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Mitogenic Effect of Fibrinogen on Hematopoietic Cells: Involvement of Two Distinct Specific Receptors, MFR and ICAM-1[1]

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In addition to its well-known functions in blood clotting and cell adhesion, fibrinogen has been reported to be a mitogen for lymphoid cell lines and for human hematopoietic progenitors. Two specific receptors, the mitogenic fibrinogen receptor (MFR) and intercellular adhesion molecule-1 (ICAM-1/CD54), have been identified as possible candidates for the mediation of the mitogenic effect of fibrinogen. However, it has been questioned whether the MFR and ICAM-1 are truly distinct molecules. Using an antiserum specific for the MFR, we demonstrate that the MFR is a cell surface molecule clearly distinct from ICAM-1. Both receptors can be expressed separately or coexpressed on different cell types. Moreover, they are regulated differently: ICAM-1 is calcium-dependent whereas the MFR is not and the MFR is down-regulated by fibrinogen whereas ICAM-1 is not. The inhibition by an anti-MFR serum of the mitogenic effect of fibrinogen confirms the mitogenic function of the MFR.

Human fibrinogen, a 340-kDa plasma protein which is also present in regenerating tissues and bone marrow, is a homodimer made up of two sets of α, β, and γ polypeptide chains linked by interchain disulfide bonds. Fibrinogen is a protein known to be involved in various cell adhesion processes such as platelet aggregation, leukocyte adhesion or leukocyte transendothelial migration. In addition to these adhesive functions, purified human fibrinogen stimulates proliferation of Tland B lymphoma-derived cell lines such as JM and Raji cells. Fibrinogen also stimulates the production of early hematopoietic progenitors in long-term Dexter type human bone marrow cultures (1, 2) and potentiates the stimulating effect of low concentrations of recombinant human interleukin-3 to promote the development of highly purified human $CD34^+$ primitive hematopoietic progenitors (3).

Lévesque *et al.* first demonstrated that the mitogenic effect of fibrinogen on Raji cells is controlled via receptor modulation and identified a specific receptor called the mitogenic fibrinogen receptor (MFR) (4, 5). More recently, Gardiner *et al.* reported that the mitogenic action for fibrinogen on Raji cells is mediated through the intercellular adhesion molecule-1 (ICAM-1/CD54) (6, 7). As these authors considered that Raji cells express only one fibrinogen receptor, it has been suggested that the MFR and ICAM-1 could be the same molecule. In addition, the MFR and ICAM-1 share various functional and biochemical properties. Both display similar molecular weights (92 ± 3 kDa), a low affinity for fibrinogen and do not belong to the integrin family. Indeed, these receptors do not recognize the RGDS sequence found on fibrinogen, fibronectin and other adhesion molecules. However, some observations suggest that the MFR and ICAM-1 are different molecules. First, the MFR is a calcium-independent receptor whereas ICAM-1 is calcium-dependent. Second, in some selected Raji cell lines, the MFR is expressed at more than 150,000 molecules per cell (2); its expression can be upregulated up to 400,000 molecules per cell. In contrast, ICAM-1 appears never to be expressed at more than 40,000 molecules per cell (7). Consequently, it is important to clarify whether the MFR and ICAM-1 are different entities and whether both of them are mitogenic.

In this paper, we demonstrate unequivocally that the MFR is distinct from ICAM-1. Both receptors present distinct antigenic properties, may be coex-

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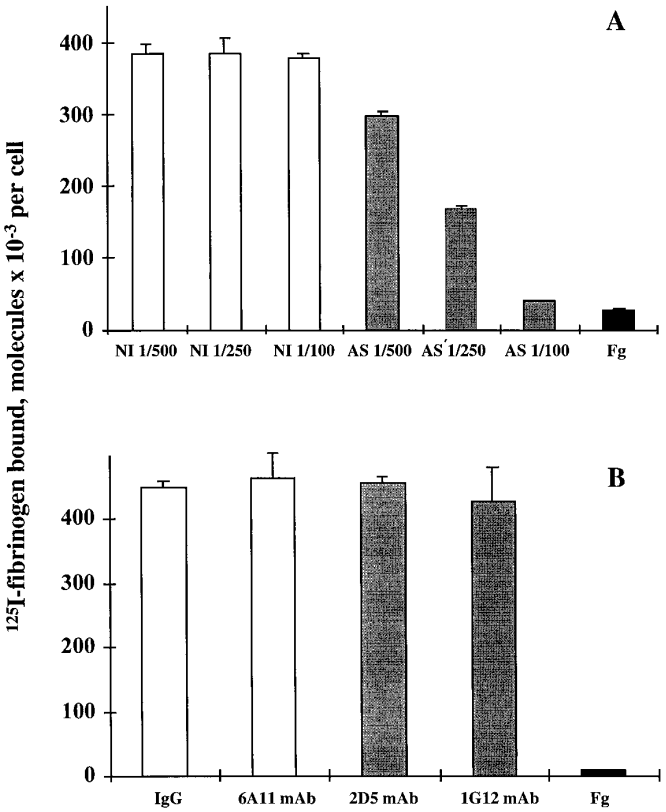

