

## BETA-GLUCAN ENHANCED KILLING OF RENAL CELL CARCINOMA MICROMETASTASES BY MONOCLONAL ANTIBODY G250 DIRECTED COMPLEMENT ACTIVATION

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**Metastases from renal cell carcinomas (RCC) are resistant to radiation and chemotherapy but are relatively immunogenic. We have investigated the possibility to eliminate human RCC micrometastases using MAb G250. G250 penetrates human micrometastases completely in a spheroid model and induces complement deposition rapidly on the outmost cell layers. However, complement dependent cytotoxicity (CDC) was barely detected using either <sup>51</sup>chromium release assays or confocal microscopy, due to relatively low expression of the G250 antigen and the effect of membrane bound complement regulatory proteins. Addition of blocking anti-CD59 MAbs enhanced formation of C5b-9 and consequently complement mediated lysis (13%). Complement assisted cellular cytotoxicity (CACC) was not detectable, although the iC3b ligand and CR3 receptor were present on respectively target and effector cells. Addition of soluble  $\beta$ -glucan induced the killing of MAb and iC3b opsonized spheroids by effector cells (6–21%). Despite a lower affinity for G250 antigen, a bispecific anti-G250\*anti-CD55 MAb enhanced cell killing in spheroids comparable to the parental G250 MAb. Our results suggest that complement-activating G250 in combination with anti-mCRP MAbs is able to kill human RCC cells in micrometastasis *in vitro*. For CACC the presence of CR3-priming  $\beta$ -glucan seems to be obligatory. *In vivo*, bi-MAb may be more effective as therapeutic agent due to its increased C5a generating properties.**

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**Key words:** renal cell carcinoma; immunotherapy;  $\beta$ -glucan; complement activation

Renal cell carcinoma (RCC) is the seventh leading cause of cancer accounting for 3% of malignancies in men. Owing to the few symptoms in early stages and the slow progression of the disease, approximately one third of the patients present with extensive local spread or metastasis at time of diagnosis. At present, radical nephrectomy is the main therapy but for advanced disease, it is mainly palliative.<sup>1</sup> Unlike other urological malignancies, RCC is resistant to both chemo- and radio-therapy.<sup>2</sup> Various clinical observations suggest that the patients immune system plays a role in the course of renal cell cancer: spontaneous tumor regression,<sup>3</sup> long dormancy of metastasis, the presence of a T-cell-mediated immune response<sup>4</sup> and tumor responses in patients receiving cytokine therapy (IL-2 and IFN- $\alpha$ ).<sup>5</sup> Therefore, RCC is a logical candidate for monoclonal antibodies (MAb) based immunotherapy.<sup>6–8</sup>

G250 is one of the most extensively studied MAbs associated with RCC. The detected antigen is also known as carbonic anhydrase IX and its expression is absent in normal kidney.<sup>9,10</sup> More than 80% of primary and metastatic renal-cell tumor samples show homogeneous G250 antigen expression on cell membranes. G250 antigen is particularly high expressed on clear-cell RCC, which comprises 90% of all renal cell carcinoma.<sup>9</sup> Clinical trials using iodine-131 labeled G250 established the usefulness of this MAb for therapeutic imaging. G250 successfully targets both primary and metastatic RCC, including both bone and soft-tissue metastasis.<sup>11,12</sup> Quantitative analysis of tissue samples 1 week after administration revealed detectable levels of MAb in metastases.<sup>13</sup> Recently cG250, a chimeric version of MAb G250 in which the murine IgG Fc region is replaced by a human Fc region, has been tested for radio-immunotherapy of RCC in patients with metastatic

RCC. The results were promising and clinical phase II/III studies are now being performed.<sup>11,14</sup> Unlabeled cG250 antibodies alone or in combination with IL-2 have been shown to stimulate immune effector functions in patients with advanced renal cell carcinoma in early-phase clinical trials.<sup>15,16</sup> cG250 induces antibody dependent cell-mediated cytotoxicity (ADCC) against several RCC cell lines, especially in combination with interleukin-2.<sup>17,18</sup> However, in another study no complement mediated lysis was found after G250 treatment,<sup>19</sup> indicating a need for optimization of the conditions, preferably in a human model system.

In our study, the possibility of using the patient's own complement system to eliminate RCC micrometastases was investigated using MAb G250. For this purpose, multicellular RCC spheroids are used as a 3D model with a close resemblance to human small tumors or micrometastases *in vivo*. A human model is preferred because of the species selectivity of complement regulating proteins.<sup>20</sup> Spheroids have been shown to be more resistant against radiotherapy, chemotherapy<sup>21</sup> and antibody induced immunotherapy<sup>22</sup> than cells growing in monolayer cultures. In general, nucleated cells are protected against complement-mediated injury by membrane bound complement regulatory proteins (mCRP). RCC cells in suspension express sufficient amounts of mCRPs to prevent G250 induced complement-dependent cytotoxicity (CDC).<sup>23</sup> Therefore, in our system specific antibodies against mCRPs (CD46, CD55 and CD59) were used to block this protection. CD46 and CD55 interfere at the level of C3/C5 convertases, preventing iC3b deposition on the target cell and hence effector cell mediated cytotoxicity. CD59, on the other hand, binds tightly into the C5b-8 complex, preventing incorporation of C9 molecules, which is necessary for generation of the lytic membrane attack complex (MAC).<sup>23,24</sup> Recently, a model has been proposed concerning how phagocytes are able to eliminate complement opsonized tumor cells. According to this model, the efficiency of the complement system against tumor cells can be enhanced by using immune response modifiers such as  $\beta$ -glucan.<sup>25</sup> These molecules, generally present in the cell walls of plants, fungi and bacteria, bind to complement receptor 3 (CR3) and induce a change in configuration that favors binding to opsonized tumor cells, similar to the mechanism of binding/phagocytosis of bacteria.<sup>26</sup> In our study, the optimal conditions for G250-mediated elimination of RCC micrometastasis were investigated using blocking MAbs against mCRPs and different batches and concentrations of  $\beta$ -glucan. The performance of G250 as an immunotherapeutic antibody was evaluated

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in comparison with W6/32, a MAb against HLA-class I, known to be an efficient activator of the complement system.<sup>23</sup>

