

Chimeric immunoglobulin E reactive with tumor-associated antigen activates human Fc ϵ RI bearing cells

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Crosslinking of immunoglobulin E molecules that are bound to the Fc ϵ receptors expressed on mast cells or basophils triggers activation of these cells, resulting in the development of a type I hypersensitivity. Targeting this potent immune reaction towards tumors by using IgE that reacts with a tumor-associated antigen, may induce a local inflammation at the tumor site, and may therefore promote tumor regression. We have previously shown that murine IgE bound to tumor cells can activate murine mast cells to release TNF- α and histamine. To further investigate the therapeutic potential of IgE-mediated immunotherapy of carcinomas, we have developed human/murine chimeric versions, containing the murine variable regions and human constant regions, of both G250 and 323/A3 IgE. These chimeric IgEs are reactive respectively with the G250 renal cell carcinoma antigen and the Ep-CAM molecule, which is highly expressed by most carcinomas. Transfection of the respective chimeric heavy and light chain genes into recipient Sp2/0 myeloma cells yielded chimeric IgE-producing clones. Chimeric G250 and 323/A3 IgE reacted with tumor cells expressing the G250 antigen or Ep-CAM, respectively. To generate a cell line that expresses Fc receptors for human or chimeric IgE, the rat basophilic leukemia cell line RBL-7 was transfected with the human Fc ϵ RI α chain (RBL-7TZ) and subsequently tested for binding of chimeric IgE. Functional assays showed that both chimeric IgEs activated RBL-7TZ cells to release TNF- α when cultured with tumor cells that express the respective specific antigen. Furthermore, both chimeric IgEs were able to activate freshly isolated human basophils. [Hum Antibod 1997; 8: 169–180]

Keywords: Chimeric immunoglobulin E; renal cell carcinoma; mast cells

Introduction

A type I hypersensitivity reaction, caused by allergen challenge, is initiated by crosslinking of immunoglobulin E (IgE) molecules bound to the Fc ϵ receptors on the membrane of mast cells or basophils. Upon receptor crosslinking the cells degranulate and release mediators,

such as histamine and cytokines, that affect vascular permeability, induce chemotaxis of other immune effector cells to the site of allergen challenge, and thereby initiate an inflammatory reaction¹. The fact that a small amount of allergen can initiate a strong inflammation at the site of challenge, indicates the power and sensitivity of an IgE-mediated immune response. This immune reaction might be beneficially applied to elicit a local inflammation in a targeted tissue, for example in tumor therapy. The inflammatory reaction may be confined to the site of the tumor, when IgE is

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used that reacts with an antigen that is highly expressed in tumors only.

In a previous study, we have demonstrated that murine G250 IgE when bound to the human renal cell carcinoma (RCC)-associated antigen G250, is capable of activating murine mast cells *in vitro*². The antigen recognized by the G250 MAb has previously been shown to be a suitable target for antibody-based immunotherapy of RCC, because of its high expression in RCC tissue, and its absence in normal kidney tissue and most other normal tissues³. In studies with patients having RCC, the injected murine G250 IgG localized preferentially in the tumor^{4,5}. Therapy of RCC, xenografted in nude mice, with G250 IgG2a resulted in tumor growth inhibition⁶.

Similar to murine G250 IgE², murine 323/A3 IgE is capable of activating murine mast cells in the presence of specific tumor cells (Luiten *et al.*, manuscript in preparation). The 323/A3 MAb⁷ recognizes the epithelial cell adhesion molecule, Ep-CAM⁸, which is highly expressed in most carcinomas⁹. The 17-1A MAb¹⁰, which also recognises the Ep-CAM molecule, has been successfully used in clinical trials for the treatment of minimal residual disease of colorectal carcinoma¹¹. However, treatment of established solid tumors with specific IgG is less effective, which is probably due to the limited number of intra-tumoral effector cells that can be activated by IgG¹².

Therapy with IgE may represent a more effective treatment of such solid tumors than IgG, because of the increased local vascular permeability and chemotaxis of granulocytes to the site of the tumor, that are induced by mast cell mediators. Furthermore, the serum level of IgE comprises less than 0.001 percent of the total serum immunoglobulin¹, and very low levels of specific IgE are sufficient to trigger a type I hypersensitivity reaction after a challenge with minute amounts of antigen. Therefore, IgE therapy may be more effective than IgG-based immunotherapy, which requires relatively high amounts of specific antibody and high antigen expression levels on the tumor cells¹³.

IgE mediates cellular responses by binding to the Fcε receptor on effector cells. Chimeric IgE (cIgE) contains the human epsilon chain constant regions, which bind to the human Fcε receptor¹⁴. As a result, cIgE may activate human Fcε receptor bearing effector cells, analogous to human IgE. In addition, chimerization of both heavy and light chains of the IgE molecule may reduce the immunogenicity of the MAb in patients, as was shown for antibodies of the IgG isotype¹⁵.

We have developed a human/murine cIgE of the G250 and 323/A3 MAbs to enable further studies on the applicability of tumor-specific IgE for immunotherapy of cancer. In addition, the rat basophilic leukemia cell line (RBL-7TZ), transfected with the human FcεRIα, was developed and used in *in vitro* studies with chimeric IgE. The cIgEs developed were characterized biochemically and tested functionally for their capacity to trigger the release of TNF-α or histamine from transfected RBL-7TZ cells or from freshly isolated human basophils.

Materials and methods

Expression vectors

The chimeric light chain expression vectors, pSV₂*gpt*323/A3V_κHC_κ and pSV₂*gpt*G250V_κHC_κ, encoding respectively the chimeric 323/A3 and G250 kappa (κ) light chain, and the *gpt* gene for resistance to mycophenolic acid, have been described previously^{16,17} and were kind gifts of Dr M.P. Velders (University of Leiden, Leiden, The Netherlands) and Dr L.R. Coney (Centocor Inc., Malvern, PA, USA) respectively.

The DNA fragments containing the genomic sequence encoding the variable region of the heavy chain (V_H) of the 323/A3 or G250 MAb, were isolated from the respective expression vectors, previously used to generate chimeric 323/A3 and G250 IgGs^{16,17}. The human epsilon chain expression vector, pSV₂*gpt*HC_ε had been derived by Dr L.R. Coney (unpublished) from the IgG1 heavy chain expression vector¹⁸, by replacing the human gamma chain genomic DNA fragment with a human epsilon chain genomic DNA fragment containing the domains CH1 to 4. The 3 kb *Eco* RI fragment encoding 323/A3 V_H was cloned into the *Eco* RI site of pSV₂*gpt*HC_ε, yielding the expression vector pSV₂*gpt*323/A3V_HHC_ε for the c323/A3 epsilon chain (Figure 1A). Likewise, the chimeric G250 epsilon chain expression vector, pSV₂*gpt*G250V_HHC_ε, was constructed by inserting the 2.3 kb *Eco* RI fragment encoding G250 V_H into the *Eco* RI site of pSV₂*gpt*HC_ε (Figure 1B).

The expression vector, FcεRIαNT-*zeo*, encoding the α subunit of the human FcεRI was constructed by excizing the full-length FcεRI α chain cDNA (a gift of Dr U. Blank, Institut Pasteur, Paris¹⁹) from the pcMV4 vector using *Mlu* I and *Xba* I and cloning blunt end, using *Eco* RV, into the vector NT-*zeo*, containing the zeocin resistance (*zeo*) gene^{20,21}.