FIG. 1. Anti-MFR serum strongly inhibits high levels of fibrinogen binding on Raji cells. Radiolabelled fibrinogen binding was determined after preincubation of Raji cells for 30 minutes : (Fig. 1A) with a non-immune mouse serum (NI) or anti-MFR serum (AS) diluted 500-fold, 250-fold, 100-fold ; (Fig. 1B) with 50 μg/ml of IgG, anti-ICAM-1 isotypic control 6A11 mAb, anti-ICAM-1 2D5 mAb and anti-ICAM-1 1G12 mAb. The non-specific binding was estimated in the presence of a 100-fold excess of unlabelled fibrinogen (Fg). Three independent experiments gave the same results. **Anti-ICAM-1 2D5, 1G12 or 84H10 mAbs displayed similar reactivity.**

pressed or expressed separately on distinct cell types and are regulated differently. The down-regulation of the MFR by its own ligand, a property exhibited by many growth factor receptors but not observed with ICAM-1, and the inhibition of fibrinogen-mediated cell proliferation by an anti-MFR serum confirm a mitogenic role for the MFR.

MATERIALS AND METHODS

Cell lines and normal cells. Cells were grown in suspension at 37°C in a humidified atmosphere with 5% CO_2. Burkitt's lymphoma Raji, T-cell leukemia JM and promyelocytic HL-60 cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine and 10% (v/v) FCS. The fibrinogen receptor expression was upregulated by culturing Raji or JM cells for 12 hours in serum-free SG*B medium (StemBio Research, France). Conversely, receptor down-regulation was achieved by incubating JM cells in FCS-containing medium supplemented with 50 μg/ml purified human fibrinogen for 24 hours as previously described (8). Fibrinogen was purified according to Kekwick *et al.* (9) modified by Lévesque *et al.* (8). Ficoll-separated human bone marrow mononuclear cells were first enriched for $CD34^+$ cells using a magnetic cell sorting protocol (*MACS*, Miltenyi Biotec, Sunnyvale, CA). Cells were stained with an anti-CD34 PE-conjugated mAb (8G12 clone; Becton Dickinson, San Jose CA) and purified using a Vantage fluorescence activated cell sorter (FACS-Vantage, Becton Dickinson). Cells were sorted in serum-free SG*A medium (StemBio Research). Sorted $CD34^+$ cells were tested in a low cell density assay; they were seeded at 2×10^3 cells/ml in 96-well plates in a total volume of 100 μl/well of SG*A medium with 0.17 U/ml rhIL-3, 10 U/ml rhIL-6, 1U/ml rhG-CSF and 10 U/ml rherythropoietin (Valbiotech, France) with or without 30 μg/ml fibrinogen, a concentration previously found to be optimal (3). Cells were counted using an inverted microscope after 48lhours of culture.

Anti-MFR serum and monoclonal antibodies. Raji cells expressing up to 400,000 fibrinogen binding sites per cell were selected. The protein fraction from 90 to 100 kDa was obtained from non-reducing preparative polyacrylamide gels according to Laemmli (10). These