#### MATERIAL AND METHODS

##### *Antibodies, sera, cell lines and chemicals*

The following MAb were used: J4-48 (anti-CD46, IgG1, Sanquin, Amsterdam, The Netherlands), MBC1 (anti-CD55 IgG1; a gift from Prof. Dr. B.P. Morgan, Cardiff, United Kingdom), BRIC229 (anti-CD59, IgG2b, BPL Commercial Department, Elstree, UK), W6/32 (anti-HLA-class I, IgG2a, ATCC, Rockville, MD), G250 (anti-MN/CAIX antigen, IgG1 and IgG2a, a class switch variant, which is able to activate the complement system, were developed in our department; chimeric cG250 in which the murine IgG1 Fc region is replaced by a human, was a kind gift from Prof. Dr. S. Warnaar, Wilex, Munich, Germany); anti-iC3b and anti C5b-9 (both IgG2b) were from Quidel Corporation (San Diego, CA). anti C5b-9 (IgG, DAKO A/S, Glostrup, Denmark), anti-Fc $\gamma$ RIII (clone 3F8, Becton Dickinson, San Jose, CA), anti-CR3 (anti-CD11b, clone 2LPM19c, IgG1, Dako), anti- $\beta$ -glucan (IgG, Biosupplies, Parkville, Australia), anti-laminin, anti-collagen type IV MAbs and FITC-labeled rabbit-anti-mouse F(ab)<sub>2</sub> fragments-were from Dako. Bi-specific MAb directed against CD55 and respectively G250 and HLA-class I were generated in our department.<sup>27</sup> C6-depleted serum (Quidel) or normal human serum (NHS) from a healthy AB+ donor was used as complement source. The following human renal carcinoma cell lines were used: SK-RC-1, SK-RC-45 and SK-RC-52, purchased from the Memorial Sloan-Kettering Cancer Center, New York, NY.<sup>28</sup> Cells were cultured in RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM glutamine and 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin (ICN Biochemicals, Irvine, CA). Paraformaldehyde, glutaraldehyde, propidium iodide and bovine serum albumin (BSA) were from Sigma Chemical Co. (Zwijndrecht, The Netherlands).

##### *Spheroid generation*

Spheroids were cultured by a modified liquid overlay technique<sup>29</sup> using 96-well plates (Flat bottom, Greiner, Frickenhausen, Germany) coated with 0.6% agarose (w/v, Invitrogen) in RPMI 1640 at 37°C in humidified air with 5% CO<sub>2</sub>. Medium was renewed every 4th day. To increase reproducibility for chromium release experiments, culture conditions of 5 days were chosen to avoid necrosis in the center of the spheroids. Mean spheroid sizes were routinely recorded by measuring 2 orthogonal diameters of individual spheroids, quantified with an inverted microscope equipped with a calibrated reticule. Only regular formed spheroids of the same size were selected for experiments. Two representative spheroids from every plate were trypsinized to verify cell number and viability using trypan blue 0.4% solution (Sigma Chemical Co.).

##### *Immunofluorescence microscopy*

Spheroids were collected from the plates, washed with RPMI, embedded in Tissue-Tek OCT and snap-frozen in liquid nitrogen-cooled 2-methyl-butane and stored at -80°C until use. Cryostat sections of 5  $\mu$ m were mounted on glass slides (Starfrost, Burgdorf, Germany), dried o/n at 37°C, fixed in acetone for 10 min and stored at -20°C until further use. The sections were incubated with MAbs in appropriate dilutions in PBS with 1% BSA (PBS-BSA) for 1 hr in a moist chamber, washed in PBS-BSA, and incubated with FITC-conjugated rabbit anti-mouse IgG (Dako) diluted in PBS-BSA. The sections were counterstained with propidium iodide when applicable and mounted in Vectashield (Vector Laboratories, Burlingame, CA) or Mowiol.<sup>30</sup> Background immunofluorescence was assessed by using irrelevant isotype matched control antibodies or omitting the primary antibodies. A Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and He/Ne (543 nm) lasers and 25 $\times$  and 63 $\times$  objectives were used to obtain the images.

##### *Reflection contrast microscopy*

Spheroids were fixed with 1% paraformaldehyde/1.5% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide, embedded in Epon and ultrathin sectioned (90 nm) with a Leica Ultracut UCT ultramicrotome as described before.<sup>31</sup> The sections were stained with 1% toluidine blue and analyzed by reflection contrast microscopy using a Leica DM microscope equipped with a RCM module.

##### *Electron microscopy*

The Epon embedded material was also used for electron microscopy. Ultrathin sections were collected on copper grids as described before.<sup>31</sup> The sections were poststained with uranyl acetate and Reynold's lead citrate (both Sigma Chemical Co.) and were examined using a Philips CM-10 electron microscope operating at 60 kV. Images were digitalized using a flatbed scanner (Minolta Image Scan Multi).

##### *<sup>51</sup>Chromium release assay*

Lysis of spheroids was determined by a <sup>51</sup>chromium release assay, performed as described previously with minor modifications.<sup>32</sup> Five days old SK-RC-1 spheroids of 10,000 cells initially were individually labeled with 1  $\mu$ Ci in 100  $\mu$ l RPMI 1640 overnight at 37°C in counting tubes. After 3 washes with RPMI, the spheroids were counted for total radioactivity. Spheroids with radioactivity under or above 10% of the average amount were eliminated. Labeled spheroids were incubated with 50  $\mu$ l antibodies (12.5  $\mu$ g/ml) on ice for 4 hr and subsequently with 50  $\mu$ l of serum (1:10 final dilution in RPMI) at 37°C. The concentration of antibodies and serum was based on previous experiments with cell suspensions.<sup>23</sup> For complement dependent cytotoxicity experiments the spheroids were incubated for 4 hr or 24 hr at 37°C. Next, the spheroids were separated from the media by pipetting and washed 2 $\times$  with medium. The washes were collected with the medium. Radioactivity of spheroids and accompanying media were counted separately in a Compugamma CS counter (Pharmacia, Gaithersburg, MD) and the total radioactivity was calculated per spheroid. Spontaneous release of chromium was determined in untreated spheroids and subtracted. For CACC, the spheroids were labeled similarly. Donor blood derived granulocytes or vitamin D<sub>3</sub>/retinoic acid stimulated U937 cells were incubated with  $\beta$ -glucan for 15 min in RPMI medium supplemented to 2 mM Mg<sup>2+</sup>/Ca<sup>2+</sup>. 100,000 treated U937 cells were added to individual spheroids (calculated ratio 10:1) and the tubes were gently turned to stimulate contact.

