Role of protein kinase C activation in synthesis of complement components C2 and factor B in interferon- γ -stimulated human fibroblasts, glioblastoma cell line A172 and monocytes

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The synthesis of C2 and factor B, the key components of complement system, is performed by various kinds of cells and is also up-regulated by interferon- γ (IFN- γ). By using human fibroblasts, human glioblastoma cell line A172 and monocytes, we investigated the signal-transduction mechanism for IFN- γ -induced synthesis of C2 and factor B. The C2 and factor B synthesis induced by IFN- γ in all three cell types was inhibited by a protein kinase C (PKC) inhibitor, 1-(5-isoquinolylsulphonyl)-2-methylpiperazine (H-7). The depletion of PKC in these cell types after treatment with phorbol 12-myristate 13-acetate (PMA) resulted in inhibition of IFN- γ -induced C2 production. In addition, IFN- γ treatment elicited a decrease in cytoplasmic PKC in A172 cells, indicating that PKC is activated by IFN- γ . These results suggest that PKC is crucial for IFN- γ -

induced C2 and factor B synthesis. Northern-blot analysis showed that the effects at H-7 were at least partly mediated by modulation of C2 and factor B mRNA abundance in A172 cells. Since treatment of fibroblasts and A172 cells with IFN- γ had no effect on intracellular Ca^{2+} concentration, and since neither EGTA nor nifedipine inhibited C2 or factor B synthesis induced by IFN- γ , we concluded that intracellular Ca^{2+} mobilization was not involved in the effect of IFN- γ . In addition, genistein, herbimycin A and *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W-7) had no inhibitory effect on IFN- γ -mediated action in any of the three cell types, which suggests that IFN- γ acts independently of tyrosine kinases and calmodulin-dependent protein kinases.

INTRODUCTION

The complement system plays an important role in host defences and inflammatory reactions. Although most of the complement proteins are synthesized primarily in the liver [1], it has also been reported that several complement proteins are produced at extrahepatic sites, such as monocytes [2], fibroblasts [3], endothelial cells [4], and cells derived from myelomonocytic tumours [5] and glial cells [6]. The synthesis of complement proteins is regulated by inflammation-promoting cytokines, such as interferon- γ (IFN- γ), tumour necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 [7–11].

Complement components C2 and factor B are essential for the classical and alternative pathways of complement activation, respectively, because they provide catalytic subunits for the C3 and C5 convertases, both of which are key enzymes for the complement system. The genes for C2 and factor B are located only 0.4 kb apart within the class III region of the human major histocompatibility complex (MHC) on the short arm of chromosome 6 and are structurally similar to each other, suggesting that C2 and factor B originate from a gene-duplication event [12]. It has been reported that C2 and factor B production by various cell types is up-regulated upon treatment with IFN- γ [7,8]. The induction of C2 and factor B genes by IFN- γ might be reasonable in the context of immune response, considering the importance of these two proteins in the complement cascade.

IFN- γ , a key substance in the cytokine network, modulates many aspects of immune response, including complement-protein synthesis. However, the signal-transduction pathways utilized by

IFN- γ are not well understood, and are probably also complicated by tissue-specificity, as in the case of IFN- γ -mediated MHC class II antigen expression. MHC class II expression by IFN- γ has been shown to be dependent on tyrosine phosphorylation, which is followed by protein kinase C (PKC) activation and Ca^{2+} mobilization in human glioblastoma cell line T98G [13,14]. However, in human promyelocytic leukaemia cell line HL60 and murine bone-marrow macrophages, the involvement of Ca^{2+} - and calmodulin-dependent protein kinases, not of PKC, was reported [15,16]. In the human monoblastoid cell line U937, both PKC and calmodulin-dependent protein kinases are not involved in the IFN- γ -mediated action [17].

