 2-amino-5-bromo-6 meta fluoro phenyl-4(3H) pyrimidinol; AIPP, 2-amino-5-iodo-6-phenyl-4 pyrimidinol; S-RPMI 1640, supplemented tissue culture medium 1640; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

with a pasteur pipette. When large numbers of cells were required, the exudate from several animals was pooled. The PE cells were then resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum, antibiotics (500 U/ml of penicillin and 50 μg/ml of streptomycin and HEPES[3] buffer (supplemented RPMI 1640 medium; S-RPMI 1640).

Treatment of effector cells.

Carbonyl iron ingestion. Twelve million PE cells suspended in 1 ml of S-RPMI 1640 were incubated with 20 mg of carbonyl iron powder (GAF Corporation, New York, NY) for 30 min at 37°C in a 5% CO_2 humidified atmosphere. After incubation, iron-ingesting cells were removed by multiple passage over a magnet.

Glass adherence. Fifty million PE cells in 10 ml of S-RPMI 1640 were incubated in 100-mm diameter glass petri dishes at 37°C for 1 hr. The nonadherent cell population was collected by washing the dishes three times with warm S-RPMI 1640. The adherent cells were removed by using a rubber policeman. Both cell populations were then washed and resuspended in S-RPMI 1640.

Silica treatment. PE cells were treated with silica *in vitro*, according to a described method (26). Briefly, 10^7 PE cells suspended in 1 ml of S-RPMI 1640 were incubated with 2 mg of silica for 90 min at 37°C. The suspension was then layered over a Ficoll-Hypaque gradient and was centrifuged at 1200 × G for 20 min (20°C). Interface cells were removed, washed, and resuspended in S-RPMI 1640. Silica particles of <5μm average size were obtained through the courtesy of Dr. Robock, Steinkohlenbergbauverein, Germany.

Nylon wool column fractionation. PE cells were passed over nylon wool columns as described by Julius et al. (27). Briefly, 50 × 10^6 PE cells were placed on columns consisting of 0.8 g of nylon wool (Fenwal Laboratories, Deerfield, IL), placed into the barrel of a 10-ml syringe, and incubated for 45 min at 37°C. After incubation, the cells not adhering to the nylon wool were eluted by washing with 20 ml of warm S-RPMI 1640 and were diluted to the required concentration.

Treatment with Thy-1.2 monoclonal antibodies. T cells were removed by treatment with monoclonal anti-Thy-1.2 antibodies (New England Nuclear, Boston, MA) and guinea pig complement (GIBCO, Santa Clara, CA) by using a one-step cytotoxicity assay. Equal volumes of PE cells (2 × 10^6/ml, suspended in S-RPMI 1640), Thy-1.2 antiserum (10^{-3} dilution), and complement (1/5 dilution) were incubated for 1 hr at 37°C.

Target cells. Murine T cell lymphoma YAC-1 (1), acute myelogenous leukemia, C1498 (28), and human acute myelogenous leukemia, K-562(29) were used in these studies. All target cells were grown as continuous cultures in S-RPMI 1640. For cytotoxicity studies, the target cells were labeled with radioactive sodium chromate as described in detail previously (30).

NK cell cytotoxicity assay. One hundred microliters of effector cells were plated in quadruplicate into the wells of round-bottomed microtiter plates (Linbro Scientific, Hamden, CT). Fifty microliters containing 10^4 target cells (1:50 target to effector cell ratio) were then added and the cultures were incubated for 4 hr at 37°C in a 5% CO_2 humidified atmosphere. After incubation, the plates were centrifuged at 250 × G for 10 min and an aliquot was removed from each well and counted in an Autogamma Scintillation Spectrometer. Standard error of the mean of replicates was less than 5%. Spontaneous release of ^{51}chromium (^{51}Cr) was determined by incubating the target cells with medium alone and ranged from 5 to 9%. Maximum release of ^{51}Cr was determined after freezing and thawing tumor cells four times and ranged from 85 to 95%. The percentage of cytotoxicity was determined according to the formula:

$$\frac{\text{Percent exp. release} - \text{percent spont. release}}{\text{Percent max. release} - \text{percent spont. release}} \times 100$$

Evaluation of tumor-binding cells. Percentage of tumor-binding PE cells was determined as described (31). Two million YAC-1 cells were mixed with 2 × 10^5 of fluorescein isothiocyanate- (Sigma, St. Louis, MO) labeled PE cells in 0.2 ml of S-RPMI 1640. The cultures were then centrifuged (200 × G) for 5 min (20°C), placed on ice for an additional 30 min, and resuspended. The percentage of fluorescing effector cells binding to nonfluorescing target cells was determined after counting 200 cells under an ultraviolet microscope.

