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Regulation of synthesis of complement protein C4 in human fibroblasts: cell- and gene-specific effects of cytokines and lipopolysaccharide

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SUMMARY

Synthesis and secretion of the class III major histocompatibility complex (MHC) gene product, C4, were detected in human skin fibroblasts by metabolic labelling, immunoprecipitation and SDS–PAGE analysis. Pro-C4 (~185,000 MW) was present in intracellular lysates, and the mature protein was present in extracellular media, with three bands of ~93,000, 75,000 and 33,000 MW, corresponding to the α, β and γ chains, respectively. C4 expression was increased in a dose-dependent manner by interferon-γ (IFN-γ), but was unaffected by interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) alone, each of which augmented the expression of factor B, C3 and other complement proteins synthesized in fibroblasts. Simultaneous incubation of fibroblasts with IFN-γ and TNF resulted in a synergistic increase in C4 synthesis. RNA blot analyses indicated that regulation of C4 synthesis by IFN-γ and the combination of IFN-γ and TNF was mediated primarily at a pretranslational level. Lipopolysaccharide (LPS) had no effect on C4 or HLA-DR synthesis in fibroblasts, either constitutive or IFN-γ-regulated. These results are in contrast to the effects of LPS in monocytes, where LPS decreased constitutive synthesis and counter-regulated the IFN-γ-enhanced expression of both C4 and HLA-DR. C2 expression in fibroblasts was also increased primarily by IFN-γ. However, C2 synthesis was increased by LPS, IL-1 and TNF, although to a lesser extent than the increase in synthesis of factor B stimulated by these mediators. These results show that up-regulation by IFN-γ is a common feature of C2 and C4 expression in human cells that constitutively synthesize these proteins. In contrast, regulation of MHC class III and class II genes by LPS, TNF, IL-1, and IL-6 is cell- and gene-specific.

INTRODUCTION

The complement system comprises more than 20 plasma proteins and is an effector of several functions associated with cytotoxicity, immune response, clearance of immunocomplexes and inflammation.[1] While plasma complement proteins are synthesized primarily in liver, a number of other cell types synthesize and secrete complement proteins at extrahepatic sites, suggesting that synthesis in local sites contributes to the inflammatory processes.[2–5] For example, skin fibroblasts synthesize and secrete at least eight separate complement proteins, including proteins in the classical activation pathway (C1q, C1r, C1s, C1 inhibitor, C2) and alternative pathway (factor B, C3, factor H).[6,7]

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C4, C2, and factor B are class III major histocompatibility complex genes (MHC), located on the short arm of chromosome 6.[8] In humans, C4 is encoded by two loci, C4A and C4B, 10 kb apart one from the other, which are located about 30 kb downstream from the C2 and factor B genes.[9] Extracellular C4 protein is composed of three disulphide-linked polypeptide chains: the α-chain (93,000 MW), β-chain (75,000 MW) and γ-chain (33,000 MW). The aminoterminal portion of the α-chain is linked by two disulphide bonds to both the β- and the γ-chain, whereas the carboxyl terminal portion of the α-chain is disulphide-linked only to the γ-chain.[10] Studies of C4 biosynthesis in cell culture have shown that C4 is synthesized as a single chain protein of approximately 185,000 MW[11] and is converted into the functional three-chain protein during post-translational transport–secretion processes.[12,13]

Early reports indicated that three fibroblasts-like cell lines, WI-38, AM and HG, incorporated ^{14}C-amino acids into C4 protein.[14] More recently, Harrison *et al.* studied the synthesis of complement proteins in fibroblasts from sclera.[15] These cells produced C4 in small amounts after prolonged culture (> 72 hr) in the presence of interferon-γ (IFN-γ). Production of C4 was assessed by haemolytic activity; the size and subunit structure of the protein were not examined.

In this study we analysed the effect of cytokines and lipopolysaccharide (LPS) on expression of C4, C2 and HLA-DR[16] in fibroblasts and monocytes to determine the cell-specific effect of the mediators. The data indicate that regulation by IFN-γ is a common feature of HLA-DR, C4 and C2 expression, whereas regulation by LPS, LPS + IFN-γ, tumour necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 are cell- and gene-specific.