The molecular mechanisms responsible for IFN- γ -mediated modulation of complement production have not been studied in detail. Although there has been a study using monocytes [18], further research using specific inhibitors of intracellular second messengers or by directly monitoring intracellular signalling events after treatment of IFN- γ is needed. Considering the tissue-specific manner of IFN- γ -mediated signal transduction, further study using other cell types should also help to clarify the mechanism. In the present study, we investigated the intracellular signal-transduction molecules essential for the IFN- γ -induced production of complement components C2 and factor B in human skin fibroblasts, glioblastoma cell line A172 and monocytes. We report that C2 and factor B production induced by IFN- γ in those cell types was suppressed by a PKC inhibitor, 1-(5-isoquinolylsulphonyl)-2-methylpiperazine (H-7), and IFN- γ -induced C2 synthesis in those cell types was inhibited by the depletion of PKC by treatment with phorbol 12-myristate 13-

Abbreviations used: IFN, interferon; PKC, protein kinase C; H-7, 1-(5-isoquinolylsulphonyl)-2-methylpiperazine; W-7, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide; TNF, tumour necrosis factor; IL, interleukin; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum (heat-inactivated for 2 h at 56 °C); SFU, site-forming units; PMSF, phenylmethanesulphonyl fluoride; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; SSC, 0.15 M NaCl/0.015 M sodium citrate.

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acetate (PMA). Furthermore, treatment with IFN- γ elicited a cytosolic PKC activation in A172 cells. We also found that C2 and factor B synthesis by A172 cells induced by IFN- γ was at least partly inhibited at the level of mRNA abundance by the PKC inhibitor H-7.

MATERIALS AND METHODS

Cells

Passage-9 human glioblastoma cell line A172 was kindly provided by the Japanese Cancer Research Resources Bank. Human fibroblasts were obtained from skin from the upper arm of a healthy 27-year-old male. These cells were cultured in DMEM (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal-calf serum (FCS) (GIBCO) and 2 mM L-glutamine and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. A172 cells were cultured from the first-generation passages and discarded after 15 passages. The fibroblasts from passage 5–11 were used. Peripheral-blood monocytes were isolated from a healthy 27-year-old male as described [19], and then were cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS under the same conditions described above.

Reagents

Recombinant human IFN- γ was kindly provided by Shionogi Pharmaceutical Co. (Osaka, Japan). H-7 was purchased from Seikagaku Kogyo (Tokyo, Japan). Genistein, EGTA and PMA were purchased from Sigma (St. Louis, MO, U.S.A.), and herbimycin A was kindly provided by Dr. M. Nakajima (Institute of Medical Science, University of Tokyo, Japan). *N*-(6-Amino-hexyl)-5-chloro-1-naphthalenesulphonamide (W-7) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Nifedipine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Guinea-pig serum was obtained from the Research Institute of Microbial Disease at Osaka University (Osaka, Japan).

Viability test

Viability of cells was assessed by the Trypan Blue dye-exclusion test.

Cell stimulation

Human fibroblasts (1.2×10^5), A172 cells (5.2×10^5) and monocytes (5×10^5) were grown to confluence in 24-well tissue-culture plates (Multidish 24; Nunc, Kamstrup, Denmark). Cells were preincubated for 30 min in 0.3 ml of the culture media with or without various concentrations of H-7, genistein, herbimycin A, W-7, or nifedipine, and then were incubated for 72 h in the absence or presence of 100 units/ml IFN- γ . At appropriate time points, the culture media were collected and assayed for C2 and factor B. For the study of factor B synthesis by monocytes, we used monocytes from day 7 of culture, because no factor B was detected within the first 3 days of culture by our e.l.i.s.a. system.

Haemolytic assay for C2

This was performed by the method described by Borsos et al. [20] with a slight modification [21]. Intermediate cells, EAC14 [sensitized sheep erythrocytes (EA) with the human first (C1) and fourth (C4) components of complement] ($T_{\max}$, the time at which greatest haemolysis is shown, 2–3 min), were prepared by reaction of sensitized sheep erythrocytes (Ishizu Pharmaceutical Co., Tokyo, Japan) with normal human serum in the presence of

triethylenetetramine-*NNN'N''N'''N'''*-hexa-acetic acid (Dojin Laboratories, Kumamoto, Japan). A 0.1 ml portion of diluted supernatants and 0.2 ml of EAC14 (7.5×10^7 cells/ml) in GVB (veronal-buffered saline containing 0.1% gelatin, 1 mM MgCl₂ and 0.15 mM CaCl₂, pH 7.5) were mixed and incubated at 30 °C for $T_{\max} + 3$ min. After standing on ice, 1.2 ml of cold cEDTA (10 mM EDTA containing 1:50-diluted guinea-pig serum, isotonic veronal-buffered saline and 0.1% gelatin) was added to the reaction mixture and then the mixture was incubated for 1 h at 37 °C. After centrifugation, haemolysis was estimated by measurement of the A_{415} . The haemolytic activity was expressed as the average number of EAC142 sites per cell [20]. Assays were performed in duplicate.