Irradiation. In some experiments, untreated ABPP or AIPP-treated mice were irradiated from a ^{60}cobalt radiation source (at the rate of 40 rads/min).

Statistical analysis. The difference between experimental and control groups was evaluated statistically with a Student's *t*-test and the probability (p) was calculated.

RESULTS

Induction of PE-NK cell cytotoxicity by pyrimidinones. As illustrated in Figure 1, NK cells from the peritoneal cavity of B6D2F$_1$ hybrid mice exhibited low levels of cytotoxicity against T cell lymphoma, YAC-1; the mean cytotoxicity value was 6.8% ± 1.0. When the mice were injected i.p. with 250 mg/kg of either of the pyrimidinones, however, a strong and consistent augmentation of NK cell cytotoxicity was demonstrated. NK cell cytotoxicity was enhanced as early as 6 hr after a single injection of pyrimidinones and progressed with time. The peak of NK cell activity was observed 2 to 4 days post-treatment, at which time the cytotoxicity reached levels as high as 60 to 90%. The high PE-NK cell levels were sustained for 12 days after AIPP or ABPP administration, and for 4 days after ABmFPP treatment. Because these were the latest time intervals tested, the exact duration of pyrimidinone-mediated NK cell augmentation in PE is currently unknown. Interestingly, the degree of NK cell augmentation was independent of the serum levels of IFN induced by each pyrimidinone (Table I); ABPP and AIPP were equally effective NK cell stimulators, despite the fact the former was a strong and the latter a poor inducer of serum IFN (25). Additionally, ABmFPP, which also induced significantly higher levels of serum IFN than AIPP, was less efficient as an NK cell augmentator.

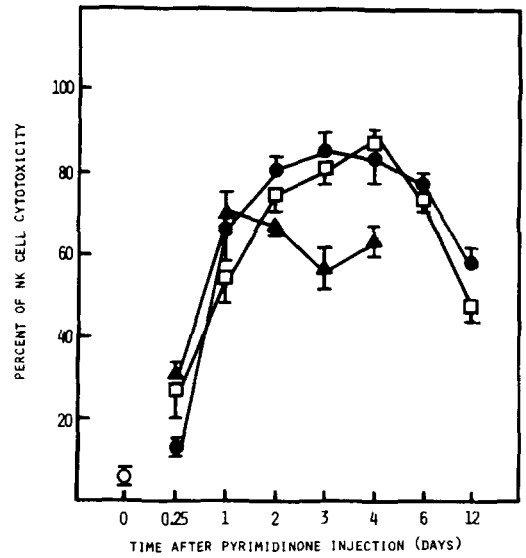

Figure 1. Induction of PE-NK cell cytotoxicity with pyrimidinones: time study. B6D2F$_1$ female mice were injected i.p. with 250 mg/kg of AIPP (●); ABPP (□); or ABmFPP (▲). Untreated or PBS-treated control mice were included in all experiments. Because there was no difference between these two types of controls, the data were pooled. Data represent the mean percent of cytotoxicity ± SE. Six to 12 mice were tested at each time interval. All cytotoxicity values of AIPP, ABPP, and ABmFPP-injected mice were significantly different from those of control mice, determined by Student's *t*-test analysis; p values were <0.001 for all groups, except for AIPP-injected mice tested 6 hr after treatment ($p < 0.002$).

TABLE I

Serum IFN levels in B6D2F$_1$ mice after treatment with pyrimidinones

Treatment	IFN Levels (U/ml)[a]						
	Time after Treatment (hr)						
	0	3	6	9	12	24	48
AIPP[b]	<10	<10	10	15	<10	<10	<10
ABPP	<10	510	6300	5000	480	<10	<10
ABmFPP	<10	200	7500	3400	1000	<10	<10

[a] The IFN induced by pyrimidinone molecules was characterized as IFN-α. The values represent the mean of serum IFN levels of five mice. Standard error of the mean was 20%. Determination of IFN levels is described in the text. A level of <10 U/ml is the lowest detectable level, because samples were not assayed at dilution lower than 1/10.

