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Immune-complex inhibition of macrophage activation is not due to an interaction with the binding or processing of IFN-γ

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SUMMARY

Preincubation of macrophages with immune complexes suppresses the interferon-gamma (IFN-γ) induction of tumouricidal activity and Ia surface expression. The studies reported in this manuscript were designed to test if immune complexes alter the interaction of IFN-γ with macrophages. The binding of IFN-γ to its specific cellular receptor, the uptake or the degradation were not affected by preincubation of macrophages with immune complexes. Preincubation of macrophages with high doses of phorbol esters mimics the inhibitory effect of immune complexes. This suggests that a strong activation of protein kinase *C* suppresses a subsequent activation of macrophages by IFN-γ.

INTRODUCTION

Immune complexes have been shown to inhibit a number of immune responses *in vivo* (Sinclair, 1979; Bolton, 1980; Virgin & Unanue, 1984; Heyman & Wigzell, 1984). The detailed mechanism of these phenomena remains obscure. Macrophoges play a central role in the initiation of antigen-specific, T-dependent immune responses by the process known as antigen presentation (Unanue, 1981). Thus, it is possible that immune complexes inhibit immune responses via a direct effect on macrophage function. Recent *in vitro* studies from several laboratories have provided hints about potential mechanisms. Esparza, Green & Schreiber (1983) have shown that engagement of the macrophage membrane by insoluble immune complexes inhibits the development of IFN-γ-mediated non-specific tumouricidal activity by murine macrophages. Other reports published recently confirmed this observation (Virgin *et al.*, 1985a) and demonstrated that immune complexes inhibit other macrophage activities, such as antigen presentation (Virgin *et al.*, 1985a), Ia expression (Virgin, Wittenberg & Unanue, 1985b; Hanaumi, Gray & Suzuki, 1984) and IL-1 production (Arend, Joslin & Massoni, 1985).

Over the past years, it has been shown that IFN-γ represents the major, if not the only, macrophage-activating factor produced by stimulated T lymphocytes (Schreiber & Celada, 1985). The binding of IFN-γ to its specific receptor initiates macrophage activation (Celada *et al.*, 1984, 1985; Schreiber *et al.*, 1985). Also, the interaction of IFN-γ with its receptor on macrophages induces activation of protein kinase *C* and mobilizes intracellular calcium (Hamilton *et al.*, 1985). Both of these molecular events are essential for the development of macrophage tumouricidal activity (Celada & Schreiber, 1986). Therefore, two alternative possibilities for immune complex inhibition of macrophage activation are: (i) an interaction of immune complexes with the binding or processing of IFN-γ by macrophages and (ii) interaction with the mediators of the signal transduction originated after the triggering of the receptor. In the present manuscript, we report that immune complexes do not inhibit binding or degradation of IFN-γ by macrophages. However, pretreatment with high doses of phorbol esters, which pharmacologically stimulate protein kinase *C*, renders macrophages unresponsive to IFN-γ.

Abbreviations: BMM, bone marrow-derived macrophages; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; E-IgG, opsonized erythrocytes with IgG; E-IgM, opsonized erythrocytes with IgM; HKLM, heat-killed *Listeria monocytogenes*; IFN, interferon; Ka, constant of affinity; PDD, 4-beta-phorbol, 12,13-didecanoate; phorbol, 4-beta-phorbol; PMA, 12-0-tetradecanoyl-phorbol 13-acetate; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Media, supplements, buffers and reagents

All media, supplements and buffers used in these experiments were purchased or prepared as described previously (Celada *et al.*, 1984) and were determined to contain less than 1 pg endotoxin/ml using the *Limulus amebocyte* lysate assay (Sigma Chemical Co., St Louis, MO).

Soluble and insoluble immune complexes of bovine serum albumin (BSA) or human IgG with the corresponding rabbit antibody were prepared as previously described (Esparza *et al.*, 1983). Aggregated IgG was prepared by incubation of purified IgG for 1 hr at 62°. Bovine erythrocytes were opsonized with IgG (E-IgG) or with IgM (E-IgM) by incubation at 37° with IgG

or IgM anti-bovine erythrocytes (Cappel Laboratories, Cochranville, PA) (Esparza *et al.*, 1983). The phorbol esters, including 12-0-tetradecanolyphorbol 13-acetate (PMA), 4-beta-phorbol,12,13-didecanoate (PDD) and 4-beta-phorbol (phorbol), were purchased from Sigma Chemical Co., dissolved in dimethyl sulphoxide (DMSO), and 1 mg/ml stock solution was stored at −20°. Stock solution or the DMSO carrier was diluted in culture medium immediately before use.

