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Synthesis and regulation of the fourth component of complement (C4) in the human monocytic cell line U937: comparison with that of the third component of complement (C3)

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SUMMARY

Production of the fourth component of complement (C4) by the human monocytic cell line U937 and its regulation were investigated in comparison with the production of the third component of complement (C3) in a cell culture system. Although no detectable C4 was produced by U937 without stimulation, U937 was induced by recombinant interferon-gamma (IFN-γ) to synthesize C4 in a dose- and time-dependent fashion. The production of C4 was reversibly inhibited by cycloheximide, indicating that it resulted from *de novo* synthesis. The C4 synthesized by U937 cells was functionally active as assessed by haemolytic assay. SDS–PAGE following biosynthetic labelling showed that subunit structure of C4 synthesized by U937 cells was identical with that of plasma C4 but that molecular weight of α-chain was greater than that of plasma C4. We compared the regulation of C4 synthesis with that of C3 synthesis. Although C3 synthesis by U937 cells was enhanced by IFN-γ, lipopolysaccharide (LPS) and phorbol myristate acetate (PMA), C4 synthesis was induced only by IFN-γ. LPS and IFN-γ induced a synergistic increase in C3 synthesis by U937 cells. U937 cells incubated with LPS and IFN-γ synthesized a greater amount of C4 than those incubated with IFN-γ alone. Thus it was demonstrated that the synthesis of C3 and C4 was independently regulated. This study shows that the U937 cell line provides a useful model for studies on the synthesis of complement proteins and on the regulation of complement production.

INTRODUCTION

The fourth component of complement (C4) is a three-chain, disulphide-linked glycoprotein with M_r ~200,000.[1] The C4 protein is synthesized by hepatocytes and monocytes as a single-chain precursor, pro-C4, and is glycosylated and cleaved to form the disulphide-bonded subunits (α, β and γ) of native extracellular C4.[2]

U937 is a human monocyte-like cell line established from human histiocytic lymphoma.[3] It has monoblastic and immature monocytic characteristics and can be induced to differentiate into monocyte/macrophage-like cells with various agents, including phorbol myristate acetate (PMA), vitamin D_3, and interferon-gamma (IFN-γ).[4]

Monocytes and macrophages may provide an important extrahepatic source of several complement components, including C4.[5,6] Unlike human monocytes, which are difficult to obtain in large numbers in pure form, U937 cells are easily available in unlimited supply. Recently, U937 cells have been shown to synthesize C1-inactivator,[7] C2,[8] C3,[9] factor B,[10] factor D[11] and factor H.[12] The synthesis of C4 by U937 cells has not yet been described. In this study, we first show that U937 cells synthesize and secrete C4, and then compare the regulation of C4 synthesis by U937 cells with that of C3.

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MATERIALS AND METHODS

Reagents

RPMI-1640 was purchased from Gibco (Grand Island, NY). Foetal calf serum (FCS) was from Sera Lab. (Crawley Down, Sussex, U.K.). Penicillin and streptomycin were obtained from Meiji Co. (Tokyo, Japan). Recombinant IFN-γ was obtained from Shionogi Co. (Tokyo, Japan). Lipopolysaccharide (LPS) (*Escherichia coli* strain, B6) was purchased from Difco (Detroit, MI). Interleukin-1 (IL-1) was obtained from Genzyme Co. (Boston, MA). Phorbol 12-myristate 13-acetate (PMA), cycloheximide and phenylmethanesulphonylfluoride (PMSF) were purchased from Sigma (St Louis, MO). [^{35}S]Methionine and Amplify were from Amersham International (Amersham, Bucks, U.K). Goat anti-human C3, goat anti-human C4, rabbit anti-human C3, rabbit anti-human C4, horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and rabbit anti-goat IgG were purchased from MBL (Nagoya, Japan). ELISA plates

(Immulon 600) were obtained from Greiner (Frikenhausen, Germany). Sensitized sheep erythrocytes were obtained from Ishizu Co. (Tokyo, Japan). Purified human C3 and C4 were from Cordis Lab. (Miami, FL).