E.l.i.s.a. for factor B

Intact factor B was measured by a solid-phase sandwich e.l.i.s.a. as described previously [22]. An affinity-purified rabbit anti-(human factor Bb) IgG was used to coat the wells of 96-well microtitre plates (Titertek; ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), and mouse monoclonal anti-(human factor Ba) IgG (FD3-20) was used as the detection reagent. Both antibodies were kindly provided by Dr. J. E. Volanakis (University of Alabama, Birmingham, AL, U.S.A.). A standard curve was constructed by using dilutions of normal human serum with a known concentration of factor B (280 μ g/ml). The lower limit of detection for this e.l.i.s.a. was 25 ng/ml. Assays were performed in triplicate.

PKC depletion by PMA treatment

Fibroblasts, A172 cells and monocytes were incubated in medium with or without PMA for 48 h, which accelerates the degradation of PKC [23]. The concentrations of PMA added to fibroblasts, A172 cells and monocytes were 800 nM, 50 nM and 10 nM respectively, which were decided according to the PMA concentrations utilized in the previous PMA depletion studies [24,25]. Then, the PMA-treated or non-treated cells were washed twice with the medium and cultured in the medium with or without 100 units/ml IFN- γ for 24 h, and the culture media were assayed for C2.

Binding of ¹²⁵I-IFN- γ to monocytes

Binding studies with ¹²⁵I-IFN- γ (478 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) were performed by the method described by Krakauer et al. [26]. Briefly, human adherent peripheral-blood mononuclear cells (1.2×10^6 cells per well) were preincubated with medium or 10 nM PMA for 48 h at 37 °C. Then the cells were washed with PBS and incubated with 0.3 ml of culture media containing serial 2-fold dilutions of ¹²⁵I-IFN- γ , ranging from 0.25 to 2 nM, at 4 °C for 80 min. Non-specific binding was determined in the presence of 500-fold molar excess of unlabelled IFN- γ and was subtracted from total binding to calculate specific binding. After washing with PBS, cells were solubilized with 0.4 ml of 0.2 M NaOH, and the radioactivity was determined.

Assay of PKC

A172 cells were grown to confluence in a 75 cm² flask (2×10^7 cells). After being rinsed with DMEM, the cells were incubated with DMEM containing 10% FCS and IFN- γ (100 units/ml). At appropriate time points, the cells were scraped and resuspended in 2.3 ml of ice-cold homogenizing buffer [50 mM Tris, pH 7.5, 2 mM EDTA 10 mM EGTA, 0.25 mM

sucrose, 1 mM phenylmethanesulphonyl fluoride (PMSF)] at a concentration of $(0.7\text{--}1.0) \times 10^7$ cells/ml, and sonicated in a Branson sonicator (Branson Sonic Power Co., Danbury, CT, U.S.A.) at 20 W for 1 min. The homogenate was centrifuged at 100 000 *g* for 1 h at 4 °C, and the supernatant served as the cytosolic fraction. Centrifuged pellets were homogenized in the same sonicator in 2.3 ml of ice-cold homogenizing buffer containing 0.5% (v/v) Nonidet P40, gently rotated for 2 h at 4 °C and centrifuged at 100 000 *g* for 1 h at 4 °C. The supernatant served as the membrane fraction. The samples were then assayed for PKC activity with a PKC enzyme-assay system (Amersham), according to the manufacturer's instructions. PKC activity was determined by measuring the incorporation of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP (3000 Ci/mmol; Amersham) into synthetic peptide KRTLRR [27]. PKC specific activity was determined by subtracting the activity measured in the absence of Ca^{2+} , phosphatidylserine and PMA from that measured in their presence, and it was expressed as nmol of ^{32}P transferred to KRTLRR peptide/min per 10^8 cells. Assays were performed in duplicate.

