

Correlation of Immunomodulatory and Therapeutic Activities of Interferon and Interferon Inducers in Metastatic Disease

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The mechanism of therapeutic activity of recombinant murine interferon-gamma (rMu IFN- γ) and the IFN inducer polyinosinic-polycytidylic acid solubilized with poly-L-lysine in carboxy methyl cellulose (pICLC) in treating metastatic disease was investigated by comparing effector cell augmentation with therapeutic activity in mice bearing experimental lung metastases (B16-BL6 melanoma). Effector cell functions in spleen, peripheral blood, and lung (the organ with tumor) were tested after 1 and 3 weeks of rMu IFN- γ or pICLC administration (intravenous, three times a week). In these studies, natural killer (NK), lymphokine-activated killer (LAK), cytolytic T lymphocytes (CTL) (against specific and nonspecific targets), and macrophage tumoricidal and tumorigenic activities were measured. rMu IFN- γ and pICLC had therapeutic activity and immunomodulatory activity in most assays of immune function examined. Specific CTL activity of pulmonary parenchymal mononuclear cells (PPMC), but not in splenocytes or peripheral blood lymphocytes (PBL), during week 3 and not during week 1, correlated with the therapeutic activity of rMu IFN- γ and of pICLC. Macrophage tumoricidal activity in PPMC, but not in alveolar macrophages, also correlated with the therapeutic activity of rMu IFN- γ , but the opposite was true for the therapeutic activity of pICLC. NK

Abbreviations used: IFN; interferon; rMu IFN- γ , recombinant murine interferon-gamma; i.v., intravenous; tiw, three times a week; NK, natural killer; LAK, lymphokine-activated killer; CTL, cytolytic T lymphocytes; PPMC, pulmonary parenchymal mononuclear cells; BRM, biological response modifiers; PBL, peripheral blood lymphocytes, HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; poly(I,C), polyinosinic-polycytidylic acid; poly(I,C)-LC or pICLC, poly(I,C) solubilized with poly-L-lysine in carboxymethyl cellulose.

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Received June 3, 1987; accepted October 23, 1987.

Published 1988 by Alan R. Liss, Inc.

activity of PPMC, but not of splenocytes or PBL, during week 1 correlated with the therapeutic activity of pICLC; in contrast, NK activity at any site did not correlate with the therapeutic activity of rMu IFN- γ . LAK activity at any site did not correlate with the therapeutic activity of either agent.

Key words: treatment of metastases, interferon-gamma, double-stranded polyribonucleotides, mechanism of therapeutic activity, preclinical models, poly (I,C)-LC, cytolytic T lymphocyte (CTL), anti-tumor activity, tumor-specific

Interferons (IFN) consist of a family of glycoproteins whose synthesis and secretion are induced by viral infections and other stimuli. The IFNs induce an antiviral state in most cell types, and they inhibit the proliferation of many different types of tumor cells. Additionally, the IFNs have immunomodulatory activity, especially for the augmentation of macrophage tumoricidal activity and of natural killer (NK) cell cytotoxicity [1-7]. Although IFN- γ shares many properties with IFN- α and IFN- β , it has greater antiproliferative [8] and immunomodulatory [7,8] activities than IFN- α and IFN- β . Additionally, the cellular receptor for IFN- γ is distinct from the receptor for IFN- α and IFN- β [9].

The double-stranded polyribonucleotides such as polyinosinic-polycytidylic acid [poly(I,C)] stimulate the synthesis and secretion of IFNs [10-16]. Poly(I,C) also has potent immunomodulatory activity in a variety of assays of immune function, including antibody production [17,18], allograft rejection [19,20], protection against viral infection [15,21-25], and against transplantable tumor challenges [21,26-33], NK activity [6,20,34-39], and macrophage tumoricidal activity [39,40]. However, the effectiveness of poly(I,C) is limited in humans and primates by its susceptibility to the action of serum ribonucleases [41-43]. However, the complex of poly(I,C) admixed with poly-L-lysine and solubilized with carboxymethyl cellulose [poly(I,C)-LC] alleviates this problem [44,45].

Both recombinant murine interferon-gamma (rMu IFN- γ) [46] and poly (I,C)-LC [33,47,48] have consistently shown significant therapeutic activity in a variety of animal tumor models. However, demonstration of this preclinical therapeutic activity depends strictly on optimal dosage, schedule, route, and duration of administration [46-48]. Possibly as a result of these limitations, recombinant human (rH) IFN [49-62] and poly (I,C)-LC [63-69] have produced mixed results in clinical trials with a variety of cancer types. The use in clinical trials of excessively high doses of these biological response modifiers (BRMs), at or near the maximum tolerated dose, levels which exceeded the optimal therapeutic dose, may explain the discrepancy between the preclinical efficacy of these agents and their less impressive effects in clinical trials.

In the present studies, we investigated the immunomodulatory activity of rMu IFN- γ and of poly(I,C)-LC in tumor-bearing animals. Levels of NK, lymphokine-activated killer (LAK), cytolytic T lymphocyte (CTL), and macrophage tumoricidal and tumoristatic activities were assessed in cells from peripheral blood, spleen, and lungs (the organ with tumor), 1 and 3 weeks after the initiation of treatment, in mice bearing B16-BL6 experimental lung metastases. The therapeutic effects of rMu IFN- γ and of poly(I,C)-LC were correlated with their immunomodulatory activities in the various assay systems in an attempt to identify the immunomodulatory activities responsible for the therapeutic activity of these agents.

MATERIALS AND METHODS

Animals

Specific pathogen-free male C57BL/6 mice were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility. Mice were 7–8 weeks old when they received tumor cells.