MATERIALS AND METHODS

Cells

Human fibroblasts were obtained from the human genetic repository (GM8399; National Institute of General Medical Science, Camdem, NJ) or initiated from skin biopsy of normal volunteers.[17] The cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 10% heat-inactivated (56°, 30 min) fetal bovine serum (FBS; Gibco-BRL) at 37° in humidified air with 5% CO_2. Media, FBS and all reagents used for cell culture were screened by the manufacturers for endotoxin content and contained $< 0{\cdot}006$ ng endotoxin/ml. Cells were periodically screened for mycoplasma contamination and used from passages 2–10 only.

Confluent monolayers of peripheral blood monocytes from normal blood donors were established by adherence of dextran-purified leucocytes on Primaria 24-well plates (Falcon, Lincoln Park, NJ).[18] Cells at a density of 3×10^7/ml were allowed to adhere for 2 hr at 37°. Adherent cells were then rinsed vigorously with warm Hanks' balanced salt solution (HBSS; Gibco-BRL) and incubated in M199 (Gibco-BRL) containing 15% human serum.

To ensure that the effects of mediators were not unique to cells derived from a single donor, the experiments were repeated with fibroblasts and monocytes derived from at least two more individuals.

Biosynthetic labelling

Human fibroblasts were grown to confluency in 24 multi-well tissue culture plates (Corning, Corning, NY) in DMEM containing 10% FBS. After adherence, monocytes were incubated in M199 supplemented with 15% human serum for 24 hr. Before each experiment the cells were washed twice with warm HBSS and incubated for 24 hr in DMEM without serum, containing 0·1% bovine serum albumin (BSA; cell culture-tested; Sigma, St Louis, MO) alone or containing mediator(s). IL-1β (Monsanto Co., St Louis, MO), IL-6, IFN-γ (Amgen Inc. Thousand Oaks, CA) and TNF-α (Genentech Inc., San Francisco, CA) were human recombinant DNA-derived, purified to homogeneity, and free of detectable endotoxin by the Limulus lysate assay. LPS, phenol extract from *Escherichia coli* 0111:B4, was purchased from Sigma.

Following incubation with the mediators, the cells were washed twice with HBSS and incubated for 4 hr in DMEM without methionine (Gibco-BRL) supplemented with 350–500 μCi/ml of ^{35}S-methionine (ICN Radiochemical, Irvine, CA; specific activity 1199 Ci/mmol).[19] At the end of the pulse period, the supernatant was recovered and the cells were washed twice with warm phosphate-buffered saline (PBS) and lysed by one freeze–thaw cycle in the same buffer containing 0·5% sodium deoxycholate (Fisher, Pittsburgh, PA), 1% w/v Triton X-100 (Sigma), 10 mM EDTA (Sigma), 2 mM phenylmethylsulphonyl fluoride (Sigma) and 100 μg/ml leupeptin (Boheringer Dia. Inc., Somerville, NJ). Lysed cells and medium supernatant were clarified by centrifugation and total protein synthesis was measured by trichloroacetic acid (Sigma) precipitation of 5-μl aliquots of cell lysates or medium supernatant .[13] SDS (Bio-Rad, Hercules, CA) was added to the cell lysates to a final concentration of 1%.

