MURINE GAMMA INTERFERON ACTIVATES THE RELEASE
OF A MACROPHAGE-DERIVED Ia-INDUCING FACTOR
THAT TRANSFERS Ia INDUCTIVE CAPACITY

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It is currently thought that accessory cell function by monocyte/macrophage lineage cells is dependent upon their expression of cell surface I region–associated (Ia) glycoprotein determinants (reviewed in 1–4). Extensive evidence exists to support the hypothesis that cell surface Ia antigens may constitute a major cell surface recognition network that regulates the genetic control of the immune response (reviewed in 5–7). Recent studies (8, 9) have demonstrated that these cell surface Ia glycoproteins can be induced on various populations of normal murine macrophages by conditioned supernatants from lectin-stimulated lymphocyte cultures. Ia expression on normal cells was shown in these experiments to be a transient event dependent upon the positive modulation of an undefined lymphokine activity in the crude supernatant preparations. Negative modulation of Ia expression has also been demonstrated by prostaglandins (10).

More recently, work from this laboratory and others (11, 12) demonstrates that conditioned medium from concanavalin A (Con A)[1]-activated mouse or rat spleen cell cultures could stimulate the cell surface expression of Ia determinants on the murine macrophage cell lines WEHI-3 and P388D1. Subsequent experiments by other workers using these same macrophage cell lines as targets for Ia induction suggest that the active Ia-inducing component of the crude Con A supernatants is interferon-gamma (INF-γ) (13, 14), and they clearly show that a cloned source of murine IFN-γ will stimulate Ia antigen expression on the macrophage cell line WEHI-3 (15).

In this report we demonstrate that cloned murine IFN-γ and the active Ia-stimulating modality of conditioned medium from Con A-activated rat spleen cell cultures both stimulate the macrophage tumor cell line P-388D1 to release a second activity which, in turn, is capable of inducing cell surface Ia expression on the monocyte tumor cell line WEHI-3. Experimental evidence is given to demonstrate that this Ia-inducing factor (IaIF) is neither shed cell surface Ia

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antigens nor any known form of murine interferon. The results clearly suggest that immune interferon may regulate Ia induction by the stimulation of the biosynthesis and/or release of IaIF that subsequently modulates both the qualitative and quantitative expression of cell surface Ia antigens.

Materials and Methods

Cell Lines. The murine monocyte/macrophage lineage tumor cell lines P-388D1 (16) and WEHI-3 (17) were maintained in RPMI 1640 supplemented with antibiotics and 10% fetal calf serum. This was the standard culture medium used in all experiments. These mononuclear phagocyte tumor cell lines are of the H-2^d haplotype and have been maintained in our laboratory in continuous culture for several years.

Murine IFN-γ and Rat Con A Supernatant. Spleen cell-derived conditioned medium was obtained by treating rat spleen cell suspension cultures for 48 h with Con A (5 μg/ml), as previously described (18). The Con A-induced supernatant was diluted to 25% concentration in fresh medium before being used in the WEHI-3 induction cultures. Cloned murine IFN-γ was provided by Dr. Patrick Gray, Genentech, Inc., South San Francisco, CA. The cloned IFN-γ was produced by transfecting the monkey cell line COS-7 with the probe specific for the DNA sequence coding for murine IFN-γ. The experimental details of the cloning procedures are described elsewhere (19). Negative control supernatants consisted of conditioned medium from normal COS-7 cultures.

Production of IaIF. IaIF was obtained from 10-ml P-388D1 suspension cultures that were initially seeded at 2.5 × 10^5 cells/ml and treated with either 25% rat Con A supernatant (vol/vol) or 50 U/ml of cloned murine IFN-γ. Induction cultures were incubated at 7% CO_2 for 48 h. Cells were then centrifuged at 200 g, washed twice in sterile culture medium, and reseeded at 2–5 × 10^5 cells/ml in fresh culture medium containing no exogeneous source of murine immune interferon for an additional 24 h. Cells were centrifuged at 200 g and the conditioned medium was collected and stored at 4°C. This was the source of IaIF in all experiments.

Ia Induction Assay. WEHI-3 was used as the target cell to assay the Ia-inducing activity of the following: (*a*) cloned murine IFN-γ, (*b*) the rat Con A supernatant or, (*c*) the P-388D1-derived IaIF. WEHI-3 was seeded at 1 × 10^5/ml in 10-ml suspension cultures containing either 50 U/ml of the cloned murine IFN-γ, 25% (vol/vol) of Con A supernatant, or 25% (vol/vol) of the P-388D1-derived IaIF supernatant. The cultures were incubated for 48 h at 7% CO_2 and the cells were then centrifuged at 200 g, washed twice in calcium- and magnesium-free 0.01 M phosphate-buffered solution containing 0.1% bovine serum albumin and 0.1% sodium azide (PAB), and stained with appropriate anti-Ia^d monoclonal antibodies (MAb) for flow cytometry (FC) analysis.

It is important to note that both macrophage tumor cell lines are capable of releasing IaIF under active stimulation by an interferon source, and both cell lines can effectively be used as targets in the Ia induction assay using IaIF supernatant from either P-388D1 or WEHI-3 (unpublished results). We elected to use P-388D1 as the "producer" cell line and WEHI-3 as the target because the observed levels of background staining with the anti-Ia MAb on uninduced P-388D1 due to Fc receptor binding and constitutive expression of cell surface Ia were significantly higher than on WEHI-3. Additionally, on a comparative basis, P-388D1 cultures generated almost five- to eightfold more IaIF activity (on a per cell basis) than WEHI-3.

