Identification of human complement factor H as a chemotactic protein for monocytes

Karim NABIL*, Bertrand RIHN†, Marie-Claude JAURAND‡, Jean-Michel VIGNAUD§, Jean RIPOCHE‖, Yves MARTINET* and Nadine MARTINET*[1]

*Institut National de la Santé et de la Recherche Médicale, Unité 14, CHU Brabois, C.O. No. 10, 54511 Vandœuvre Les Nancy, France, †Institut National de la Recherche et de la Sécurité, 54511 Vandœuvre Les Nancy, France, ‡Institut National de la Santé et de la Recherche Médicale, Unité 139, 8 rue Général Sarail, 94010 Créteil Cedex, France, §Service d'Anatomo-pathologie, Hôpital Central, 54000 Nancy, France, and ‖Unité de Recherche Associée du Centre National de la Recherche Scientifique 1456, 146 rue Léo-Saignat, 33076 Bordeaux Cedex, France

We used chromatographic separation to purify to homogeneity a monomeric monocyte chemotactic protein of 150 kDa contained in mesothelioma pleural effusions. It was identified by N-terminal amino acid sequencing and immunoblotting as complement factor H, an inhibitor of the alternative complement pathway. Specific antibodies against factor H inhibited the monocyte chemotactic activity of the purified protein, which was most active at 10 nM. Factor H is a restrictive factor of alternative complement pathway activation. The new chemotactic function assigned to factor H in recruiting monocytes to the mesothelioma site might contribute to malignant cell phagocytosis via the iC3b/complement receptor type 3 pathway. These functions link the humoral and cellular immune systems.

INTRODUCTION

A wide range of micro-organisms and immune complexes activate the alternative complement pathway. The initial activation event is the deposit of a C3b molecule on the surface of a potential activator [1,2]. The bound C3b then binds factor B to form C3bB, which is subsequently cleaved by factor D to form Ba and Bb. Bb remains associated with C3b, producing C3bBb, the alternative complement pathway C3 convertase (reviewed in [3,4]). The complement pathway proceeds to form a lytic complex that inserts itself in the lipid bilayer membrane, thereby inducing membrane disruption [3,4]. Factor H has a key role in regulating the activation of the alternative complement pathway C3 convertase by acting as a cofactor for factor I-mediated cleavage of C3b to iC3b [5] and by accelerating the dissociation of C3bBb by competing with factor B for binding to C3b [6]. Factor H is also a restrictive factor of classical complement pathway activation on the surface of nucleated cells through its cofactor activity for C3b degradation [7].

Human factor H is a 155 kDa monomeric glycoprotein of 1213 amino acid residues [8], of which the entire sequence is built up from 20 contiguous complement control protein (CCP) modules, also called short consensus repeats [9]. The CCP module is the most common structural unit of all the cofactors of factor I [10]. In factor H the C3b-binding site is located within CCP modules 3–5. Furthermore, testing of factor H CCP deletion mutants in CHO cells has demonstrated that CCP1–4 were required for full functional cofactor activity [11]. Currently, a large family of serum proteins structurally related to factor H are being characterized [12,13]. The human factor H gene has been mapped to the regulation complement activation gene cluster on chromosome 1q [14]. Alternative splicing of the factor H gene gives rise to a truncated form of factor H of 40–43 kDa that includes the N-terminal seven CCP modules followed by four unique amino acid residues. This shorter form retains cofactor activity [15–17]. Factor H is usually purified by immunoaffinity. The resulting factor H can be further fractionated by hydrophobic affinity chromatography on phenyl-Sepharose into two forms, termed ϕ_1 and ϕ_2, that are indistinguishable on SDS/PAGE and have identical cofactor activities [18]. However, only the ϕ_2 form binds to a factor H receptor on B lymphoblastoid cells and demonstrates aggregation at low ionic strength [18,19]. ϕ_2 possibly differs by a post-translational modification involving tyrosine sulphation [20].

