Production and interferon- γ -mediated regulation of complement component C2 and factors B and D by the astrogloma cell line U105-MG

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In this paper, we demonstrate the synthesis of the complement component C2 and factors B and D by the human astrogloma cell line U105-MG. All three components were structurally and antigenically similar to their serum counterparts, as determined by biosynthetic labelling studies or Western blot analysis. Northern blot analysis demonstrated that the mRNAs of all three components had the same apparent sizes as the equivalent mRNAs from hepatocyte and monocyte cell lines. Interestingly, U105-MG cells produce two C2 transcripts with sizes of ~ 2.8 and 2.3 kb. Interferon- γ (IFN- γ) enhanced the expression of C2 and factor B mRNA and protein in a dose- and time-dependent fashion, while factor D expression was refractory to IFN- γ . IFN- γ appeared to predominantly enhance the expression of the large (2.8 kb) C2 transcript. Kinetic studies demonstrated peak C2 and factor B expression in 48 h in response to IFN- γ , similar to the acute-phase response of factor B in serum. These data are the first to demonstrate the synthesis of C2 and factor D by astrogloma cells. Combined with previous reports documenting the synthesis of C3 by astrocytes, our data suggest that endogenous synthesis of complement proteins, and particularly of alternative pathway activation components (C3, factors B and D), may play an important role in host defence in the central nervous system.

INTRODUCTION

Complement proteins C2 and factor B are essential components of the classical and alternative pathways respectively of complement activation. These proteins provide the catalytic subunits of the C3 and C5 convertases of complement. C2 and factor B are structurally similar and their genes are tandemly located on chromosome 6 within the class III region of the major histocompatibility complex (reviewed in [1,2]). The expression of these proteins is regulated in a tissue-specific fashion, with synthesis restricted to hepatocytes, monocytes and fibroblasts (reviewed in [3,4]). Both molecules are also regulated transcriptionally, with expression being stimulated by lipopolysaccharide (LPS) and cytokines, including interferons, interleukin-1 (IL-1) and IL-4 [5–7]. We have presented evidence that C2 is also regulated at the level of translation [8].

Factor D is a key enzyme in the alternative pathway of complement activation, cleaving factor B to its proteolytically active state and leading to the formation of the C3 convertase, C3bBb [9]. As with C2 and factor B, factor D expression is also regulated in a tissue-specific fashion, with synthesis restricted primarily to adipocytes [10,11], monocytes [12,13] and the sciatic nerve [11]. Transcriptional regulation of factor D is less well understood. Tumour necrosis factor has been shown to down-regulate expression of mouse factor D, also termed adipsin, by adipocytes [14], and retinoic acid treatment of 3T3-F442A adipocytes down-regulates factor D expression by decreasing the stability of the transcript [15].

Recent studies have demonstrated that complement proteins are synthesized by astrocytes or astrogloma cell lines [16,17]. Astrocytes are the major glial cells in the central nervous system (CNS) and function in maintaining a balanced homeostatic environment. There is growing evidence that astrocytes function

as immunocompetent cells in the CNS, acting as antigen-presenting cells and responding to and producing several cytokines (for review, see [18]). Production of complement proteins, and cytokines that regulate complement gene expression, by astrocytes would provide an endogenous source of complement in the CNS. We report here the synthesis of C2 and factors B and D by the astrogloma cell line U105-MG. C2 and factor B synthesis by these cells was upregulated by interferon- γ (IFN- γ). These results suggest that endogenous complement production in the CNS may be important in host defence in the immunologically privileged CNS.

MATERIALS AND METHODS

Cell culture

The astrogloma cell line U105-MG has been described previously [19]. These cells stain positively for glial fibrillary acidic protein, an intracellular protein unique to astrocytes [20]. Cells were maintained in a 50:50 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; CellGro; Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 2 mM-L-glutamine, 1.5 g of D-glucose/litre and a mixture of 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 0.25 μ g of fungizone/ml (Whittaker Bio-products Inc.). Cells in near-confluent monolayers [(1–2) $\times$ 10⁷ cells/75 mm² flask] were passaged by treatment with trypsin/EDTA (1:250.002%; Sigma Chemical Co.) in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS), pH 7.2, followed by neutralization with media containing FBS.

