Brief Definitive Report

ROLE OF INTERLEUKIN 2, INTERLEUKIN 4, AND α, β, AND γ INTERFERON IN STIMULATING MACROPHAGE ANTIBODY-DEPENDENT TUMORICIDAL ACTIVITY

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Macrophage antibody-dependent cellular cytotoxicity (ADCC) to tumor targets is subject to in vivo and in vitro regulation (reviewed in reference 1). Among endogenous macrophage-stimulating factors, γ interferon (IFN-γ) is well known for inducing nonspecific tumoricidal activity (e.g., reference 2). Using individual purified factors and neutralizing antibodies, we show here that murine IFN-α, IFN-β, rIFN-γ, and rIL-2, but not rIL-4, stimulate ADCC.

Materials and Methods

Effector Cells. Adherent peritoneal exudate macrophages from protease peptone-injected C3H/HeN mice (Charles River Breeding Laboratories, Inc., Kingston, NY) were prepared as described (3). The adherent cells were >95% macrophages (3). The cells were incubated for 2 d in medium ± factors (1); the cell number did not change during this time.

Lymphokines. Mitogen-induced lymphokine was the supernatant of 2×10^6/ml normal mouse spleen cells cultured 2 d with 10 μg/ml Con A (Sigma Chemical Co., St. Louis, MO). Mock lymphokine supernatant collected 1 h after addition of Con A to cells did not stimulate ADCC. Murine rIFN-γ (a gift from Schering-Plough Corp., Kenilworth, NJ), purified rIL-4 (4), IFN-α (Lee BioMolecular, San Diego, CA), IFN-β (Lee BioMolecular), and human rIL-2 (Cetus Corp., Emeryville, CA) were used as stimulators. Rabbit anti-human IL-2 raised to human rIL-2 produced in *Escherichia coli* (neutralizing titer of 3,100 n.u./ml, M. V. Doyle, Cetus Corp.), R4-6A2 monoclonal anti-mouse IFN-γ (Lee BioMolecular), and 11B11 monoclonal anti-IL-4 (5) were used. 11B11 antibody was produced from the hybridoma cells serum free, purified by ammonium sulfate precipitation and protein A–agarose chromatography, coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA), and used as an immunoaffinity column to deplete Con A lymphokine of IL-4 activity. IFN was assayed by vesicular stomatitis virus infection of L929 and IL-2 by proliferation of HT-2 cells ± anti-IL-4. IL-4 in lymphokine was determined by T. Mossman (DNAX, La Jolla, CA) using the HT-2 proliferation assay ± anti-IL-4 and anti-IL-2.

Cytotoxicity Assays. ADCC was measured by adding 2×10^4 mouse thymoma R1 cells to effector cells at an E/T ratio of 1:1 to 6:1. Rabbit anti-mouse brain (anti-Thy-1) (Accurate Chemical & Scientific Corp., Westbury, NY) was used at 1:2,000 in the assay, except as noted. Controls lacking antibody, or macrophages, or both, were run in replicate in all experiments (3). ADCC was determined at 9 or 24 h by vigorously pipetting nonadherent cells and performing a viable count in a hemacytometer. Results are stated as means ± SEM. Cultures containing factors ± antiserum but lacking macrophages did not differ from R1 only cultures by >10% on average. Percent ADCC is calculated as $(1 - A/C) \times 100$, where A and C are target cell counts from wells with similarly treated macrophages with and without antiserum, respectively. Percent ADCC induced by the 2-d pretreatment is calculated as $100 \times [(X - Y)/(100 - Y)]$, where X and Y are percent ADCC values for factor-stimulated and medium control macrophages, respectively.