MAb/Fluorescent Reagents. The two anti-Ia^d MAb used in the assay were MK-D6 (anti-I-A^d), developed by Dr. P. Marrack and Dr. J. Kappler (20), and an anti-I-E/$C^{d,k}$ (anti-Ia.7) MAb probe, clone 13/18, developed by Dr. G. Hammerling (21). The anti-I-A^k MAb, clone 10-3.6, was developed in the laboratory of Dr. Leonard Herzenberg (22) and was generously provided to us for this study. The MK-D6 and anti-Ia.7 MAb were obtained from the Becton-Dickenson Monoclonal Antibody Center, Inc., Mountain View, CA. All fluorescence staining was carried out using an indirect immunofluorescence procedure. The "second-step" antisera was a fluorescein isothiocyanate (FITC)-conjugated

(fluorescein/protein ratio of 6.24), affinity-purified, goat anti-mouse IgG heteroantiserum (No. 6250; Tago, Inc., Burlingame, CA).

Immunofluorescent Staining. WEHI-3 target cells (5×10^5 in 50 μl) were aliquoted into 10×75-mm borosilicate glass tubes. Four separate samples were aliquoted from each test culture and stained in the following manner: (*a*) The first sample tube received no antibody treatment and was analyzed to determine background autofluorescence. (*b*) The second FC sample tube was first stained with a saturating concentration of the inappropriate anti-I-A^k (γ2a) MAb, followed by staining with the FITC-conjugated goat anti-mouse IgG antiserum. This sample served as the "negative control" since any antibody binding was due to nonspecific Fc receptor binding of the inappropriate anti-I-A^k MAb that has the same γ2a heavy chain isotype as the anti-Ia^d MAb. (*c*) The last two sample tubes in each test group were stained with saturating concentrations of either MK-D6 or anti-Ia.7 MAb, respectively, followed by staining with the FITC-conjugated goat antiserum (as above). Positive fluorescence emission from these samples represented staining of either I-A^d or I-E/C^d determinants. All antibody incubations were carried out at 4°C for 30 min and cells were washed twice in PAB after each antibody incubation.

FC Analysis. FC analysis was performed using either a FACS III instrument (B-D FACS Systems, Mountain View, CA) or the FC systems at the Los Alamos National Laboratory (LANL), Los Alamos, New Mexico. The details of FC analysis using the FACS III (23) and the LANL system (24) have been described previously. It is important to note that the fluorescence staining profiles of a given cell sample are displayed on the FACS III instrument on a linear scale of 256 channels (fluorescence intensity increasing from left to right), whereas the fluorescence emission on the LANL instrument is recorded over a 255 channel, 3 decade log scale (fluorescence intensity increasing from left to right). The raw data from all test samples is gated to eliminate either dead or aggregated cells from analysis as described previously (24). The negative control for each test sample consists of cells stained with the inappropriate anti-I-A^k (γ2a) MAb and the FITC-conjugated second antibody. Data is presented in two ways: (*a*) as the percentage of positive cells in each test sample minus the percentage of positive cells (background staining) in the negative control, and (*b*) as the difference between the mean fluorescence intensity (mean fluorescence channel number) of cells stained with either of the two anti-Ia^d MAb and the FITC-conjugated antibody and cells stained with the anti-I-A^k MAb and the FITC-coupled second antibody. The mean fluorescence channel number (MN) indicates the degree to which a given population of cells are positive for a given Ia antigen.

Interferon Assay. Interferon was assayed by a modification of the plaque reduction assay of McGhee et al. (25). Briefly, mouse L-929 cells were grown in modified Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). Serial twofold dilutions were made of each unknown sample in medium supplemented with 5% FBS. One ml of each dilution was added to confluent monolayers of L cells in 25-mm² wide-mouth tissue culture flasks. Each dilution was inoculated into replicate flasks. The flasks were incubated for 24 h at 37°C at 5% CO$_2$. The sample dilutions were removed and the flasks were rinsed with Hank's balanced salt solution. The cells were challenged with vesicular stomatitis virus (Indiana strain) which was allowed to adsorb for 1 h. Monolayers were overlayed with 5 ml of medium containing 5% FBS and 0.4% agarose and again incubated for 24 h. Monolayers were then fixed in 10% formalin in phosphate-buffered saline (PBS) and stained with 0.25% crystal violet and the number of plaques was determined. The interferon titer was defined as the reciprocal of the dilution of unknown sample that reduced the number of viral plaques by 50% from that observed in untreated cultures.

Protein A Column. Staphylococcal protein A, covalently linked to Sepharose CL-4B (protein A-Sepharose) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The protein A-Sepharose was swollen in 10 mM PBS, pH 8.0, containing 0.1% sodium azide and was packed (5-ml bed volume) in a 10-ml plastic pipette. The elution buffer was 3 M sodium thiocyanate (NaSCN) and the elution procedure was as described for the specific elution of murine γ2 immunoglobulin (26). The selective binding of murine immunoglobulin isotypes to protein A-Sepharose is well-characterized (27) and the pro-

cedure has been used extensively in this laboratory for the purification of isotype-specific murine immunoglobulins.

Affinity Column. Affinity-purified MK-D6 (γ2a) and Leu-1 (γ2a) monoclonal antibodies (Becton-Dickenson Monoclonal Antibody Center, Inc.) were chemically linked to poly-acrylic hydrazide-agarose (PAH) (Miles Scientific, Naperville, IL) by a sodium borohydride procedure as described (28). Two mg of purified antibody was coupled to 1 ml (packed volume) of PAH for each of the two columns. After washing the columns with 0.01 M PBS to remove azide, the 2.5-ml test samples were applied and mixed with the column bed to form a slurry. The column was allowed to settle by gravity at room temperature for 5 min before elution. The sample was eluted at a flow rate of 0.5 ml/min; after the initial sample was below the level of the gel bed, a final 6–7 ml of fresh culture medium was washed over the column and collected in the sample tube.

