THE ROLE OF MANNOSE-BINDING RECEPTOR IN THE INNATE IMMUNITY OF HUMAN EPIDERMAL KERATINOCYTES

PhD Dissertation

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PUBLICATIONS

I. Gy. Szolnoky, Zs. Bata-Csörgő, A. Sz. Kenderessy, M Kiss, A. Pivarcsi, Z. Novák, K. Nagy Newman, G. Michel, T. Ruzicka, L. Maródi, A. Dobozy, L. Kemény: A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans. *J. Invest. Dermatol.* 2001 (accepted for publication)

II. A. Dobozy, **Gy. Szolnoky**, R. Gyulai, A. Kenderessy Szabó, L. Maródi, L. Kemény: Mannose receptors are implicated in the Candida albicans killing activity of epidermal cells. *Acta Microbiol. Immunol. Hung.* 43: 93-95, 1996

III. L. Kemény, **Gy. Szolnoky**, A. Kenderessy Szabó, R. Gyulai, M. Kiss, G. Michel, K. Nagy-Newman, T. Ruzicka, A. Dobozy: Identification of a soluble interleukin-8 inhibitor in the supernatant of polymorphonuclear leukocytes. *Immunol Lett* 64: 23-29, 1998.

IV. K Nagy, I Szőke, I Sonkodi, E Nagy, A Mari, **Gy Szolnoky**, HN Newman: Inhibition of microflora associated with oral malignancy. *Oral Oncol* 36: 32-36, 2000.

V. Kemény Lajos, Kenderessy Szabó Anna, Kiss Mária, **Szolnoky Győző**, Gyulai Rolland, Farkas Árpád, Szabad Gábor, Günter Michel, Thomas Ruzicka: Interleukin-8 inhibitor izolálása polimorfonukleáris leukociták felülúszójából. *Bőrgy. Vener. Szle.* 75, 107-111, 1999.

VI. Szolnoky Győző, Kemény Lajos, Kenderessy Szabó Anna, Kiss Mária, Bata Zsuzsanna, Dobozy Attila: A mannóz kötő receptorok szerepe a humán keratinociták Candida albicans killing aktivitásában.. *Bőrgyógy. Vener. Szle.* 75: 151-154, 1999.

VII. Szolnoky Győző, Kemény Lajos, Kenderessy Szabó Anna, Kiss Mária, Bata Zsuzsanna, Dobozy Attila: A humán keratinocita mannóz kötő receptorok jellemzése. *Bőrgyógy. Vener. Szle.* 76: 317-320, 2000.

1. INTRODUCTION

The epidermis, the outermost skin layer constitutes a major barrier to the enviroment and provides the first line of defense against microorganisms. In the human epidermis keratinocytes represent a physical and biological protective barrier against external pathogens. Additionally, keratinocytes play an important role in inflammatory and immunologic responses of the human body. In both experimental and naturally occurring cutaneous *Candida albicans* infections, the infecting *Candida albicans* organisms are confined to the upper portion of the epidermis (31, 49, 53). Deep or systemic *Candida* infections are rare in patients with chronic mucocutaneous candidiasis, despite extensive superficial infections (28, 41). The formation of neutrophilic infiltrates in the epidermis and epidermal hyperproliferation are characteristic cutaneous changes in *Candida albicans* infections of the skin. Each of these cutaneous responses has been suggested to be important for the defense against superficial fungal infections (52, 54). Although polymorphonuclear (PMN) leukocytes easily phagocytize *Candida albicans* in vitro, ultrastructural examination of *Candida*-infected skin failed to show fungal elements in regions of microabscesses (49). On the other hand, in that same study, the majority of the fungal cells were found among epithelial cells of the stratum corneum and they could not be detected in noncornified cells of the malphigian layer. In a more recent investigation using cyclophosphamide-treated mice it was shown that even in the absence of epidermal neutrophil infiltrates *Candida albicans* is confined to the epidermis. Removal of the epidermis by scraping prior to inoculation with the yeast, however, resulted in *Candida* pseudohyphae invasion of the dermis, indicating that suprabasal keratinocytes may play a role in the defense against cutaneous *Candida* infection (19). Vaginal epithelial cells of both rodents and macaques have recently been shown to be predominantly responsible for innate immunity against yeast cell colonization (57, 58).

In previous work it has been shown that, epidermal cells have direct candidacidal

activity and this activity can be increased through the stimulation of epidermal cells with ultraviolet (UV) light (11, 12), α-melanocyte stimulating hormone (α-MSH) (13) and interleukin-8 (IL-8) (29). IL-1, prostaglandin E_2 (PGE_2) and platelet-activating factor (PAF) have also been demonstrated to be involved in *Candida* killing by human epidermal cells (14), but the mechanism of killing remains unknown.

Mononuclear phagocytes are believed to play an important role in combating fungal infections. Optimal phagocytosis of *Candida albicans* requires opsonization, but unopsonized yeast can also be internalized by macrophages through the mannose receptor (27). The major components (80 to 90%) of the cell wall of *Candida albicans* are carbohydrates: (i) mannan or polymers of mannose covalently associated with proteins to form glycoproteins, also referred to as mannoproteins; (ii) glucans that are branched polymers of glucose containing 1,3 and 1,6 linkages; and (iii) chitin, which is an unbranched homopolymer of N-acetyl-D-glucosamine (GlcNAc) containing 1,4 bonds. Proteins (6 to 25%) and lipids (1 to 7%) are present as minor wall constituents. Yeast cells and germ tubes are similar in their cell wall composition, although the relative amounts of glucans, chitin, and mannan may vary with the morphology. Glucans and chitin are the structural components of the wall. Quantitatively, glucans contribute to 47 to 60% by weight of the cell wall. Chitin is a minor (0.6 to 9%) but structurally important component, particularly associated with cell-cell connections in the ring between parent and daughter cells, in the bud scar, and in the septa of dividing independent cells. Mannose polymers do not exist as such but are found in covalent association with proteins (mannoproteins). They are the main material of the amorphous cell wall matrix in which the structural polymers (glucans and chitin) are embedded, and they represent 40% of the total cell wall carbohydrate. The mannoproteins of *Candida albicans* share several general features with the mannoproteins of Saccharomyces cerevisiae, a model organism that is one of the most thoroughly investigated yeasts in this regard. The structure of the carbohydrate

component of yeast mannoproteins is described in detail below. Although mannose is the main monosaccharide component of *Candida albicans* cell wall glycoproteins, there is evidence that other sugar residues are also present in some wall glycoprotein species.Electron microscopy studies have shown the existence of several layers in the cell wall of *Candida albicans*. The microfibrillar polysaccharides, glucan and chitin, appear to be more concentrated in the inner cell wall layer, adjacent to the plasma membrane. Proteins and glyco(manno)proteins fill the network of structural polymers and appear to be dominant in the outermost cell wall layer, which has a fibrillar or flocculent aspect.

Although glucans are present in greater abundance than mannan in the wall of this fungal species, they are immunologically less active. On the characterization of antigen patterns for the serologic classification or identification of medically important *Candida* species demonstrated that cell wall mannan is the main candidal antigen responsible for the specificity of the different serologic reactions (7, 39). The macrophage mannose receptor (MMR) recognizes carbohydrates expressed on the surfaces of micro-organisms and is particularly well-suited to direct particles to phagolysosomes and trigger a respiratory burst (38).

It has been shown that human keratinocytic cells from squamous carcinoma (SCL-1) and multiply passaged cultured keratinocytes from normal skin express sugar-binding proteins on their surface (8, 10). However, relevant physiological importance could not be attributed to these sugar-binding structures. Human keratinocytes also possess the ability to synthetize and express cell surface moieties characteristic of effector and/or accessory cells of the immune system (22, 23).

2. AIMS

The immunological research group of the Department of Dermatology, University of Szeged firstly described that human keratinocytes belong to the group of killer cells and since then numerous relevant data on the regulation of killing mechanism have been obtained, although the exact mechanism has not been clarified.

Our aims were:

1. To investigate the effect of mannan, Mannose-BSA, other carbohydrates and an interleukin-8 inhibitor on the *Candida albicans* killing activity of human keratinocytes.

2. To investigate, that mannose-binding structures play a role in the adherence of *Candida* to keratinocytes.

3. To demonstrate the presence of mannose-binding receptors on the surface of keratinocytes.

4. To characterize the mannose-binding structures on the surface of human keratinocytes with radioligand binding studies and flow-cytometric analysis.