Reagents

Recombinant human IFN- γ (specific activity 1 $\times$ 10⁷ units/ml) was a gift from Dr. Etty Benveniste (UAB, Birmingham, AL,

Abbreviations used: CNS, central nervous system; FBS, fetal bovine serum; RIMA, radioimmunometric assay; SSC, 0.15 M-NaCl, 0.015 M-sodium citrate; SSPE, 0.15 M-NaCl, 10 mM-NaH₂PO₄, 1 mM-EDTA; TBS, Tris-buffered saline, pH 7.2; LPS, lipopolysaccharide; IL, interleukin; IFN- γ , interferon- γ ; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

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U.S.A.). Rabbit polyclonal neutralizing antibody to human IFN- γ (1×10^4 neutralizing units/ml) was purchased from Endogen (Boston, MA, U.S.A.). Recombinant human IL-1 β (specific activity 5×10^8 units/mg) was purchased from Amgen Biologicals (Thousand Oaks, CA, U.S.A.). [35 S]Methionine was purchased from Amersham Corporation (Arlington Heights, IL, U.S.A.; 10 mCi/ml; > 1000 Ci/mmol). Methionine-free DMEM was purchased from Flow Laboratories (McLean, VA, U.S.A.). Proteinase inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.) [leupeptin, aprotinin and phenylmethanesulphonyl fluoride (PMSF)] or Sigma Chemical Co. (pepstatin A). Staph A cells (Immunoprecipitin; Cowan strain, formalin-fixed and heat-killed) were from Gibco/BRL (Bethesda, MD, U.S.A.).

Cell stimulation

For dose-response studies, flasks of near-confluent U105-MG cells [$1-2 \times 10^7$] were rinsed with PBS and then cultured in media containing increasing amounts of IFN- γ (125–1000 units/ml) for 48 h. Control cultures were grown in medium alone. For kinetic studies, paired flasks of cells were incubated without or with IFN- γ (250 units/ml) for 24–120 h. At the appropriate time points, supernatants were removed and assayed immediately or frozen at -70°C until assayed. Cumulative complement protein production was measured at all time points. The specificity of the IFN- γ enhancement of complement protein production was ascertained in experiments in which U105-MG cells were incubated for 48 h with 250 units of normal IFN- γ /ml, or with IFN- γ (250 units/ml) that had been preincubated with a 10-fold excess of anti-(human IFN- γ) neutralizing antibody for 2 h at 37°C . The inclusion of this antibody reproducibly inhibited the effect of IFN- γ on both C2 and factor B expression by 70–100% (results not shown).

Radioimmunoassays (RIMAs)

To quantify the C2, factor B and factor D secreted by U-105-MG cells, solid-phase RIMAs were performed as previously described [21,22]. All three RIMAs utilize rabbit polyclonal antibodies in the solid phase and ^{125}I -labelled mouse mAb as reporters. In the C2 and factor B RIMA, the polyclonal antibodies are specific for C2a and Bb respectively, while the mAbs are specific for C2b and Ba respectively. Therefore these two assays measure only intact C2 or factor B. Cell supernatants were appropriately diluted (usually 1:5, v/v) in Tris-buffered saline (TBS, pH 7.2), containing 0.1% BSA before use in the assays. A standard curve and controls consisting of wells coated with BSA only (1%), or polyclonal antibody only, were included with each assay. All samples were assayed in duplicate. Non-specific binding in all assays was less than 1% and was subtracted from experimental values.