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TABLE I

Stimulation of Macrophage ADCC by rIFN-γ, IL-2, IFN-α, and IFN-β

Exp.	Pre-treatment	U/ml	9 h				24 h			
			−As*	+As*	% ADCC	% Δ‡	−As*	+As*	% ADCC	% Δ‡
1	Medium		19 ± 1	12 ± 0	39 ± 6		43 ± 1	29 ± 1	33 ± 3	
	IFN-γ	50	21 ± 0.5	1 ± 1	95 ± 5	92§	41 ± 1.5	4 ± 1.5	90 ± 6	85§
		5	21 ± 0	2 ± 2	86 ± 9	77§	47 ± 2.5	5 ± 0	89 ± 7	84§
		1	21 ± 1	9 ± 1	57 ± 7	30	44 ± 1.5	23 ± 2	48 ± 6	22
	LK 20%		18 ± 0	2 ± 0	91 ± 0	85§	40 ± 1.5	3 ± 0	92 ± 5	88§
	5%		20 ± 0.5	3 ± 1	85 ± 8	75§	43 ± 1	7 ± 0.5	84 ± 3	76§
	1%		20 ± 0.5	9 ± 1.5	55 ± 8	26	42 ± 1.5	21 ± 1	50 ± 4	25
	IL-2	50	20 ± 2	6 ± 1	70	51§	41 ± 0	5 ± 0	88	82§
		5	22 ± 1	8 ± 0.5	64	41§	43 ± 1.5	8 ± 0.5	81	72§
		1	20 ± 0	10 ± 0.5	50	18	45 ± 1	23 ± 1	49	24
2	Medium		14 ± 0.5	14 ± 1.5	0		45 ± 2	47 ± 5	0	
	IFN-α	50	15 ± 1.5	8 ± 1	47	47§	44 ± 0	22 ± 1	50	50§
		10	14 ± 0	11 ± 2	21	21	45 ± 2	32 ± 2	29	29
		5	13 ± 0	12 ± 1.5	9	9	48 ± 3	47 ± 2	2	2
		1	16 ± 1.5	16 ± 1	0	0	45 ± 1	49 ± 2	0	0
	IFN-β	50	13 ± 1	8 ± 0	39	46§	42 ± 1	20 ± 1	52	52§
		5	14 ± 0.5	15 ± 0.5	0	0	47 ± 2	45 ± 2	6	6
		1	15 ±0.5	14 ± 0.5	7	7	49 ± 1	46 ± 3	6	6
	IFN-γ	5	12 ± 1	7 ± 0.5	42	42§	45 ± 01	24 ± 1	47	47§

Macrophages were tested 2 d with medium, Con A–induced lymphokine (LK), or factors, and then assayed for killing R1 targets at an E/T ratio of 6. Targets were not affected by antiserum or LKs in the absence of macrophages.

* Viable targets ($\times 10^4$/ml) at 9 and 24 h.

‡ % ADCC induced by agent pretreatment was determined by comparison with media control.

§ Significantly different from medium control, $p < 0.05$.

Results

ADCC-stimulating Effect of Individual IFNs and IL-2. We previously showed that a 2-d incubation of mouse peritoneal exudate macrophages in culture with Con A–induced lymphokine was optimal for stimulating ADCC (1). We followed this protocol to study the effect of individual factors. The R1 thymoma cell line was used as the target since it is very sensitive to macrophage ADCC, but fairly resistant to nonspecific killing and NK lysis (3). The assay used visual counting of target cells surviving macrophage coculture at 9 and 24 h. The R1 targets are easily distinguished from peritoneal cells by size. A single batch of Con A–induced lymphokine was used throughout these experiments, and it contained 35 U/ml of IFN activity, 92 U/ml IL-2, and 58 U/ml IL-4, and <0.2 ng/ml LPS. Table I shows that recombinant murine IFN-γ greatly potentiates ADCC at 5 and 50 U/ml, with moderate effects seen at 1 U/ml. Comparison of the titration of Con A–induced lymphokine and IFN-γ in three experiments (Table I and not shown) suggests that 5% lymphokine has the activity of ~2–4 U/ml IFN-γ in this assay. Rat rIFN-γ (Amgen, Thousand Oaks, CA) had almost the identical effect as mouse IFN-γ on murine macrophage ADCC.