Immunoprecipitation and SDS–PAGE

Goat polyclonal antibodies (IgG fraction) to complement proteins factor B, C4, and C2 were purchased from Atlantic Antibodies (INCSTAR, Scarborough, MA). Antibodies to HLA-DR antigen were purchased from Becton Dickinson (Mountain View, CA). Sequential immunoprecipitation for C4, factor B, C2 and HLA-DR was performed as described elsewhere.[13] Briefly, samples were precleared with *Staphylococcus aureus* protein A (Immunoprecipitin; Gibco-BRL) and incubated overnight at 4° with excess antibody. After the incubation, the immunocomplexes were precipitated with an excess of protein A, washed twice with PBS containing 1% SDS, 1% w/v Triton X-100, 0·5% Na deoxycholate and 0·5% BSA (ICN Immunobiologicals, Costa Mesa, CA) and four times with the same buffer without BSA. After washing, the immunoprecipitates were subjected to SDS–PAGE analysis under reducing conditions (5% 2-mercaptoethanol), according to the method of Laemmli.[20] Unlabelled molecular weight standards (Sigma) were analysed in parallel lanes and visualized by Coomassie Brilliant Blue R-250 staining. ^{14}C-methylated molecular weight standards were purchased from Amersham Corporation (Arlington Height, IL). The gels were fixed in a solution of 46% methanol and 8% acetic acid in water, equilibrated in water for 30 min, and treated with Fluoro-Hance (RPI, Mount Prospect, IL) for 30 min, dried and exposed with an intensifier screen at −70° to Kodak XAR-5 film (Eastman, Rochester, NY). To determine the incorporation of ^{35}S-methionine into the individual immunoprecipitated proteins, gels slices corresponding to the autoradiographed bands and gel slices of corresponding blank spaces in the gel for subtracting the background, were cut and digested in 15% hydrogen peroxide (18 hr at 65°). Radioactivity was then measured with Bio-Safe II scintillation fluid (RPI) in an LKB liquid scintillation counter (Model 1215).

Under the conditions of the experiments described in this paper, intracellular levels of complement protein paralleled those in the extracellular compartment (Fig. 1). Therefore, autoradiographs of cell lysate immunoprecipitations only are shown in the other figures.

RNA extraction and Northern blot analysis

For RNA isolation, fibroblasts were incubated for 24 hr in the presence of mediators in 100-mm culture dishes. After the treatment, the cells were rinsed twice with warm HBSS and lysed with guanidium isothiocyanate. Total RNA was isolated by CsCl density gradient ultracentrifugation[21] and quantified by absorbance at 260 nm. RNA samples (20 μg) were denatured and subjected to electrophoresis in 1% agarose gel containing formaldehyde.[22,23] Equal amounts of loading of total RNA were insured by ethidium bromide staining. The gel was rinsed overnight and the RNA transferred to a nitrocellulose sheet (Bio-Rad Lab, Hercules, CA). The membranes were baked,

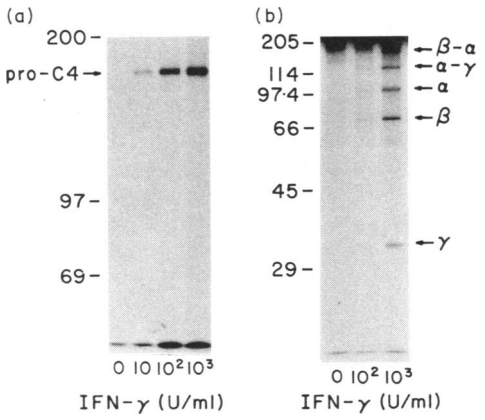

Figure 1. C4 synthesis in human fibroblasts: effect of IFN-γ. Human fibroblasts were incubated for 24 hr with medium alone as control or with increasing concentrations of IFN-γ and then radiolabelled with ^{35}S-methionine for 4 hr in the absence of IFN-γ. (a) Autoradiograph of SDS–PAGE (6%) (see the Materials and Methods) of C4 immunoprecipitates of cell lysates. (b) Autoradiograph of SDS–PAGE (10%) of C4 immunoprecipitates from medium supernatant from the same cells. Molecular weight markers ($\times 10^{-3}$) are indicated in the left margins.

prehybridized and hybridized overnight at 65° in 5 × SSC (1 × SSC = 0·15 M NaCl, 0·015 M sodium citrate, pH 7), 0·2% Ficoll 400, 0·2% BSA, 0·2% polyvinyl pyrrolidone, 0·2% SDS, 0·05% Na pyrophosphate, 0·1 mg/ml boiled, sonicated salmon sperm DNA (Sigma, pH 6·5). After hybridization, the blots were washed twice in 0·2% SDS, 0·2 × SSC for 20 min at 65° and autoradiographed as described above.

The C4 cDNA probe was a 556 bp *Pst*I fragment from the 3′ end of the cDNA pC4A11 clone,[24] corresponding to portions of both C4A and C4B. The probe was radiolabelled[25] with 100 μCi of α-[^{32}P]dCTP (NEN, Boston, MA), using the Random Primed DNA labelling kit (Boehringer-Mannheim, Somerville, NJ) according to the manufacturer's protocol.

