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Reversible binding of a guinea-pig lymphokine to gelatin and fibrinogen: possible relationship of macrophage agglutination factor and fibronectin

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Summary. Macrophage agglutination factor (MAggF) is a T-cell-dependent guinea-pig lymphokine with pH stability, heat stability and isoelectric point similar to fibronectin (see preceeding paper for details). Further observations confirm the similarity between MAggF and fibronectin. MAggF in unconcentrated lymph node cell culture supernatants bound reversibly to gelatin and fibrinogen. On gel filtration chromatography, most MAggF activity in a pooled concentrated lymphokine preparation was associated with molecules of 370,000 Daltons; lesser amounts of activity were found at 240,000 and 50,000 Daltons. All molecular weight forms of MAggF bound reversibly to gelatin. Guinea-pig plasma fibronectin prepared by affinity chromatography over gelatin had a molecular weight of about 450,000, a sub-unit on reduction of about 240,000 Daltons, and showed partial antigenic identity with human plasma fibronectin. Human and guinea-pig plasma fibronectin preparations showed MAggF activity when tested using guinea-pig peritoneal macrophages, but their potencies relative to a culture supernatant standard did not correlate with the content of immunoprecipitable fibronectin measured by anti-human fibronectin antiserum. However, anti-human fibronectin immunoadsorbents specifically and reversibly bound MAggF activity in culture supernatants. On the basis of our observations, we suggest MAggF is a guinea-pig tissue fibronectin.

Abbreviations: CIG, cold insoluble globulins; GPFN, guinea-pig fibronectin; HEPES, N-2-hydroxethylpiperazine-N'-2-ethanesulphonic acid; HuFN, human fibronectin; Ig, immunoglobulin; IgG, immunoglobulinG; IEP, immunoelectrophoresis; LNC, lymph node cell(s); MAggF, macrophage agglutination factor; NGG, normal goat globulins, PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7·2; PPD, purified protein derivative of tuberculin; SDS, sodium dodecyl sulphate; Tris, tris (hydroxymethyl)aminomethane.

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INTRODUCTION

Macrophage agglutination factor (MAggF) is a T-cell-dependent, guinea-pig lymphokine produced by antigen- or mitogen-activated lymph node cells (LNC); it is antigenically distinct from migration inhibition factor and Ig (Godfrey, 1976; Postlethwaite & Kang, 1976; Godfrey & Geczy, 1978; Godfrey & Koch, 1980). MAggF activity is associated with a heat-stable protein (Postlethwaite & Kang, 1976; Godfrey, 1980) whose electrophoretic mobility, isoelectric point and pattern of denaturation by heat and pH (Godfrey, 1980; Godfrey & Purohit, 1982) are similar to those of human plasma fibronectin (FN; Vuento, Salonen, Stenman, Pasanen & Stenman, 1980; Mosher, 1980). The fibronectins are a family of gelatin- and fibrinogen-binding glycoproteins found in plasma and attached to cells which apparently mediate cell adhesion to the extracellular matrix and regulate cell

morphology (Mosher, 1980; Yamada, 1980). These physical similarities of MAggF and FN encouraged us to determine if MAggF could bind reversibly to gelatin and fibrinogen and if FN preparations from human and guinea-pig plasma contained appreciable amounts of MAggF activity when tested on guinea-pig macrophages. The results of these studies and their bearing on the relationship of MAggF and FN form the basis of this report.

MATERIALS AND METHODS

Animals and sensitization

Male Hartley guinea-pigs, 350±50 g at time of sensitization, were obtained from Dutchland Farms (Germantown, PA.) and injected in multiple sites with 1 ml of Freund's complete adjuvant containing 1 mg of heat-killed mixed strains of human *Mycobacterium tuberculosis* (Godfrey, 1976; Godfrey & Purohit, 1982).

Culture of LNC and preparation of culture supernatants

Lymph nodes were removed 30–60 days after sensitization and LNC prepared (Godfrey, 1976). Viability was greater than 80% by dye exclusion. LNC 50×10^6 were cultured for 20 hr at 37° in 2 ml of serum-free modified RPMI-1640 (Godfrey, 1976; Godfrey & Purohit, 1982) in sterile screw-capped culture tubes (NUNC, from Vangard, Piscataway, N.J.). Stimulated LNC were cultured with PPD (tuberculin), 20 μg/ml, control LNC were cultured without antigen. After culture, culture supernatants were harvested by centrifugation, PPD, 20 μg/ml, was added to control LNC supernatants, and the supernatants divided into aliquots, quick-frozen in dry ice-acetone and stored at −70°.

The preparation of thirty-fold concentrates of stimulated and control culture supernatants has been described in detail (Godfrey & Purohit, 1982).