IFN-α and IFN-β also stimulate ADCC, but at higher concentrations than for IFN-γ (Table I). In three experiments with each type, 50 U/ml IFN-α or IFN-β had about the same potentiating activity as 5 U/ml IFN-γ. Human rIL2 also

TABLE II
Role of IL-4 in Macrophage ADCC

Pretreatment	U/ml	9 h				24 h			
		−As*	+As*	% ADCC	% Δ	−As*	+As*	% ADCC	% Δ
Medium		23 ± 0.5	18 ± 1	22		46 ± 3	38 ± 2	17	
IL-4	100	25 ± 1.5	20 ± 0	20	−3	46 ± 0.5	36 ± 1	22	6
	10	23 ± 0	17 ± 2	26	5	42 ± 2.5	32 ± 2.5	24	8
	1	24 ± 2	19 ± 0.5	21	−1	44 ± 1	36 ± 2.5	18	1
LK 20%		22 ± 1.5	4 ± 0.5	82	77‡	43 ± 1	2 ± 1	95	94‡
5%		24 ± 1	9 ± 1	62	51‡	48 ± 2.5	9 ± 0.5	81	77‡
1%		22 ± 0.5	13 ± 0.5	41	24	48 ± 0.5	30 ± 2	37	24
IL-4–depleted LK 20%		21 ± 0.5	5 ± 0.5	76	70‡	45 ± 1.5	4 ± 0	91	89‡
5%		22 ± 0	9 ± 0	59	47‡	44 ± 1	7 ± 0.5	84	81‡
1%		22 ± 1	12 ± 1	45	29	46 ± 1	30 ± 1.5	35	22

Macrophages were pretreated with IL-4, LK, or IL-4–depleted LK and assayed for ADCC as in Table I. The LK contained 58 U/ml IL-4, and IL-4 in the depleted LK was undetectable (<5 U/ml). The results suggest that IL-4 does not stimulate ADCC.
* Viable targets surviving ($\times 10^4$/ml).
‡ % ADCC induced by pretreatment significantly different from medium control.

TABLE III
Anti-IFN-γ Antibody Blocks the ADCC-stimulating Effect of LK

Exp.	Pretreatment	−As*	+As*	% ADCC	% Δ‡	+ anti-IFN-γ (130 n.u.)			
						−As*	+As*	% ADCC	% Δ‡
1	Media	16 ± 0.5	12 ± 1	25		14 ± 0	10 ± 0.5	29	5
	γ-IFN 5 U/ml	14 ± 1	6 ± 2	57	43	15 ± 0.5	12 ± 1	20	−7
	LK 20%	14 ± 0.5	4 ± 2	71	61	12 ± 0	8 ± 1.5	33	11
2	Media	23 ± 0.5	18 ± 1	22		22 ± 1	16 ± 2	27	6
	LK 20%	22 ± 1.5	4 ± 0.5	82	77	20 ± 1	15 ± 0	25	4
	IL-2 50 U/ml	25 ± 0.5	6 ± 1	75	68	21 ± 2	5 ± 0.5	76	69
	IL-2 + a-IL-2	21 ± 0	15 ± 1	29	9				
	LK + IL-2	22 ± 0	3 ± 0	86	82	21 ± 0.5	6 ± 0	71	63
	LK + a-IL-2	21 ± 1.5	5 ± 1	76	69				
	a-IL-2	26 ± 1	20 ± 1.5	23	1				

Macrophages were pretreated with lymphokines ± neutralizing antibodies for 2 d and then tested for ADCC as in Table I. The anti-rIFN blocked the ADCC-stimulating activity of LK. The anti-human IL-2 (50 n.u.) does not neutralize mouse IL-2; it blocked the ADCC-stimulating activity of rhIL-2 but not that of the murine LK. The cultures containing the combination of LK, IL-2, and anti-IFN-γ show that the antibody did not nonspecifically block increased ADCC since a strong augmentation was seen due to the IL-2.
* Viable targets ($\times 10^4$/ml) at 9 h.
‡ Percent ADCC induced by pretreatment.

stimulated ADCC in the range of 5–50 U/ml (Table I). When nonadherent cells from the peritoneal exudate population were similarly incubated with IL-2, they did not have any ADCC activity (not shown).