Anti-IFN-γ–blocking Antibody. The anti-murine IFN-γ–blocking antibody used was a rabbit anti-IFN-γ IgG raised by repeated subcutaneous immunization with a partially purified (sp act > 10^5 μm/mg of protein), phytohemagglutinin-induced murine spleen cell-derived IFN-γ. The anti-IFN-γ heteroantiserum had a neutralizing titer of 5,000 U/ml (defined as the reciprocal of the highest antiserum dilution that neutralized 10 U of murine IFN-γ). The antiserum was purchased from Enzo Biochem, Inc., New York (cat. No. EAB-473, lot No. 2/09/001).

Results

FC Analysis of Ia Induction of WEHI-3 by Cloned IFN-γ, Rat Con A Supernatant, and P-388D1-derived IaIF.   Fig. 1 gives a summary of typical experimental results using indirect immunofluorescence and quantitative FC analysis to examine WEHI-3 cells for the expression of Ia cell surface antigens after incubation in
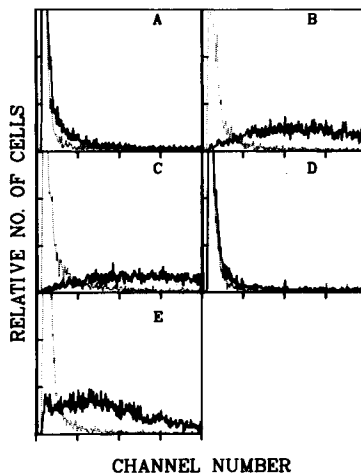

CHANNEL NUMBER

FIGURE 1.   Overlays of gated fluorescence histograms for WEHI-3 cells stained with MK-D6 (anti-I-A^d) MAb followed by FITC-conjugated goat anti-mouse IgG compared with the same test sample stained with an inappropriate anti-I-A^k MAb (10-3.6) and FITC-conjugated goat anti-mouse IgG antiserum. The test groups are made up of 5 × 10^5 WEHI-3 target cells taken from suspension cultures grown in: (*A*) medium only [RPMI 1640 (10% FCS)], (*B*) medium plus 50 U/ml of cloned murine IFN-γ, (*C*) 25% (vol/vol) rat Con A supernatant, (*D*) medium plus 25% (vol/vol) of P-388D1-conditioned medium, (*E*) medium plus 25% (vol/vol) of P-388D1-generated IaIF (after activation by 50 U/ml of cloned murine IFN-γ). Analysis was carried out on the Becton-Dickenson FACS III. The x axis indicates a 256 channel linear scale, increasing fluorescence intensity left to right; y axis indicates the number of cells in each channel. Voltage and gain settings on the fluorescence amplifier were the same for all samples. 10,000 cells were analyzed for each test sample.

either medium alone (Fig. 1*A*), 50 U/ml of cloned IFN-γ (Fig. 1*B*), 25% (vol/vol) rat Con A supernatant (Fig. 1*C*), 25% (vol/vol) P-388D1-conditioned medium (Fig. 1*D*), or 25% (vol/vol) IaIF supernatant derived from P-388D1 cultures after the cells had been stimulated by murine IFN-γ (Fig. 1*E*). As displayed in Fig. 1, WEHI-3 does have some weak background expression of I-A antigen; however, after culture in rat Con A supernatant or the cloned murine immune interferon, there is a profound increase in fluorescence due to the increased expression of cell surface I-A determinants. Fig. 1*D* clearly shows that conditioned medium taken from P-388D1 before stimulating the macrophage cell line with an exogenous source of IFN-γ had no effect in inducing the expression of cell surface Ia antigens on WEHI-3 target cells. By contrast, WEHI-3 target cells cultured in IaIF supernatant taken from P-388D1 cells that had been initially activated by a 48-h preincubation in cloned murine IFN-γ (Fig. 1*E*) were again strongly induced for the expression of I-A antigens. Thus, rat Con A supernatant, cloned murine IFN-γ, and supernatant taken from the macrophage tumor cell line P-388D1 after its activation by cloned immune interferon stimulate quantitatively significant increases in the expression of cell surface Ia antigens on the macrophage tumor cell target WEHI-3. The data in Table I show that there is an increase in the cell surface expression of the I-E/C

TABLE I

FC Analysis of Ia Induction of WEHI-3 by Cloned IFN-γ, Rat Con A Supernatant, and P-388D1-derived IaIF

WEHI-3 test culture	MAb	ΔMN*	Percent positive cells[‡]
Culture medium	MK-D6	11	13
	Ia.7	11	10
Rat Con A supernatant (25%)	MK-D6	110	94
	Ia.7	108	92
IFN-γ (50 U/ml)	MK-D6	106	96
	Ia.7	103	95
Control P-388D1-conditioned medium	MK-D6	5	8
	Ia.7	6	10
IaIF (IFN-γ induced)	MK-D6	80	86
	Ia.7	67	79

* ΔMN values indicate the difference in the mean channel fluorescence (on a linear scale) between a gated population of WEHI-3 target cells stained with an inappropriate anti-I-A^k (γ2a) MAb and the enhancing, FITC-conjugated goat anti-mouse second antibody and a sample stained with either the anti-I-A^d (MK-D6) or the anti-I-E/C^d (Ia.7) MAb and theFITC-labeled goat anti-mouse second antibody. Fluorescence was measured on the 256 channel linear fluorescence scale of the Becton-Dickenson FACS III; 10,000 cells were analyzed per sample.