Measurement of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

This was done by measuring the fluorescence of Fura-2 [28] with a 2- λ -Microscopic Fluorometer (CAM-220, Japan Spectroscopic Co., Tokyo, Japan) and an Axiovert 35 M microscope (Carl Zeiss, Oberkochen, Germany) with an Axiovert 40 $\times$ fluor objective at 37 °C, and CA-2000DP analysis system (Japan Spectroscopic Co.). Cells were plated on a glass cover-slip, and grown to near-confluence in Phenol-Red-free DMEM (GIBCO) supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2 . The cells were loaded with Fura-2 AM (5 μM ; Dojin Laboratories) in Phenol-Red-free DMEM (without NaHCO_3) supplemented with 10% FCS at 37 °C in normal air for 1 h. Then the cells were rinsed twice with the medium, and $[\text{Ca}^{2+}]_i$ after treatment with IFN- γ was measured in the presence of 0.5 ml of the medium.

Effect of EGTA on the stimulation of C2 synthesis by IFN- γ in A172 cells

A172 cells were incubated in DMEM containing 10% FCS and 1 mM EGTA for 1 h. Then IFN- γ was added to the culture medium containing EGTA and the cells were cultured for an additional 72 h. The C2 synthesis was assayed by haemolytic assay.

RNA isolation and analysis

Total cellular RNA was isolated by the hot-phenol method [29]. Then 12 μg of total RNA was denatured in formaldehyde for 15 min at 55 °C, size-fractionated by electrophoresis on a 1.2% (w/v) agarose/formaldehyde gel, transferred to a nitrocellulose membrane filter (Schleicher and Schuell, Dassel, Germany) and pre-hybridized for 5 h at 42 °C in a solution of 50% (v/v) formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 1 $\times$ Denhardt's solution, 0.1% (w/v) SDS, 25 mM KH_2PO_4 and 200 μg of denatured salmon sperm DNA/ml. Hybridization was performed overnight at 42 °C in a solution containing 46.5% formamide, 4 $\times$ SSC, 4 $\times$ Denhardt's solution, 0.1% SDS, 25 mM K_2HPO_4 , 10% (w/v) dextran sulphate and 200 μg of denatured salmon sperm DNA/ml, containing denatured ^{32}P -labelled probe (1.0×10^6 c.p.m./ml). The membrane was washed in 1 $\times$ SSC/0.5% SDS at 65 °C and subjected to autoradiography. Quantification of autoradiographs was performed by scanning with a densitometer (Digital Densitrol DMU-33C; Toyo Science Industries Co., Tokyo, Japan). Density

values were standardized to the value for β -actin hybridization within each experiment.

DNA and cDNA probes

A full-length C2 cDNA and a 1.7 kb *Eco*RI fragment of human factor B cDNA were utilized as probes [22,30]. The β -actin DNA probe was purchased from Wako Pure Chemical Industries. All probes were labelled with [$\alpha\text{-}^{32}\text{P}$]dCTP (3000 Ci/mmol; ICN Biomedicals Inc., Irvine, CA, U.S.A.) by the random-primed DNA-labelling kit (United States Biochemical Co., Cleveland, OH, U.S.A.).

Statistical analysis

All experiments were performed three or four times, and the data are expressed as means $\pm$ S.E.M. *P* values were calculated by the paired *t* test.

RESULTS

Kinetics of IFN- γ -mediated up-regulation of C2 and factor B synthesis

Kinetic experiments were performed by culturing nearly confluent fibroblasts, A172 cells and monocytes in the absence or presence of 100 units/ml IFN- γ for 24–96 h. The ratio of cumulative synthesis of both C2 and factor B in IFN- γ -treated cells to that in untreated cells increased during the first 72 h, and then decreased slightly by 96 h (Figure 1). We therefore chose 72 h as the ideal time period for incubation with IFN- γ for the three cell types.

Viability test

Concentrations of reagents used in all our experiments had no effect on viability of cells, as assessed by Trypan Blue exclusion and the absence of detached or floating cells (results not shown).

Effects of H-7 on IFN- γ -induced C2 and factor B synthesis

The effect of H-7 on the stimulation of C2 synthesis by IFN- γ is shown in Figure 2(a). After treatment with 100 units/ml IFN- γ , C2 synthesis increased 6.7-fold in fibroblasts, 4.3-fold in A172 cells and 4.2-fold in monocytes compared with that in control cells cultured in medium alone. However, the PKC inhibitor H-7 inhibited this IFN- γ -induced C2 synthesis dose-dependently in both fibroblasts and A172 cells, and the maximal inhibition, found at 10 μM , was 38% in fibroblasts, 50% in A172 cells and 17% in monocytes. In Figure 2(b) the effect of H-7 on IFN- γ -induced factor B synthesis is shown. Factor B synthesis after treatment with 100 units/ml IFN- γ was 1.6 times greater in fibroblasts, 1.5 times greater in A172 cells and 2.9 times greater in monocytes than in control cells cultured in medium alone. H-7 also inhibited factor B synthesis induced by IFN- γ dose-dependently and, at a dose of 10 μM , almost completely inhibited it to the control level in fibroblasts and A172 cells, and inhibited it by 34% in monocytes. These data suggest that PKC is involved in the IFN- γ -induced C2 and factor B synthesis.