RESULTS

Effects of cytokines on the expression of C4 and C2 in human fibroblasts

Human fibroblasts synthesized and secreted C4, as indicated by the presence of pro-C4 in intracellular lysates and mature C4 protein in extracellular media (Fig. 1a and b). Under reducing conditions, intracellular pro-C4 was present as a single chain protein of ~180,000 MW (Fig. 1a);[12] extracellular C4 was detected as three bands of ~93,000, 75,000 and 33,000 MW, corresponding to the α, β and γ chains. Two additional radiolabelled bands of ~168,000 and 125,000 MW, corresponding to the β–α and α–γ intermediates of C4, were also detected (Fig. 1b). All these C4 polypeptides have been characterized previously,[26,27] and have been detected in cell culture media of human hepatoma cells[13] and of mouse fibroblasts transfected with the human C4 gene.[28] Synthesis of C4 was increased in a dose-dependent manner by IFN-γ, with stimulation detected at concentrations as low as 10 U/ml (Fig. 1). Mean values of two separate experiments revealed increases of C4 synthesis of 3·2-, 6·0-, and 8·3-fold for IFN-γ concentrations of 10, 100 and 1000 U/ml, respectively. Constitutive expression of C4 varied somewhat from one experiment to another (Fig. 1 versus Figs 2 and 5), but IFN-γ always increased C4 expression over resting levels. As previously noted,[29] C2 and factor B synthesis was also up-regulated by IFN-γ.

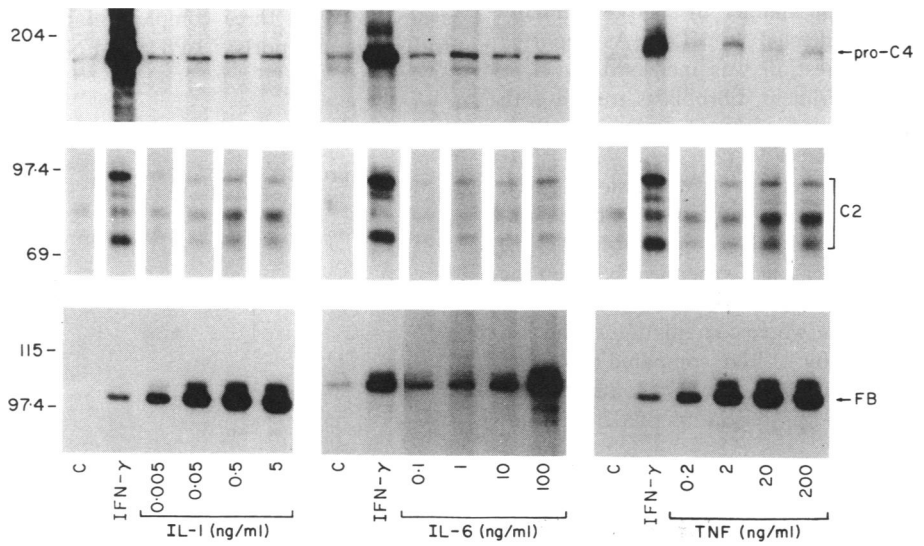

Figure 2. Effects of IL-1β, IL-6 and TNF-α on the synthesis of C4, C2 and factor B in human fibroblasts. Autoradiographs of SDS–PAGE (first two rows 6%, third row 7·5%) of C4, C2 and factor B immunoprecipitates derived from cell lysates of human fibroblasts incubated for 24 hr in the presence of medium alone (C), IFN-γ 1000 U/ml (lane 2), or increasing concentrations of IL-1 (left column), IL-6 (central column), and TNF (right column). In this experiment, lysates from a single set of radiolabelled fibroblasts were sequentially immunoprecipitated first for C4, then factor B, and finally C2. As reported elsewhere,[44] C2 is detected in the cell lysate as three intracellular bands of ~84,000, 79,000 and 70,000 MW. A fourth protein of ~74,000 MW represents a degradation product due to the process of sequential immunoprecipitation. Molecular weight markers ($\times 10^{-3}$) are indicated in the left margin.