Effect of IL-4 and IL-4-depleted Lymphokine. Treatment of macrophages with rIL4 did not stimulate ADCC, whereas Con A–induced lymphokine depleted of IL-4 retained its activity (Table II). The results were seen, respectively, in three and four separate experiments. This suggests that IL-4 does not play a major role in this macrophage tumoricidal mechanism.

Anti-IFN-γ Blocks Most of the Activity in Lymphokines. A neutralizing mAb was used to assess the contribution of IFN-γ to the activity in lymphokine. Table III shows that 130 neutralizing units/ml of antibody almost completely blocked the

TABLE IV

Effect of Antiserum Concentration and E/T Ratio on Stimulated ADCC

E/T	Pretreatment	−As*	2,000*	% ADCC	% Δ‡	20,000*	% ADCC	% Δ‡	200,000*	% ADCC	% Δ‡
5	Medium	31 ± 0.5	17 ± 1	45		28 ± 2	10		34 ± 2	0	
	IFN-γ 50	29 ± 1	2 ± 1.5	93	87	8 ± 0	72	69	22 ± 2	24	24
	5	25 ± 0	3 ± 0.5	88	78	17 ± 0	32	24	30 ± 0	0	0
	1	32 ± 2	10 ± 1	69	44	26 ± 2	19	10	31 ± 1	2	2
1	Medium	26 ± 2	21 ± 0	19		22 ± 1	15		24 ± 2	8	
	IFN-γ 50	23 ± 1	10 ± 1.5	57	47	11 ± 1.5	52	44	21 ± 0	9	1
	5	26 ± 2	17 ± 1	35	20	18 ± 2	31	19	24 ± 1.5	8	0
	1	24 ± 2.5	19 ± 0.5	21	2	19 ± 0.5	21	7	n.d.		

Macrophages were pretreated with medium or 1, 5, or 50 U/ml IFN-γ for 2 d and assayed for ADCC at 5:1 or 1:1 ratio in the presence of 1:2,000, 1:20,000, or 1:200,000 dilution, or no antiserum.

* Viable targets ($\times 10^4$/ml) at 9 h.

‡ Percent ADCC induced by pretreatment.

ADCC-stimulating activity of the Con A–induced lymphokine and of 5 or 50 U/ml IFN-γ. It did not block the activity of IL-2. One concern with adding a neutralizing antibody into a reaction with macrophages is that nonspecific effects due to Fc binding may occur. The ability of IL-2 to boost ADCC in the presence of Con A–induced lymphokine and neutralizing anti-IFN-γ antibody suggests that the antibody specifically abrogates the effect of IFN-γ in the lymphokine. Anti–human IL-2 blocked the activity of human rIL-2. These results suggest that IFN-γ is the major factor in Con A–induced lymphokine which promotes macrophage ADCC, although IL-2 must also be considered since its concentration in lymphokine may be high enough to have some effect.

Effect of Antiserum Concentration and E/T Ratio on Stimulated ADCC. The previous experiments used a single E/T ratio and a relatively high antibody concentration. The enhancement of killing by IFN-γ is evident at 1:2,000 dilution of antiserum, which gives considerable spontaneous ADCC. Enhancement is also seen at 10-fold and marginally at 100-fold lower amounts of antiserum for which there is no spontaneous activity; results typical of three experiments are shown in Table IV. An E/T ratio of 5:1 allows enhanced cytotoxicity of 70 to >90% of targets, but enhancement by IFN-γ is seen even at a ratio of 1:1 (Table IV).

Discussion

Our assay for ADCC, visual counts of surviving targets, is possible due to the extreme sensitivity of the R1 targets to this type of macrophage killing. The results read at 24 h are very similar to the 9-h point, although the targets in the nonmacrophage and no antibody controls frequently double in this time span. This suggests that most of the killing occurs in the first 9 h and that the subsequent growth of the survivors is similar to that of control tumor cells. We defined "cytotoxicity" in terms of fewer targets compared to controls; tumorilysis is only demonstrated when the number of surviving targets is less than the initial number. This was the case with the higher concentrations of the active lymphokines studied.