Indirect MAggF assay

MAggF assays were performed in duplicate or triplicate on serially diluted coded aliquots of culture supernatants or fractionated materials in modified RPMI-1640 with 10% foetal calf serum (lowest dilution, 1/10, final volume, 1·8 ml) as described (Godfrey, 1976; Godfrey & Purohit, 1982). After 4–6 hr at 37°, the tubes were gently swirled to dislodge loosely adherent clumps and scored as − to ++ depending on the number of clumps of aggregated macrophages on culture tube walls (Godfrey & Purohit, 1982). MAggF titre was defined as the reciprocal of the last dilution to show a + response. Results are reported as either geometric mean titres or as geometric mean relative titres, $(MAggF_S/(MAggF)_C$, where S and C refer to titres of stimulated and control cultures, respectively. As control preparations were essentially devoid of MAggF activity, the use of relative titres is a convenient way to summarize results.

Chromatography of concentrated culture supernantants

Ten millilitres of concentrated supernatants of stimulated and control cultures were dialysed against 500 volumes of phosphate-buffered saline (PBS), pH 7·2, at 4°. Dialysed supernatants were applied to a 2·5 cm × 100 cm column of beaded acrylamide-agarose (AcA34, LKB Products, Bethesda, Md) which had been equilibrated with PBS, and 10 ml fractions collected. Absorbance at 280 nm was monitored continuously (Uvicord, LKB). MAggF activity of each fraction was determined in triplicate and geometric mean titres computed. The column had been previously calibrated with materials of known molecular weight: blue dextran (Pharmacia), 2,000,000; thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; aldolase, 158,000 (Sigma Chemical Co., St. Louis, Mo.); 2,4-dinitrophenylglycine, 241 (Chromatographically pure, BDH Ltd, Poole, Dorset). The molecular weights of MAggF activity were estimated from a plot of K_{av} against the logarithm of molecular weight (Determann, 1969).

Human fibronectin (HuFN) and antiserum to HuFN (anti-HuFN)

HuFN isolated from citrated plasma by the method of Engvall & Ruoslahti (1977) and goat anti-HuFN were purchased (Calbiochem-Behring Corp., La Jolla, Calif.). Purity of HuFN was assessed by diffusion in 1% agarose gel against anti-HuFN and anti-human fibrinogen (obtained from Dr D. Galanakis), and by sodium dodecylsulphate-polyacrylamide electrophoresis (SDS-PAGE) in 5% gels, pH 8·8 at 1·5 ma/gel, in the presence and absence of dithiothreitol (White, Janoff & Godfrey, 1980). No sample or stacking gels were used (Porzio & Pearson, 1977). Normal goat serum was a gift from Professor J. Elias. Specificity of anti-HuFN was monitored by double diffusion in agarose gel and immunoelectrophoresis (IEP; White *et al.*, 1980) using HuFN, human fibrinogen (obtained from Dr D. Galanakis) and normal human plasma.

Preparation of insolubilized proteins and arginine

Agarose beads (4%, Bio-Gel A-15m, Bio-Rad Laboratories, Riverside, Calif.) were activated with CNBr (March, Parikh & Cuatrecases, 1974). They were conjugated with the 50% saturated $(NH_4)_2 SO_4$ globulin fraction of goat anti-HuFN (0·1 mg or 1 mg/ml settled bead volume) or with normal goat globulins (NGG; 0·1 mg/ml settled bead volume). The activity of bound immunoglobulins was assessed using rocket IEP (Weeke, 1973) to determine the amount of HuFN in solutions before and after their absorption with agarose conjugates of NGG or anti-HuFN. Specific binding of HuFN by agarose-anti-HuFN (1 mg/ml) was 46–49 μg/ml settled beads.

Guinea-pig fibronectin (GPFN; 1 mg/ml settled bead volume), guinea-pig fibrinogen (1 mg/ml settled bead volume), and guinea-pig α_2-macroglobulin (α_2M) (1 mg/ml settled bead volume) were purified as described below. They were conjugated to 4% agarose beads activated by the above method. Binding of protein was assessed indirectly by spectrophotometry of unbound protein in the washings (Hudson & Hay, 1976) and confirmed by treating an aliquot of beads with excess sodium trinitrophenylsulphonate (1% in methanol) and observing the development of a dark orange colour indicative of trinitrophenyl-protein conjugates when compared with the pale orange of similarly treated unconjugated beads.

Gelatin (1 mg/ml settled bead volume; Type 1, Sigma) or arginine were bound to 4% agarose beads activated by the above method and the amount bound determined as described (Vuento & Baheri, 1979).

Preparation of GPFN

Nine volumes of freshly drawn guinea-pig blood was mixed with 1 volume of 3·8% sodium citrate-50 mM benzamidine (Sigma) -5 mg hexadimethrine bromide (Polybrene, Sigma). Cells were removed by centrifugation and GPFN was prepared from plasma by a modification of the method of Vuento & Vaheri (1979). Briefly, an aliquot of plasma was chromatographed over 4% agarose beads at room temperature, eluted with 50 mM Tris/HCl, pH 7·5, and fractions giving precipitates with HuFN (gel diffusion) were pooled and concentrated. GPFN was adsorbed to agarose-gelatin and eluted after washing with 1 M arginine at pH 7·5; this eluate was adsorbed to agarose-arginine at low ionic strength and eluted with 0·1 M NaCl. This preparation is referred to as GPFN-1.