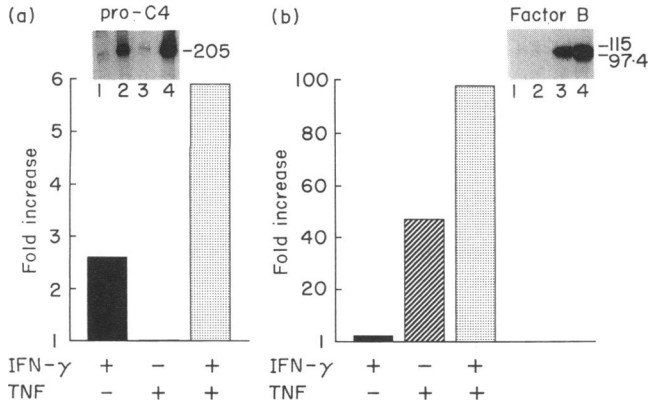

Figure 3. Effects of TNF-α and IFN-γ on the expression of C4 and factor B in human fibroblasts. The cells were incubated 24 hr with medium alone as control, IFN-γ (100 U/ml), TNF (20 ng/ml), or IFN-γ (100 U/ml) plus TNF (20 ng/ml) and radiolabelled with ^{35}S-methionine for 4 hr. Cell lysates were sequentially immunoprecipitated for C4 (a) or factor B (b), and analysed by SDS–PAGE. Radioactive bands corresponding to the specific proteins were cut, digested and counted for radioactivity. Results were calculated after subtraction of background c.p.m. (see the Materials and Methods) and expressed as fold increase (experimental c.p.m. divided by control c.p.m.) for IFN-γ alone (solid bars), TNF alone (hatched bars), and IFN-γ and TNF (stippled bars). Inserts are autoradiographs from SDS–PAGE analyses of C4 (left) and factor B (right), for control (lane 1), IFN-γ (lane 2), TNF (lane 3), IFN-γ and TNF (lane 4).

To study the effects of other cytokines on the synthesis of the class III MHC complement gene products C4, C2 and factor B, human fibroblasts were incubated for 24 hr with IL-1β, IL-6, or TNF-α at varying concentrations (Fig. 2). None of the cytokines at any of the concentrations tested appreciably increased expression of C4. Expression of C2 was increased in a dose-dependent manner by TNF, minimally enhanced by IL-1 and unaffected by IL-6. As reported elsewhere,[29–32] factor B expression was increased by IL-1, TNF and IL-6. In each experiment, fibroblasts treated with IFN-γ as positive control showed increased expression of C2, C4 and, to a lesser extent, factor B.

While TNF alone had no effect on expression of C4, addition of TNF to IFN-γ increased synthesis of C4 by sixfold, appreciably more than the effect of IFN-γ alone (twofold) in the same experiment (Fig. 3), indicating a synergism between the two cytokines. Synergism between TNF and IFN-γ was also observed for factor B synthesis, where combination of the two mediators increased synthesis by 90-fold, compared to 46-fold for TNF alone and twofold for IFN-γ alone. In contrast, there was no effect of TNF on IFN-γ up-regulation of C2 (data not shown).

Steady-state levels of C4 mRNA: effects of IFN-γ and TNF

IFN-γ alone increased C4 mRNA content, and TNF plus IFN-γ in combination induced a synergistic increase in C4 mRNA (Fig. 4). These mRNA results paralleled the changes observed in protein synthesis, indicating that the effects of the IFN-γ and TNF on C4 synthesis are mediated at one or more pretranslational steps, similar to the mechanism accounting for the effect of IFN-γ on other complement proteins.[29,33,34]

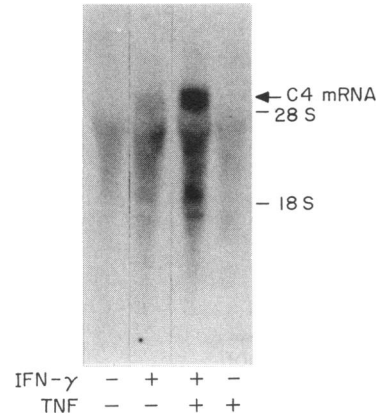

Figure 4. Effects of IFN-γ and TNF-α on C4 mRNA from human fibroblasts. Northern blot of total cellular RNA from human fibroblasts treated with IFN-γ (100 U/ml) or with TNF (20 ng/ml), and IFN-γ (100 U/ml) plus TNF (20 ng/ml). 20 ng of total RNA was electrophoresed in agarose gel and transferred to nitrocellulose filters. Due to the low abundance of C4 mRNA, the autoradiographs were exposed for 14 days.

