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Immunology

Autocrine secretion of tumor necrosis factor under the influence of interferon-γ amplifies *HLA-DR* gene induction in human monocytes

(macrophages/HLA class II/monokine/lymphokine/synergism)

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ABSTRACT **Recombinant interferon-γ (IFN-γ) induced *HLA-DR* gene expression in both U937 and THP-1 human monocytic cell lines, although the former was only very weakly inducible. Combination of recombinant tumor necrosis factor (TNF) and IFN-γ resulted in a synergistic enhancement of DR mRNA and protein induction in both cell lines. TNF alone increased the constitutive expression of the *DR* gene in THP-1 cells. In the HLA class II-negative U937 cells, TNF used alone was not able to induce *DR* gene expression. Such a negative result was not due to a lack of TNF receptor expression in U937 cells, since TNF clearly induced *HLA* class I and TNF gene expression in this cell line. THP-1, but not U937, cells secreted TNF under the influence of IFN-γ. Neutralization of TNF by a specific antibody decreased IFN-γ-induced DR antigen expression in THP-1 cultures. These observations indicate that TNF is not able to directly induce *DR* gene expression, but rather amplifies ongoing expression of this gene, whether constitutive or induced by IFN-γ. In the two cell lines tested, the level of DR inducibility under the influence of IFN-γ used alone depended on a different inducibility of TNF secretion by IFN-γ. Altogether, our observations indicate that TNF, whether exogenous or endogenously produced under the influence of IFN-γ, amplifies *DR* gene expression in monocytes, a phenomenon that may provide to such antigen-presenting cells a selective sensitivity to the *DR*-inducing effects of IFN-γ.**

It is now well established that interferon-γ (IFN-γ), when used in its recombinant form and in the apparent absence of other natural immunoregulatory mediators, can induce the expression of *HLA* class II genes, as shown in human skin fibroblasts (1), endothelial cells (1), thymic epithelial cells (2), synovial cells (3), peripheral blood monocytes (4, 5), and monocytic cell lines (6). Such inducibility, however, is not seen in other cell types such as B lymphocytes (7) and some colon carcinoma cells (8). The level of HLA class II inducibility by IFN-γ treatment is indeed variable among different cell types, even within a single lineage such as monocytes. For example, we have previously reported that the THP-1 monocytic cell line is highly sensitive to the DR antigen-inducing effect of recombinant IFN-γ (6), whereas others have shown that the U937 cell line is poorly DR inducible (9). The mechanisms underlying this variable sensitivity to the HLA class II-inducing effects of IFN-γ are not understood.

There is evidence that crude lymphokine preparations contain a mediator, different from IFN-γ, which participates in the full induction of class II DR antigens in monocytes by such preparations (10, 11). Among the known immunoregulatory mediators, many candidates for a role of cofactor in IFN-γ-induced HLA class II expression could be envisaged. However, IFN-α and -β have been shown to enhance class I, but not class II, HLA antigens (4, 6). Moreover, IFN-α and -β appear to decrease (rather than amplify) class II antigen expression induced by IFN-γ (12, 13). Interleukin 1 appears to be unable to synergize with IFN-γ for DR induction *in vitro* (14). In contrast, tumor necrosis factor (TNF), a monokine with pluripotent effects (15), synergizes with IFN-γ in antiviral (16), antiproliferative (17), and DR-inducing effects on colocarcinoma cells (18) and pancreas islet cells (14). Looking at the combined effects of IFN-γ and TNF in monocytes is of special interest, since it has been shown that IFN-γ enhances the expression of TNF receptors on TNF-responder cells (19), enhances transcription of the TNF gene in mouse macrophages (20), and that IFN-γ and TNF synergize in their differentiation-inducing effects in human myeloid cell lines (21).