Animals

C3HeB/FeJ mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Lymphokine preparations

IFN-γ-containing lymphokine supernatants were prepared by stimulating cultures of the murine T-cell hybridoma 24/G1 with Concanavalin A as described previously (Celada *et al.*, 1984).

Purified recombinant IFN-γ

Escherichia coli-derived murine IFN-γ was a gift from Genentech Inc. (So. San Francisco, CA). A preparation (lot number 2446/48) was used that had been purified to greater than 97% homogeneity and displayed specific anti-viral activity of 8×10^6 IRU/mg. Purified recombinant murine IFN-γ was radioiodinated using ^{125}I-Bolton-Hunter reagent (ICN, Cleveland, OH) as described (Celada *et al.*, 1984). The amount of IFN-γ after the radiolabelling procedure was measured using a specific ELISA test as described previously (Schreiber *et al.*, 1985). Typical preparations were labelled to a specific activity of 7 Ci/g.

Macrophages

Resident and protease peptone-elicited peritoneal macrophages were obtained by lavage with RPMI-1640 and isolated by adherence as described previously (Celada *et al.*, 1984). Bone marrow-derived macrophages (BMM) were produced *in vitro* using L cell-conditioned medium as described elsewhere (Celada *et al.*, 1984).

Tumouricidal activity

IFN-γ-dependent induction of tumourilytic activity in protease-peptone-elicited C3HeB/FeJ peritoneal exudate macrophages toward ^{111}Inoxine chelate-labelled P815 target cells was measured as described elsewhere (Celada *et al.*, 1984). One unit of MAF activity is defined as the amount that induces 50% maximal lysis of the target cells. Viability of the phorbol ester, heat-killed *Listeria monocytogenes* (HKLM) or immune complex-treated macrophage populations was determined by trypan blue dye exclusion and release of lactic dehydrogenase. No reduction in cell viability was noted in the experimental conditions used.

Ia expression

Induction of Ia antigen expression on macrophages was measured by using the WR19M.1 macrophage cell line as previously described (Schreiber *et al.*, 1985). In brief, 5×10^5 cells in 1 ml of IFN-γ-containing medium were incubated for 48 hr at 37°. The cells were detached by gentle scraping and fixed in paraformaldehyde. Macrophages were incubated with anti-I-A^d monoclonal antibody MK-D6. An excess of murine IgG (250 μg/ml) was added simultaneously to block Fc receptors. As indicator antibody, we used an F(ab′)$_2$ fragment of goat anti-mouse IgG conjugated with fluorescein isothiocyanate. Cellular fluorescence was measured by flow cytometry. As a control, anti-I-A^k antibody (10·2-16) was used in parallel.

Receptor-binding assays

Quantification of ^{125}I-IFN-γ binding to macrophages was performed as described elsewhere (Celada *et al.*, 1984). In brief, 5×10^6 macrophages were incubated at 4° for 2 hr with different amounts of ^{125}I-IFN-γ. Cell-associated and free-radiolabelled IFN-γ were separated by centrifugation over pthalate oil. Specific binding was defined as the difference between total binding and the non-specific binding occurring in the presence of a 200-fold excess of unlabelled IFN-γ.

IFN-γ degradation by macrophages

Quantification of the IFN-γ processed by macrophages was carried out as described previously (Celada & Schreiber, 1987). In brief, 5×10^6 macrophages were incubated with 1 ml of medium containing 200 ng ^{125}I-IFN-γ for various periods of time at 37°. At the indicated times, medium containing unbound ^{125}I-IFN-γ and its degradation products was collected from each well. Then, 500 μl of fetal calf serum and 1 ml 20% trichloroacetic acid (TCA) were added at 4° and the samples centrifuged for 20 min at 4° at 2000 ***g***. TCA-soluble and insoluble materials were collected and counted. As a control, an aliquot of ^{125}I-IFN-γ was incubated at 37° in an empty well, and processed as above. The levels of TCA soluble counts in the control were used as background levels for the experimental tubes.

RESULTS

Immune complexes inhibit the IFN-γ induction of tumouricidal activity in macrophages

Based on our previous experiments (Esparza *et al.*, 1983), we tested the amount of IgG-anti-IgG and BSA–anti-BSA immune complexes needed to inhibit the IFN-γ induction of tumouricidal activity. Table 1 demonstrates that after incubation with insoluble (IgG-anti-IgG) immune complexes, macrophages expressed a reduced IFN-γ-dependent tumouricidal response and Ia expression. Inhibition by the insoluble immune complexes was dose-dependent, and viability was unaffected by phagocytosis of the immune complexes. No inhibition of macrophage activation (defined in this study as the cytotoxic activity and Ia surface expression) was observed when cells were preincubated with soluble immune complexes (IgG–anti-IgG), human IgG, or heat-aggregated IgG. The inhibitory capacity of the insoluble immune complexes is not related to the antigen used. Inhibition of tumouricidal activity was also affected by insoluble immune complexes of BSA–anti-BSA.