RNA isolation and analysis

Total cellular RNA was isolated by the method of Chirgwin *et al.* [23]. Samples of RNA (20 μg) were denatured in formaldehyde for 15 min at 55°C and then size-fractionated by electrophoresis in 1.0% agarose/formaldehyde gels containing ethidium bromide for visualization of the 28 S and 18 S ribosomal bands. The RNA was then transferred to nylon membrane filters (MSI; Fisher Scientific in $12 \times \text{SSC}$ (3 M-NaCl/0.3 M-sodium citrate) overnight. After transfer, the RNA was cross-linked to the membrane in a u.v. Stratalinker (Stratagene, La Jolla, CA, U.S.A.). The membrane was prehybridized for at least 4 h at 42°C in a solution of 50% formamide, $5 \times \text{SSPE}$, $1 \times \text{Denhardt's}$ solution, 0.2% SDS and 100 μg of denatured salmon sperm DNA/ml. Hybridization was performed in the same solution overnight at 42°C by adding denatured ^{32}P -labelled probe ($\sim 1.0 \times 10^6$ c.p.m./ml). The mem-

brane was washed successively in $4 \times \text{SSC}$ and $2 \times \text{SSC}$ at room temperature, and then with $1 \times \text{SSC}/0.1\%$ SDS and $0.5 \times \text{SSC}/0.1\%$ SDS at 42°C . The blots were autoradiographed for various time periods at -20°C . Blots were stripped between probings in $0.1 \times \text{SSC}/1.0\%$ SDS at 95°C for 15–30 min. Stripped blots were exposed for 4 days to ensure that all probe had been removed, and then reprobbed for cyclophilin. Quantification of autoradiographs was by scanning densitometry with a Bio-Rad model 620 video densitometer. Density values were normalized to the value for cyclophilin hybridization within each experiment.

cDNA probes

To probe for C2 mRNA, a 1 kb *EcoRI* fragment (C2HL5-3) from the 3' end of the human C2 cDNA was used [24]. For factor B, a 2 kb *EcoRI* fragment (B9HL4-1) from the 5' end of the human factor B cDNA, isolated and characterized in our laboratory, was used. For factor D, a 788 bp *BamHI-HindIII* fragment of human adipsin/D cDNA MTN 31/40 clone (a gift from Dr. Tyler White, Metabolic Biosystems, Mountain View, CA, U.S.A.) was used. A cDNA for rat cyclophilin (pIB15) [25] was kindly provided by Dr. Etty Benveniste, and a 700 bp *EcoRI-PstI* fragment was used. All probes were labelled with [α - ^{32}P]dCTP (Amersham) by the random-priming method [26]. The specific radioactivity of labelled probes ranged from 1×10^8 to 1×10^9 cpm/ μg .

Biosynthetic labelling, immunoprecipitation and autoradiography

U105-MG cells were metabolically labelled with [^{35}S]methionine as described below. Cells [$(1-2) \times 10^7$] were first treated for 48 h with 250 units of IFN- γ /ml, then washed extensively and incubated in Met-free DMEM for 10 min, followed by incubation in Met-free DMEM containing [^{35}S]methionine (8 ml, 250 $\mu\text{Ci}/\text{ml}$, 1058 Ci/mmol) for 30 min at 37°C in 5% $\text{CO}_2/95\%$ humidified air. Cells were then washed with Dulbecco's PBS and either lysed in 4 ml of buffer (containing 0.5% sodium deoxycholate, 1% SDS, 1% Triton X-100, 10 mM-EDTA, 1 μg of each of leupeptin, pepstatin A and aprotinin/ml and 2 mM-PMSF in Dulbecco's PBS, pH 7.2, or incubated for 2 h in complete media without radiolabel (chase) before collecting the supernatant and lysing the cells. Lysates and supernatants were cleared of cellular debris by centrifugation for 15 min in a Microfuge, and aliquots (1 ml) of the samples were stored at -90°C . Trichloroacetic acid precipitation of the samples demonstrated approx. 10% incorporation of radiolabel in the supernatant and 80–95% incorporation in the lysates.

