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ORIGINAL ARTICLE

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Differential lytic and agglutinating activity of the anti-Lewis^x monoclonal antibody FC-2.15 on human polymorphonuclear neutrophils and MCF-7 breast tumor cells. In vitro and ex vivo studies

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Abstract The Lewis^x (Le^x) trisaccharide (CD15) linked to proteins and glycolipids is highly expressed on the surface of normal human polymorphonuclear neutrophils (PMN) and several human neoplasias, such as breast and gastrointestinal carcinomas and chronic myeloid leukemias. FC-2.15 is an IgM murine mAb that specifically recognizes Le^x and has been previously shown to mediate the in vitro lysis of Le^x(+) cells by human complement. In a phase I clinical trial of FC-2.15, a temporary neutropenia was the main toxicity, and antitumor responses were observed. In order to characterize FC-2.15 further and determine the physiological relevance of Le^x binding, the reactivity of FC-2.15 on PMN was investigated under several conditions. Flow cytometry revealed a strong reactivity of FC-2.15 with almost 100% of PMN, and Scatchard analysis demonstrated an affinity constant of 5.14×10^9 M^{-1} and 1.11×10^6 antigen sites/cell. In vitro, the binding of Le^x epitopes by FC-2.15 induced PMN homotypic aggregation, only 28.4 ± 4.1% remaining as single cells. When PMN and the Le^x(+) MCF-7 breast cancer cells were co-incubated, FC-2.15 induced heterotypic aggregation. In ^{51}Cr-release assays employing human complement, FC-2.15 lysed 93.4 ± 7.9% of PMN and 87.8 ± 10.7% of MCF-7 cells. However, when the effect of FC-2.15 was tested in ex vivo circulating blood, no lytic activity against PMN was detected, whereas MCF-7 cells were still lysed. Blood smears demonstrated that FC-2.15 induced PMN agglutination and heterotypic aggregates when MCF-7 cells were present. A pretreatment of PMN with colchicine impaired PMN agglutination both in vitro (single PMN = 81.15 ± 4.35%) and in ex vivo circulating blood. In the latter condition, FC-2.15-lytic activity was restored, suggesting that PMN homotypic aggregation by FC-2.15, but not lysis, is dependent on microtubule integrity and that PMN agglutination hinders their lysis. Moreover, when ^{51}Cr-release assays were performed following agglutination, FC-2.15 cytotoxicity was restricted to isolated PMN. It is suggested that crosslinking of Le^x epitopes by FC-2.15 induces PMN to form homotypic aggregates. It is suggested that the neutropenia observed in FC-2.15-treated patients would be due to PMN agglutination and margination, rather than lysis. In addition, FC-2.15 appears to be able to lyse Le^x(+) tumor cells in circulation.

Key words FC-2.15 · Monoclonal antibody · Lewis X · neutrophils · neutropenia

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Introduction

The migration of peripheral polymorphonuclear neutrophils (PMN) into tissues is the central event in the inflammatory response. Both leukocyte trafficking and recruitment to sites of injury are mediated by adhesion molecules [32]. The carbohydrate antigen sialyl-Lewis^x (sialyl-Le^x) [NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc] (CD15s) is highly expressed on the surface of PMN and appears to be the ligand of the endothelial-cell–leukocyte adhesion molecule-1 (ELAM-1), exclusively expressed on the cell surface of activated human endothelial cells [1, 23, 31, 32, 40, 44]. PMN also bear another fucose-containing carbohydrate, the related Lewis^x (Le^x) (3-fucosyl-lactosamine) (CD15), both on glycolipids and

at the termini of N- and O-linked oligosaccharides [8, 19, 39, 40]. Although a function for Le^x in intercellular recognition is also suggested [11, 15, 16, 34], the roles of Le^x and sialyl-Le^x would be different, as appears from their different expression in neutral cells in culture [34] and in several tissues [16, 26]. Therefore, the physiological role of Le^x remains obscure.

FC-2.15 is an IgM murine mAb raised against a human breast carcinoma, which reacts with the majority of breast and digestive carcinomas and with chronic myeloid leukemias [24]. FC-2.15 specifically recognizes the Le^x trisaccharide epitope and, therefore, also cross-reacts with normal cells and tissues expressing Le^x, mostly PMN and bone marrow myeloid progeny [24]. In vitro assays have shown that FC-2.15 mediates a strong human complement-dependent lytic activity against Le^x-positive cells [3]. A phase I clinical study of FC-2.15, performed in 11 patients with advanced cancer, demonstrated that the most consistent toxicity was a profound but rapidly reversible neutropenia, frequently accompanied by fever and chills that were easily managed. Therefore, and although FC-2.15 mediates in vitro lysis of PMN [3], the paucity of symptoms suggested that the neutropenia could be rather ascribed to PMN margination. In the same study, an objective remission of hepatic metastases of breast carcinoma was observed [25]. On the basis of these results, it was considered of interest to characterize further the interaction of FC-2.15 with PMN and with a tumor model, such as the strongly $Le^x(+)$ breast cancer cell line MCF-7 [3]. In the present study the FC-2.15 interaction with PMN and MCF-7 cells was assayed both in vitro and in ex vivo circulating blood (EVCB), as an approach to in vivo conditions.

Materials and methods

Reagents

Tissue-culture media and fetal bovine serum (FBS) were from Life Technologies (Gaithersburg, Md., USA). The radioisotopes ^{125}I ($Na^{125}I$) and ^{51}Cr ($Na_2{}^{51}CrO_4$) were purchased from New England Nuclear (Boston, Mass., USA). The iodogen reagent was from Pierce (Rockford, Ill., USA). The Axioplan microscope was from Carl Zeiss (Göttingen, Germany) and the Kontron KS300 imaging system was from Kontron Elektronik (München, Germany).