‡ The percent positive cells for each test culture indicates the difference between the percentage of cells that stain for positive fluorescence with the anti-I-A^k (negative control) MAb and the percentage of the test sample that stained by the MK-D6 or Ia.7 MAb. The negative control sample from the medium-only test culture was gated such that ~10% of the stained cells showed fluorescence emission brighter than the channel number selected as the lower fluorescence window. This channel number was used as the lower limit of positive fluorescence for all test samples.
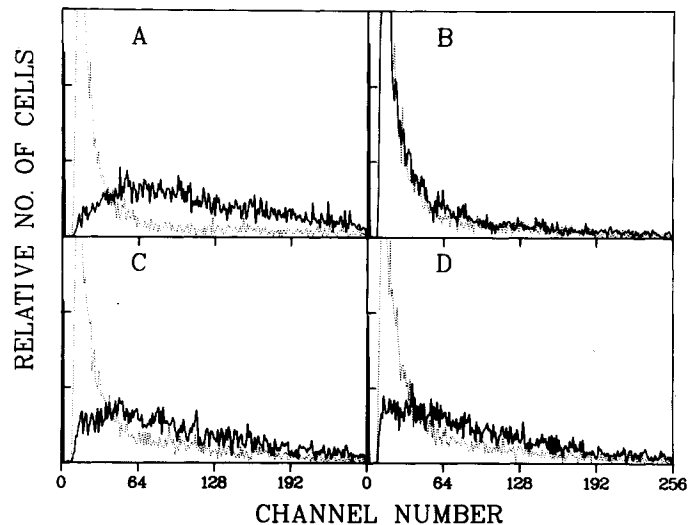
FIGURE 2. WEHI-3 was grown in 25% (vol/vol) rat Con A supernatant alone (A), 25% (vol/vol) rat Con A supernatant preincubated with a 5,000 U equivalence of anti-murine IFN-γ heteroantiserum (B), 25% (vol/vol) P-388D1-derived IaIF (C), 25% (vol/vol) of P-388D1-derived IaIF preincubated with a 5,000 U equivalence of an anti-murine IFN-γ heteroantis-erum (D). All the IaIF supernatants were harvested from P-388D1 cultures initially activated with 50 U/ml of cloned murine IFN-γ. Overlays demonstrate a comparison of staining histograms for MK-D6 (I-A^d) MAb staining to those of WEHI-3 cells stained with an inappropriate anti-I-A^k MAb (10-3.6) and FITC-conjugated goat anti-mouse IgG second antibody. 10,000 cells were analyzed per test sample on the Becton-Dickenson FACS III. Raw histograms were gated to eliminate dead and aggregated cells. X axis indicates a 256 channel linear scale of increasing fluorescence emission from left to right; y axis indicates the number of collected cells in each fluorescence channel.

antigen density comparable to that observed for the I-A determinant after treating WEHI-3 with cloned immune interferon, rat Con A supernatant, or IaIF derived from IFN-γ-activated P-388D1 cells. Thus, there was a quantitatively similar increase in both the amount of anti-Ia staining, as measured by the increase in MN, and the present positive cells for the I-A (MK-D6), and the I-E/C (Ia.7) antigens after the target cells are activated by any of the three Ia induction regimens.

Anti-murine IFN-γ–blocking Experiments. Since IaIF was collected from P-388D1 supernatants after cellular activation by either rat Con A supernatant or cloned murine interferon, it was important to show that the Ia induction observed after treatment of WEHI-3 targets by IaIF was not due simply to carryover of IFN-γ that might have adhered to the P-388D1 cells when they were removed from the initial activation cultures. Additionally, although macrophages are not commonly thought to release IFN-γ, it was important to eliminate the possibility that P-388D1 could be mediating Ia-inducing function via the release of endogenously generated immune interferon. Thus 2.5 ml of rat Con A supernatant was incubated (12 h, 4°C) with a 5,000 U equivalence of an anti-murine IFN-γ–specific heteroantiserum. The antibody-treated rat Con A supernatant was subsequently added at 25% (vol/vol) to a 10-ml culture of WEHI-3 cells. Our analysis of the interferon concentration of the crude rat Con A supernatant (see

below) indicated it had ~25–30 U/ml of activity. In a similar fashion, 2.5 ml of P-388D1-derived IaIF was also preincubated with a 5,000 U equivalence of the same anti-IFN-γ heteroantibody before its addition to the WEHI-3 induction culture (25% vol/vol). Fig. 2 shows the results of a representative experiment. WEHI-3 in 25% rat Con A supernatant showed a positive fluorescence shift for the I-A^d marker, MK-D6 (Fig. 2A). Preincubation of the rat Con A supernatant before use in the induction culture completely blocked the Ia induction response on WEHI-3, as observed in panel Fig. 2B. Identical results were obtained using cloned murine IFN-γ (Table II) and the same anti-murine IFN-γ antiserum. In contrast, the Ia-inducing effects of P-388D1-derived IaIF were essentially unaffected by any attempt to block them with preincubation of the active supernatant with the anti-murine interferon antiserum. Thus, anti-I-A^d fluorescence staining produced a strong positive shift in immunofluorescence for cells cultured with IaIF in the absence of any anti-IFN-γ–blocking regimen (Fig. 2C) and a quantitatively comparable positive shift in anti-I-A^d fluorescent staining for cells cultured in IaIF that was pretreated with the blocking antibody (Fig. 2D). Table II gives the values for the mean channel fluorescence differences (ΔMN) and the differences in the percent of positive cells between the negative control sample and the anti-I-A^d- and anti-I-E/C^d-stained samples from each test culture in the experiment represented by the FC histograms in Fig. 2. The data in Table II shows that the immunofluorescence staining profile for I-E/C^d determinants is the same as that for the anti-I-A^d fluorescent staining shown of Fig. 2. This data clearly suggests that, although the Ia-inducing activity of rat Con A supernatant and purified (cloned) murine IFN-γ can be totally abrogated by an anti-IFN-γ antiserum, the Ia-inducing activity demonstrated by P-388D1-derived IaIF is essentially unaffected by the same blocking regimen.

