

Regulation of immunoglobulin secretion by Factor H of human complement

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Summary. As human B lymphocytes and macrophages carry surface receptors for Factor H (B1H), we investigated the possibility that this complement component regulates their function. Factor H inhibits immunoglobulin secretion by peripheral mononuclear cells (MNC) stimulated with pokeweed mitogen if present at the initiation of the cultures and at concentrations greater than 50 µg/ml. Factor H also inhibited stimulation and differentiation of purified B cells into immunoglobulin-secreting cells by Epstein-Barr virus (EBV). The inhibitory effect of Factor H was abrogated if anti-Factor H antibody was present in the cultures. EBV-transformed B-cell lines secreted less immunoglobulin if Factor H was present in the culture for at least 4 days. Culture of MNC with Factor H did not lead to the generation of suppressor T cells or macrophages. In contrast, Factor H did not cause proliferation of human peripheral total MNC or enriched T-cell or B-cell subpopulations. Also, Factor H did not inhibit the proliferation of MNC in response to several mitogens and antigens. Our results strongly indicate that Factor H is able to block human B-cell differentiation *in vitro* without blocking the proliferative ability of the cells. Factor H seems to act directly on the B cells through its receptor on their surface, since it inhibited T-dependent and T-independent

B-cell differentiation but generated no suppressor cells.

INTRODUCTION

Complement is classically considered to be part of the effector branch of the immune system by virtue of its cytotoxic mechanism. Certain complement factors, as well as complement breakdown products, actively participate in the regulation of the immune responses. Examples are the effects of C3a on proliferative responses of lymphocytes, production of migration inhibitory factor and humoral immune response (Hobbs *et al.*, 1981; Morgan *et al.*, 1983), the effects of C3b, C3c and C3d on lymphocytic proliferation and antibody production (Dukor *et al.*, 1974; Möller & Coutinho, 1975; Hartmann & Bokish, 1975; Tsokos, Berger & Balow, 1984), the effects of C4 on human and guinea-pig immune responses (Burger & Shevach, 1979; Ochs *et al.*, 1983), the effect of C5 and complement Factor B on lymphocyte activation (Sundsmo, 1983) and, finally, the effects of Factor B on monocyte/macrophage function (Sundsmo & Götze, 1980, 1983).

Factor H is a regulatory protein of the alternative pathway of complement activation. It regulates the complement cascade by binding to C3b (Conrad, Carlo & Ruddy, 1978; Fearon, 1978) and serving as a cofactor of Factor I-induced cleavage of C3b to iC3b (Pangburn, Schrieber & Müller-Eberhard, 1977; Ross

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et al., 1982). Factor H is synthesized by human peripheral blood monocytes (Whaley, 1980) and has been found in human platelets and lymphocytes (Kennedy & Davies, 1981; Wilson *et al.*, 1982; Schulz *et al.*, 1984). Factor H binds to specific cell surface membrane receptors (Lambris & Ross, 1982) and triggers several cellular functions like blastogenesis of murine splenocytes (Hammann *et al.*, 1981), release of Factor I by lymphocytes (Lambris, Dobson & Ross, 1980) and enhancement of the respiratory burst in human peripheral monocytes (Schopf *et al.*, 1982). We designed the present experiments to investigate the effect of Factor H on proliferative and humoral responses of human peripheral lymphocytes *in vitro*.

The results indicate that, although Factor H neither induces proliferation nor inhibits proliferative responses to antigens and mitogens, it inhibits the differentiation of peripheral B lymphocytes to immunoglobulin-secreting cells after stimulation with pokeweed mitogen (PWM) or Epstein-Barr virus (EBV).

MATERIALS AND METHODS

Mononuclear cells (MNC) isolation

Human peripheral venous blood was drawn into heparinized syringes. After dilution (1:2) with phosphate-buffered saline (PBS), it was centrifuged over Ficoll-Paque (Pharmacia, Piscataway, NJ) and the interface containing MNC was collected and washed with RPMI-1640.