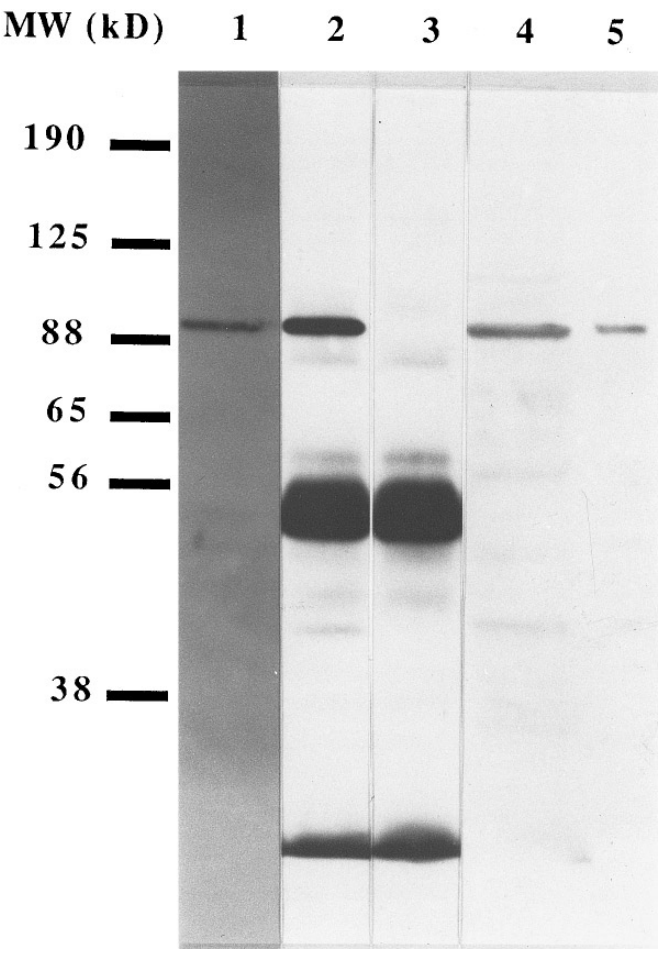

FIG. 2. Comparison by Western blot analysis of anti-MFR serum and anti-ICAM-1 mAbs reactivities. Proteins purified from Raji cells on fibrinogen-Sepharose (lane 1), Raji cell plasma membrane protein fraction immunoprecipitated with anti-MFR serum (lanes 2 and 3), whole Raji cell extracts (lane 4) and TPA-treated HL-60 cell extracts (lane 5) were run on 8% SDS-PAGE. Proteins electrotransferred to nitrocellulose were probed with anti-MFR serum (lanes 1 and 2) or anti-ICAM-1 LB-2 mAb (lanes 3 to 5) in a standard ECL test. The scale corresponds to molecular weight standards. Three independent experiments using anti-ICAM-1 2D5, LB-2 and 84H10 mAbs gave the same results.

conditions avoided co-migration of proteins with the 90 kDa-reduced transferrin receptor chains, a major cell surface protein of proliferating cell lines. Extracted proteins were concentrated to 1 ml by ultrafiltration on a PM10 Amicon membrane (Danvers, MA). Protein extracts were extensively dialysed against PBS and filtered on a 0.22 μm filter before injection into 6 week-old male Balb/c mice. Immunizations were carried out by intraperitoneal injections of extracted proteins. Last injections consisted of a protein fraction purified by affinity chromatography on fibrinogen-Sepharose previously developed for MFR isolation (5). An antiserum was selected for its ability to suppress a high level of ^{125}I-fibrinogen binding on Raji cells in a dose-dependent manner with a binding assay as previously described (2). It will be referred as to anti-MFR serum. Anti-ICAM-1 2D5 mAb which inhibits fibrinogen ICAM-1 interaction (11) and isotypic control 6A11 mAb specific for ICAM-1 intracellular domain were kindly provided by Dr Alain Duperray (INSERM U217, Grenoble, France). Anti-ICAM-1 LB-2 mAb and 84H10 mAb were from Becton-Dickinson and Immunotech (Marseille, France) respectively.

Immunofluorescence staining and FACS analysis. Approximately 100,000 cells in 50 μl PBS containing 0.2 % BSA were incubated for 15 minutes at 4°C with 200 μg/ml rat γglobulins (Jackson Immunoresearch, West Grove PE) and then for 30 minutes at 4°C with 50 μg/ml anti-ICAM-1 2D5 mAb or a 1/100 dilution of anti-MFR serum. Unrelated mouse serum or anti-ICAM-1 isotypic control 6A11 mAb (50 μg/ml) were used as controls. Cells were washed in PBS/BSA, incubated for 30 minutes at 4°C with 10 μg/ml PE-conjugated rat anti-mouse IgG (Becton Dickinson), washed again in PBS/BSA and analyzed on FACS-Vantage; 10^4 events were stored in list mode files.

Proteins, SDS PAGE and Western blot analysis. Plasma membrane proteins were prepared according to Uze *et al.* (12) followed by dialysis against PBS. Affinity chromatography on fibrinogen-coupled CNBr-activated Sepharose was set up as previously described (5) except that the basal medium of lysis, washing and elution buffers was PBS instead of IMDM. Proteins were immunoprecipitated according to Héron *et al.* (13). Proteins were analyzed by SDS-PAGE according to Laemmli (10). Prestained molecular weight markers (33.5 to 190 kDa) SDS-7B from Sigma Chemicals Co. were routinely included in each run. After transfer of the proteins, the Hybond-C nitrocellulose sheets (Amersham Intl.) were incubated for 2 hours in PBS containing 5% (w/v) dry skimmed milk (Bio-Rad Lab. Hercules, CA) and then for 2 hours in PBS supplemented with 0.05% (v/v) Tween-20 (PBST), 5% (v/v) normal goat serum and 0.5 μg/ml normal goat γ globulins to eliminate non-specific background. Membranes were treated with specific antibodies for 16 hours at 4°C. Antibodies