Antiviral Activity of P-388D1-derived IaIF. Although macrophage are not com-

Table II

FC Analysis of the Effects of Anti-IFN-γ Heteroantibody on the Ia-inductive Capacity of Rat Con A Supernatant, Cloned Murine IFN-γ, and IaIF

WEHI-3 test culture	MAb	ΔMN*	Percent positive cells‡
Medium control	MK-D6	10	<5
	Ia.7	8	<5
Rat Con A supernatant (25% vol/vol)	MK-D6	68	85
	Ia.7	64	81
Rat Con A supernatant (25% vol/vol)	MK-D6	6	10
(treated with anti-IFN-γ Ab)	Ia.7	5	8
IFN-γ (50 U/ml)	MK-D6	80	90
	Ia.7	74	87
IFN-γ (50 U/ml) (treated with anti-	MK-D6	7	12
IFN-γ Ab)	Ia.7	8	10
IaIF	MK-D6	45	59
	Ia.7	46	69
IaIF (treated with anti-IFN-γ Ab)	MK-D6	40	59
	Ia.7	41	57

*‡ See legend to Table I.

monly known to produce immune interferon, they are known to generate and release alpha interferon (reviewed in 29). It was of interest, therefore, to determine if the IaIF activity observed in IFN-γ-activated P-388D1 supernatants was attributable to another known molecular species of interferon. This was done in two ways. Indirectly, we evaluated the ability of purified alpha (α) and beta (β) murine interferon to stimulate Ia expression, using up to 1,000 U/ml of either purified alpha or beta murine interferon (Lee Biomolecular, San Diego, CA) in the standard WEHI-3 induction system. As reported by others (29), we observed no quantitatively significant increase in Ia expression after culturing the target cells with these purified interferon species (data not shown).

Using a more direct method of analysis, we tested IaIF active supernatants from IFN-γ-activated P-388D1 cultures for the presence of any antiviral activity. As described in Materials and Methods, the assay was carried out using a standard plaque reduction assay (25) using murine L929 cells and vesicular stomatitis virus. Table III shows the data collected from two individual experiments. Cloned murine IFN-γ and rat Con A supernatant both showed quantitatively significant levels of antiviral activity, 1,500 U/ml and 20–25 U/ml, respectively. The P-388D1-derived IaIF supernatant was negative for any antiviral activity (<1 U/ml) in both experiments. Since an undiluted supernatant of IaIF showed virtually

TABLE III

Comparative IFN Titers of Active Ia-inductive Supernatants

WEHI-3 test culture	I-A induction (ΔMN)*		Interferon concentration	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
			U/ml[‡]	
Medium alone	8	10	<1	<1
Rat Con A supernatant	130	110	25	22
Cloned murine IFN-γ	136	NT[§]	1,500	NT
IaIF (P-388D1 derived)	110	86	<1	<1
New Castle disease virus[‖]	NT	NT	NT	1,024

* Ia induction activity was determined by using the standard Ia induction assay described in Materials and Methods. Anti-I-A^d (MK-D6) fluorescence was measured on the Los Alamos National Laboratory flow system and ΔMN values represent the differences in mean channel fluorescence on a log scale between a negative control sample of WEHI-3 target cells stained with the anti-I-A^k MAb (10-3.6) and FITC-conjugated second antibody and a test sample of cells stained with the anti-I-A^d (MK-D6) MAb and FITC-conjugated second antibody. Ia induction cultures were stimulated with 25% (vol/vol) rat Con A supernatant, 25% (vol/vol) IaIF, and 50 U/ml IFN-γ, respectively.

[‡] Interferon units are calculated as described in Materials and Methods. Units of activity in this assay only reflect the relative interferon activity of the various undiluted supernatants compared with each other in a single experiment, and do not represent units of activity calculated as a result of comparison with an international reference standard of interferon activity. Interferon data represent the average titer results from replicate flasks.

[‖] New Castle disease virus was used to induce interferon in murine 3T3 cells and this supernatant was the internal control in the second experiment.

[§] Not tested.

no antiviral activity, but a 1:4 dilution of the same supernatant shows potent Ia-inducing activity, the results of the antiviral function experiments clearly suggest that the Ia-inducing activity of P-388D1-generated supernatant is not due to any known species of murine interferon.

Analysis of P-388D1-derived IaIF for Shed Ia Antigen (MK-D6 Affinity Column). Since the IFN-γ- or rat Con A supernatant-activated P-388D1 cells used as the source of IaIF are highly Ia positive before being recultured in fresh medium (12, 13), we carried out experiments to demonstrate that the IaIF activity was not simply attributable to shed Ia determinants that were being transferred in culture to the WEHI-3 target cells. Table IV shows experimental results obtained when we attempted to test the ability of an anti-Ia affinity column to deplete an IaIF-positive supernatant of its Ia-inducing activity. 2 mg of affinity-purified MK-D6 (anti-I-A^d) MAb was coupled to 1 ml (packed volume) of PAH (see Materials and Methods). We determined from previous titration experiments (data not shown) that 2.5 ml of IaIF usually provided optimal induction of up to 10^7 target cells in a standard 10-ml, 48-h induction culture, and that 1.0 μl (1 mg/ml) of purified anti-I-A^d (MK-D6) MAb provided saturation binding for >10^7 WEHI-3 cells grown in such cultures. Thus, 2 mg of MK-D6 antibody represented a 2,000-fold antibody excess if one assumed that all the Ia-inducing capacity of 2.5 ml of IaIF supernatant was due to shed Ia antigen. A 2.5-ml sample of IaIF was mixed in a slurry with the PAH column bed, allowed to settle by gravity, and incubated at room temperature for 5 min before being eluted off the column with 6.5 ml of fresh medium. 1 ml of FBS was added to the 9-ml eluent and the 10-ml sample was filter sterilized and added to WEHI-3 cells seeded at 1 × 10^5/ml. After the standard 48-h incubation, the target cells