We present evidence here that exogenous IFN-γ and TNF cooperate for induction of *DR* gene expression in human myelomonocytic cell lines at both the mRNA and protein levels. To analyze the signals regulating *HLA* class II gene expression under the influence of each mediator, we have taken advantage of our previous observation that one monocytic cell line (THP-1) is constitutively positive for membrane DR antigen expression, whereas another (U937) is not (6). We now show that recombinant TNF used alone does not behave as a direct *HLA-DR* inducer, but rather amplifies an ongoing *DR* gene expression, whether constitutive or IFN-γ-induced. Finally, we have used a neutralizing antibody to TNF to demonstrate a role for IFN-γ-induced endogenously produced TNF in the amplification of *DR* gene expression induced by IFN-γ.

MATERIALS AND METHODS

Cell Cultures and Reagents. The human myelomonocytic cell lines U937 and THP-1 were grown in tissue culture flasks (Corning) at an initial cell density of 2×10^5 cells per ml in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (Boehringer Mannheim)/2 mM L-glutamine/25 mM Hepes/100 units of penicillin per 100 μg of streptomycin per ml. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma.

Cytokines. The cloning and expression of human recombinant IFN-γ in Chinese hamster ovarian cells has been described (22). The preparation used had a specific activity of 1.8×10^7 antiviral units per mg of protein. Human recombinant TNF, (specific activity, 2.4×10^7 cytotoxic units of protein as determined by cytotoxicity in murine L929 cells)

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Abbreviations: IFN-γ, interferon-γ; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; NRS, nonimmune rabbit serum.

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was cloned and expressed in *Escherichia coli* as described (23).

Antibodies. Murine monoclonal antibody BM50 (IgG3) directed against HLA-DR was kindly provided by D. Charron (Paris). It was used as an $F(ab')_2$ fragment. Fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin was purchased from Nordic (Tilburg, The Netherlands). Crude rabbit anti-human recombinant TNF antiserum was prepared by repeated injection in rabbits of recombinant TNF in complete Freund's adjuvant. It had a titer of 5×10^5 units in a TNF neutralization assay in L929 cells (unpublished data). A nonimmune rabbit serum (NRS) was used as a control.

cDNA Probes. The cDNA clone of HLA-DR chain (24) was a kind gift from D. Piatier (Paris). A cDNA encoding human TNF was phTNF-1 clone, as described (23). The cDNA encoding HLA class I (HLA-B7) was provided by B. Jordan (Marseilles, France).

Analysis of HLA-DR Antigen Expression. Cell cultures were either left untreated or were stimulated with IFN-γ (1–100 units/ml), TNF (100 units/ml), or a combination of the above concentrations of IFN-γ and TNF as indicated in individual experiments. In some experiments, IFN-γ induction was done in the presence of either rabbit anti-human TNF antibody or NRS at a final dilution of 1:150. After 24–48 hr of incubation, the cells were washed three times in cold medium containing 0.1% sodium azide and then incubated on ice for 30 min with the $F(ab')_2$ fragment of monoclonal anti-DR antibody or medium followed by incubation with goat anti-mouse immunoglobulin. The fluorescence of 10^4 cells was analyzed with either an EPICS C (Coulter) or a Cytofluorograf 50-HH (Ortho Diagnostics). Both EPICS C and Cytofluorograf 50-HH instruments use the 488 nm line from an argon ion laser at 200 mV of light power. The exciting light was excluded by a dicroic filter and narrow band pass filter (514–540 nm). The EPICS C and the Cytofluorograf 50-HH used a logarithmic or a linear scale, respectively. Results are presented as mean fluorescence intensity (MF) and/or as percentage of positive cells, representing the difference between cells stained with anti-DR antibody plus goat anti-mouse immunoglobulin and cells stained with goat anti-mouse immunoglobulin alone. It should be noted that the results presented histograms and values for the two cell lines cannot be directly compared, since the gain used for U937 was always set considerably higher than that used for THP-1, to be able to record the very modest induction of DR antigen by IFN-γ in U937 cells. The low-angle light scattering was used for gating, thus eliminating the contribution of debris from histograms.