Cell lines

The murine myeloma cell line Sp2/0, obtained from Centocor Inc. (Malvern, PA, USA), was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin. The renal cell carcinoma cell line A704, that expresses the G250 antigen, and the human colon carcinoma cell line LS180, that expresses Ep-CAM, were obtained from the American Type Culture Collection (ATCC). The cell line B16C215 was derived from the murine melanoma B16 cell line transfected with human EpCAM cDNA, and was kindly provided by Dr M. Dohlsten (Pharmacia, Lund, Sweden)²². The cell line RBL-7 is a subclone of the rat basophilic leukemia cell line RBL-2H3²³ that had previously been selected for high serotonin release upon stimulation with murine IgE anti-DNP 2682-I (a gift of Dr U. Blank, Institut Pasteur, Paris) and DNP-BSA. The cells were cultured in Dulbecco's modified minimal essential medium (DMEM), supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin.

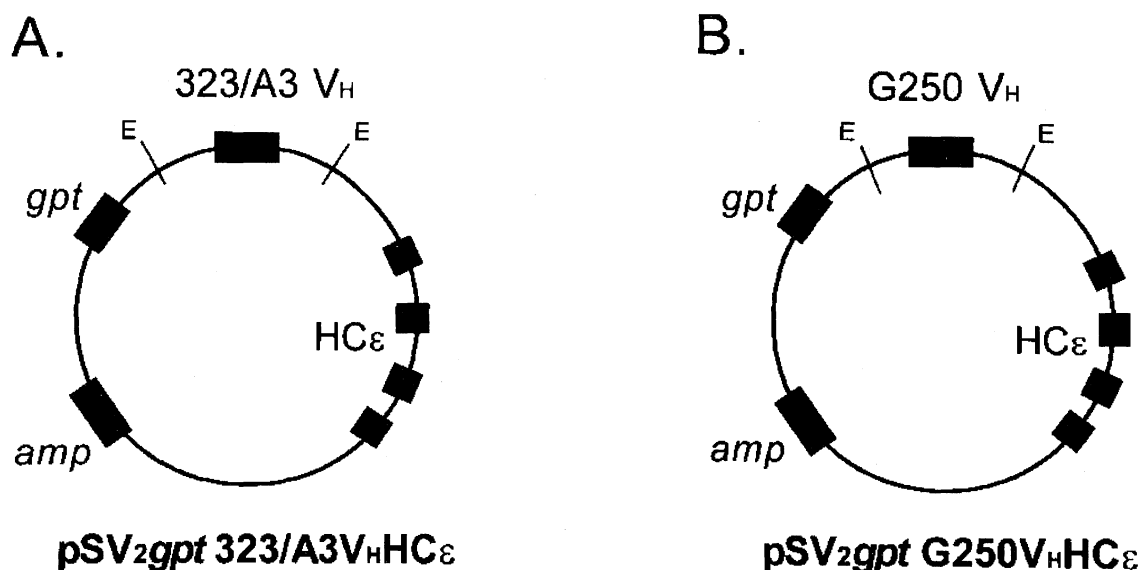


Figure 1: Structural map of the chimeric epsilon heavy chain expression vectors. Murine genomic DNA fragments containing the variable regions of the heavy chain of the 323/A3 MAb (323/A3 V_H) or G250 MAb (G250 V_H), were cloned into the human epsilon heavy chain expression vector, yielding expression vectors for the chimeric 323/A3 epsilon chain (A, pSV₂gpt323/A3V_HHC_ε) or G250 epsilon chain (B, pSV₂gptG250V_HHC_ε). E, *Eco* RI; *amp*, ampicillin; *gpt*, xanthine-guanine phosphoribosyl transferase, HC_ε, human epsilon heavy chain exons

Transfection

Sp2/0 cells, seeded at a density of 2×10^5 cells per ml one day prior to transfection, were transfected with 5 μ g heavy and 5 μ g light chain plasmid DNA using 70 μ l DOTAP transfection reagent (Boehringer Mannheim BV, Almere, The Netherlands) in hepes-buffered saline (HBS) at a final volume of 200 μ l, according to the instructions supplied with the transfection reagent. After culturing the cells for 16–18 h in the presence of the DOTAP/DNA complexes, the medium was replaced, and the cells were cultured further for 24 h. Subsequently, the cells were plated into flat-bottom 96 well plates at a density of 4000 or 1000 cells per well in medium containing 0.25 μ g ml⁻¹ mycophenolic acid, 1.25 μ g ml⁻¹ hypoxanthine and 25 μ g ml⁻¹ xanthine (Sigma Aldrich, Bornem, Belgium). Mycophenolic acid-resistant cells were selected in 2 to 3 weeks. cIgE production by the transfectants was determined by ELISA. For both IgE specificities, antibody producing transfectants were selected and subcloned by limiting dilution. Clones were tested for cIgE production by ELISA, and clones with relatively high antibody production as compared to the other clones, were selected for further culture.

RBL-7 cells were transfected with Fc ϵ RI α NT-zeo by electroporation in a BioRad Gene Pulser at 260 V and 960 μ F. The transfected cells (RBL-7TZ) were cultured for 48 h, after which 0.5 mg ml⁻¹ zeocin (Cayla, Toulouse, France) was added. Surviving cells were cloned using sterile steel cylinders as described²⁴. The expression of the human Fc ϵ RI α chain was assessed by flow cytometry.

Immunoassay for cIgE

ELISA plates were coated for 24 h with rabbit anti-human IgE-Fc ϵ specific antibody (DAKO Immuno-globulins, Glostrup, Denmark) at a concentration of 1 μ g/well in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.55) at 4°C. The plates were washed with PBS, 0.05% Tween and incubated with 50 μ l culture supernatant of the transfectants for 1–1.5 h at 37°C. After washing, the plates were incubated with 50 μ l/well horseradish peroxidase-conjugated goat anti-human kappa chain (1:4000, Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1 h at 37°C. IgE-bound peroxidase activity was assayed using tetramethylbenzidine substrate (70 μ g ml⁻¹, 0.03% H₂O₂ in 0.1 M NaAc pH 5.5, 100 μ l/well), followed by the addition of 50 μ l/well 2 N H₂SO₄ to stop the enzyme reaction. The cIgE concentration in the culture supernatant of two clones was determined by a radio-immunoassay using sheep anti-human IgE coupled to CNBr-activated sepharose and ¹²⁵I-labeled sheep anti-human IgE as described²⁵.

Flow cytometry

The expression of the human Fc ϵ RI α chain on transfected RBL-7 cells (RBL-7TZ) was determined by flow cytometry using the 6F7 antibody (a kind gift of Dr J. Hakimi, Hoffman-LaRoche, Nutley, NJ, USA)²⁶ directed against the human Fc ϵ RI α chain, and FITC-labeled F(ab)₂ fragments of goat anti-murine immunoglobulin antibody (Jackson ImmunoResearch, West-

grove, PA, USA). The binding of cIgE to target cells was measured by incubating A704, B16C215, RBL-7 and RBL-7TZ cells, detached by trypsin/EDTA treatment, with culture supernatant of the cIgE transfectants for 1 h at 4°C. Bound IgE was detected with rabbit anti-human IgE-Fc ϵ specific antibody (DAKO), and goat anti-rabbit IgG/FITC conjugate (Southern Biotechnology Associates Inc.). The relative fluorescence of the cells was measured on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). 1×10^4 events were recorded per sample and analyzed using the WinList 3.0 software program after gating on a fluorescence-1 (FITC) and fluorescence-3 (propidium iodide) two parameter dot plot, excluding dead cells permeable for propidium iodide. Membrane-bound cIgE was measured using rabbit anti-human IgE-Fc ϵ specific antibody and FITC-labeled goat anti-rabbit IgG.