Cell lines

The human breast cancer cell line MCF-7 [37] was grown in Dulbecco's modified Eagle medium (DMEM)/Ham's nutrient mixture F-12 (1:1) supplemented with 10% FBS, 2 mM glutamine, 10 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. The human breast cancer cell line IIB-BR-G [7] was grown in the same medium supplemented with 20 nM sodium selenite, 100 μM ascorbic acid, 5 ng/ml epidermal growth factor, 5 μg/ml transferrin, 5 μg/ml prolactin, 1.4 μM hydrocortisone and 0.1 nM 17β-estradiol. For experiments, exponentially growing cells were harvested by a detachment solution containing 15 mM sodium citrate/135 mM KCl.

Isolation of PMN

PMN were obtained from fresh blood of volunteer normal donors as previously described [8]. Briefly, heparinized whole blood was fractionated by dextran sedimentation, followed by a Ficoll-Hypaque gradient centrifugation. PMN were recovered in the pellet and, after lysis of the remaining erythrocytes by a 10-min incubation with an erythrocyte lysis solution (0.1 M EDTA, 10 mM KCO_3H, 0.155 M NH_4Cl) they were centrifuged and resuspended in phosphate-buffered saline (PBS).

Antibodies

The murine mAb FC-2.15 (IgM) was purified from ascitic fluid developed in Balb/c mice as previously described [25]. Other antibodies used as controls were murine mAb 436 (IgM, ascites), which recognizes the protein core of the polymorphic epithelial mucin [27], and murine mAb B1.1 (IgM, ascites), which recognizes the protein moiety of carcinoembryonic antigen (CEA) [10]. mAb 436 and B1.1 were generously provided by Dr. M. Nuti (University of Pisa, Italy).

Flow cytometry assay

Isolated PMN were incubated with 50 μg/ml FC-2.15 (60 min at 4 °C), washed three times with PBS and incubated for 60 min at 4 °C with fluorescein-isothiocyanate-labeled $F(ab)_2$ goat anti-(mouse IgM) (Zymed Laboratories Inc., South San Francisco, Calif., USA). Finally, they were washed three times with PBS and fixed with 2% formaldehyde, and Le^x expression was analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, Calif., USA). In control experiments, PBS was used instead of FC-2.15.

Iodination of FC-2.15

FC-2.15 was labeled with ^{125}I (Na^{125} I, 368 μCi/μl) by the iodogen method [13]. Briefly, 100 μg FC-2.15 was added to 1 mCi $Na^{125}I$ in a glass tube coated with 40 μg iodogen reagent for 10 min at room temperature. Free $Na^{125}I$ was removed by gel filtration on a Sephadex G-50 column. Eluted fractions containing ^{125}I-labeled FC-2.15, as determined by gamma counting, were pooled and stored at 4 °C. The specific activity of the labeled antibody was about 2.4 μCi/μg.

Scatchard plot analysis

Isolated PMN were used as target antigens PMN (3×10^5/100 μl) were incubated for 3 h at 4 °C in 100 μl PBS containing increasing concentrations of ^{125}I-FC-2.15 (range 0.05–30 μg/ml). To determine non-specific uptake, control incubations were performed in the presence of a 100-fold excess of unlabeled FC-2.15. After two washings, the radioactivity bound to the suspended cells was counted in a gamma counter. The Scatchard plot analysis [35] was used to calculate the affinity constant (K_a) and the average number of antigen sites per cell.

In vitro agglutination assay

Suspensions of PMN at 3×10^6 cells/ml PBS or DMEM were incubated with FC-2.15, at a concentration indicated in each experiment, for 1 h at 37 °C with gentle agitation. After that, the suspension was observed under a phase-contrast microscope (Axioplan, Carl Zeiss). The percentage of total cells remaining as single cells was determined, taking as 100% the number of single cells of a control experiment performed in the absence of FC-2.15. When indicated, a pretreatment of PMN with 2.5 μM colchicine for 1 h at 37 °C was performed and the drug was maintained during the

agglutination assay. Similar experiments were performed with mixed suspensions of PMN and MCF-7 [$Le^x(+)$] or IIB-BR-G [$Le^x(-)$] cells [8, 13] (3×10^6:3×10^5 cells/ml PBS or DMEM). Control mAb were B1.1 (ascites 1/100), reactive with PMN but not with MCF-7 cells, and 436 (ascites 1/100), reactive with MCF-7 cells but not with PMN (data not shown).

Antibody-dependent complement-mediated cytotoxicity

A ^{51}Cr-release assay was used to test the ability of FC-2.15 to mediate complement-dependent cytotoxicity. Target cells (PMN or MCF-7) (2.5×10^6/ml) were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$, sp. act. 550 mCi/mg; 150 μCi/ml) for 2 h at 37 °C, and washed three times with PBS or culture medium. ^{51}Cr-labelled cells (10^5) in 100 μl DMEM plus 100 μl human serum as the source of complement were incubated at different FC-2.15 concentrations (0, 5, 20 μg/ml) and for different intervals at 37 °C. Parallel incubations containing target cells resuspended in 200 μl medium alone or in 200 μl 0.1 M HCl were performed to determine spontaneous and total ^{51}Cr release respectively. At the end of the incubation period, the cells were centrifuged at 1200 rpm for 10 min and the radioactivity in the supernatant was determined in a gamma counter. Each point was assayed in triplicate and the percentage specific cytolysis was calculated as previously described [3]. When indicated, the assay was performed in the presence of different concentrations of heparin (from 0 U/ml to 100 U/ml) and the specific cytolysis was calculated as above.