3. MATERIALS AND METHODS

3.1 Cells

3.1.1 Separation of human epidermal keratinocytes

Human epidermal cells were obtained from healthy individuals undergoing plastic surgery after giving their informed consent in conformity with the requirements of the Institutional Review Board (IRB) of the Albert Szent-Györgyi Medical Center, University of Szeged. After removal of the subcutaneous tissue and much of the reticular dermis, the tissue samples were cut into small strips and incubated with 0.25% trypsin (Sigma, Budapest, Hungary) overnight at 4 °C. Subsequently, the epidermis was peeled off from the dermis. Dissociation of the epidermal layer into a single cell suspension was accomplished by gentle aspiration with a Pasteur pipet. The cell suspension was filtered through a 100 μm cell filter (BioDesign Inc. of New York, Carmel, NY). A suspension of single cells (10^6) was prepared in Dulbecco's modified Eagles medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 20% fetal bovine serum (FBS), 20 mM Hepes buffer, 100 U/ml penicillin and 100 μg/ml streptomycin (each from Gibco). The viability of the cells was always >85%, as determined by trypan blue exclusion. The separated cells were characterized by immunohistochemistry on cytospin preparations (Cytospin, Shandon-Elliot, Frankfurt, Germany). Keratinocytes were labeled with the monoclonal antibody Lu 5 (Boehringer Mannheim Biochemicals, Mannheim, Germany), that reacts with an epitope common to all cytokeratins (1-19), Langerhans cells were labeled with anti-CD1a antibody (Boehringer), endothelial cells were labeled with the monoclonal antibody BMA 120 (Behring Werke, Marburg, Germany), which reacts with a 200 kDa antigen which is different from the von Willebrand factor (vWf) molecule, but is also highly specific for endothelial cells (1). Fibroblasts and melanocytes were identified with anti-vimentin antibody (Boehringer) (2). Immunohistochemical analyses of the separated cells demonstrated that over 95% were keratinocytes; the rest were contaminating fibroblasts and

melanocytes; no endothelial or Langerhans cells were detected.

3.1.2 Culturing of HaCaT keratinocytes

Human HaCaT keratinocytes (kindly provided by Dr. N. E. Fusenig, Heidelberg, Germany) were grown in 75 cm^3 cell culture flasks (Costar, Cambridge, MA, USA) and maintained in high glucose DMEM supplemented with 10% FBS, L-glutamine, penicillin/streptomycin and fungizone (Sigma, Budapest, Hungary) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every second day. Cells were harvested through trypsinization.

3.1.3 Culturing and separation of Candida albicans

Candida albicans (0656 CBS Delft) was cultured on Sabouraud agar and transferred onto fresh agar 24 h prior to the killing assay. *Candida* cells were harvested with phosphate-buffered saline (PBS) and counted in a hemocytometer. Under these conditions sufficient numbers of *Candida* cells can be obtained without germ tubes and hyphae. Their viability was checked with trypan blue.

For the adherence assay, *Candida albicans* cells were resuspended in PBS at a final concentration of 10^7 blastospore cells/ml and then fixed with 70% ethanol for 1 h at room temperature and washed twice in PBS. The *Candida* cells were next incubated with 0.1 mg/ml fluorescein-isothiocyanate (FITC) (Sigma, Budapest) in a 0.5 M carbonate/bicarbonate buffer (pH 9.5) for 30 min on a moving plate in the dark and finally washed 5 times in PBS (46).

3.1.4 Separation of human polymorphonuclear (PMN) leukocytes

Human PMN leukocytes were isolated from venous blood freshly drawn from healthy donors after obtaining their informed consent in accordance with the requirements of the IRB of the Medical and Health Science Center, University of Debrecen by dextran sedimentation and centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), as previously described (Arenberger *et al*, 1992). Contaminating erythrocytes were lysed with hypotonic

saline and the PMN leukocytes were then washed with PBS and counted under a microscope.

3.2 Killing assay

3.2.1 Candida albicans killing of human keratinocytes and HaCaT cells

A modified method originally introduced for blood leukocytes was employed to evaluate *Candida* killing by keratinocytes (12). Briefly, 10^6 human keratinocytes and $2x10^6$ *Candida* cells, or *Candida* alone in DMEM, were incubated for 4 h at 37 °C in an atmosphere of 5% CO_2 in a volume of 1 ml. After the incubation period Triton X-100 (1%) (Sigma) was added to the mixture and to the *Candida* control (*Candida* alone) for 5 min at 4 °C in order to lyse the keratinocytes. The *Candida albicans* cells were then stained with methylene blue (0.1%) for 45 min at 4 °C and the killed, non-viable *Candida* cells were counted in a hemocytometer under the microscope. The number of *Candida* killed by the keratinocytes was calculated by subtracting the number of dead *Candida* cells in the control from the number of dead *Candida* cells in the reaction mixture. The percentage of non-viable *Candida* cells was defined as the killing activity. To ascertain the accuracy of our methylene blue assay we have also determined the colony forming units (CFU) (35, 48) in parallel with the *Candida albicans* cell counts in a separate experiment. A strong correlation was found between the results of the two assays: r=0.984, $p<0.05$.

3.2.2 Candida albicans killing of human PMN leukocytes

In several experiments PMN leukocytes were used to measure their *Candida* killing activity. For the killing assay of PMN leukocytes, *Candida* yeasts were opsonized with 2.5% normal human serum for 30 min prior to each assay and then washed with PBS. 10^6 leukocytes were incubated with $2x10^6$ opsonized *Candida* cells, or *Candida* alone in DMEM was incubated for 30 min at 37 °C in an atmosphere of 5% CO_2 in a volume of 1 ml. After the incubation period, Triton X-100 (1%) (Sigma) was added to the mixture and to the *Candida* control (*Candida* alone) for 5 min at 4 °C in order to lyse the granulocytes. The

Candida albicans cells were then stained with methylene blue (0.1%) for 45 min at 4 °C and the killed, non-viable *Candida* cells were counted in a hemocytometer under the microscope. The number of *Candida* cells killed by the PMN leukocytes was calculated by subtracting the number of dead *Candida* cells in the control from the number of dead *Candida* cells in the reaction mixture. The percentage of non-viable *Candida* cells was defined as the killing activity.

3.2.3 Modulation of Candida killing by UV-B, α-MSH and IL-8

Keratinocytes in suspensions were exposed to UV radiation up to 220 mJ/cm^2 using a Saalman-SUP lamp (Gerhard Saalman, Herford, Germany) with a spectrum of 290-330 nm without any specific filtration subsequent to a 20 min of warm-up period. Spectroradiometry was employed to determine the spectral output before each experiment. The viability of the cells after UV irradiation never dropped below 80%. After UV exposure, keratinocytes were incubated in DMEM at 37 °C at 5% CO_2 for 3 h before use in the above-described *Candida* killing assay. In other experiments keratinocytes were stimulated for 12 h with $2x10^{-8}$ M recombinant human IL-8 (Hermann Biermann, Bad Nauheim, Germany) or with 20 μg/ml α-MSH (Sigma) for 1 h (37 °C, 5% CO_2) prior to the killing assay.

3.2.4 Preparation of PMN leukocyte supernatant containing IL-8 inhibitory (IL-8INH) activity

Human neutrophils ($2x10^8$/ml) were incubated for 30 min at 37 °C in PBS, and then centrifuged (1500xg for 10 min). In another experiment, $2x10^6$ /ml neutrophils were used for the generation of IL-8INH activity. Pooled cell supernatants from different healthy individuals were next centrifuged again (15.000xg for 30 min), and concentrated by ammonium sulfate precipitation, followed by dialysis against PBS.

3.2.6 Inhibition of Candida killing

100 or 500 μg/ml mannan (Sigma, >99% pure), mannosylated-BSA (Man-BSA) (E/Y Labs,

San Mateo, CA) or 300 μg/ml galactose-BSA (Gal-BSA) (Sigma) dissolved in DMEM was added to a mixture of human keratinocytes and *Candida* cells to investigate the inhibition of killing activity.

In other experiments the resultant PMN leukocyte supernatant, containing 100 μg/ml protein, referred to as IL-8INH, was tested for its ability to block the the effect of IL-8 of stimulating *Candida* killing of human keratinocytes compared to that of human PMN leukocytes. In the *Candida albicans* killing assay, 0.1-20 nM IL-8 stimulated PMN leukocytes or human keratinocytes were incubated with *Candida albicans* cells (dissolved in PBS containing 0.25% BSA) in a ratio of 1:2 for 90 min, with or without the presence of concentrated IL-8INH.

To probe the hypothesis that Man-BSA may interfere with the killing of *Candida* by cells that do not express mannose-binding receptors, the killing of *Candida albicans* by PMN leukocytes was studied in the presence or in the absence of 1 mg/ml Man-BSA.

3.2.6 The investigation of the role of mannan in chemically induced cell death

To examine a possible non-specific protective effect of mannan on *Candida albicans,* making it more resistant to chemically induced cell death, $5x10^5$ yeast cells were pretreated with 3 mg/ml mannan for 120 min prior to incubation with the fungicide ciclopirox olamine (Hoechst, Budapest, Hungary) in a concentration of 2.5 or 5 mg/ml for 120 min in a final volume of 1 ml PBS. Dead, non-viable *Candida* cells were determined as described above.

3.3 Adherence assays In the adherence assay, $5x10^5$ freshly separated human keratinocytes were mixed with $2x10^6$ FITC-conjugated *Candida* cells in a final volume of 1 ml PBS and incubated for 120 min on a rotator at 37 °C. The cells were then examined by fluorescence microscopy (Opton, Germany) and by flow cytometric analysis using a FACStar Plus Flow Cytometer (Becton Dickinson). Data were collected by using a linear amplifier for side scatter (SSC) and logarithmic amplifiers for forward scatter (FSC) and fluorescence

intensity (FL1). FSC/SSC proved to be efficient in discriminating between human keratinocytes and *Candida albicans* cells, due to the substantial differences in structure and size. To determine the extent of adherence, non-adherent yeasts were gated out and the green fluorescence of the keratinocytes was measured by using the FL1 channel. This allows a distinction of *Candida*-binding (FL1-positive) and non-binding FL1-negative keratinocytes. Flow cytometric data (an average of $5x10^3$ events) were analyzed by the Cell Quest 3.1 F software (Becton Dickinson). To study the effect of mannan on the ability of keratinocytes to bind *Candida* cells, keratinocytes were incubated with 3 mg/ml mannan dissolved in PBS for 60 min before the adherence assay.