##### *Isolation of effector cells*

Granulocytes were extracted from the ficoll-pellet of a buffy coat (Sanquin Blood Bank, Leiden, The Netherlands) by Dextran separation. Erythrocytes were hypotonically lysed with lysis buffer (Pharmacy of the LUMC, Leiden, The Netherlands) and diluted in RPMI medium containing 2 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>.

##### *Stimulation of pro-monocytic cells*

U937 or THP-1 cells were stimulated with 100 nM retinoic acid and 100 nM vitamin D<sub>3</sub> (both from Sigma Chemical Co.) for 3 days to induce macrophage-like properties, including enhanced CR3 expression.<sup>33,34</sup>

##### *TNF- $\alpha$ ELISA*

The TNF- $\alpha$  ELISA was performed with antibodies and standards from a human TNF- $\alpha$  ELISA development kit (PeproTech EC, London, UK) according to the manufacturers recommended procedures (range 0.001–2 ng/ml). All samples were measured in duplicates.

##### *Flow cytometry*

Flow cytometric analysis on single cells was performed as described previously.<sup>35</sup> In brief, cells (2.5  $\times$  10<sup>5</sup>) were incubated with a 100  $\mu$ l mixture of primary antibodies diluted in PBS/0.5% BSA (30 min, 4°C). Cells were washed twice with PBS/0.5% BSA and incubated with 100  $\mu$ l of a mixture of secondary antibodies

(30 min, 4°C). After washing, the cells were resuspended in 250 µl of PBS/0.5% BSA containing 1 µg/ml propidium iodide (PI) to stain dead cells. Samples were measured with a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA). Ten thousand events of PI-negative cells were counted. Data are expressed in MESF values. MESF values were calculated on the basis of a flow cytometry standardization kit (Quantum 25 FITC; Flow Cytometry Standards Europe, Leiden, The Netherlands).

#### Generation of soluble $\beta$ -glucans

Batches of  $\beta$ -glucan isolated from *S. cerevisiae* were respectively Auxoferm YGT plus (kindly provided by Deutsche Hefewerke GmbH, Hamburg, Germany), Imucell WGP (Biopolymer Engineering, Minnesota) and Natural Soluble Glucan (NSG, kindly provided by R. Hansen and G. Ross).  $\beta$ -glucan from *P. ostreatum*, Seaweed (Laminarin) and Barley were from Sigma. Insoluble  $\beta$ -glucans were solubilized using different methods: sonification for 15 min to 24 hr, sonification in DMSO,<sup>36</sup> sulfation<sup>37</sup> and NaOH/HCl treatment.<sup>38</sup> Finally, all batches were dissolved in PBS.  $\beta$ -glucan from barley and seaweed and a preparation of  $\beta$ -glucan consisting of equal amounts (w/w) of 4 different batches of dextran (15–20,000, 40,000, 70,000 and 200,000 from Fluka, Buchs, Switzerland) were boiled in PBS for 10 min. The latter mixture was used as control. The carbohydrate content of all  $\beta$ -glucan batches were normalized by measurement of their concentration with the phenol-sulfuric acid colorimetric method.<sup>39</sup> In brief, respectively, 15 µl phenol 5% and 120 µl concentrated sulphuric acid were added to 20 µl sample diluted in water in a 96-well plate. After shaking and incubation for 10 min, the plates were read at 492 nm. The  $\beta$ -glucan preparations were added for 24 or 48 hr to myeloid cell lines (U937 or THP-1) that were previously stimulated with retinoic acid and vitamin D<sub>3</sub>.

#### Statistical analysis

Data are given as group means  $\pm$  standard deviation. Differences in group means were tested for significance using Student's *t*-test, considering  $p < 0.05$  significant.

## RESULTS

#### Model validation

**Spheroid formation.** RCC cell lines SK-RC-1, SK-RC-45 and SK-RC-52 formed regular round-shaped spheroids after 2–3 days of culture on agarose coated tissue culture plates (Fig. 1a). The spheroids were resistant to mechanical disruption and needed extensive trypsinization and mechanical pipetting to be separated into single cells. No substantial necrosis/apoptosis was found in the center of spheroids after 7 days of culturing, as determined by propidium iodide staining. Trypan blue staining of trypsinized spheroids showed that more than 95% of the cells were viable under these conditions. After 5 days of growth, the spheroids were regularly shaped, with diameters of  $778 \pm 68 \times 782 \pm 67$  µm for SK-RC-1 cells ( $p < 0.0001$ ,  $n=96$ , paired *t*-test). To obtain uniformity, only regular formed spheroids, consisting of approximately 10,000 cells, were initially selected after 5 days of culturing and the smallest spheroids (10%) and largest spheroids (10%) were discarded. All cell lines formed extracellular matrix components in spheroid culture similar to what is found in RCC tumors *in vivo* (Fig. 1b,c).<sup>40</sup> Figure 1d–f shows circumferential membranous staining for G250 on spheroids from all cell lines except SK-RC-45, which is known not to express G250.<sup>23</sup> Cell line SK-RC-1 was selected for further study in our model because of the intermediate level of G250 expression. mCRPs CD46, CD55 and CD59 are present on the cell membranes and the staining is homogeneous throughout the spheroids (Fig. 1g–i). The intensity of staining of mCRPs on SK-RC-1 cells was CD59 > CD46 > CD55, comparable with previous results from flow cytometric analyses of single cells.<sup>23</sup> The intensity of G250 staining was higher than for CD46 and CD55 but was lower than for CD59 and HLA-class I (Fig. 1j–l).

**Antibody penetration.** Monoclonal antibodies against G250 [G250 (IgG1), G250 (IgG2a) and cG250], CD55, CD59 and HLA-class I were tested for their ability to bind to the cells of spheroids and their ability to penetrate the spheroids in culture. Although all antibodies were able to penetrate the spheroids, significant differences were noticed. All G250 types and anti-HLA-class I were able to penetrate the spheroids completely within 24 hr (Fig. 2a), whereas the anti-CD55 and anti-CD59 antibodies needed up to 48 hr to completely penetrate the spheroids. Differences in diffusion speed of the antibodies were to be expected considering the variation in affinity. Antibodies with higher affinity are known to diffuse more slowly.

**Complement deposition.** Deposition of complement on SK-RC-1 spheroids was analyzed after incubation with G250 (IgG2a) MAb for 4 hr, followed by incubation with human serum as source of complement (24 hr). iC3b and C5b-9 deposits were present on the outer layers of the spheroids already after 30 min of incubation at 37°C. Prolongation of serum incubation resulted in more intense staining of iC3b on the peripheral cells of the spheroid, accompanied by a patchy staining in the center of the spheroid, whereas the C5b-9 deposits stayed confined to the peripheral cell layers (Fig. 2b). Similar incubations using spheroids from SK-RC-45 cells, which lack G250 expression, did not show complement depositions.