Ammonium sulphate precipitation of cIgE

Chimeric IgE transfectants were adapted to grow on serum free culture medium (RPMI, 1% Nutridoma-SP low protein serum replacement, Boehringer Mannheim BV, Almere, The Netherlands). Culture supernatant was concentrated fifteen times using a ProVario-3 filter system with a 70 kD membrane cassette (Filtron Technology, Terheijden, The Netherlands) and clarified by centrifugation at 15,000 rpm for 30 min in a Sorvall centrifuge (Sorvall Centrifuges, Du Pont, Wilmington, DE, USA). cIgE was precipitated at 4°C by adding ammonium sulphate to 60%, spun down at 15,000 rpm for 45 min, and dissolved in PBS. After a passage through a G50 gel filtration column, the IgE concentration was determined by OD measurements at 280 nm and by a radioimmunoassay, as described²⁵.

Gel electrophoresis and immunoblotting

Gel electrophoresis was performed according to Laemmli²⁷, using nonreducing and reducing (1% β -mercaptoethanol) sample buffers. 10 μ g of protein containing cIgE was electrophoresed through a 12.5% acrylamide gel, stained with Coomassie brilliant blue or blotted onto a PVDF membrane (Hybond-PVDF, Amersham Life Science, Buckinghamshire, UK). Protein blots were incubated with either horseradish peroxidase-conjugated goat anti-human kappa chain (Southern Biotechnology Associates Inc.) or rabbit anti-human IgE-Fc fragment specific antibody (DAKO), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Science). Bound antibody conjugates were visualized by incubation with chemiluminescent substrate (ECL) and autoradiography (ECL Western blotting detection reagents, Amersham Life Sciences).

Serotonin release

RBL-7TZ and RBL-7 cells, resuspended in RPMI medium supplemented with 10% FCS (RPM/FCS) at a

density of 1×10^6 cells per ml, were incubated with 3 μ Ci ml⁻¹ [³H]serotonin (Amersham Life Science) for 1 h at 37°C, washed and resuspended in RPMI/FCS. After another incubation of 1 h at 37°C to remove noninternalized [³H]serotonin, the cells were washed, distributed in 96-wells microtiter plates at 2×10^5 cells per well and incubated for 1 h at 37°C with purified anti-nitrophenacetyl (NP) cIgE (kindly provided by Dr M.S. Neuberger, MRC Centre, Cambridge, UK)¹⁴ in a final volume of 50 μ l. Anti-NP IgE has similar affinity to the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl caproate (NIP) as to NP. Control incubations were performed with culture supernatant containing murine anti-dinitrophenyl (DNP) IgE produced by the hybridoma cell line H1-DNP-e-26²⁸. After washing the cells four times with 200 μ l HBSS to remove nonadherent cells, 25 μ l RPMI/FCS was added to each well, and incubated at 37°C for 15 min. Cells were challenged with 25 μ l prewarmed NIP-BSA (3 μ g ml⁻¹) or DNP-BSA (1 μ g ml⁻¹) for 30 min at 37°C. In parallel, cells were incubated with the 6F7 antibody (anti-human Fc ϵ RI α) without IgE preincubation. Reactions were stopped by adding 50 μ l ice-cold medium and placing the plates on ice. Serotonin release was assayed by counting 50 μ l supernatant mixed with 200 μ l Aqualuma-Plus scintillation fluid (Lumac, The Netherlands) in a β -plate counter (Pharmacia, Uppsala, Sweden). [³H]serotonin release was calculated as a percentage of the release by cells lysed in 0.5% SDS and 0.5% NP-40 (maximal release).

TNF- α release assay

RBL-7 and RBL-7TZ cells were assayed for the release of TNF- α upon activation by cIgE in the presence of specific antigen-expressing tumor cells, using the activation assay as described earlier^{2,29}. Briefly, A704, LS180, B16 or B16C215 cells were seeded in a 24 well plate at a density of 2.5×10^4 cells per well, cultured overnight and incubated with a series of ten-fold dilutions of G250 or 323/A3 cIgE culture supernatant in DMEM for 1 h at 4°C. Control assays were performed with murine G250 or 323/A3 IgEs. After a washing with DMEM, 1×10^5 RBL-7 or RBL-7TZ cells were added per well in a final volume of 0.5 ml in DMEM and cultured overnight. In parallel experiments, the RBL to tumor cell ratio was varied by adding 10- or 100-fold less RBL cells to the wells. Supernatants of these cocultures were assayed for TNF- α content using the WEHI bioassay³⁰.

Basophil activation assay, histamine release

Human mononuclear cells were enriched to approximately 2% human basophils by collecting the interphase after centrifugation of peripheral blood cells over 1.077 g/cm³ Percoll (Pharmacia). Membrane-bound IgE was removed from the basophils by lactic acid treatment as described³¹. The basophils were then sensitized with culture supernatant containing 323/A3 or G250 cIgE and washed to remove unbound cIgE. Membrane-bound

cIgE on the basophils was crosslinked by an incubation with sheep anti-human IgE antiserum (SH25P01, CLB Amsterdam, The Netherlands), as previously described³¹. To check the efficacy of the lactic acid treatment, the basophils were incubated with sheep anti-human IgE without prior IgE sensitization. As a parameter for basophil activation by cIgE and sheep anti-human IgE, the amount of histamine released was measured by fluorometric analysis as described³². Experimental histamine release was calculated as a percentage of the total amount of histamine present in the cells.

Results

Generation of G250 and 323/A3 cIgE

We have generated chimeric versions of both G250 and 323/A3 IgE, in which both heavy and light chains are composed of human constant regions and murine variable regions. Chimeric epsilon heavy chain genes were constructed by inserting the genomic DNA fragment encoding either the G250 or the 323/A3 heavy chain variable gene segment, into an expression vector containing the human epsilon chain constant gene segment (*Figure 1*). The G250 or 323/A3 chimeric epsilon heavy chain expression vectors were transfected into Sp2/0 myeloma cells together with the respective chimeric light chain expression vector. The transfections gave rise to 13 G250 cIgE producing transfectants and 19 transfectants producing 323/A3 cIgE. After 1 month of culture, two clones producing the highest level of G250 or 323/A3 cIgE were selected and cultured for two months in medium without the selection drug. All subclones obtained from a limiting dilution subcloning of these two transfectants produced cIgE, indicating that the two parental clones selected stably expressed the cIgE. Subclones with the highest production levels, as measured by ELISA, were selected for antibody purification and adapted to low protein serum free culture medium. The amounts of antibody present in ten day spent culture medium, as determined by the radioimmunoassay, of subclone cG250E13.9, producing G250 cIgE, and subclone c323/A3E11.7, producing 323/A3 cIgE, were $0.5 \mu\text{g ml}^{-1}$ and $1.9 \mu\text{g ml}^{-1}$ respectively.

Biochemical characterization of cIgE

Chimeric IgE in spent culture supernatant of clone cG250E13.9 and clone c323/A3E11.7 that had grown in serum free medium, was precipitated with 60% saturated ammonium sulphate and analyzed by reduced and nonreduced SDS gel electrophoresis. After staining with Coomassie brilliant blue, for the 323/A3 cIgE two major protein bands were seen (*Figure 2A*), which were confirmed to represent the κ light chain and ϵ heavy chain by immunoblotting with anti-human κ chain Ab (*Figure 2B*) and anti-human IgE-Fc ϵ specific Ab (*Figure*

2C), respectively. *Figure 2A* also shows the protein bands of reduced chimeric 323/A3 IgG1, that contains the same κ light chain as in cIgE, and a $\gamma 1$ heavy chain, that is smaller than the ϵ chain. Likewise, the presence of κ light chains and ϵ heavy chains in G250 cIgE was analyzed (see *Figure 2B,D*). The extra ϵ chain band of reduced G250 cIgE in lane 9 might be due to differences in glycosylation. Precipitation of the G250 cIgE from culture supernatant was less efficient as compared to the 323/A3 cIgE, probably due to a lower production level of G250 cIgE. Therefore, the Coomassie staining pattern did not show the G250 light chain as a major band (not shown). The blots of nonreduced cIgE containing samples in *Figure 2C* and *2D* show one major high molecular weight protein band corresponding to whole IgE.