Tumors

The tumor cell lines used in these studies were B16-BL6 [70], a highly invasive variant derived from the B16 malignant melanoma of C57BL/6 origin, 3LL-M2 [71], a metastatic variant of the Lewis lung carcinoma line of C57BL/6 origin, YAC-1 [72], a lymphoma induced by Moloney virus in A/SN mice, and P815 [73], a mastocytoma induced by methylcholanthrene in DBA/2 mice. All cell lines were propagated by serial passage in vitro in complete Eagle's minimum essential medium with Earle's salts, supplemented with 5% fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, and twofold vitamin solution. All cell lines were tested to ensure freedom from mycoplasma and murine viruses. All medium components were routinely tested for endotoxin contamination with a *Limulus* amoebocyte lysate test, and only components with levels of endotoxin less than 0.125 EU/ml were used.

rMu IFN- γ

rMu IFN- γ , supplied through the courtesy of Dr. Michael Shepard (Genentech, South San Francisco, CA), had a specific activity of $\approx 2 \times 10^7$ U/mg. rMu IFN- γ was diluted in saline solution containing 0.5% normal mouse serum prior to injection. Poly(I,C)-LC was supplied through the courtesy of Dr. Hilton Levy (NIAID, NIH, NCI-FCRF, Frederick, MD) and was diluted with saline solution prior to injection.

Tumoricidal Assays

Single-cell suspensions of spleen cells were prepared by passing crushed spleens through a wire mesh sieve. Mononuclear cells from collagenase-dissociated pulmonary parenchyma, referred to as pulmonary parenchymal mononuclear cells (PPMC), were obtained by centrifugation on a Ficoll-Hypaque density gradient. Blood, anticoagulated with EDTA, was obtained from the retroorbital sinus, and mononuclear cells were purified from whole blood by density gradient centrifugation on colloidal silica (Sepracell-MN, Sepratech, Oklahoma City, OK). The NK and LAK activities of single-cell suspensions of peripheral blood lymphocytes (PBL), splenocytes, and PPMC from each group were determined simultaneously. NK and LAK activities were routinely assessed in a 4-hr ^{51}Cr release assay, using YAC-1 cells as the targets for NK activity and P815 cells as targets for LAK activity, as previously described [74]. CTL activity of PBL, spleen cells, and PPMC was tested in an 18-hr [^{75}Se]methionine release assay [74]. B16-BL6 cells were used as specific targets in these assays, with syngeneic Lewis lung carcinoma cells (3LL-M2) serving as a specificity control. Macrophages, purified by 3 cycles of adherence at 37°C and washing, were obtained from lung lavage (alveolar macrophages) and from PPMC (pulmonary macrophages). Macrophage tumoricidal activity was determined in a 72-hr in vitro assay, with [^{125}I]UdR-labeled B16-BL6 cells as targets [74]. Macrophage tumoricidal activity was determined simultaneously in a 72-hr in vitro assay, in which

reduction in [^3H]TdR uptake by B16-BL6 cells (terminally pulsed for the last 18 hr of culture) was measured. Macrophage tumoricidal and tumoristatic assays were performed in the presence and absence of added exogenous bacterial lipopolysaccharide (LPS), which functions as a second signal for macrophage activation [75,76], at a concentration of 5 ng/ml.

Experimental Design

Groups of 40 mice were inoculated intravenously (i.v.) with 40,000 viable B16-BL6 cells. After 24 hr, i.v. treatment with either rMu IFN- γ or poly(I,C)-LC was initiated. rMu IFN- γ doses ranging from 0.5–5 $\times 10^6$ U/kg and poly(I,C)-LC doses ranging from 0.005 to 1.25 mg/kg were used, and treatment was maintained three times a week (tiw) for 4 weeks. One and three weeks after inoculation of tumor cells, 10 mice from each group were sacrificed, and we assessed NK, LAK, and CTL activities of splenocytes; NK, LAK, CTL, and macrophage tumoricidal and tumoristatic activities in PPMC; tumoricidal and tumoristatic activities of alveolar macrophages, and NK and CTL activities of PBL. To compare levels of lytic activity in different experimental conditions, levels of NK, LAK, and CTL activity were expressed as lytic units (LU)/ 10^7 cells with 1 LU defined as the number of effector cells required to produce 20% specific release. Four weeks after tumor inoculation, ten mice from each group were sacrificed, and their lungs were removed, washed, and preserved in buffered formalin. The number of metastatic foci on the lungs was enumerated with the aid of a dissecting microscope. The remaining ten animals in each group were maintained until death to evaluate the effect of rMu IFN- γ or poly(I,C)-LC on survival. Prolongation of survival and reduction in the number of pulmonary metastases were used as measures of the therapeutic efficacy of the treatment protocols. Each experiment was performed three times with similar results, and data from the three experiments were pooled.

Statistical Analyses

The survival of cohorts of mice receiving different treatment regimens is presented as Kaplan-Meier survival curves, and the statistical significance of these differences was analyzed with the Kruskal-Wallis test [77]. The statistical significance of the differences in the number of lung metastases was analyzed by the Mann-Whitney U-test [77]. The correlations of therapeutic efficacy, expressed as the median survival time or as the median number of lung metastases, with the various measures of effector cell function were analyzed by Pearson's correlation coefficient [77]. NK, LAK, and CTL activities were expressed as LU/ 10^7 cells. Macrophage tumoricidal activity was expressed as % specific release, and macrophage tumoristatic activity was expressed as % reduction in uptake of [^3H]TdR. For these analyses, therapeutic activity (median survival time or median number of lung metastases) at different doses of the BRMs was compared with immunomodulatory activity at the different BRM doses in a stepwise 2 \times 2 correlation matrix. Simply put, demonstration of a correlation between the therapeutic and immunomodulatory activities of a BRM depends on there being a similar dose dependence for both activities.