[b] Pyrimidinones were injected i.p. in the dose of 250 mg/kg.

Strain distribution of pyrimidinone-induced PE-NK cell activity. To determine whether augmentation of PE-NK cell cytolytic activity was restricted to B6D2F$_1$ hybrids or represented a more general phenomenon, seven inbred strains of mice with

identical or different H-2 haplotypes were tested for their sensitivity to pyrimidinone stimulation. In these studies, NK cell cytotoxicity was tested against three different tumor target cell lines of syngeneic, allogeneic, and xenogeneic origin, and ABPP and AIPP were used as prototypes of strong and poor inducers of circulating IFN, respectively. The results of these experiments are illustrated in Table II. The NK cell cytotoxic potential in the peritoneal cavity of all untreated mouse strains studied, was very low or nonexistent, regardless of the tumor target cell line employed. As was the case with B6D2F$_1$ mice, 3 days after i.p. administration of 250 mg/kg of ABPP or AIPP, substantial increases in PE-NK cell activity were observed in each strain of mice. With regard to the tumor target, NK cell cytotoxicity was more pronounced to YAC-1 than to C1498 and K-562. Interestingly, and perhaps most importantly, PE cytotoxicity was induced not only against allogeneic and xenogeneic target cells but also against syngeneic tumors. Anti-syngeneic target cell reactivity was demonstrated by a 17-fold increase of NK cell cytotoxicity in PE from A and B6 mice to YAC-1 and C1498, respectively. The latter observation may be particularly significant for cancer treatment. As shown earlier in B6D2F$_1$ hybrids, ABPP and AIPP were equally effective NK cell stimulators, despite their differential capacity to induce IFN (although not shown in each mouse strain, AIPP and ABPP induced in other mouse strains levels of IFN similar to those presented in Table I). In fact, AIPP was more potent than ABPP in stimulating NK cells of B6, BALB/c, and DBA/2 strains of mice against YAC-1, and BALB/c mice against C1498. Induction of PE-NK cell cytotoxicity was independent of H-2 haplotype, was not tumor target-specific, and did not exhibit any histocompatibility or species-specific restrictions.

Characterization of pyrimidinone-induced PE effector cells. Even though the characteristics of PE cytolytic cells, such as prompt cytotoxicity against a variety of targets and no requirement of sensitization or antibody "assistance" for function, suggested that the cytotoxicity induced by pyrimidinones was mediated by NK cells, we performed additional characterization studies. The AIPP and ABPP-induced PE cytotoxicity was not affected by treatment with carbonyl iron or silica, treatments resulting in fewer than 1% phagocytic cells when evaluated by latex ingesting technique (32). This indicates that the cytolytic cells lacked macrophage properties (Fig. 2). This was further substantiated by the observation that cytotoxicity was not removed by glass adherence, and was fully expressed in the fraction nonadherent to glass. There was, however, still some residual cytotoxicity present in the glass-adherent fraction. To determine whether this residual cytotoxicity could be mediated by macrophages, the glass-adherent fraction was further treated with carbonyl iron. Because the cytotoxicity was not depleted, but rather was enriched after this treatment, the glass-adherent cytolytic cells do not appear to be of macrophage nature; residual cytotoxicity was most likely due to incomplete removal of NK effector cells. In fact, it has been reported that activated NK cells are more adherent than non-activated NK cells (33).

Further characterization studies demonstrated that pyrimidinone-induced cytotoxic potential was not abolished by treatment with Thy-1.2 monoclonal antibodies and complement (Fig. 3), despite the fact that such treatment was effective in removing more than 95% of thymocytes and 30% of splenocytes. On the basis of these experiments, mature cytotoxic T cells most likely did not mediate pyrimidinone-stimulated cytotoxicity. A slight decrease in cytotoxic potential observed after Thy-1.2 treatment was compatible with sensitivity of a subpopulation of NK cells to Thy-1.2 antisera (34). Nylon wool fractionation experiments (Fig. 3) revealed that cytotoxic cells were present in the nylon wool-filtered fraction. Because this fraction was composed of less than 2% B cells, determined by direct immunofluorescence (32), the latter cells do not appear to be the effectors of pyrimidinone-induced cytotoxicity in PE. Moreover, cytotoxicity was fully expressed after exposure of PE cells to combined multiple treatments, including glass adherence, nylon wool fractionation, and Thy-1.2 antiserum and complement treatment (see Fig. 3). These treatments resulted