3.4 Immunohistology Skin biopsies were taken from disposed tissues of healthy individuals undergoing plastic surgery operations. The samples were embedded in cryomatrix (Shandon, Life Sciences International, England), 7 μm sections were cut and fixed in acetone (10 min, 4 °C), and the sections were stained with a monoclonal antibody (mAb_{15}) against the human MMR (mouse IgG_1) and with the goat antiserum against the purified human MMR (both kindly provided by Philip D. Stahl, Washington University, St.Louis, MO).

The incubation step with the primary antibodies (1:50 dilutions) and the appropriate controls (mouse IgG_1, preimmune goat serum) for 60 min at room temperature was followed with incubation of biotin-conjugated secondary antibodies, then streptavidin-biotin peroxidase, using the DAKO Strept ABC complex kit and its protocol (DAKO A/S, Denmark). 3-amino-9-ethylcarbazole (AEC, Sigma) was used as a peroxidase substrate. 2.5 ml AEC (4 mg AEC dissolved in 1ml N,N-dimethylformamide) was added to 17 ml 0.1 M acetate buffer (pH 5.2) and 10 μl 30% H_2O_2. The sections were counterstained with hematoxylin.

3.5 Isolation of KcMR

Freshly separated human keratinocytes were collected from healthy individuals undergoing

plastic surgery operations as described above. $2.4x10^8$ keratinocytes were homogenized with a handheld Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in loading buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), 1.25 M NaCl, 15 mM $CaCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (all from Sigma). Debris was removed by centrifugation (12.000 x g, 35 min). The receptor was bound to a mannose-coupled epoxy activated mannose- Sepharose 6B column (Pharmacia Biotech, Uppsala, Sweden) in loading buffer and subsequently eluted with the same buffer containing 0.1 M mannose. The partially purified mannose-binding protein was subjected to electrophoresis in 7.5% polyacrylamide gel in sodium dodecyl sulfate (SDS) under non-reducing conditions. Protein was visualized with silver staining (45).

3.6 Radioligand binding study

3.6.1 Saturation assay

Man-BSA was trace-labeled with ^{125}I by the chloramine T method (44). $5x10^5$ keratinocytes were incubated with increasing concentrations of ^{125}I-Man-BSA (0.25-5 μg/ml, $1x10^6$ cpm/μg) in DMEM containing 1 % BSA, 10 mM Hepes, and 1.2 mM $CaCl_2$ in a final volume of 400 μl. The incubation lasted for 90 min at 4 °C under continuous shaking, and was terminated by washing the cells twice with ice-cold PBS. Cell-associated radioactivity was detected in a Packard gamma-spectrometer (Packard Instrument Co. Inc., Downers Grove, IL). Mannan-independent binding was determined in the presence of 3 mg/ml unlabeled mannan. The mannan-specific binding was defined as the difference between the total and the mannan-independent ^{125}I-Man-BSA binding (47). Each assay was carried out in duplicate in three separate experiments. For determination of the dissociation constant (K_D) and the number of binding sites per cell (B_{max}), saturation curves were analyzed with the computerized non-linear curve-fitting program MxN-FIT (6).

3.6.2 Competition assay

In competition studies the cells were incubated with 0.15 μg/ml ^{125}I-Man-BSA and with increasing concentrations of unlabeled ligand in the range of 10-3000 μg/ml. Competition studies were performed under the same conditions as in the saturation assays.

3.7 Internalization of ^{125}I-Man-BSA Man-BSA was again trace-labeled with ^{125}I by the chloramine T method. To determine the kinetics of internalization of ^{125}I-Man-BSA, a modified radioligand binding study was performed at 37 °C (15). Briefly, keratinocytes ($5x10^5$/well) were incubated with 10 μg ($1.0x10^6$ cpm/μg) ^{125}I-Man-BSA in the presence or absence of 3 mg/ml mannan in a final volume of 400 μl. After different time intervals (10, 20 and 30 min), the binding was stopped by washing the cells 4 times with ice-cold PBS, and the cell-bound radioactivity was measured with a gamma-counter. The mannan-specific binding was defined as the total binding minus the mannan-independent binding. To examine how much of the cell-bound radioactivity was internalized, cells were treated for 3 min with acetic acid (pH 3) and then washed twice with ice-cold PBS. The acid-stable radioactivity represents internalized ligand, while the acid-labile radioactivity is equivalent to surface-bound ligand (2).

3.8 Ca^{2+} and trypsin effects To determine the Ca^{2+} dependence of the Man-BSA binding sites, the radioligand binding study was performed in the presence of 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma) at 37 °C.

To investigate the trypsin sensitivity of the mannose-binding receptors, cells were incubated with 10 μg/ml trypsin (type I from bovine pancreas, Sigma) for 10, 60 or 120 min prior to the binding assay.

3.9 Flow cytometric detection of the KcMR $1x10^6$ freshly separated human keratinocytes were incubated with 100 μl goat anti-human MMR antiserum dissolved in 0.5% BSA-PBS at a dilution of 1:25 for 1 h on ice. For isotype control, non-immune goat serum was used. Cells were washed twice in PBS and incubated for another 1 h on ice with

biotinylated mouse anti-goat IgG at a 1:200 dilution. After washing again twice in PBS cells were incubated with 100 µl streptavidin-PE (Becton Dickinson, Heidelberg, Germany) at 1:100 dilution for 1 h on ice. The cells were then fixed in 1% paraformaldehyde and analyzed by flow cytometry using a FACStar Plus Flow Cytometer (Becton Dickinson). Data (an average of 10000 events per sample) were analyzed with the Cell Quest 3.1 F program.

3.10 Statistical analysis The paired Student's t-test was used for statistical evaluation, a level $p<0.05$ being taken as significant. The significance of the correlation between the *Candida albicans* cell counts and the colony forming units was determined by analysis of variance.

4. RESULTS

4.1 Inhibition of *Candida* killing by carbohydrates

4.1.1 Spontaneous and stimulated human keratinocyte Candida albicans killing is inhibited by mannan and Man-BSA and not by Gal-BSA. The spontaneous *Candida* killing activity of freshly separated keratinocytes was decreased by more than 50% by mannan at both 100 μg/ml and 500 μg/ml concentrations, indicating a potent inhibitory activity (**Figure 1a**). Pretreatment with α-MSH, IL-8 or UV-B light exposure induced a nearly 3-fold increase in the *Candida* killing activity of epidermal cells. Incubation with mannan resulted in a significant reduction in the percentage of all stimulated *Candida*. The mannan inhibition of the stimulated killing activity was higher at 500 μg/ml than at 100 μg/ml mannan concentrations. The presence of 100 μg/ml or 500 μg/ml Man-BSA inhibited the spontaneous killing activity very similarly to mannan (**Figure 1b**), but Man-BSA exhibited a higher inhibition than mannan at the corresponding concentrations of the activated killing. Our results are analogous to those obtained in experiments with macrophages (36). The spontaneous *Candida* killing activity of freshly separated keratinocytes was not influenced by the addition of Gal-BSA, suggesting that the inhibitory effect of mannan was specific (9.7±1.2% and 9.3±2.3% *Candida* cell death, respectively in the presence or absence of 300 μg/ml Gal-BSA). Data were obtained from two independent experiments performed in triplicate.

4.1.2. α-MSH stimulated Candida killing of HaCaT cells is inhibited by mannan

0.1 and 0.5 mg mannan significantly diminished the Candida albicans killing activity of HaCaT cells (data not shown).

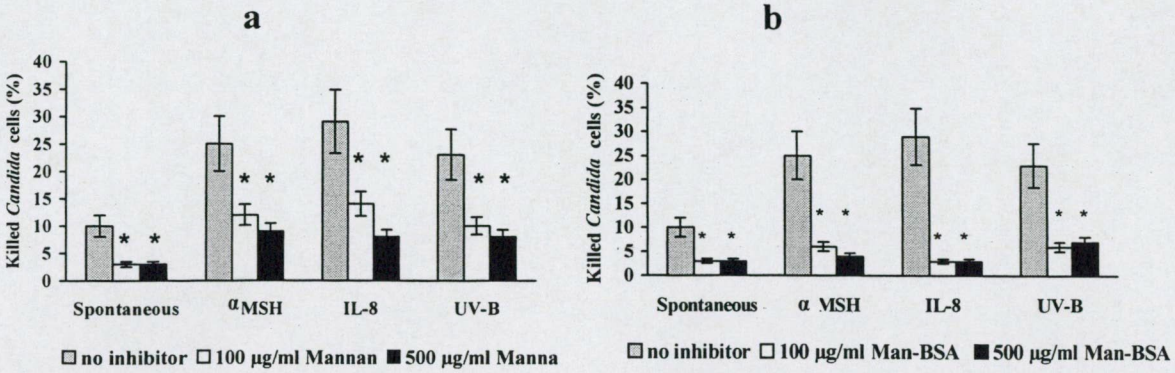

Figure 1. Mannan (a) and Man-BSA (b) inhibit *Candida* killing by keratinocytes. Spontaneous and stimulated (α-MSH, IL-8 and UV-B) *Candida* killing is represented by gray bars. Addition of 100 µg/ml mannan or 100 µg/ml Man-BSA (white bars) or 500 µg/ml mannan or 500 µg/ml Man-BSA (black bars) to the killing assay resulted in a significant reduction of both the spontaneous and the stimulated *Candida albicans* killing. Error bars represent the means ± SEM of the results of six separate experiments. *Candida* killing activity is expressed as the percentage of killed yeast cells. * denotes significant difference ($p<0.05$).