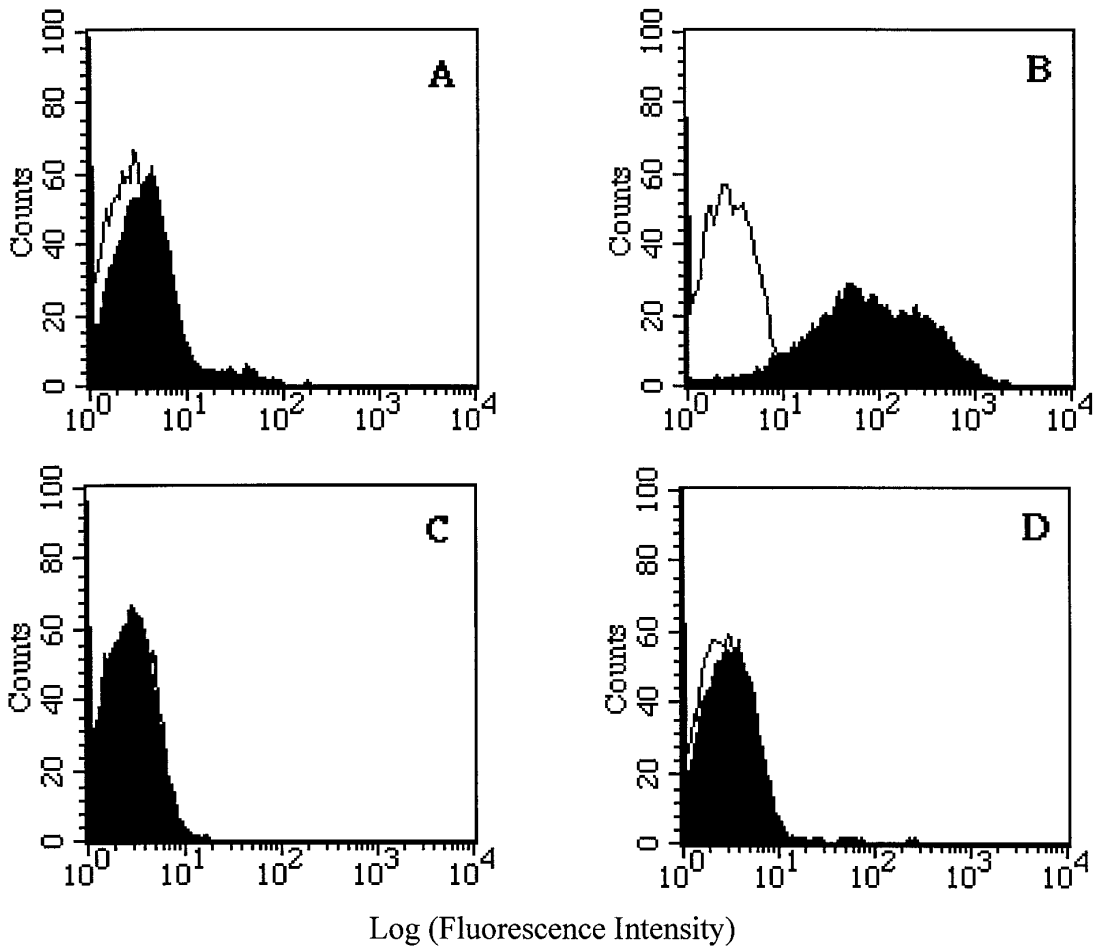

FIG. 3. Flow cytometry staining obtained with the anti-MFR serum was unrelated to ICAM-1 expression. HL-60 cells were cultured for 48 hours in the absence (A and C) or in the presence of 50 ng/ml TPA (B and D). Cells were indirectly stained using anti-ICAM-1 2D5 mAbs (A and B) or anti-MFR serum (C and D) and analysed by FACS (black histogram). Control 6A11 mAb specific for the intracellular domain of ICAM-1 and irrelevant mouse serum were used as isotypic controls (open histogram).

were diluted between 1/500 to 1/5,000 for anti-ICAM-1 mAbs and 1/20,000 for the anti-MFR serum in PBST supplemented with 3% BSA. After extensive washing in PBST, membranes were incubated for 1 hour at room temperature with horse-radish peroxidase conjugated goat anti-mouse IgG (Immunotech) diluted 1/10,000 in PBST supplemented with 3% BSA. Antigens were detected by enhanced chemiluminescent detection (ECL , Amersham Intl.).

