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Dr. habil. Anna Teresa Salek
MILAN, Institute of Microbiology
Passau, Germany

INFLUENCE OF YEAST KILLER TOXINS ON THE CYTOTOXICITY OF SHIGA-LIKE TOXINS.
I. EFFECT OF KILLER TOXINS ON MAMMALIAN CELLS

1. INTRODUCTION

It has been observed that an increasing number of pathogens are becoming resistant to antibiotics in current use. Therefore, biotechnology is turning to the natural product to find new biotherapeutic agents, active against pathogenic bacteria or yeasts. Recently, a prophylactic and therapeutic anti-microbial strategy, based on a specific physiological target, has become effective due to the use of killer yeasts directed against their natural competition.

The killer phenomenon has been reported for strains of the genera *Saccharomyces, Kluyveromyces, Hansenula* (or *Pichia*), *Hanseniaspora, Candida, Torulopsis, Debaromyces, Cryptococcus* and *Ustilago* (1-8). The above mentioned yeasts produce toxins, which are mostly glycoproteins and similar in structure to lectins. They act against sensitive strains of the same or closely related species as well as against unrelated microorganisms, including pathogenic yeasts or bacteria such **e**ntero**h**emorrhagic ***E****scherichia* ***c****oli* (EHEC), producing Shiga-Like-Toxins (SLT). The activity of yeast killer toxins are lethal due to the presence of specific cell wall receptors. Therefore, they have been proposed as a potentially useful biotherapeutic agents for improvement of the human or animals health (9–15) as well as for environmental control (16–17).

The toxic effect of killer protein can not be comprehensively utilised without a very specific study. Such studies aim at characterising:

- an influence of yeast killer proteins on standard mammalian cells (examination of cell viability/cytotoxicity),
- the biochemistry of killer toxins as well as
- receptors (ligands) from pathogens or which bind the killer glycoprotein.

Cytotoxicity is a complex event *in vivo,* where its expression may be manifest in a wide spectrum of effects, from simple cell death, as in a toxic effect of anticancer drugs on both the cells of the cancer and normal cells of the bone marrow, skin, or gut, to complex metabolic aberrations such as neuro- or nephrotoxicity, where no cell death may occur, only functional change. Of course, the definition of cytotoxicity will tend to vary depending on the nature of the study, whether cells are killed or simply have their metabolism altered.

Current legislation demands that new veterinary medicine go through extensive cytotoxicity testing before they are released. This usually involves a large number of experiments on animals, which are very costly and raise considerable public concern. Therefore, more human and economic is to perform at least part of cytotoxicity testing *in vitro* on specialised mammalian cell lines or embryo cells to explain a mechanism of proved drug.

Experiments in these studies were carried out *in vitro* to determine the potential cytotoxicity of different yeast killer toxins (proteins) to mammalian cells (or some change in their activity). Since killer toxins can be used as a veterinary agent for oral application during

a therapy of microbial infections (18), they must shown to be non-toxic, without antigenic effect and harmless for intestinal as well as kidney cells.

2. PRINCIPLE AND METHODS

The choice of cytotoxicity assay (the short-term-perturbation of a specific metabolic pathway) depends on the agent/drug under study, the nature of the response, and the particular target mammalian cells.

Alterations in respiration, assayed by the MTT-test (**m**icro**t**i**t**ration test), were used to measure the metabolic response to potentially toxic yeast killer proteins. The MTT-test measures the activity of mitochondrial succinate dehydrogenase in living cells, independent of whether they are carrying out DNA synthesis or not. The method has the advantage of rapid completion of large experiments with counting of death cells.

After a weak-yellow-coloured, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) has diffused into cells, then dehydrogenases of active (live) mitochondria are able to open the tetrazolium ring (reduction of MTT), and the water-insoluble dark-blue formazan is produced. Cells are lysed with DMSO (**d**i**m**ethyl-**s**ulfo**o**xid), setting the water-insoluble MTT-formazan crystals free (dissolved), and the intensity of the blue colour is assayed by spectrophotometric means. A large number of samples can be measured and the results evaluated according to various criteria with the help of an on-line computer.

In the context, eventually, of killer toxin cytotoxicity and activity tested cells, it should be kept in mind that effects of killer toxin on metabolism must be interpreted only as such. To establish an irreversible effect on cell survival, the culture should be continued in the absence of that agent.

3. MATERIALS AND METHODS

3.1 Cultivation of mammalian cells

Material:

- Mammalian cell lines: **HeLa** (human uterus cancer cells), **PK-15** (pork kidney cells), **Vero** (monkey kidney cells), **Hep 2** (human liver cells), **V-79** (Chinese hamster kidney cells).
- Embryo cells: **EBL** (embryo bovine lung) cells.
- Growth medium: for normal mammalian cells D´MEM (**D**ulbecco´s **m**odified **E**agle **M**edium), supplemented with 1% penicillin/streptomycin (each 1000 μg/ml), 5 or 10% FCS (**F**oetal-**C**alf-**S**erum) and 1 or 2% L-glutamine.
  For PK-15, Vero, HeLa and EBL cells, D´MEM supplemented with 5% FCS, 1% antibiotic mixture (streptomycin + penicillin) and 1% L-glutamine was used.
  For Hep-2 cells D´MEM with 1% antibiotic mix, 10% FCS and 2% l-glutamine was used.
  Cells V-79 were grown on RPMI-1640 AUTO MOD™ commercial medium (Sigma, Cell Culture Catalog No. R-7755).
- T 175 (Nunck) flasks: containing 20 ml of the appropriate medium.
- Thawing of mammalian cells: For very rapid thawing of cells, a warm water bath (35°C) was used. The DMSO (**d**i**m**ethyl **s**ulf**o**xide) was washed out by washing and centrifugation with 8 ml D´MEM (+FCS). The cells were re-suspended in a small volume of medium.