Generation and characterization of RBL-7 transfectants expressing the human Fc ϵ RI α chain

To generate a cell line of mast cell or basophilic lineage that can be activated by human or cIgE, RBL-7 cells were transfected with the human Fc ϵ RI α chain, since a human mature basophilic cell line that binds human IgE was not available. RBL-7TZ cells, stably transfected with cDNA encoding the human Fc ϵ RI α subunit, expressed membrane receptors that were recognized by the anti-human Fc ϵ RI α chain antibody 6F7 as was assessed by flow cytometry (*Figure 3*). Parental RBL-7 cells did not react with the 6F7 antibody (*Figure 3*). Direct receptor crosslinking by 6F7 antibody also triggered serotonin release by the RBL-7TZ cells (not shown).

Sensitization with murine anti-DNP IgE triggered serotonin release by both RBL-7TZ (*Figure 4A*) and parental RBL-7 cells in response to DNP-BSA. The amount of activation mediated by the transfected Fc ϵ RI α , was measured by sensitizing the cells with anti-NP cIgE, which activates human Fc ϵ RI bearing cells when crosslinked by NIP-BSA¹⁴. The transfectants (*Figure 4B*), but not parental RBL-7 cells, released serotonin when sensitized with anti-NP cIgE and challenged with NIP-BSA. These results show that RBL-7TZ cells expressed a functional Fc ϵ RI containing the human α chain, and could bind cIgE and subsequently trigger a secretory response when cell-bound cIgE was crosslinked.

Binding of 323/A3 and G250 cIgE to tumor cells and to Fc ϵ receptor expressing cells

The binding of G250 or 323/A3 cIgE to respectively A704 tumor cells, expressing the G250 antigen, or to Ep-CAM transfected B16C215 cells, was evaluated by flow cytometry as described in Materials and methods. *Figure 5* shows that G250 cIgE reacted with the A704 cells, whereas the 323/A3 cIgE did not, indicating that the reaction of G250 cIgE with A704 cells did not result

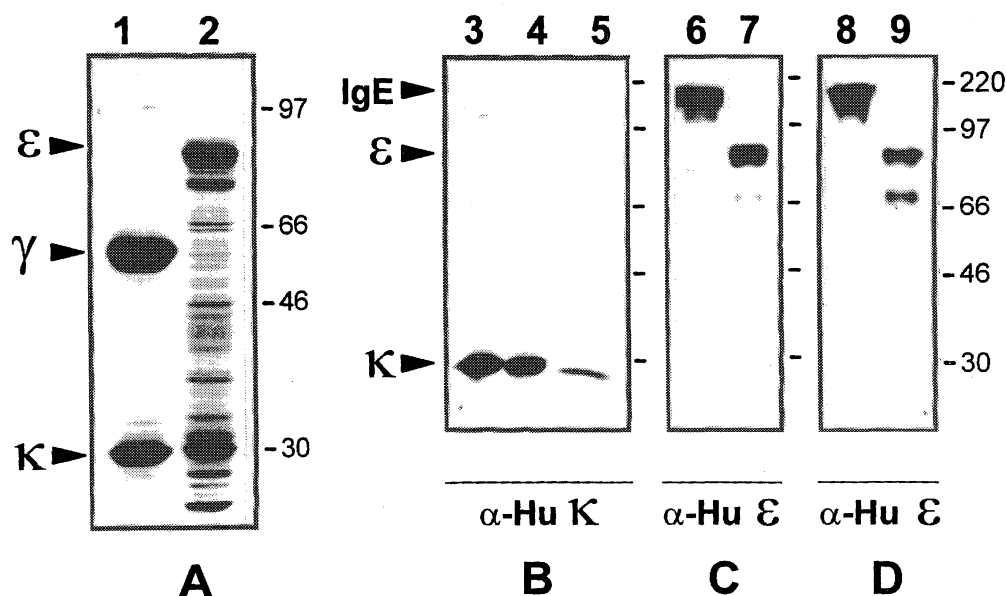


Figure 2: Biochemical characterization of the cIgE. Protein precipitated from culture supernatant containing 323/A3 or G250 cIgE was electrophoresed under reducing and non-reducing conditions on a 12.5% acrylamide gel, stained with Coomassie brilliant blue, **A**; or transferred to a PVDF membrane and incubated with goat anti-human κ chain Ab, **B**; or rabbit anti-human IgE Fc ϵ specific Ab, **C**; **D**: respectively. Lane 1, chimeric 323/A3 IgG1; lane 2, 323/A3 cIgE; lane 3, chimeric 323/A3 IgG1; lane 4, 323/A3 cIgE; lane 5, G250 cIgE; lanes 6 and 7, 323/A3 cIgE; lanes 8 and 9, G250 cIgE; lanes 1–5, 7 and 9, reduced; lanes 6 and 8, nonreduced

from an aspecific reaction of cIgE with the tumor cells. The 323/A3 cIgE, as expected, reacted with the B16C215 cells, that express the Ep-CAM molecule, but not with A704 cells, which lack Ep-CAM expression.

In addition to secreted immunoglobulins, hybridoma cells may also express immunoglobulin in a membrane-bound form³³. The presence of cell membrane-bound IgE on both the G250 and the 323/A3 cIgE producing transfectants, was detected by flow cytometry using rabbit anti-human IgE-Fc ϵ specific antibody. A positive shift in fluorescence was observed for the entire cell population, suggesting that all transfected cells express membrane-bound cIgE (Figure 6).

The binding of 323/A3 and G250 cIgE to the human Fc ϵ RI α chain was evaluated, using the transfected RBL-7TZ cells, which express Fc ϵ receptors containing the human Fc ϵ RI α chain and rat Fc ϵ RI β and γ chains. Figure 7 shows that both cIgEs bound to the RBL-7TZ cells, but not to the untransfected RBL-7 cells.

Activation of rat basophilic leukemia (RBL) cells transfected with the human Fc ϵ RI α

323/A3 and G250 cIgE were tested functionally for their capacity to activate cells of the basophilic lineage in the presence of tumor cells expressing specific antigen. The

RBL-7TZ cells, transfected with the human Fc ϵ RI α chain, and human basophils (see next section) were used for cIgE-mediated activation assays.