Complement factor H and the plasma precursor (PMCFS-1) of the major monocyte chemotactic factor (MCFS-1) were found in the skin site of the delayed hypersensitivity reaction induced in the guinea pig [21]. By functional and immunological studies it was shown that factor H is identical with PMCFS-1. This suggests that factor H is converted into a monocyte chemotactic protein like MCFS-1 by proteolytic cleavage by an endogenous trypsin-like protease [21]. Thrombin activity is present in the delayed hypersensitivity reaction; factor H digested by thrombin was found to express monocyte chemotactic activity (MCA) [22]. This digestion product was capable of separation by reverse-phase chromatography into two protein peaks, H_1 and H_2. The H_2 form was found to be more hydrophobic. In addition, H_2 had a potent MCA, whereas H_1, like intact factor H, showed none. H_1 and H_2 were indistinguishable by SDS/PAGE and it was suggested that H_2 was identical with ϕ_2 except that it arose from proteolysis of factor H by thrombin [22].

Previously we reported that malignant pleural effusions con-

Abbreviations used: CCP, complement control protein; DMEM, Dulbecco's modified Eagle's medium; FMLP, *N*-formyl-Met-Leu-Phe; MCA, monocyte chemotactic activity; MCAF, monocyte chemotactic and activating factor; MCFS-1, major monocyte chemotactic factor 1 (skin); MCP-1, monocyte chemotactic protein 1; PEP, pleural effusion pool; PMCFS-1, plasma precursor of MCFS-1; TBS, Tris-buffered saline; TBST, TBS containing Tween-20; TFA, trifluoroacetic acid.

[1] To whom correspondence should be addressed.

tained MCA that were especially potent in mesothelioma effusions [23]. We now report the purification to homogeneity of the main MCA from mesothelioma fluids that is very probably factor H.

EXPERIMENTAL

Sample collection

Pleural effusions from patients presenting mesothelioma were collected by standard sterile clinical procedures at the department of Pneumology of Nancy. They were centrifuged at 500 ***g*** and 4 °C for 15 min to remove the cellular debris. A small aliquot of the supernatant was tested for MCA while the remaining part was divided into aliquots and frozen at −20 °C until further use. All patients had histologically documented mesothelioma reviewed by the National French Pathologists Board for the Study of Mesothelioma. Blood was collected on heparin (10 i.u./ml) from normal blood bank donors and the monocytes were separated by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation as previously described [24].

Monocyte chemotactic assay

The presence of MCA was detected, as previously described [25], with a 48-well chemotaxis chamber [26] (Neuro Probe, Cabin John, MD, U.S.A.), with normal blood monocytes used as the target cells. *N*-Formyl-Met-Leu-Phe (FMLP; Sigma Chemical Co., L'Isle d'Abeau Chesnes, La Verpillière, France) was used as a positive control at 10 nM in Dulbecco's modified Eagle's medium (DMEM; Sigma), or in DMEM alone as the negative control. The migratory response was then quantified by reading the attenuance at 605 nm after the extraction of stain from the cells with 125 mM HCl. Each sample was tested in triplicate and the results were expressed as a percentage by using the following formula: $100 \times (D_{605}$ of the sample minus D_{605} in the presence of DMEM alone)/(D_{605} in the presence of FMLP minus D_{605} in the presence of DMEM alone).

The same assay was done with the purified protein by using granulocytes as the target cells with a filter with a pore size of 8 μm.

The purified protein and the Sigma factor H MCA were tested at concentrations ranging between 1 and 50 nM.

Protein purification

A pleural effusion pool (PEP) including seven mesothelioma effusions was prepared and divided into 200 ml portions that were processed each time to begin a purification. The steps of the purification were performed at room temperature with buffers containing 1 mM PMSF (Sigma). An aliquot of each effluent and eluent fraction was dialysed in PBS before being tested for MCA. Purification was achieved by sequential chromatography, consisting of anion-exchange chromatography, then cation-exchange, reverse-phase and finally molecular-sieve chromatographies. At each step, the eluted fractions containing the highest MCA specific activity were selected for further purification.

Step 1: anion-exchange standard chromatography

DEAE-Trisacryl M resin (IBF–Sepracor, Villeneuve La Garenne, France) (600 ml) was equilibrated in buffer A [4 mM EDTA/10 mM Tris/HCl (pH 8.5)] and mixed at room temperature for 30 min with 200 ml of PEP diluted 1:27 with distilled and deionized water to reach the same conductivity as that of buffer A. The slurry was then poured into a Pharmacia chromatography column XK 50/60® (Pharmacia, Saint Quentin les Yvelines, France). Next the absorbent was washed with 3 litres of buffer A (2.5 ml/min). Elution was performed with a 640 ml linear gradient of NaCl from 0 to 1 M in buffer A. The active fractions were then concentrated 12-fold on an Amicon cell (Grace, Epernon, France) and divided into 200 μl fractions that were kept frozen at −20 °C until used.