The differences between soluble and insoluble immune complexes may be related to the different interaction with macrophages. At the dose of immune complexes used in the assay, after 45 min of incubation less than 5% of soluble immune complexes were phagocytosed in contrast with insoluble immune complexes, in which more than 50% were phagocytosed. This may explain the different inhibitory activity observed when IgG- or IgM-opsonized erythrocytes were preincubated with macrophages (Table 1). Murine macrophages do not express Fc receptor for IgM on the cell surface, and therefore they are unable to ingest E-IgM.

Table 1. Inhibition by immune complexes of IFN-γ induction of macrophage activation

Immune complexes	Dose	% specific ^{111}In release	% Ia-positive cells
Control	—	52±6*	92±3
Insoluble	0·05 ng/cell	22±7	49±12
	0·15 ng/cell	4±1	15±8
Soluble	0·05 ng/cell	55±8	90±6
	0·15 ng/cell	53±6	92±4
E-IgG	5 E/cell	28±4	39±10
	10 E/cell	2±2	6±4
E-IgM	5 E/cell	53±7	93±5
	10 E/cell	57±5	94±6

* Each value represents the mean and the SD of three independent experiments. For cytotoxicity, 10^5 protease peptone-elicited peritoneal exudate macrophages were incubated with the indicated amounts of immune complexes (Ig–anti-IgG) for 45 min at 37°. Cultures were then washed, and macrophages were incubated in the presence of IFN-γ (0·5 IRU/ml) for 4 hr at 37°. Cultures were washed and 10^4 ^{111}In-labelled P815 target cells and 10^6 heat-killed *Listeria monocytogenes* (second signal) were added. After 18 hr of culture at 37°, culture supernatants were harvested, counted, and the percentage specific ^{111}In release calculated. For Ia expression, 5×10^5 cells were incubated with the indicated amounts of immune complexes and IFN-γ (5 IRU/ml) for 48 hr at 37°. The cells were then fixed and the percentage of cells expressing Ia determined.

Table 3. Degradation of IFN-γ by macrophages incubated with immune complexes

	ng IFN-γ degraded/5×10^6 cells		
	Time of incubation (hr)		
Immune complexes	2	4	6
Control	0·6±0·1*	4·2±0·3	7·6±0·5
Insoluble (0·15 ng/cell)	0·3±0·1	4·0±0·4	7·8±0·7
Soluble (0·15 ng/cell)	0·5±0·3	4·5±0·2	7·2±0·5
E-IgG (10 E/cell)	0·2±0·1	4·6±0·6	7·9±0·4
E-IgG (10 E/cell)	0·3±0·0	4·1±0·3	7·9±0·8

* Each value represents the mean and the SD of two independent experiments. Elicited peritoneal macrophages, 5×10^6, were incubated in a six-well tissue culture plate in the presence of immune complexes or opsonized erythroyctes. Cultures were then washed, and macrophages were incubated at 37° in the presence of 200 ng of ^{125}I-IFN-γ for the periods shown. Supernatants were harvested and trichloroacetic acid-non-precipitable radioactivity was counted as a measure of degraded IFN-γ.

Table 2. Effect of immune complexes on the number of IFN-γ receptors and Ka

Immune complexes	Receptors/cell	Ka (10^9 M)
Control	9500±500*	0·9±0·1
Insoluble (0·15 ng/cell)	9800±300	1·2±0·1
Soluble (0·15 ng/cell)	10,000±200	1·0±0·1
E-IgG (10 E/cell)	9500±600	1·4±0·3
E-IgM (10 E/cell)	9800±300	0·9±0·3

* Each value represents the mean and the SD of two independent experiments. BMM, 5×10^6, were preincubated with immune complexes as indicated in Table 1. After washing, macrophages were incubated at 4° for 2 hr with various amounts of radiolabelled recombinant IFN-γ. Cell-associated and free ^{125}I-IFN-γ were separated by centrifugation over pthalate oil (Celada *et al.*, 1984). Specific binding was defined as the difference between total binding and the non-specific binding occurring in the presence of a 200-fold excess of unlabelled IFN-γ. Similar results were obtained using soluble or insoluble immune complexes made with BSA–anti-BSA.