Cell culture

U937 cells, provided by Japanese Cancer Research Resources Bank, were grown in RPMI-1640 medium with 10% heat-inactivated (56°, 2 hr) FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2/95% air atmosphere at 37°. For C3 and C4 assay, U937 cells (1×10^5 cells/ml) were cultured in the absence or presence of varying concentrations of IFN-γ, LPS, IL-1 or PMA for 72 hr. Culture supernatants were used for the assay. Cell viability were assessed by trypan blue exclusion.

Assay of C3 and C4 by ELISA

C3 and C4 were assayed by our previous method with some modification using ELISA.[13] Briefly, the wells in ELISA plates were coated with goat anti-human C3 or C4. After blocking with bovine serum albumin (BSA), samples or known concentrations of purified C3 or C4 were added and incubated. After washing, the plates were treated with rabbit anti-human C3 or C4, horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and 2,2′-azino-*bis* (3-ethylbenzothiasoline-6-sulphonic acid) (ABTS) as a substrate for HRP. The enzyme activity was detected under an autoreader (SLT-Lab Instruments, Groedich, Austria). The value of the supernatants was compared with a standard curve obtained from purified C3 or C4.

Assay of C4 haemolytic activity

C4 haemolytic activity was assayed by a one-step method using C4-deficient serum of guinea-pig (C4D).[14] 0·25 ml of sensitized sheep erythrocytes ($1{\cdot}5 \times 10^8$ cells/ml) was mixed with 0·25 ml of 50-fold-diluted C4D and 0·25 ml of samples for 2 hr at 37°. Reactions were stopped with 2·5 ml ice-cold EDTA buffer. A range of dilutions of normal human serum was assessed in parallel as a control. The erythrocyte lysis was estimated by measurement of the OD_{415} after centrifugation.

Metabolic labelling of intracellular and secreted proteins

U937 cells (5×10^6 cells/ml) were incubated in methionine-free RPMI-1640 at 37 for 1 hr. [^{35}S]Methionine (500 μCi/ml) was added and incubated for 16 hr. The culture supernatant was collected by centrifugation for immunoprecipitation. The cells were washed twice in PBS and lysed with 2 ml cold lysis buffer [0·05 M Tris/HCl/1 mM PMSF/0·4% (w/v) Triton X-100, pH 7·5]. The lysate was collected by centrifugation for 15 min at 10,000 ***g*** and analysed by immunoprecipitation.

Immunoprecipitation, gel electrophoresis and autoradiography

Immunoprecipitation was done by solid phase immunoprecipitation technique (SPIT).[15] The wells of ELISA plate were coated with rabbit anti-goat IgG and treated with goat anti-human C4. After blocking with BSA, culture supernatants or lysates were added and incubated. The wells were washed with PBS by 1% NP-40 and immunoprecipitates were dissolved in Laemmli's sample buffer [0·05 M Tris/HCl/1% SDS/10% glycerol/5% 2-mercaptoethanol (2-ME)/0·01% bromophenol blue, pH 6·8] and boiled for 5 min. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli,[16] a resolving gel of 9% acrylamide being used. The gel was treated with Amplify, dried and exposed to Kodak XAR film at −70° for autoradiography.

RESULTS

Induction of C4 production by IFN-γ

U937 cells were cultured for 72 hr in the absence or presence of varying concentrations of IFN-γ (Fig. 1). Unstimulated U937 cells produced a small amount of C3 but no measurable C4. It was found, however, that in the presence of IFN-γ, U937 cells produced C4 at a concentration of 1 U/ml IFN-γ; IFN-γ enhanced the C4 production in a dose-dependent manner. IFN-γ also enhanced the C3 production by U937 cells in a dose-dependent manner but the effect of IFN-γ was greater in C4 production.

Kinetics of production of C4 by U937 cells

U937 cells were cultured for 24, 48, 72 hr in the presence of 1000 U/ml of IFN-γ and C4 in the culture supernatants was measured by ELISA. As shown in Fig. 2, U937 produced measurable C4 at 24 hr and produced increasing amounts of C4 up to 72 hr.