Effect of PKC depletion by PMA on IFN- γ -induced C2 synthesis

As the effect of H-7 on IFN- γ -induced C2 synthesis of monocytes was not as great as its effect on fibroblasts and A172 cells, we further characterized the role of PKC in IFN- γ -mediated C2 production in monocytes. We studied whether up-regulation of

Figure 1 Kinetics of IFN- γ -mediated up-regulation of C2 and factor B synthesis by fibroblasts (●), A172 cells (■) and monocytes (▲)

Confluent fibroblasts, A172 cells and monocytes were cultured in the medium with or without 100 units/ml IFN- γ . At the appropriate time points, the culture media were assayed for C2 and factor B. Factor B synthesis by fibroblasts at 24 h was not detected in this study. Results are expressed as a percentage of the control and are means $\pm$ S.E.M. of three experiments.

Figure 2 Effects of H-7 on IFN- γ -induced C2 and factor B synthesis

Confluent fibroblasts, A172 cells and monocytes were incubated for 30 min in media containing various concentrations of H-7. Then, after treatment with 100 units/ml IFN- γ for 72 h, the culture media were assayed for C2 (a) and factor B (b). The rates of C2 synthesis by fibroblasts, A172 cells and monocytes in medium alone (control) were 5.50 ± 0.75 , 0.66 ± 0.15 and 9.90 ± 0.87 SFU/ 10^7 cells per 72 h respectively. The rates of factor B synthesis by these cell types in medium alone (control) were 1515 ± 50 , 720 ± 21 and 575 ± 45 ng/ 10^7 cells per 72 h respectively. Results are expressed as a percentage of the control assay and are the means $\pm$ S.E.M. of four experiments: * $P < 0.05$.

C2 by IFN- γ was inhibited in PKC-depleted monocytes as well as fibroblasts and A172 cells by 48 h of pretreatment with PMA. As shown in Table 1, the increase in C2 synthesis by treatment with IFN- γ was completely inhibited by PMA pretreatment in monocytes. In addition, PMA pretreatment resulted in the inhibition of IFN- γ -induced C2 synthesis in both fibroblasts and A172 cells, by 30% and 80% respectively. These results suggest that PKC is involved in IFN- γ -induced C2 synthesis in all three cell types. Moreover, the amount of IFN- γ -induced C2

expression in the PKC-depleted monocytes was less than that of basal C2 expression in monocytes.

Effect of long-term PMA treatment on IFN- γ receptors on monocytes

To rule out the possibility that the effect of PMA treatment shown in Table 1 was due to alterations in IFN- γ receptor numbers/coupling after chronic PKC activation, we next studied

Table 1 Effect of PKC depletion by PMA on IFN- γ -induced C2 synthesis

The rates of C2 synthesis by fibroblasts, A172 cells and monocytes in control (not treated with PMA and IFN- γ) were 0.80 ± 0.14 , 0.12 ± 0.02 and 0.82 ± 0.09 site-forming units (SFU)/ 10^7 cells per 24 h respectively. Results are expressed as a percentage of the control assay, and are means $\pm$ S.E.M. of three experiments: * significantly different ($P < 0.05$) compared with cells not treated with PMA.

Cells	PMA ... IFN- γ ...	C2 haemolytic activity (% of control)	
		Untreated +	Treated +
Fibroblasts		319 $\pm$ 17	252 $\pm$ 10*
A172		274 $\pm$ 16	135 $\pm$ 17*
Monocytes		304 $\pm$ 12	33 $\pm$ 13*

the effect of long-term PMA treatment on specific binding of 125 I-IFN- γ to monocytes, which showed a marked decrease in IFN- γ -induced C2 synthesis after PKC depletion by PMA. As shown in Figure 3, treatment of monocytes with PMA for 48 h did not alter specific binding of 125 I-IFN- γ to monocytes. This result that PMA treatment does not induce modifications on the binding of IFN- γ to the receptor is consistent with the previous reports using fibroblasts and macrophages [31].