Immune complexes do not interfere with the binding or processing of IFN-γ by macrophages

In order to determine whether the endocytosis of immune complexes inhibits the IFN-γ interaction with macrophages, the molecule that induces activation of these cells (Schreiber & Celada, 1985), two types of studies were performed. In the first experiment, macrophages were incubated with immune complexes, cooled at 4°, and the binding of radiolabelled IFN-γ was measured. As shown in Table 2, insoluble or E-IgG, at doses that inhibit macrophage activation, did not affect the number of surface receptors expressed by macrophages or the affinity for the ligand (Ka). No modifications of the IFN-γ binding were found even in the presence of immune complexes when macrophages were not washed after the preincubation period.

In the second experiment, after incubation with immune complexes, macrophages interacted with ^{125}I-IFN-γ at 37°. After various lengths of time, the amount of degraded IFN-γ released by macrophages preincubated with immune complexes was not different from the controls (Table 3). Also, no differences were found if the immune complexes were Ig–anti-Ig or BSA–anti-BSA. The determination of IFN-γ degradation products in culture supernatants detects not only the uptake but also the catabolic rate of the IFN-γ (Celada & Schreiber, 1987). Together, these results indicate that immune complex inhibition of cytotoxic activity is not due to a direct effect on IFN-γ–macrophage interaction.

Table 4. Inhibition of IFN-γ induction of macrophage tumouricidal activity by preincubation with phorbol esters that activate protein kinase *C*

Treatment	Dose	% specific ^{111}In Release	% Ia-positive cells
Medium control	—	46±8*	94±6
TPA	10 ng/ml	32±12	—
	200 ng/ml	5±3	18±10
PDD	10 ng/ml	28±16	—
	200 ng/ml	8±5	21±8
Phorbol	10 ng/ml	42±10	—
	200 ng/ml	47±14	95±3

* Each value represents the mean and the SD of three independent experiments. Phorbol esters at the indicated doses were incubated with 10^5 protease peptone-elicited peritoneal exudate macrophages for 45 min at 37° in a volume of 100 μl. After washing, induction of tumouricidal activity and Ia expression by IFN-γ was determined as described in Table 1.

Preincubation of macrophages with phorbol esters abolishes the tumouricidal activity induced by IFN-γ

The transduction of signals mediated by the triggering of IFN-γ with the specific receptor includes an activation of the protein kinase *C* and an intracellular calcium mobilization (Hamilton *et al.*, 1985; Celada & Schreiber, 1986). The calcium- and phospholipid-dependent protein kinase *C* enzyme is translocated upon activation from the cytosol to the membrane and catalyses the phosphorylation of numerous proteins (Nishizuka, 1986). We considered the possibility that immune complexes may stimulate and translocate the protein kinase *C* to the membrane. If this is the case no protein kinase *C* can be activated after the triggering of the IFN-γ receptor by the ligand.

In an attempt to prove this hypothesis, we preincubated macrophages with pharmacologic agents, such as phorbol esters, which directly activated protein kinase *C*. As shown in Table 4, esters which bind and activate protein kinase *C* (TPA and PDD) (Niedel, Kunn & Vandenbark, 1983), inhibit the ability of IFN-γ to induce tumouricidal activity and Ia expression. This effect was not observed by preincubation with phorbol, which is not a protein kinase *C* activator. Depending on the IFN-γ concentration used, TPA displayed an ID_{50} of 60–550 ng/ml.

Similarly, HKLM used to trigger the induction of cytotoxicity of IFN-γ, when preincubated in larger doses with macrophages (10^8 bacteria/ml), inhibits the subsequent activation by IFN-γ. This 'second signal' is also considered a protein kinase *C* activator (Hamilton *et al.*, 1986). Phorbol esters or HKLM do not interfere with either the binding or the processing of IFN-γ by macrophages (Celada *et al.*, 1985; Celada & Schreiber, 1987).

DISCUSSION

The experiments described herein were designed to study the mechanism of inhibition of IFN-γ-dependent activation of macrophage tumouricidal activity by immune complexes. This observation was initially reported by Esparza *et al.* (1983) and may provide hints about the mechanism of immune complexes inhibition of immune responses (Sinclair, 1979; Bolton, 1980; Virgin & Unanue, 1984; Heyman & Wigzeu, 1984). The hypothesis to be tested was that immune complexes alter the IFN-γ interaction with macrophages. However, the binding of IFN-γ to its specific cellular receptor, the uptake, or catabolism were not affected by preincubation with immune complexes.

