

FUNCTIONAL STUDY OF A MONOCLONAL ANTIBODY TO  
IgE Fc RECEPTOR (Fc<sub>ε</sub>R2) OF EOSINOPHILS, PLATELETS,  
AND MACROPHAGES

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Several characteristics of human eosinophil heterogeneity in some diseases associated with eosinophilia have been reported. They concerned eosinophil morphology with reduced numbers of granules and increased membrane receptors (1), cell density, and cytotoxic functions (2) or metabolism (3). The more interesting topic related to this point is the functional role of these altered eosinophils in human pathology, as suspected in a variety of diseases characterized mainly by cardiac or lung injury (4). Our previous works showed that the presence of receptors for IgE (Fc<sub>ε</sub> receptors), directly involved in the effector function of human eosinophils against IgE-coated parasite targets, was restricted to a subpopulation of eosinophils (5). This eosinophil subpopulation of abnormally low density, named for this reason "hypodense" (2), was only present in the blood of highly hypereosinophilic patients (eosinophil counts >3,000/mm<sup>3</sup>) or in the tissues, mainly in some cases of lung diseases (6). In addition, a recent report indicating that pulmonary eosinophils could kill lung parenchymal cells (7), suggested a particularly effective function of these eosinophils, not only in antiparasite cytotoxicity but also in various pathological situations.

Like eosinophils, human platelets were discovered to be effector cells against schistosomula of *Schistosoma mansoni*, in vitro and in vivo, in the presence of IgE antibodies (8). Both mechanisms of killing involved Fc<sub>ε</sub> receptors, which bind IgE immunoglobulins with a relatively low affinity (9). Antigenic similarities between Fc<sub>ε</sub>R of eosinophils and platelets were suggested by the parallel inhibition of both IgE-dependent cytotoxicity and IgE binding, with polyclonal antibodies also able to inhibit Fc<sub>ε</sub>R on human lymphocytes and monocytes (5, 9–11).

The original aim of this study was to prepare an mAb specifically directed against the hypodense subpopulation of human eosinophils in order to obtain a phenotypic marker that could characterize and quantify these cells both in the blood and tissues of hypereosinophilic patients. Moreover, since recent data suggested that these hypogranular cells were activated in vivo (6), the presence

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of such a marker could be useful in following activation and/or maturation of eosinophils. We report here on an IgM mAb named BB10 raised against hypodense blood and lung eosinophils, which turns out to be an antibody to FcεR of eosinophils, platelets, and macrophages (FcεR2).

### Materials and Methods

*Preparation of mAbs.* Balb/c mice were immunized with two doses of  $10^7$  human eosinophils at 3 wk intervals, in the presence of CFA. Hypodense blood eosinophils were used for immunization and were purified as described below ("cell purification"). The first immunization was given subcutaneously and the second intraperitoneally. 2 wk after the second injection, animals were bled and a first control on this polyclonal antiserum was performed. 3 d before the hybridization, two mice were boosted intravenously with  $5 \times 10^6$  lung eosinophils obtained by bronchoalveolar lavage.

For hybridization, spleen cells were removed and fused with the SP<sub>2</sub>O myeloma cell line, using PEG and DMSO according to the technique previously described (12). Culture supernatants were tested by indirect fluorescence, using human eosinophils coated on microscope slides, according to the immunofluorescence slide assay (IFSA)<sup>1</sup> technique detailed below (13). Among 11 hybridoma cultures containing antibodies reacting with eosinophils, three were selected and cloned twice by the limiting dilution technique. Subsequently, the clones were injected subcutaneously into BALB/c mice to induce solid tumors. After homogenization of these tumors, cells were injected into the peritoneal cavities of BALB/c mice; the ascitic fluids were collected 2 wk later and assayed by indirect fluorescence on various cell populations. One given clone (named BB10) was selected for further investigation. This BB10 mAb was found to be an IgM immunoglobulin by Ouchterlony analysis using antimouse immunoglobulins antisera (Bionetics Laboratory Products, Charleston, SC).

A second mouse IgM mAb (G10-54) with a different specificity was used as a control in all the experiments. It was obtained, by a procedure similar to that used for the preparation of BB10, from mice immunized with a soluble antigenic extract of *Echinococcus multilocularis*.

*Purification of IgM Fraction.* The same procedure was used to purify the IgM fraction of BB10 and control G10-54. Ascitic fluids from mice injected with either BB10 or G10-54 tumor cells were submitted to gel filtration using ACA34 gel (IBF, Villeneuve-La-Garenne, France) and K50/100 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The IgM fractions were stored at +4°C, at a concentration of 2 mg/ml, without further dialysis.

*Cell Purification.* Human eosinophils were purified from the venous blood of patients with hypereosinophilia of various etiologies by centrifugation upon discontinuous metrizamide gradients (Nyegaard, Oslo, Norway), according to the technique previously described (14). Several cell fractions containing more than 85% pure eosinophils were collected from the various gradients, according to their density (2). They were referred to as hypodense cells when obtained in the lower density layers, corresponding to metrizamide concentrations <23% and "normodense" cells when collected from the top of the 25% and 24% layers. Lung eosinophils were purified with the same procedure from bronchoalveolar lavage (BAL) performed under fiber-optic bronchoscopy on patients with the pulmonary infiltration with eosinophil (PIE) syndrome (15). For mice immunization, hypodense blood and lung eosinophils from the same patient with hypereosinophilia-associated drug hypersensitivity were used. Human monocytes and neutrophils were purified by the same technique, from peripheral blood leucocytes of normal or slightly hypereosinophilic patients. They were recovered respectively from the top of the 18% step for monocytes, and from the top of the 22% and 23% steps for neutrophils (14).

<sup>1</sup> *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; DMSI, dimethylsuberimidate; FMF, flow microfluorometry; IFSA, immunofluorescence slide assay; MEM/FCS, minimum essential medium supplemented with FCS; NRS, normal rat serum; PGE1, prostaglandine E1; PIE, pulmonary infiltration with eosinophils; PLL, poly-L-lysine; RBL, rat basophil leukemia.

Washed human platelets were purified from venous blood of normal subjects collected on citric acid/dextrose medium, and platelet-rich plasma was washed in saline supplemented with citric acid (36 mM), glucose (5 mM), calcium (2 mM), magnesium (1 mM), BSA (0.35%), and prostaglandin E1 (PGE1) (100 nM), according to a previous technique (16). Alveolar macrophages were obtained from nonhypereosinophilic patients by BAL, and the alveolar cell population, consisting of nearly 80% alveolar macrophages, was washed as reported earlier (17).