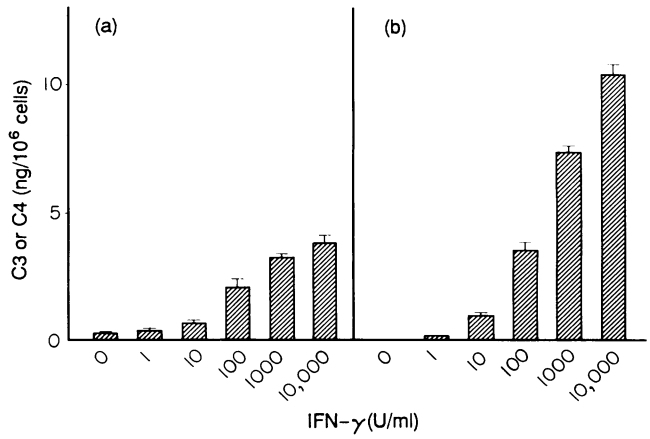

Figure 1. Effect of varying concentrations of IFN-γ on the production of C3 (a) or C4 (b) by U937 cells over 72 hr. C3 or C4 concentrations in the culture supernatants were measured by ELISA. The results were corrected according to the cell concentrations at 72 hr. The data represent the mean ± SEM of triplicate measurements.

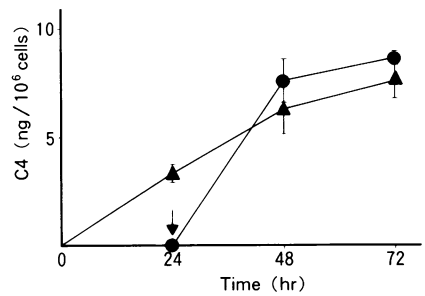

Figure 2. Kinetics of production of C4 by U937 cells. The cells were cultured in the presence of 1000 U/ml of IFN-γ and with (●) or without (▲) cycloheximide (5 μg/ml). After 24 hr (arrow), the cycloheximide-treated cells were washed and recultured in cycloheximide-free medium. Each point is the mean ± SEM of the triplicate determinations.

Table 1. Stimulatory effects of IFN-γ on the synthesis of haemolytically active C4 by U937 cells

IFN-γ (U/ml)	C4 activity (effective mol × 10^{-7}/10^6 cells)	Specific activity (effective mol × 10^{-6}/ng C4 protein)
0	0	0
1	0	0
10	0·21 ± 0·02	2·1
100	1·54 ± 0·09	4·2
1000	3·10 ± 0·01	4·2
10,000	4·54 ± 0·36	4·4

U937 cells (1×10^5/ml) were cultured for 72 hr in RPMI-1640 with 10% FCS in the presence or absence of various concentrations of IFN-γ. C4 activity in the supernatants is the mean ± SEM calculated from values obtained from triplicate cultures.

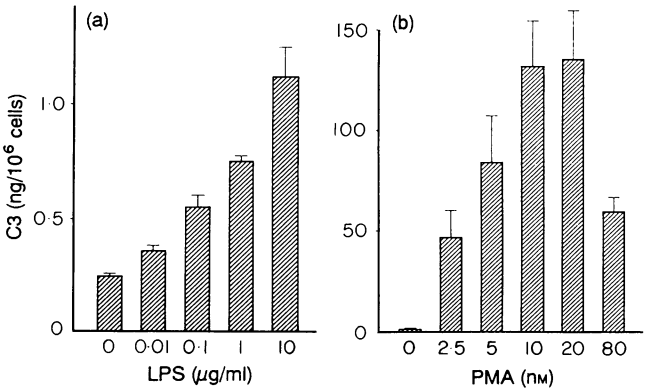

Figure 3. Effect of varying concentrations of LPS (a) or PMA (b) on the production of C3 by U937 cells. The data represent the mean ± SEM of triplicate measurements.

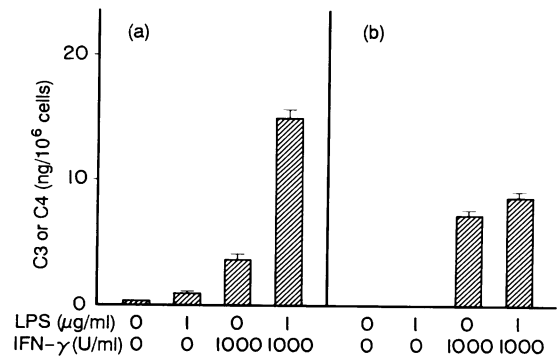

Figure 4. Effect of LPS and IFN-γ on the production of C3 (a) or C4 (b) by U937 cells. The data represent the mean ± SEM of triplicate measurements.