#### Complement dependent cytotoxicity (CDC) of spheroids induced by G250 (IgG2a)

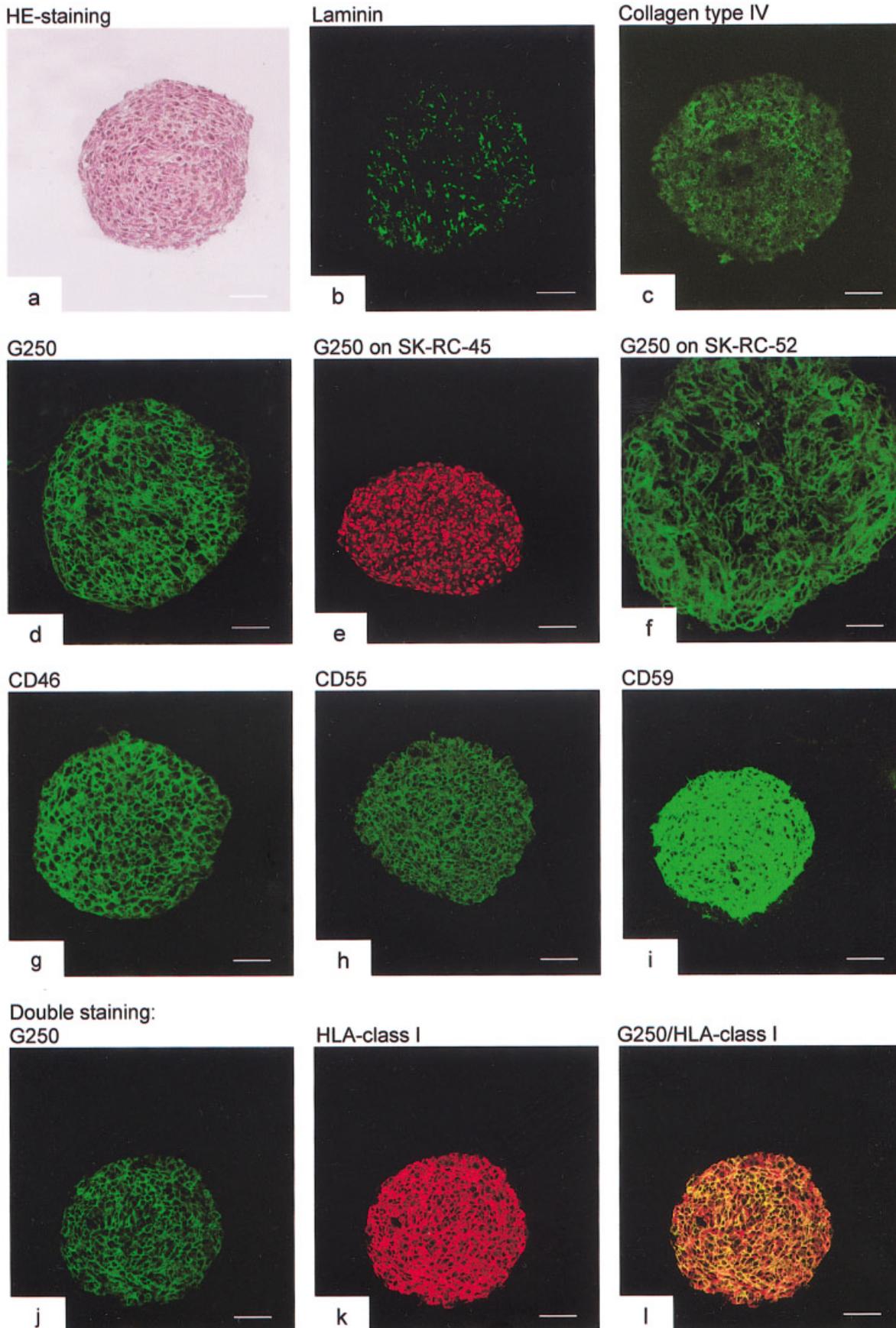
CDC of SK-RC-1 renal carcinoma spheroids was measured using <sup>51</sup>chromium release assays. Incubation of individual spheroids with only MAb G250 (IgG2a) followed by NHS for 4 to 24 hr did not result in significant more lysis of spheroids compared with incubation with NHS only (Fig. 3, mean of 5 experiments, using at least 4 individual spheroids per condition per experiment). The same result was observed for anti-CD46 (J4-48), anti-CD55 MAb (MBC1) or anti-CD59 MAb (Bric229). Combining G250 (IgG2a) and anti-CD59 antibody lead to significant more lysis, whereas combinations of G250 (IgG2a) with anti-CD46 or anti-CD55 MAb did not. G250 (IgG1), alone or in combination with anti-CD59 did not induce cell killing. The anti-G250 (IgG2a)/anti-CD59/NHS mediated lysis of RCC spheroids was less effective than lysis of RCC cells in suspension (14 vs. 21% specific lysis, respectively). The effect of anti-HLA-class I MAb in combination with anti-CD59 is also shown in Figure 3 for comparison. The morphologic changes of complement dependent lysis on spheroids using anti-G250/anti-CD59/NHS and HLA-class I/anti-CD59/NHS for 24 hr are shown in Figure 4. Lysis of cells is visible throughout the spheroids.

#### Role of antibody dependent cellular cytotoxicity (ADCC) and complement assisted cellular cytotoxicity (CACCC) in lysis of spheroids