TABLE II

Strain distribution of pyrimidinone-induced peritoneal exudate NK cell cytotoxicity to syngeneic, allogeneic, and xenogeneic tumors

Tumor Target	Mouse Strain	H-2 Haplotype	Percent of NK Cell Cytotoxicity[a]		
			Control	AIPP[b]	ABPP[b]
YAC-1	A/He	a	3.4 ± 0.9 (12)	57.7 ± 5.9 (8)	60.3 ± 4.6 (6)
	B6	b	2.9 ± 0.9 (11)	58.8 ± 4.5 (6)	31.8 ± 3.6 (5)
	BALB/c	d	6.4 ± 2.0 (6)	77.3 ± 4.0 (6)	47.4 ± 5.4 (4)
	DBA/2	d	0.9 ± 0.5 (8)	64.8 ± 2.2 (5)	46.4 ± 2.9 (4)
	C3H/Anf	k	3.3 ± 0.5 (8)	69.8 ± 2.5 (7)	64.8 ± 5.4 (4)
	CBA	k	2.6 ± 0.5 (8)	77.3 ± 4.2 (4)	62.9 ± 3.1 (4)
	AKR	k	1.6 ± 0.5 (8)	51.6 ± 9.5 (6)	54.4 ± 4.5 (4)
	B6D2F$_1$	b/d	5.6 ± 0.6 (7)	85.5 ± 4.4 (6)	80.9 ± 3.7 (6)
C1498	A/He	a	−0.1 ± 0.05 (2)	14.7 ± 0.8 (2)	24.6 ± 1.0 (2)
	B6	b	−0.4 ± 0.4 (5)	16.6 ± 1.6 (5)	ND
	BALB/c	d	−1.7 ± 1.5 (6)	19.3 ± 2.2 (3)	8.6 ± 1.1 (4)
	DBA/2	d	−0.6 ± 0.5 (9)	19.3 ± 1.4 (4)	16.1 ± 0.4 (4)
	C3H/Anf	k	−0.4 ± 0.4 (5)	51.1 ± 3.6 (4)	44.0 ± 9.4 (4)
	CBA	k	−0.3 ± 0.2 (8)	25.7 ± 3.1 (4)	44.2 ± 3.1 (4)
	AKR	k	0.4 ± 0.2 (8)	14.0 ± 2.5 (4)	10.2 ± 1.6 (4)
	B6D2F$_1$	b/d	1.0 ± 0.5 (10)	55.3 ± 3.1 (9)	52.6 ± 7.0 (4)
K-562	A/He	a	−2.5 ± 0.4 (3)	ND	21.2 ± 2.2 (3)
	BALB/c	b	0.7 ± 1.0 (7)	39.7 ± 1.1 (3)	34.8 ± 0.4 (4)
	DBA/2	d	−1.6 ± 0.6 (7)	32.2 ± 3.6 (3)	31.4 ± 0.9 (4)
	C3H/Anf	k	−0.2 ± 0.3 (5)	34.6 ± 4.2 (4)	37.6 ± 5.6 (4)
	CBA	k	−1.7 ± 0.3 (4)	ND	28.2 ± 4.9 (4)
	AKR	k	−1.1 ± 0.4 (4)	ND	31.4 ± 2.2 (4)

[a] Values represent mean percent of cytotoxicity ± SE; the number of mice tested is indicated in parentheses; ND = not done.

[b] Injected i.p., 250 mg/kg, 3 days before cytotoxicity assay. Cytotoxicity of all AIPP and ABPP-treated mice was significantly different from that of untreated mice, determined by a Student *t*-test analysis. P values comparing the difference in cytotoxicity of AIPP or ABPP-treated mice against that of control mice were <0.001 for all mouse strains tested against all targets, except of ABPP-treated BALB/c mice against C-1498 (<0.005), and A/He and CBA mice against K-562 (<0.002).