Step 2: cation-exchange FPLC chromatography

Concentrated active fractions (200 μl) from step 1 were diluted in 8 ml of equilibration buffer B [4 mM EDTA/10 mM trisodium citrate (pH 6)] and were filtered on an Acrocap filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) before being applied to a MonoS HR 5/5® column (Pharmacia FPLC system) equilibrated in buffer B. Elution was performed with a 20 ml linear gradient of NaCl from 0 to 400 mM in buffer B.

Step 3: reverse-phase FPLC chromatography

Pooled active fractions from step 2 were dialysed with distilled and deionized water, then adjusted to 0.1 % (v/v) trifluoroacetic acid (TFA) before being applied to a Pro RPC HR 5/10® column (Pharmacia) previously equilibrated in 0.1 % (v/v) TFA in water. After extensive washing of the column, elution was performed with a 40 ml linear gradient of 0–100 % (v/v) acetonitrile containing TFA as before. To test for MCA, 100 μl of each collected fraction was mixed with 10 μl of an aqueous solution of 1 % (w/v) BSA (Sigma) and freeze-dried before resuspension in 100 μl of DMEM alone. For comparison, BSA was added to the positive and negative controls to the same final concentration.

Step 4: exclusion HPLC chromatography

A Waters 712 WISP HPLC system (Millipore, St. Quentin Yvelines, France) was used. The active fractions from step 3 were pooled, freeze-dried and resuspended in 100 μl of 100 mM phosphate buffer, pH 6.7, containing 100 mM Na_2SO_4 and 0.05 % sodium azide (buffer C) and then injected on a TSK G 3000 SW column with a guard column (Merck–Clevenot, Schelles, France), previously equilibrated in buffer C. Elution was performed with the same buffer.

SDS/PAGE

Protein of total PEP and concentrated selected active fractions from each step were measured with the Bradford [27] or micro bicinchoninic acid protein assay (Pierce, Interchim, Montluçon, France) methods. Fractions of 800 ng were dried under vacuum and finally resuspended in 1 μl of electrophoresis sample buffer. They were analysed by PAGE [4–15 % (w/v) gel] and then silver-stained (Pharmacia Phast System).

N-terminal amino acid sequencing

HPLC purified protein (60 pmol) was dialysed in distilled and deionized water and adsorbed on a Prosorb® cartridge (Perkin–Elmer, Roissy, France). The N-terminal amino acid sequencing was realized on a microsequencing system 476 A from Applied Biosystems (Lincoln Centre Drive, Foster City, CA, U.S.A.) equipped with an on-line reverse-phase HPLC, in accordance with the manufacturer's instructions.

Western blotting

As described before, samples of step 2, peak II concentrated active fractions and of the purified protein, reduced (by 3 mM 2-mercaptoethanol) or unreduced, were separated by SDS/PAGE

with the prestained molecular markers from Sigma in a last well. The proteins were transferred to a PVDF membrane (Immobilon®-P; Millipore) with the Phast System apparatus. The membrane was blocked with 3% (v/v) gelatin fish (Sigma) in Tris-buffered saline (TBS) containing Tween-20 [TBST; 10 mM Tris/HCl (pH 8)/150 mM NaCl/0.05% (v/v) Tween-20] for 1 h and incubated further for 1 h with rabbit purified antibodies: L740 (1/200 in TBST) raised against human factor H (kindly given by Dr. M. Fontaine, Rouen, France). After the membrane had been washed three times in TBST, it was incubated for 1 h with biotinylated goat anti-(rabbit IgG) (Dako, Trappes, France; diluted 1:500 in TBST). After three more washes in TBST, the membrane was incubated for 30 min with Dako peroxidase-conjugated streptavidin (diluted 1:400 in TBST). After washing the membrane in TBS, protein bands were revealed by the addition of 0.05% 4-chloro-1-naphthol in 20% (v/v) methanol in TBS and 3.5 μl/ml 30% (w/w) H_2O_2 solution. A duplicate of the gel was stained with silver.