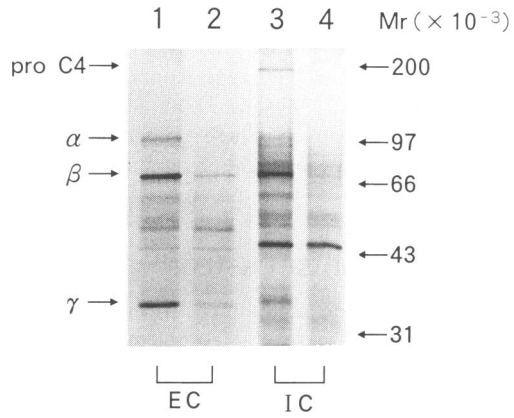

Figure 5. Autoradiograph of SDS–PAGE of U937 extracellular supernatant (EC) and intracellular lysate (IC) after biosynthetic labelling of [^{35}S]methionine and immunoprecipitation with anti-C4. (1) supernatant; (2) supernatant immunoprecipitated with unlabelled C4; (3) lysate; (4) lysate immunoprecipitated with unlabelled C4.

Synthesis of C4 was completely blocked in the presence of 5 μg/ml of cycloheximide. However, when the cells were washed after 24 hr and recultured in cycloheximide-free medium, increasing amounts of C4 production were found at 48 and 72 hr. U937 cells preincubated with IFN-γ and cycloheximide produced greater amounts of C4 during the first 24 hr than those without cycloheximide.

Haemolytic activity of secreted C4

The functional activity of the newly synthesized C4 protein was investigated by haemolytic assay (Table 1). Haemolytic activity in the supernatant of cultured U937 cells was detected when U937 cells were stimulated by 10 U/ml of IFN-γ and it increased in a dose-dependent manner. The specific activity of C4 produced by U937 cells was similar to that of fresh normal human serum determined in parallel ($4{\cdot}0 \times 10^6$ effective molecules/ng of C4).

Induction of C3 or C4 production by U937 by various stimulants

U937 cells were cultured for 72 hr in the presence of varying concentrations of LPS, PMA, IL-1 and IFN-γ. LPS and PMA stimulated C3 production in a dose-dependent fashion (Fig. 3) but had no stimulatory effect on C4 production. On the other hand, IL-1 had no effect on either C3 or C4 production (data not shown). A synergistic enhancement of C3 production was observed when U937 cells were incubated with LPS (1 μg/ml) and IFN-γ 1000 U/ml). U937 cells incubated with LPS and IFN-γ synthesized greater amounts of C4 than those incubated with IFN-γ alone (Fig. 4). Although the enhancement by LPS was small, we obtained the same results by repeated experiments.

Biosynthetic labelling and SDS–PAGE analysis of C4

C4 was immunoprecipitated with anti-human C4 antibody from the supernatants or lysates of U937 cells metabolically labelled with [^{35}S]methionine and analysed by SDS–PAGE (under reducing conditions) and autoradiography (Fig. 5). The extracellular C4 was characterized by three radioactive bands of M_r

99,000, 74,000 and 34,000 corresponding to the α-, β- and γ-chains respectively. The intracellular lysates showed a major radioactive band of M_r 200,000 corresponding to the pro-C4 and those of M_r 99,000, 74,000 and 34,000 corresponding to the α-, β- and γ-chains respectively. These radioactive bands with C4 were blocked when they immunoprecipitated with excess of unlabelled purified C4.

DISCUSSION

There is increasing evidence that cells from monocyte/macrophage lineage synthesize several complement components.[17] Recently, Kulics *et al.*[6] demonstrated the biosynthesis of C4 in human monocytes.