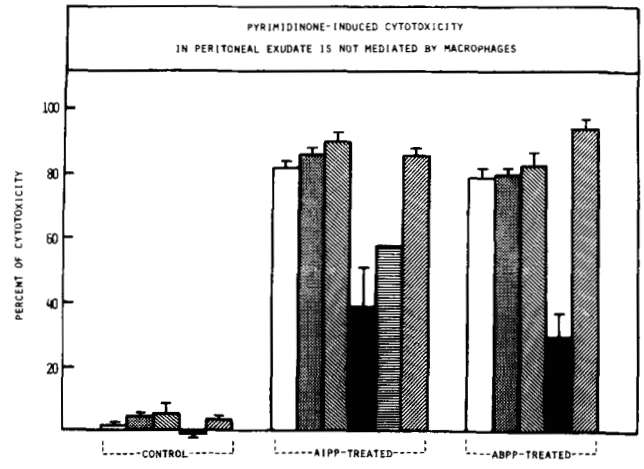

Figure 2. Effect of macrophage depletion on pyrimidinone-induced cytotoxic cells in PE of B6D2F$_1$ mice. Unseparated PE (□); carbonyl iron treatment (▨); glass-nonadherent (▧); glass-adherent (■); glass-adherent and carbonyl iron-treated (▤); silica treatment *in vitro* (▨). All separations were performed with both control and ABPP or AIPP-treated (250 mg/kg, i.p.) PE 3 days after treatment. *Bars*, mean percent of cytotoxicity ± SE of five different experiments, composed of pooled PE. As evaluated by a Student *t*-test analysis, there was no significant difference in cytotoxicity after macrophage removal in control, ABPP, or AIPP-treated mice.

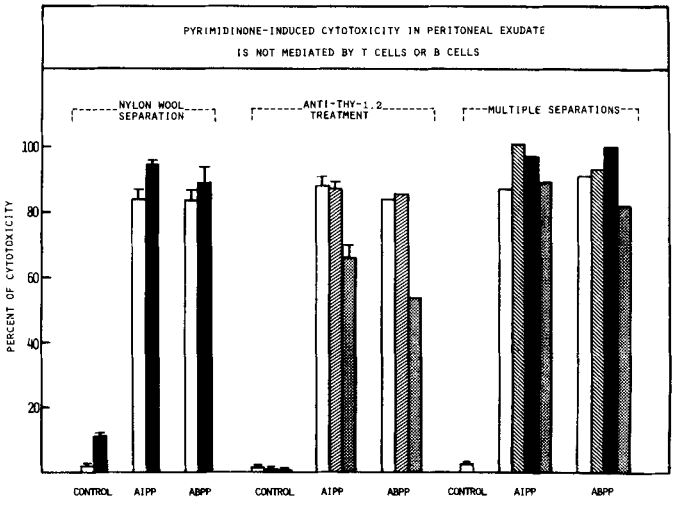

Figure 3. Effect of T cell and B cell-depleting procedures on pyrimidinone-induced cytotoxic cells in PE of B6D2F$_1$ mice. Unseparated (□); nylon wool-nonadherent (B cell-depleted) fraction (■); complement-treated (▨); monoclonal Thy-1.2 antiserum and complement-treated (T cell-depleted) (▩). In multiple separation experiments, the same population of PE cells was depleted first of macrophages by adherence (▧), followed by removal of B cells by nylon wool separation (■), and finally, the nylon wool-filtered fraction was depleted of T cells with Thy-1.2 monoclonal antibodies (▩). Mice were either untreated or were injected with ABPP or AIPP (250 mg/kg, i.p., 3 days before cytotoxicity test). Bars, mean percent of cytotoxicity ± SE of three different experiments of pooled exudates. Bars without SE represent a single experiment. As determined by a Student *t*-test analysis, there was no significant difference in PE cytotoxicity between pyrimidinone-induced, unseparated *vs* nylon wool-filtered cells. Significant difference was observed between complement-treated and Thy-1.2 antibodies and complement-treated pyrimidinone-induced PE cells ($p < 0.01$ for AIPP and <0.001 for ABPP).

in the removal of a majority of macrophages, T cells, and B cells, and a substantial enrichment of null cell population (s). Results from all of these experiments indicated that NK cells are the cytolytic cells.