Interactions between LPS and IFN-γ in the expression of C4 and C2 in human fibroblasts

Previous experiments indicated that, in monocytes, LPS abrogated IFN-γ-induced increases in C4 synthesis. This effect of LPS was specific since LPS enhanced C3 and factor B up-regulation induced by IFN-γ.[33] In fibroblasts, synthesis of C4 was not affected by LPS (Fig. 5), and LPS did not counter-regulate the IFN-γ-enhanced expression of C4 (Fig. 6). In the same experiments, LPS induced a dose-dependent increase in the expression of factor B, with only a minimal effect on C2 synthesis at the highest concentration tested (Fig. 5). (Cells treated with IFN-γ as positive control showed increased synthesis of all three complement proteins.) In accordance with previous reports,[29] LPS and IFN-γ had a synergistic effect

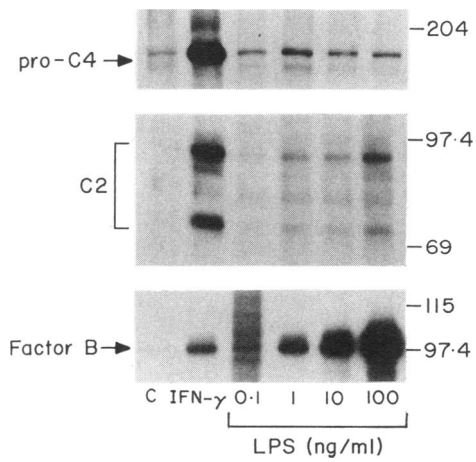

Figure 5 Effects of LPS on C4, C2 and factor B expression in human fibroblasts. SDS–PAGE (6% top, and 7·5% middle and bottom panels) of immunoprecipitates from cell lysates of fibroblasts incubated for 24 hr in medium alone (C), medium containing IFN-γ (1000 U/ml), or increasing concentrations of LPS. Molecular weight standards ($\times 10^{-3}$) are indicated in the right margin.

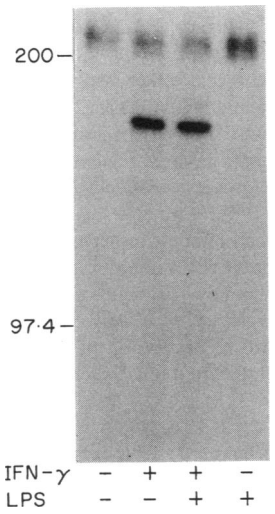

Figure 6. Effects of LPS and IFN-γ on C4 synthesis. Human fibroblasts were incubated for 24 hr in medium alone as control, or in medium supplemented with IFN-γ (1000 U/ml), LPS (100 ng/ml), or with IFN-γ (1000 U/ml) plus LPS (100 ng/ml). Autoradiographs of SDS–PAGE (6%) were prepared for C4 from intracellular lysates as described in the legend for Fig. 1. Molecular weight markers ($\times 10^{-3}$) are indicated in the left margin. As previously described,[29,31] a band of ~220,000 MW, which is present in all immunoprecipitates from fibroblast cell lysates, appeared to bind aspecifically to protein A. The intensity of this band was not affected by preclearing (see the Materials and Methods) and varied according to the culturing conditions of the cells, but did not correlate with change of any of the specific proteins analysed.

on the expression of factor B, but only a modest additive effect in increasing the synthesis of C2 (data not shown).