GPFN was also prepared from cold insoluble globulins (CIG) of heparinized blood. Blood was anticoagulated with 20 u./ml heparin (Heparin sodium solution, 1000 u./ml ICN Nutritional Biochemicals, Cleveland, Ohio). The cells were removed by centrifugation at room temperature and additional heparin (Stathakis & Mosessen, 1977), benzamidine and Polybrene were added to a final concentration of 20 u./ml, 5 mM and 0·5 mg/ml, respectively. After incubation at 4° for 18–24 hr, CIG were removed from plasma by centrifugation at 4°, washed once with cold 50 mM Tris/HCl, pH 7·5, and solubilized in 250 mM Tris/phosphate, pH 7·5 by incubation at 37° for 24 hr. The solubilized CIG were passed over agarose beads at room temperature to remove agarose binding materials and dialysed against 50 mM Tris/HCl, pH 7·5. GPFN was then isolated from CIG as described (Vuento & Vaheri, 1979). This preparation is referred to as GPFN-2.

A third GPFN preparation (GPFN-3), also from CIG, was made by a modification of the above procedure. CIG were prepared, washed and solubilized as above. Aliquots were chromatographed on a 4% beaded agarose column (2·5 cm × 100 cm) at room temperature in 250 mM Tris/phosphate, pH 7·5 and fractions collected. Fractions reacting with anti-HuFN in gel diffusion were pooled, dialysed against 50 mM Tris/HCl, pH 7·5, and applied to gelatin-agarose. GPFN was then isolated as described (Vuento & Vaheri, 1979). This preparation (GPFN-3) was conjugated to agarose beads as described above.

Purity of GPFN preparations 1, 2 and 3 was evaluated by double diffusion in agarose gel to determine relative antigen concentrations. Several dilutions of each preparation and known concentrations of HuFN and human fibrinogen were diffused against constant amounts of anti-HuFN and anti-human fibrinogen, and concentrations of FN and fibrinogen estimated from the position of the precipitin line in the unstained slides (Hudson & Hay, 1976). GPFN-1 and GPFN-2 were also analysed by SDS-PAGE in 5% gels in the presence and absence of reducing agent.

Preparation of guinea-pig fibrinogen

Guinea-pig fibrinogen was prepared as a by-product of the third GPFN preparation described above. Material not binding to gelatin-agarose was repassaged two additional times on gelatin-agarose and then passed twice over agarose-anti-HuFN. Affinity substrates were eluted after each passage. Purity of guinea-pig fibrinogen was evaluated by double diffusion in agarose gel against anti-HuFN and

anti-human fibrinogen. The preparation reacted only with anti-fibrinogen and not with anti-HuFN.

Preparation of guinea-pig α_2M

α_2M was prepared from heparinized plasma containing benzamidine, 5 mM and Polybrene, 0·5 mg/ml, by the method of Roberts, Reisen & Hall (1974) as modified by White, Habicht, Godfrey, Janoff, Barton & Fox (1981). Purity was monitored by SDS-PAGE in 5% gels and by immunodiffusion against anti-α_2M antiserum (White *et al.*, 1981). This preparation reacted strongly with anti-α_2M antiserum and did not react with anti-fibrinogen or anti-HuFN.

Determination of protein

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.

Determination of immunoprecipitable GPFN

This was determined by rocket immunoelectrophoresis (IEP; Weeke, 1973) using goat anti-HuFN. HuFN was used as a standard. Its concentration for this purpose was determined from the reported absorbance coefficient $A^{280\ nm}_{1\%-1\ cm} = 12{\cdot}8$ (Mosessen & Umfleet, 1970).

Use of insolubilized proteins and arginine as absorbents of MAggF activity

Immunoadsorption of unconcentrated culture supernatants was performed by incubating 1 ml with 0·6 ml of a 50% suspension of washed beads for 24 hr at 4°. The mixtures were gently rotated during incubation. After incubation, supernatants were removed, beads were washed at room temperature with RPMI-1640 medium and washes added to the removed supernatants. Beads were then eluted at room temperature three times with equal volumes of 100 mM acetic acid. The eluates were pooled and dialysed against 1500 volumes of RPMI-1640 medium at 4° until pH had returned to neutrality as judged by phenol red colour. The specificity of immunoadsorbents was shown by determining the ability of washed agarose anti-HuFN beads to adsorb MAggF after previous exposure to fifteen-fold excess of HuFN, GPFN-1 or α_2M.

To demonstrate binding of MAggF to insolubilized fibrinogen, unconcentrated culture supernatants (0·3 ml) were incubated with equal volumes of washed beads conjugated with guinea-pig fibrinogen, GPFN or guinea-pig α_2M for 18 hr at 4°. Supernatants were removed, beads were washed extensively at room temperature with PBS, and washes pooled with the removed supernatant material. The beads were eluted three times with 100 mM acetic acid at room temperature as described above, the eluates pooled, and dialysed extensively against RPMI-1640 at 4° until pH had returned to neutrality.