The radiation experiments demonstrated that the pyrimidinone-induced cytotoxic cells, like NK cells, were relatively radioresistant, because they were functional after total body irradiation of B6D2F$_1$ mice with 1000 rads (Table III).

Effect of pyrimidinones on NK cell tumor-binding properties. We also tested the effect of AIPP and ABPP on the capacity of PE-NK cells to bind to YAC-1 tumor. Nylon wool-filtered PE population was used in these studies to avoid possible nonspecific tumor cell binding, which was observed in unseparated cells from various tissues (31). The efficacy of NK cells to bind to YAC-1 tumor (Table IV) was significantly augmented by pyrimidinones; the augmentation index was 1.5 and 1.8 after the administration of AIPP and ABPP, respectively. These experiments demonstrated that pyrimidinone molecules can also increase the number of tumor-binding cells.

DISCUSSION

We show that three different 5-halo-6-phenyl pyrimidinones induced significant and relatively long-lasting cytotoxicity in PE of mice. The cells exhibiting such cytotoxicity displayed typical NK cell characteristics, such as natural occurrence, rapid reactivity to a wide variety of target tissues (of syngeneic, allogeneic, and xenogeneic origin), no histocompatibility and species-specific restriction, lack of macrophage properties, and partial sensitivity to Thy-1.2 monoclonal antibodies. Furthermore, cytolytic cells were relatively radioresistant, another NK cell characteristic.

Pyrimidinone-induced PE cytotoxicity or its degree was not mouse strain-specific, in that it was demonstrated in eight different strains of mice. Differences in the degree of potentiation, however, were observed when different target cells were compared; YAC-1 was found to be the most sensitive cell line.

TABLE III

Effect of irradiation on pyrimidinone-induced peritoneal exudate NK cell cytotoxicity of B6D2F$_1$ mice

Treatment	Percent of NK Cell Cytotoxicity[a]		
	Control	AIPP[b]	ABPP[b]
None	2.2 ± 0.4 (6)	83.9 ± 2.5 (4)	75.6 ± 3.7 (4)
1000rads[c]	0.9 ± 0.3 (6)	78.3 ± 3.5 (5)	64.3 ± 3.9 (4)

[a] Values represent the mean percent of cytotoxicity ± SE. Number of mice tested is indicated in parentheses.
[b] 250 mg/kg, i.p., 60 hr before irradiation.
[c] Mice were irradiated 17 hr before cytotoxicity test. There was no significant difference in NK cell cytotoxicity between PE of pyrimidinone-treated and pyrimidinone-treated and irradiated groups, determined by Student's *t*-test analysis.

TABLE IV

Effect of pyrimidinone molecules on tumor-binding and killing properties of peritoneal exudate NK cells

Treatment	Percent of Tumor-Binding Cells[a]	Percent of Cytotoxicity
Control	19.4 ± 2.7	12.3 ± 1.9
AIPP[b]	29.9 ± 0.8[c]	92.7 ± 3.7[d]
ABPP[b]	35.5 ± 2.7[e]	95.5 ± 6.5[d]

[a] Data represent the mean percent of tumor-binding cells ± SE of three different experiments.
[b] Pyrimidinones were injected in the dose of 250 mg/kg, 3 days before tumor-binding assay and cytotoxicity test; the same PE cell suspension was used for both tests.
[c] $p < 0.05$, [d] $P < 0.01$, and [e] $p < 0.02$ in comparison to control mice.

That the high level of pyrimidinone-induced cytotoxicity detected in the peritoneal cavity was indeed a result of induction of NK cells and not a consequence of their mobilization from other tissues was indicated by concomitant increase of NK cytotoxicity in the other organs, such as the spleen, bone marrow, and peripheral blood (35). Analyses of NK cell tumor-binding and tumor-killing properties after treatment with pyrimidinones indicated that NK cell augmentation was accomplished both via NK cell activation and NK cell recruitment.