323/A3 cIgE activated RBL-7TZ cells to release TNF- α in the presence of B16C215 cells (Figure 8A), while parental RBL-7 cells, which did not bind cIgE (Figure 7), were not activated. Control assays showed that RBL-7 cells were able to release TNF- α upon activation by murine 323/A3 IgE (not shown). The parental B16 cells lack Ep-CAM expression and did not trigger activation of RBL-7TZ cells even at high concentrations of cIgE, that triggered maximal TNF- α release in the presence of B16C215 (Figure 8A). The Ep-CAM expressing cell line LS180 precoated with 323/A3 cIgE, activated RBL-7TZ, but not RBL-7 cells, to release TNF- α (not shown). In the absence of either cIgE, or specific tumor cells, the level of TNF- α release was comparable to the background release of RBL-7TZ cells. The cIgE-mediated activation depended on the concentration of 323/A3 cIgE present in the coculture of mast cells and tumor cells (Figure 8B). A cIgE concentration as low as 0.1 ng per ml resulted in a significant release of TNF- α . Figure 8B also shows that with a mast cell to tumor cell ratio of 4 or 0.4 and a 323/A3 cIgE concentrations of 0.1 ng ml⁻¹ detectable amounts of TNF- α were released. G250 cIgE at a concentration of 0.2 ng per ml or higher was capable of activating RBL-7TZ cells in the presence of A704 cells, which express the G250 antigen. 323/A3 cIgE did

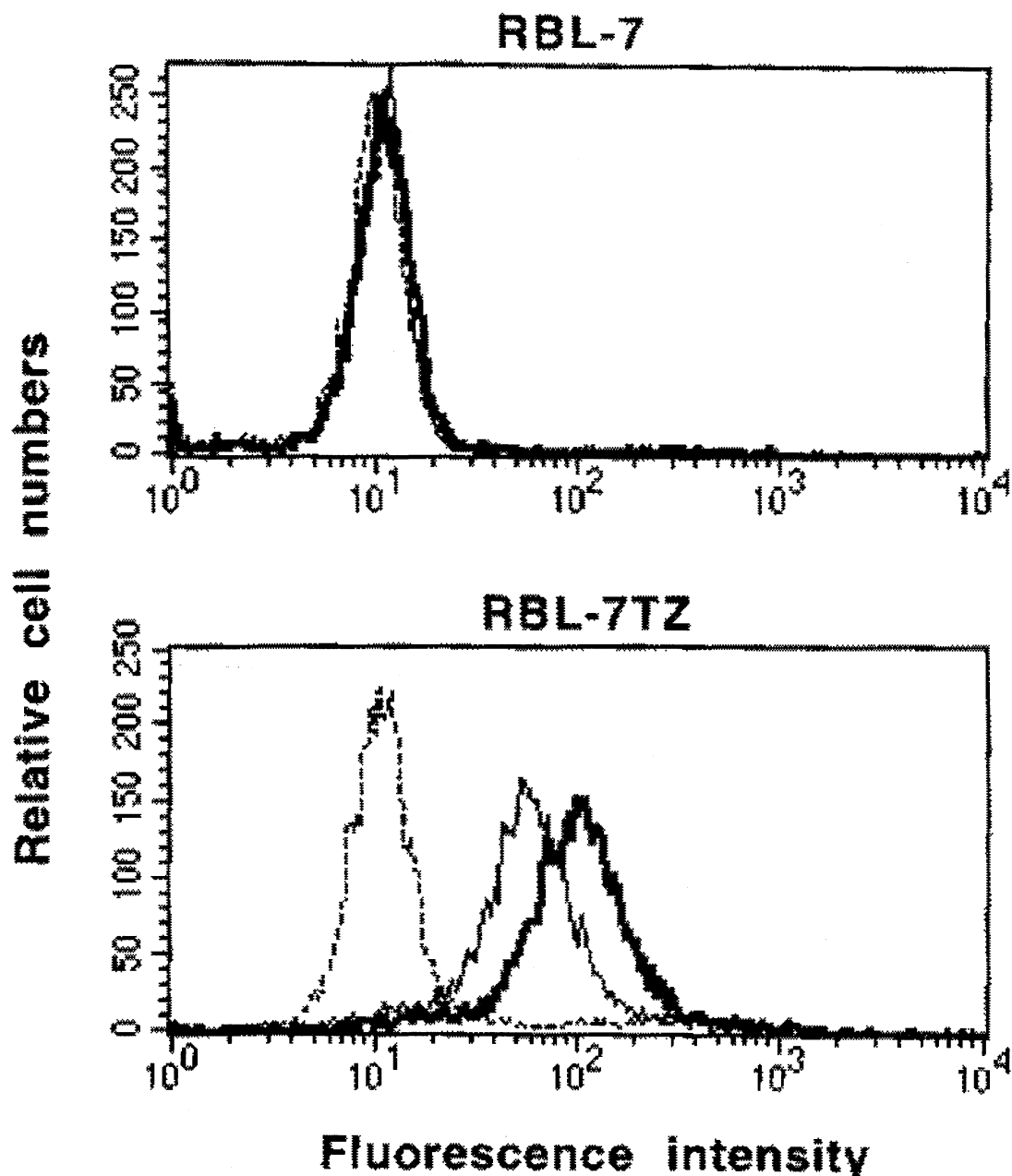


Figure 3: Expression of human Fc ϵ RI α chain by transfected RBL-7 cells. RBL-7 and transfectant RBL-7TZ were incubated with 0 (dotted histograms), 1 (thin histograms) or 10 μ g ml $^{-1}$ (bold histograms) anti-human Fc ϵ RI α chain antibody 6F7. Bound antibody was detected with FITC-labeled goat anti-murine Ig F(ab) $_2$ fragments. The relative fluorescence of the cells was analyzed by flow cytometry. Vertical axis: cell number; horizontal axis: log channel number ranging from 10 ^{0} to 10 ^{4} (FITC fluorescence intensity)

not bind to the A704 cells, and did not trigger activation of the RBL-7TZ cells (Figure 9).

Activation of freshly isolated human basophils by cIgE

Freshly isolated human mononuclear cells, enriched to contain 2% basophils, were used to test the capacity of 323/A3 and G250 cIgE, to activate native human

effectors cells when crosslinked with anti-IgE Ab. The basophil preparation was first treated with lactic acid to remove endogenous IgE from the Fc ϵ receptors. The amount of histamine released by the basophils after incubation with 323/A3 or G250 cIgE and anti-IgE Ab was higher than after treatment with anti-IgE Ab only (Figure 10). The histamine release observed when basophils were incubated with anti-IgE MAb without cIgE sensitization, was probably due to the crosslinking of incompletely removed endogenous IgE. The increase

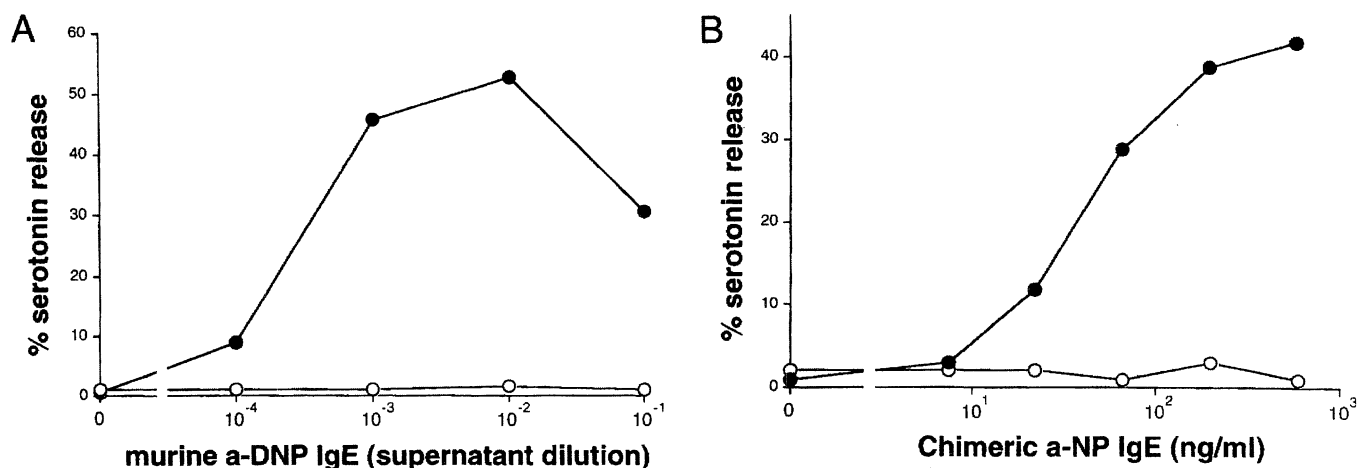


Figure 4: Serotonin release triggered by the aggregation of human Fc ϵ RI α chain in RBL-7 transfectants. **A:** RBL-7TZ cells were sensitized with ten-fold dilutions of culture supernatant containing murine anti-DNP IgE (●) or medium only (○) and challenged with 0 or 1 $\mu\text{g ml}^{-1}$ DNP-BSA. **B:** RBL-7TZ cells were sensitized with 0, 10, 100 or 1000 ng ml^{-1} anti-NP clgE and challenged with 0 (○) or 3 (●) $\mu\text{g ml}^{-1}$ NIP-BSA. The graphs present the percentage serotonin released as a function of the IgE concentration used for sensitization