For immunoprecipitation, samples (1 ml) were diluted with 1 ml of 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 20 mM-EDTA and 4 mM-PMSF in Dulbecco's PBS, pH 7.2, for culture medium, or with 10 mM-EDTA and 2 mM-PMSF in Dulbecco's PBS, pH 7.2, for lysates, and then precleared by incubation with Staph A cells alone and then with Staph A cells pretreated with normal rabbit IgG (200 μl of a 10% suspension of cells; 1 h rotation at 4°C). Affinity-purified rabbit anti-(human factor Bb) or rabbit anti-(human C2a) antiserum (1 ml) was incubated with Staph A (500 μl of 10% suspension) cells for 1 h at room temperature on a shaker. These cells were washed in lysis buffer without proteinase inhibitors and resuspended in 500 μl of TBS, pH 8.0, containing 0.1% Triton X-100 and 0.025% NaN_3 , and stored at -20°C until used. C2 or factor B was immunoprecipitated from precleared samples (2 ml total volume) by incubation with Staph A cells pretreated with rabbit anti-(human C2a) or anti-(human Bb) antibody (50 μl of cells; 18 h rotation at 4°C). The Staph A cells were pelleted, washed twice with 1 ml of 10 mM-TBS, pH 8.0, containing 0.1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate and 0.025% NaN_3 , once

with 10 mM-TBS, pH 8.0, containing 0.025% NaN_3 , and once with 50 mM-TBS, pH 6.8. The cells were then resuspended in 40 μl of SDS sample buffer, boiled for 3 min, pelleted and the supernatants loaded on 5–20% gradient polyacrylamide gels in the presence of SDS. Purified factor B (4 μg) was run along with high- and low-molecular-mass standards. After electrophoresis, the gels were stained, destained, dried and exposed to Kodak AR X-Omat film at -90°C .

Western blot analysis

Aliquots of culture media from U105-MG cells, controls consisting of freshly prepared culture media, and factor D-containing urine from a patient with Fanconi's syndrome [27] were fractionated by electrophoresis on a 5–20% gel by SDS/PAGE under reducing conditions and transferred to a nitrocellulose filter by electroblotting [28]. The filter was preincubated with 5% non-fat dried milk in TBS, pH 7.2, to block reactive sites, then incubated with polyclonal antisera to factor D. Protein bands were developed using alkaline-phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad).

Statistical analysis

All experiments were performed two to four times, and the data are expressed as the means $\pm$ S.E.M. *P* values were calculated by the paired Student's *t*-test using the StatView 512+ software program.

RESULTS

C2 and factor B mRNA and protein expression in response to IFN- γ

We have reported previously that a number of astroglioma cell lines produce variable amounts of C2 and factors B and D [17]. For the present experiments we selected U105-MG cells because they produce substantial amounts of all three proteins. Initial experiments indicated that stimulation with IFN- γ increased production of C2 and factor B by these cells. In parallel experiments, IL-1 β , over a concentration range of 0.001 to 1.0 ng/ml, had no effect on the production of C2, factor B or factor D (results not shown).

To determine the optimal concentration of IFN- γ for C2 production, we performed dose-response studies. U105-MG cells $[(1-2) \times 10^7]$ were incubated without or with increasing amounts of human recombinant IFN- γ (125–1000 units/ml) for 48 h. Both C2 protein, quantified by RIMA, and mRNA levels, assessed by Northern blot analysis, were determined. Northern blot analysis (Fig. 1a) demonstrated that peak levels of C2 mRNA were observed between 250 and 500 units of IFN- γ /ml. The blot was stripped and re-probed for cyclophilin mRNA, which is not modulated by IFN- γ treatment [29]. The lower portion of Fig. 1(a) shows that essentially equivalent amounts of cyclophilin mRNA were present in each lane. Densitometric scanning of both autoradiograms, to quantitatively compare relative amounts of mRNA in each lane, indicated that C2 mRNA levels were increased between 4- and 6-fold in response to IFN- γ concentrations between 250 and 1000 units/ml. Similar increases in C2 protein production were quantified by RIMA (Fig. 1b). There were no statistically significant differences among the amounts of C2 produced in response to 250, 500 or 1000 units of IFN- γ /ml.