TABLE IV

Effect of Anti-I-A Affinity Column Passage on Ia-inductive Capacity of IaIF

Test cultures	Antibody staining*	MN[‡]	Percent positive cells[§]
WEHI-3 + 25% IaIF	Negative control stain	30	—
	MK-D6	98	54
	Ia.7	100	58
WEHI-3 + 25% IaIF after MK-D6 affinity column passage	Negative control stain	21	—
	MK-D6	63	32
	Ia.7	74	52
WEHI-3 + 25% IaIF after Leu-1 affinity column passage	Negative control stain	20	—
	MK-D6	54	21
	Ia.7	78	49

* Samples labeled with the anti-I-A^k MAb (10-3.6) and FITC-conjugated second antibody are the negative controls. Background fluorescence is due to nonspecific binding of the inappropriate anti-I-A^k reagent to WEHI-3 target cells.

[‡] MN values are measured on a 255 channel, three decade log fluorescence scale (Los Alamos National Laboratory flow system) and represent the mean fluorescence due to binding of anti-Ia MAb and FITC-conjugated second antibody (see Materials and Methods for immunofluorescence staining procedure) by a gated population of WEHI-3 target cells selected to exclude dead and aggregated cells.

[§] See footnote [‡] to Table I; percent positive cells equals the percent difference between the negative control sample and the test sample.

were harvested and stained for cell surface Ia expression. A negative control column made up of coupled Leu-1 MAb (γ2a) was used in exactly the same fashion and the eluent from this column passage was similarly tested for Ia-inducing capacity. As Table IV shows, there is a quantitatively significant diminution of the ability of an active IaIF sample to induce I-A^d expression on WEHI-3 after passage over the MK-D6 affinity column. However, there is a comparable loss of I-A^d induction potential of the same IaIF supernatant after its passage over the Leu-1 affinity column. The quantitatively similar loss of activity after passage on the negative control column clearly suggests that the abrogation of I-A^d-inductive capacity observed after passage over either column was due to some form of nonspecific binding of the active component. Additionally, IaIF samples passed over either column showed a quantitatively similar loss of I-E/C^d-inductive capacity, as demonstrated by the comparable loss of MN and the percent of positive cells due to anti-Ia.7 MAb staining in the two test cultures, as compared to the anti-Ia.7 staining profile for WEHI-3 cells cultured with unprocessed IaIF. Again, this clearly suggests that any observed loss of Ia-inductive potential subsequent to column passage of IaIF is directly attributable to nonspecific binding of the active factor to the columns, and is not due to specific binding of the column-complexed anti-Ia antibody to the appropriate molecular species of shed Ia antigen. After the nonspecific loss of activity due to MK-D6 affinity column passage, there is still a quantitatively significant amount of I-A induction activity in the eluent, as shown by a ΔMN of a 42 channel shift (log scale) between the negative control sample and the sample stained with the anti-I-A^d MAb.

Analysis of P-388D1-derived IaIF for Shed Ia Antigen (Protein A-Sepharose Col-

TABLE V

Effects of Incubation with an Anti-I-E/C^d MAb and Protein A-Sepharose Column Passage on the Ia-inductive Capacity of IaIF

WEHI-3 test culture	Antibody staining*	MN[‡]	Percent positive cells[§]
WEHI-3 in medium alone	Negative control stain	38	—
	MK-D6 (>I-A^d)	34	0
	Ia.7 (>I-E/C^{d,k})	33	0
WEHI-3 + 25% IaIF	Negative control stain	34	—
	MK-D6 (>I-A^d)	76	78
	Ia.7 (>I-E/C^{d,k})	69	72
WEHI-3 + 25% preincubated column-passed IaIF	Negative control stain	39	—
	MK-D6 (>I-A^d)	79	60
	Ia.7 (>I-E/C^{d,k})	76	58

* Anti-I-A^k (10-3.6) MAb and FITC-goat anti-mouse IgG antibody labeling is used as the negative control stain and measures only nonspecific binding of the inappropriate anti-I-A^k (γ2a) antibody and the fluoresceinated, enhancing second antibody to the target cells. MK-D6 and Ia.7 MAb fluorescence are compared with the negative control sample and show the increase in fluorescence staining due to increased cell surface Ia expression.

[‡] MN values are measured on a 256 channel linear fluorescence scale of the Becton-Dickenson FACS III and represent the mean fluorescence emission for all the cells within a gated population of WEHI-3 targets from which dead and aggregated cells are excluded. Increases in MN values over the negative control sample indicate an increase in cell surface Ia antigen expression.