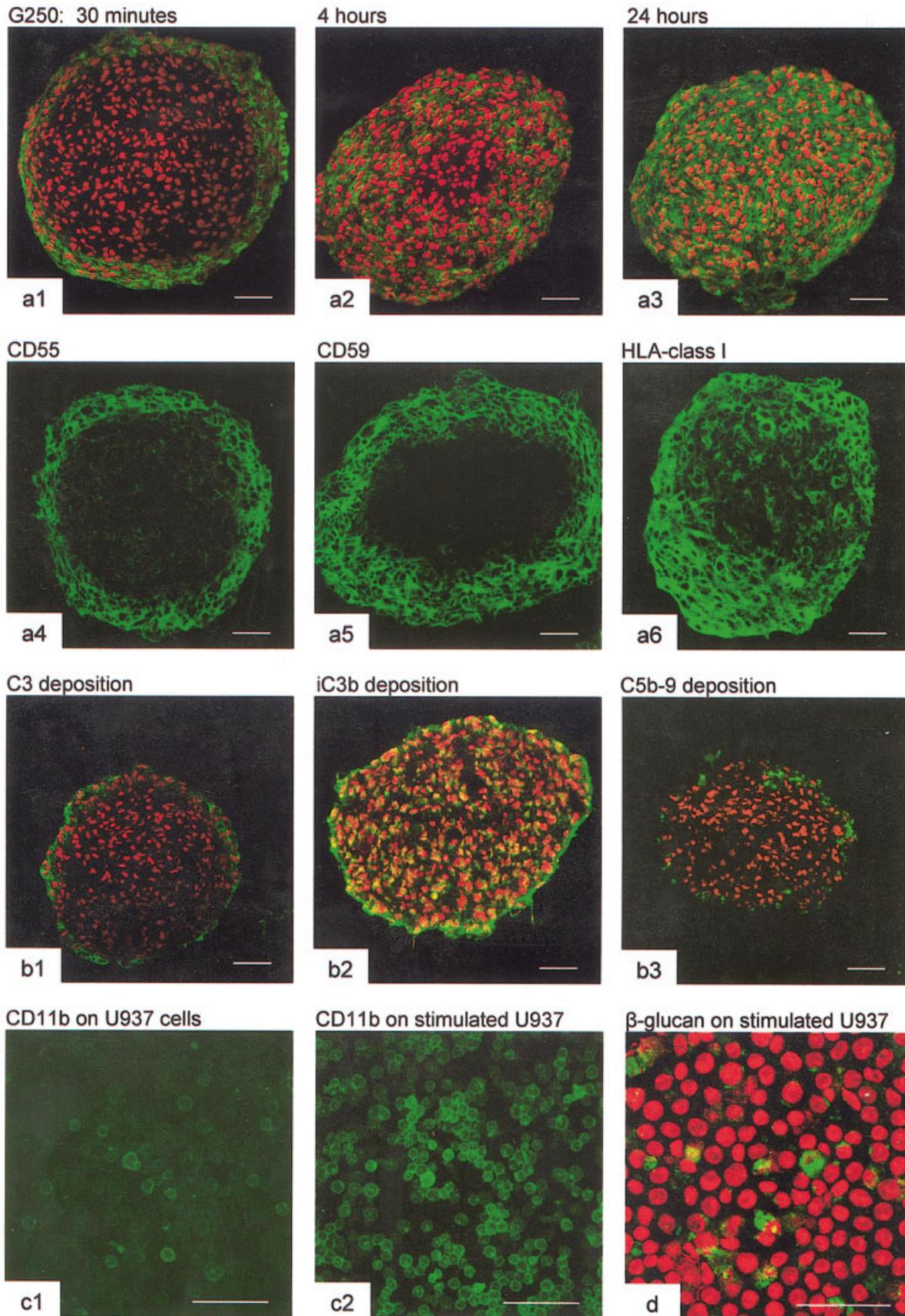
RCC spheroids (SK-RC-1 and SK-RC-52) or RCC cells in suspension were treated with different combinations of MAbs and serum. Especially the combination of G250 with anti-CD55 was effective in generation of C3/iC3b deposits on the target cells in the presence of serum. However, the addition of granulocytes to opsonized spheroids or single cells did not result in significant enhanced <sup>51</sup>chromium release compared to the addition of effector cells alone (<sup>51</sup>chromium release data not shown, morphological alteration shown in Fig. 4d). Also the anti-HLA-class I MAb in combination with anti-CD55 and anti-CD59 showed no effect on lysis.

#### Testing of bio-activity of different batches of $\beta$ -glucan

According to Ross and colleagues,<sup>25</sup> the efficiency of elimination of opsonized tumor cells by phagocytic cells can be enhanced by certain forms of  $\beta$ -glucan. For this purpose different batches of soluble  $\beta$ -glucan, prepared from various sources and using different solubilization methods were tested for their activity in a TNF- $\alpha$  ELISA and <sup>51</sup>chromium release assays. Enhanced cytokine secre-



**FIGURE 1** – Characteristics of renal cell carcinoma SK-RC-1. (a) Haematoxylin-Eosin-staining (b) Immunofluorescence (IF) staining of laminin, (c) IF staining of collagen type IV, (d) IF staining of G250, (e) IF staining of G250 on SK-RC-45, (f) IF staining of G250 on SK-RC-52, (g) IF staining of CD46, (h) IF staining of CD55, (i) IF staining of CD59 and (j,k,l) IF double staining of G250 and HLA-class I. Spheroids were frozen, sectioned and incubated with primary antibodies (b–l), followed by staining with FITC-conjugated secondary antibodies unless otherwise indicated. For intensity comparison, IF pictures were taken using identical settings. Scale bars = 100  $\mu$ m.



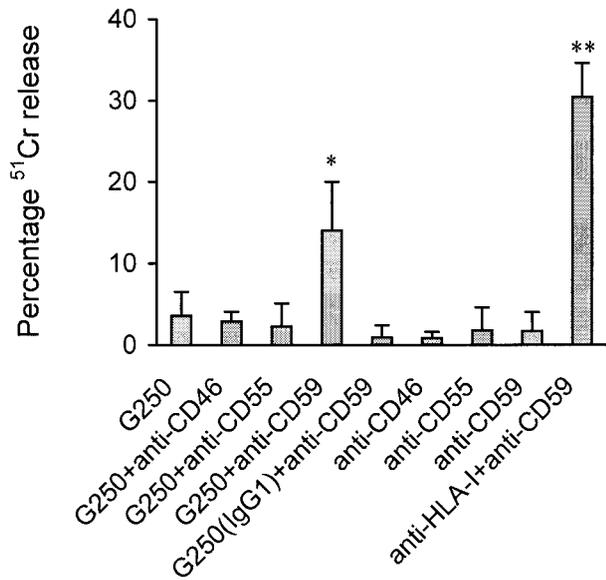
**FIGURE 2** – (a) Diffusion of MAbs anti-G250, anti-CD55, anti-CD59 and anti-HLA-class I MAb into renal carcinoma cell spheroids. MAbs were demonstrated on cryostat sections of SK-RC-1 spheroids that were incubated for different time intervals, using confocal microscope imaging. (a1–3) anti-G250 for, respectively, 30 min, 4hr and 24 hr. (a4–6), respectively, anti-CD55, anti-CD59 and anti-HLA-class I for 8 hr. (b) Immunofluorescence staining of C3, iC3b and C5-9 deposition on SK-RC-1 renal carcinoma cells grown as multicellular spheroids and incubated with G250 (4 hr on ice) and human serum (24 hr, 37°C). (b1) C3, (b2) iC3b and (b3) C5b-9. (c) CR3 expression on U937 cells with and without treatment of vitamin D<sub>3</sub> and retinoic acid. (d) Staining of barley β-glucan on stimulated U937 cells. The nuclei of cells were stained with propidium iodide (red) for a1–3. Scale bars = 100 μm.