To demonstrate binding of MAggF to insolubilized gelatin, unconcentrated supernatants or fractionated materials were incubated with equal volumes of packed agarose or agarose-gelatin for 18 hr at 4°. At the end of this time, supernatants were removed, beads were washed extensively with PBS, and washes were pooled with the removed supernatant material. Beads were eluted twice at room temperature with equal volumes of 1 M arginine in 50 mM Tris/HCl, pH 7·5 for 30 min (with rotation) and the eluates pooled. Removed supernatants (including PBS washes) and arginine eluates were dialysed extensively at 4° against RPMI-1640 if no further adsorptions were to be performed. If the arginine eluates of agarose-gelatin were to undergo a second cycle of adsorption with agarose-arginine, the eluates were dialysed against 50 mM Tris/HCl, pH 7·5. Dialysed arginine eluates were mixed with equal volumes of washed, packed agarose-arginine beads and incubated at 18 hr at 4°. At the end of this incubation, supernatants were removed, beads were washed with Tris/HCl, pH 7·5, and the washings added to the removed supernatants. The beads were then eluted twice at room temperature with 100 mM NaCl in 50 mM Tris/HCl, pH 7·5, as above, and eluates were pooled and dialysed.

All materials were sterile filtered after dialysis. They could be frozen at −20° without reducing MAggF activity.

Statistical treatment

Statistical significance of the results was determined by the use of Student's *t* test. Statistical significance of dose activity curves and relative MAggF potency was determined by a parallel-line assay modified from Colquhoun (1971). For this purpose, the activity of the concentrated supernatant of PPD stimulated LNC was arbitrarily defined to contain 100 u./ml of activity.

RESULTS

Comparison of HuFN and GPFN preparations

SDS-PAGE analysis of the unreduced HuFN preparation showed it to consist of a single protein with a mol. wt of about 440,000 (Fig. 1). When reduced, this

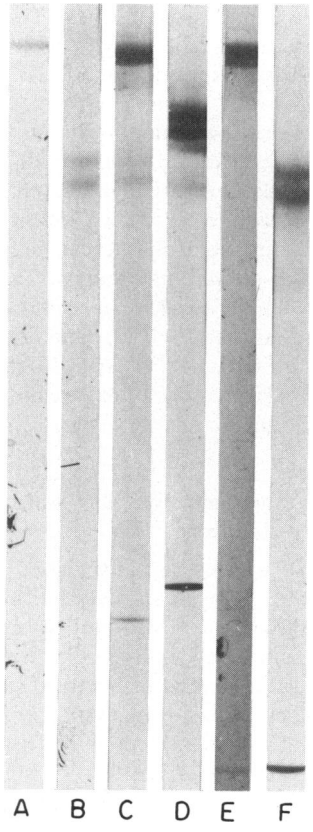

Figure 1. SDS-PAGE of 15 μg of two guinea-pig FN preparations (GPFN-1 and GPFN-2) run under non-reducing and reducing conditions. Migration is from top to bottom; the origins of all gels have been aligned and the gels cut off at the solvent front. A, Unreduced HuFN; B, reduced HuFN; C, unreduced GPFN-1; D, reduced GPFN-1; E, unreduced GPFN-2; F, reduced GPFN-2.

material yielded a doublet with an approximate mol. wt of 220,000 as described by others (Mosher, 1980). The major bands in unreduced and reduced GPFN-1 and GPFN-2 were similar in molecular weight to those found in HuFN, but these GPFN preparations also contained traces of proteins with larger and smaller molecular weights than that of the major component.

Anti-HuFN antiserum gave a single immunoprecipitation line against HuFN (Fig. 2) and detected an antigen in guinea-pig plasma, GPFN and CIG (not shown), which showed partial identity with HuFN, presumably GPFN. Anti-HuFN did not react in gel diffusion against human fibrinogen, but gave a single arc of β mobility when tested against human or guinea-pig plasma on IEP (data not shown).

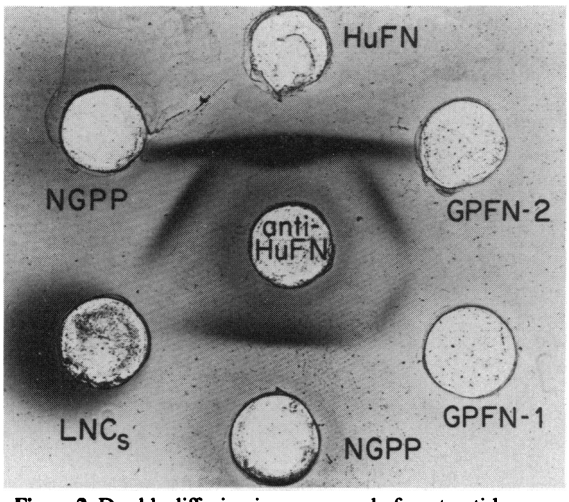

Figure 2. Double diffusion in agarose gel of goat anti-human fibronectin (anti-HuFN) against HuFN, normal guinea-pig plasma (NGPP), two guinea-pig fibronectin preparations (GPFN-1 and GPFN-2) and concentrated, antigen-stimulated LNC culture supernatant (LNCs). See Materials and Methods section for details.