RESULTS AND DISCUSSION

An antiserum was selected for its ability to suppress, in a dose-dependent manner, a high level of ^{125}I-fibrinogen binding observed on some Raji cell lines. This antiserum (AS) was able to inhibit more than 50 % of the fibrinogen binding after a 250-fold dilution and 95% after a 100-fold dilution as compared to the control serum from a non-immunized mouse (NI) (Fig. 1A). In contrast, high concentrations (up to 50 μg/ml) of anti-ICAM-1 2D5 and 1G12 mAbs, which suppress the fibrinogen-ICAM-1 interaction (11), did not significantly inhibit the high level of fibrinogen binding (Fig. 1B). **These results strongly suggested that the antiserum blocked the fibrinogen binding on receptors distinct from ICAM-1,** in particular the MFR which has been described as being highly expressed under these conditions. For the sake of clarity, the antiserum will be subsequently referred to as anti-MFR serum.

We compared by Western blot analyses the specificity of anti-MFR serum and anti-ICAM-1 antibodies (Fig. 2). The anti-MFR serum revealed a single 92 kDa band in Raji cell plasma membrane protein fraction purified by affinity chromatography on fibrinogen-Sepharose previously developed for MFR isolation (5) (Fig. 2, lane 1). When the MFR immunoprecipitated with anti-MFR serum (Fig. 2, lane 2) was blotted with anti-ICAM-1 mAbs (Fig. 2, lane 3), it was not detectable. The only common proteins detected in both cases were antiserum immunoglobulin heavy and light chains (Fig. 2, lanes 2 and 3); these bands confirmed that the same