[§] See footnote [‡] to Table I.

umn). As a result of the high degree of nonspecific binding of the IaIF activity to the antibody-coupled PAH columns, we carried out a second control experiment to test for the presence of shed Ia antigens in IaIF supernatant. 2.5 ml of active IaIF supernatant from P-388D1 was preincubated with 200 μl (1 mg/ml) of affinity-purified anti-Ia.7 (I-E/C) MAb (12 h, 4°C). By calculations similar to those previously described (see above), 200 μl of affinity-purified anti-Ia.7 MAb represents a 200-fold neutralizing excess of antibody if the Ia-inducing capacity of 2.5 ml of IaIF is simply due to shed Ia molecules. The 2.5 ml of anti-Ia.7 MAb-treated IaIF was poured over a 5-ml protein A-Sepharose 4B column (binding capacity, 100 mg protein) and eluted slowly (0.5 ml/min) with 6.5 ml of fresh medium. 1 ml of fetal calf serum was added to the eluent and it was filter sterilized before being added to a standard WEHI-3 Ia induction culture. The data in Table V show the MN values and the percent of Ia-positive cells for gated populations of WEHI-3 tumor target cells grown in 25% IaIF before and after the supernatant was treated with anti-Ia.7 MAb and passed over the protein A-Sepharose column. The data show that there was only marginal loss of I-A^d determinant induction activity in the column-passed material compared with the untreated sample of IaIF as measured by the percentage of I-A^d-positive cells in the two cultures. I-E/C induction activity was also relatively undiminished in the anti-Ia.7–treated, column-passed sample of IaIF. These results strongly suggest that the Ia-inducing activity of IaIF is not attributable to shed Ia determinants, due to the inability to absorb out Ia-inducing activity using a standard procedure of anti-Ia MAb treatment and protein A-Sepharose column separation used in the isolation of Ia glycoprotein species (27). The column released >95% of the bound, purified anti-Ia.7 MAb used in the initial absorption step of the experiment upon elution with 3 M NaSCN (data not shown).

Discussion

In this study we describe a series of observations that support the hypothesis that immune interferon (IFN-γ) regulates the expression of macrophage cell surface Ia antigens by first stimulating the release of a second macrophage-derived factor activity which, in turn, controls the induction of Ia determinant expression. Thus we show that after in vitro stimulation by either Con A-activated rat spleen cell supernatant or cloned murine IFN-γ, the murine macrophage tumor cell line P-388D1 releases a second factor activity, Ia-inducing factor (IaIF), which will then induce the expression of Ia cell surface determinants on the murine monocytic tumor cell line, WEHI-3. We further demonstrate that the P-388D1-derived IaIF activity cannot be blocked by an anti-murine IFN-γ antiserum, which almost completely blocks the Ia-inducing effect of either cloned immune interferon or Con A-activated rat spleen cell supernatant. Additionally, we give experimental results that clearly suggest that IaIF activity is not simply the effect of shed Ia determinants, since neither the passage over an anti-Ia affinity column nor treatment of the IaIF supernatant with an excess of anti-Ia MAb and subsequent passage over a protein A-Sepharose column specifically removed any of the Ia-inducing capacity of the P-388D1-generated IaIF.

Since many of the murine B lymphoma and macrophage tumor cells used in our earlier studies of antigen presentation by tumor cell lines (30) are known to

shed Ia antigen complexes, the controls used to demonstrate that the IaIF activity was not due to shed Ia determinants were very critical. It is important to point out that the protein A column released >95% of the purified anti-Ia 7 MAb (γ2a) used in the control experiment upon elution with 3 M NaSCN. Thus there was complete binding of the anti-Ia MAb and the lack of significant diminution in Ia-inducing capacity of the anti-Ia.7-treated IaIF after column passage was not the result of any abrogation of the normal immunoglobulin-binding properties of the protein A (27). The loss of Ia-inducing activity of the IaIF sample after passage over the MK-D6 (anti-I-A^d) affinity column was clearly the result of nonspecific binding, since loss of activity was observed for both I-A and I-E/C determinant induction and was observed to occur to a quantitatively similar degree after passage on the negative control (Leu-1) column. In addition to the control experiments demonstrated in this paper, there are early studies by Emerson and Cone (31, 32) that show that shed Ia antigen complexes from normal murine spleen cells in suspension culture optimally attach to either syngeneic or allogeneic murine T cells, B cells, or macrophages within 30 min (at 37°C), and are catabolized by the absorbing cells with a half-life of ~4 h. These observations do not fit the kinetics of Ia induction after IaIF treatment since Ia expression first appears on WEHI-3 target cells after ~8–12 h and is not optimal until 48–72 h in culture (data not shown).

This report also clearly demonstrates that IaIF activity is not due to the release of bound exogeneous IFN-γ by the P388.D1 tumor cell line after pulsing the macrophage cell line in an induction culture containing cloned IFN-γ. Additionally, IaIF activity is not due to P388.D1-generated IFN-γ, since the IaIF functional activity cannot be blocked by an anti-IFN-γ heteroantibody and has no demonstrable antiviral activity. However, these results do not eliminate the theoretical possibility that IaIF activity is due to some degradation product of IFN-γ produced by the "processing" of IFN-γ by P388.D1. This breakdown product could conceivably have lost both antiviral activity and the epitope(s) recognized by the neutralizing heteroantiserum, while retaining Ia-inducing activity. That this is probably not the case is best demonstrated by the fact that we have very recently isolated a clone of P388.D1 that constitutively releases IaIF activity without any requirement for prior IFN-γ stimulation (data not shown). This clone is highly Ia positive and it is our untested hypothesis that the constitutive production of IaIF activity by such macrophage tumor cell lines and possible normal cellular counterparts may regulate the IFN-γ-independent, constitutive expression of Ia antigens by certain macrophage subpopulations. We are presently testing this hypothesis.