4.1.3 Stimulated Candida killing of human freshly separated PMN leukocytes and keratinocytes is inhibited by IL-8INH

The IL-8 induced *Candida albicans* killing activity of PMN leukocytes were inhibited nearly completely by the IL-8INH obtained from the supernatant of freshly separated PMN leukocytes. The IL-8 very efficiently induced *Candida* killing of freshly separated human keratinocytes, similarly to that of neutrophils, were nearly totaly blocked by the IL-8INH, suggesting that IL-8INH can modulate IL-8-induced effects on different cell-types.

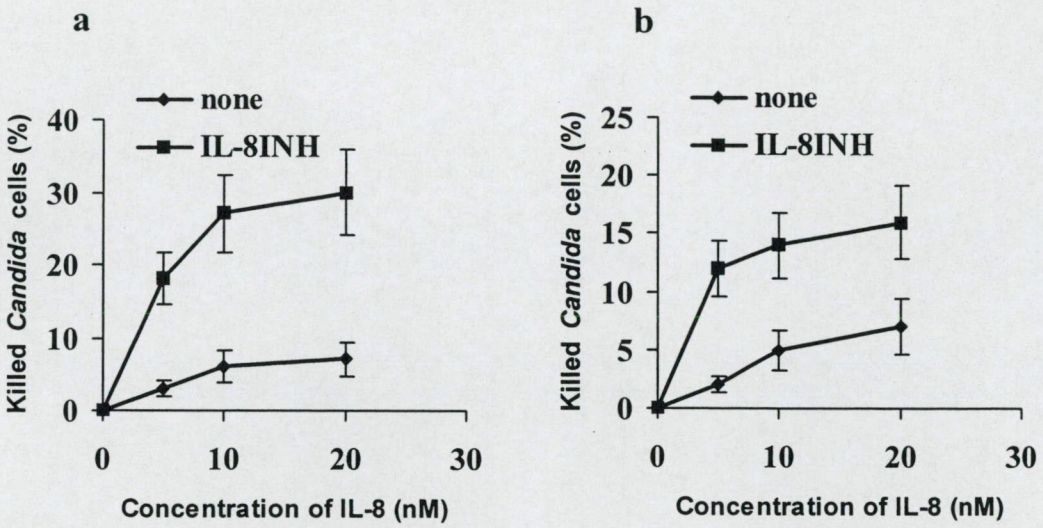

Figure 2. The IL-8INH suppresses the IL-8-induced *Candida* killing activity of human PMN leukocytes (a) and keratinocytes (b). IL-8INH significantly decreases IL-8 stimulated killing activity applied in increasing concentrations in human PMN cells and keratinocytes. Values are means±SD of the results of four independent experiments.

4.1.4 Mannan has no protective effect on chemically induced Candida cell death

Mannan did not exert a non-specific protective effect on ciclopirox olamine-induced *Candida albicans* cell death. The addition of 5 mg/ml ciclopirox olamine killed the total number of *Candida* cells both in the presence and in the absence of mannan. In the presence of mannan 6.3 ±0.6%, and in the absence of mannan 6.7±0.6% of yeast cells were killed by 2.5 mg/ml ciclopirox olamine. These results were obtained from two independent experiments performed in triplicate.

4.1.5 Man-BSA does not inhibit Candida killing of PMN leukocytes

To probe the hypothesis that Man-BSA may interfere with the killing of *Candida* by cells that do not express mannose-binding receptors, the killing of *Candida albicans* by peripheral blood granulocytes was studied. There was no difference in the killing of *Candida albicans*

opsonized with 2.5% normal human serum in the presence or absence of Man-BSA (45±7% and 39±5%, respectively in the presence or absence of 1 mg/ml Man-BSA after a 30 min of incubation) These data were obtained from four independent experiments performed in triplicate.

4.2 *Candida albicans* adheres to human keratinocytes and this adherence is inhibited by mannan To visualize the adherence of *Candida albicans* to keratinocytes, FITC-conjugated yeast cells were incubated with keratinocytes. **Figure 3a** shows that FITC-positive *Candida* cells attached to the surface of keratinocytes. The addition of 3 mg/ml mannan produced a significant decrease in the number of keratinocytes which bound FITC-conjugated *Candida* (**Figure 3b**). *Candida* adherence was quantitated by means of flow cytometric analysis. Unattached FITC-labeled *Candida* cells were electronically gated out on the basis of light scattering analysis. After a 120 min-incubation 16% of the keratinocytes were FITC-positive, indicating the adherence of the yeasts to the keratinocytes (**Figure 3c**). Following mannan pretreatment of human keratinocytes for 60 min prior to incubation with *Candida albicans*, 6% of keratinocytes were FITC-positive, revealing a marked decrease in their *Candida*-binding capacity (**Figure 3d**).

4.3 Immunohistology of human skin sections The monoclonal antibody specific for the human MMR (Lew *et al*, 1994) showed the clear staining of individual cells in the dermis, whereas no positive cells were seen in the epidermis (**Figure 4a**). The polyclonal antibody raised against the human MMR displayed a similar staining pattern in the dermis, but it also showed the staining of keratinocytes, which was more pronounced, affecting cells in the suprabasal layers (**Figure 4b**). With both controls, weak, diffuse, cytoplasmic staining was observed throughout the epidermis and dermis (not shown), indicating that the antibody staining was specific. These results suggest that the KcMR differs from the MMR.

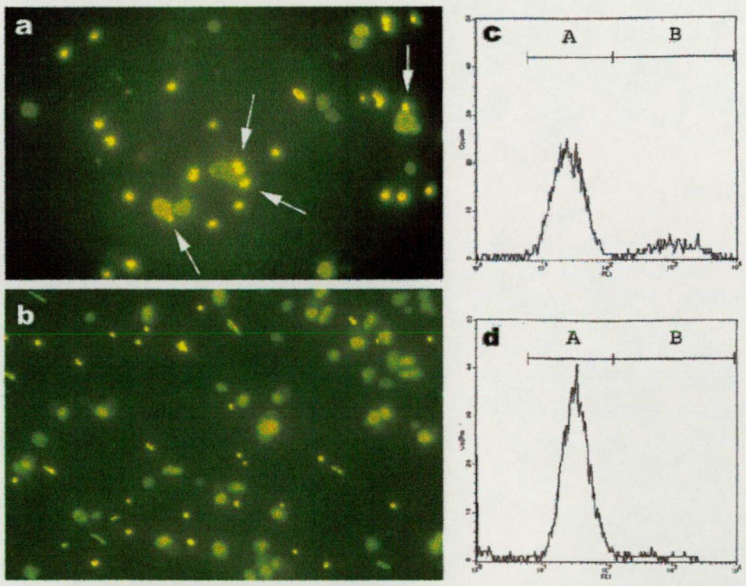

Figure 3. *Candida albicans* adheres to the surface of keratinocytes. Keratinocytes with moderate autofluorescence bound *Candida* cells (arrow) (a). Incubation with mannan reduced the number keratinocytes which bound *Candida* (b). *Candida* attachment was quantitated by flow cytometry (c,d). The abscissa depicts yeast cell fluorescence intensity on a logarithmic scale and the ordinate the relative cell number. (A) Autofluorescence of keratinocytes without FITC-labeled yeasts; (B) fluorescence of *Candida* cells adhered to the surface of keratinocytes. After the incubation 16% of the keratinocytes were FITC-positive (c). Following mannan (3 mg/ml) pretreatment of human keratinocytes for 60 min prior to incubation with *Candida albicans*, 6% of keratinocytes were FITC-positive (d).

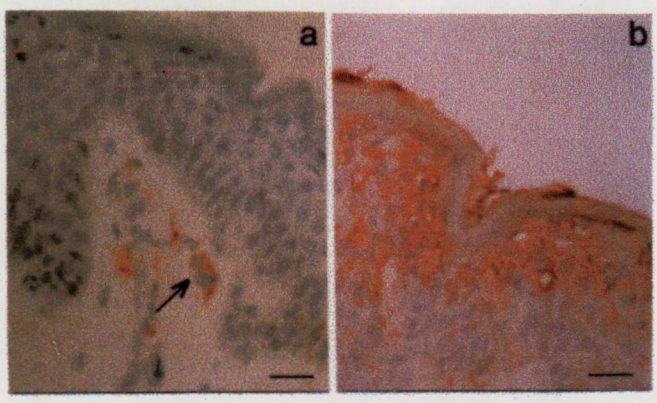

Figure 4. Sections of normal human skin stained with antibodies against the human macrophage mannose receptor. With the monoclonal antibody (mAb_{15}) a few individual cells showed positive staining in the dermis (arrow) (a). The polyclonal antibody stained not only the dermal cells, but also the keratinocytes in the epidermis (b). The staining was more pronounced on the suprabasal cells and the pattern indicated a membrane localization. Scale bar: 20 μm.