To study the FC-2.15-dependent complement-mediated cytotoxicity in circulating whole blood, the ^{51}Cr-labeled cells (450 000 PMN/ml or 150 000 MCF-7 cells/ml) were resuspended in heparinized whole blood (10 U/ml) and the blood was subjected to flow conditions in an infusion set with a peristaltic pump (see below). When indicated, different concentrations of FC-2.15 were added and, at the times specified, blood samples (500 μl) were withdrawn with a 1.0-ml insulin syringe (27-gauge × 1.25 cm; Terumo Medical Corporation, Eckton, USA). Experimental ^{51}Cr release was determined by diluting 1:2 the blood with PBS and centrifuging at 2000 rpm for 20 min. Total release was measured by diluting 1:2 the blood with 0.15 M HCl and centrifuging as indicated above. In both cases, radioactivity in the supernatant was counted in a gamma counter. Spontaneous release was determined only at time zero, before the addition of mAb FC-2.15. At each time assayed, blood smears were prepared and dyed with May-Grünwald-Giemsa stain. When indicated, a pretreatment of isolated ^{51}Cr-labelled PMN or ^{51}Cr-labelled MCF-7 and of whole blood with 2.5 μM colchicine for 1 h at 37 °C was performed before they were mixed, to perform the ^{51}Cr-release assay.

To study the FC-2.15-dependent complement-mediated cytotoxicity against PMN homotypic aggregates, suspensions of 5×10^6 ^{51}Cr-labeled PMN/ml were first incubated in PBS with FC-2.15 (0, 5, 20 μg/ml) for 1 h at 37 °C with gentle agitation, and the percentage of total cells remaining as single cells was calculated as indicated above. Then, human serum as source of complement (1:1, v:v) was added and cytolysis was calculated after 90 min incubation at 37 °C in the presence of 20 μg/ml FC-2.15.

Ex vivo circulating blood (EVCB) system

Heparinized blood (10 U/ml) was obtained from normal volunteers. The flow conditions were established by introducing the blood into an infusion set (BIOM, B14, 4 mm diameter, Buenos Aires, Argentina) connected to a peristaltic pump (LKB-Pump P1, Pharmacia Uppsala, Sweden) at a controlled flow rate of 730 ml h^{-1} (1.61 cm/s). The system was maintained at 37 °C and oxygenation during circulation was provided through a 25-gauge G needle placed at the access port of the infusion set. Through this access port, the samples were obtained as indicated before. The percentage of spontaneous hemolysis at different times of circulation was determined as follows: 50 μl blood was diluted 1:1 with PBS, incubated for 10 min at room temperature, and centrifuged at 2000 rpm for 10 min and the absorbance of the supernatant (diluted 30:500 with distilled water) at 416 nm was determined as a measure of hemoglobin release. To calculate total hemolysis, 50 μl blood was diluted 1:1 with erythrocyte lysis solution and incubated, centrifuged and measured as indicated above.

Imaging analysis

The size of the clumps was measured using an Axioplan microscope and a Kontron KS300 imaging system

Electron microscopy

Cells were fixed with 10% glutaraldehyde in PBS for 1 h at room temperature, post-fixed in 1% osmium tetroxide in barbital acetate buffer, pH 7.4 for 2 h at 4 °C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone and finally embedded in Epon 812.

Results

Reactivity of PMN with FC-2.15

We have previously reported that FC-2.15 reacts with peripheral PMN [24]. In order to quantify this reactivity, isolated PMN were evaluated by flow cytometry and the K_a and the number of antigen sites per cell were determined by Scatchard plot analysis. As shown in Fig. 1, 99.9% of PMN were strongly positive with mAb FC-2.15 (Fig.1A) as compared to a control experiment with PBS where only 0.4% of cells were positive (Fig. 1B). The Scatchard plot analysis (Fig. 2), considering a 1/1 stoichiometry for the antibody/antigen reaction, showed a linear relationship, indicating a homogeneous population of binding sites with a K_a of 5.14×10^9 M^{-1} and a binding capacity of 1.11×10^6 antigen sites/cell. It is interesting to mention that this affinity is considerably higher than the value of 6.9×10^7 M^{-1} obtained for MCF-7 cells [3].

FC-2.15 agglutinates PMN in vitro and forms heterotypic aggregates between PMN and tumor $Le^x(+)$ cells

Two main possibilities exist to explain the neutropenia induced after FC-2.15 injection in vivo: PMN lysis or agglutination and margination. It has been reported that PMN are normally agglutinable by lectins, such as concanavalin A or phytohemagglutinin, [2, 5, 36] and by some antibodies [14, 21, 22]. In order to test whether FC-2.15 agglutinates PMN, the assay described in Materials and methods was performed. When isolated PMN were incubated with 50 μg/ml FC-2.15 for 1 h, PMN homotypic aggregates were formed and only 28.4 ± 4.1% of the total cells remained isolated (Table 1). Interestingly, FC-2.15 was not able to aggregate MCF-7 $Le^x(+)$ tumor cells (single cells = 96.0 ± 4.7%), although when PMN and MCF-7 cells were co-incubated, heterotypic aggregates formed and only 27.0 ± 5.3% of the PMN and 35.1 ± 7.4% of MCF-7