Rat peritoneal cells were purified by a method of centrifugation through discontinuous Percoll gradients, adapted from the technique used for human eosinophils (18). Briefly, a Percoll solution (Pharmacia Fine Chemicals) was diluted to obtain the following densities: 1.105, 1.095, 1.085, 1.080, 1.075, 1.065. 2 ml of each solution were layered on top of each other. The peritoneal cell suspensions stimulated 48 h previously by injection of 0.9% sterile physiological saline were obtained from normal rats, and were suspended in minimum essential medium supplemented with 1% heat-inactivated normal rat serum (MEM/NRS) at a concentration of  $25 \times 10^6$ /ml. 2 ml of the cell suspension were layered over the Percoll gradient and the tubes were then spun at 400 g at +4°C for 60 min. Cytocentrifuge smears were prepared for differential counts and stained with Giemsa. Eosinophils (>80% purity) were recovered at the interface between the layers of  $\rho = 1.095$  and  $\rho = 1.085$ , whereas macrophages (>90% purity) were obtained at the density layer of 1.065. Lymphocytes were obtained from mesenteric lymph nodes of normal rats, according to previously described techniques (19). Purified mast cells (>90% purity) were recovered after centrifugation of normal rat peritoneal cells over 22.5% metrizamide (19).

Human cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS. Two U937 cell lines varying in their IgE binding capacity were donated by Dr. B. Stadler (Institut für Klinische Immunologie, Inselspital, Bern, Switzerland) and Dr. R. Van Furth (Department of Microbial Diseases, University Hospital, Leiden, Netherlands).

*Reagents.* Highly purified human IgE myeloma protein (P.S.) was kindly donated by Dr. H. L. Spiegelberg (Scripps Clinic, La Jolla, CA). Human IgG was purified from normal human serum by ion exchange chromatography. Rat IgE myeloma protein was obtained from Dr. Bazin (Louvain University, Brussels, Belgium). For competition experiments, IgE was previously aggregated with dimethyl suberimidate (DMSI) (5). Fluorescein-labeled F(ab')<sub>2</sub> fragment of antimouse IgG and IgM goat antisera were purchased from Cappel Laboratories (Cochranville, PA). A goat antiserum directed against a lymphoblastoid B cell line and able to inhibit FcεR of lymphocytes and monocytes was kindly donated by Dr. H. L. Spiegelberg, Scripps Clinic (11). An mAb (BC4) directed against the Fcε receptor of rat basophilic leukemia (RBL-2H<sub>3</sub>) cells was obtained from Dr. R. Siraganian (National Institutes of Health, Bethesda, MD).

*Immunofluorescence Slide Assay (IFSA).* Because of the difficulties encountered in the regular supply of eosinophils from hypereosinophilic patients, we adapted a method using eosinophils and other cell populations coated on microscope slides (a technique previously described for lymphocytes [13]). To perform the screening of hybridoma supernatants Teflon-coated slides (10 reaction areas per slide) were coated overnight at room temperature with poly-L-lysine (PLL, Sigma Chemical Co., St Louis, MO) at 0.5 mg/ml in PBS. After washing with HBSS, 20 μl-aliquots of cell suspensions ( $10^6$  cells/ml in HBSS without protein) were deposited onto each reaction area. After 10 min at room temperature, the coated cells were fixed with glutaraldehyde (0.05% in 0.06 M Sorensen's buffer, pH 7.4) for 15 min at room temperature. The slides were then washed with 0.3 M glycine buffer in order to block free aldehyde groups. After coating with cells, the slides could be kept at +4°C in humidified atmosphere for 1 mo. Before their use in the fluorescence assay, the degree of purity and the morphological aspects of the cells were verified using interference contrast microscopy (Polyvar, Reichert, Federal Republic of Germany). Before the addition of reagents, the reaction areas were incubated with HBSS containing 0.2% gelatin, to avoid nonspecific binding.

For the fluorescence procedure on these coated slides, reaction areas were first incu-

bated with 20  $\mu$ l of the mAbs (purified IgM fractions at 100  $\mu$ g/ml, in HBSS-gelatin). After 60 min at room temperature and three washings in HBSS without gelatin, 20  $\mu$ l of fluorescein-conjugated F(ab')<sub>2</sub> fragment of antimouse IgM immunoglobulins (1:20 dilution in 0.02 M PBS + 0.2% gelatin) were added to each reaction area. The slides were then incubated for 30 min at room temperature, in the dark, and washed twice with PBS. A third washing with Evans blue diluted 1:10,000 in PBS was performed to abolish autofluorescence of eosinophil granules.

**Flow Microfluorometry Analysis.** The various cell populations ( $5 \times 10^6$  cells/ml; except for platelets,  $10^8$ /ml) were incubated with BB10 or control G10-54 IgM fractions at 200  $\mu$ g/ml (final concentration). After 30 min of incubation (at room temperature for platelets and +4°C for the other cell populations), the cells were then reacted with FITC-labeled anti-mouse IgM antibodies (1:20 dilution for 30 min).

After two washings, stained cell suspensions were then analyzed for surface fluorescence using an Ortho Cytofluorograph System H50 (Ortho Diagnostic Systems Inc., Raritan, NJ). The results are expressed as the percentage of cells with a fluorescence intensity exceeding the threshold, obtained with the fluorescent reagent only.

**Cytotoxicity Assays.** *S. mansoni* schistosomula prepared according to the skin penetration procedure, were the targets of the cytotoxicity assays (20). For IgE-mediated eosinophil-dependent cytotoxicity, 50- $\mu$ l aliquots of the schistosomula suspension containing 50 targets in MEM supplemented with 1% heat-inactivated FCS (MEM/FCS), were incubated in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark). 50  $\mu$ l of unheated sera from *S. mansoni*-infected patients or normal subjects were added at a final dilution of 1:32, together with 100  $\mu$ l of hypodense eosinophils purified from highly hypereosinophilic patients (5,000:1 E/T ratio, according to previous reports) (5). The IgG-mediated killing assay was performed as previously described (14) with normodense eosinophils from slightly eosinophilic individuals and heat-inactivated pooled human immune sera, as a source of IgG antibodies. For platelet-dependent cytotoxicity, 50 schistosomula in 80  $\mu$ l MEM medium were incubated in flat-bottomed plates with 20  $\mu$ l unheated serum (final dilution 1:10) from *S. mansoni*-infected patients or normal subjects, and 100  $\mu$ l MEM containing  $7.5 \times 10^7$  platelets from normal subjects (8). In both assays, the percentage of cytotoxicity was evaluated by microscopic examination after 24–48 h at 37°C in 5% CO<sub>2</sub> atmosphere. For inhibition experiments, eosinophils or platelets were preincubated with various concentrations of ascitic fluids (dilution 1:100) or purified mAbs for 60 min at +4°C for eosinophils and 37°C for platelets. The results of inhibition by BB10 were compared with results obtained with cells incubated with medium or control IgM.

**Rosette Assays.** For the study of Fc receptors, a rosette assay using aldehyde-treated erythrocytes coated with human IgE or human IgG was performed as previously described (5). Cell suspensions (30  $\mu$ l of a solution containing  $4 \times 10^6$  cells/ml in MEM/FCS) were incubated with 30  $\mu$ l of Ig- or BSA-sensitized erythrocytes ( $2 \times 10^8$ /ml in MEM/FCS). After centrifugation at 100 g at +4°C for 10 min, the cells were further incubated at +4°C for 30 min. For complement (CR1) receptors, one volume (30  $\mu$ l) of sheep erythrocytes bearing human C3b (E<sup>c</sup>C3b), prepared according to a previous technique (21) with  $10^8$ /ml HBSS, was added to an equal volume of cell preparations ( $4 \times 10^6$ /ml in HBSS). After centrifugation at 100 g for 5 min at +20°C, the sedimented cells were incubated for 30 min at 37°C. In both assays (FcR or CR1), the pellets were gently resuspended and the percentage of rosette forming cells was evaluated microscopically, as previously described (5). For inhibition experiments, the various cell populations were preincubated with the mAbs for 60 min at +4°C before rosette formation, similar to the cytotoxicity assay.