Inhibition assays of the MCA

The purified protein (75 μl at 4 μg/ml) in PBS was diluted in the same volume of anti-C5a antibodies (Serotec/Realef, Paris, France) already diluted in PBS. All the samples were incubated at 25 °C for 30 min before testing for MCA. As a comparison the purified protein alone was treated in the same way. An anti-[human monocyte chemotactic protein 1 (MCP-1)/monocyte chemotactic and activating factor (MCAF)] monoclonal antibody (500-M71, Pepro Tech, Rocky Hill, NJ, U.S.A.) and the same anti-(factor H) polyclonal antibodies as mentioned above were also tested in the same experimental conditions.

Thrombin digestion

The purified protein (500 μl at 6 μg/ml) in PBS was mixed with 3 μl of a 10 μg/ml thrombin (Sigma) solution in PBS and was then incubated for 120 min at 37 °C [22]. The digestion was stopped with 10 μl of 50 mM (w/v) PMSF in propan-2-ol. The digest was then dialysed in PBS before testing it at several dilutions in PBS (1:2, 1:3 and 1:6) for MCA.

RESULTS

Protein purification

Step 1

As shown in Figure 1, on passage of PEP through a column of DEAE-Trisacryl M resin we observed three main peaks of MCA. The first peak eluted at approx. 260 mM NaCl (peak I; fractions 8–12) and represented 21.0 ± 11.0% of the starting MCA, the second eluted at approx. 630 mM NaCl (peak II; fractions 23–28, 38.0 ± 20.0% of the starting MCA) and the third at approx. 790 mM NaCl (peak III; fractions 30–35, 41.0 ± 22.0% of the starting MCA). There was no MCA in the effluent.

Step 2

A different elution pattern with two peaks of MCA activity was seen after Mono S chromatography of the pooled active fractions of peak II from step 1 (Figure 2). The first peak eluted at approx. 120 mM NaCl (peak I; fractions 4–6), whereas the second eluted at approx. 230 mM NaCl [peak II; fractions 9–11, with the highest specific activity: 390 ± 186 units/mg (as defined in Table 1)]. A small additional MCA was found for the unbound fraction.

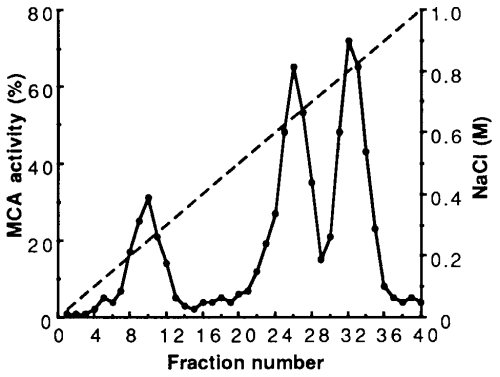

Figure 1 Anion-exchange chromatography

Purification step 1. DEAE-Trisacryl M resin (600 ml) was equilibrated in buffer A and mixed with 200 ml of PEP diluted 1:27 with deionized water. The slurry was deposited in a Pharmacia XK 50/60 column. After the column had been washed with 3 litres of buffer A, elution was performed with a 640 ml linear gradient from 0 to 1 M NaCl in buffer A (broken line) at 2.5 ml/min; 40 fractions of 16 ml each were collected and samples of 1 ml from each fraction were dialysed in PBS and assayed for MCA (●).

Step 3

Again two MCA peaks were observed after chromatography, on a Pro RPC column, of the pooled active fractions from peak II of step 2 (Figure 3). The first peak eluted at 33% acetonitrile (peak I; fractions 13–15); the second eluted at approx. 42% (peak II; fractions 17–19). There was no MCA in the effluent.

Step 4

After exclusion chromatography on a TSK G 3000 SW column of the pooled fractions from peak I of step 3 (Figure 4), only one main MCA eluted at approx. 300 kDa in fractions 12 and 13. A