IFN-γ is well known as a stimulator of macrophage nonspecific tumoricidal activity (2). The results presented here demonstrate that IFN-γ is also the major factor in mitogen-induced lymphokine which stimulates macrophage ADCC,

although IL-2, IFN-α, and IFN-β have this activity as well. The effect is dose dependent and is seen with a range of antibody concentrations. The IL-2–stimulated ADCC appears to be a direct effect of the lymphokine on macrophages since nonadherent peritoneal cells did not manifest this activity. The possibility that IL-2 acted indirectly via the few percent of lymphoid cells in the macrophage fraction was not investigated. Malkovsky et al. (6) reported that IL-2 also stimulates human monocyte nonspecific tumoricidal activity.

IL-4 mediates multiple biological activities that affect various cell lineages. With respect to macrophages, IL-4 has been shown to coregulate their CSF-dependent growth, to increase Ia expression, and to augment their ability to present antigen (reviewed in references 7 and 8). Crawford et al. (8) recently showed that IL-4 stimulates peritoneal macrophages for increased nonspecific cytotoxicity, but we found no effect of this LK in our ADCC system (Table II). Crawford et al. (8) also showed that IL-4 increases the Fc receptor expression (attributed to FcR type II) of bone marrow–derived macrophages. ADCC reactions may differ in effector mechanisms and method of triggering, reviewed by Johnson et al. (9). Therefore, our study of ADCC in one population of peritoneal macrophages, that elicited by proteose peptone, should be expanded to other types of cells and assays.

Other cytokines that interact with macrophages include IL-1, TNF, and CSF. Macrophage-CSF induces nonspecific tumoricidal activity in murine macrophages and enhances that activity induced by crude sources of lymphokine factors (10) and by IFN-γ (Ralph, P., and I. Nakoinz, manuscript in preparation). M-CSF alone does not modulate macrophage ADCC, but it enhances the effect of mitogen-induced lymphokine, and the IFNs and IL-2 (Ralph, P., and I. Nakoinz, manuscript in preparation). M-CSF is not detectable in mouse spleen lymphokine (10). A preliminary investigation of IL-1 (Genzyme, Boston, MA) and human rTNF (Cetus Corp.) showed no effect on ADCC at 1, 5, or 50 U/ml in our assay (unpublished observations).

We showed previously that LPS could stimulate ADCC similarly to lymphokines, but only at high concentrations of 1 μg/ml (1). The activity of mitogen-induced lymphokine and individual factors studied here is not due to LPS because of their low content measured by the Limulus assay, because polymyxin B does not abrogate the effect, and because LPS-hyporesponsive C3H/HeJ mice respond to protein factors (Ralph, P., and I. Nakoinz, manuscript in preparation).

This study used a polyclonal antiserum. All four murine IgG classes are capable of directing macrophage ADCC to tumor targets (3, 9), although the most active mAbs tend to be of the IgG2a isotype (9). Immune lymphokine is reported to increase macrophage expression of Fc receptors for IgG2a but not IgG2b (11). The macrophage capacity for cytolysis must also be considered in the stimulation of ADCC. In this respect, the difference between ADCC and "nonspecific" killing of tumor targets is remarkable. With ADCC, maximum activity increases through day 2 of culture with LK while nonspecific killing is close to zero at this time (1, 10). In addition, the ADCC-sensitive R1 target is resistant to lymphokine-induced, nonspecific killing. Further study is required to determine the contributions of Fc receptors and "cytolytic capacity" of lymphokine-activated macrophages in ADCC.

Summary

Pretreatment of murine peritoneal exudate macrophages with 1–5 U/ml rIFN-γ or rIL-2, or higher concentrations of IFN-α or IFN-β greatly stimulated ADCC to Rl lymphoma targets. The assay was direct counting of viable target cells after 9 and 24 h using an E/T ratio of 5:1. 2d of pretreatment was optimal for enhancing ADCC. rIL-4 was inactive and IL-4-depleted Con A–induced spleen lymphokine retained its ADCC-stimulating activity. Antibody to IFN-γ blocked the ADCC-promoting effect of the lymphokine, suggesting a major role for this factor.

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