RESULTS

Therapeutic Activity of rMu IFN- γ and of Poly(I,C)-LC

rMu IFN- γ doses of 1.5 and 2.5 $\times 10^6$ U/kg significantly reduced the number of pulmonary metastases, compared to saline treatment [1.5×10^6 U/kg ($P = 0.0002$)

and 2.5×10^6 ($P = 0.0014$)], as shown in Table I. rMu IFN- γ doses of 0.5 and 5×10^6 U/kg had no significant therapeutic activity. Additionally, the two doses of rMu IFN- γ with therapeutic activity (1.5 and 2.5×10^6 U/kg) did not differ significantly from each other, but both doses had significantly greater therapeutic activity when compared to the 0.5 or 5×10^6 U/kg doses (Table I). When therapeutic activity was expressed as prolongation of survival, the therapeutic effects of rMu IFN- γ were observed at dosage levels identical with those for reduction in the number of long metastases, with therapeutic activity at doses of 1.5 and 2.5×10^6 U/kg and no therapeutic activity at doses of 0.5 and 5×10^6 U/kg (data not shown). Poly(I,C)-LC doses of 0.05, 0.5, and 1.25 mg/kg all significantly prolonged survival, compared to saline treatment, of animals bearing B16-BL6 experimental lung metastases [0.05 mg/kg ($P = 0.0016$), 0.5 mg/kg ($P = 0.0005$), and 1.25 mg/kg ($P = 0.0001$)], as shown in Figure 1. The lowest poly(I,C)-LC dose tested, 0.005 mg/kg, had no significant therapeutic activity compared with saline treatment, and this dose had significantly less therapeutic activity than the other three doses of poly(I,C)-LC (Fig. 1). The same three doses of poly(I,C)-LC had therapeutic activity, compared to saline treatment, when therapeutic activity was expressed as reduction in the number of pulmonary metastatic nodules (data not shown).

Effect of BRMs on NK and LAK Activities

The NK and LAK activities of splenocytes, PPMC, and PBL were examined 1 and 3 weeks after tumor inoculation. With rMu IFN- γ , no striking dose-response effects were seen because all the doses of rMu IFN- γ tested boosted the levels of NK activity (data not shown). Poly(I,C)-LC also boosted NK activity at most tested doses, but dose response effects could be seen during week 1 (Fig. 2). NK activity in PPMC peaked at a dose of 0.5 mg/kg of poly(I,C)-LC, and NK activity in splenocytes and PBL peaked at a dose of 0.05 mg/kg of poly(I,C)-LC. During week 3, poly(I,C)-LC did not augment NK activity in any site effectively (Fig. 2B). Little or no LAK activity was detected in any tested organ site, at any dose of either BRM, or at either time examined (data not shown).

Effect of BRMs on CTL Activity

The CTL activity of splenocytes, PPMC, and PBL was tested 1 and 3 weeks after tumor inoculation. Specific targets—B16-BL6 melanoma cells, the same cell type as the primary tumor—and nonspecific control targets—syngeneic Lewis lung

TABLE I. Therapeutic Activity of rMu IFN- γ on Experimental Lung Metastases[†]

BRM	Dose (U/kg)	No. of metastases		P values*			
		Median	Range	vs. saline	vs. 0.5×10^6	vs. 1.5×10^6	vs. 2.5×10^6
Saline		> 300	18->300				
rMu IFN- γ	0.5×10^6	167	8->300	0.063			
rMu IFN- γ	1.5×10^6	15	3->300	0.0002	0.0003		
rMu IFN- γ	2.5×10^6	41	0->300	0.0014	0.04	0.095	
rMu IFN- γ	5×10^6	>300	58->300	0.31	0.014	0.0003	0.001

[†]Mice received i.v. inoculations of 40,000 B16-BL6 melanoma cells. rMu IFN- γ was administered i.v., *tiw* beginning 1 day later. After 4 weeks, mice were sacrificed, and the number of metastatic nodules on the lungs was enumerated with the aid of a dissecting microscope.

*Determined by Mann-Whitney U-test.