PKC translocation in A172 cells induced by IFN- γ

We next examined whether IFN- γ activated PKC in A172 cells. Because PKC translocates from cytosol to membrane when it is activated, PKC activation results in a rapid decrease in its cytoplasmic activity and a coincident increase in the membrane activity [32,33]. Therefore, A172 cells were treated with 100 units/ml IFN- γ for various time periods, and the PKC activity in both the cytosolic and the membrane fraction was measured. As shown in Figure 4, cytosolic PKC activity was maximally decreased at 20 min by 50%, followed by a rapid increase to the control level by 40 min after IFN- γ treatment. On the other hand, little PKC activity was detected in the membrane fraction during the 60 min period.

Role of Ca^{2+} on the IFN- γ -induced C2 and factor B synthesis by fibroblasts and A172 cells

As the evidence given above indicates that PKC plays an important role in the IFN- γ effect, we next investigated the IFN- γ -mediated mobilization of intracellular Ca^{2+} , which interacts synergistically with PKC in a variety of cellular responses to external stimuli [32]. After up to 20 min of IFN- γ treatment, there was no increase in $[\text{Ca}^{2+}]_i$ in fibroblasts and A172 cells treated with 100 units/ml IFN- γ (90.0 ± 4.2 nM and 73.7 ± 3.3 nM respectively) or with 1000 units/ml IFN- γ (88.0 ± 3.2 nM and 74.5 ± 2.9 nM respectively) compared with that in untreated cells (90.5 ± 5.5 nM and 72.4 ± 2.4 nM respectively). In addition, depletion of extracellular Ca^{2+} by 1 mM EGTA in A172 cells, or inhibition of Ca^{2+} influx through the Ca^{2+} channels by nifedipine (up to 10 μM), in fibroblasts and A172 cells did not show any inhibitory effect on IFN- γ -mediated C2 and factor B synthesis (results not shown). Taking these results together, we suggest that intracellular Ca^{2+} mobilization is not involved in IFN- γ -mediated C2 and factor B synthesis.

Figure 3 Specific binding of 125 I-IFN- γ to IFN- γ receptors on untreated and PMA-treated monocytes

Monocytes (1.2×10^6) were incubated with medium alone ($\circ$) or with 10 nM PMA ($\bullet$) for 48 h at 37 $^\circ\text{C}$ before 125 I-IFN- γ binding. Results are expressed as means $\pm$ S.E.M. of three experiments.

Effects of genistein, herbimycin A and W-7 on IFN- γ -induced C2 and factor B synthesis

The tyrosine kinase inhibitor genistein did not have any inhibitory effect on IFN- γ -induced C2 and factor B synthesis in fibroblasts, A172 cells or monocytes (results not shown). Likewise, the more specific tyrosine kinase inhibitor herbimycin A did not show any inhibitory effect in any of these three cell types (results not shown). Owing to their susceptibility to genistein and herbimycin A, monocytes were administered lower concentrations of genistein (up to 3 μM) and herbimycin A (up to 0.03 $\mu\text{g}/\text{ml}$) than the other two cell types (10 μM and 0.1 $\mu\text{g}/\text{ml}$ respectively). A calmodulin antagonist, W-7 (10 μM), also had no effect on IFN-

Figure 4 Time course of PKC activity in A172 cells after treatment with IFN- γ

A172 cells (2×10^7) were treated with 100 units/ml IFN- γ for the time indicated, and then the cells were lysed and assessed for the activity of cytoplasmic ($\bullet$) and membrane ($\circ$) PKC. The PKC activity of control cells (0 min) was 6.47 ± 0.47 nmol of ATP/min per 10^8 cells for the cytoplasmic fraction and 0.04 ± 0.03 nmol of ATP/min per 10^8 cells for the membrane fraction. Results are expressed as means $\pm$ S.E.M. for three experiments: * $P < 0.05$.