tion (TNF- $\alpha$  and IL-6) by macrophages has been described to occur in parallel with increase in CR3 expression.<sup>25,26</sup> Therefore all  $\beta$ -glucan batches were prescreened by measuring the presence of TNF- $\alpha$  in the supernatant of macrophage like cells (U937 or THP-1 cells pretreated with vitamin D<sub>3</sub> and retinoic acid) exposed to different concentrations of  $\beta$ -glucan with an ELISA. In addition, enhanced CR3 expression of the macrophage-like cells was confirmed by flow cytometric analysis (MESF 10.9 vs. 41.0 and 9.9 vs. 21.4 for U937 and THP-1, respectively) and confocal laser scanning microscopy (Fig. 2c1–2). The different methods of solubilization did not show a consistent best method. In general, sulfation<sup>37</sup> gave the best results with respect to TNF- $\alpha$  release, but for some batches, sonification for 2 hr was equally efficient. Based on the results of the TNF- $\alpha$  ELISA (Fig. 5a), barley  $\beta$ -glucan was chosen for further study and laminarin was chosen as a reference. To determine the optimal concentration and conditions for com-

plement assisted cellular cytotoxicity, these 2 preparations were subsequently tested in <sup>51</sup>chromium release assays using W6/32 opsonized SK-RC-1 spheroids as targets (Fig. 5b). Because of the more reproducible CR3 levels, U937 cells pretreated with vitamin D<sub>3</sub>/retinoic acid were chosen as effector cells instead of freshly isolated granulocytes. In these experiments, C6-depleted serum was used to prevent direct complement dependent cell lysis. From these experiments 10  $\mu$ g/ml barley  $\beta$ -glucan was chosen as optimal concentration for further experiments. The presence of barley  $\beta$ -glucan on U937 cells was low but detectable as confirmed by flow cytometry and confocal laser microscopy (data not shown, Fig. 2i).

*Phagocytes mediated lysis of spheroids*

Figure 6 shows that G250 MAb alone or in the presence of C6-depleted serum does not induce U937-mediated lysis. Addition of anti-CD55 enhances the lysis to 20% for G250 and to more than 30% for anti-HLA-class I MAb. Omitting  $\beta$ -glucan results in no lysis for all antibody combinations tested. To investigate whether complement deposition can be enhanced by blocking CD55, a bispecific antibody (anti-G250\*anti-CD55) was used. As shown in Figure 6, use of this bi-Mab resulted in enhanced lysis compared to the parental G250 MAb.



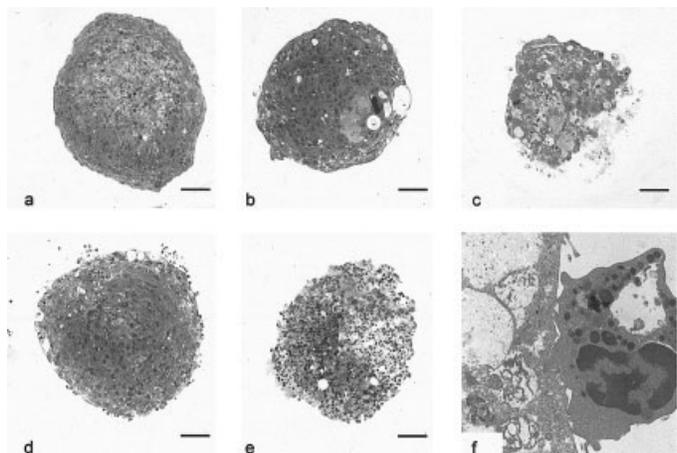
**FIGURE 3** – Effect of anti-G250, anti-CD46, anti-CD59 and anti-CD55, alone or in combination on the complement mediated lysis of SK-RC-1 spheroids. Lysis was measured using a <sup>51</sup>chromium release assay after 24 hr. The indicated percentage lysis is compared to the presence of NHS only. The values are the mean out of 5 experiments, using at least 4 individual spheroids per condition per experiment, except for anti-CD46 (1 experiment and 4 spheroids). The combination of anti-HLA-class-I MAb with anti-CD59 is shown as positive control. Significant differences vs. negative control: \* $p=0.02$ , \*\* $p < 0.0001$ .