**Binding of Radiolabeled BB10 and IgE.** Purified IgM fractions of the mAbs BB10 or control G10-54, and rat or human IgE were radioiodinated by the chloramine T technique (22). Two hundred  $\mu$ g of BB10 or G10-54, or IgE were labeled with 0.2 mCi of <sup>125</sup>I-Na to a sp act of 0.5–0.7  $\mu$ Ci/ $\mu$ g. Radioiodinated antibodies were stored at +4°C in PBS containing 1% BSA. For the quantitation of BB10 binding, the various cell populations were incubated at  $10^7$ /ml for 60 min at +4°C, except for platelets ( $10^9$ /ml for 60 min at 37°C), with varying concentrations of labeled BB10 or control G10-54 in a total volume

of 200  $\mu$ l PBS-BSA 1%. After washings, the cell suspensions were centrifuged at 8,500  $g$  for 2 min through 1 ml of 25% sucrose, and the radioactivity of the pellets was measured in a gamma scintillation spectrometer (Inter technique CG4000, Plaisir, France).

The specificity of the binding of radiolabeled BB10 was determined at each point and for all cell populations by subtracting the cpm bound to cells after addition of equal concentrations of labeled, control IgM G10-54. In some experiments, cells were incubated with a 50-fold excess dose of cold BB10 before addition of radiolabeled BB10. The inhibition ranged between 71 and 87% ( $78.5 \pm 0.6\%$  for eosinophils and  $80 \pm 8\%$  for platelets;  $n$ , 6 experiments), which indicated between 13 and 30% of nonspecific binding.

For competition experiments, rat eosinophils, human monocytes ( $10^6$  cells/tube), or human platelets ( $8 \times 10^7$ /tube) were incubated with various concentrations (100  $\mu$ g–5  $\mu$ g per tube) of unlabeled reagents (control G10-54, BB10, or aggregated IgE) for 30 min at +4°C (except for platelets, room temperature). The radiolabeled reagents were then added (1  $\mu$ g BB10/tube, 60 min at +4°C, or 1  $\mu$ g IgE/tube, 90 min at +4°C, or room temperature for platelets). The cell suspensions were centrifuged over 20% sucrose, as above. In all cases, the background radioactivity of controls without cells was subtracted.

*Molecular Weight Determination of the Molecules Eluted from BB10 or IgE Affinity Column.* Membrane proteins were solubilized by treating the cells ( $75 \times 10^9$  platelets or  $\times 10^8$  U937/ml) with a nonionic detergent mixture consisting of 0.5% NP-40 in PBS (0.14 M NaCl; 0.01 M PO<sub>4</sub>, pH 7.4) in the presence of 2 mM PMSF and 2 mM EDTA. After a 30 min incubation at +4°C, the insoluble material was removed by centrifugation at 22,000  $g$  for 40 min. The detergent extracts were then radiolabeled with <sup>125</sup>I by using Iodogen (Pierce Chemical Co., Rockford, IL).

For isolation of the molecule reactive with BB10, the lysates were passed over a CNBr-activated Sepharose 4B column (1.5 ml) to which the mAb BB10, at a concentration of 0.870 mg/ml, was coupled. Controls included cell lysates passed over Sepharose 4B to which G10-54 purified IgM was coupled and also control supernatants without cells. The columns were first washed with PBS (plus NP-40, PMSF, and EDTA) and the adsorbed material was eluted with 3 M NaSCN. Fractions of 0.5 ml were collected and dialyzed.

For isolation of the IgE reactive molecules, 35–70  $\mu$ l of the detergent extract were first agitated on a test tube rotator with BSA immunosorbent for 30 min at 20°C. Then the extracts were passed over a CNBr-activated Sepharose column (200  $\mu$ l) to which 1 mg human myeloma IgE was coupled, for 16 hr at +4°C. The affinity column was washed with PBS containing 0.5% NP-40, 2 mM PMSF, and 2 mM EDTA. Bound, radiolabeled material was eluted with 3 M NaSCN by fractions of 32  $\mu$ l, dialyzed, and concentrated by centrifugation on Centricon (Amicon Corp., Danvers, MA).

SDS-PAGE (13% weight/vol polyacrylamide) was then performed. The gels were stained with silver in the case of material eluted from BB10 column or submitted to autoradiography in the case of IgE immunosorbent. All the techniques used for this molecular weight determination have been adapted from previous papers (23–24).

*Expression of the Results.* All results were presented as mean  $\pm$  SEM. Student's  $t$  test for paired experiments was used to compare the values obtained in the presence and in the absence of BB10.

## Results

*Characteristics of BB10 Fluorescence Staining.* For practical reasons (storage of cells fixed on PLL-coated slides), the IFSA was used both to screen the hybridoma supernatants and also to make a survey of cell types reactive with BB10. Intense membrane fluorescence was detected on eosinophils (human and rat) and on macrophages (both human alveolar and rat peritoneal). The staining of human eosinophils was more pronounced with degranulated hypodense blood and tissue purified cells, than with the fully granulated normodense populations (Fig. 1 and Table 1). Intensity of fluorescence staining of blood monocytes was inferior to that observed for human alveolar macrophages (Table I). These results and