Expression of class II MHC protein HLA-DR in human fibroblasts and monocytes: effects of LPS and IFN-γ parallel the effects on C4 in the same cells

The two-chain HLA-DR molecule (~34,000 and 29,000 MW, corresponding to the α- and β-chain, respectively) was detected constitutively in monocytes (Fig. 7b), but only upon stimulation with IFN-γ in fibroblasts (Fig. 7a), consistent with previous observations.[35–37] For fibroblasts (Fig. 7a), the IFN-γ-induced expression of HLA-DR was unaffected by LPS, while in monocytes (Fig. 7b) LPS decreased the constitutive expression of HLA-DR and counter-regulated the enhanced expression induced by IFN-γ. Thus, the effects of IFN-γ and LPS on HLA-DR expression paralleled the effects of these mediators on the synthesis of C4 in these cells.

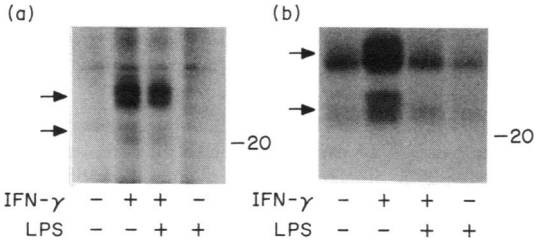

Figure 7. Effects of IFN-γ and LPS on HLA-DR expression in human fibroblasts and monocytes. Autoradiograph of 10% SDS–PAGE of HLA-DR immunoprecipitates from cell lysates of fibroblasts (a) and monocytes (b) incubated as described in Fig. 6. Arrows represent α- (34,000 MW) and β- (29,000 MW) chains of the HLA-DR antigen. Molecular weight standards ($\times 10^{-3}$) are indicated in the right margin.

DISCUSSION

Extrahepatic expression of proteins involved in the two pathways of complement activation provides a mechanism for supply of rate limiting components in the complement cascade and a rapid response to tissue injury or microbial invasion. This provides a trigger for recruitment of cells and soluble mediators of inflammation and constituents of the subsequent specific immune response. Tissue- and cellular-specific regulation of complement components at sites of injury or infection would therefore offer a biological advantage in this local response.

Previous studies[2] have established cellular specificity and developmentally determined regulation of complement gene expression as a function of specific cytokines and other extracellular stimuli. In particular, studies[38–40] using a human hepatoma-derived cell line (HepG2), primary skin fibroblasts, and an intestinal epithelial cell line (CaCo2) suggested that complement proteins produced at hepatic and extrahepatic sites are regulated independently. For example, in human hepatoma cells HepG2, C2 synthesis is not affected by IL-1[38] nor by TNF,[39] while our results show that in fibroblasts C2 expression is increased by TNF and, although to a lesser extent, by IL-1. Moreover recent reports show that in intestinal epithelial cells, CaCo2[40] synthesis of C4 was enhanced by IL-6, but in fibroblasts C4 expression was not affected by this cytokine. None of these differences can be accounted for by the absence of receptor or differences in global signal transduction mechanisms because other genes are responsive to these cytokines in the various cell types.

In fibroblasts, synthesis of C4 is increased synergistically by IFN-γ and TNF by a pretranslational mechanism. Cell-surface receptors for IFN-γ are known to be increased by some pro-inflammatory cytokines.[41,42] However, it is unlikely that synergism between IFN-γ and TNF for synthesis of C4 in fibroblasts is mediated by a change in the number of the IFN-γ receptors, as IFN-γ and TNF have only additive effects on C2 synthesis. Thus, the synergism for synthesis of C4 is distal to the receptor–ligand interaction.

The data in this report also demonstrate cellular specificity of the effect of LPS on MHC class II and III gene expression. That is, in monocytes LPS counter-regulated constitutive and IFN-γ-increased expression of C4 and HLA-DR. In contrast, in fibroblasts endotoxin had no effect on either constitutive nor IFN-γ-enhanced expression of these proteins. Endotoxin was, however, capable of up-regulation of other complement genes in the fibroblast. Counter-regulation by LPS of IFN-γ-regulated class II and III MHC gene expression, and the suppression by granulocyte–macrophage colony-stimulating factor (GM-CSF) of constitutive and LPS-regulated C3 expression[43] in mononuclear phagocytes, may reflect the importance of these cells in antigen presentation and the temporal and/or spatial requirement for expression of these gene products at different stages of inflammation.

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