Recent experiments by Steeg et al. (8) and King et al. (15) offer convincing data to support the concept that immune interferon regulates the biosynthesis and cell surface expression of Ia antigenic determinants. The experimental data described in our study confirm the observations of these earlier experiments describing the role of IFN-γ in the regulation of Ia expression and extend our knowledge about the actual mechanism whereby IFN-γ controls the positive expression of Ia glycoproteins. Based on the experimental evidence offered in this report, we argue that immune interferon regulates the biosynthesis and cell surface expression of macrophage Ia antigens by stimulating the release (and

possible biosynthesis) of an intermediate Ia-inducing factor(s) (IaIF) by the macrophages, which subsequently modulate the actual positive induction of Ia antigen biosynthesis and expression in the plasma membrane.

There are well-documented examples of experimental proofs of the general concept that the generation of intermediate modulators of cellular activity by IFN-γ may be a ubiquitous mechanism whereby this interferon species functions. Several recent papers (33–35) show that cells treated with IFN-γ and virus-induced interferon can transfer viral resistance to cells of the homologous species and to cells of a heterologous species insensitive to that interferon. The transfer of viral resistance takes place in the complete absence of detectable interferon and is dependent upon cell to cell contact. More recently, a study by Lloyd et al. (36) determined that co-cultivation of murine L1210 lymphoblastoid cells with human interferon-treated WISH cells results in the transfer of quantitatively significant inhibition of proliferation of the murine cell line as measured by [³H]-thymidine incorporation. The transfer of antiproliferative activity was not due to the carryover of interferon by the interferon-treated cells, and the transfer was dependent upon cell to cell contact. Recombinant human IFN-γ and purified human IFN-α and IFN-β all had the same effect. Additional experiments showed that murine IFN-stimulated L cells transferred antiproliferative activity to human lymphoblastoid Daudi cells. It was once again clearly shown in these experiments that there was a phylogenetic barrier for the direct effect of interferon, since neither murine nor human interferon directly induced antiproliferative activity in the heterologous species. Recently, we have completed preliminary experiments that suggest that, like the transfer of post–interferon-stimulated antiviral or antiproliferative activity, IaIF activity can also be transferred across a species barrier. Thus neither purified human IFN-γ nor conditioned medium from U937 induces Ia expression on WEHI-3 target cells, but conditioned medium taken from the human macrophage cell line U937 after its activation by a source of human IFN-γ induces Ia determinant expression on WEHI-3 cells. Present work continues in an effort to confirm these observations.

Unlike the transfer of post–interferon-activated antiviral or antiproliferative activity, the transfer of P-388D1-derived Ia-inductive function is not dependent upon cell to cell contact, but is mediated by a soluble factor released into the culture medium. There are several possible reasons for this observation: (a) The transfer mechanisms for post–IFN-γ-stimulated IaIF activity and antiviral or antiproliferative function may simply be different. (b) Preliminary characterization analysis of IaIF activity suggests it is extremely hydrophobic and that supernatant activity is readily lost by dialysis (>200,000 mol wt), passage over filtering devices, or simply by storage in glass or plastic containers. If the active molecular species responsible for the cell contact-dependent transfer of antiviral or antiproliferative function is similarly hydrophobic, it may prove difficult to demonstrate its presence. (c) Under normal physiological conditions, the transfer of IaIF may be similar to the transfer of antiviral or antiproliferative function, and the phenomenon of P-388D1 may simply be an anomaly produced by this particular transformed macrophage cell line. In support of this notion is the observation that, while WEHI-3 is highly inducible for Ia expression by IFN-γ, it produces very little soluble IaIF activity by comparison with P-388D1. Thus,

IaIF may normally function as an integral component of the cytoplasm or plasma membrane compartments. (d) Alternatively, P-388D1 may represent a unique normal macrophage phenotype in which IaIF is released as a soluble mediator, while other more immature cells of the mononuclear phagocyte lineage may not make the factor or may mediate the effects of IaIF via cell to cell contact. All of these possibilities are the focus of our present research efforts in this area.

We presently have limited data concerning the biochemical nature and the physiochemical traits of IaIF, and we are actively engaged in these characterization studies. Our preliminary results indicate that the active material is trypsin sensitive, has a molecular weight of ~200,000 as measured by gel filtration chromatography using Sephacryl S-300 (Pharmacia Fine Chemicals), and appears very hydrophobic (data not shown). The large molecular weight active material may represent some aggregated form of the IaIF molecule; more detailed analysis is required to fully describe the biochemical/physical properties of IaIF.

In conclusion, it is important to point out that the generation and release of IaIF activity after IFN-γ activation may represent an important mechanism whereby macrophage, and perhaps other cells of the lymphoid system (37) amplify the direct effects of IFN-γ stimulation. Such a transducing molecular vector could potentiate the amplified expansion of the number of Ia-positive cells involved in the modulation of immune responsiveness after an initial triggering signal from immune interferon (IFN-γ).

Summary

In this report we demonstrate that when the murine macrophage tumor cell line P-388D1 is incubated for 48–72 h with either concanavalin A-stimulated rat spleen cell supernatant or cloned murine immune interferon (IFN-γ), the cultured cells release a cell-free factor activity that in turn induces the cell surface expression of Ia antigen on the murine monocyte cell line WEHI-3. This IFN-γ-stimulated, Ia-inducing activity cannot be blocked with an anti-IFN-γ heteroantiserum that does block the induction of Ia expression on WEHI-3 by both cloned murine IFN-γ and rat Con A supernatant. The Ia-inducing factor (IaIF) generated from P-388D1 after stimulation by IFN-γ does not demonstrate any antiviral activity. The P-388D1-derived IaIF is not shed plasma membrane Ia glycoprotein molecules, as demonstrated by the inability of the active component to bind specifically to an anti-I-A^d affinity column or to a protein A column after the active supernatant is first treated with an excess of anti-I-E/C^{d,k} monoclonal antibody.

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