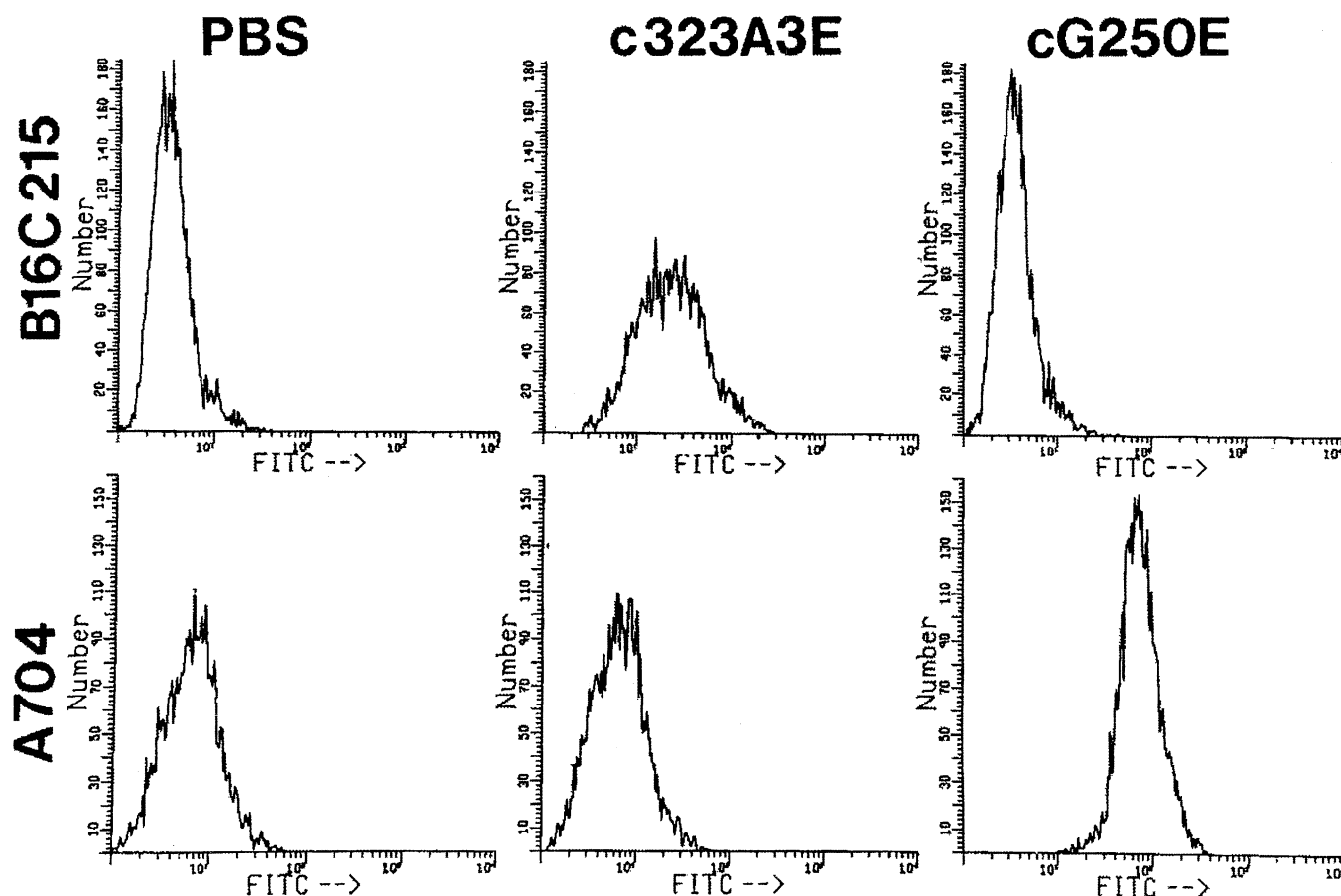


Figure 5: Binding of G250 and 323/A3 clgE to tumor cells. B16C215 or A704 cells were incubated with PBS (left) or with culture supernatant containing 19 $\mu\text{g ml}^{-1}$ 323/A3 clgE (middle) or 0.5 $\mu\text{g ml}^{-1}$ G250 clgE (right), respectively. Binding of clgE was detected by incubation with rabbit anti-human IgE Fc ϵ specific Ab and goat anti-rabbit IgG-FITC conjugate, and analyzed by flow cytometry. A704 does not express Ep-CAM, which is recognized by 323/A3 clgE, whereas B16C215 does not express the G250 antigen