Interestingly, the Northern blot analysis (Fig. 1a) demonstrated two C2 mRNA bands. The larger band was ~ 2.8 kb in size, while the smaller band was ~ 2.3 kb. The relative intensities of the two bands were nearly identical in unstimulated U105-MG cells. However, treatment of the cells

Fig. 1. C2 mRNA and protein expression by U105-MG cells: IFN- γ dose-response

Cells $[(1-2) \times 10^7]$ were untreated or treated with increasing amounts of human recombinant IFN- γ as shown (125–1000 units/ml) for 48 h. Total RNA was prepared and 20 μg portions were analysed by Northern blot analysis using the C2 probe (a). The positions of ribosomal RNA size standards are shown to the left. At the bottom of (a) is the same blot stripped and re-probed for cyclophilin. The blots were exposed at -20°C for 3 days (C2) and 1 day (cyclophilin), with two intensifying screens. Supernatants were collected at 48 h and assayed for C2 by RIMA (b); shown are means $\pm$ S.E.M. from four experiments.

Fig. 2. Factor B mRNA and protein expression by U105-MG cells: IFN- γ dose-response

Cells $[(1-2) \times 10^7]$ were untreated or treated with increasing amounts of human recombinant IFN- γ as shown (125–1000 units/ml) for 48 h. Total RNA was prepared and 20 μg portions were analysed by Northern blot analysis using the factor B probe (a). The position of rRNA size standards are shown to the left. At the bottom of (a) is the same blot stripped and re-probed for cyclophilin. The blots were exposed at -20°C for 8 days (factor B) and 1 day (cyclophilin), with two intensifying screens. Supernatants were collected at 48 h and assayed for factor B by RIMA (b); shown are means $\pm$ S.E.M. from three experiments.

with IFN- γ increased the intensity of the larger band by approx. 5-fold over the smaller band, while the intensity of the smaller band increased only slightly.

Similar studies were performed to examine IFN- γ -induced modulation of factor B expression by U105-MG cells. Fig. 2(a) demonstrates that peak levels of factor B mRNA were observed at between 500 and 1000 units of IFN- γ /ml. The blot was

Fig. 3. Kinetics of C2 mRNA and protein levels in U105-MG cells in response to IFN- γ

Cells $[(1-2) \times 10^7]$ were incubated with (+) or without (-) 250 units of human recombinant IFN- γ /ml for 24 h periods up to 120 h. Total RNA was prepared from the cells for Northern blot analysis of C2 mRNA levels using 20 μ g of total RNA (a). The blots were exposed at -20°C for 6 days (C2) or 1 day (cyclophilin; bottom), with two intensifying screens. Supernatants from the same cells were assessed for C2 protein by RIMA (b); shown are the means $\pm$ S.E.M. from two experiments.

stripped and re-probed for cyclophilin mRNA; the resulting autoradiograph is shown in the lower portion of Fig. 2(a). Densitometric scanning of both autoradiograms to normalize the relative amounts of mRNA between lanes demonstrated that levels of factor B mRNA increased by 4-5-fold in response to IFN- γ concentrations between 250 and 1000 units/ml. In contrast, a maximum 3-fold increase in secreted factor B was measured by RIMA (Fig. 2b). There were essentially no differences in the amounts of factor B secreted when IFN- γ concentrations between 250 and 1000 units/ml were used. Factor B and C2 mRNA levels were 2.5-fold and 16-fold lower respectively in U105-MG cells than in HepG2 cells (a human hepatoma cell line; results not shown).

Kinetics of up-regulation of C2 and factor B expression by U105-MG cells in response to IFN- γ treatment

Kinetic experiments were performed by culturing nearly confluent U105-MG cells (1×10^7 cells) in the presence of

Fig. 4. Kinetics of factor B mRNA and protein levels in U105-MG cells in response to IFN- γ

Cells $[(1-2) \times 10^7]$ were incubated with (+) or without (-) 250 units of human recombinant IFN- γ /ml for 24 h periods up to 120 h. Total RNA was prepared from the cells for Northern blot analysis of factor B mRNA levels using 20 μ g of total RNA (a). The blots were exposed at -20°C for 4 days (factor B) or 1 day (cyclophilin; bottom), with two intensifying screens. Supernatants from the same cells were assessed for factor B protein by RIMA (b); shown are means $\pm$ S.E.M. from three experiments.