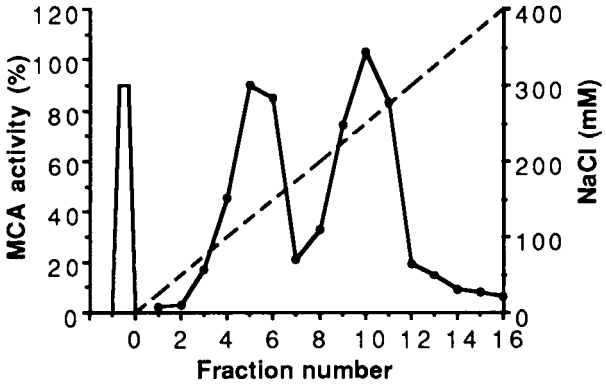

Figure 2 Cation-exchange chromatography

Purification step 2. A 200 μl portion of the concentrated fractions of peak II from step 1 was diluted in 8 ml of buffer B and applied to a Mono S HR 5/5 column previously equilibrated in buffer B. Elution was performed, after washing through all the unbound proteins, with a 20 ml linear gradient from 0 to 400 mM NaCl in buffer B (broken line) at 0.4 ml/min. Collected fractions (16 fractions of 1.25 ml each) were dialysed before testing for MCA (●).

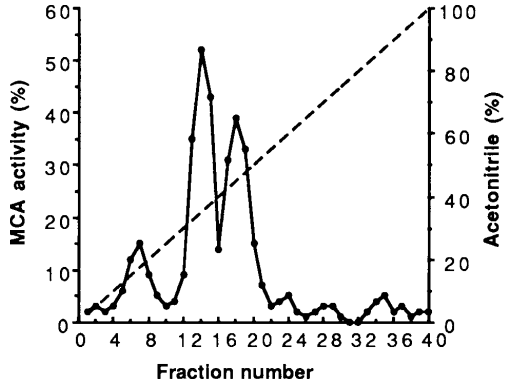

Figure 3 Reverse-phase chromatography

Purification step 3. A 3.75 ml portion of active fractions from peak II of step 2 were dialysed in deionized water and adjusted to 0.1% (v/v) TFA before being injected on a Pro RPC HR 5/10 column equilibrated in 0.1% (v/v) TFA in water. The absorbent was extensively washed with the same TFA buffer. Elution was performed with a 40 ml linear gradient of acetonitrile from 0 to 100% in the TFA buffer (broken line) at 0.3 ml/min. Collected fractions (40 fractions of 1 ml each) were treated as described in the Experimental section before testing for MCA (●).

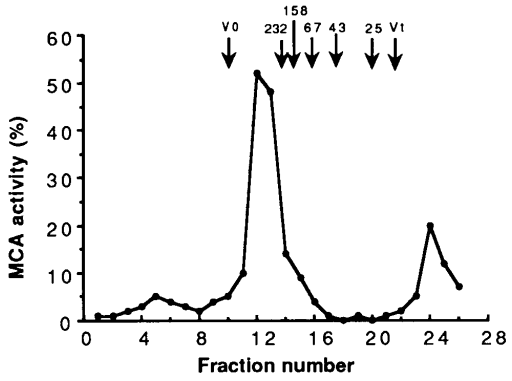

Figure 4 Exclusion chromatography

Purification step 4. Active fractions of peak I from step 3 were freeze-dried and resuspended in 100 μl of buffer C before being injected on a TSK G 3000 SW column equilibrated with buffer C. Elution with the same buffer was performed at 0.6 ml/min and fractions of 0.6 ml were collected. The calibration proteins used were: catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; α-chymotrypsinogen A, 25 kDa; their positions of elution are indicated at the top. The void volume (V_0) was estimated with Blue Dextran 2000; V_t is the total column volume. The fractions were dialysed as usual before testing for MCA (●).

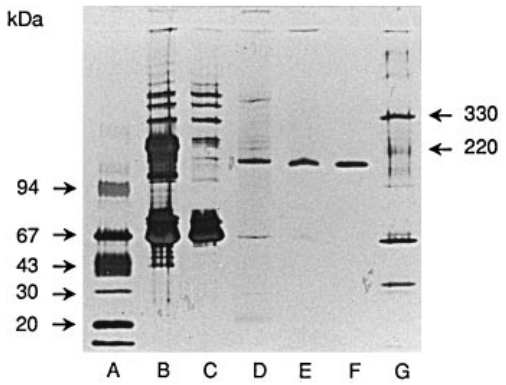

Figure 5 SDS/PAGE analysis

Electrophoresis was performed under non-reducing conditions with the Pharmacia Phast system as described in the Experimental section. The designated molecular mass markers were: trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa), phosphorylase B (94 kDa), ferritin (220 kDa) and thyroglobulin (330 kDa); their positions are indicated at the left. Lanes A and G were loaded respectively with the low- and high-molecular-mass markers from Pharmacia; lane B, PEP; lane C, step 1 peak II; lane D, step 2 peak II; lane E, step 3 peak I; lane F, step 4 fractions 12 and 13.

small MCA in fractions 24 and 25 with an apparent molecular mass smaller than 5 kDa was also observed.