Plaque assay

B-cell responses were estimated by the use of a reverse haemolytic plaque assay modified in our laboratory (Tsokos & Balow, 1981). SeaPlaque agarose (FMC Corp., Rockland, ME) was dissolved (0.8%) in RPMI-1640 containing 2 mM HEPES buffer (Microbiological Associates, Walkersville, MD) by heating to boiling, and then left to equilibrate at 42° in a water-bath. Agarose, 0.85 ml, was mixed with sheep red blood cells (SRBC) coupled with 0.05 ml of *Staphylococcus* protein A by use of chromic chloride and with 0.1 ml of MNC to be tested. The mixture was vortexed, then poured into 15 × 66 mm petri-dishes (Falcon, Oxnard, CA) prelayered with 1.2% SeaKem agarose (FMC Corp) in PBS. After the gel had solidified, 1 ml of developing antiserum was added. This developing antiserum was the IgG fraction of

rabbit anti-human immunoglobulins (IgA + IgG + IgM) purchased from Cappel Laboratories (Downingville, PA), diluted 1:50 in RPMI-1640. The petri-dishes were incubated for 2 hr at 37° and then the developing antiserum was replaced with 1 ml of guinea-pig complement (Cappel Laboratories) diluted 1:10 in veronal-buffered saline containing 0.5% gelatin.

Fractionation of MNC

MNC were fractionated into SRBC rosette-forming (E⁺) and non-SRBC rosette-forming (E⁻) cells by the use of SRBC pretreated with 2-aminoethylisothiourea bromide hydrobromide (AET; Sigma, St Louis, MO). SRBC were incubated for 15 min at 37° with 0.15 M AET (pH 9.0) and then washed six times with saline solution and resuspended in PBS containing 10% fetal calf serum (FCS, Gibco, Grand Island, NY) at a concentration of 4%. Equal volumes of SRBC and MNC (10 × 10⁶ ml) suspensions were incubated for 10 min at room temperature, spun at 200 g for 5 min, further incubated at 4° for 30 min, layered over Ficoll-Paque cushions and spun for 25 min at 400 g. The interface containing the E⁻ cells and the pellet containing the E⁺ cells were collected in separate tubes. The E⁺ cells were incubated for 10 min at room temperature with 10 ml of ACK lysing buffer (NIH, Media Unit) and then washed twice with RPMI-1640. E⁻ cells were re-rosetted on most occasions and the percentage of E⁺ cells was always less than 1%. E⁺ and E⁻ cells were stained (indirect immunofluorescence) with Leu 1 monoclonal antibody (Becton-Dickinson, Sunnyvale, CA): the former were >95% positive, whereas the latter showed less than 2% of cells with surface staining. MNC were depleted of monocytes by passage through Sephadex G-10 columns as previously described (Tsokos, Magrath & Balow 1983).

Culture of human MNC

Cultures were carried out in 24-well flat-bottomed plates (Costar, Cambridge, MA), each well containing 2 ml of MNC or E⁻ cell suspension (0.5 × 10⁶ ml) in RPMI-1640 with 50 µg/ml streptomycin and 50 units/ml penicillin (NIH, Media Unit) and 10% heat-inactivated FCS. Pokeweed mitogen (PWM; Gibco) was used at a final concentration of 1% of the stock solution. EBV-containing supernatants of a lymphoblastoid cell line (B95) infected with EBV were used at a concentration of 5% of the final culture media, which

proved to provide optimal plaque-forming cell (PFC) responses.

Proliferative responses

Cultures for the estimation of the proliferative responses were carried out in 96-well round-bottomed plates (Linbro Division, Flow Laboratories, Inc., Hamden, CT) in 0.2 ml final volume with four replicates for each experimental point. Cell concentration was 1×10^6 /ml in RPMI-1640 containing 10% FCS. Concentrations of Factor H, phytohaemagglutinin (PHA), concanavalin A (Sigma), tetanus toxoid (TT), PWM, and OKT3 monoclonal antibody (Ortho Pharmaceutical Company, Raritan, NJ) were used at the indicated concentrations. Cultures were harvested on Day 3 or 6, as indicated, by the use of a multichannel harvester following a 6-hr pulse with 0.5 μ Ci tritiated thymidine (New England Nuclear, Boston, MA). Results are expressed as counts per minute (c.p.m.).