4.4 Isolation of KcMR

4.4.1 Isolation of mannose-binding receptor with mannose-Sepharose affinity chromatography

To further characterize the KcMR, a human keratinocyte extract was subjected to mannose-Sepharose affinity chromatography. Gel electrophoresis of the eluted protein revealed a single band at ~200 kDa (**Figure 5**). The human MMR has a molecular weight of around 175 kDa.

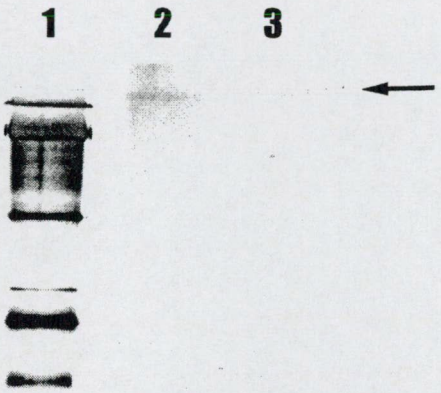

Figure 5. SDS-gel electrophoresis of the human keratinocyte mannose-binding receptor. The eluates with the two highest protein contents (lanes 2 and 3) from epoxy-activated mannose-Sepharose 6B column were concentrated and subjected to SDS-gel electrophoresis under non-reducing condition. Proteins were stained with silver. The separation revealed a single protein with a mass of ~200 kDa (arrow). Lane 1: molecular weight marker.

4.5 ^{125}I-Man-BSA binding to freshly separated human keratinocytes

4.5.1 Competition assay

Radioligand binding experiments were performed with ^{125}I-Man-BSA ($6x10^4$ cpm/well) at 4 °C to prevent internalization and degradation of the ligand. Competition studies revealed that unlabeled mannan dose-dependently inhibited the ^{125}I-Man-BSA binding to freshly separated human keratinocytes, suggesting that the ^{125}I-Man-BSA binding to the cells was mannan-specific (**Figure 6**).

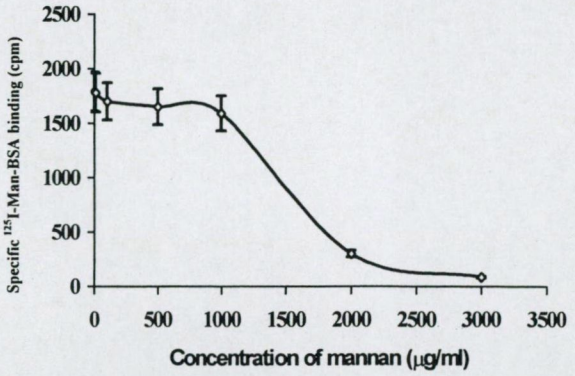

Figure 6. Competition study of ^{125}I-Man-BSA binding to human keratinocytes. Cells were incubated at 4 °C for 90 min with ^{125}I-Man-BSA ($6x10^4$ cpm/well) and with increasing concentrations of unlabeled ligand (mannan) in the range of 10-3000 μg/ml. Mannan dose-dependently inhibited the ^{125}I-Man-BSA binding to freshly separated keratinocytes. Error bars represent the means±SEM of the results of six separate experiments.

4.5.2 Saturation assay

Incubation of keratinocytes with increasing concentrations of ^{125}I-Man-BSA resulted in increasing ligand binding to the cells (**Figure 7**). Analysis of the binding data via a nonlinear curve-fitting program (MxN-FIT) indicated a single class of mannose-binding sites with a K_D of $1.4x10^{-8}$ M and a B_{max} of $1x10^4$ binding sites per cell. The Scatchard transformation of the data was linear, suggesting a single population of receptors (**Figure 7**, *inset*).

4.6 Internalization of ^{125}I-Man-BSA In separate experiments, keratinocytes were incubated with $4x10^6$ cpm/well of ^{125}I-Man-BSA, with or without 3 mg/ml mannan at 37 °C for 10, 20 or 30 min. The mannan-specific binding was calculated by subtracting the measured radioactivity in the presence of mannan (mannan-independent binding) from the radioactivity measured in the absence of mannan (total binding) (**Figure 8**). The mannan-independent binding detected after a 10 min-incubation at 37 °C did not vary considerably on further incubation (20, 30 min), whereas the mannan-specific binding increased on longer incubation. After a 10 min-incubation there was no difference in acid-labile and acid-stable specific radioactivity (**Figure 9**). After 30 min-incubation the considerable increase (8-fold)

in mannan-specific binding was acid-precipitable to almost 90%, while the acid-stable fraction exhibited only a slight change. This suggested that most of the bound ^{125}I-Man-BSA remained cell surface-associated and only a very small portion was internalized.

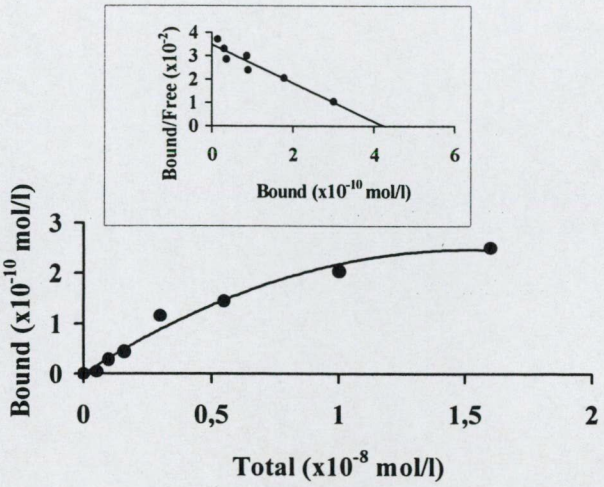

Figure 7. Saturation study of ^{125}I-Man-BSA binding to human keratinocytes. Cells were incubated with increasing concentrations of ^{125}I-Man-BSA (10^5-$2x10^6$ cpm/well). Mannan-independent binding was determined in the presence of 3 mg/ml unlabeled mannan. Mannan-specific binding was defined as the difference between the total and the mannan-independent ^{125}I-Man-BSA binding. Each assay was carried out in duplicate in three separate experiments. The inset shows the Scatchard analysis of the data: K_D=1.4x10^{-8} M, and B_{max}=10^4 binding sites per cell.

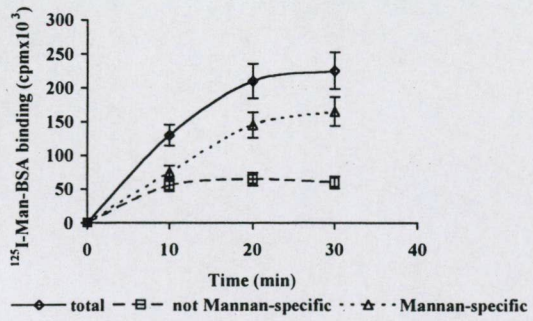

Figure 8. Radioligand binding of human keratinocytes at 37 °C. The binding of ^{125}I-Man-BSA ($4x10^6$ cpm in each well) was measured at 37 °C after 10, 20 or 30 min. The difference between the total (solid line) and the mannan-independent activity (measured in the presence of 3 mg/ml unlabeled mannan) (dashed line) is shown by the dotted curve and represents the mannan-specific binding. Error bars are the means of the results of four separate experiments ± SEM.

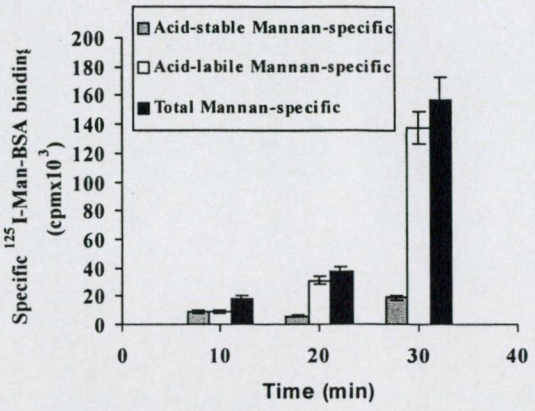

Figure 9. Binding of ^{125}I-Man-BSA to human keratinocytes at 37 °C is drastically decreased after acetic acid (pH 3) treatment. The mannan-specific binding, calculated as the difference between the total and the mannan-independent binding is shown by the black bars. The mannan-specific binding after acetic acid treatment is demonstrated by the gray bars (acid stable). The difference between the total and the acid-stable binding represents the acid-labile binding (white bars). Error bars are the means of the results of four separate experiments ± SEM.

4.7 Effect of Ca^{2+} depletion on radioligand binding To determine the Ca^{2+} dependence of carbohydrate recognition, the effect of EGTA, a Ca^{2+}-chelating agent was examined on the ligand binding. In the radioligand binding assay, human keratinocytes ($5x10^5$) were incubated with ^{125}I-Man-BSA ($4x10^6$ cpm/well) in the presence or absence of 10 mM EGTA for 30 min at 37 °C. As shown in **Figure 10**, EGTA almost completely inhibited the radioligand binding to the keratinocytes, suggesting that the binding was Ca^{2+}-sensitive.