Figure 5 Northern-blot analysis of C2, factor B and β -actin mRNA from A172 cells

A172 cells (2×10^7) were treated with 100 units/ml IFN- γ in the absence or presence of 10 μ M H-7 for 72 h. Lane 1 shows total RNA (12 μ g) from untreated A172 cells; lane 2 shows total RNA (12 μ g) from A172 cells treated with 100 units/ml IFN- γ ; lane 3 shows total RNA (12 μ g) from A172 cells treated with 100 units/ml IFN- γ and 10 μ M H-7.

γ -induced C2 and factor B synthesis in any of the three cell types (results not shown).

Northern-blot analysis of C2 and factor B mRNA from A172 cells

As shown in Figure 5, the amounts of C2 and factor B mRNA increased after 72 h of treatment with IFN- γ (lane 2) compared with the basal level of mRNAs in untreated cells (lane 1). In the presence of 10 μ M H-7, both C2 and factor B mRNA levels were inhibited (lane 3). To compare quantitatively the relative amounts of mRNA, densitometric scanning of the autoradiograms was performed. When standardized by the amount of β -actin in each lane, the relative amounts of C2 mRNA from lanes 1, 2 and 3 were 1, 4.2 and 3.0 respectively, whereas those of factor B mRNA from lanes 1, 2 and 3 were 1, 5.6 and 4.3 respectively.

DISCUSSION

Gene expression and biosynthesis of complement proteins are under the regulation of different cytokines and hormones, such as IFN- γ , IL-1, IL-6, TNF and glucocorticoids [8,9,11,34]. However, the intracellular signals responsible for regulation by these agents are not well understood. Although it has been suggested that PKC is involved in IFN- γ -induced C2 production by monocytes, because PMA mimics part of the IFN- γ action [18], the intracellular signals responsible for complement production mediated by IFN- γ have not been investigated in detail. Moreover, considering the tissue-specific effects of IFN- γ , IFN- γ may utilize different intracellular signals to induce complement production in different cell types.

In the present study, we investigated the intracellular signals for the IFN- γ -mediated C2 and factor B synthesis, the key enzymes in the complement system, by using three different cell

types: fibroblasts, glioblastoma cell line A172 and monocytes. On the basis of the results that (1) the PKC inhibitor H-7 inhibited IFN- γ -induced C2 and factor B synthesis in all three cell types (Figure 2), (2) PKC depletion by PMA treatment resulted in the inhibition of IFN- γ -induced C2 synthesis in all the three cell types (Table 1), and (3) IFN- γ treatment elicited a rapid activation of cytosolic PKC of A172 cells (Figure 4), we concluded that IFN- γ induces C2 and factor B synthesis through intracellular PKC-dependent mechanisms, irrespective of tissue specificity. As PKC depletion produced by pretreatment with PMA suppressed not only the IFN- γ -induced C2 synthesis, but also the basal expression of C2 by monocytes (Table 1), we suggest that PKC is also involved in the steady-state level of C2, at least in monocytes. As the specific binding curve of 125 I-IFN- γ of PMA-treated monocytes was identical with that of untreated monocytes (Figure 3), we suggest that the inhibition of the IFN- γ effect by PMA is caused by PKC depletion, not by the alterations in IFN- γ receptor numbers and affinity. There was a difference in the rate of decrease in IFN- γ -induced C2 synthesis among the three cell types upon PKC depletion by PMA (Table 1). The tissue-specific difference is conceivable, because it has been reported that PKC isoenzymes which display different modes of down-regulation in response to PMA treatment are distributed in a tissue-specific manner [33,35]. Although PKC is known to translocate from cytosol to membrane upon activation [36], we were not able to demonstrate an increase in PKC activity of the membrane fraction of A172 cells complementarily to the decrease in PKC activity in the cytosolic fraction (Figure 4). This can be explained by the rapid degradation of cytosolic PKC shortly after its activation, which results in a decrease in cytosolic PKC without a coincident increase in membrane PKC. This high turnover of PKC is reported in several cell types [37–39]. At least nine PKC isoenzymes have been discovered so far [40]. They can be divided into two large groups, Ca^{2+} -dependent conventional PKCs (cPKCs) and Ca^{2+} -independent novel PKCs (nPKCs), which differ not only in their mode of activation, but also in their structures, tissue distributions and substrate specificities. Ca^{2+} -dependent cPKCs include α , β I, β II and γ isoenzymes, whereas Ca^{2+} -independent nPKCs are δ , ϵ , η , ζ and θ . As no intracellular Ca^{2+} mobilization occurred in response to IFN- γ treatment in fibroblasts or in A172 cells, the IFN- γ effect is probably mediated either exclusively by Ca^{2+} -independent nPKCs or by PKC activated by diacylglycerol generated from pathway(s) which do not disturb intracellular Ca^{2+} levels. One example of such a pathway is the hydrolysis of phosphatidylcholine, instead of phosphatidylinositol, induced by IFN- α [41].