All fibronectin preparations prepared by affinity chromatography were contaminated to some degree by fibrinogen. As estimated by the position of precipitation lines in gel diffusion against anti-human fibrinogen using known concentrations of human fibrinogen, the contamination was 1% in HuFN and GPFN-3, 2%–5% in GPFN-2, and 5%–8% in GPFN-1 (data not shown).

Molecular weight of MAggF activity

The vast majority of guinea-pig MAggF in LNC culture supernatants eluted from a calibrated AcA34 (beaded acrylamide-agarose) column with molecules of 370,000 Daltons (Fig. 3). Small amounts of activity were associated with molecules of mol. wt $> 1 \times 10^6$, 240,000 and about 50,000. Pools A, B. and C were formed from fractions corresponding to material with mol. wt of 370,000, 240,000 and about 50,000, respectively, and concentrated by ultrafiltration. Approximately 10% of the applied activity was recovered; pool A accounted for 90% and pool C 9% of recovered activity. Because MAggF activity was destroyed by reduction (Godfrey & Purhohit, 1982), it was not possible to perform chromatography on reduced supernatants.

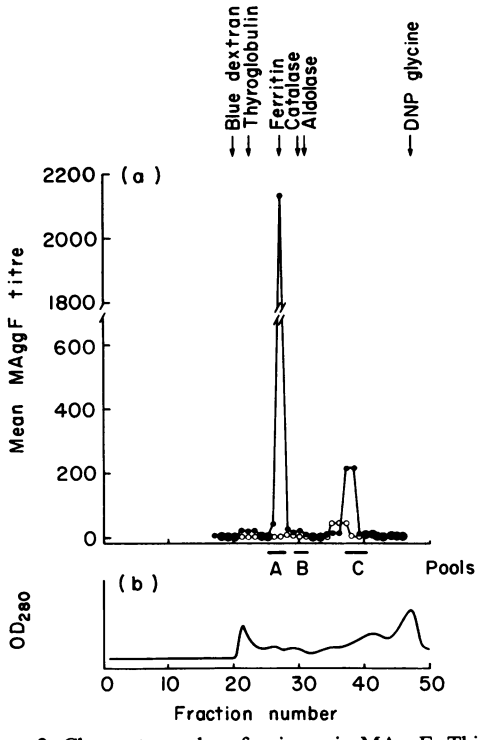

Figure 3. Chromatography of guinea-pig MAggF. Thirty-fold concentrated antigen stimulated (LNCs ●) or control (LNCc ○) LNC culture supernatants were dialysed against PBS, pH 7·2, and 10 ml applied to a 2·5 cm × 100 cm column previously calibrated with proteins of known molecular weight. The column was eluted with PBS at 4°; 10 ml fractions were collected. Each fraction was assayed for MAggF activity in triplicate and geometric mean MAggF activity was computed. (a) MAggF activity. See Materials and Methods section for details. Fractions in the indicated regions were pooled to form pools A, B and C. (b) Absorbance at 280 nm of LNCs. LNCc contained smaller amounts of protein but was qualitatively similar.

MAggF activity binds reversibly to fibrinogen and gelatin

Table 1 shows that MAggF activity in unconcentrated LNC supernatants bound reversibly to fibrinogen but not to fibronectin or α_2M. Conversely, significant activity ($P < 0.05$) could be eluted only from incubated fibrinogen beads and not from incubated fibronectin or α_2M conjugated beads. The increase in titre after adsorption with GPFN and the decrease in titre seen after adsorption with α_2M were not significant ($P > 0.05$). Table 2 shows that MAggF activity in unconcentrated LNC supernatants (a different group from those used in the experiments of Table 1) bound reversibly to gelatin. Activity could be eluted with 1 M arginine and subsequently adsorbed to agarose-arginine at low ionic strength. The activity could be eluted from agarose-arginine by 100 mM NaCl. The increase in titres after adsorption with agarose or elution from gelatin or arginine was not significant. MAggF in concentrated LNC supernatants also bound to gelatin (data not shown) as did activity fractionated by gel chromatography. Table 3 shows that MAggF activity of each molecular weight range bound significantly ($P < 0.05$) and reversibly to agarose-gelatin, but not to agarose beads alone. Again, observed increases in titre were not statistically significant ($P > 0.05$).

Guinea-pig MAggF activity binds specifically to anti-HuFn immunoadsorbents

A possible antigenic relationship between MAggF and FN was investigated using anti-HuFN. This antiserum used in gel diffusion revealed HuFN and GPFN to have partial antigenic identity but gave no precipitin lines when tested against concentrated stimulated or control LNC cultures supernatant (Fig. 2). Since gel diffusion is not a sensitive method for detecting partial indentity, the ability of anti-HuFN immunoadsorbents to bind MAggF was therefore studied directly (Table 4). Preparations of anti-HuFN immunoadsorbents containing either 0·1 mg or 1 mg globulins/ml packed agarose gave essentially similar results and results from both preparations were averaged together. MAggF activity was significantly bound ($P < 0.05$) by anti-HuFN immunoadsorbents but not by agarose-NGG or by agarose beads alone. Similarly, significant titres ($P < 0.05$) of MAggF activity could be released only from incubated anti-HuFN and not from incubated NGG or agarose alone. The titre of acid eluted activity was significantly lower than the titre of the untreated materials ($P < 0.05$). The specificity of MAggF adsorption by anti-HuFN was further shown by the observation that pretreatment of anti-HuFN with either HuFN or GPFN blocked the ability of anti-HuFN to bind MAggF activity, while pretreatment of anti-HuFN with the unrelated guinea-pig plasma protein α_2M did not affect immunoadsorbent activity.