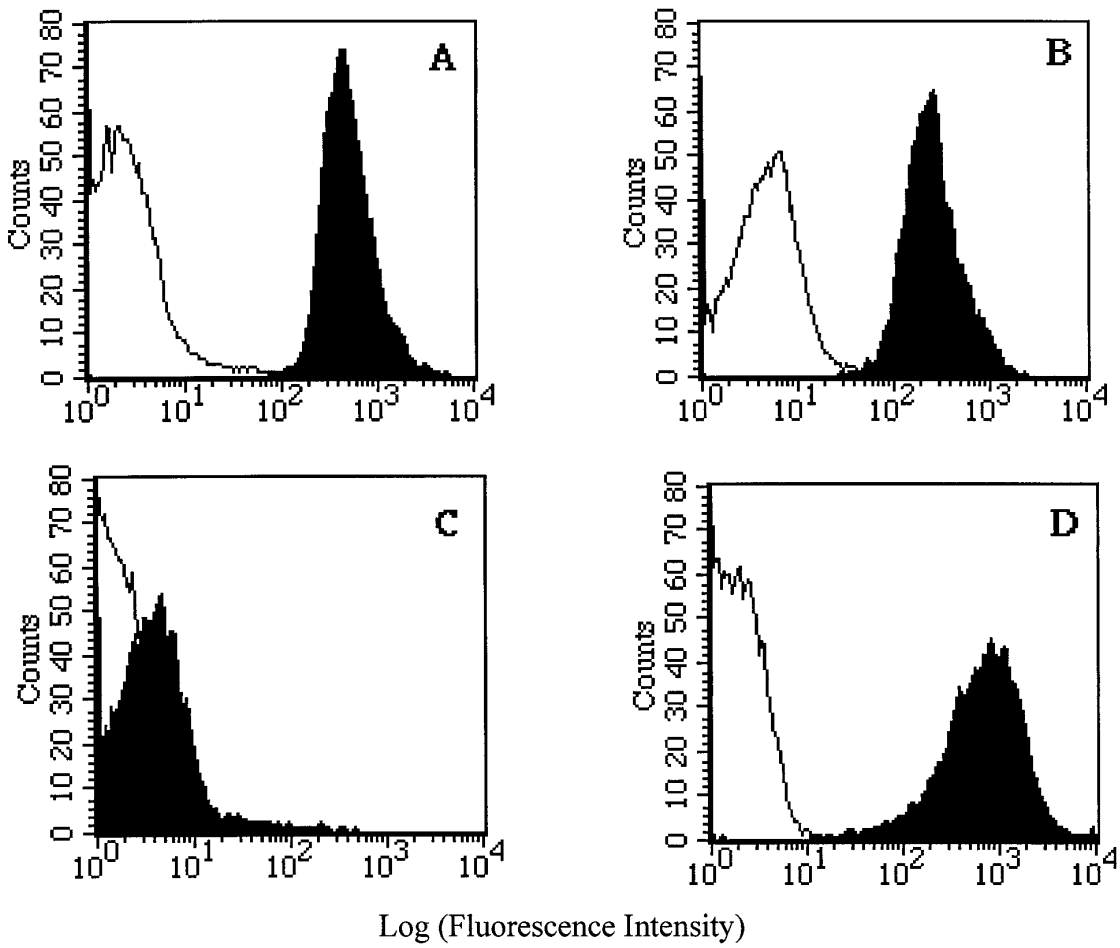

FIG. 4. Some Raji strains expressed ICAM-1 but not the MFR. One Raji strain (A and C) and another (B and D) were cultured in serum-free conditions. Cells were indirectly stained using anti-ICAM-1 2D5 mAbs (A and B) or anti-MFR serum (C and D) and analysed by FACS (black histogram). Control 6A11 mAb specific for the intracellular domain of ICAM-1 and irrelevant mouse serum were used as isotypic controls (open histogram).

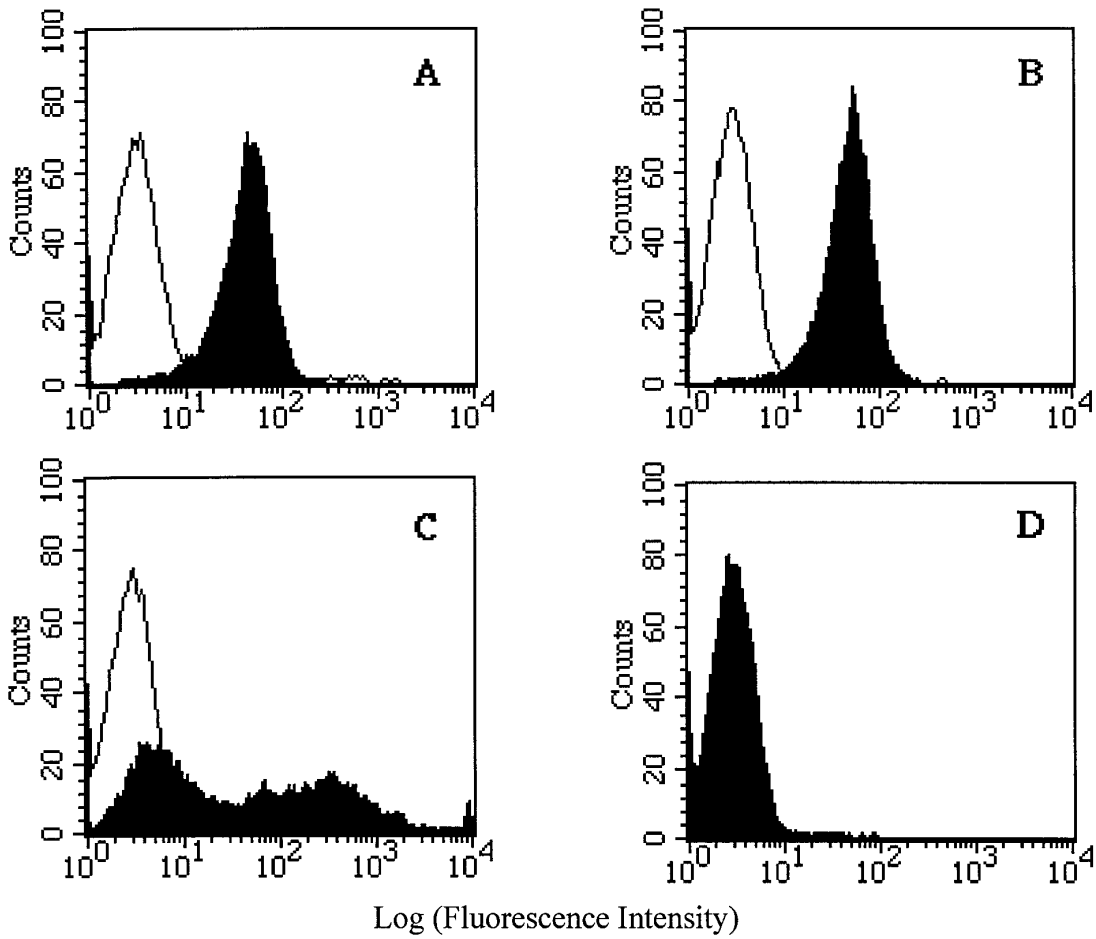

FIG. 5. Fibrinogen-induced down-regulation correlated only with MFR expression. JM cells were cultured in serum-free medium (A and C) or in FCS-containing medium and 50 μg/ml of purified human fibrinogen (B and D). Cells were indirectly stained using anti-ICAM-1 2D5 mAbs (A and B) or anti-MFR serum (C and D) and analysed by FACS (black histogram). Control 6A11 mAb specific for the intracellular domain of ICAM-1 and irrelevant mouse serum were used as isotypic controls (open histogram).