RNA Blot Analysis of mRNA. Cell cultures were incubated with IFN-γ, TNF, or both for 18 hr and were washed in cold medium. Extraction of cytoplasmic RNA was as described by Perbal (25). Equal amounts of RNA (15 μg) were denatured at 65°C for 10 min in sample buffer and then size-fractionated through 1.2% agarose gels containing 2.2 M formaldehyde and 1 μg of ethidium bromide per ml. RNA was transferred overnight onto Hybond-N nylon membranes (Amersham) using 20× SSC (1× SSC is 0.15 M NaCl/0.75 M sodium citrate). The efficiency of the transfer was verified by UV transillumination. After transfer, membranes were exposed to UV illumination for 5 min and prehybridized at 42°C for 18–24 hr in a solution containing 50% deionized formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% $NaDodSO_4$, 0.75 M NaCl, 1 mM EDTA (pH 8.0), 10 mM phosphate buffer (pH 7.0), 500 μg of denatured salmon sperm DNA per ml, and 10% dextran sulfate. Hybridization was performed at 42°C for 18 hr in a similar solution containing 2×10^6 cpm of ^{32}P-labeled plasmid DNA per lane. The cDNA probes were labeled with a nick-translation kit (N5500; Amersham) according to the manufacturer's instructions. Specific activity was in the order of $2–5 \times 10^8$ cpm per μg of DNA. After washing at increasing stringency (final wash, 0.1× SSC/0.1% $NaDodSO_4$ at 65°C for 5 min), the [^{32}P]DNA bound to the filters was visualized by autoradiography at −70°C with intensifying screens.

TNF Assay. TNF activity was detected in culture supernatants by a cytotoxicity assay using L929 cells treated with 1 μg of actinomycin D per ml (Calbiochem) in 96-well microplates. One unit was the reciprocal of the last dilution of culture supernatant giving 50% cytotoxic effect. Our test was calibrated against our preparation of recombinant human TNF. When needed, cytotoxic activity of supernatants was neutralized by an excess of our rabbit antiserum to human recombinant TNF.

RESULTS

Membrane expression of DR antigen in U937 and THP-1 cells cultured for 24 hr in control medium or medium containing 100 units of IFN-γ per ml is shown in Fig. 1. It should be noted that the gain used for cytofluorographic analysis in U937 was much higher than that used for THP-1 cells to disclose the very modest effect of IFN-γ in U937 cultures. U937 cells were found to be constitutively HLA-DR antigen-negative, whereas THP-1 was clearly constitutively positive, in the representative experiment shown in Fig. 1 as in all experiments done (see Tables 1 and 3). IFN-γ induced a modest increase of DR antigen expression in U937 cells, and a much clearer increase of this antigen in THP-1 cells. This difference in inducibility is clearly seen in histograms (as shown in Fig. 1 and also in Fig. 4) as well as in percentage of DR-positive cells (as seen in Table 3).

Table 1 shows the effect of incubation with IFN-γ only, TNF only, or a combination of the two mediators. In U937 cells, high concentrations (100 units/ml) of IFN-γ induced a modest enhancement of DR expression, whereas a clear enhancement was seen in THP-1 cultures treated with IFN-γ concentrations as low as 1 unit/ml. Addition of TNF only did not induce any detectable HLA-DR expression in U937. In contrast, TNF alone consistently enhanced expression of this antigen in THP-1 cells. Simultaneous addition of the two mediators resulted in more intense DR expression, superior