Even though the mechanism of action of pyrimidinone molecules on NK cells is still under investigation, serum levels of IFN, induced by these agents, did not correlate with NK cell induction. This statement is based on the observation demonstrating that AIPP, despite being a poor inducer of circulating IFN, was as effective in NK cell augmentation as ABPP, which induced high levels of serum IFN. This apparent discrepancy, however, cannot be taken to indicate that IFN does not play a role in NK cell induction by pyrimidinones, because locally produced (in the peritoneal cavity) rather than circulating IFN, may be involved. In fact, our preliminary results indicate that AIPP, although not an inducer of circulating IFN, induces significant levels of IFN in the peritoneum 3 days after the treatment (manuscript in preparation).

Our finding that pyrimidinone molecules are powerful stimulators of NK cells, together with the observation that they exhibit antitumor properties (23, 24, 36, unpublished observations) suggest these agents may be of particular importance in cancer therapy. Indeed, it is plausible to postulate that the antitumor properties of pyrimidinone molecules observed in experimental animals may, at least in part, be mediated by NK cells. Because of these characteristics, the studies on pyrimidinones are of great importance.

Acknowledgments. The authors acknowledge Dr. Evan Hersh for reviewing the manuscript, A. Khan for technical assistance, and R. Prather for excellent secretarial assistance.

REFERENCES

1. Kiessling, R., and O. Haller. 1978. Natural killer cells in the mouse: an alternative immune surveillance mechanism. Contemp. Top. Immunobiol. 8:171.