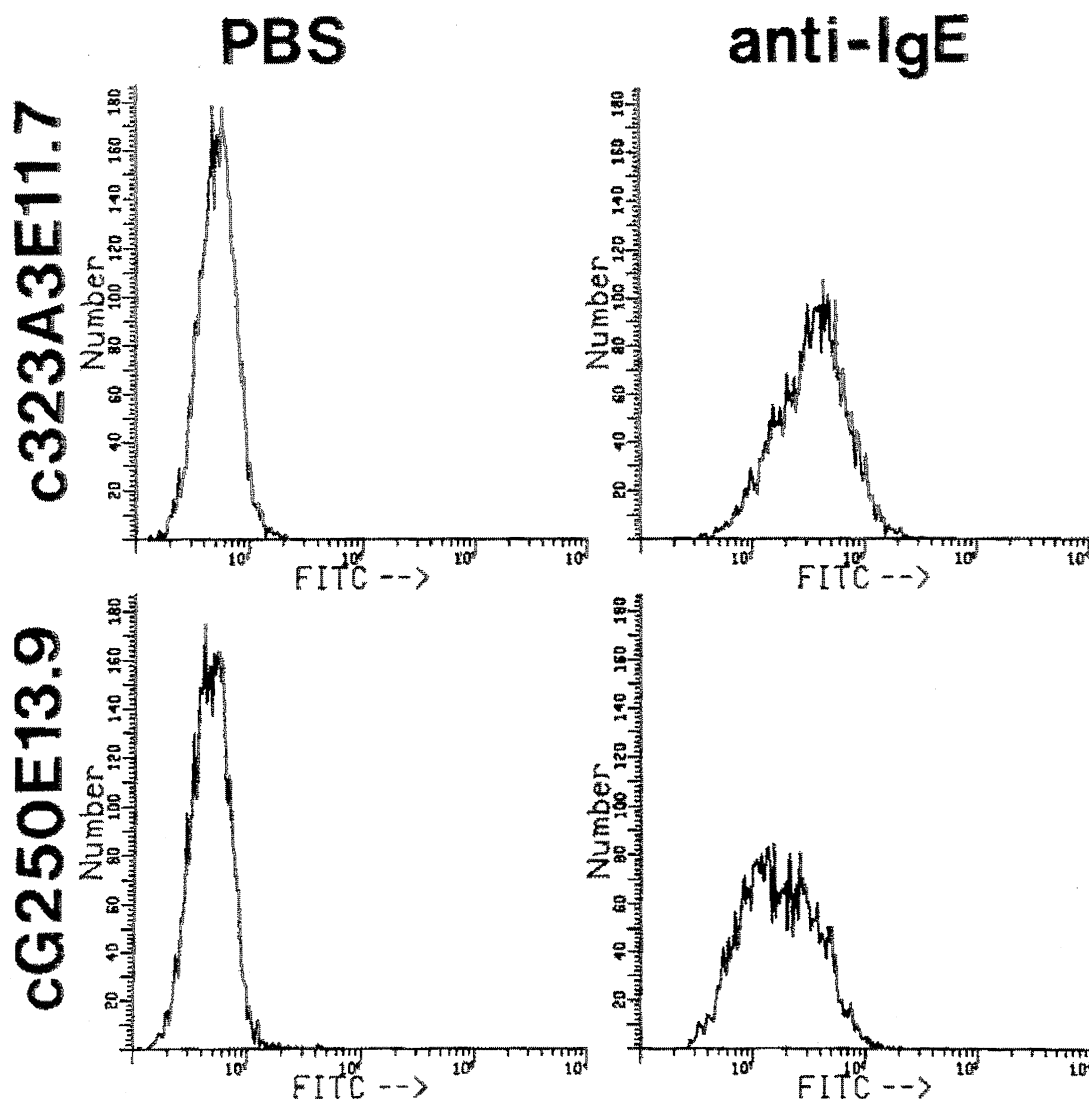


Figure 6: Presence of membrane-bound cIgE on the transfected cells. 323/A3 cIgE (c323/A3E) or G250 cIgE (cG250E) producing transfectants were incubated with PBS (left) or with rabbit anti-human IgE Fc ϵ specific Ab (right), followed by goat anti-rabbit IgG-FITC conjugate, and analyzed by flow cytometry

in histamine release by basophils sensitized by cIgE indicates that both cIgEs can mediate human basophil degranulation.

Discussion

Many monoclonal antibodies directed against tumor-associated antigens have been developed during the past decade^{12,34}. Most hybridomas produce antibodies of the IgG isotype. Isotype-switching of a hybridoma to an IgE producing hybridoma can be induced experimentally³⁵, but remains largely dependent on the chance of a switch to the IgE isotype. Therefore, only a limited number of IgE producing hybridomas, mostly of murine origin, are available. We have previously generated such IgE-isotype switch variants of the G250 MAbs against human

RCC³⁶, and also of the 323/A3 MAbs, which reacts with the Ep-CAM molecule (unpublished results).

Here, we report the generation of chimeric human/murine versions of both the G250 IgE and the 323/A3 IgE. Transfection of Sp2/0 cells with genes encoding the chimeric epsilon heavy chain and the chimeric light chain respectively, yielded transfectants that produced G250 or 323/A3 cIgE. The cIgEs bound to the rat basophilic RBP-7TZ cells, which were transfected with the α chain of the human Fc ϵ RI and expressed functional Fc ϵ receptors for human IgE.

The functional activity of the cIgEs was confirmed by their capacity to activate RBL-7TZ cells in the presence of specific antigen-expressing tumor cells, and by their capacity to activate human basophils when crosslinked by anti-IgE Ab. G250 cIgE mediated activation of RBL-

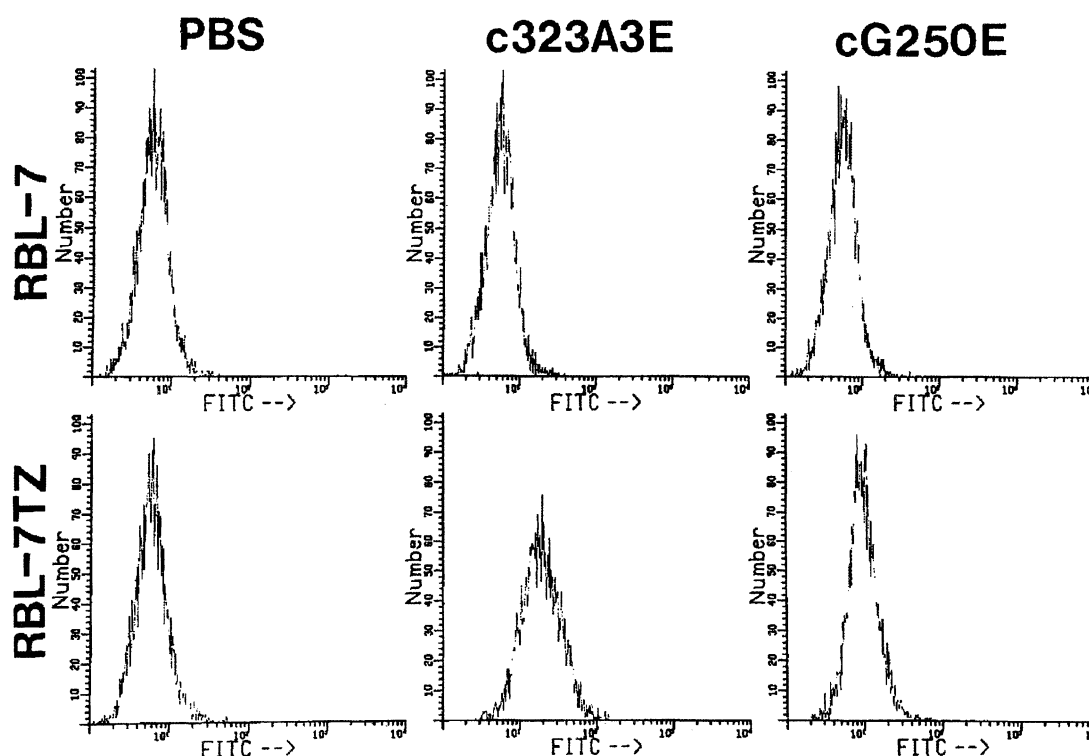


Figure 7: Binding of cIgE to FcεRI-positive cells. RBL-7 or RBL-7TZ cells were incubated with PBS (left) or with culture supernatant containing $19 \mu\text{g ml}^{-1}$ 323/A3 cIgE (middle) or $0.5 \mu\text{g ml}^{-1}$ G250 cIgE (right). Binding of cIgE was detected by incubation with rabbit anti-human IgE Fcε specific Ab and goat anti-rabbit IgG-FITC conjugate and flow cytometry

7TZ, as measured by the release of TNF- α , when cultured with the A704 RCC cell line, that expresses the G250 antigen. Likewise, coculturing RBL-7TZ with the B16C215 cell line that expresses Ep-CAM, in the presence of 323/A3 cIgE resulted in TNF- α release. A low concentration of cIgE ($0.1\text{--}0.2 \text{ ng ml}^{-1}$) was sufficient to trigger a significant TNF- α release. Even when the effector to tumor cell ratio was titrated down to 0.4, chimeric cIgE-mediated activation of the RBL-7TZ cells was observed. Human mononuclear cells enriched for basophils were used to investigate the capacity of our chimeric IgE to activate human FcεR expressing cells. Both G250 and 323/A3 cIgE activated these human basophils to release histamine when cell-bound cIgE was crosslinked by an anti-IgE Ab. In conclusion, the binding and activation experiments show that the transfections of cIgE heavy and light chains resulted in the production of structurally and functionally intact 323/A3 and G250 cIgE.

Immunoglobulin E is produced at low levels in humans, which complicates the isolation of pure IgE from serum samples. cIgE closely resembles human IgE, since the constant regions, that mediate the IgE function, are of human origin, and might therefore be very useful in studies of human IgE. Furthermore, both the cDNAs for the G250 antigen (Dr E. Oosterwijk, personal communication), and for the Ep-CAM molecule have been cloned^{37–39}, and are available for the generation of appropriate activation models. As was evident from our

activation assays, the expression of antigen on the membrane of cells can serve as a 'solid' phase antigen source for crosslinking of IgE bound to Fcε receptors on effector cells.