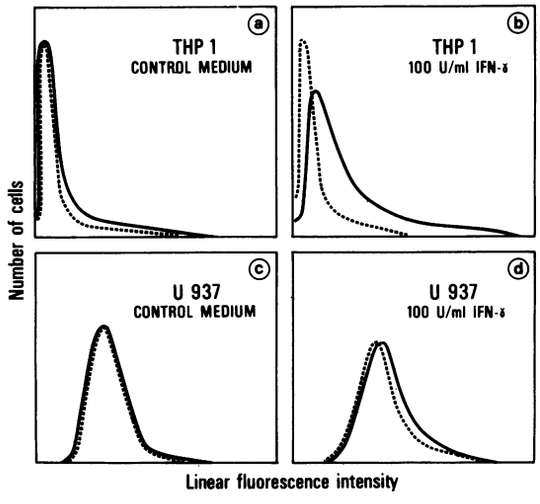

FIG. 1. Fluorographic analysis of DR antigen membrane expression on U937 or THP-1 cells incubated for 24 hr in control medium or IFN-γ (100 units/ml) and stained with the $F(ab')_2$ fragment of the anti-DR monoclonal antibody BM50 and goat anti-mouse immunoglobulin. Fluorescence intensity was calculated on a Cytofluorograf 50-HH and is represented on a linear scale. ----, goat anti-mouse immunoglobulin staining; ——, $F(ab')_2$ anti-DR plus goat anti-mouse immunoglobulin staining.

Table 1. Cytofluorographic analysis of DR antigen expression in U937 and THP-1 cells treated with IFN-γ and/or TNF

Cell line	Culture conditions	MF Exp. 1	MF Exp. 2
U937	Control medium	5	0
	IFN-γ (100 units/ml)	11	10
	TNF (100 units/ml)	4	−1
	IFN-γ (100 units/ml) + TNF (100 units/ml)	38	45
THP-1	Control medium	37	80
	IFN-γ (1 unit/ml)	62	134
	TNF (100 units/ml)	58	139
	IFN-γ (1 unit/ml) + TNF (100 units/ml)	78	169

Results are from two representative experiments, with an incubation time of 24 hr. MF (mean fluorescence) was calculated as described in *Materials and Methods*. Analysis was made on a Cytofluorograf 50-HH.

to that of each mediator used alone, in both cell types. It should be noted that the combined effect was truly synergistic in U937 (since TNF alone had no effect), whereas it was less striking in THP-1 cells, in which each mediator used alone was able to enhance DR expression.

The synergistic effect of the two mediators seen at the membrane antigen level was also observed at the DR mRNA level. As shown in Fig. 2, IFN-γ and TNF each were able to increase DR mRNA expression in THP-1 cells. When cells were treated with both mediators, detection of DR transcripts was much enhanced, especially at suboptimal IFN-γ concentrations such as 1 or 10 units/ml. A striking synergism between the two mediators was also observed in U937 cells, where induction of DR mRNA by each mediator used alone was not detectable.

The effects of TNF on two other genes in U937 cells are shown in Fig. 3. Incubation of such cells with 100 units of TNF per ml induced both TNF gene transcription and an increase in the steady-state level of HLA class I mRNA.