250 units of IFN- γ /ml for 24-120 h. This amount of IFN- γ was chosen as optimal as the dose-response experiments (Figs. 1 and 2) suggested that greater amounts of IFN- γ would not significantly enhance the response. Control cells were grown for the same time periods in the absence of IFN- γ . The cumulative production of C2 in response to IFN- γ is shown in Fig. 3. The Northern blot (Fig. 3a) demonstrates an increase in C2 mRNA levels, compared with untreated control cultures, throughout the 120 h time period. Densitometric scanning and normalization for cyclophilin mRNA indicated that IFN- γ -induced C2 mRNA levels were about 7-fold higher than those in the untreated controls at 48 h; this decreased to about 3-fold higher than controls throughout the remaining time periods. Essentially no change in C2 mRNA levels was seen in untreated cultures at any time point. C2 protein production increased continuously during the first 72 h. At that time point, IFN- γ -stimulated cells had produced 5 times more C2 than control cells. The ratio of C2 protein production in IFN- γ -treated versus untreated cells then decreased slightly through the remaining culture period.

The cumulative production of factor B mRNA and protein in response to IFN- γ is shown in Fig. 4. Factor B mRNA in stimulated cells, assessed by densitometric scanning, reached a 4-fold higher level than in controls at the 48 h time point, and then

Fig. 5. Factor D mRNA and protein levels in U105-MG cells in response to IFN- γ

Cells [$(1-2 \times 10^7)$] were incubated with or without 250 units of human recombinant IFN- γ /ml for 24 h periods up to 120 h. Total RNA was prepared from the cells for Northern blot analysis of factor D mRNA levels using 20 μ g of total RNA (a). The blots were exposed at -20°C for 13 days (factor D) or 1 day (cyclophilin; bottom), with two intensifying screens. Supernatants from the same cells were assessed for factor D protein by RIMA (b); shown are means $\pm$ S.E.M. from four experiments. For Western blot analysis of factor D, cells (2×10^7) were collected and analysed as described in the Materials and methods section. The blot was probed with a polyclonal anti-(human factor D) antibody (c) and developed using an alkaline phosphatase-conjugated rabbit anti-human IgG. Lane 1, freshly prepared culture media; lane 2, supernatant from U105-MG cells; lane 3, urine from a patient with Fanconi's syndrome.

declined throughout the remainder of the culture period (Fig. 4a). Factor B protein levels increased to a maximum of 2.4-fold higher than controls at 48 h, and then declined to 1.6-fold higher than controls at 120 h (Fig. 4b).

Factor D mRNA and protein expression in U105-MG cells

In experiments to determine the optimal concentration of IFN- γ for factor D production, we found factor D gene expression to be refractory to stimulation by IFN- γ (Fig. 5a). However, as determined by RIMA, U105-MG cells synthesize and secrete significant amounts of factor D ($\sim 6-8$ ng/ml per 10^6 cells per day; Fig. 5b). Kinetic studies also demonstrated no response to 250 units of IFN- γ /ml over a 5 day period (results not shown). Western blot analysis of factor D secreted by U105-MG cells demonstrated that factor D produced by these cells appears as a doublet which may represent slight degradation of the protein or a pro-D and mature D molecule (Fig. 5c).