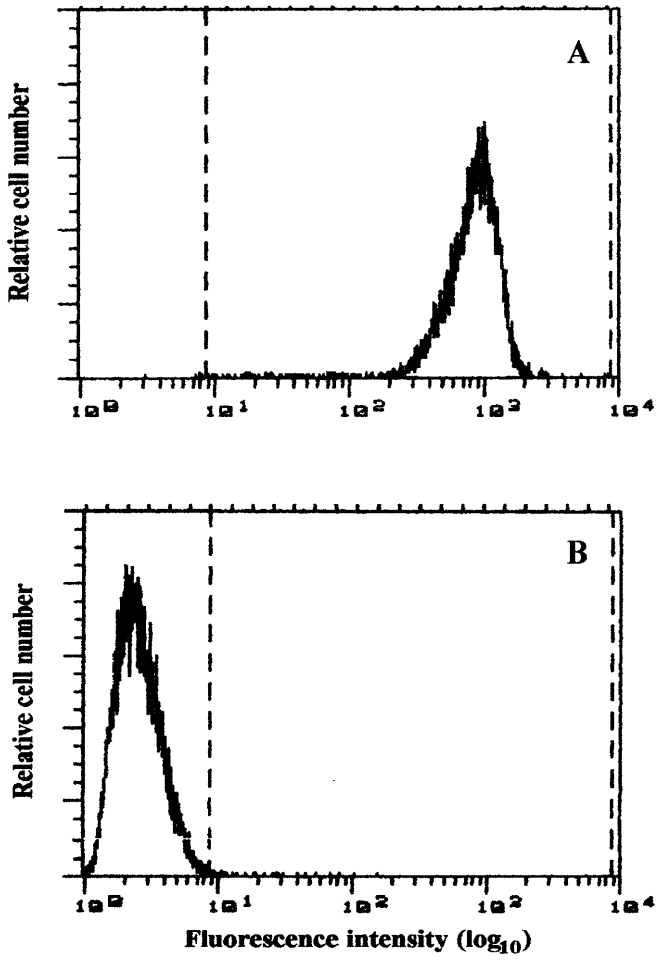

Fig. 1A, B Flow cytometry analysis of polymorphonuclear neutrophils (PMN) with mAb FC-2.15. **A** Reactivity of FC-2.15 with PMN. **B** Control experiment with phosphate-buffered saline (PBS)

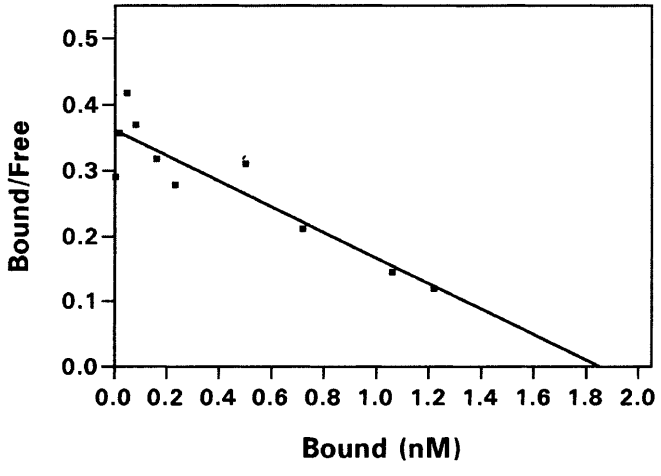

Fig. 2 Scatchard plot analysis of FC-2.15 on PMN. Scatchard plot analysis of FC-2.15 was performed on isolated PMN as described in Materials and methods. A representative graph from two different experiments is shown and each point is the mean of triplicate determinations

Table 1 FC-2.15 in vitro agglutination assay. Target cells [polymorphonuclear neutrophils (*PMN*), MCF-7, IIB-BR-G or a combination of them] were incubated with FC-2.15 (50 μg/ml) or control mAb 436 (ascitis, 1/100) or B1.1 (ascitis, 1/100) for 1 h at 37 °C. The percentage of remaining single cells was calculated for each incubation. Determinations were performed in triplicate

Cells incubated	mAb added	Single cells (%)
PMN	–	100
PMN	FC-2.15	28.4 ± 4.1
MCF-7	–	100
MCF-7	FC-2.15	96.0 ± 4.7
PMN + MCF-7	FC-2.15	27.0 ± 5.3 (PMN)
		35.1 ± 7.4 (MCF-7)
IIB-BR-G	FC-2.15	100
IIB-BR-G + PMN	FC-2.15	90.3 ± 4.3 (IIB-BR-G)
		15.6 ± 3.3 (PMN)
MCF-7	436	53.3 ± 6.1
PMN	436	91.4 ± 6.3
MCF-7 + PMN	436	48.5 ± 9.5 (MCF-7)
		96.4 ± 6.3 (PMN)
MCF-7	B1.1	108.2 ± 12.9
PMN	B1.1	61.4 ± 10.6
MCF-7 + PMN	B1.1	111.2 ± 14.3 (MCF-7)
		54.7 ± 16.7 (PMN)

remained as single cells (Table 1). Several control experiments were performed with the cell line IIB-BR-G (negative for Le^x expression) for co-incubation experiments, or mAb 436 (reactive with MCF-7 but not with PMN) or B1.1 (reactive with PMN but not with MCF-7) instead of FC-2.15 (Table 1). None of these conditions reproduced the effect of FC-2.15 on PMN and PMN/MCF-7 cells.

In Fig. 3, a heterotypic aggregate obtained after co-incubating MCF-7 and PMN cells in the presence of 50 μg/ml FC-2.15, as observed by transmission electron microscopy, is shown. A tight binding between PMN and MCF-7 cells is observed.

FC-2.15 lyses PMN in vitro

It has been previously described that FC-2.15 exerts a rapid and strong cytotoxic effect against MCF-7 cells in the presence of human serum as source of complement [3]. In order to analyze the cytotoxic effect against PMN, an in vitro ^{51}Cr-release assay with different FC-2.15 concentrations (5–20 μg/ml) and different periods of incubation (0, 30, 60, 90 min) was performed. As a positive control, the same experiment was performed with ^{51}Cr-labeled MCF-7 cells. Figure 4A shows that FC-2.15 exerted a strong lytic activity against PMN. The maximal lysis of 93.4 ± 7.9% was reached with 20 μg/ml FC-2.15 and 90 min incubation, although considerable lysis (59.8 ± 11.7%) was also detected with 5 μg/ml after 30 min incubation. Longer incubation times did not increase the FC-2.15 lytic activity (data not shown). The lysis values obtained with MCF-7 cells were similar to those previously reported (Fig. 4A) [3].