The purification protocol is described in Table 1, where the average results obtained for five complete procedures are shown. The specific activity was multiplied by approx. 9000-fold after step 4. According to these results, there is approx. 0.2 μg of this monocyte chemotactic factor/ml of PEP.

Identification of the monocyte chemotactic factor as factor H

MCA purification to homogeneity was achieved. After SDS/PAGE and silver staining, only a single band of protein was visible (Figure 5, lane F), indicating that the MCA migrates with an apparent molecular mass of 150 kDa, which is slightly increased on reduction (figure 6, lane C).

The following N-terminal sequence was identified: EDCNE-LPPRRNTEI. The cysteine residue was difficult to identify even after reduction and derivatization. It was obtained as the most likely result by the analysis computer program. The purified protein was also found to be pure to approx. 97%. We then searched the protein data bank (EMBL FASTA Server: fasta@ebi.ac.uk) for similar proteins and found a 100% match with the N-terminal amino acid sequence for human complement factor H.

In Figure 6 it is shown that the anti-(factor H) antibodies

Table 1 Purification of the monocyte chemotactic factor

Total protein was determined by the methods of Bradford and Lowry. MCA units were calculated from the formula: $\{[D_{605}(X) - D_{605}(DMEM)] \times \text{total volume (ml)}\}/\{[D_{605}(FMLP) - D_{605}(DMEM)] \times \text{test volume (ml)}\}$, where X was the sample under test.

Step	Total protein (mg/ml)	Total MCA (units)	Specific activity (MCA units/mg)	Yield (%)	Purification (fold)
1. PEP	53.000	1840	0.17	–	–
2. DEAE eluate	2.600	702	3	38.2	18
3. Mono S eluate	0.029	212	390	12.5	2294
4. Pro RPC eluate	0.009	107	789	5.8	4641
5. TSK G 3000 SW eluate	0.007	64	1529	3.5	8994

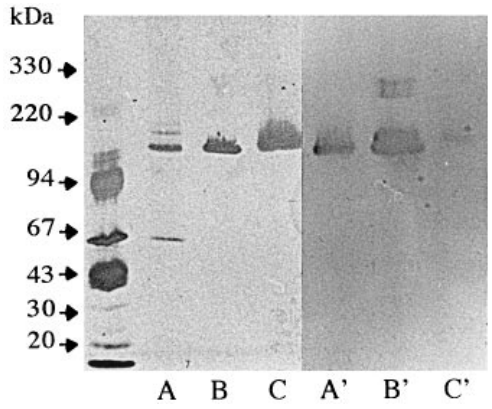

Figure 6 Western-blot analysis

Concentrated active fractions from step 2 peak II and step 4 purified protein, both non-reduced and reduced, were separated by SDS/PAGE and further blotted on PVDF (Immobilon®-P) membrane (lanes A′, B′ and C′ respectively). The blot was treated as described in the Experimental section. A duplicate gel with the same samples, labelled A, B and C respectively, was stained with silver. In the the left-hand lane were loaded the low-molecular-mass markers from Pharmacia.

recognized, during Western blotting, a single band of protein at approx. 150 kDa in lanes A′, B′ and C′ with a slight shift when the purified protein was reduced. In lane A′ (step 2, peak II), the protein was recognized in an impure mixture of proteins; in lane B′ the purified protein formed aggregates recognized by the antibodies. Interestingly, there were no detectable factor H-related or truncated forms.