Correlation of relative MAggF potency with FN content

The relationship of MAggF activity to FN was further

Table 1. Reversible binding of MAggF activity in unconcentrated LNC culture supernatants to guinea-pig fibrinogen

Treatment	Mean relative MAggF titre
None	1,800,000 (0·25)*†
Adsorption with agarose-GPFN	2,400,000 (0·24)†‡§
Acid eluate of incubated agarose-GPFN	18 (0·55)
Adsorption with agarose-fibrinogen	42 (0·70)‡
Acid eluate of incubated agarose-fibrinogen	91,000 (0·72)
Adsorption with agarose-α_2M	160,000 (0·83)§
Acid eluate of incubated agarose-α_2M	24 (0·34)

* Geometric mean (standard error in log units). Number of observations = 8. Relative MAggF titre = $(\text{MAggF titre})_S/(\text{MAggF titre})_C$, where S and C refer to titres of antigen stimulated and control cultures, respectively. See Materials and Methods section for details of treatment and assay.

† Means do not differ significantly, $P > 0{\cdot}05$.

‡ Means differ significantly, $P < 0{\cdot}05$.

§ Means do not differ significantly, $P > 0{\cdot}05$.

Table 2. Reversible binding of MAggF activity in unconcentrated LNC culture supernatants to agarose-gelatin and agarose-arginine beads

Treatment	Mean relative MAggF titre
None	46,000 (0·53)*
Adsorption with agarose	150,000 (0·36)†
Arginine eluate of incubated agarose	32 (0·28)
Adsorption with agarose-gelatin	22 (0·35)†
Arginine eluate of incubated agarose-gelatin	95,000 (0·04)
Arginine eluate of incubated agarose-gelatin after adsorption with agarose-arginine	2·2 (0·33)
NaCl eluate of incubated agarose-arginine	100,000 (0·58)

* Geometric mean (standard error in log units). Number of observations = 8. See Materials and Methods section and footnote to Table 1 for details of treatments and assay.

† Means differ significantly, $P < 0{\cdot}05$.

Table 3. Reversible binding of MAggF activity of various molecular weights to agarose-gelatin

Treatment	Relative MAggF titre		
	Pool A	Pool B	Pool C
None	46,000 (0·35)*	220 (1·2)	1000 (0·75)§
Adsorption with agarose	10,000 (0·75)†	1000 (1·7)‡	220 (1·6)¶
Arginine eluate from incubated agarose	4·6 (0·67)	2·2 (0·33)	2·2 (0·75)
Adsorption with agarose-gelatin	46 (0·88)†	10 (0·58)‡	10 (1·4)¶
Arginine eluate from incubated agarose-gelatin	46,000 (0·33)	2200 (1·4)	10,000 (0·58)§

* Geometric mean (standard error in log units). Peak activity in Pool A corresponds to 370,000 Daltons, in Pool B to 240,000 Daltons and in Pool C to 50,000 Daltons. (See also Fig. 3). See Materials and methods section and footnote to Table 1 for details of treatments and assay.

† Means differ significantly, $P < 0{\cdot}05$.

‡ Means differ significantly, $P < 0{\cdot}05$.

§ Means do not differ significantly, $P > 0{\cdot}05$.

¶ Means differ significantly, $P < 0{\cdot}05$.

Table 4. Specific adsorption of MAggF activity from unconcentrated LNC culture supernatants by anti-HuFN immunoadsorbents

Treatment	No. of observations	Mean relative MAggF titre
None	8	120,000 (0·12)§
Adsorption with agarose	4	75,000 (0·13)
Acid eluate from incubated agarose	4	18·8 (0·25)
Adsorption with NGG	6	83,000 (0·42)†
Acid eluate from incubated NGG	6	22·5 (0·67)‡
Adsorption with anti-HuFN	16	6·0 (0·35)†
Acid eluate from incubated anti-HuFN	12	18,000 (0·35)§
Adsorption with anti-HuFN pretreated with:		
HuFN	4	75,000 (0·28)
GPFN-1	4	100,000 (0·50)
GP α_2M	4	18·8 (0·25)

* Geometric mean (standard error in log units). See Materials and Methods section for details of treatments and assay.
† Means differ significantly, $P < 0{\cdot}05$.
‡ Means differ significantly, $P < 0{\cdot}05$.
§ Means differ significantly, $P < 0{\cdot}05$.