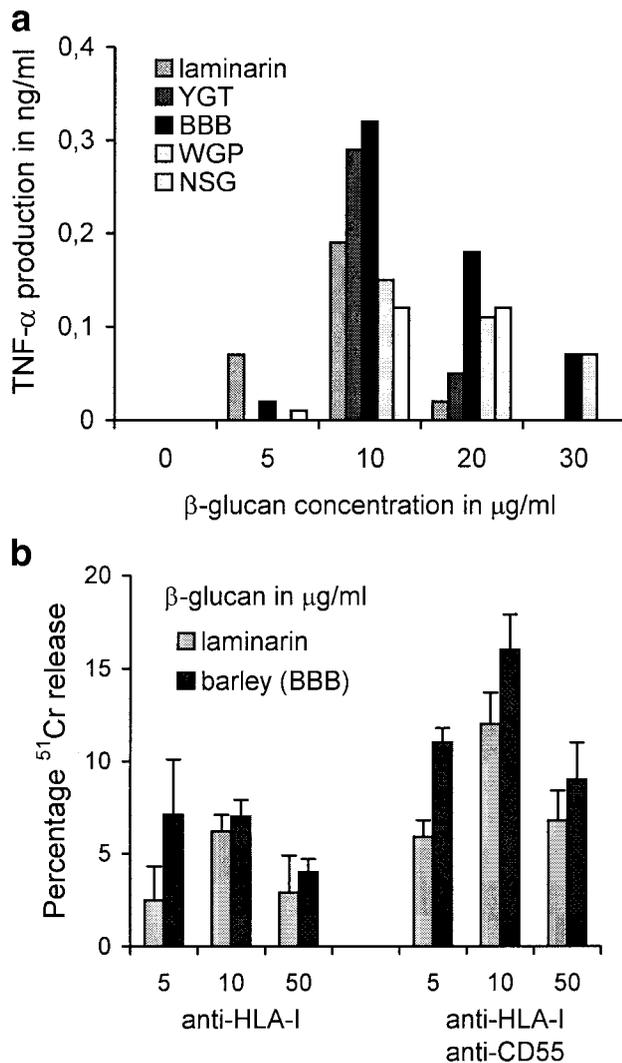
DISCUSSION

The management of metastatic renal cancer remains a therapeutic challenge. Because RCC are resistant against radio- and chemotherapy,<sup>1</sup> attention has been focused on immunotherapy. Despite promising laboratory results, so far immunotherapy of solid tumors with cytokines (e.g., IL-2 or TNF- $\alpha$ ) has met little success with responses in only 15–20% of the patients.<sup>41</sup> Attempts to combine immuno- and chemo-therapy are associated with enhanced systemic toxicity but did not improve survival.<sup>41</sup> A more direct approach to stimulate the immune system is to administer MAb against cell-surface proteins specifically found on tumor cells. Antibodies can mediate tumor cell destruction by antibody-dependent cellular cytotoxicity (ADCC) *via* the Fc portion<sup>42</sup> and, depending on their isotype, are also potent activators of the complement system.<sup>43</sup> In our study, we investigated the role of the complement system in the destruction of RCC micrometastasis using complement activating mouse IgG2a MAb.<sup>43</sup>

The multicellular spheroid model of RCC cells resembles closely the situation *in vivo*. The cells are densely packed and enveloped by the same extracellular matrix proteins that are abundantly present in small tumors and micrometastases *in vivo*.<sup>40</sup> These spheroids, treated with MAb G250 (IgG2a) in the presence of serum, were resistant against CDC. This phenomenon was already shown in earlier experiments using RCC suspensions.<sup>23</sup> Resistance to lysis was partly overcome by adding blocking anti-

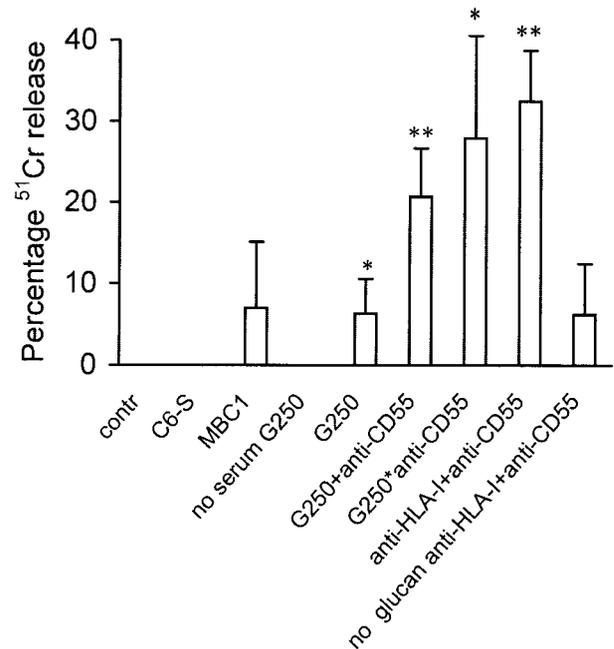
**FIGURE 4** – Morphological changes in SK-RC-1 spheroids after MAb and complement treatment. Multicellular spheroids were treated with NHS and MAbs for 24 hr and fixed in paraformaldehyde. (a) no treatment, (b) anti-G250+anti-CD59, (c) anti HLA-class I + anti-CD59, (d) anti-HLA-class I + anti-CD-55 plus granulocytes, (e) idem with  $\beta$ -glucan treated granulocytes and (f)  $\beta$ -glucan treated granulocyte attached to SK-RC-1 renal spheroid (electron microscopy  $\times 8,900$ ). Scale bars = 100  $\mu$ m.





**FIGURE 5** – Activity tests of different batches of solubilized  $\beta$ -glucan. (a) TNF- $\alpha$  excretion of vitamin D<sub>3</sub>/retinoic acid-stimulated U937 cells treated with different batches of  $\beta$ -glucan for 24 hr as determined by ELISA. For every source of  $\beta$ -glucan different solubilization methods were used and they were preselected for TNF- $\alpha$  excretion. The different batches used in this assay were preselected for highest TNF- $\alpha$  inducing activity. Values are the means of duplicates. BBB: boiled Barley beta-glucan. (b) Cytotoxicity enhancing effect of  $\beta$ -glucans using  $^{51}$ chromium release assays, SK-RC-1 renal cell spheroids, anti-HLA-class I complement inducing antibody, C6-depleted serum and with or without anti-CD55. All values are expressed against the control conditions including  $\beta$ -glucan, C6-depleted serum and anti-HLA-class I MAb.

mCRP MAb against CD59, while anti-CD46 and anti-CD55 were less efficient. The inefficiency of the latter MAbs to block lysis was already established in experiments using cell suspensions of different carcinoma cell lines.<sup>44,45</sup> Anti-CD55 and, to a lesser extent, anti-CD46 enhance C3 deposition on the target cells and increase the amount of released C5a, stimulating a local inflammatory reaction. The relatively poor effect of these antibodies on pore formation is probably caused by the much higher expression of CD59 on most (tumor) cell types.<sup>23,44,45</sup> Although addition of MAb against CD59 lead to measurable lysis of tumor cells after 24 hr of incubation, cell killing was decreased compared to what was found using single cells. This phenomenon has also been shown for spheroids of other tumor types<sup>22,32</sup> and can only partly be explained by the difference in accessibility of the antibodies to the



**FIGURE 6** – Complement assisted cellular cytotoxicity of SK-RC-1 renal cell spheroids treated with complement stimulating G250 (IgG2a) MAb in combination with anti-CD55 and C6-depleted serum, using stimulated U937 cells primed with 10  $\mu$ g/ml barley  $\beta$ -glucan as effector cells. The data are obtained with a  $^{51}$ chromium release assay counting the spheroids after extensive washing. They are expressed as the amount of  $^{51}$ Cr in spheroids to which only effector cells were added subtracted with the amount in treated spheroids ( $n=5$ ). Data for anti-HLA-class I MAb are added for comparison. Significant differences vs. negative control: \* $p \leq 0.03$ , \*\* $p \leq 0.001$ .