EBV-transformed B-cell lines

MNC were depleted of E⁺ cells and were subsequently infected with EBV and grown in culture flasks. Cells were divided and fed with fresh media (RPMI-1640 containing 10% FCS) every 3–4 days. The B-cell lines used in the experiments presented here were 10 weeks old and consisted of cells that produced (tested by cytoplasmic staining), carried on their surface (tested by indirect immunofluorescence), and secreted (tested by reverse haemolytic plaque assay) IgG.

Preparation of Factor H

Factor H was prepared as previously described (Lambris *et al.*, 1980) from human plasma. Alternatively, after passage through a Sepharose CL-6B column, the Factor H pool was loaded onto a Mono Q column using the FPLC system (Pharmacia) equilibrated with 20 mM phosphate buffer, pH 7.5. The Factor H retained in the column was eluted with a 30 ml gradient of NaCl (0–500 mM). A representative profile of the eluate is given in Fig. 1. The pooled Factor H was homogenous when tested by both sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunodiffusion analysis with several antisera. Rabbit anti-factor H antibody was prepared as previously described and purified by affinity chromatography (Lambris & Ross, 1982).

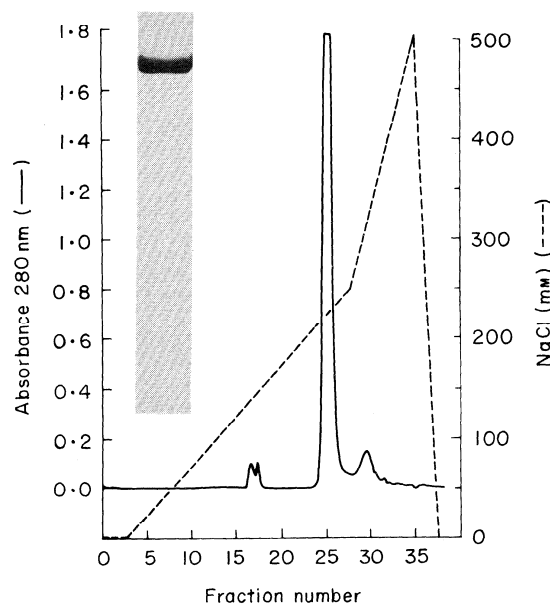


Figure 1. Purification of human Factor H by FPLC. The CL-6B sepharose Factor H pool was concentrated to 8 mg/ml, then dialysed against 20 mM phosphate buffer, pH 7.5, and 1 ml was injected into a Mono-Q column (5 × 50 mm) and equilibrated with 20 mM phosphate buffer, pH 7.5. Factor H was eluted with a 30 ml gradient of NaCl (0–500 mM) at a flow rate of 2 ml/min.

RESULTS

Inhibition of PFC responses of MNC to PWM

Factor H effectively inhibited the development of PFC after stimulation of MNC with PWM in a dose-dependent manner (Fig. 2). At a concentration of 50 μ g/ml, Factor H inhibited this response by 50%, and at 100 μ g/ml by 80%; 10 μ g/ml was not inhibitory. MNC cultured in the presence of FCS, but not mitogen, developed lesser but appreciable PFC responses which seemed to be inhibited by Factor H (Fig. 2). Optimal responses were obtained on Days 6 or 7 using 1% PWM in cultures with or without Factor H (results not shown).

In order to exert its inhibitory effect on the PFC response, Factor H had to be present at the initiation of the cultures (Fig. 3). If we delayed adding Factor H until 2 days after culturing began, inhibition was limited to 40%; if added 4–6 days later, no inhibition occurred.