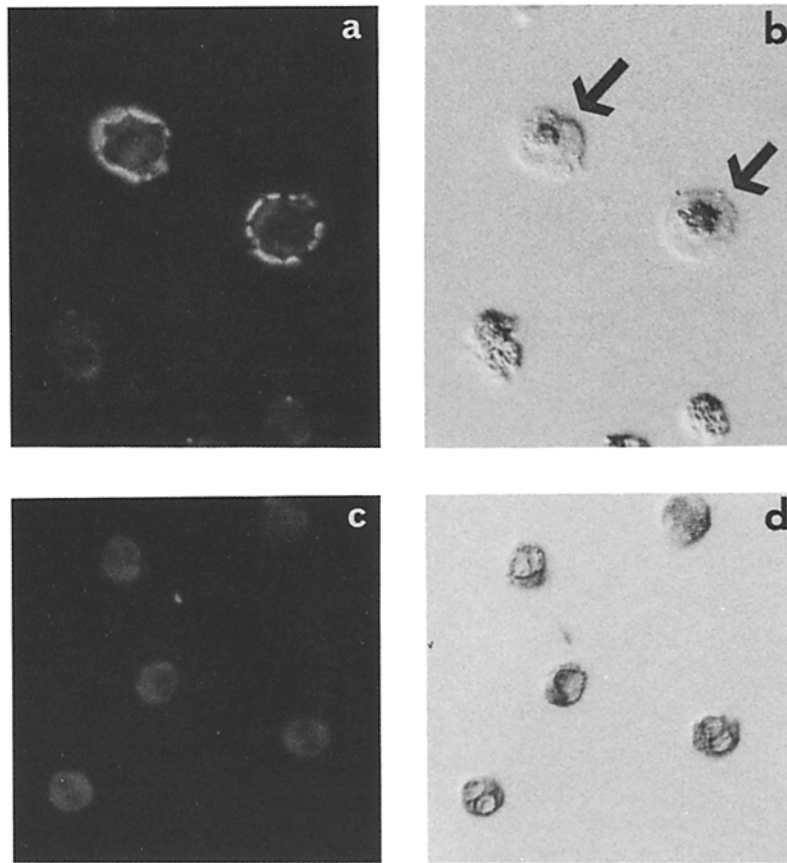


FIGURE 1. Binding of BB10 to human eosinophils (*a* and *b*) "hypodense" cells; (*c* and *d*) "normodense" eosinophils coated on PLL-slides; (*a* and *c*) immunofluorescence slide assay (IFSA); (*b* and *d*) interferential contrast microscopy. The less granulated ("hypodense") eosinophils (*arrows*) stained more brightly than the fully granulated ones.

especially the variation in the intensity of fluorescence staining between normodense and hypodense eosinophils and between monocytes and alveolar macrophages, suggested a coincident distribution of the BB10 antigen and the low affinity Fc $\epsilon$ R2. In addition, quantitative analysis of various cell populations by FMF both confirmed the qualitative analysis by IFSA and suggested that the BB10 antigen was present only on a significant proportion of hypodense eosinophils from hypereosinophilic patients and monocytes and platelets from normal subjects. An increase in the percent of positive cells was detected in alveolar macrophages, whereas neutrophils were negative. Among the rat peritoneal cells, eosinophils and a large proportion of macrophages were positive, whereas lymphocytes were negative (Table I, Fig. 2). Moreover, a preliminary experiment revealed that the polyclonal mouse antisera obtained before the hybridization could induce high levels of inhibition of IgE rosettes formed by hypodense human eosinophils, indicating some relationship with the IgE Fc receptors.

*Inhibition of IgE-dependent Cytotoxicity.* To investigate the effect of BB10 on

TABLE I  
 Reactivity of BB10 with Various Cell  
 Populations by Immunofluorescence

Cells used	IFSA* (intensity of fluorescence)	FMF† (percent positive cells)
Human cells:		
Hypodense blood eosinophils	+++	37.6 ± 8.9
Normodense blood eosinophils	+	10.6 ± 5.5
Hypodense lung eosinophils	+++	ND
Neutrophils	±	4.6 ± 0.5
Blood monocytes	+	9.4 ± 2.1
Alveolar macrophages	+++	16.9 ± 0.1
Platelets	ND	10.8 ± 6.4
Rat peritoneal cells:		
Eosinophils	+++	17.3 ± 1.9
Macrophages	+++	90.9 ± 0.3
Lymphocytes	+	2.9 ± 0.2

\* The various cell preparations were deposited onto PLL-coated microscope slides and successively incubated with the purified IgM fractions of BB10 or G10-54 (100 µg/ml), and with fluorescein-conjugated anti-mouse IgM antibodies (1:20). The results are estimated according to the intensity of membrane fluorescence.

† Cells were incubated in suspension with BB10 or G10-54 at 200 µg/ml for 30 min at +4°C and further stained with fluorescein-conjugated anti-mouse IgM antibodies (percent positive cells ± SEM). On all cell populations, control G10-54 antibodies gave results <5%.

the IgE-dependent effector function of eosinophils, inhibition experiments were performed by preincubation of hypodense eosinophils (purified from blood leukocytes of highly hypereosinophilic patients according to their low density) with medium or with various concentrations of BB10 or control G10-54. Ascitic fluids (1:100) or decreasing concentrations (200–10 µg/ml) of purified IgM fractions were incubated with eosinophils for 60 min at +4°C before addition to schistosomula and antischistosome IgE antibodies; there was no washing. As shown in Fig. 3, preincubation of hypodense eosinophils with ascitic fluids of BB10 induced a high level of inhibition of IgE-dependent cytotoxicity, and a dose-dependent inhibition was obtained with the purified IgM fraction of BB10. Student's *t* test for paired experiments revealed highly significant results ( $p < 0.001$ , *n*, 16 experiments), except in the case of the lower dose of purified BB10.

Since the IgE-dependent cytotoxicity of both human eosinophils and platelets was recently shown to be inhibited at the same level with polyclonal anti-FcεR antibodies (5, 9, 10), we performed inhibition experiments of platelet-mediated killing by BB10 (Fig. 4). When human platelets were preincubated with ascitic fluid of BB10 before addition of anti-*S. mansoni* immune sera and schistosomula targets, a very significant inhibition of the killing was obtained compared with that of platelets incubated with medium (Student's *t* test for paired experiments:  $p < 0.01$ , *n*, 9 experiments). Similarly to eosinophils, a dose-dependent inhibition of IgE-mediated killing was also obtained with the purified IgM fractions of BB10 ( $p < 0.0001$ , *n*, 9 experiments), whereas control IgM G10-54 did not lead

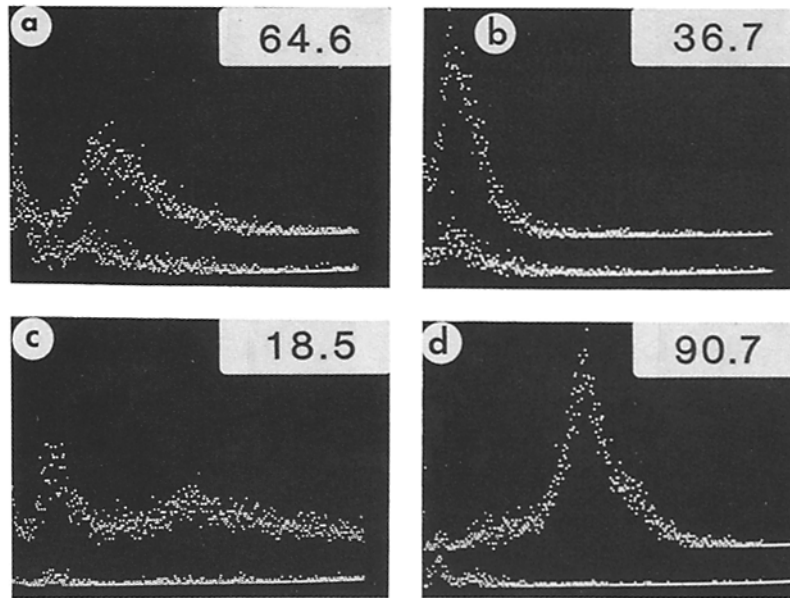


FIGURE 2. Binding of BB10 (*upper curve*) or control G10-54 (*lower curve*) to various cell populations by flow cytometry. (*a*) Hypodense eosinophils from a patient with HES; (*b*) from a patient with filarial infection. (*c*) Normal human platelets; (*d*) normal rat macrophages. Vertical, relative cell number; Horizontal, relative fluorescence intensity. Values given in the corners are the percent positive cells in the peak for one representative experiment.