2. Herberman, R. B., and H. T. Holden. 1978. Natural cell-mediated immunity. Adv. Cancer Res. 27:305.
3. Lotzová, E., and K. B. McCredie. 1978. Natural killer cells in mice and man and their possible biological significance. Cancer Immunol. Immunother. 4:215.
4. Herberman, R. B. 1980. Natural killer cells and cells mediating antibody-dependent cytotoxicity against tumors. Clinical Immunobiol. 4:73.
5. Herberman, R. B., and J. R. Ortaldo. 1981. Natural killer cells: their work in defenses against disease. Science 214:24.
6. Klein, G. O., G. Klein, R. Kiessling, and K. Karre. 1978. H-2-associated control of natural cytotoxicity and hybrid resistance against RBL-5. Immunogenetics 6:561.
7. Riccardi, C., A. Santoni, P. Barlozzari, P. Puccetti, and R. B. Herberman. 1980. *In vivo* natural reactivity of mice against tumor cells. Int. J. Cancer 25:475.
8. Karre, K., G. O. Klein, R. Kiessling, G. Klein, and J. C. Roder. 1980. Low natural *in vivo* resistance to syngeneic leukaemias in natural killer-deficient mice. Nature 284:624.
9. Talmadge, J. E., K. M. Myers, D. J. Preiur, and J. R. Starkey. 1980. Role of NK cells in tumor growth and metastasis in beige mice. Nature 284:622.
10. Hanna, N. 1980. Expression of metastatic potential of tumor cells in young nude mice is correlated with low levels of natural killer cell-mediated cytotoxicity. Int. J. Cancer 26:675.
11. Hanna, N., and I. J. Fidler. 1980. Role of natural killer cells in the destruction of circulating tumor emboli. J. Natl. Cancer Inst. 65:801.
12. Lotzová, E., K. B. McCredie, J. A. Maroun, K. A. Dicke, and E. J. Friereich. 1979. Some studies on natural killer cells in man. Transplant. Proc. XI:1390.
13. Hersey, P., A. Edwards, M. Honeyman, and W. H. McCarthy. 1979. Low natural-killer-cell activity in familial melanoma patients and their relatives. Br. J. Cancer 40:113.
14. Ziegler, H. W. (Loms), N. E. Kay, and J. M. Zarling. 1981. Deficiency of natural killer cell activity in patients with chronic lymphocytic leukemia. Int. J. Cancer 27:321.
15. E. Lotzová, C. A. Savary, J. U. Gutterman, and E. M. Hersh. 1982. Modulation of natural killer cell-mediated cytotoxicity by partially purified and cloned interferon. Cancer Res. 42:2480.
16. Klein, M., J. Roder, T. Haliotis, *et al.* 1980. Chediak-Higashi gene in humans. II. The selectivity of the defect in natural-killer and antibody-dependent cell-mediated cytotoxicity function. J. Exp. Med. 151:1049.
17. Savary, C. A. and E. Lotzová. 1978. Suppression of natural killer cell cytotoxicity by splenocytes from *Corynebacterium parvum*-injected, bone-marrow-tolerant, and infant mice. J. Immunol. 120:239.
18. Lotzová, E. 1980. *C. Parvum*-mediated suppression of the phenomenon of natural killing and its analysis. *In* Natural Cell-Mediated Immunity Against Tumors. Edited by R. B. Herberman. Academic Press, New York. P. 735.
19. Wolfe, S. A., D. E. Tracey, and C. S. Henney. 1976. Induction of "natural killer" cells by BCG. Nature 262:584.
20. Herberman, R. B., M. E. Nunn, H. T. Holden, S. Staal, and J. Y. Djeu. 1977. Augmentation of natural cytotoxic activity of mouse lymphoid cells against syngeneic and allogeneic target cells. Int. J. Cancer 19:555.
21. Gidlund, M., A. Orn, H. Wigzell, A. Senik, and I. Gresser. Enhanced NK cell activity in mice injected with interferon and interferon inducers. 1978. Nature 273:759.
22. Djeu, J. Y., J. A. Heinbaugh, H. T. Holden, and R. B. Herberman. 1979. Role of macrophages in the augmentation of mouse natural killer cell activity by poly I:C and interferon. J. Immunol. 122:182.
23. Weed, S. D., G. D. Kramer, and D. A. Stringfellow. 1980. Antiviral properties of 6-arylpyrimidines. *In* Current Chemotherapy and Infectious Disease. Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy. Vol. II. Edited by John D. Nelson and Carlo Grassi. The American Society for Microbiology, Washington, D.C. P. 1408.
24. Stringfellow, D. A. 1981. 6-arylpyrimidinones: interferon inducers-immunomodulators-antiviral and antineoplastic agents. *In* Augmenting Agents in Cancer Therapy. 1981. Edited by E. M. Hersh and M. A. Chirigos. Raven Press, New York. P. 215.
25. Stringfellow, D. A., H. C. Vanderberg, and S. D. Weed. 1980. Interferon induction by 5-halo-6-phenyl-pyrimidinones. J. Interferon Res. 1:1.
26. Tracey, D. E. 1979. The requirement for macrophages in the augmentation of natural killer cell activity by BCG. J. Immunol. 123:840.
27. 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.
28. Goldie, H., and M. D. Felix. 1951. Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. Cancer Res. 11:73.
29. Lotzová, E., K. B. McCredie, L. Muesse, K. A. Dicke, and E. J. Freireich. 1979. Natural killer cells in man: their possible involvement in leukemia and bone marrow transplantation. *In* Experimental Hematology Today. Edited by S. J. Baum and G. D. Ledney. Springer Verlag, New York. P. 207.
30. Lotzová, E., and C. A. Savary. 1981. Parallelism between the effect of cortisone acetate on hybrid resistance and natural killing. Exp. Hematol. 9:766.
31. Roder, J. C., and R. Kiessling, 1978. Target-effector interaction in the natural killer cell system. I. Covariance and genetic control of cytolytic and target cell-binding subpopulations in the mouse. Scand. J. Immunol. 8:135.
32. Preud'homme, J. L., and S. Labaume. 1976. Detection of surface immunoglobulins on human cells by direct immunofluorescence. *In In Vitro* Methods in Cell-Mediated and Tumor Immunity. Edited by B. R. Bloom and J. R. David. Academic Press, New York. P. 155.
33. Roder, J. C., K. Karre, and R. Kiessling. 1981. Natural killer cells. Prog. Allergy 28:66.
34. Mattes, M. J., S. O. Sharrow, R. B. Herberman, and H. T. Holden. 1979. Identification and separation of Thy-1-positive mouse spleen cells active in natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J. Immunol. 123:2851.
35. Lotzová, E., C. A. Savary, E. M. Hersh, A. A. Khan, and D. A. Stringfellow. Potentiation of murine natural killer cell-mediated tumor killing by antiviral pyrimidine molecules. Proc. AACR 22:279.
36. Milas, L., E. M. Hersh, D. A. Stringfellow, and N. Hunter. 1982. Studies on the antitumor activities of pyrimidinone-interferon inducers. I. Effect against artificial and spontaneous lung metastases of murine tumors. J. Natl. Cancer Inst. 68:139.