Inhibition assays of the MCA

To test for any contamination of the purified protein with C5a or MCP-1, which could have accounted for the MCA, we incubated anti-C5a antibodies alone or with our purified protein and measured the resulting MCA. No antibody dilution alone demonstrated an MCA above 10 % of the positive control. In the presence of varied dilutions of anti-C5a antibodies there was no

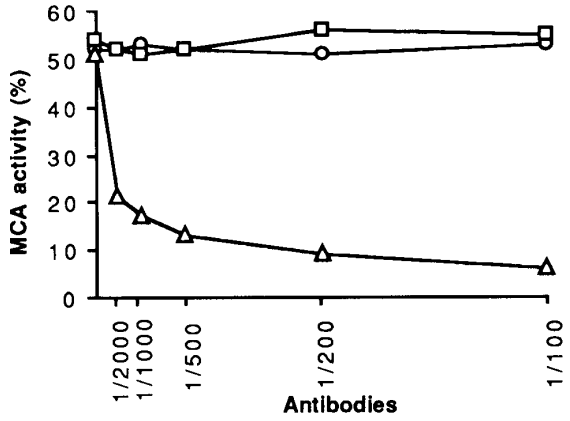

Figure 7 Inhibition assays of the MCA

The purified protein was diluted with PBS or with several dilutions in PBS of anti-C5a (○), anti-(MCP-1/MCAF) (□) or anti-(factor H) (△) antibodies (the purified protein final concentration was 2 μg/ml). The samples were then incubated for 30 min at 25 °C before their MCA was tested.

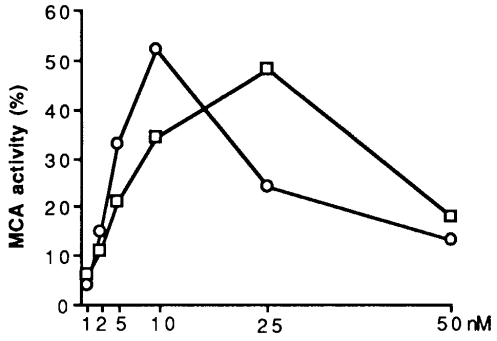

Figure 8 Characterization of the purified protein and Sigma factor H MCA

The purified protein (○) and Sigma factor H (□) were diluted in PBS at different concentrations (shown on the abscissa) and tested for their MCA.

significant loss of the purified protein MCA. The same negative results were obtained when using the anti-(human MCP-1/MCAF) antibody. Anti-(factor H) antibodies inhibited completely and specifically the purified protein MCA (Figure 7).

Thrombin digestion

Thrombin treatement modified neither the electrophoretic behaviour of the purified protein nor its MCA. It was found to reach 13 ± 5 % (three experiments) at all dilutions, whereas thrombin plus PMSF had an MCA of approx. 15 ± 6 %. PMSF alone had no MCA. The same results were obtained with the factor H purchased from Sigma (results not shown).

Characterization of the purified protein MCA

As described for chemotactic proteins, when the purified protein was tested for MCA from 1 to 50 nM, a bell-curved MCA dose response was obtained with a maximum at 10 nM (approx. 2 μg/ml) (Figure 8). The Sigma factor H response was similar, with a maximum at 25 nM. Checkerboard analysis demonstrated that the purified protein is chemotactic and not chemokinetic, mainly for monocytes but with a 50 % chemotactic activity for granulocytes also (results not shown).

DISCUSSION

We previously reported that mesothelioma pleural effusions demonstrated significant MCA [23]. We have now purified the main MCA contained in mesothelioma effusions. At the end of the purification procedure an apparent molecular mass of 300 kDa for the purified protein was calculated. Sequencing analysis indicated that the purified protein shared with factor H the 14 N-terminal amino acids. The purified protein was further resolved by SDS/PAGE as a monomeric protein of approx. 150 kDa, slightly lowered by protein reduction, whereas the formation of higher-molecular-mass protein aggregates (approx. 300 kDa) were observed. The purified protein and the aggregates were both recognized by the anti-(factor H) antibodies (Figure 6). Importantly, the purified protein had *in vitro* a specific MCA that was completely inhibited by the anti-(factor H) antibodies. In previous findings, factor H showed the same aggregation characteristics as the purified protein and particularly the formation of dimers of approx. 300 kDa under conditions of low ionic strength [19]. Western blot and SDS/PAGE analysis excluded the presence of truncated forms of factor H or related proteins that could have contaminated the purified protein preparation.