investigated by comparing dose activity curves of concentrated culture supernatants of stimulated and control LNC, with curves derived from two preparations of GPFN, HuFN and α_2M (Fig. 4 and Table 5). α_2M showed no MAggF activity at the highest dose tested, 1 mg (data not shown), nor at any lower dose. Control LNC culture supernatant contained negligible MAggF activity; its potency relative to that of stimulated LNC culture supernatant could not be calculated. In contrast, the dose activity curves of HuFN, GPFN-1 and GPFN-2 were statistically parallel to the stimulated LNC culture supernatant and relative potencies of these preparations could be calculated. The potency of HuFN, GPFN-1 (from plasma) and GPFN-2 (from CIG) relative to that of stimulated LNC culture supernatants was 2·3%, 470% and 11,000%, respectively. This enormous range in MAggF potency did not correlate with immunoprecipitable FN

Table 5. Comparison of MAggF potency of several preparations with their relative concentration of immunoprecipitable FN

Preparation	MAggF potency* (arbitrary units/ml)	Immunoprecipitable FN† (% HuFN standard)
LNC_C	NC	$<3{\cdot}2$
LNC_S	100	$<3{\cdot}2$
HuFN	$2{\cdot}3 \pm 1{\cdot}3$	100
GP FN-1	470 ± 200	72 ± 7
GP FN-2	$11{,}000 \pm 5000$	79 ± 8
GP α_2M	NC	$<3{\cdot}2$

* MAggF potency ± SD calculated by parallel line assay modified from Colquhoun, 1971. See also Fig. 4. NC, not calculable.
† Immunoreactive FN determined by rocket IEP using anti-HuFN. HuFN concentration (from $A^{280\ nm}_{1\%-1\ cm} = 12{\cdot}8$, Mosessen & Umfleet, 1970) was 623 mg/ml. Number of observations = 5.

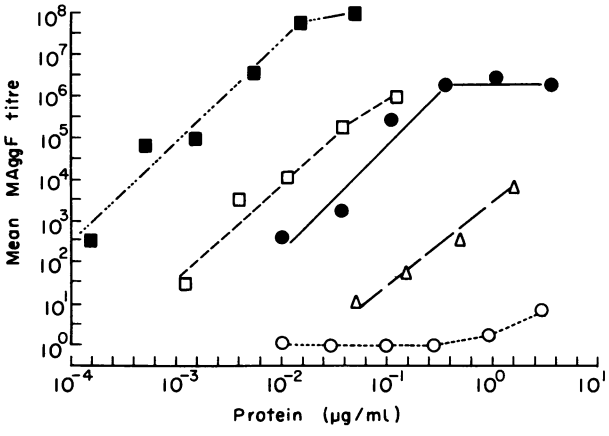

Figure 4. MAggF activity of concentrated, antigen stimulated (LNC_S) or control (LNC_C) LNC culture supernatants, human fibronectin (HuFN) and two guinea-pig fibronectin preparations (GPFN-1 and GPFN-2) plotted against protein concentration measured by Lowry technique (BSA standard). Each point is the mean of three–five determinations in duplicate. LNC_c (O···O); LNC_s (●—●); HuFN (△– –△); GPFN-1 (□ - - - □); GPFN-2 (■···–··■).

measured by rocket IEP using anti-HuFN (Table 5). Neither culture supernatant contained any FN by this assay while all three FN preparations had similar amounts of FN.

DISCUSSION

The studies reported in this and the accompanying paper show MAggF and FN to have many properties in common. Both MAggF and FN are gelatin- and fibrinogen-binding proteins with similar resistance to denaturation by heat and pH (Mosher, 1980; Vuento *et al.*, 1980). They have similar isoelectric points and electrophoretic mobilities (Godfrey, 1980; Mosher, 1980). Most MAggF activity is associated with molecules similar in molecular weight to native FN (Mosher, 1980). The small amounts of MAggF activity found at lower molecular weights recall the reported polydispersity of FN molecular weights (Chen, Amrani & Mosesson, 1977). The biological activity of both MAggF and FN is labile to reduction (Ali & Hynes, 1978; Wagner & Hynes, 1979). Even more important, MAggF and FN appear to show partial antigenic identity, and anti-FN antiserum reversibly and specifically binds MAggF activity. Finally, GPFN and HuFN preparations show MAggF activity, and dose–response plots of this activity are parallel to similar plots derived from stimulated LNC culture supernatant MAggF activity. On the basis of our observations, we suggest that MAggF is a guinea-pig FN.

There are some differences between the properties of MAggF and FN. The mol. wt of MAggF (370,000) is slightly lower than that of FN (450,000, Mosher, 1980); this difference is within the error of measurement of molecular weight determination by gel filtration chromatography. MAggF seems somewhat more soluble at 4° than HuFN, or, at least, there was no obvious loss of MAggF activity in culture supernatants held at 4° for prolonged periods, under conditions where HuFN would be expected to be relatively insoluble (Stathakis & Mosesson, 1977; Godfrey & Purohit, 1982). The low recovery of activity after gel filtration carried out at 4° may, however, be a reflection of a degree of cold insolubility. We have found that guinea-pig plasma FN seems to be more soluble at 4° than is human plasma FN (unpublished observations), and the relative solubility of MAggF at 4° may be due to a species difference. The observation that MAggF is released into the culture supernatant rather than remaining tightly bound to its cell of origin (presumably a T cell), cannot be taken as a point of difference between MAggF and tissue FN, since many tissue FN have been shown to be released from the cells synthesizing them (Wagner & Hynes, 1979; Mosher, 1980).