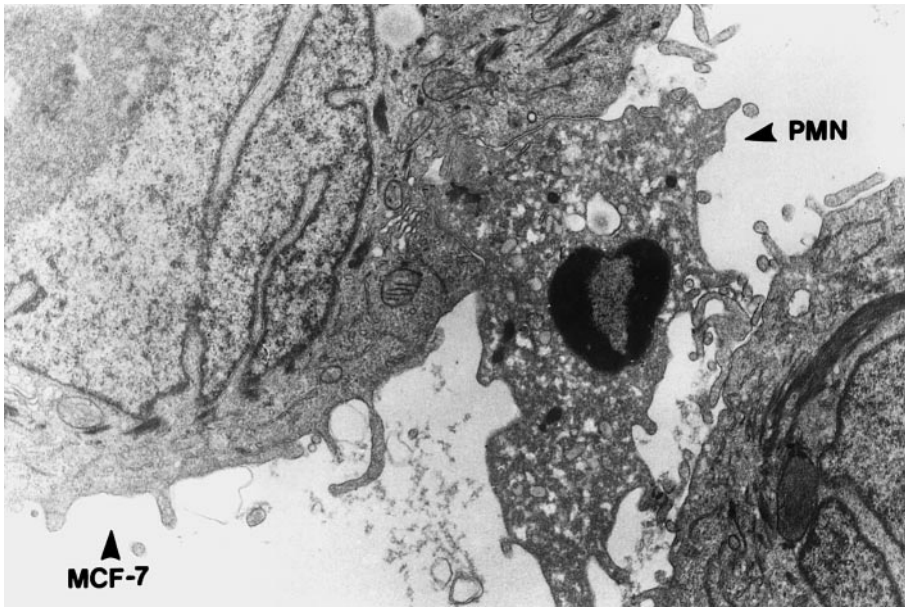

Fig. 3 Electron microscopy of a heterotypic aggregate. An electron microscope observation of a heterotypic aggregate between PMN and MCF-7 cells is shown. The specimen was processed as indicated under Materials and methods. Original enlargement: 10 000×

FC-2.15 agglutinates, but does not lyse, PMN in EVCB

In order to approximate in vivo conditions, an assay was performed in which ^{51}Cr-labeled PMN were resuspended in an EVCB system (see Materials and methods). Under these conditions, completely different results were observed (Fig. 4B). No lytic effect was observed at 5 μg/ml FC-2.15 and only a weak lytic activity was obtained at 20 μg/ml after 90 min incubation (lysis = 21.87 ± 6.5%). Controls performed with ^{51}Cr-labeled PMN in the absence of FC-2.15 did not show spontaneous PMN lysis during the incubation under the flow conditions applied. It is important to mention that the experiment was not extended beyond 90 min because longer periods of circulation produced increasing hemolysis (more than 10% hemolysis at 120 min rising to 84% at 24 h) (data not shown).

In order to discount the possibility that the lack of PMN lysis in whole blood was due to heparin, an in vitro lysis assay was performed with ^{51}Cr-labeled PMN in the presence of different heparin concentrations encompassing the 10 U/ml used in the EVCB. The results obtained indicate that heparin did not inhibit the FC-2.15 cytotoxic effect against PMN (Fig. 5).

In order to determine whether the lack of lysis in EVCB is restricted to PMN or is common to other $Le^x(+)$ cells, the same experiment was performed with ^{51}Cr-labeled MCF-7 cells. Contrary to what was observed with PMN (Fig. 4B), the lysis of MCF-7 cells by FC-2.15 after 90 min was equal to that in the conventional in vitro ^{51}Cr-release assay, although the values

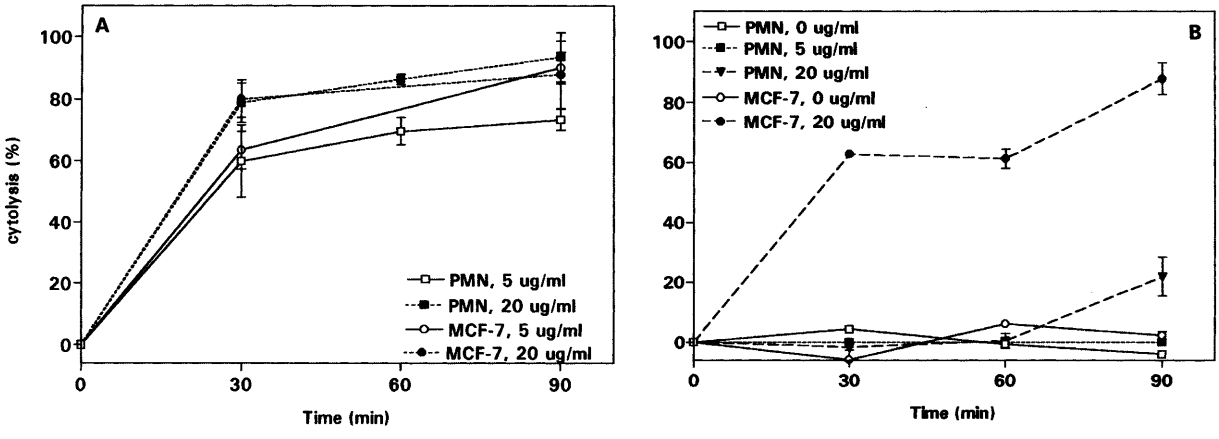

Fig. 4A, B FC-2.15-dependent complement-mediated cytotoxicity on PMN and MCF-7 cells. **A** ^{51}Cr-labeled PMN or MCF-7 cells were incubated for different times at 37 °C with human serum (diluted 1/2) plus FC-2.15 at 5 μg/ml or 20 μg/ml. After centrifuging, supernatants were counted for radioactivity and the percentage of specific cytolysis was calculated. **B** ^{51}Cr-labeled cells were resuspended in whole blood and the FC-2.15 -specific cytolysis (for 5 μg/ml and 20 μg/ml) was calculated after different times of circulation. Each point represents the mean of triplicate determinations (*bars* ± SD)