In the present study, we first demonstrated that the biosynthesis of C4 by U937 cells was induced by IFN-γ. Although unstimulated U937 cells synthesized no measurable C4, IFN-γ induced U937 cells to synthesize C4 in a dose- and time-dependent fashion. The synthesis of C4 by U937 cells was reversibly inhibited with cycloheximide, indicating that C4 produced in the culture supernatant resulted from *de novo* synthesis. U937 cells preincubated with IFN-γ and cycloheximide produced greater amounts of C4 during the first 24 hr than those without cycloheximide. Lappin *et al.*[18] reported that in the presence of cycloheximide, the abundances of C1-inhibitor messenger RNA (mRNA), C2 mRNA and factor B mRNA increased in human monocytes. The abundance of C4 mRNA may also increase during the incubation with cycloheximide in U937 cells and result in the rapid raise of C4 production after removal of cycloheximide. The C4 synthesized by U937 cells was functional as assessed by haemolytic assay.

SDS–PAGE following biosynthetic labelling showed that extracellular C4 synthesized by U937 cells had the same subunit structure as the plasma form of C4. Although the size of β- and γ-chains were identical to those of plasma C4, the size of α-chain ($M_r \sim 99{,}000$) was greater than that of plasma C4 ($M_r \sim 94{,}000$) but was identical to that previously reported from hepatocytes and macrophages.[2] Previous studies showed that α-chain of C4 circulating in human plasma was ~5000 MW smaller than that of C4 secreted by hepatocytes and macrophages and that the structural differences resided at the COOH terminus.[2] Intracellular C4 showed partial processing of pro-C4 to α-, β-, and γ-chains.

We compared the regulation of C4 synthesis with that of C3 synthesis. U937 cells synthesized C3 spontaneously and IFN-γ enhanced C3 synthesis in a dose-dependent fashion similar to C4 synthesis. Previous studies showed that IFN-γ produced increases in the abundance of C1-inh, C2, factor B and C4 binding-protein mRNA in association with increases in secretion rates of these four proteins in human peripheral blood monocytes.[18,19] IFN-γ increased the secretion rate of factor H without increases in factor H mRNA.[19] In contrast, IFN-γ reduced the C3 synthesis by human monocytes.[18,20] Although the mechanism of the discrepancy with our results of U937 is unclear, it may be due to the difference of cell maturation. It is of interest that LPS and PMA induce the synthesis of C3, but not C4, by U937 cells. LPS, however, when added together with IFN-γ in the culture of U937 cells, enhanced the production of C4 more than IFN-γ alone. Kulics *et al.*[6] showed that LPS and IFN-γ have counter-regulatory effects on C4 synthesis by human monocytes. This difference may be a reflection of a different stage of cell maturation. Likewise, LPS and IFN-γ induced synergistic increase in C3 synthesis by U937 cells. IFN-γ and LPS have also been reported to induce a synergistic increase in factor B synthesis by U937 cells.[10] The basis for this synergism in C3 synthesis is unclear. Vitamin D_3 and granulocyte–Macrophage colony-stimulating factor (GM-CSF) have been reported to up-regulate IFN-γ receptors on U937 cells.[21] LPS might have a similar effect on U937 cells.

As described above, it was demonstrated that the synthesis of C3 and C4 were independently regulated. C3 is a component of both classical and alternative pathways and C3 synthesis is enhanced by several compounds which directly stimulate monocyte/macrophages such as LPS, PMA and IFN-γ. The regulation of C3 synthesis resembles that of factor B which is a component of the alternative pathway. Factor B synthesis by U937 cells is enhanced by LPS, PMA, IL-1 and IFN-γ.[10] On the other hand, C4 is a component of the classical pathway, and in this study, C4 synthesis by U937 cells was regulated only by IFN-γ which is produced mainly by activated T cells as a result of immune response.[22] It seems that the components of the alternative pathway are quickly synthesized as an initial host response at local sites of infection or inflammation, followed by the synthesis of components of the classical pathway which are involved in the immunological events.

The local synthesis of complement proteins by mononuclear phagocytes may play an important role in the inflammatory response.[5] On the other hand, U937 is a convenient model and source of immature human monocytes and has been used for a variety of investigations. Here we show that the U937 cells have a capacity to synthesize C4 and this suggests that the cells of monocyte/macrophage lineage can synthesize C4 even at an early stage of differentiation. Further studies are warranted about the relationship between monocyte/macrophage differentiation and complement synthesis, and about the molecular mechanisms of regulation of complement synthesis by U937 cells.

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