Biosynthetic labelling studies

Because we detected two C2 transcripts on Northern blot analysis of unstimulated and IFN- γ -stimulated U105-MG cells, we performed biosynthetic labelling studies with [^{35}S]methionine to determine whether the cells also synthesized and secreted two C2 proteins. As a control, biosynthetically labelled factor B was also examined. In these experiments, cells were stimulated with 250 units of IFN- γ /ml for 48 h prior to labelling, to enhance

Fig. 6. Autoradiograph of SDS/PAGE of [^{35}S]methionine-labelled U105-MG cells immunoprecipitated with antibodies to C2 and factor B

U105-MG cells were labelled with [^{35}S]methionine as described in the Materials and methods section, and labelled C2 and factor B were immunoprecipitated using rabbit anti-(human C2a) or -(human Bb) antibodies bound to Staph A cells. Precipitated proteins were separated on 5–20% gradient SDS gels, and the gels were stained, dried and exposed to Kodak AR X-Omat film for 2–3 days at -90°C . The resulting autoradiographs are shown. (a) Immunoprecipitation with antibodies to C2 from cell lysates at time zero (lane 1), 2 h after chasing with unlabelled methionine (lane 2), or from culture supernatants (lane 3). (b) Same as (a), except the immunoprecipitation was performed with antibodies to factor B. Also shown are the positions of molecular mass markers. The arrows denote the position of authentic factor B.

detection of the proteins, and then treated as described in the Materials and methods section. ^{35}S -labelled C2 and factor B from cell lysates (at time zero and 2 h after chasing with unlabelled methionine), and from culture medium (2 h post-chase) were immunoprecipitated with polyclonal anti-(human C2) or -(factor B) antibody and subjected to SDS/PAGE. The resulting autoradiographs are shown in Fig. 6. Fig. 6(a) shows biosynthetically labelled C2 intracellularly at both time zero and 2 h post-chase (lanes 1 and 2) and in the culture supernatant at 2 h post-chase (lane 3). The apparent molecular mass of C2 synthesized by the U105-Mg cells was slightly higher than that of C2 in serum (~ 105 kDa versus ~ 100 kDa). A lower-molecular-mass band (~ 70 kDa) was present intracellularly at both time points (lanes 1 and 2), but not in the supernatant. For factor B, two bands of very similar molecular mass were present intracellularly at time zero; however, only a single band was seen at 2 h in the lysate and extracellularly (Fig. 6b). The apparent molecular mass of factor B produced by U105-MG cells is similar to that of C2 (~ 105 kDa) and is also slightly higher than that of its serum-derived counterpart. The additional lower-molecular-mass bands in the 30–45 kDa range seen in both panels of Fig. 6 are non-specifically bound proteins. These bands

are also seen when using unrelated IgG to pre-clear biosynthetically labelled samples (results not shown).

DISCUSSION

We have shown production of complement component C2 and factors B and D by the human astrogloma cell line U105-MG. Western blot analysis and biosynthetic labelling studies demonstrated that all three proteins secreted by U105-MG cells were comparable in size to authentic C2 and factors B and D in serum (Figs. 5 and 6). Similarly Northern blotting indicated that the sizes of the mRNAs for each of the three proteins in U105-MG cells were comparable to those present in HepG2 cells. IFN- γ treatment up-regulated C2 and factor B gene expression, but factor D expression was not affected by IFN- γ . These are the first data to demonstrate the production of factor D and C2 by astrogloma cells, although factor B has been previously shown to be synthesized by murine and rat astrocytes and glioma cell lines [16].

Factor D is limited in its cellular expression, with blood monocytes/macrophages [12,13], adipocytes [10,11] and sciatic nerve [11] being the only sites of synthesis currently known. The latter two sites are unique for complement proteins, which are produced predominantly by hepatocytes, monocytes/macrophages, fibroblasts and epithelial and endothelial cells [4,5,30]. Stimulation of U105-MG cells by IFN- γ had no effect on the expression of factor D mRNA or protein levels (Fig. 5). These data, combined with previous studies from our laboratory demonstrating no effects of vitamin D, phorbol 12-myristate 13-acetate, dibutyryl cyclic AMP or supernatants from concanavalin A-treated monocytes (S. R. Barnum & J. E. Volanakis, unpublished work) and down-regulation of factor D mRNA levels in response to treatment with tumour necrosis factor- α or retinoic acid [14,15], suggest that the regulation of factor D gene expression is different from that of most complement components.