Negative results were also obtained when testing further the purified protein for traces of C5a or MCP-1, known monocyte chemotactic proteins [28,29] (Figure 7). The comparison between the purified protein MCA and the Sigma factor H indicated a stronger MCA for the purified protein (Figure 8) but demonstrated for both a bell-shaped dose–response curve, characteristic of chemotactic proteins. Sigma plasmatic factor H is a mixture of ϕ_1 and ϕ_2 factor H forms that are usually further separated by their different hydrophobicities [18]. A prerequisite for a monocyte chemotactic protein is to bind to monocytes; ϕ_2, but not ϕ_1, has been shown to be the form of factor H able to bind to monocytes [20,30]. We therefore conclude that we have very probably purified the ϕ_2 form of factor H. As Sigma factor H is a mixture of ϕ_1 and ϕ_2, where only the latter could have an MCA, it becomes easier to interpret the lowest MCA observed for the Sigma factor H. An MCA for factor H had been previously attributed to an H_2 form of factor H obtained only by thrombin digestion of factor H [22]. H_2 was not subjected to peptide sequencing but had the same biochemical characteristics as ϕ_2. We observed that the purified protein and the Sigma factor H are insensitive to thrombin digestion with no resultant changes in electrophoretic behaviour or MCA. However, it was found that thrombin had a moderate MCA that was unchanged when thrombin was inhibited by PMSF. An MCA for thrombin has been reported before [31] and it is possible that thrombin is responsible for this factor H MCA. Although ϕ_2 has never been obtained by proteolysis of factor H [18] we cannot exclude that the protein we have purified has not been proteolysed in the pleural space or during the purification procedure. To elucidate this point more comparison between H_2 and ϕ_2 would be required. Some of the other MCAs contained in pleural effusions have been partly characterized. For example, in peak II of step 3 the MCA was mainly due to MCP-1, which has been found before in malignant effusions [32]. MCP-1 belongs to the C-C chemokine family [33], in which the first two conserved cysteine residues are juxtaposed (reviewed in [34,35]). It has a chemotactic specificity for mononuclear cells, including monocytes [29,36,37]. MCP-2 and MCP-3 are also C-C chemokines, chemotactic for monocytes, basophils and eosinophils [38,39]. Interleukin 8, which is a member of a second family of chemoattractants (the C-X-C chemokines, in which the first two conserved cysteines are separated by one amino acid residue [35]), is active mainly on neutrophils [40–42]. Factor H belongs to a different family of human serum proteins [12] made up of CCP modules that contain four cysteine residues forming disulphide bonds between Cys I–III and Cys II–IV, thereby maintaining the three-dimensional protein structure [43]. This is reminiscent of the C motifs in C-C and C-X-C chemokines [34]. The concentration of factor H in the bloodstream, where blood monocytes are usually found, is 1000-fold that in the pleural fluid. Therefore a significant reservoir of factor H must be present outside the blood to allow the blood monocytes to migrate to the pleural space along a positive concentration gradient of factor H. This could occur through a specific microenvironment's existing outside the capillary wall. However, it is conceivable that monocytes extravasate first to the pleural space via an another chemoattractant, but are further attracted towards the mesothelioma site by factor H produced locally. In addition, membrane-bound factor H [44] might form a solid-phase gradient that is more stable than the fluid-phase one, which needs to be fed by the continuous secretion of chemotactic factor H and thus might be difficult to regulate. Lastly, during their migration outside the blood stream, monocytes could become activated or responsive to factor H. Indeed it has been shown that the binding of factor H to polymorphonuclear leucocytes is increased by their activation with FMLP [45]. All these mechanisms have been previously suggested to explain the mechanisms regulating the activities of the chemotactic proteins [35]. The model that we used *in vitro* to purify the mesothelioma-fluid MCA has enabled the successful purification of nearly all the known monocyte chemoattractants active *in vivo*, including MCP-1 [29,36]. This is a strong indication of the physiological relevance of the chosen model. However, more experiments will be necessary before conclusions can be drawn about the role of factor H MCA *in vivo*.

Factor H is a cofactor in the cleavage of C3b by factor I. The end product of the reaction is iC3b, a very potent opsonin that binds to complement receptor type 3 and possibly complement receptor type 4 on phagocytic cells [46]. During mesothelioma progression, factor H could recruit monocytes and granulocytes to the tumour site and also contribute to malignant cells' phagocytosis via the deposition of iC3b on the tumour. This type of interaction could represent a new link between the humoral immune system, involved in the complement response, and the cellular immune reaction, involved in the chemotactic response.

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