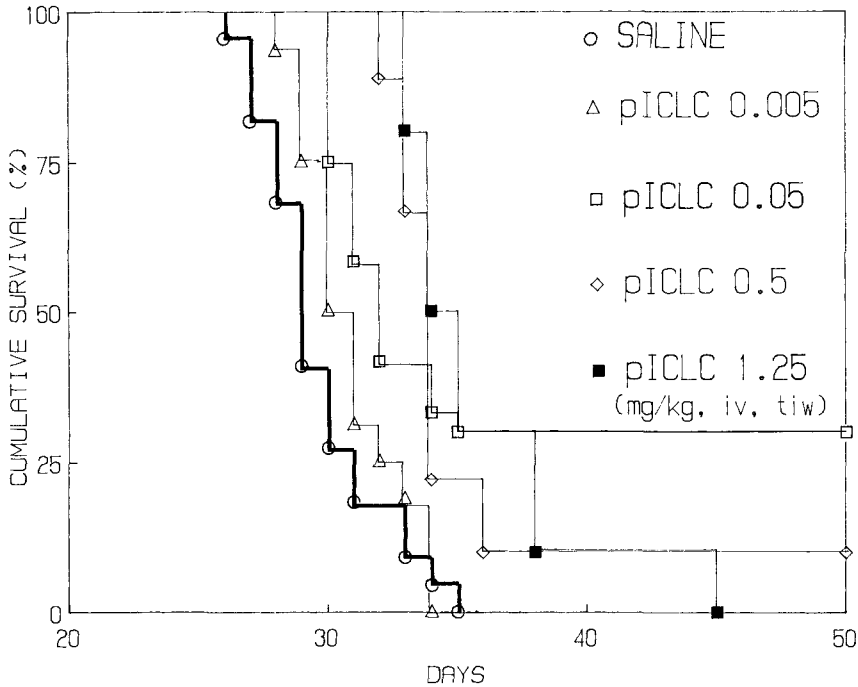


Fig. 1. Effect poly(I,C)-LC on survival of mice inoculated i.v. with 40,000 B16-BL6 melanoma cells. Treatment with poly(I,C)-LC (i.v., tiw) was initiated 1 day later. The statistical significance of the differences in survival was analyzed with the Kruskal-Wallis test. Results (P values) are presented in Table II.

TABLE II. Survival Data

Poly(I,C)-LC (mg/kg)	Median survival time (days)	Range	P values			
			vs. saline	vs. 0.005 mg/kg	vs. 0.05 mg/kg	vs. 0.5 mg/kg
0	29	26-35				
0.005	30	28-34	0.18			
0.05	32	30-> 84	0.0016	0.048		
0.5	34	32-> 84	0.0005	0.0022	0.32	
1.25	34	33-45	0.0001	0.0003	0.17	0.31

carcinoma (3LL-M2) cells—were tested. During week 1, lytic activity against B16-BL6 targets was seen in PBL from animals treated with all doses of rMu IFN- γ (Fig. 3A). PPMC displayed low levels of lytic activity against B16-BL6 targets at some doses of rMu IFN- γ , but splenocytes had little or no lytic activity against B16-BL6 targets (Fig. 3A). This activity does not represent specific CTL activity because PPMC had similar levels of lytic activity against the nonspecific control target, Lewis lung carcinoma (Fig. 3C), as against the specific target, B16-BL6 (Fig. 3A). In contrast, no lytic activity was seen against either target by effector cells from any organ of animals treated with poly(I,C)-LC during week 1 (data not shown). However during week 3, the pattern of lytic activity was quite different from that seen during week 1 for both poly(I,C)-LC and rMu IFN- γ . During week 3, lytic activity against

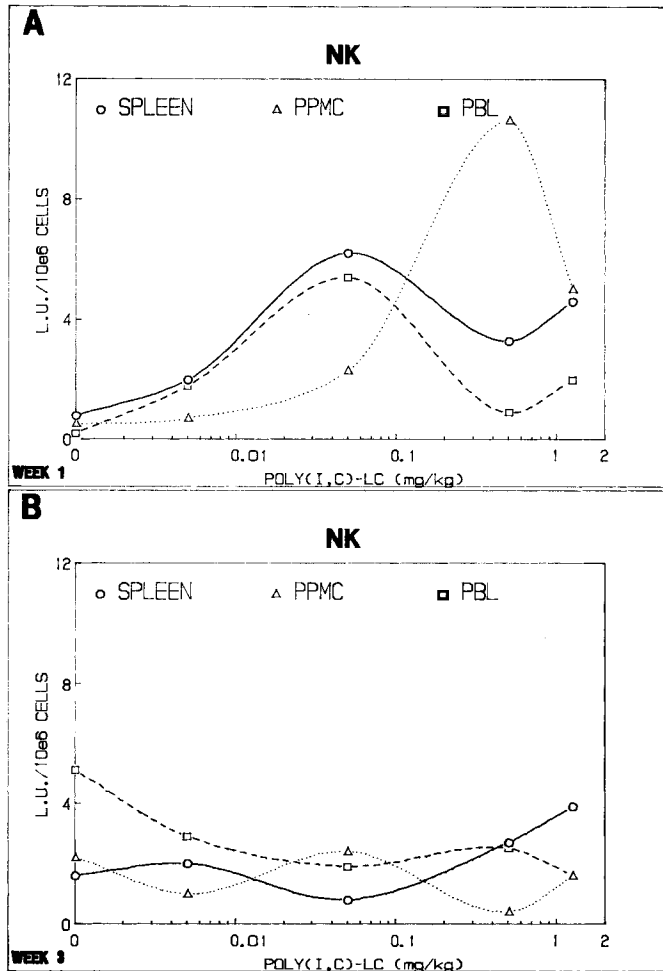


Fig. 2. Effect of i.v. poly(I,C)-LC on NK activity in mice bearing B16-BL6 experimental metastases. NK activity, expressed as lytic units/ 10^6 cells, was measured 1 (A) and 3 (B) weeks after tumor inoculation.

the specific target, B16-BL6, was observed only in PPMC, not in splenocytes or PBL (Figs. 3B, 4A). This lytic activity peaked at doses of 2.5×10^6 U/kg of rMu IFN- γ (Fig. 3B) and 0.5 mg/kg of poly(I,C)-LC (Fig. 4A), respectively. The lack of lytic activity by PPMC against the specificity control targets, Lewis lung carcinoma (Fig. 3D), during week 3, strongly suggests that this activity is mediated by specific CTL and was found only in the tumor-bearing organ, the lung. Furthermore, the PPMC did not mediate LAK activity during week 3 (data not shown). The identity of the effector cells in PPMC as CTL is further strengthened by the demonstration that treatment of PPMC with anti-Thy 1.2 plus complement, but not anti-asialo GM1 plus complement, ablated their ability to kill B16-BL6 target cells (Fig. 4A). With NK activity, the opposite effects were observed (Fig. 4B). These results confirm the T cell nature of the effector cells in PPMC and provide further support for their identity as specific CTL.