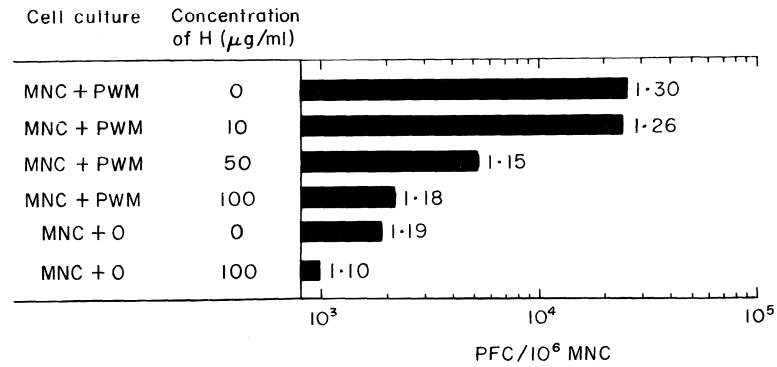


Figure 2. Inhibition of PWM-induced PFC responses of MNC by Factor H. Factor H was added to MNC when incubation began. Bars represent the geometric mean of six experiments, and the numbers at the right of each bar are the standard errors of the geometric means. Cultures were harvested on Day 6.

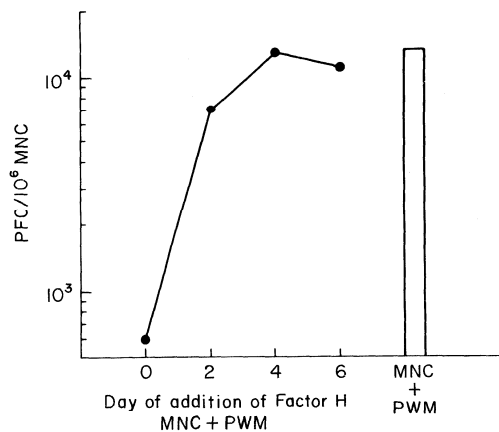


Figure 3. Factor H (100 $\mu\text{g/ml}$), added to MNC only at early phases of the cultures, inhibited the PWM-induced PFC. Points represent the mean of two experiments. Cultures were harvested on Day 7.

Inhibition of EBV-induced PFC responses of human B cells

Human B cells (MNC depleted of E^+ cells) transform into immunoglobulin-secreting cells if infected with EBV. Significant numbers of PFC (immunoglobulin-secreting cells) can be detected as early as 3 days after the infection, with further expansion later (Tsokos, Magrath & Balow, 1983). Factor H added at a concentration of 100 $\mu\text{g/ml}$ at the initiation of such cultures abrogated the transformation of B cells into PFC (Table 1). Similar results were obtained (data not shown) when the E^- cells had been further depleted of

monocytes by passage through a Sephadex G-10 column. Therefore, Factor H seemed to inhibit B-cell differentiation by direct action on B cells rather than through accessory cells.

In order to examine if factor H can inhibit the secretion of immunoglobulin by established EBV-transformed B-cell lines, we cultured such cells in medium containing 100 μg Factor H and estimated the PFC responses 2 and 4 days later. Although the number of PFC was unchanged on Day 2, by Day 4 the number of PFC in cultures containing Factor H decreased significantly (Table 2). The need for the continuous presence of Factor H for at least 4 days to express its inhibitory effect on transformed B cells duplicated that observed when PWM was used to induce PFC in cultures of MNC.

Anti-H antibody added to cultures containing Factor H subsequently abolished this inhibitory effect.

Table 1. Factor H inhibits EBV-induced PFC responses of human B cells

	Factor H ($\mu\text{g/ml}$)	Anti-Factor H*	Exp. 1	Exp. 2
B cells	0	0	66†	246
B cells	EBV	0	8200	23,400
B cells	EBV	100	620	3800
B cells	EBV	100	+	8800
B cells	EBV	0	+	6500

* 50 $\mu\text{g/ml}$.

† PFC/ 10^6 cells, means of duplicates.

Table 2. Continuous presence of Factor H is necessary to inhibit the secretion of immunoglobulin by EBV-transformed B-cell lines

Culture	Day 2	Day 4
Medium	20,000*	16,000
Factor H	19,500	5900

* PFC/10⁶ B cells; numbers are the means of two experiments involving two different cell lines.

Table 1 shows that anti-H antibody abrogated the inhibitory effect of Factor H in the EBV-induced B-cell PFC response, but that the antibody itself lacked any effect. Similar observations were made when anti-H antibody was added to cultures of MNC driven by PWM and inhibited by Factor H (Table 3).