We next investigated the possibility of the contribution of another Ca^{2+} -dependent pathway, calmodulin-dependent kinases, by using a calmodulin antagonist, W-7. The effect of IFN- γ was not inhibited by W-7, indicating that calmodulin-dependent protein kinases might not be involved in the IFN- γ effect. This result is inconsistent with a previous finding that trifluoperazine, a calmodulin antagonist, abrogated C2 synthesis in monocytes stimulated by IFN- γ [18]. The discrepancy can be explained by the fact that W-7 has been shown to be a more specific inhibitor to calmodulin than is trifluoperazine [42]. Therefore our result may reflect more specifically the effect of calmodulin in IFN- γ -mediated action.

It has been shown that tyrosine kinases are implicated in cellular proliferation and carcinogenesis. Recently, inflammatory cytokines, such as IL-1 and IL-6, have been shown to mediate their signals through tyrosine kinases, too [43,44]. Moreover, IFN- γ -induced MHC class II antigen expression in the glioblastoma cell line T98G has also been reported to be dependent on tyrosine kinases [13]. Because C2 and factor B genes are

located in the MHC class III region, close to the class II region, we investigated the possibility of tyrosine kinase involvement in the IFN- γ induction of C2 and factor B synthesis. However, two tyrosine kinase inhibitors, genistein and herbimycin A, did not inhibit the effect of IFN- γ on any of the three cell types, which suggests that tyrosine kinases make no contribution to IFN- γ -mediated C2 and factor B synthesis.

It has been reported that IFN- γ dose-dependently increased the transcriptional rate of the factor B gene, as well as the half-life of the mRNA, resulting in increased abundance of factor B mRNA and protein, whereas IFN- γ increased the abundance of C2 mRNA and protein by producing a dose-dependent increase in the transcriptional rates of C2 gene despite a decrease in half-life of mRNA in various cell types, such as monocytes, Hep G2 cells, fibroblasts and human umbilical vein endothelial cells [8]. As shown in Figure 5, C2 and factor B mRNA abundances were up-regulated in response to IFN- γ , which was inhibited by 38 % and 28 % respectively after treatment with 10 μ M H-7. The effect of H-7 may be through the inhibition of transcriptional rates and/or the mRNA stability of these proteins. The increased abundance of factor B mRNA (5.6-fold) in the presence of IFN- γ was in excess of the amount of protein synthesis (1.5-fold), whereas there was no discrepancy between the effect of IFN- γ -induced C2 mRNA increase (4.2-fold) and protein synthesis (4.3-fold). The discrepancy in the level of factor B mRNA and protein synthesis might indicate that factor B synthesis induced by IFN- γ is regulated not only at the level of abundance of mRNA, but also at the post-transcriptional level, such as rate of translation, intracellular protein processing, catabolism and secretion. In fact, we have previously shown that the expression of C2, a structural and functional homologue of factor B, is regulated translationally in a tissue-specific manner [45]. In addition, post-translational glycosylation is reported to affect C2 and factor B secretion [46]. The decrease produced by H-7 in the IFN- γ -induced enhancement in protein synthesis, 50 % for C2 and 100 % for factor B, was not the same as the changes in the mRNA abundance level, approx. 38 % for C2 and 28 % for factor B. This may suggest that H-7 not only affects the abundance level of mRNA, but also inhibits the post-transcriptional stages of IFN- γ -induced C2 and factor B synthesis.

We have demonstrated evidence that PKC is involved in IFN- γ -mediated C2 and factor B synthesis in fibroblasts, A172 cells and monocytes. Considering the selective activation of PKC β in HeLa cells and PKC ϵ in Daudi cells after IFN- α treatment [41,47], it is possible that the induction of C2 and factor B synthesis by IFN- γ may also be mediated by activation of particular PKC isoenzyme(s) in a tissue-specific manner. In addition, it is possible that other signal-transduction pathways, which we did not investigate, may be involved in IFN- γ -mediated C2 and factor B synthesis in these cell types.

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