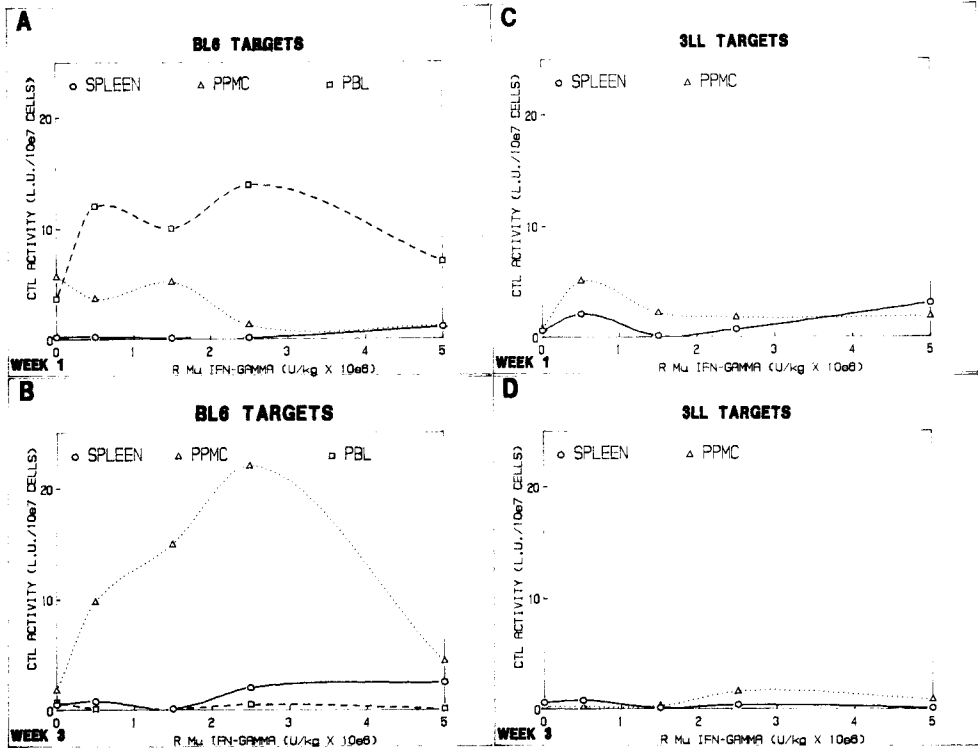


Fig. 3. Effect of i.v. rMu IFN- γ on CTL activity against specific (B16-BL6; A,B) and nonspecific (3LL; C,D) targets in mice bearing B16-BL6 experimental metastases. CTL activity, expressed as lytic units/ 10^7 cells, was measured 1(A,C) and 3(B,D) weeks after tumor inoculation.

Effect of BRMs on Macrophage-Mediated Cytotoxicity and Cytostasis

Macrophages were obtained from alveoli by lung lavage and from PPMC after 1 and 3 weeks of BRM treatment, and their tumoricidal and tumoristatic activities assessed. The results in Figure 5 demonstrate that rMu IFN- γ , administered i.v. tiw, augmented macrophage-mediated tumoricidal and tumoristatic activities. During week 1, rMu IFN- γ augmented tumoricidal activity in pulmonary macrophages with a peak at 1.5×10^6 U/kg (Fig. 5A). Also during week 1, rMu IFN- γ augmented macrophage tumoristatic activity, with a peak at 0.5×10^6 U/kg (Fig. 5C). During week 3, the tumoricidal (Fig. 5B) and tumoristatic (Fig. 5D) activities of pulmonary macrophages peaked at a dose of 2.5×10^6 U/kg. Alveolar macrophage activities did not show similar responses to rMu IFN- γ during week 3. In contrast, during week 1, poly(I,C)-LC boosted alveolar macrophage tumoricidal activity more effectively than pulmonary macrophage tumoricidal activity, with a peak at 0.5 mg/kg (Fig. 6A). There was some discrepancy between results from tumoricidal and tumoristasis assays because poly(I,C)-LC boosted the tumoristatic activity of pulmonary macrophages more effectively than that of alveolar macrophages, with a peak at 0.5 mg/kg (Fig. 6C). During week 3, however, macrophage tumoricidal and tumoristatic activities were not significantly boosted by poly(I,C)-LC (Fig. 6B,D).