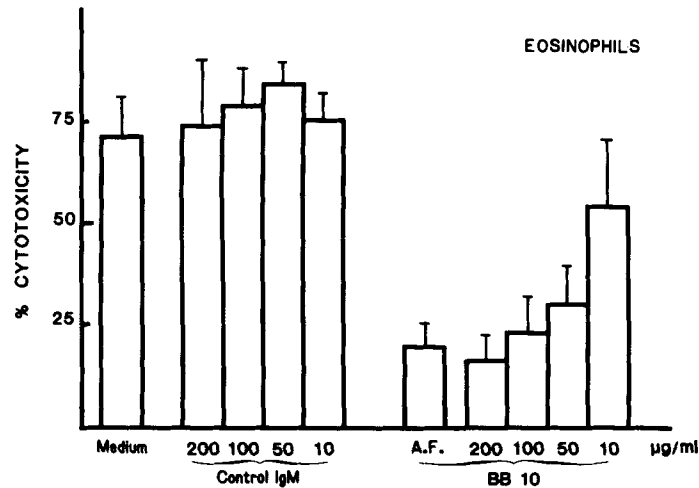


FIGURE 3. Inhibitory role of BB10 on IgE-dependent killing mediated by human eosinophils. Hypodense eosinophils were preincubated for 60 min at +4°C, either with medium or with ascitic fluids (AF), or purified IgM fractions of BB10 or control G10-54. Without washings, the cells were added to schistosomula and IgE-containing immune sera and the cytotoxicity was measured after 48 h contact.

to any significant inhibition of the cytotoxicity. In both cases, eosinophils and platelets, microscopical observations revealed that BB10 was not cytotoxic for the effector cells.



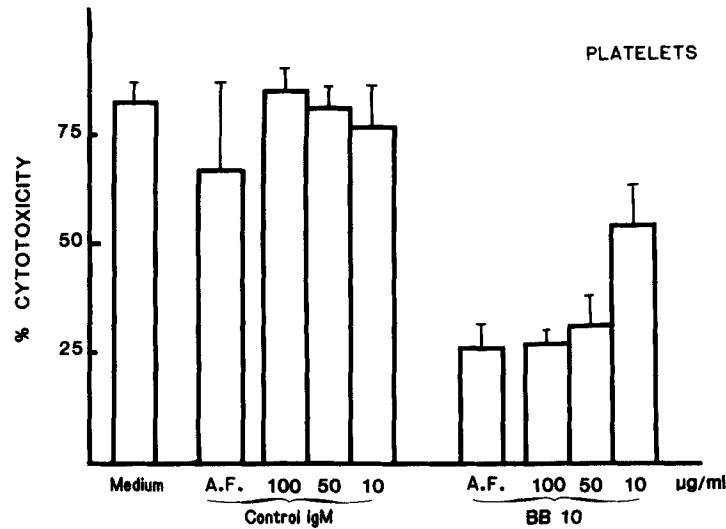


FIGURE 4. Inhibition by BB10 of the IgE-dependent killing by human platelets. Human platelets were preincubated for 60 min at 37°C with medium or with ascitic fluids (AF) or purified IgM fractions of control IgM G10-54 or BB10. Schistosomula and unheated anti-*S. mansoni* immune sera were then added and the percentage of cytotoxicity was measured after 24 h contact.

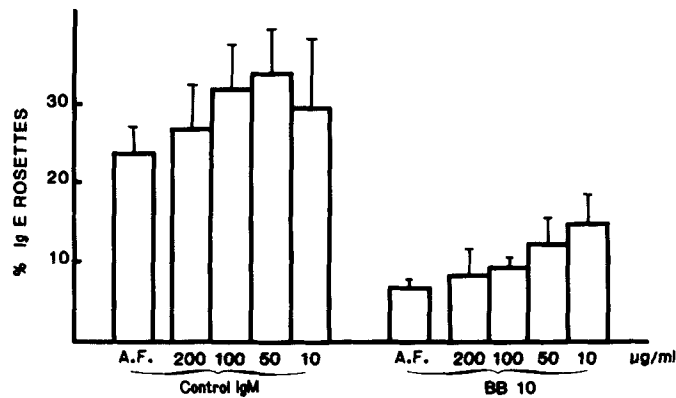


FIGURE 5. Role of BB10 on IgE rosette formation by eosinophils. Human hypodense eosinophils were incubated for 60 min at +4°C with control IgM or with BB10 (AF, ascitic fluids or IgM fractions), before the addition of IgE-coated erythrocytes ( $2 \times 10^8$ /ml). The percentage of rosette forming eosinophils was estimated on suspensions after staining with Discombe's diluent.

*Role of BB10 on Surface Receptors.* The expression of such ADCC mechanisms required functional Fc receptors able to bind specific antibodies. Among the various factors potentially involved in ADCC, we investigated the inhibitory role of BB10 on several membrane receptors, including Fc $\epsilon$  or Fc $\gamma$  receptors and C3 receptors. Rosette assays were therefore performed with human eosinophils and sheep erythrocytes coated with IgE immunoglobulins, to investigate the inhibitory role of BB10 on the IgE Fc receptors. As shown in Fig. 5, the IgE rosettes formed by hypodense human eosinophils were inhibited either in the presence of ascitic fluids or decreasing concentrations of purified BB10 (Student's *t* test

TABLE II  
Effect of BB<sub>10</sub> on Various Surface Receptors

Leukocyte type	Assay	Percent positive with:		Percent inhibition
		BB10	Control G10-54	
Eosinophils:	IgG rosettes	36.3 ± 3.8	35.4 ± 5.5	0
	IgG-dependent cytotoxicity	84.6 ± 6.6	81 ± 4	0
	C <sub>3b</sub> rosettes	43 ± 17	46.5 ± 6.4	7.6
Neutrophils:	IgG rosettes	77.7 ± 10.6	69.5 ± 13.1	0

The cells (purified human blood eosinophils or neutrophils) were preincubated with BB<sub>10</sub> or control G10-54 for 30–60 min at +4°C before addition to indicator erythrocytes in the rosette assays or to parasite targets and heat-inactivated immune serum in the cytotoxicity assay. The IgG rosettes were performed by using sheep erythrocytes coated with human normal IgG. Sheep erythrocytes bearing C<sub>3b</sub> were prepared by sequential deposition of C<sub>3b</sub> by fluid-phase and cell-bound amplification convertases.

for paired experiments:  $p < 0.0001$ ,  $n$ , 21 experiments; except for the lower dose:  $p < 0.05$ ,  $n$ , 8 experiments).

Because these results suggested a direct inhibitory role of BB10 on the Fc $\epsilon$  receptors of eosinophils, it was interesting to know the effect of BB10 on other surface receptors. When human eosinophils were preincubated with BB10 or with control G10-54, no difference in the proportion of IgG-rosettes was detected. Similarly, no inhibition of the IgG-mediated, eosinophil-dependent cytotoxicity was observed. In addition, the preincubation of eosinophils or neutrophils with BB10 did not lead to any significant inhibition of rosettes with C3b or IgG-coated erythrocytes, respectively (Table II).