4.8 Effect of trypsin on ^{125}I-Man-BSA binding The trypsin sensitivity of the mannose-binding sites was investigated by incubating the freshly separated human keratinocytes with 10 μg/ml of trypsin at 37 °C for different time intervals prior to the radioligand binding study. In line with previous observations involving the use of macrophages (56), the binding sites appeared to be highly sensitive to trypsin treatment. Trypsin induced a time-dependent loss of radioligand binding (**Figure 11**). Incubation of cells with trypsin for 120 min resulted in a loss of >90% of the radioligand binding.

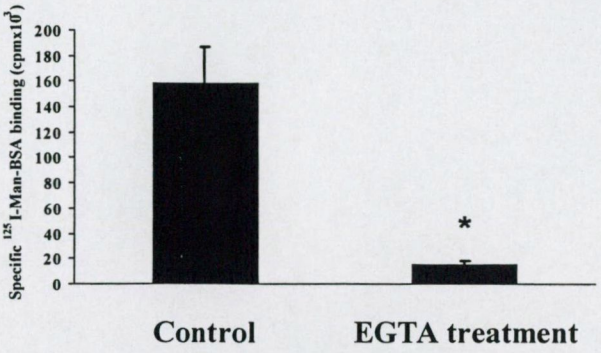

Figure 10. Ca^{2+} depletion inhibits ^{125}I-Man-BSA binding to human keratinocytes. In the radioligand binding assay, human keratinocytes were incubated with ^{125}I-Man-BSA in the presence or absence of 10 mM EGTA for 30 min at 37 °C. The Figure shows the mannan-specific binding. Error bars represent means ± SEM, n=4, *, denotes a significant decrease ($p<0.01$) for cells treated with EGTA as compared with cells without EGTA treatment.

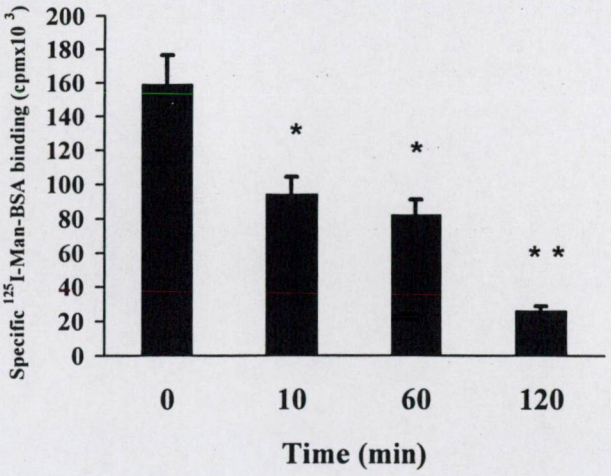

Figure 11. Limited proteolysis reduces ^{125}I-Man-BSA binding to human keratinocytes. Trypsin treatment at 37 °C was used to assess the sensitivity of the keratinocyte mannose-binding receptors to proteolytic digestion. ^{125}I-Man-BSA binding was measured both in the presence and in the absence of mannan. Incubations were stopped and binding was evaluated after an incubation period of 0, 10, 60 or 120 min with 10 μg/ml trypsin. The mannan-specific ^{125}I-Man-BSA binding underwent significant time-dependent decrease. Error bars represent means ± SEM, n=4, * and ** denote significant decreases ($p<0.05$ and $p<0.001$) on trypsin-treated cells relative to cells without trypsin treatment.

4.9 Flow cytometric analysis of KcMR Although the keratinocytes were exposed to trypsin during cell preparation in our *Candida* killing and ^{125}I-Man-BSA binding assays, the results of the experiments indicated that the receptors recovered quickly. Kang and Schlesinger also found that the removal of surface expressed MMR activity by trypsin incubation at 4 °C was followed by a fast (10 min) recovery of the receptor activity at 37 °C (25). To determine the recovery of the KcMR after trypsinization during the preparation of a cell suspension, freshly separated keratinocytes were incubated in PBS at 37 °C for 10, 30, 60 or 120 min after trypsin exposure, then stained with the goat polyclonal serum and analyzed by flow cytometry. Both the percentage of positive cells and ΔMCF (the mean channel fluorescence of cells stained with the goat immune serum minus the mean channel fluorescence of cells stained with the non-immune goat serum) showed a rapid, time-dependent increase in KcMR re-expression on keratinocytes. After the shortest (10 min) incubation, 32.5% of the cells already exhibited positive staining relative to the isotype control, and the ΔMCF was 8.36 (**Figure 12**), indicating that the KcMRs on the keratinocytes can recover very rapidly after trypsinization. Following prolonged incubation, time-dependent increases were detected in both the number of mannose receptor-positive cells (30 min: 49.38%, 60 min: 56.18%, 120 min: 65.81%) and ΔMCF (30 min: 12.47, 60 min: 14.08, 120 min: 17.05).

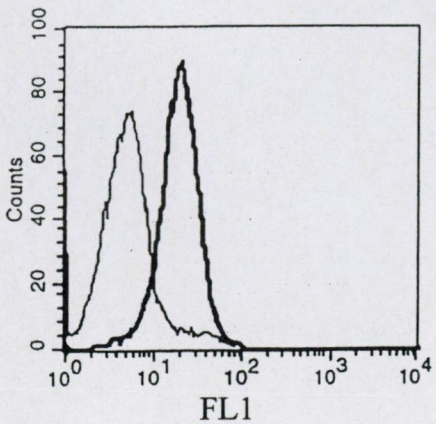

Figure 12. Flow cytometric detection of human keratinocyte mannose-binding receptor on freshly separated human keratinocytes after trypsin treatment. Cells were stained with the goat anti-human macrophage mannose receptor antiserum and analyzed by flow cytometry. For isotype control, non-immune goat serum was used. 10 min after the trypsin treatment 32.5% of the cells already exhibited positive staining (FL1, thick line) relative to the isotype control (FL1, thin line), and the ΔMCF was 8.36, indicating that the keratinocyte mannose-binding receptors on the keratinocytes can recover very rapidly after trypsinization.

5. DISCUSSION

Innate immunity is a phylogenetically ancient defense system that provides a first barrier against pathogens through nonspecific effector mechanisms. Resistance (immunity) to invasion is present from birth, and is therefore called "innate" (natural) immunity. Response to various micro-organisms is antigen-independent, while in a specific response is antigen-dependent. In the natural immunity there is immediate maximal response that results in no immunologic memory compared to specific immunity, where a lag time between exposure and maximal response can be detected.

Epithelial surfaces (skin, respiratory tract) are constantly exposed to various microorganisms. Infectious diseases can occur when micro-organisms invade these epithelial surfaces. In mammals, neutrophils, macrophages, and natural killer cells are primarily involved in these mechanisms, however keratinocytes have recently proved to play an effective role in the innate immunity beyond their barrier function. Immunocompetent cells use a variety of proteins and peptides as effector molecules that are able to kill or to inactivate microbial pathogens. Proteins of complement cascade, a variety of lectins, polypeptides present in neutrophil granules, such as the iron-binding lactoferrin, the bactericidal permeability-increasing protein, secretory phospholipase A2, serprocidins and lysozyme, are endowed with microbicidal activity.

The mechanism of the primary host defense against pathogenic micro-organisms in the skin is not completely understood. It has been demonstrated earlier that human epidermal cells exert candidacidal activity mediated by keratinocytes (11, 12).

A plasma membrane receptor for ligands with terminal mannose residues, the MMR, has already been well defined on human macrophages (56), and other cell types, as well (32). Mannose receptors can mediate the endocytotic clearance of glycoproteins, including

enzymes from neutrophils, tissue-type plasminogen activator, glucocerebrosidase and ricin and the uptake of IgE, and they are involved in the phagocytosis of various micro-organisms (9, 34, 42, 43, 47, 55). Marodi *et al* reported that monocyte-derived macrophages are able to ingest and kill *Candida* through the mannose receptor (35, 37). The expression of the surface MMR receptor can be downregulated during macrophage activation with *Bacillus-Calmette-Guerin* (15) or with LPS and phorbol esters (51), and upregulated by corticosteroids, PGE_2, and IL-4 (50, 56, 59). Although IFN-γ treatment decreases the number of mannose receptors on the macrophages, their capacity for the mannose receptor-mediated phagocytosis is increased, suggesting a dissociation between the effects of IFN-γ on the numbers of receptors expressed on the surface and the function of these receptors (36, 38).

In earlier studies, it has been shown that *Candida albicans* killing by human keratinocytes could be enhanced by UV-B light, α-MSH (12, 13) and IL-8 (29). The present study has revealed that mannan and Man-BSA are very effective inhibitors of both spontaneous and UV-B, α-MSH and IL-8 stimulated *Candida* killing by freshly separated keratinocytes, indicating that mannose-binding structures are present on the keratinocytes and play a role in the killing. Gal-BSA did not have any influence on the *Candida* killing activity of freshly separated human keratinocytes, suggesting that mannan and Man-BSA have specific inhibitory effects. Pretreatment of *Candida* cells with mannan did not protect the yeast from the fungicidal effect of the antimycotic drug, ciclopirox olamine, ruling out the possibility that mannan and Man-BSA exerted a non-specific protective effect on *Candida albicans* cells, making them more resistant to killing. Similarly, mannan did not exert any influence on the *Candida* killing activity of human PMN leukocytes that lacked mannose receptor. These results confirm that mannan has a specific effect on the *Candida*/keratinocyte interaction.