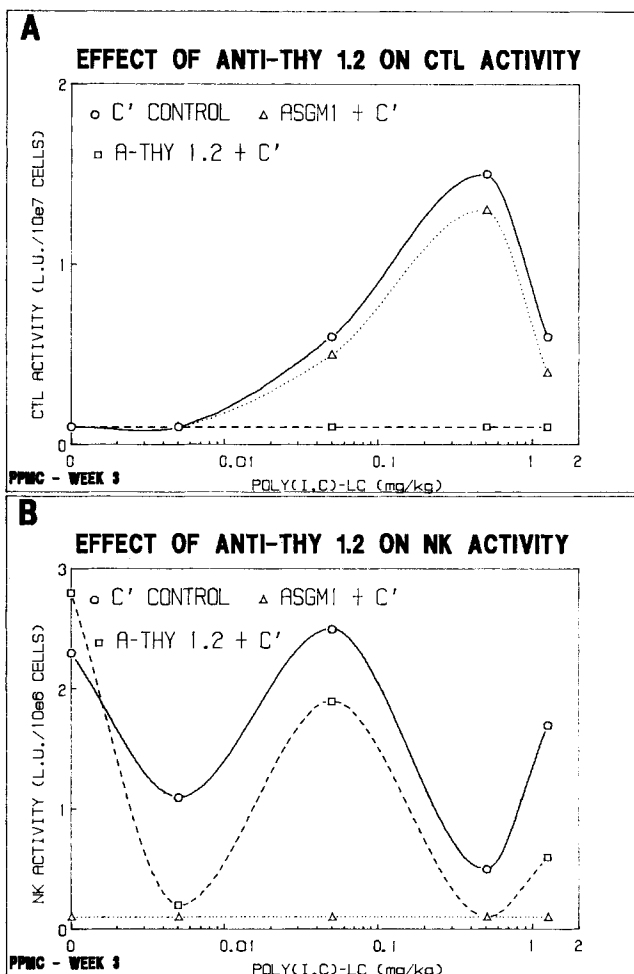


Fig. 4. Effect of antibody depletion with anti-Thy 1.2 (New England Nuclear, Boston, MA) or anti-asialo GM1 (Wako Chemical) on specific CTL (A) and NK (B) activity of PPMC from mice bearing experimental lung metastases, after 3 weeks of poly(I,C)-LC administration. Treatment with antibody plus complement (C') was performed as previously described [74]. B16-BL6 melanoma cells were the targets for specific CTL activity (A), and YAC-1 cells were the targets for NK activity (B).

Correlation of Therapeutic Activity With Effector Cell Function

In order to determine which effector cell functions were responsible for the therapeutic activity of rMu IFN- γ and of poly(I,C)-LC, the various effector cell functions in the different anatomical sites were correlated with therapeutic activity using Pearson's correlation coefficient [77]. This analysis revealed that the therapeutic activity of these BRMs correlated with augmented effector functions only in the organ with tumor, the lung, but not in the spleen or PBL (Tables III, IV). Specific CTL in PPMC at week 3 (with B16-BL6 as targets) correlated with the therapeutic activity of both rMu IFN- γ ($P = 0.0088$, Table III) and of poly(I,C)-LC ($P = 0.037$, Table IV), but CTL activity in splenocytes or PBL at week 3 did not show a similar correlation. However, the CTL activity at any site tested during week 1 did not show a similar

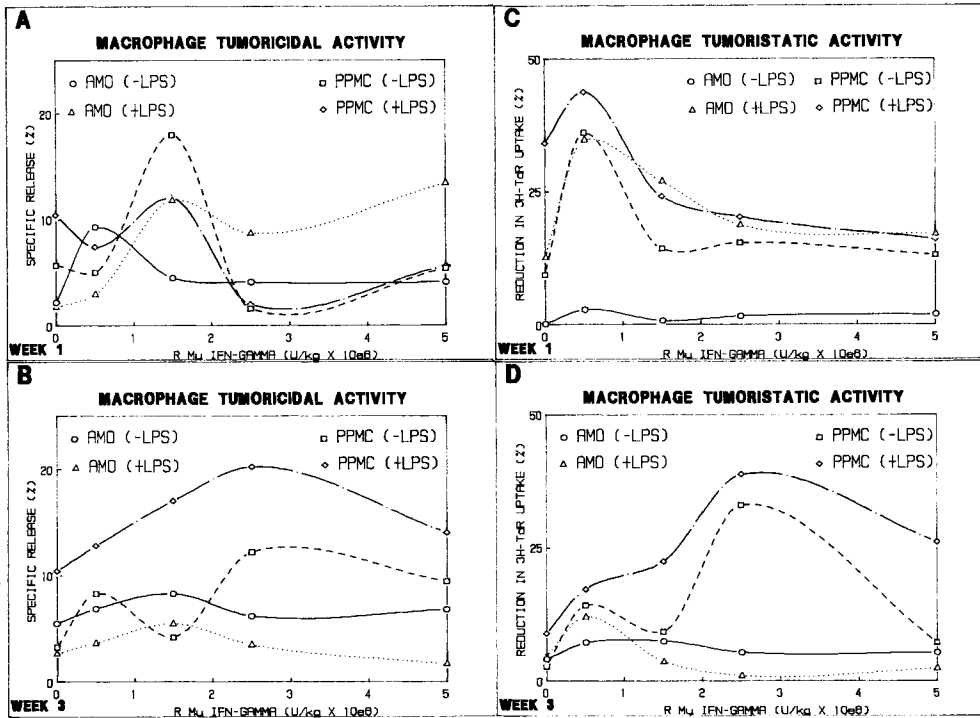


Fig. 5. Effect of i.v. rMu IFN- γ on macrophage tumoricidal (A,B) and tumoricidal (C,D) activities in mice bearing B16-BL6 experimental metastases. Macrophage tumoricidal activity, measured as specific release of [¹²⁵I]UdR from B16-BL6 targets in a 72-hr assay, and macrophage tumoricidal activity, expressed as reduction in [³H]TdR uptake by B16-BL6 cells in a 72-hr assay, were measured 1(A,C) and 3(B,D) weeks after tumor inoculation.

correlation (Table III, IV) because no specific CTL activity was seen then (Fig. 3A,C). Macrophage tumoricidal and tumoricidal activities in PPMC but not in alveoli, also correlated with the therapeutic activity of rMu IFN- γ (Table III). With poly(I,C)-LC, the opposite was true, and its therapeutic activity correlated with alveolar macrophage tumoricidal activity but not with pulmonary macrophage tumoricidal activity (Table IV). The NK activity in PPMC, but not in splenocytes or PBL, correlated with the therapeutic activity of poly(I,C)-LC (Table IV) only during week 1, but not with the therapeutic activity of rMu IFN- γ (Table III). LAK activity in any tested site at either time did not correlate with therapeutic activity of either BRM (Tables III, IV).