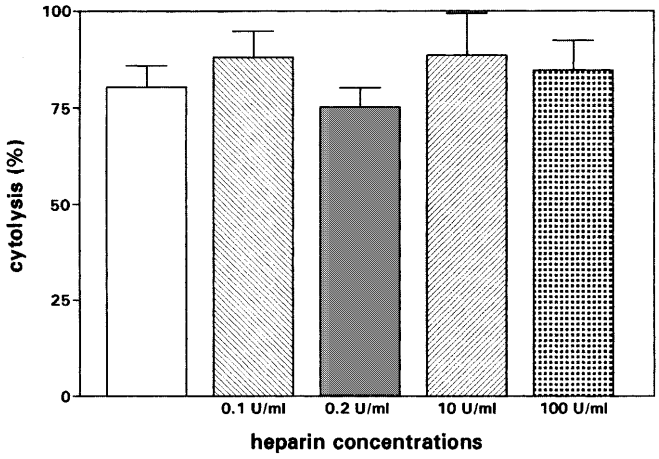

Fig. 5 Effect of heparin on the FC-2.15-dependent complement-mediated cytotoxicity on PMN. ^{51}Cr-labeled PMN were incubated for 90 min at 37 °C with human serum (diluted 1/2) plus FC-2.15 at 20 μg/ml in the presence of different concentrations of heparin. After centrifuging, supernatants were counted for radioactivity and the percentage of specific cytolysis was calculated. Each *bar* represents the mean of triplicate determinations

obtained at shorter times were around 30% lower. Analysis of blood smears prepared during the EVCB experiment demonstrated that FC-2.15 induced PMN homotypic aggregates, the size of which increased at longer circulation times. Clumps of 4–8 PMN were observed throughout the smear and larger aggregates along the edges. The areas of 45 representative PMN aggregates were measured and the average areas were determined as a function of circulation time. The results, shown in Fig. 6, demonstrate that the mean aggregate size increased steadily with time, reaching about 900 μm^2 (around 113 PMN) at 90 min. In the experiments carried out with ^{51}Cr-labeled MCF-7 in EVCB, the blood smears showed heterotypic PMN-MCF-7 cells aggregates, as observed in the in vitro agglutination assay.

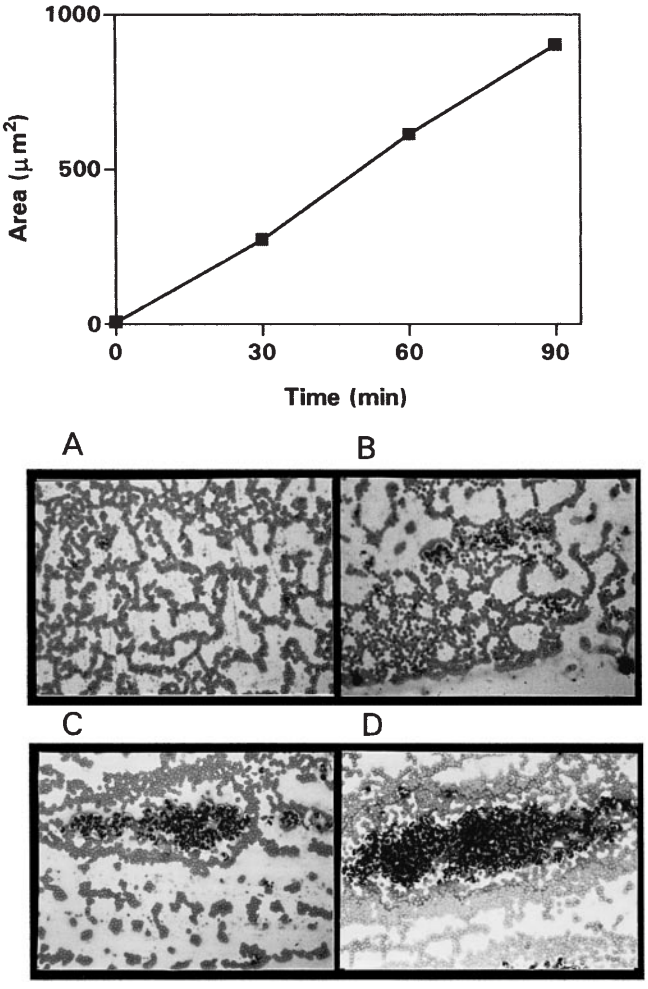

Fig. 6A–D FC-2.15 agglutination in ex vivo circulating blood. ^{51}Cr-labeled PMN were resuspended in whole blood and subjected to flow as described under Materials and methods. At different times, smears were prepared and dyed with May-Grünwald-Giemsa stain. The area of 45 homotypic aggregates was measured for each time and the mean obtained represented as a function of time. Pictures of representative aggregates for each time are shown at the bottom. **A** 0 min, **B** 30 min, **C** 60 min, **D** 90 min. Original magnification: 200×

FC-2.15 does not lyse PMN homotypic aggregates

The lack of lysis in EVCB, where PMN are clumped in the presence of FC-2.15, could be due to a steric hindrance to the access of complement factors to the C region of FC-2.15. To test this possibility, ^{51}Cr-labeled PMN were first aggregated with FC-2.15 for 1 h at 37 °C and then human serum was added as a source of complement. The percentage of isolated PMN was determined before addition of human serum and the cytolysis was measured after 90 min at 37 °C. Figure 7 shows a strong correlation between the percentage of single cells and the percentage of cytolysis, suggesting that only isolated PMN are lysed. This assumption is supported by the fact that, when the cells were observed under phase-contrast microscopy, isolated PMN were non-viable whereas aggregated PMN were alive (data not shown).