Moreover, comparison experiments between the inhibitory role of BB10 and other anti-IgE Fc receptor antisera were performed on rat eosinophils and mast cells (Table III). Since BB10 could bind to rat eosinophils (Table I), its inhibitory role on rat eosinophil Fc $\epsilon$ R could be confirmed by inhibition of IgE rosettes, whereas the IgE rosettes formed by rat peritoneal mast cells were not significantly inhibited (Table III). Similar results were obtained with the polyclonal goat antiserum, already known to inhibit the IgE receptors of human lymphocytes, monocytes, and eosinophils (11). In contrast, the mAb directed against the rat basophil leukemia Fc $\epsilon$ R could only inhibit IgE rosettes formed by rat mast cells.

*Binding of Iodinated BB10 and IgE.* The binding of radioiodinated BB10 to various human blood cell populations was first studied. As shown in Figure 6*a*, one could notice that only hypodense eosinophils were able to specifically bind labeled BB10, whereas no significant binding was detected on normodense eosinophils or on neutrophils (three patients were studied in each case). In parallel, experiments performed on normal human platelets revealed specific binding of BB10, similar to hypodense eosinophils (Fig. 6*b*).

Experiments of competition of binding between BB10 and IgE were performed on purified eosinophils from normal rats to obtain the more homogeneous populations. As shown in Table IV, when rat eosinophils were preincubated with unlabeled BB10, a dose-dependent inhibition of binding of both radioiodinated BB10 and IgE was observed. Very high levels of inhibition were similarly observed when the cells were preincubated with aggregated IgE before incubation with iodinated BB10 or IgE. Experiments performed on human monocytes

TABLE III  
*Comparison of Various Anti-IgE Fc Receptor Antisera on Rat Eosinophils  
 and Mast Cells*

Cells incubated with:	Percent IgE rosettes with:	
	Rat Eosinophils	Rat mast cells
Control	14.2 ± 1.2	69.2 ± 4.3
BB10 (µg/ml)		
200	3.7 ± 0.5*	65.5 ± 2.5
100	2.5 ± 0.9*	56.2 ± 3.2
50	4.3 ± 0.9*	73.8 ± 3.8
10	5.3 ± 1.6*	66.0 ± 3.8
Polyclonal goat antiserum (final dilution)		
1/10	3.0 ± 1.0*	67.1 ± 7.4
1/25	3.2 ± 1.2*	68.9 ± 2.2
1/50	11.2 ± 6.2	64.3 ± 5.2
Monoclonal anti-RBL (µg/ml)		
5	11.3 ± 0.6	8.2 ± 3.5*
1	10.9 ± 0.7	8.0 ± 1.0*
0.5	12.3 ± 1.4	22.6 ± 5.1*
0.1	14.6 ± 0.9	46.5 ± 2.5‡

Rat eosinophils or mast cells were preincubated (60 min at +4°C) with BB<sub>10</sub>, with goat antiserum directed against a B lymphoblastoid cell line, or with anti-RBL antibodies (purified mAb directed against the IgE Fc receptor of RBL [25]). The percent of IgE rosettes was compared on purified eosinophils or mast cells from normal rats incubated either with the controls (respectively G10-54, normal goat serum, or medium) or with the antireceptor antibodies. Results are the mean ± SEM of at least four experiments.

\* Inhibition was significant at  $p < 0.001$ .

‡ Inhibition was significant at  $p < 0.02$ .

and platelets clearly confirmed the inhibitory role of BB10 on the binding of radioiodinated human IgE.

*Comparison by SDS-PAGE Analysis of the Platelet Proteins Eluted from BB10 or IgE Affinity Columns.* The detergent lysates from purified human platelets were passed over insolubilized mAb BB10 and eluted with high salts. As seen in Fig. 7A (lane 1), several bands can be identified in this affinity-enriched material, which are not present in the controls without cell lysate (Fig. 7A, lane 2). Four bands were observed at  $M_r$  of 32,000, 43,000, 45,000, and 94,000. In addition, a nonspecific band was detected at 67,000.

In the case of the labeled platelet extract eluted from IgE affinity column (Fig. 7B, lane 1), a very similar pattern was obtained in autoradiographic SDS-PAGE analysis, with four bands at 31,500, 43,000, 45,000, and 97,000. In both immunosorbents, the 43,000 band was the most prominent band. Fig. 7B (lane 2) shows an autoradiograph of radiolabeled U937 (FcεR+) extract eluted from IgE column. The two main bands detected at 23,000 and 43,000 were similar to those already reported (24). The control FcεR<sup>-</sup> U937 cells submitted to the same procedure did not show any labeled material (Fig. 7B, lane 3).

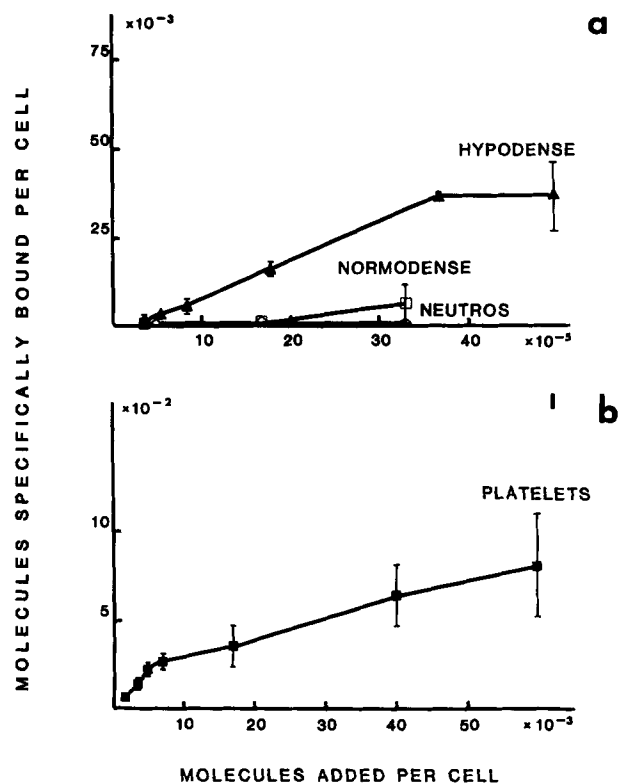


FIGURE 6. Binding of  $^{125}\text{I}$ -BB10 to various cell populations. Each line represents the specific binding of varying concentrations of BB10, obtained by subtracting the nonspecific binding of equal concentrations of radiolabeled G10-54 (mean  $\pm$  SEM of three experiments). (a) Specific binding to hypodense eosinophils,  $\blacktriangle$ ; normodense eosinophils,  $\square$ ; and neutrophils,  $\diamond$ . (b) Specific binding to normal human platelets,  $\blacksquare$ .

### Discussion

In this study we describe an mAb able to inhibit the IgE-dependent killing of parasite targets by human eosinophils and platelets. The effector function of these cells was previously shown to require a specific binding site for IgE immunoglobulins with a relatively low affinity for the ligand (9). This mAb, BB10, is an IgM immunoglobulin and was obtained after immunization of mice with hypodense eosinophils. Such eosinophils were purified from the blood of highly hypereosinophilic patients (with the hypereosinophilic syndrome or parasitic diseases, for instance), or from tissues (lungs), according to their abnormally low density (2, 3, 6). This eosinophil subpopulation was recently shown to exhibit increased IgE-dependent cytotoxicity against schistosomula targets compared with that of eosinophils with normal density (normodense) (5).