DISCUSSION

Identifying agents with therapeutic activity against established metastatic disease, optimizing the administration protocols, and understanding the mechanism of their therapeutic activity are the primary goals of the preclinical studies of BRMs. Previous studies have demonstrated the importance of dosage, route, duration, and schedule of administration in optimizing the therapeutic activity of BRMs for the treatment of metastatic disease in preclinical models [46-48,74]. rMu IFN- γ and

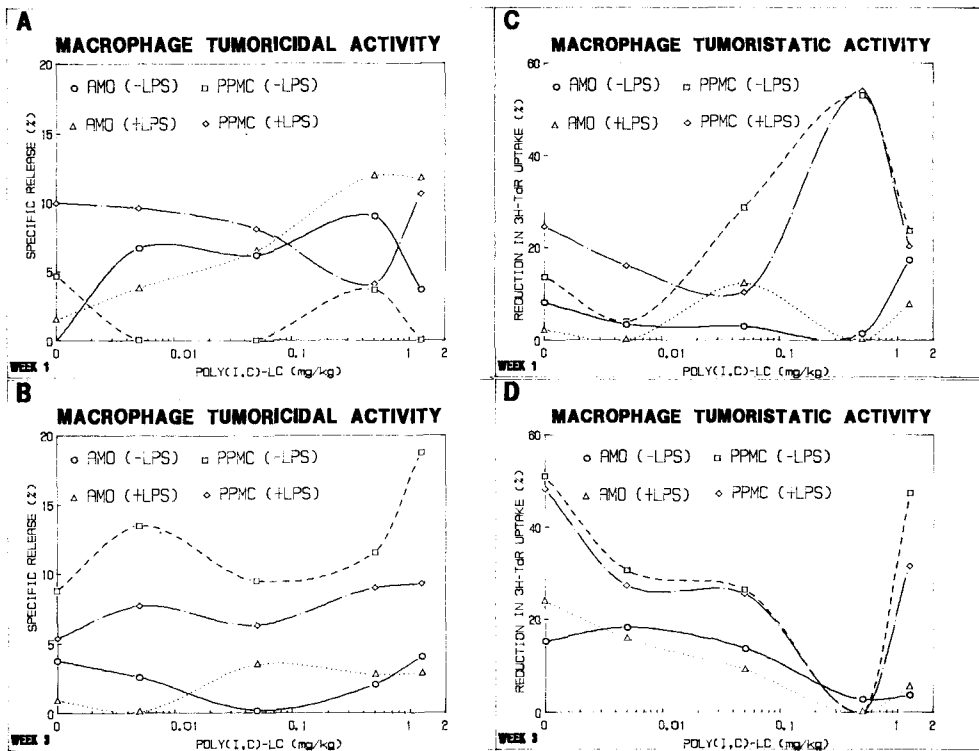


Fig. 6. Effect of i.v. poly(I,C)-LC on macrophage tumoricidal (A,B) and tumoristatic (C,D) activities in mice bearing B16-BL6 experimental metastases. Macrophage tumoricidal activity, measured as specific release of [125 I]UdR from B16-BL6 targets in a 72-hr assay, and macrophage tumoricidal activity, expressed as reduction in [3 H]TdR uptake by B16-BL6 cells in a 72-hr assay, were measured 1(A,C) and 3(B,D) weeks after tumor inoculation.

poly(I,C)-LC have consistently shown significant therapeutic activity in animal models of experimental and spontaneous metastases, but this therapeutic activity depends on utilization of optimal therapeutic protocols as discussed above. For example, i.v. administration of rMu IFN- γ produced greater therapeutic benefit compared with intraperitoneal or intramuscular administration [46], owing presumably to the higher, but less sustained serum levels of rMu IFN- γ obtained following i.v. administration compared with the other routes [46,78,79].

The results of the present studies are consistent with these concepts as therapeutic activity depended strongly on the dose of the BRM administered. Both rMu IFN- γ and poly(I,C)-LC also had immunomodulatory activity in most of the assay systems employed, with several exceptions. NK activity was not boosted, and may even have been depressed, after 3 weeks of poly(I,C)-LC (Fig. 2B) administration. This finding is consistent with other reports of NK hyporesponsiveness following repeated BRM administration [7,46,80-88]. LAK activity, measured by release of 51 Cr in a 4-hr assay with P815 cells as targets, was not boosted by treatment with either rMu IFN- γ or poly(I,C)-LC at any dosage level. In fact, LAK activity was not detected during these studies. Similarly, during week 3, rMu IFN- γ and poly(I,C)-LC did not augment CTL activity in splenocytes or PBL against either the specific or nonspecific target.

TABLE III. Correlation of Therapeutic Activity of rMu IFN- γ With Effector Cell Functions

	Effector cell function ^a							
	NK			LAK		CTL		
	Spleen	PPMC	PBL	Spleen	PPMC	Spleen	PPMC	PBL
Week 1	0.30 ^b	0.18	0.15	0.44	0.60	0.10	0.51	0.58
Week 3	0.14	0.41	0.36	0.02	0.55	0.47	0.84 (0.0088)	0.41

	Macrophage activity							
	[¹²⁵ I]UdR release				Reduction in [³ H]TdR uptake			
	AMO ^c		PPMC		AMO		PPMC	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
Week 1	0.41	0.24	0.17	0.37	0.26	0.35	0.74 (0.039)	0.82 (0.011)
Week 3	0.27	0.04	0.72 (0.036)	0.20	0.37	0.66	0.26	0.78 (0.023)

^aMice received i.v. inoculations of 40,000 B16-BL6 melanoma cells. rMu IFN- γ was administered i.v. beginning 1 day later. After 1 and 3 weeks, effector functions in various organs were tested. Effector cell functions were compared with median number of lung metastases using Pearson's correlation coefficient for medians.