It has been demonstrated that the supernatant of PMN leukocytes contains a protein

that suppresses the *Candida albicans* killing activity of PMN leukocytes and human keratinocytes in vitro. Other data supported, that this IL-8INH exerts its effect in vivo, as well. IL-8INH binds specifically to IL-8 and is not a product of a proteolytic cleavage of the preexisting IL-8 receptors, hence the application of protease inhibitors during the preparation of IL-8INH did not affect the potency of IL-8INH. Furthermore, IL-8INH activity could not be absorbed out with antibodies against the IL-8 receptor, suggesting that it is not a fragment of the IL-8 receptor.

The adherence of *Candida* to keratinocytes was visualized by fluorescence microscopy and flow cytometric analysis. These data proved that freshly separated human keratinocytes bind fluorescein-labeled *Candida albicans* cells on their surface, and this can be inhibited by the addition of mannan. To characterize the mannose-binding sites on the surface of the keratinocytes, radioligand binding assays were performed with ^{125}I-Man-BSA at 4°C. Mannan exhibited a potent dose-dependent inhibitory effect on the ^{125}I-Man-BSA binding of the keratinocytes. The binding assays indicated that the number of binding sites per keratinocytes is 10 000/cell with a K_D=1.4 x 10^{-8} M. At 37 °C, the trace-labeled Man-BSA binding displayed a time-dependent increase and the binding was again inhibited by mannan. The majority (nearly 90%) of the cell-bound ^{125}I-Man-BSA was precipitable with acetic acid, indicating that most of the Man-BSA remained on the surface of the keratinocytes and only a small portion was internalized. The depletion of Ca^{2+} decreased the Man-BSA-binding capacity of the keratinocytes. Proteolytic digestion with trypsin at 37 °C considerably reduced the binding activity, but removal of the surface receptors with trypsin was followed by a rapid recovery.

Characteristic features of the mannose-binding structures of keratinocytes are compared with the characteristics of the MMR in Table I. These two structures exhibit similarities in the number of binding sites per cell, in the dissociation constants, in their

sensitivity to proteolysis and in their Ca^{2+}-dependent activity. Macrophages internalize mannose efficiently, but a similar phenomenon can not be observed with keratinocytes. While a monoclonal antibody against MMR did not stain keratinocytes, a goat anti-human MMR immune serum reacted with suprabasally localized keratinocyte membrane sructures. Mannose-Sepharose chromatography of keratinocyte extracts isolated a single protein with an apparent molecular weight of ~200 kDa, and on Western blot, the goat anti-human MMR immune serum also recognized a band of around 200 kDa. The human MMR has a molecular weight around 175 kDa. These data indicate that the mannose-binding receptors on human keratinocytes could be different from the human MMR.

	KcMR	MMR
Number of sites per cell	$1x10^4$	$2x10^4$
K_D(M)	$1.8x10^{-8}$	10^{-8}
Internalization	Not efficient	Efficient
Ca^{2+} dependency	Dependent	Dependent
Sensitivity to proteolysis	High	High
Recovery after proteolysis	Rapid	Rapid
Molecular weight	~200 kDa	~175 kDa

Table I. Comparison of keratinocyte mannose receptor (KcMR) with macrophage mannose receptor (MMR)

The ability of mannose-binding is shared by a number of different Ca^{2+}-dependent (type C) lectins (61). While most of the members of the type C lectin group contain only a single carbohydrate-binding domain (40), the known receptors have either 8 (MMR and phospholipase A_2 receptor) or 10 (DEC 205 receptor) lectin domains, and it is likely that these domains cooperate with each other to enhance ligand avidity (16, 60). All three of these receptors appear to mediate various endocytotic phenomena (21, 24, 62). Our results indicate

that the KcMR is probably not a member of the endocytotic type C lectins. Although our data clearly suggest that the KcMR plays a role in the *Candida* killing activity of the cells, in light of the differences between the MMR and the KcMR the mechanism of killing may be different.

Our data indicate that the human KcMR is responsible for the initial phase of anti-*Candida* activity. Various effector mechanisms can restrict *Candida* growth in infected hosts. It has recently been demonstrated that nitric oxide (NO) plays a crucial role in the *Candida* killing mechanism of keratinocytes (30). The adherence of *Candida* cells to the KcMR could induce NO production. Numerous studies have reported that NO synthesis is a necessary component of non-specific defense mechanisms against various bacteria, viruses and fungi (33). Human keratinocytes are sources of constitutive and inducible NO isoforms. It has been shown that IL-8, α-MSH and UV-B irradiation can induce NO production by keratinocytes (4, 5). Several pathogens, such as *Escherichia coli*, *Candida albicans*, and *Mycobacteria*, have been shown to be sensitive to NO, and the chemical generation of NO on the skin surface may provide a protective mechanism against various pathogens (17). NO synthesis could be mediated through MMRs in chicken (26). Mannose receptors may also be involved in the induction of human-beta-defensin-2 (hBD-2 peptide antibiotic), which is highly effective against *Candida albicans, Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa* (20). It has been observed that the human skin contains a shield of endogenous peptide antibiotics (18). Besides the constitutively expressed antimicrobial peptides, a group of these proteins are inducible and can be recruited by contact with microorganisms.

Our results reveal the presence and for the first time the physiological importance in the host defense against *Candida* infection of mannose receptors on human keratinocytes. Further work is needed for a better characterization of its structure and function.

6. SUMMARY

1. We have shown that both stimulated and unstimulated *Candida* killing activity of human keratinocytes and the HaCaT immortalized keratinocyte cells can be inhibited by the mannan and mannose-BSA.

2. We proved that the supernatant of human polymorphonuclear leukocytes contained an IL-8 inhibitor, that suppressed the IL-8 stimulated *Candida* killing activity of keratinocytes.

3. We have demonstrated that mannose-binding receptors are present mainly on the suprabasal layer of epidermal keratinocytes.

4. We have characterized the mannose-binding receptors on the surface of human keratinocytes.

5. We have proved, that human keratinocyte mannose-binding receptors, unlike classical macrophage mannose receptors, do not mediate phagocytosis.

7. REFERENCES

1. Alles JU, Bosslet K: Immunocytochemistry of angiosarcomas. A study of 19 cases with special emphasis on the applicability of endothelial cell specific markers to routinely prepared tissues. *Am J Clin Pathol* 89:463-471, 1988

2. Arenberger P, Kemeny L, Ruzicka T: Characterization of high-affinity 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) binding sites on normal human keratinocytes. *Epithelial Cell Biol* 2:1-6, 1993

3. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106: 761-771, 1988

4. Bruch-Gerharz D, Fehsel K, Suschek C, Michel G, Ruzicka T, Kolb-Bachofen V: A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. *J Exp Med* 184:2007-2012, 1996

5. Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V: Nitric oxide in human skin: current status and future prospects. *J Invest Dermatol* 110:1-7, 1998

6. Bürgisser EJ: *MxN-FIT. An IBM-PC program for the analysis of complex binding data according to the law mass action.* Rheienfeld, Germany, Buerco AG, 1988

7. Cassone A: Cell wall of Candida albicans: its functions and its impact on the host. *Curr Top Med Mycol* 3:248-314:248-314, 1989

8. Cerdan D, Grillon C, Monsigny M, Redziniak G, Kieda C: Human keratinocyte membrane lectins: characterization and modulation of their expression by cytokines. *Biol Cell* 73:35-42, 1991

9. Chung KN, Shepherd VL, Stahl PD: Swainsonine and castanospermine blockade of mannose glycoprotein uptake by macrophages. Apparent inhibition of receptor-mediated endocytosis by endogenous ligands. *J Biol Chem* 259:14637-14641, 1984

10. Condaminet B, Redziniak G, Monsigny M, Kieda C: Ultraviolet rays induced expression of lectins on the surface of a squamous carcinoma keratinocyte cell line. *Exp Cell Res* 232:216-224, 1997

11. Csato M, Bozoky B, Hunyadi J, Dobozy A: Candida albicans phagocytosis by separated human epidermal cells. *Arch Dermatol Res* 279:136-139, 1986

12. Csato M, Kenderessy AS, Dobozy A: Enhancement of Candida albicans killing activity of separated human epidermal cells by ultraviolet radiation. *Br J Dermatol* 116:469-475, 1987

13. Csato M, Kenderessy AS, Dobozy A: Enhancement of Candida albicans killing activity of separated human epidermal cells by alpha-melanocyte stimulating hormone [letter]. *Br J Dermatol* 121:145-147, 1989

14. Csato M, Kenderessy AS, Judak R, Dobozy A: Inflammatory mediators are involved in the Candida albicans killing activity of human epidermal cells. *Arch Dermatol Res* 282:348-350, 1990

15. Ezekowitz RA, Austyn J, Stahl PD, Gordon S: Surface properties of bacillus Calmette-Guerin-activated mouse macrophages. Reduced expression of mannose-specific endocytosis, Fc receptors, and antigen F4/80 accompanies induction of Ia. *J Exp Med* 154:60-76, 1981

16. Ezekowitz RA, Williams DJ, Koziel H, Armstrong MY, Warner A, Richards FF, Rose RM: Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. *Nature* 351:155-158, 1991

17. Fierro IM, Barja-Fidalgo C, Cunha FQ, Ferreira SH: The involvement of nitric oxide in the anti-Candida albicans activity of rat neutrophils. *Immunology* 89:295-300, 1996

18. Gallo RL, Huttner KM: Antimicrobial peptides: an emerging concept in cutaneous biology. *J Invest Dermatol* 111:739-743, 1998

19. Hahn BL, Sohnle PG: Characteristics of dermal invasion in experimental cutaneous candidiasis of leucopenic mice. *J Invest Dermatol* 91:233-237, 1988

20. Harder J, Bartels J, Christophers E, Schroder JM: A peptide antibiotic from human skin [letter] *Nature* 387:8611997

21. Harris N, Super M, Rits M, Chang G, Ezekowitz RA: Characterization of the murine macrophage mannose receptor: demonstration that the downregulation of receptor expression mediated by interferon-gamma occurs at the level of transcription. *Blood* 80:2363-2373, 1992

22. Hunyadi J, Simon M, Jr., Dobozy A: Immune-associated surface markers of human keratinocytes. *Immunol Lett* 31:209-216, 1992.