We do not believe that guinea-pig MAggF is related to macromolecular insoluble cold globulin (T-MICG), a cold-insoluble surface protein of murine

and human T cells (Hauptman, 1978; Hauptman, Kansu, Serno & Godfrey, 1979). This protein is not a T-cell-secretory protein, antisera raised against it are not cross-reactive with any plasma protein, and antisera with specificities against a large number of plasma proteins (including Ig, FN and α_2M) show no cross-reactivity with it. When isolated, T-MICG is a single chain molecule of 225,000 Daltons containing no disulphide-linked subunits. Unlike FN, it does not exist in solution as a dimer; its molecular weight on gel chromatography is the same as on SDS-PAGE (225,000 Daltons). Although there are a few superficial similarities between MAggF and MICG, the difference between these two materials are such that it seems unreasonable to identify MAggF with MICG.

The observed parallelism of the dose activity curves of FN preparations and stimulated LNC culture supernatant implies a similarity in the mechanism of action of the active principles in these materials (Colquhoun, 1971). Since the two GPFN preparations contained similar amounts of immunoprecipitable FN, but differed nearly twenty-fold in their MAggF potency, it is not possible to identify MAggF activity with immunoprecipitable FN. It is not unreasonable to ascribe differences in potency between GP and HuFN to species specificity of lymphokine activity, (Godfrey & Gell, 1978), since guinea-pig macrophages were used to detect MAggF activity. The lack of immunoprecipitation between MAggF (tissue FN) in stimulated LNC culture supernatants and anti-HuFN might be ascribed to the absence of an immunodominant epitope on MAggF which was present on plasma FN in both guinea-pig and man (Ruoslahti & Engvall, 1978). It is reasonable to expect that antisera whose specificity was directed against a less immunodominant determinant present on both guinea-pig plasma FN and MAggF (tissue FN) would show better correlation between immunoreactive FN and MAggF potency than was obtainable using antiserum directed against a heterologous plasma FN.

One other aspect of the dose activity curves requires comment. GPFN-2 (isolated from CIG) displayed a maximum titre nearly a 100-fold greater than GPFN-1 (isolated directly from plasma) or the culture supernatant from stimulated LNC. This occurrence could be due to either the presence of MAggF active substances in GPFN-2 missing from the other preparations or to the presence of MAggF inhibitory substances in the other preparations or to some combination of these circumstances. The current studies do not permit a clear choice between these alternatives; the frequent increases in MAggF titre after several of the various adsorptions and elutions were not significant but might indicate that concentrated and unconcentrated culture supernatants contained MAggF inhibitors. Technical error seems an unlikely explanation: the data of Fig. 4 were derived from three to five assays for each preparation.

We are not the first to report the molecular weight of MAggF to be poly-disperse. Rouveix, Badenoch-Jones & Turk (1979) found MAggF activity at both 150,000 Daltons and at 48,000 Daltons with the majority of activity associated with the smaller molecules. We found MAggF activity at comparable mol. wt (240,000 and 50,000) and, indeed, there was more activity associated with the 50,000 Dalton than with the 240,000 Dalton fractions. However, 90% of the total recovered MAggF activity in our preparations was found at 370,000 Daltons. Since our findings are based on a single pooled concentrated preparation they are not definitive and will require confirmation. The reasons for the difference in results between the two laboratories is unknown and will also require further study. It might result from the method of preparation of LNC culture supernatants: ours were concentrated by ultrafiltration, those of Rouveix *et al.* (1979) by salt precipitation and freeze-drying. These different methodologies might lead to differences in state of aggregation or dissociation, or, alternatively, to differences in degrees of proteolytic or other degradative processes. Studies on the association and dissociation of various molecular forms of MAggF and their stability under various methods of concentration offer a means to resolve the reported differences in molecular weights.

The discovery of the gelatin-binding properties of MAggF has several immediate consequences. Firstly, it opens the possibility of rapid one-step purification of MAggF from unconcentrated LNC culture supernatants by affinity chromatography over insolubilized gelatin. Secondly it suggests that an immunoassay, (either radio- or enzyme-linked) for MAggF could be developed using insolubilized gelatin and anti-FN. Thirdly, and most intriguing, it provides a basis for developing hypotheses to explain the biochemical events of delayed hypersensitivity. The ordered secretion of tissue FN (MAggF), inducers of procoagulants, and inducers of plasminogen activator by antigen-stimulated sensitized T cells might account for the immobilization of macrophages and elicitation of fibroblasts in tissues at the site of the delayed reaction and the subsequent resolution of the inflammatory

process (Godfrey & Gell, 1978; Clark, Dvorak & Colvin, 1981; Geczy & Hopper, 1981; Tsakamoto, Helsel & Wahl, 1981). Experiments directed towards each of these possibilities are now in progress.

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