Increased C2 and factor B protein and mRNA levels in response to IFN- γ have been documented previously in a number of cell types and is tissue-specific [5,7,31,32]. The increases we report here for C2 (4–6 fold) and factor B (4–5 fold) are comparable with those reported for monocytes, monocyte cell lines (U937) and fibroblasts [5,7,31,32], at both the protein and mRNA levels. Fewer studies have examined the kinetics of C2 and factor B synthesis in response to IFN- γ ; however, Hamilton and co-workers [33] have reported kinetics of monocyte responses similar to those described here. The kinetic studies shown in Figs. 3 and 4 demonstrate that the increased synthesis of C2 and factor B peaks at approx. 48 h. This response is characteristic of most acute-phase proteins [34,35]. Factor B has been previously shown to be an acute-phase protein, as determined by elevated serum levels in response to infection [21]. Although serum levels of C2 are not elevated in the acute-phase response, increased C2 synthesis by macrophages and fibroblasts in response to LPS and IFN- γ [5] suggests that C2 gene expression is up-regulated at tissue sites during the acute-phase response.

The detection of two C2 transcripts (Figs. 1 and 3) is unique for human tissues; however, multiple primary translation products have been identified in primary hepatocyte cultures and HepG2 cells [36]. The larger 2.8 kb C2 transcript is similar in size to that detected previously in human liver [24,37]. Expression of this transcript was responsive to IFN- γ treatment in a dose- and time-dependent fashion (Figs. 1 and 3), while the smaller ~2.3 kb band responded only weakly to IFN- γ treatment. Biosynthetic labelling studies (Fig. 6) demonstrated only a single C2 translation product, suggesting that only the larger transcript is translated and secreted. The smaller transcript seen in U105-MG

cells may arise through alternative splicing and code for a truncated C2 protein. However, no smaller C2 protein is seen extracellularly in the biosynthetic labelling experiments shown in Fig. 6(a). Smaller, cell-associated, forms of C2 have been reported in HepG2 cells and primary hepatocytes [36]; thus we may be seeing similar translation products in U105-MG cells. Cell-free translation studies using both total RNA and poly(A)⁺ mRNA are necessary to address these possibilities. RNAase protection experiments have indicated the presence of two C2 transcripts differing in the size of the 5' untranslated region [8]. The size difference between these alternative C2 mRNAs was estimated to be approx. 150 nucleotides, considerably less than the ~500 nucleotide size difference observed in the Northern blots of U105-MG RNA. Similarly, sequence analysis of murine cDNA clones has demonstrated two C2 transcripts in liver arising from alternative splicing mechanisms [38]. However, the size difference (21 nucleotides) is again too small to account for the transcripts we observed. Although multiple transcripts have been reported for murine factor B [39,40] in kidney and intestine, we observed a single protein band for factor B secreted into the supernatants of U105-MG cells (Fig. 6b). The doublet seen intracellularly (Fig. 6b, lane 1) probably reflects various levels of glycosylation of factor B. Biosynthetic studies using tunicamycin will help to address this question and to determine if the increased size of the secreted C2 and factor B synthesized by U105-MG cells (Fig. 6) is due to additional carbohydrate.

In addition to these three complement components, we have demonstrated the synthesis of C3 from several astrogloma cell lines and primary rat astrocytes (Y. Ishii, S. R. Barnum and J. E. Volanakis, unpublished work). The synthesis of C3 and factors B and D by astrocytes provides the CNS with an endogenous source of proteins sufficient for the assembly of the alternative pathway C3 convertase. Thus it is likely that the alternative pathway may play an important role in host defences in the immunologically privileged CNS. Even though endogenous CNS synthesis of the terminal complement components (C5–C9) has not been demonstrated, C3a generated by activation of the alternative pathway could increase vascular permeability, leading to recruitment of additional plasma proteins important in host defence. It is also tempting to speculate that the hormone-like C3a and/or other complement activation fragments may have additional effects on brain cells.

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