The inhibition by immune complexes of IFN-γ development of tumouricidal activity and Ia expression is dose-dependent and correlated with the amount of material bound to the macrophages (Esparza *et al.*, 1983; Virgin *et al.*, 1985b). This may explain the different ability of soluble or insoluble immune complexes to inhibit cytotoxic activity. In fact, soluble immune complexes have been reported to trigger events in macrophages, such as superoxide anion production (Tamoto & Koyama, 1980; Baxter, Leslie & Reeves, 1983) and complement synthesis (McPhaden & Whaley, 1982). Previous studies have shown that IgG that has been polymerized binds to Fc receptors on cells with higher avidity than monomeric IgG (Knutson, Kijilstra & Vanes, 1977). In our system, for the same input of immune complexes, 10-times more insoluble than soluble immune complexes were phagocytosed by macrophages. In the present manuscript, immune complexes were made with human IgG and rabbit antibodies against IgG or BSA. Both human and rabbit immunoglobulins are recognized by the Fc receptors of murine macrophages (Haeffner-Cavaillon, Klein & Dorrington, 1979).

The different capacity to inhibit IFN-γ induction of activation by IgG- and IgM-coated erythrocytes is also related to the distinct interaction with macrophages. These cells exhibit receptors for the Fc portion of immunoglobulins of the IgG1, IgG2a, IgG2b, IgG3 and IgE, but probably not for the IgM or IgA isotypes (Unkeless, Fleit & Melman, 1981). Virgin *et al.* (1985a) have shown that immune complexes prepared with IgG1, Ig2a, IgG2b and IgE but not IgM or IgA inhibit the Ia expression by IFN-γ. Moreover, no inhibition was found if immune complexes were prepared with the $F(ab')_2$ fragment of IgG. Together, these results indicate that immune complexes act via the Fc receptor to inhibit macrophage activation by IFN-γ.

The signal transduction mechanism utilized by the Fc or IFN-γ receptors appears to be similar to that employed in many other systems (Nishizuka, 1986). Ligand binding is followed rapidly by the formation of diacyglycerol and a rapid rise in the level of free intracellular Ca^{2+}. The diacyglycerol activates the Ca^{2+} phospholipid-dependent protein kinase *C*, and this results in a translocation of this enzyme from cytosol to membrane (Hamilton *et al.*, 1985). The fact that both receptors use the same pathway may explain the inhibitory effect of immune complexes on macrophage activation. Following the interaction of immune complexes with macrophages, protein kinase *C* may be activated, and then the triggering of IFN-γ receptor may not have the substrate for this action.

This explanation is supported by several indications. First, immune complexes inhibit IFN-γ-induced changes in the macrophage rather than inhibiting expression of already synthesized mRNA or protein. Once a protein is induced, immune complexes have little modulatory effect. Consistent with this, immune complexes do not inhibit Ia expression or cytotoxicity of activated macrophages (Virgin *et al.*, 1985a). Second, cyclic

AMP that inhibits phospholipase *C* activity, and thereby suppresses diacylglycerol formation and subsequent protein kinase *C* activation (Nishizuka, 1986), inhibits Ia expression (Snyder, Beller & Unanue, 1982) and the induction of cytotoxicity (Celada & Schreiber, 1986) by IFN-γ on murine macrophages. Finally, a strong activation of protein kinase *C* by high doses of phorbol esters abolishes the macrophage capacity to respond to IFN-γ (Keller, 1979; Murray, 1982; this manuscript).

It is noteworthy that as the IFN-γ effect on macrophages can be mimicked by a combination of low doses of protein kinase *C* activators with calcium ionophores (Celada & Schreiber, 1986; Somers *et al.*, 1986), these compounds mimic the activation of resting B cells by anti-IgM (Monroe & Kass, 1985). Preincubation of resting B cells with high doses of phorbol esters inhibits the activation mediated by anti-IgM (Mizuguchi *et al.*, 1986). Inhibition of the anti-IgM stimulation of DNA synthesis in B cells is due to the suppression by phorbol ester of the increases in inositol phospholipid metabolism that normally results from the cross-linkage of membrane IgM. This may reflect a feedback inhibition mechanism by which activated protein kinase *C* limits the magnitude and duration of receptor signaling (Gold & De Franco, 1987).

The results presented in this manuscript thus demonstrate that immune complex inhibition of IFN-γ activation of macrophages is not due to a direct interaction with the binding and/or catabolism of IFN-γ. Preactivation of protein kinase *C* induces an inability of macrophages to respond to IFN-γ. This mechanism may be implicated for the inhibitory effects of immune complexes on macrophage cytotoxicity. However, more work will be needed to confirm this hypothesis.

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