cells in the spheroids because G250 penetrated spheroids completely within 8 hr. The microenvironment within the spheroids, for instance the presence of extracellular matrix components such as laminin and collagen IV, and the different expression of integrins and growth factors<sup>46</sup> will presumably also play a role. According to the “binding site barrier model”, diffusion of antibodies into spheroids depends on the affinity and concentration of antibody<sup>47</sup> and high affinity antibodies show retarded diffusion. Our antibody penetration experiments show the presence of MAb G250 throughout the whole spheroid within 8 hr, which was similar to (anti-HLA-class I), or faster than other MAbs (anti-CD46, anti-CD55, anti-CD59). A barrier for diffusion of complement components with a high molecular weight may be more important to explain the diminished susceptibility, as shown in the distribution patterns of C5b-9 on spheroids. Also other mechanisms such as a different pattern of mCRP expression compared to single cells might be involved. Although the combination anti-G250/anti-CD59 was able to induce cell killing, anti-HLA class I MAb (W6/32), with similar spheroid penetration characteristics, was 2 times more efficient in inducing complement mediated cell death under the same conditions. HLA-class I is 2–3 times more abundant on SK-RC-1 cells than G250.<sup>23</sup> Previous experiments on RCC cell suspensions indicated that a threshold level of antigen expression is required to trigger complement activation.<sup>23</sup> Moreover, if C5b-9 complement complex formation is limited, nucleated cells are able to escape cell death by endocytosis.<sup>48</sup> Our results show that, in principle, it is possible to mediate substantial lysis of RCC micrometastases using antibody induced complement activation but that the efficiency of cell killing is largely depending on the abundance of the antigen. Although the expression of antigen is found to be enough for G250 imaging of micrometastases of 8 mm *in vivo*, the distribution is heterogeneous and a statistical correlation between cG250 binding and antigen expression was

found.<sup>49,50</sup> In our model system, using SK-RC-1 cells with intermediate G250 expression levels, it seems to be too low to induce direct complement mediated lysis. Experiments using cells with (artificially) enhanced G250 expression would presumably increase lysis but would also remove our model system from the *in vivo* situation.

Osmotic lysis due to pore formation (CDC), as induced by the MAb combination G250/anti-CD59, might not be the principal effector mechanism of complement *in vivo*. Pore formation does not increase C5a release or C3 deposition, whereas the MAb combination G250/anti-CD55 does. The generation of anaphylatoxin C5a will induce or enhance an inflammatory reaction at the tumor site and recruit effector cells, which in principle could attack opsonized cells *via* CR3. Therefore our CDC model underestimates the effectiveness of MAb immunotherapy. Because previous studies had shown that addition of anti-CD55 was most effective in enhancing the G250-stimulated deposition of C3 on target cells,<sup>23,27,44</sup> we focused on the combination G250 and anti-CD55 in association with effector cells, using conditions in which CDC does not occur (C6-depleted serum). Although complement mediated elimination of gram-positive bacteria and yeasts by iC3b-receptor expressing phagocytes and NK-cells is an efficient process *in vivo*,<sup>25</sup> we did not find an additional effect of granulocytes or macrophage-like cells on the killing of opsonized RCC spheroids. Deposition of iC3b on the cell surface of tumor cells apparently does not promote phagocytosis in a similar way as for the elimination of microbes and yeasts.<sup>24</sup> It has been suggested that this is caused by an inactive state of CR3, the most important receptor for iC3b on phagocytes.<sup>26</sup> CR3 activation apparently requires the engagement of 2 sites on its  $\alpha$ -subunit (CD11b). The iC3b-binding site located at the N-terminus and a lectin site at the C-terminus, which binds to carbohydrate structures present in the cell walls of microbes and yeasts. Addition of soluble  $\beta$ -glucan, isolated from yeasts, mushrooms or plants, to phagocytic cells seems to function as a signal necessary to prime CR3 for binding of iC3b on opsonized tumor cells. Although the mechanisms are still largely unknown, the immunomodulatory role for  $\beta$ -glucan has been documented for over 40 years. Recent experiments in which  $\beta$ -glucan, alone or in combination with chemotherapy or MAbs, was used as cancer therapy showed promising results.<sup>51–54</sup> Orally administered yeast  $\beta$ -glucan inhibited the growth of mouse colon tumors in mice and pulmonary metastasis of Lewis lung carcinoma, presumably by enhancing the production of IL-2, IFN- $\gamma$  and TNF- $\alpha$  by macrophages.<sup>51,55</sup> Anti-tumor antibodies in

combination with soluble  $\beta$ -glucan therapy of mice implanted with mouse mammary tumors showed more than 57% reduction in tumor weight, whereas therapy failed in C3 or CR3 deficient mice.<sup>54</sup> In another study, oral  $\beta$ -glucan isolated from barley, enhanced the anti-tumor effects of different MAbs against human neuroblastoma, melanoma, lymphoma and breast carcinoma xenografts in mice irrespective of antigen or tumor sites.<sup>52,53</sup> It should be noted that in the latter study, the positive therapeutic effect is probably overestimated due to the species selectivity of mCRP, resulting in increased C3b and C5a generation in xenogeneic models. Our study, using a human model system, shows that similar results might be obtained in RCC carcinoma patients, provided that suitable MAbs are used and that mCRPs are inhibited at the same time. While  $\beta$ -glucans are not used by Western oncologists, in Asian countries  $\beta$ -glucan containing mushroom preparations are used clinically to treat cancer with varying results, but mostly under conditions with insufficient clinical validation.  $\beta$ -glucan consists solely of D-glucose and carries a Generally Regarded As Safe (GRAS) rating from the American Food and Drug Administration (FDA), which means there is no known toxicity for an oral dose of the purified form.

The next step should be to increase the effect of G250. In our model, blocking of mCRPs is required for effective complement induced lysis. The anti-mCRP MAbs used in this study were less well penetrating the spheroids than G250, hence probably limiting the cell killing effects. Therefore, bispecific MAbs with a high affinity arm directed against the tumor antigen and a low affinity arm against CD55 might be more potent for MAb immunotherapy. The affinity for the tumor is decreased compared to the bivalent parental antibody, thus enhancing its penetration properties. Moreover, the simultaneous blocking of CD55 provides increased levels of C5a, to attract phagocytic cells, and C3b, to enable these effector cells to bind the tumor cells *via* CR3. Our results using anti-G250/anti-CD55 bispecific MAb showed enhanced lysis compared to the parental G250 MAb, indicating the possibilities of this system and the need for further investigation.

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