Mice were therefore immunized by hypodense blood eosinophils and boosted with hypodense lung eosinophils from the same patient, who was shown to give high levels of IgE-dependent cytotoxicity against schistosomula targets (5). With the IFSA procedure used to screen the hybridoma supernatants and control the specificity of purified antibodies, the strong membrane fluorescence with BB10, the negativity of unrelated IgM mAbs (anti-*Echinococcus multilocularis*), the

TABLE IV  
*Competition Experiments Between BB10 and IgE*

Cell types	Preincubated with unlabeled:	Cell-bound radioactivity (cpm/tube) after incubation with:	
		<sup>125</sup> I-BB10	<sup>125</sup> I-IgE
Rat eosinophils:	Control IgM	9142 ± 1790	5101 ± 388
	BB10 (× 100)	1451 ± 342* (84) <sup>‡</sup>	1460 ± 252* (71)
	(× 50)	4119 ± 847 <sup>§</sup> (55)	2350 ± 377* (54)
	(× 25)	7194 ± 1672 (21)	3854 ± 780 (24)
	(× 10)	10020 ± 1528 (0)	4266 ± 607 (16)
	IgE (× 50)	2259 ± 356* (75)	1897 ± 317* (63)
	(× 25)	2037 ± 466* (77)	1778 ± 983* (65)
	(× 10)	1944 ± 359* (79)	2152 ± 930 <sup>§</sup> (58)
	(× 5)	2147 ± 255* (76)	1538 ± 979* (70)
	Human monocytes:	Medium	
BB10 (× 50)		ND	1356 ± 489* (72)
IgE (× 20)			854 ± 490* (82)
Human platelets:	Medium		11352 ± 1359
	BB10 (× 50)	ND	5526 ± 687 <sup>§</sup> (51)
	IgE (× 10)		2083 ± 813* (81)

The various cell populations ( $10^6$ /tube for eosinophils and monocytes and  $8 \times 10^7$  platelets/tube) were preincubated with various concentrations of unlabeled reagents (from 100- to 5-fold excess labeled products) before incubation with <sup>125</sup>I-BB10 or the respective rat or human <sup>125</sup>I-IgE myeloma protein (1 μg/tube). After centrifugation over sucrose, the radioactivity of the pellets was determined. The cell-bound radioactivity was estimated by subtracting the background cpm obtained in control tubes without cells.

\* Significant inhibition at  $p < 0.01$  level.

<sup>‡</sup> Parentheses indicate the percent inhibition.

<sup>§</sup> Significant inhibition at  $p < 0.05$  level.

decrease in fluorescence intensity between hypodense and normodense eosinophils, the negativity of neutrophils and the inhibitory role of mouse immune serum (before the hybridization) on IgE rosette formation by eosinophils allowed us to consider the possibility that BB10 was an mAb directed against the eosinophil Fcε receptor. FMF analysis, moreover, confirmed that the percentage of cells stained by BB10 was closely related to the percentage of cells expressing IgE receptors by the rosette assay, as previously reported (5, 11).

The specificity of BB10 for FcεR was settled by several types of evidence. First, the high level and the dose-dependence of inhibition of IgE-mediated effector function of human hypodense eosinophils suggested that the epitope recognized by BB10 was involved in the mechanism of cell activation for killing.

However, the evidence was not sufficient to include Fcε receptors, since other membrane proteins known to play a role in cell adhesion to targets might also be involved in this mechanism. Therefore, three results confirmed the direct effect of BB10 on the IgE receptor: (a) the parallel inhibitory function of BB10 on IgE rosette formation and on IgE-mediated killing by eosinophils, (b) the lack of inhibition of other surface receptors such as eosinophil IgG or C3b receptors, or neutrophil IgG receptors, and (c) a similar inhibitory role in IgE-mediated killing by human platelets. This last point deserves some consideration. Indeed,

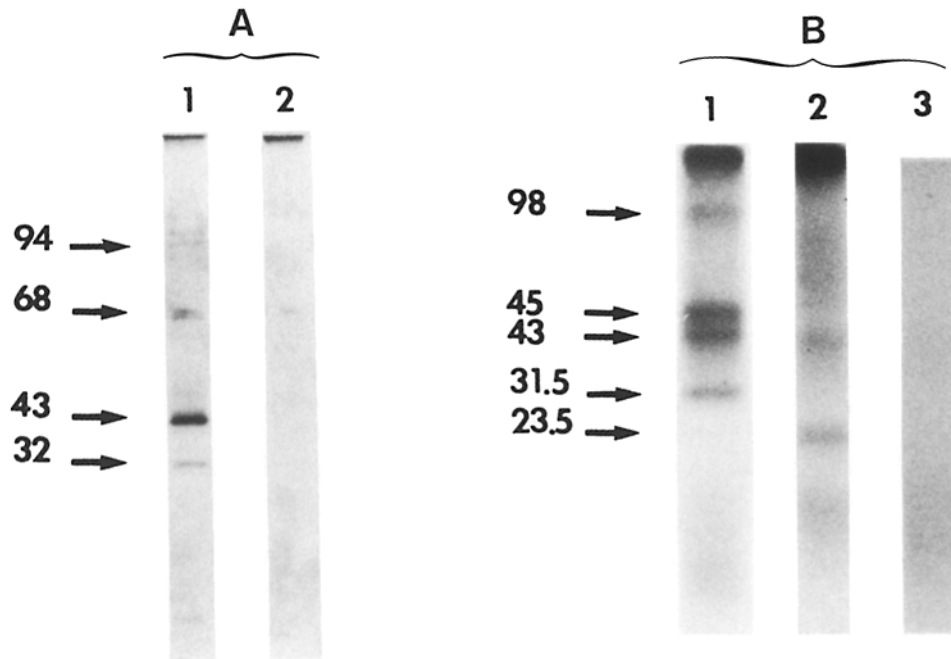


FIGURE 7. (A) SDS-PAGE analysis of platelet lysate eluted from BB10 affinity columns (lane 1); control without cells (lane 2). (B) SDS-PAGE analysis of platelet (lane 1), U937 Fc $\epsilon$ R<sup>+</sup> (lane 2), and U937 Fc $\epsilon$ R<sup>-</sup> (lane 3) extracts eluted from IgE affinity columns. Apparent  $M_r$  of the bands are indicated by arrows (A and B, lane 1).

the inhibitory role of polyclonal antibodies in IgE-dependent cytotoxicity mediated by eosinophils (5) and platelets (10) had previously revealed that a common antigenicity was shared between the IgE receptors on human eosinophils and platelets (9). However, the two killing mechanisms seemed different, since, in contrast to the eosinophil situation, adherence of platelets to their targets was not required (25). Therefore, it is likely that the inhibitory role of BB10 acted through the IgE receptors, rather than by an indirect effect on the killing mechanism itself. The parallel inhibition by an mAb of IgE-dependent effector function of eosinophils and platelets precisely confirmed the presence of a common structure between the Fc $\epsilon$ R of human eosinophils and platelets, Fc $\epsilon$ R<sub>2</sub>. Moreover, preliminary results with human monocytes indicated similar inhibition of IgE-dependent function against parasites and IgE binding by BB10.