23. Hunyadi J, Simon M, Jr., Kenderessy AS, Dobozy A: Expression of monocyte/macrophage markers (CD13, CD14, CD68) on human keratinocytes in healthy and diseased skin. *J Dermatol* 20:341-345, 1993

24. Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC: The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* 375:151-155, 1995

25. Kang BK, Schlesinger LS: Characterization of mannose receptor-dependent phagocytosis mediated by Mycobacterium tuberculosis lipoarabinomannan. *Infect Immun* 66:2769-2777, 1998

26. Karaca K, Sharma JM, Nordgren R: Nitric oxide production by chicken macrophages activated by Acemannan, a complex carbohydrate extracted from Aloe vera. *Int J Immunopharmacol* 17:183-188, 1995

27. Karbassi A, Becker JM, Foster JS, Moore RN: Enhanced killing of Candida albicans by murine macrophages treated with macrophage colony-stimulating factor: evidence for augmented expression of mannose receptors. *J Immunol* 139:417-421, 1987

28. Kauffman CA, Shea MJ, Frame PT: Invasive fungal infections in patients with chronic mucocutaneous candidiasis. *Arch Intern Med* 141:1076-1079, 1981

29. Kemeny L, Kenderessy AS, Arenberger P, Peter RU, Dobozy A, Ruzicka A: Interleukin-8 induced chemotaxis and increased *Candida albicans* killing activity of normal human epidermal cells. *Arch Dermatol Res* 284:26, 1992(abstr.)

30. Kenderessy AS, Kemeny L, Dobozy A: Nitric oxide is involved in the *Candida albicans* killing activity of keratinocytes. *J Invest Dermatol* 107:452,.1996(abstr.)

31. Kirkpatrick CH, Rich RR, Bennett JE: Chronic mucocutaneous candidiasis: model-building in cellular immunity. *Ann Intern Med* 74:955-978, 1971

32. Lew DB, Songu-Mize E, Pontow SE, Stahl PD, Rattazzi MC: A mannose receptor mediates mannosyl-rich glycoprotein-induced mitogenesis in bovine airway smooth muscle cells. *J Clin Invest* 94:1855-1863, 1994

33. Liew FY, Cox FE: Nonspecific defence mechanism: the role of nitric oxide. *Immunol Today* 12:A17-21, 1991

34. Magnusson S, Berg T: Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells. *Biochem J* 291:749-755, 1993

35. [a]Marodi L, Forehand JR, Johnston RB, Jr. Mechanisms of host defense against Candida species. II. Biochemical basis for the killing of Candida by mononuclear phagocytes. *J Immunol* 146:2790-2794, 1991

36. Marodi L, Kaposzta R, Campbell DE, Polin RA, Csongor J, Johnston RB, Jr. Candidacidal mechanisms in the human neonate. Impaired IFN-gamma activation of macrophages in newborn infants. *J Immunol* 153:5643-5649, 1994

37. [b]Marodi L, Korchak HM, Johnston RB, Jr. Mechanisms of host defense against Candida species. I. Phagocytosis by monocytes and monocyte-derived macrophages. *J Immunol* 146:2783-2789, 1991

38. Marodi L, Schreiber S, Anderson DC, MacDermott RP, Korchak HM, Johnston RB, Jr. Enhancement of macrophage candidacidal activity by interferon-gamma. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. *J Clin Invest* 91:2596-2601, 1993

39. Martínez JP, Luisa Gil M., López-Ribot JL, LaJean Chaffin W: Serologic response to cell wall mannoproteins and proteins of Candida albicans. *Clin Microbiol Rev* 11:121-141, 1998

40. Mullin NP, Hall KT, Taylor ME: Characterization of ligand binding to a carbohydrate-recognition domain of the macrophage mannose receptor. *J Biol Chem* 269:28405-28413, 1994

41. Ray TL, Wuepper KD: Experimental cutaneous candidiasis in rodents; II. Role of the stratum corneum barrier and serum complement as a mediator of a protective infalmmatory response. *Arch Dermatol* 114:539-543, 1978

42. Richardson DR, Cameron K, Robinson B, Turner KJ: The mechanisms of IgE uptake by human alveolar macrophages and a human B-lymphoblastoid cell line (Wil-2wt). *Immunology* 79:305-311, 1993

43. Rijken DC, Otter M, Kuiper J, van Berkel TJ: Receptor-mediated endocytosis of tissue-type plasminogen activator (t- PA) by liver cells. *Thromb Res Suppl* 10:63-71:63-71, 1990

44. Salacinski PR, McLean C, Sykes JE, Clement-Jones VV, Lowry PJ: Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). *Anal Biochem* 117:136-146, 1981

45. Sammons DW, Adams LD, Nishizawa EE: Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. *Electrophoresis* 2:135, 1981

46. Sarasella M, Roda K, Speciale L, Taramelli D, Mendozzi E, Guerini F, Ferrante P: A rapid evaluation of phagocytosis and killing of Candida albicans by CD13+ leukocytes. *J Immunol Methods* 210:227-234, 1997

47. Sato Y, Beutler E: Binding, internalization, and degradation of mannose-terminated glucocerebrosidase by macrophages. *J Clin Invest* 91:1909-1917, 1993

48. Sawyer RT, Garner RE, Hudson JA: Effect of lectins on hepatic clearance and killing of Candida albicans by the isolated perfused mouse liver. *Infect Immun* 60:1041-1046, 1992

49. Scherwitz C: Ultrastructure of human cutaneous candidosis. *J Invest Dermatol* 78:200-205, 1982

50. Schreiber S, Blum JS, Chappel JC, Stenson WF, Stahl PD, Teitelbaum SL, Perkins SL: Prostaglandin E specifically upregulates the expression of the mannose- receptor on mouse bone marrow-derived macrophages. *Cell Regul* 1:403-413, 1990

51. Shepherd VL, Abdolrasulnia R, Garrett M, Cowan HB: Down-regulation of mannose receptor activity in macrophages after treatment with lipopolysaccharide and phorbol esters. *J Immunol* 145:1530-1536, 1990

52. Sohnle PG, Hahn BL: Epidermal proliferation and the neutrophilic infiltrates of experimental cutaneous candidiasis in mice. *Arch Dermatol Res* 281:279-283, 1989

53. Sohnle PG, Hahn BL: The fate of individual organisms during clearance of experimental cutaneous Candida albicans infections in mice. *Acta Derm Venereol* 72:241-244, 1992

54. Sohnle PG, Kirkpatrick CH: Epidermal proliferation in the defense against experimental cutaneous candidiasis. *J Invest Dermatol* 70:130-133, 1978

55. Speert DP, Wright SD, Silverstein SC, Mah B: Functional characterization of macrophage receptors for in vitro phagocytosis of unopsonized Pseudomonas aeruginosa. *J Clin Invest* 82:872-879, 1988

56. Stahl PD: The macrophage mannose receptor: current status. *Am J Respir Cell Mol Biol* 2:317-318, 1990

57. [a]Steele C, Ozenci H, Luo W, Scott M, Fidel PL, Jr.: Growth inhibition of Candida albicans by vaginal cells from naive mice. *Med Mycol* 37:251-259, 1999

58. [b]Steele C, Ratterree M, Fidel PL, Jr.: Differential susceptibility of two species of macaques to experimental vaginal candidiasis. *J Infect Dis* 180:802-810, 1999

59. Stein M, Keshav S, Harris N, Gordon S: Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 176:287-292, 1992

60. Taylor ME, Bezouska K, Drickamer K: Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. *J Biol Chem* 267:1719-1726, 1992

61. Weis WI, Taylor ME, Drickamer K: The C-type lectin superfamily in the immune system. *Immunol Rev* 163:19-34:19-34, 1998

62. Zvaritch E, Lambeau G, Lazdunski M: Endocytic properties of the M-type 180-kDa receptor for secretory phospholipases A2. *J Biol Chem* 271:250-257, 1996

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