Table 3. Abrogation of the inhibiting effect of Factor H on PWM-induced PFC response by goat anti-H antibody

	Factor H	Anti-H antibody*	PFC/10 ⁶ MNC
MNC+PWM	0	0	12,200†
MNC+PWM	100 µg	0	1100
MNC+PWM	100 µg	+	11,150
MNC+PWM	0	+	12,560
MNC+0	0	0	1300
MNC+0	100 µg	0	605
MNC+0	0	+	560

* Added at a final concentration of 50 µg/ml.

† Mean of two experiments.

Factor H does not induce the generation of suppressor cells

MNC cultured with Factor H (100 µg/ml) for 2 or 4 days did not express any suppressor cell activity when added to fresh autologous MNC stimulated with PWM. Factor H-pretreated MNC allowed the induction of PFC responses of fresh MNC stimulated with PWM at levels comparable to those of MNC pretreated with medium alone. In contrast, MNC pretreated with Con A profoundly suppressed the PWM-induced PFC response of MNC, as expected (Table 4).

Table 4. Factor H does not stimulate the generation of suppressor cells

Target culture	Addition of MNC pretreated with	PFC/10 ⁶ MNC*
MNC+0	0†	950
MNC+PWM	0	13,950
MNC+PWM	Medium (2 days)‡	15,100
MNC+PWM	Con A (2 days)	1800
MNC+PWM	Factor H (2 days)	14,500
MNC+PWM	Medium (4 days)	11,300
MNC+PWM	Factor (4 days)	10,800

* Mean of two experiments.

† No precultured cells added.

‡ MNC were precultured with medium, Con A (10 µg/ml) or Factor H (100 µg/ml) for 2 or 4 days, washed three times with medium containing 2 mM alphanethylmannoside, and then added to the target cells at a cell ratio of 1:1.

Factor H neither induces nor inhibits the proliferation of peripheral MNC

When Factor H isolated from human plasma by FPLC was tested at concentrations up to 100 µg/ml, it failed to induce DNA synthesis in human MNC within 3 or 6 days of culture (Fig. 4a). Similarly, neither T cells (E⁺) nor non-T cells (E⁻) proliferated in response to Factor H (Fig. 4b, c). Mitogens, PWM and Con A were used in these experiments as positive controls to certify the proliferative ability of the cells used on Day 3 (Con A) and Day 6 (PWM). Simultaneously, these mitogens indicated the purity of our E⁻ cell populations since their unresponsiveness to Con A is well known.

Factor H failed to inhibit the proliferative response of MNC to several stimuli. In particular, up to 100 µg/ml of Factor H did not inhibit the proliferative response of MNC to PHA (harvested on Day 3), PWM or TT (harvested on Day 6). Similarly, Factor H failed to modify the proliferative response of T lymphocytes to OKT3 monoclonal antibody. EBV-induced B-cell proliferation was not affected by Factor H; finally, the proliferation of established EBV-transformed lymphoblastoid cell lines was not inhibited by up to 100 µg/ml of Factor H.

DISCUSSION

The major conclusion derived from these studies is that Factor H inhibits the differentiation of human B

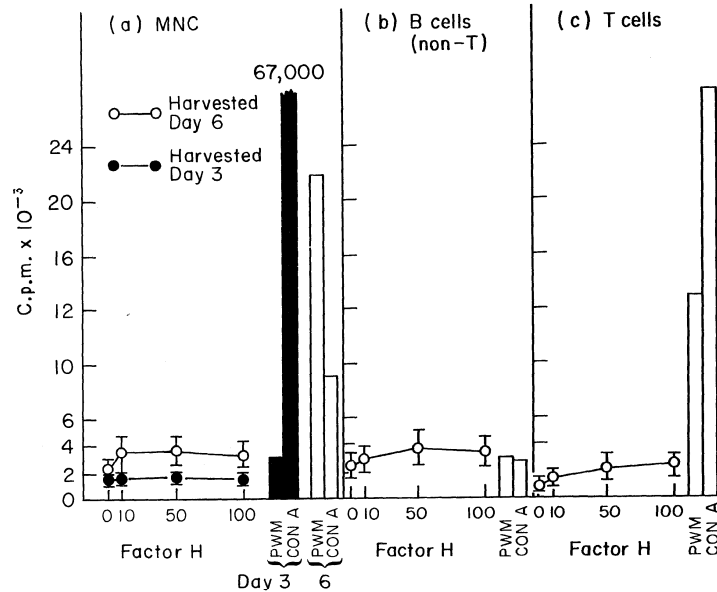


Figure 4. Proliferative responses of human (a) peripheral MNC; (b) B (non-T) cells, and (c) T cells. Cultures were harvested on Days 3 and 6, as indicated, after having added [^3H]thymidine ($0.5 \mu\text{Ci}$) prior to that. PWM was used at a concentration of 1%, and Con A at $10 \mu\text{g/ml}$.