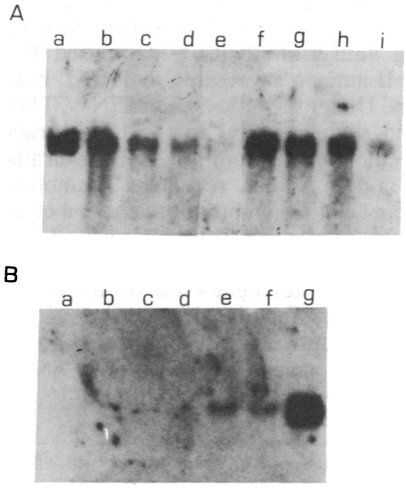

FIG. 2. Detection of DR mRNA by RNA blot analysis of cell lysates from THP-1 (*A*) or U937 (*B*) cultures incubated for 18 hr with different concentrations of IFN-γ, TNF, or both. A positive control from a constitutively DR-positive B-lymphoblastoid cell line (Namalwa) is also shown. (*A*) Lanes: a, Namalwa; b, IFN-γ (100 units/ml); c, IFN-γ (10 units/ml); d, IFN-γ (1 unit/ml); e, control medium; f, IFN-γ (100 units/ml) and TNF (100 units/ml); g, IFN-γ (10 units/ml) and TNF (100 units/ml); h, IFN-γ (1 unit/ml) and TNF (100 units/ml); i, TNF (10 units/ml). (*B*) Lanes: a, control medium; b, IFN-γ (100 units/ml); c, TNF (1000 units/ml); d, TNF (100 units/ml); e, IFN-γ (100 units/ml) and TNF (1000 units/ml); f, IFN-γ (100 units/ml) and TNF (100 units/ml); g, Namalwa.

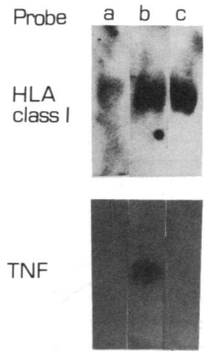

FIG. 3. Detection by RNA blot analysis of TNF or HLA class I mRNAs in U937 cells incubated for 18 hr with control medium or TNF (100 units/ml). Lanes: a, control medium; b, TNF (100 units/ml); c, Namalwa.

Induction by exogenous TNF of TNF mRNA was also observed in THP-1 cells (data not shown).

TNF activity could not be demonstrated by a sensitive bioassay in the supernatants of unstimulated U937 or THP-1 cultures. As shown in Table 2, addition of IFN-γ (100 units/ml) induced detectable TNF activity in supernatants of THP-1, but not U937 cultures, 18 hr or 42 hr after IFN-γ addition. Addition of a known TNF inducer such as PMA resulted in the early (18 hr) appearance of high TNF activity in the culture supernatant of both cell types.

To analyze the postulated role of endogenous production of TNF proteins in induction of class II genes by IFN-γ, we have used an antiserum to human TNF able to neutralize both recombinant and naturally produced TNF. As shown in Fig. 4 and Table 3, addition of this antiserum clearly decreased induction of DR membrane antigens by IFN-γ in THP-1 cells. This was observed at the level of fluorescence intensity and in percentage of DR-positive cells. In contrast to what was observed in THP-1 cell cultures, addition of antibody to TNF did not modify the modest DR antigen induction observed in U937 cells treated with IFN-γ. A role of contaminating endotoxins could be ruled out in all experiments described above, since similar results were obtained with culture medium containing either 10% fetal calf serum or 5% human AB serum (data not shown).

DISCUSSION

Using preparations of purified recombinant IFN-γ and TNF, we have shown that the two mediators synergize in the induction of HLA-DR antigen expression in human monocytic cell lines. This was shown by fluorographic immunofluorescence techniques for membrane antigen expression, and

Table 2. Spontaneous or induced TNF activity in supernatants of U937 and THP-1 cell cultures

Cell line	Culture conditions	TNF activity, units/ml 18 hr	TNF activity, units/ml 42 hr
U937	Control medium	<0.1	<0.1
	IFN-γ	<0.1	<0.1
	PMA	2048	1024 (<0.1)
THP-1	Control medium	<0.1	<0.1
	IFN-γ	4	8 (<0.1)
	PMA	256	256 (<0.1)

The TNF bioassay was performed as described in *Materials and Methods*, and supernatants of 5×10^5 cells were collected 18 or 42 hr after induction. Concentrations used were 100 units/ml for IFN-γ and 10 ng/ml for PMA. Numbers in parentheses indicate TNF activity after neutralization with our antibody to TNF (final dilution, 1:150).