^bCorrelation coefficients (*P* values for significant correlations).

^cAMO, alveolar macrophages.

TABLE IV. Correlation of Therapeutic Activity of Poly(I,C)-LC With Effector Cell Functions

	Effector cell function ^a							
	NK			LAK		CTL		
	Spleen	PPMC	PBL	Spleen	PPMC	Spleen	PPMC	PBL
Week 1	0.62 ^b	0.88 (0.049)	0.16	0.73	0.67	0.26	0.12	0.74
Week 3	0.61	0.45	0.77	0.58	0.79	0.02	0.90 (0.037)	0.15

	Macrophage activity							
	[¹²⁵ I]UdR release				Reduction in [³ H]TdR uptake			
	AMO ^c		PPMC		AMO		PPMC	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
Week 1	0.62	0.99 (0.0006)	0.35	0.54	0.17	0.06	0.80	0.50
Week 3	0.12	0.69	0.50	0.82	0.92 (0.025)	0.96 (0.0079)	0.55	0.72

^aMice received i.v. inoculations of 40,000 B16-BL6 melanoma cells. Poly(I,C)-LC was administered i.v. beginning 1 day later. After 1 and 3 weeks, effector functions in various organs were tested. Effector cell functions were compared with median survival time using Pearson's correlation coefficient for medians.

^bCorrelation coefficients (*P* values for significant correlations).

^cAMO, alveolar macrophages.

However, specific CTL activity against B16-BL6 in PPMC was augmented by both these agents in a dose-dependent manner, with peaks at 2.5×10^6 U/kg for rMu IFN- γ and at 0.5 mg/kg for poly(I,C)-LC. It should be emphasized that these effector cell populations in PPMC did not show any lytic activity against the nonspecific control target cells, syngeneic 3LL-M2 carcinoma. Furthermore, the effector cells in PPMC were sensitive to treatment with anti-Thy 1.2, but not anti-asialo GM1, plus complement. These results demonstrate that the effector cells responsible for the lytic activity against B16-BL6 targets in PPMC during week 3 are indeed specific CTL, as had been suggested by the target specificity of this lytic activity and by the absence of LAK cell activity. Furthermore, the restriction of specific CTL activity to one anatomic site, the lungs, may result from local antigen processing at the tumor site, local cytokine production, or chemotactic attraction of specific CTL to the tumor.

These studies also correlated the therapeutic and immunomodulatory effects of these BRMs in an effort to identify the mechanism of their therapeutic activity in treating metastatic disease. CTL in PPMC during week 3, but not during week 1, correlated with the therapeutic activity of both these BRMs, but CTL activity in splenocytes or PBL did not show a similar correlation. This difference in lytic activity between weeks 1 and 3 is predictable because about 10 to 14 days are required to produce a specific CTL response. Therefore, specific CTL activity would not have been expected during week 1. Macrophage antitumor activities also correlated with the therapeutic activity of these BRMs. However, the therapeutic activity of rMu IFN- γ correlated with macrophage activities in PPMC, while the therapeutic activity of poly(I,C)-LC correlated with alveolar macrophage activities. This difference may be associated with augmentation of the activities of different macrophage populations. The rMu IFN- γ may augment histiocytes (in situ tissue macrophages) while poly(I,C)-LC may augment monocytes whose activity appears as alveolar macrophages, but not histiocytes. This difference could represent a different mechanism of activation, with direct activation by rMu IFN- γ and indirect activation by poly(I,C)-LC, with the result that a mediator is diluted or inactive within parenchymal tissue.

Another discrepancy between the two BRMs' mechanisms of therapeutic activity occurred in NK activity. NK activity, at any site, at either time did not correlate with the therapeutic activity of rMu IFN- γ , but pulmonary NK activity during week 1, but not week 3, did correlate with the therapeutic activity of poly(I,C)-LC. It should be emphasized, however, that the lack of correlation of NK activity with the therapeutic activity of rMu IFN- γ is not necessarily due to a lack of NK augmentation because rMu IFN- γ boosted NK activity, but with a dose dependence different from that of rMu IFN- γ 's therapeutic activity. For both these BRMs, pulmonary CTL activity during week 3 correlated with their therapeutic activity. Otherwise, effector functions only in the organ with tumor, the lung, showed correlations with therapeutic activity of either of these agents. These analyses revealed that both the particular effector function and the site sampled represent important factors in understanding the mechanism of therapeutic action of BRMs.

In summary, these studies support and extend the concept of optimal administration of BRMs for treating metastatic disease, and they provide information on the mechanism of therapeutic action of rMu IFN- γ and poly(I,C)-LC. In particular, monitoring immune functions in the tumor-bearing organ appears to be critical for identifying the mechanism of BRMs' therapeutic effects and for predicting such activity. Obviously it is not possibly routinely to obtain samples of tumor-bearing

organs for immune monitoring of effector cell functions in patients during clinical trials of BRMs, and monitoring of effector functions in readily accessible sites such as peripheral blood may not accurately reflect the immunotherapeutic activity of BRMs. However in some situations, such as peritoneal effusions, this approach may be feasible and could prove highly informative.

ACKNOWLEDGMENTS

This research was sponsored by the DHHS, under contract No. NO1-23910 with Program Resources, Inc.

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