cells into immunoglobulin-secreting cells in response to either T-cell dependent (PWM) or T-cell independent (EBV) stimuli. Factor H had to be present in the early phases of the cultures to inhibit B-cell differentiation. In repeated efforts, we were unable to demonstrate any proliferative response of human peripheral MNC to FPLC-purified Factor H; similarly, MNC depleted of E^+ cells ($< 1\%$ E^+ cells unresponsive to the T-cell mitogen Con A) did not exhibit proliferative responses to Factor H. Similar results were obtained, whether FCS or heat-inactivated human serum (data not shown) was used. Yet Hammann *et al.* (1981) have reported that murine spleen cells carrying complement receptors exhibited significant proliferative response if cultured with human Factor H. Several factors may be responsible for the differences in our results: (i) we used human cells as responders to human Factor H; (ii) Factor H used in this study was FPLC-purified, and thus purer than their material obtained by standard chromatographic procedures; (iii) the possibility that contaminants caused the proliferative responses observed by Hammann *et al.* (1981) is supported by the fact that they failed to observe binding of B1H onto the surfaces of murine B lymphocytes.

The probable route by which Factor H exerted its

inhibitory effect on the development of immunoglobulin-secreting cells is through direct action on B cells. This is our conclusion for the following reasons: (i) human B cells carry receptors on their surface membranes that bind human Factor H (Lambris *et al.*, 1980; Schmitt *et al.*, 1981; Lambris & Ross, 1982); (ii) EBV (strain B-95)-induced B-cell transformation is monocyte and T-cell independent (Tsokos, Magrath & Balow, 1983). The experiments presented here show that Factor H could inhibit B-cell differentiation induced by EBV, and one can assume that this phenomenon was caused by direct action on B cells rather than by interference with the function of monocytes and helper T cells; and (iii) Factor H failed to induce the generation of suppressor cells from peripheral MNC (Table 4). This experiment excluded the possibility that the observed inhibition of PWM-induced PFC responses was secondary to the generation of suppressor cells. Other direct effects of Factor H on B cells have also been described. By binding to its specific receptor on human B cells and B-cell lines, Factor H directly conveys functional messages such as the signal regulating release of Factor I (Lambris *et al.*, 1980).

The recent explosion of information of B-cell

biology has established that growth and differentiation of B cells are governed by independent factors (Howard & Paul, 1983). Conceivably, the action of each is regulated independently, so substances that inhibit the action of the B-cell differentiation factor do not affect that of B-cell growth factor. Within this setting, Factor H could play a selective role. The presence of specific receptors for Factor H on lymphoid cells and the apparent effects of this protein on antibody production (present report) strongly suggest that Factor H is involved in immunoregulation. Several possibilities can explain the fact that lower concentrations of Factor H than those present in normal human serum (500 $\mu\text{g}/\text{nl}$) were needed to inhibit antibody production. It has been shown that self-aggregation of Factor H increases its binding affinity to the H receptor (Lambris & Ross, 1982). It was recently shown, using hydrophobic affinity chromatography, that two molecular populations of Factor H exist. These two populations were different only in their ability to aggregate in low- and iso-ionic strength media (Ripoche *et al.*, 1984). Aggregated, or complex-bound, Factor H might be the biologically active form of this protein which, upon binding and cross-linking of its receptors on the surface membrane of B cells, leads to inhibition of antibody production. Alternatively, Factor H isolation procedures might have amplified the generation of a biologically active form of Factor H similar to that observed for properdin (Whaley, Ward & Ruddy, 1979). Such an active form might be present in serum or in the microenvironment of lymphoid organs at low concentrations.

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