In addition, the comparison between the effect of BB10 and two other anti-Fc $\epsilon$ R antibodies on mast cells and eosinophils indicated that the epitope recognized by BB10 and by the polyclonal goat anti-Fc $\epsilon$ R lymphocyte antiserum was also present on rat eosinophils, but did not interfere with IgE binding to rat mast cells. On the other hand, the anti-RBL mAb that could inhibit IgE binding to RBL and rat mast cells (26), did not inhibit IgE binding to rat eosinophils. These findings suggest the existence of a second receptor for IgE on eosinophils, platelets, and monocytes, which is distinct from Fc $\epsilon$ R1 present on basophils and mast cells (27).

Experiments of binding of radiolabeled BB10 were performed on various cell

populations to quantify the epitope recognized by BB10 and investigate the crosscompetition between BB10 and IgE. The present results showed that similar levels of specific binding were obtained with hypodense eosinophils and normal platelets, but not with normodense eosinophils. Since BB10 is a pentameric IgM molecule, the precise numbers of binding sites for BB10 could not be measured by this technique; however, experiments now in progress with the monomeric form of BB10 revealed a  $K_a$  of  $10^7/M$  for both eosinophils and platelets, and a number of binding sites around  $10^5/cell$  for eosinophils and  $10^3/cell$  for platelets, which are close to the numbers of IgE receptors evaluated by using radiolabeled IgE (9) (Jouault, T., manuscript in preparation). Competition experiments also suggested that not only BB10 exhibited a strong inhibitory effect on IgE binding, but that preincubation of cells with aggregated IgE seemed to saturate the Fcε receptor and to inhibit the subsequent binding of BB10. This fact has to be taken into consideration in the case of human eosinophils from patients with high levels of circulating IgE immunoglobulins, a situation where the FcεR have been shown to be occupied by cytophilic IgE (15). Indeed in these cases no binding of BB10 could be detected (data not shown).

SDS-PAGE analysis of the molecules eluted from BB10 affinity column was performed with human platelets because of their availability in large quantities and high degree of purity. As recently shown for a mAb anti-B cell FcεR (28), several bands could be identified by this technology. The similarities between the SDS-PAGE patterns of platelet extracts eluted from BB10 or IgE immunosorbents suggest that the proteins retained on BB10 could very well be derived from the IgE receptor. Their  $M_r$  are closely related to protein components of the IgE receptor on WIL-2WT (23) and on the macrophage-like U937 cell line (24). Preliminary results on radiolabeled eosinophil extracts showed a pattern similar to that observed with U937 FcεR+ with two major bands at 23,000 and 45,000–50,000. Experiments are now in progress to compare platelet and eosinophil IgE receptors with the structure of the rat macrophage IgE receptor identified by using IgE and crosslinking reagents (29).

Whereas the inhibition of IgE receptors by a polyclonal antiserum indicated that FcεR on lymphocytes, macrophages, eosinophils, and platelets were all antigenically related and differed from those on basophils (9–11), BB10 did not seem to inhibit IgE receptors on two B cell lines, WIL 2 or RPMI 8866 (Spiegelberg, H. L., Scripps Clinic, La Jolla, CA and Delespesse, G., University of Manitoba, Winnipeg, Canada, personal communications). These results suggest that the FcεR on lymphocytes, already individualized from others by its higher affinity for IgE ( $10^8/M$ ) could also be structurally different. The lack of inhibition of B lymphocyte FcεR by BB10 could be due to either the increased affinity of this receptor for IgE (which is 10 times higher than affinity for BB10) or to a structural difference. In this respect, no clearcut evidence is supplied on the identity of FcεR on BLc and macrophages (11), and it would be very interesting to compare BB10 with other mAbs that can inhibit Fcε receptors on B lymphocytes or B cell lines (30).

Finally, according to the survey of cells functionally reacting with BB10, this mAb seemed different from other mAbs directed against human eosinophils. Eo 1, which, in contrast to BB10, reacted with neutrophils and lymphocytes, did

not react with platelets and had few functional effects (31). On the other hand, Eo 1-7 all bound to neutrophils also (32). One interesting characteristic between three of these mAb (Eo 4, 5, and 6) that detected membrane antigens strongly expressed on hypodense cells, and BB10, was that they were all of IgM isotype. This seems to represent a common feature of mAbs with regulatory functions and implies that multivalent binding of the appropriate receptors is a prerequisite in the triggering of functional effects (33).

Besides the difficulties encountered in studies on FcεR-bearing eosinophils because of their presence on a small proportion of cells, the fact that these cells are only present in the blood of patients with hypereosinophilia, and also because of their low affinity for IgE, it is worthwhile to recall that cytophilic IgE immunoglobulins were recently detected on blood eosinophils and, to a greater extent, on lung eosinophils (15). This was a clear demonstration that these Fcε receptors were not an *in vitro* artefact, but had sufficient affinity to bind IgE *in vivo*, especially those present on lung eosinophils. Moreover, the lung eosinophils were shown to participate to cytolysis of lung epithelial cells (7). Therefore, the presence of FcεR might represent one marker of pathological eosinophils, and BB10 could be used as an interesting reagent to identify those cells in human pathology and to investigate their susceptibility to various therapeutics in order to prevent the tissue lesions induced by eosinophils.

### Summary

An IgM mAb (BB10) was produced by immunization of mice with human eosinophils purified according to their abnormal low density ("hypodense" cells), and previously shown to exhibit increased IgE-dependent antiparasite cytotoxicity. This BB10 antibody, selected for positive fluorescence staining of hypodense blood or lung eosinophils and low or negative staining of normodense eosinophils or neutrophils, could strongly inhibit IgE-dependent cytotoxicity of human eosinophils and platelets. The specificity for the IgE Fc receptor was suggested by the high levels of inhibition of IgE rosettes formed by eosinophils after incubation with the purified IgM fraction of BB10, whereas other receptors (FcγR, CR1) were not affected. On the other hand, BB10, able to inhibit rat eosinophil FcεR, did not react with the IgE Fc receptor on mast cells or basophils. A technique using radioiodinated BB10 allowed us to quantify the specific binding of BB10 to human eosinophils and platelets. Competition experiments revealed a crossinhibition between the binding of BB10 and IgE, suggesting the specificity of BB10 for the IgE binding site of eosinophil, platelet, and monocyte FcεR. Three proteins having extrapolated  $M_r$  of 32,000, 43,000-45,000, and 97,000 were found in the platelet extract eluted from a BB10 or from an IgE immunosorbent column. These findings confirm the similarities between IgE Fc receptors on human eosinophils, platelets, and macrophages, already observed with polyclonal antibodies directed against the B lymphocyte Fcε receptor. They suggest, moreover, that the mAb BB10 can represent a good reagent for further investigations on the structure and the functions of this IgE Fc receptor (FcεR2).

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