amount of immunoprecipitate was loaded on these lanes. To assess the immunoreactivity of anti-ICAM-1 mAbs in Western blot analyses, a whole Raji cell extract was directly analyzed, and a 92 kDa protein was then detected with these antibodies (Fig. 2, lane 4). This band showed the same molecular weight and antigenic specificity as the ICAM-1 molecule detected on HL-60 cells (Fig. 2, lane 5) when these cells were differenciated with 12-O-tetradecanoyl phorbol 13-acetate (TPA), a treatment known to induce ICAM-1 expression (14). The absence of cross-reactivity between the anti-MFR serum and some anti-ICAM-1 mAbs, as shown by these Western blot analyses, confirmed that Raji cells express two distinct cell surface fibrinogen receptors, ICAM-1 and MFR. As expected, both displayed a similar molecular weight (92 ± 3 kDa).

We confirmed by flow cytometry analyses that the expression of the MFR and ICAM-1 could be distinguished on various cell lines. For instance, in standard culture conditions, HL-60 cells did not express ICAM-1 (Fig. 3A). However, when HL-60 cells were pretreated with TPA, we clearly observed the strong expression of ICAM-1 (Fig. 3B). On the contrary, the anti-MFR serum reacted neither with untreated nor with pretreated HL-60 cells (Figs. 3C and 3D). We also showed that some Raji strains express ICAM-1 but not the MFR (Fig. 4). Indeed, flow cytometry analyses revealed a similar expression of ICAM-1 in two Raji strains cultured in serum-free conditions (Fig. 4A and 4B). On the contrary, the MFR was not expressed in the first strain (Fig. 4C) but was strongly expressed in the second (Fig. 4D). **Western blot analyses confirmed the absence of MFR in plasma membrane protein fractions from Raji cells with low anti-MFR reactivity (results not shown).** We also studied by flow cytometry analyses (Fig. 5), the serum-dependent down-regulation of fibrinogen receptors previously detailed on JM cells (8). The concept of serum-dependent

down-regulation was first observed in 1980 by Sato and his colleagues (15) for the epidermal growth factor (EGF) receptor on HeLa cells. They demonstrated that EGF, a high affinity growth factor, promotes EGF receptor down-regulation in the presence of serum but not in serum-free conditions. We showed that ICAM-1 was expressed on JM cells cultured in serum-free conditions (Fig. 5A). When JM cells were grown in FCS-supplemented medium in the presence of fibrinogen, ICAM-1 expression was not down-modulated (Fig. 5B). In contrast, these analyses confirmed that the MFR expressed on JM cells (Fig. 5C) was strongly down-regulated by its own ligand in FCS-supplemented medium (Fig. 5D). Therefore, the decrease in fibrinogen binding previously observed (8) is due to the disappearance of the MFR from the cell surface.

The flow cytometry analyses showed that the expression of ICAM-1 was constitutive on Raji cells as opposed to the MFR. **This could explain why anti-ICAM-1 blocking antibodies could prevent the total fibrinogen binding on Raji cells which do not express the MFR and led Gardiner *et al.* to conclude that ICAM-1 constitutes the only fibrinogen receptor on Raji cells** (7). On the other hand, fibrinogen down-modulated up to 95% of the fibrinogen binding on Raji cells which strongly express the MFR and led Lévesque *et al.* to conclude that the MFR constitutes the only fibrinogen receptor on Raji cells (8). However, these two fibrinogen receptors are clearly distinct. As we were not able to select cell lines which express only the MFR without ICAM-1, the mitogenic function of the MFR remained to be confirmed. The MFR which is a low affinity receptor, was down-regulated by its own ligand, as are many growth factor receptors, in serum containing medium but not in serum-free medium (Fig. 5). We tested the ability of anti-MFR serum to inhibit fibrinogen induced proliferation of Raji cells in serum-free medium with transferrin only as previously detailed (1). Without fibrinogen for 48 hours (Fig. 6), only 7% of Raji cells developed clones (14 per 200 cells). In contrast, the presence of fibrinogen induced the growth of 63% of the cells. When Raji cells were pretreated with anti-MFR serum at a dilution which strongly inhibited fibrinogen binding (100 fold dilution, Fig. 1A), fibrinogen induced growth was reduced to 33%. The pretreatment of cells with anti-MFR serum inhibited the development of large clones; untreated cells developed in 48 hours significantly more 8-cell clones than pretreated cells (47 versus 7). **In addition, we observed a differential mitogenic response to fibrinogen in variant Raji strains which display either high or low anti-MFR reactivity, while having similar levels of ICAM-1 expression (results not shown). These results confirm that the MFR mediates a mitogenic function of fibrinogen on Raji cells but do not exclude that other molecules, such as ICAM-1 which is constitutively expressed on Raji cells [ref. 7], are implicated in this function.**