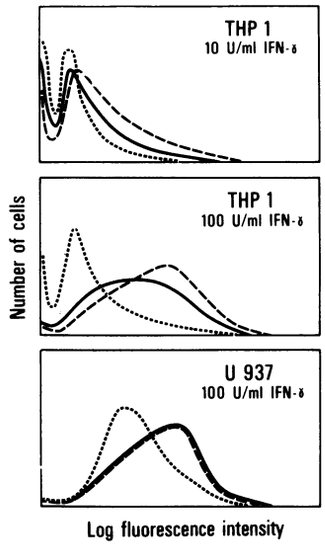

FIG. 4. Fluorographic analysis of DR antigen membrane expression on THP-1 or U937 cells incubated for 48 hr with either control medium or IFN-γ in the presence of NRS or antiserum to TNF. Cells were stained as described in Fig. 1. Fluorescence intensity was calculated on an EPICS C fluorograf (Coulter) and are represented on a logarithmic scale. -----, goat anti-mouse immunoglobulin staining; ---, $F(ab')_2$ anti-DR and goat anti-mouse immunoglobulin staining in cells treated with IFN-γ and NRS; —, $F(ab')_2$ anti-DR and goat anti-mouse immunoglobulin staining in cells treated with IFN-γ and antibody to TNF. Data are from one experiment representative of five consistent experiments.

by RNA blot analysis for detection of the steady-state level of HLA-DR mRNA. Preliminary experiments indicate that IFN-γ and TNF also synergize in induction of HLA-DQ mRNA and antigen in both cell lines (data not shown).

We have analyzed the role of each mediator used separately and taken advantage of the different constitutive expression of HLA-DR antigens in the two cell lines used. Interestingly, TNF used alone enhanced DR membrane antigen expression and mRNA levels in the constitutively positive THP-1 cell line, but not in the constitutively negative U937 line. The inability of TNF to induce class II gene expression in U937 cells was not due to a lack of sensitivity to the biological effects of TNF, since TNF treatment of these cells was able to induce the appearance of TNF and HLA class I mRNA, a finding that implies a normal membrane expression of TNF receptors on U937 cells. This is reminiscent of the observation that human monocytes treated with TNF show an enhanced cytotoxic activity that can be abrogated by antiserum to TNF (26) and confirms that TNF is able to induce its own gene in monocytes. In light of our finding that TNF synergizes with IFN-γ for *HLA-DR* gene induction but has no detectable effects on the gene when used alone in U937, it appears that TNF does not behave as a direct *HLA* class II gene inducer, but rather amplifies an ongoing expression of such genes, either constitutive (as in THP-1 cells) or IFN-γ induced (as in both U937 and THP-1 cells). This concept may help reconcile apparently conflicting results in the recent literature. In DR-negative human fibroblasts, Collins *et al.* (27) have conclusively shown that TNF treatment does not induce HLA class II mRNAs or antigens. In contrast, others have reported that treatment of a murine myelomonocytic cell line (WEHI-3) with human TNF enhances the expression of Ia mRNA and antigens (28). The different level of basal expression of class II antigens in fibroblasts and macrophages is likely to account for such differences in the effect of TNF. How exactly TNF amplifies class II gene expression is not understood. An intriguing possibility would be that TNF acts on one or more of the four control sequences recently described by Boss and Strominger (29) in the regulatory regions situated upstream of *HLA* class II genes.

While these conclusions apply to exogenous TNF, they open up the possibility that endogenous TNF amplifies the HLA class II-inducing effect of IFN-γ used in the apparent absence of other immunoregulatory mediators. This hypothesis may be especially valid when monocytes are used, since such cells are able to produce TNF. We have addressed this question in our system by using a specific antiserum able to neutralize naturally produced and recombinant TNF. Indeed, addition of such antibody clearly decreased IFN-γ induction of DR antigens in THP-1 cells, demonstrating a role for endogenous TNF in increasing the effects of IFN-γ. This prompted us to look for TNF activity in culture supernatants. No detectable TNF activity was found in the supernatants of either cell line cultured in control medium. The potential for TNF secretion was found to be similar in the two cell lines, since PMA stimulation induced an intense cytotoxic activity in supernatants that could be neutralized by our specific antiserum to TNF. A striking difference, however, was found when IFN-γ was used as a TNF inducer, since THP-1, but not U937, produced detectable cytotoxic activity in culture supernatant. This is likely to explain why addition of a neutralizing antiserum to TNF decreased IFN-γ-induced HLA class II antigen expression in THP-1, but not in U937 cell cultures. Under the influence of IFN-γ, THP-1 cells thus secrete (and probably bind to their membrane receptors) TNF molecules. Antiserum to TNF neutralizes secreted molecules and may also recognize membrane-associated TNF. The concept of membrane association of another monokine—namely, interleukin 1—is now well documented (30). Moreover, recent evidence indicates that TNF can indeed be demonstrated on the membrane of mouse macrophages, where it keeps its functional cytotoxic properties, in the absence of measurable secreted TNF (31).