FC-2.15 lyses PMN in EVCB, if their agglutination is blocked

It has been previously stated that drugs disrupting microtubular structures, such as colchicine or vinblastine, inhibit PMN agglutination by concanavalin A [2, 5, 36]. In order to test whether microtubule integrity was necessary for lysis or agglutination by FC-2.15, the effect of colchicine was determined. The in vitro agglutination assay was performed by treating isolated PMN with 2.5 μM colchicine for 1 h before the addition of FC-2.15 (20 μg/ml). Under these conditions, the agglutinating effect of FC-2.15 was significantly lower, 81.1 ± 4.3% of PMN remaining as single cells instead of 28.4% when colchicine is omitted (Fig. 8A). By contrast, colchicine did not inhibit PMN lysis by human complement, as

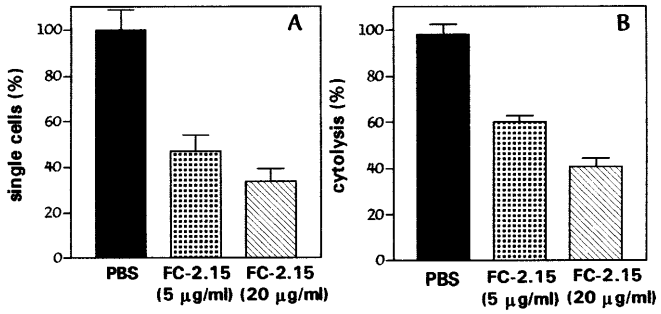

Fig. 7A, B FC-2.15-dependent complement-mediated cytotoxicity on PMN homotypic aggregates. **A** Suspensions of 5×10^6 ^{51}Cr-labeled PMN/ml were incubated with FC-2.15 (0, 5, 20 µg/ml) for 1 h at 37 °C and the percentage of single cells calculated. **B** After addition of human serum (1:1; v:v) and FC-2.15 to a final concentration of 20 µg/ml, the FC-2.15-specific cytolysis was determined

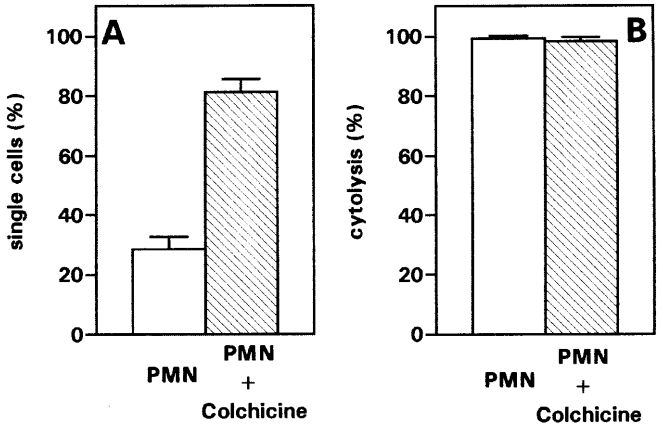

Fig. 8A, B Effect of colchicine pretreatment on the agglutination of PMN by FC-2.15 (**A**) and on the lytic activity of FC-2.15 on PMN (**B**). **A** Isolated PMN were pretreated with PBS (control) or 2.5 µM colchicine and incubated with 50 µg/ml FC-2.15 for 1 h at 37 °C and the percentage of single cells was calculated. **B** The pretreated cells were incubated with 20 µg/ml FC-2.15 in the presence of 50% human serum for 90 min at 37 °C and the percentage of cytolysis was calculated

shown in Fig. 8B. Thus, agglutination and lysis are independent effects and may be dissociated. On the basis of the previous results, an assay with ^{51}Cr-labeled PMN pretreated with colchicine and resuspended in whole blood (also pretreated with colchicine) and subjected to flow conditions was performed. In this case (Fig. 9) FC-2.15 was able to lyse the ^{51}Cr-labeled PMN, the maximal lysis being 78 ± 1.1% with 20 µg/ml FC-2.15 after 90 min circulation. This lytic activity is somewhat lower than that observed in vitro (93.4 ± 7.9%) (Fig. 4A) but the fact that the inhibitory effect of colchicine on PMN agglutination was not total should be taken into account (almost 20% of PMN aggregated in the presence of colchicine). When this experiment was performed with ^{51}Cr-labeled MCF-7 and whole blood pretreated with colchicine, it could be observed (Fig. 9) that FC-2.15 lysed MCF-7 cells to the same extent as in vitro. Control experiments performed with pretreated ^{51}Cr-labeled cells

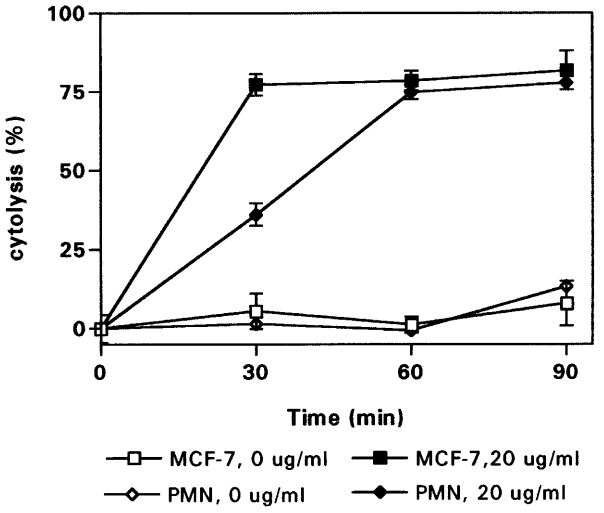

Fig. 9 Effect of colchicine pretreatment on the FC-2.15-dependent complement-mediated cytotoxicity on PMN and MCF-7 cells in ex vivo circulating blood. ^{51}Cr-labeled cells were pretreated with $2.5 \times$ µM colchicine for 1 h and resuspended in colchicine-pretreated whole blood. The FC-2.15-specific cytolysis was calculated after different times of circulation. Each point represents the mean of duplicate determinations (*bars* = ±SD)

in the absence of FC-2.15 demonstrated that these cells were not spontaneously lysed under the flow conditions applied.