Preliminary data from the therapy studies with murine 323/A3 IgE in mice bearing syngenic B16C215 tumors suggest a therapeutic effect of 323/A3 IgE (Luiten *et al.*, manuscript in preparation), which supports the hypothesis that induction of a type I hypersensitivity reaction at the tumor site may be an effective therapy of carcinomas. The generation of the 323/A3 and G250 cIgEs, reactive with tumor-associated antigens, allows further evaluation of the potential of IgE in tumor therapy involving human effector cells.

Nomenclature

RCC	renal cell carcinoma
cIgE	chimeric immunoglobulin E
RBL-7TZ	RBL-7 cells transfected with human FcεRI α

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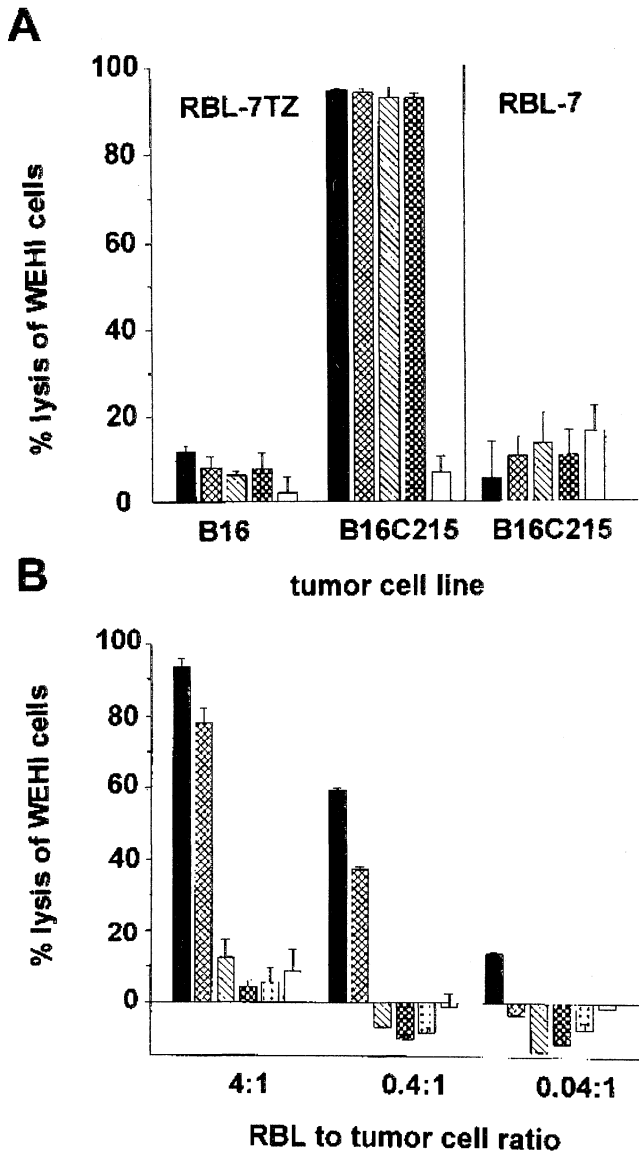


Figure 8: Activation of RBL-7TZ cells by 323/A3 clgE. **A:** Specificity of 323/A3 clgE-mediated activation B16 (left) or B16C215 cells (middle and right) were cultured overnight with a 4-fold excess of RBL-7TZ cells (left and middle) or RBL-7 cells (right) in the presence of 1000 (■), 100 (▨), 10 (▧), 1 (▩) or 0 (□) ng ml⁻¹ 323/A3 clgE. Supernatants of the cocultures were assayed in triplicate for TNF- α activity using the WEHI bioassay. **B:** Titration of RBL-7TZ cells with B16C215 cells in ratios of 4:1, 0.4:1 or 0.04:1 in the presence of 1 (■), 0.1 (▨), 0.01 (▧), 0.001 (▩), 0.0001 (▪) or 0 (□) ng ml⁻¹ 323/A3 clgE. Supernatants of the cocultures were assayed as in **A**

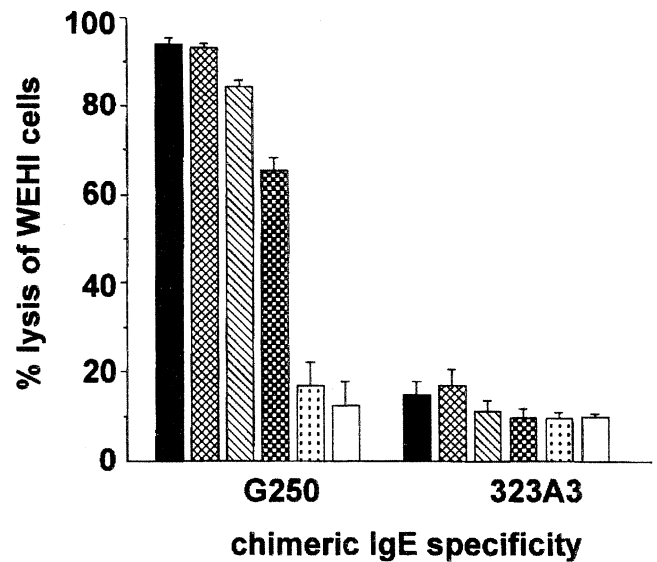


Figure 9: Activation of RBL-7TZ cells by G250 clgE. A704 cells were cultured with a 4-fold excess of RBL-7TZ cells in the presence of 200 (■), 20 (▨), 2 (▧), 0.2 (▩), 0.02 (▪) or 0 (□) ng ml⁻¹ G250 clgE (left) or 323/A3 clgE (right). Supernatants of the cocultures were assayed in triplicate for TNF- α activity using the WEHI bioassay

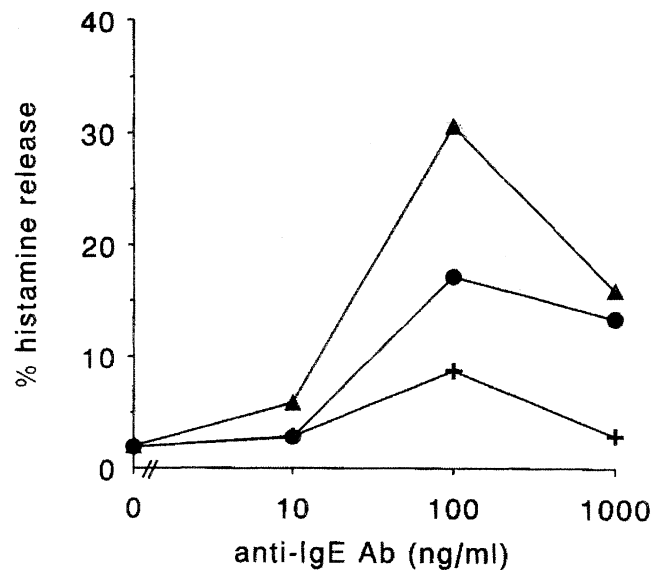


Figure 10: Activation of a freshly purified human basophil containing preparation by clgE. Human basophils were stripped from membrane-bound IgE, and incubated with 0.4 μ g ml⁻¹ 323/A3 clgE (●), G250 clgE (▲) or with buffer only (+), followed by an incubation with anti-human IgE ϵ chain Ab at 10, 100 or 1000 ng ml⁻¹. Histamine release is presented as a percentage of the maximal release

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