Table 3. Effect of antibody to TNF on IFN-γ-induced DR antigen expression in U937 and THP-1 cells

		Basal DR expression		IFN, units/ml	IFN-induced DR expression			
					+ NRS		+ Anti-TNF	
Cell line	Exp.	MF	% positive		MF	% positive	MF	% positive
U937	1	1	0.7	100	8.8	3.1	8.2	3.3
	2	1	0.8	10	6.9	2.4	7.1	2.5
THP-1	1	25	18.5	100	74	71.5	59	58.3
				10	68	64.0	56	54.1
				1	50	45.6	39	40.1
	3	9	8.8	10	33	25.8	14	10.5
	4	7	6.7	10	20	15.2	11	10.1

U937 and THP-1 cultures were incubated for 48 hr (Exps. 1 and 2) or 24 hr (Exps. 3 and 4) in control medium (basal DR expression) or IFN-γ with addition of either a control serum (NRS) or antibody to TNF. MF (mean fluorescence) and % positive cells were calculated as described in *Materials and Methods* on an EPICS C.

Our observation that endogenous TNF synergizes with IFN-γ for *HLA* class II gene expression implies that cells able to produce and respond to TNF, such as monocyte macrophages, may have a selective advantage in terms of inducibility of such antigens by IFN-γ over other cell types. There is no doubt that IFN-γ itself provides the necessary signals for class II gene induction, since U937 cells that do not secrete detectable TNF are inducible by IFN-γ used alone even in the presence of an antiserum to TNF. However, U937 cells were less readily inducible by IFN-γ than THP-1, and this correlates with the observed ability of THP-1, but not U937 cells, to respond by TNF secretion to IFN-γ treatment. There is thus a clear indication in our system that high inducibility of DR antigens by IFN-γ is associated with the initiation of an autocrine TNF-mediated amplification of class II gene expression in monocytes. This may represent an efficient "double-lock" system permitting the selection, among many cell types with IFN-γ receptors, of specialized antigen-presenting cells with a high sensitivity to the HLA class II-inducing effects of IFN-γ due to their intrinsic ability to produce TNF. This is in keeping with the observations that *in vivo* administration of IFN-γ in experimental animals leads to intense class II antigen induction in a few cell types, including macrophages and dendritic cells, whereas most parenchymal cells are less readily induced (32, 33).

It is also possible to envisage that, in inflammatory lesions, production of TNF by activated macrophages enhances IFN-γ-induced *HLA* class II gene expression in various cell types with receptors for TNF. We have indeed observed that exogenous TNF and IFN-γ synergize for class II induction in epithelial cell cultures (data not shown), and others have reported similar observations in tumor cells (18) and parenchymal cells of the pancreas (14). It is possible that the "hyperexpression" of class II antigens that we have recently analyzed in synovial cells of joints from rheumatoid patients (3) is due to the local release, among other inflammatory monokines, of TNF able to facilitate class II antigen induction by IFN-γ secreted in low amounts by infiltrating T lymphocytes. The collaboration of T lymphocytes (through IFN-γ secretion) and monocytes (through TNF secretion) in *HLA* class II gene induction would thus be yet another example of the potent immunoregulatory functions of the recently discussed "IFN–macrophage alliance" (34).

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