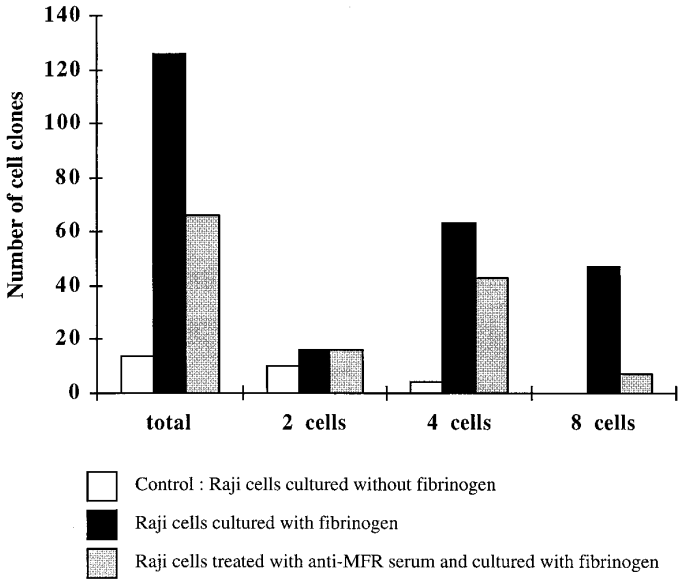

FIG. 6. Anti-MFR serum partially decreased the proliferative effect of fibrinogen on Raji cells. Two hundred Raji cells were cultured in a low cell density assay with or without 5 μg/ml of fibrinogen. Some cells were previously treated in the presence of anti-MFR serum diluted 100-fold. Fibrinogen-dependent dividing cells (cell clones) were counted after 48 hours. Three independent experiments with different concentrations of fibrinogen (1 to 10 μg/ml) gave similar results.

To further detail the physiological role of the MFR, we tested the ability of anti-MFR serum to inhibit fibrinogen induced proliferation of normal hematopoietic progenitors. Anti-MFR serum inhibited in short term culture the mitogenic effect of fibrinogen on human bone marrow $CD34^+$ cells which comprise 15 to 30% of clonogenic hematopoietic progenitors (table 1). In the presence of irrelevant serum, addition of fibrinogen increased significantly ($p = 0.04$) the number of two-cell clones (2.75, 5.6 and 7.25 times increase with fibrinogen added for experiments 1, 2 and 3 respectively). In contrast, in the presence of anti-MFR serum, addition of fibrinogen did not significantly increase the number of two-cell clones ($p = 0.91$). Therefore, anti-MFR serum abolished fibrinogen induced cell proliferation. This inhibition was dose-dependent and was not due to the toxicity of the serum since it did not reduce the number of two-cell clones in the absence of fibrinogen. In addition, cell death was consistently lower than 5% in both culture conditions.

In conclusion, we have clearly demonstrated that the MFR and ICAM-1 represent two distinct fibrinogen receptors. These two receptors have distinct antigenic properties, may be expressed on different cell types and are regulated differently. Proliferation experiments performed with anti-MFR serum confirm that the MFR is mitogenic as well as ICAM-1.

TABLE 1

Anti-MFR Serum Decreased the Proliferative Effect of Fibrinogen on Normal Human Hematopoietic Progenitors

		Culture conditions			
		With irrelevant serum		With anti-MFR serum	
	Exp. No.	Without fibrinogen	With fibrinogen	Without fibrinogen	With fibrinogen
Two-cell clones/well	1	4	11	4	5
	2	5	28	10	5
	3	4	29	3	8
Percentage of proliferative cells	mean	2.16% ± 0.28	11.33% ± 5.05	2.83% ± 1.89	3% ± 0.86

Two hundred normal human $CD34^+$ progenitors were cultured with or without 30 μg/ml of fibrinogen in the presence of irrelevant mouse serum or anti-MFR serum diluted 1000-fold. Fibrinogen-dependent dividing cells (two-cell clones) were counted after 48 hours. Cell death was lower than 5% in both culture conditions. Three independent experiments were performed using bone marrow cells from 3 different donors. The Student's t test was applied to determine the significance of the mitogenic effect of fibrinogen (i) in the presence of irrelevant serum (p=0.04) and (ii) in the presence of anti-MFR serum (p=0.91). The percentage of proliferative cells was calculated as follow: (two-cell clones ×100)/(two-cell clones + single cells).

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