Discussion

We have shown that FC-2.15 strongly reacts with almost 100% of peripheral PMN with high affinity (K_a 5.14×10^9 M^{-1}).

The use of FC-2.15 in a phase I clinical trial [25] produced two main observations: (1) an intense neutropenia took place, that was rapidly reversible after completion of mAb infusion, with appearance of PMN juvenile forms in increasing proportions after each mAb cycle; (2) clinical responses were observed, suggesting that the mAb could efficiently reach tumor sites and lyse tumor cells. The induction of neutropenia by mAb has been previously observed when an anti-CEA mAb was used for radioimmunodetection [12]. A priori, several possibilities could explain neutropenia: PMN lysis, agglutination and margination or opsonization and sequestration by the reticuloendothelial system [41]. The finding that FC-2.15 is able to lyse PMN in vitro in the presence of human complement would appear to favor the first hypothesis [3]. However, this was opposed by the relative mildness of the symptoms observed early during mAb infusion, which were less than expected if more than 90% PMN were lysed in circulating blood, although the lysis detected by ^{51}Cr release may not be accompanied by a massive release of hydrolases and other toxic soluble factors. However, this matter had to be further investigated in order to determine the safety and possibilities of treatment with anti-Le^x mAb, spe-

cially in chronic myeloid leukemia, where large amounts of circulating Le^x tumor cells maybe found [8, 17]. The experiments reported in this paper demonstrate a clear-cut difference when PMN are treated with FC-2.15 in vitro or in EVCB. In vitro, FC-2.15 induces homotypic aggregation, but if human complement is added, PMN lysis is almost total. In EVCB, PMN are clumped and lysis is almost nil. Interestingly, FC-2.15 efficiently lyses MCF-7 cells under both conditions, and heterotypic aggregates with PMN are formed.

It is well known that homotypic aggregation of PMN requires their stimulation with chemoattractants (fMet-Leu-Phe, tumor necrosis factor α, lipopolysaccharide, granulocyte/macrophage-colony-stimulating factor) in order to activate the β2-integrin (CD11b/CD18) cyto-adhesion pathway [4, 33, 43]. Reported data demonstrating (i) that crosslinking of surface antigen CD43 with mAb BS1 induces PMN activation by promoting aggregation [18], (ii) that some anti-Le^x mAb can stimulate PMN to form homotypic aggregates in an active way associated with β 2-integrin activation [38] and (iii) that an anti-Le^x IgM mAb triggers a rise in intracellular calcium in PMN [20], support the possibility that PMN homotypic aggregation induced by FC-2.15 is due to granulocytes activation. In addition, it has also been reported that chemoattractant stimulated PMN in heparinized human blood incorporate human breast cancer cells into aggregates [30]. Therefore, the experiments reported in this paper suggest that crosslinking of Le^x epitopes with mAb FC-2.15 stimulates PMN, but not $Le^x(+)$ tumor cells such as MCF-7, to aggregate. However, when PMN and MCF-7 are incubated with FC-2.15, activated PMN incorporate MCF-7 into mixed aggregates. It is worth mentioning that not every anti-Le^x mAb tested stimulates PMN to form homotypic aggregates. Stockl et al. reported this effect for 3 out of 12 different anti-Le^x mAb assayed [38]. The K_a of FC-2.15 for PMN ($5.14 \times 10^9\ M^{-1}$), considerably lower than that described for other anti-Le^x mAb, probably explains its activating effect on PMN.

Why are PMN lysed in vitro but not in EVCB? We can only speculate at the present time. Taking into account that the physiological range of shear stress induces PMN homotypic adherence by a different mechanism dependent on LFA-1(CD11a)–ICAM-3 interaction [28] and actin polymerization in cell–cell contact regions [29], a plausible explanation is that, in the EVCB used in this work, flowing blood would induce higher agglutination by the addition of both mechanisms. As aggregates are being formed, there would be a progressive steric hindrance to complement factor access to the constant region of FC-2.15, and the clumped PMN would not be lysed. Thus, agglutination would be favored over lysis. The ^{51}Cr-release assay performed in the presence of colchicine, where the lytic activity of FC-2.15 in EVCB was restored, along with the observation that performed PMN aggregates were not lysed by FC-2.15 in vitro, support this possibility. Moreover, agglutination and lysis are independent effects since only PMN aggregation by FC-2.15, but not lysis, appears to be highly dependent on microtubule integrity. That MCF-7 cells can still be lysed in EVCB would be due to their incomplete aggregation with PMN. According to this model, it may be speculated that, when FC-2.15 is injected in vivo, PMN rapidly form clumps which are sequestered from circulation, enlarging the normally marginated PMN pool. The location and fate of these clumps is presently ignored, but they do not produce significant clinical symptoms. As soon as mAb infusion is stopped, clumping would cease and PMN release from bone marrow would explain the fast recovery of PMN and the appearance of juvenile forms in circulation. This model is supported by an experimental study of neutropenia following mAb administration [41]. Neutropenia may also occur spontaneously as a result of the presence of anti-PMN antibodies. In a case report, Carr et al. reported an IgM-dependent neutrophil agglutination in a patient with colon cancer [9]. It has also been reported that patients with schistosomiasis suffering neutropenia develop high titers of specific IgM antibodies to a circulating cathodic antigen that cross-react with the repeating Le^x units on the granulocyte surface [42].

The lack of PMN lysis by FC-2.15 in circulation is reassuring and, since $Le^x(+)$ tumor cells are efficiently lysed, FC-2.15 could be useful to eliminate circulating $Le^x(+)$ tumor cells.

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