Methods:

- <u>Trypsinization</u>: after 24 hours of cultivation the old medium (20 ml) was removed and 2.5 ml of STV (**<u>S</u>**aline-**<u>T</u>**rypsin-**<u>V</u>**ersen Solution) was added, followed by incubation at 37°C for 3-5 min. D´MEM medium with FCS was used to stop the trypsinization. Then, cells were centrifugated (1200 rpm for 5 min) and thoroughly re-suspended in fresh D´MEM (+FCS) medium.
- <u>Cell suspension</u>: a high concentration of live cells, i.e. 10^6 per 1 ml of proper medium was prepared, with assay by hemocytometer (e.g. Thoma): trypsinized cells were re-suspended (100-times) in trypan blue, i.e. 0.99 ml PBS (**<u>p</u>**hosphate-**<u>s</u>**aline-**<u>b</u>**uffer)-Dulbecco´s medium + 0.010 ml 0.5% trypan blue); only unstained cells were counted (10x objective).
- <u>Preparation of microtitration plates with cultures</u>: 96-well plates (each well having 28-32 mm^2 growth area) were seeded with 100 µl of the suspension of cells (10^6 cells/ml) prepared above, and incubated for 24 hours at 37°C in 5% CO_2 incubator until the mid-log phase of growth.

3.2 Outline of MTT test for viability of cells (ev. cytotoxicity of toxins)

Materials:

- Cells to be tested, adherent or in suspension from the log-phase of a stock culture.
- MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS, sterile filtered.
- PBS without Ca^{2+} or Mg^{2+}.
- DMSO for cell lysis.
- 96-well Plates.
- Multi-pipette.
- Mikroplate shaker.
- ELISA (**<u>E</u>**nzyme-**<u>L</u>**inked **<u>I</u>**mmuno **<u>S</u>**orbent **<u>A</u>**ssay)-reader, 492 nm.

Methods:

Monolayer mammalian cultures in microtitration plates were incubated (at 37°C) in different concentration ranges of yeast killer toxin. Then, MTT was added to each well and after that plates were incubated in the dark for 4 h. Next, the medium with killer toxins and MTT were removed. The water-insoluble MTT-formazan crystals were dissolved in DMSO. Absorbance was recorded using an ELISA reader. The procedure of determination was follow:

- Cells (in T 175 Flask) were trypsinised as above and pipetted into the wells of a microtitration plate at 1×10^6 cells/ml in a volume of 100 µl: it is best to allow the outside wells to remain empty! Four rows of 10 wells are set up.
- The plate was incubated for ca. 24 h at 37°C, 5% CO_2 and 95% humidity.
- 100 µl of the killer toxin to be tested was added to each well. Controls contain culture medium (e.g. D´MEM, Dulbecco´s Modified Eagle Medium).
- The cells were cultivated for a further 24 h.
- 20 µl MTT-solution (5 mg/ml) was added to each well and the cells were incubated for a further 4 h in the incubator.
- The culture medium was pipetted away.
- Then the cells were lysed by the addition of 100 µl DMSO, which also dissolves the dye. This process was favoured by 5 min shaking.
- After thorough mixing (all cell fragments should have disappeared), the absorption at 492 nm was measured by ELISA reader.

3.3 Yeast killer toxin preparations

Killer toxins from strains:

- *Williopsis mrakii* NCYC 500 or AS/15ρ^-, grown in YNB-glu 2% medium (**Y**east-**N**itrogen-**B**ase medium+ **G**lucose),
- *Pichia anomala* UCSC 25F, grown as *Williopsis mrakii* NCYC 500,
- *Pichia subpelliculosa* NCYC 16, grown as *Williopsis mrakii* NCYC 500,
- *Saccharomyces globosus* BKM y 438, grown in YNB-glu 2% + 0.5% peptone medium,
- *Hanseniaspora valbyensis* 13cs/6ρ^-, grown in YNB-glu 2% medium.

Preparation of killer toxins:

- After purification by IEX (ion exchange) chromatography the following fractions of killer toxins were collected:
  - *W. mrakii* NCYC 500: fractions 12-33, with activity 160 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain);
  - *P. anomala* UCSC 25F: fractions 12-23, with activity 190 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain);
  - *P. subpelliculosa* NCYC 16: fractions 12-22, with activity 160 Units/ml (against *S. cerevisiae*, S-6/1 sensitive strain);
  - *S. globosus* BKM y 438: fr. 21-26, with activity 255 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain). The killer toxins in the above fractions were about 2000 times concentrated (with respect to the fresh culture).
- Preparations of killer toxin from *Hanseniaspora valbyensis* 13cs/6ρ^- which were obtained after gel filtration chromatography (a second purification method). For purification, the 2000-times concentrated and dialysed culture-supernatant (from YNB-glu 2% medium) was used.
  For measurement of potential cytotoxicity the solutions which derived from collected gel filtration fractions were used:
  - 10-15 (sample S-III),
  - 16-20 (sample S-IV),
  - 21-25 (sample S-V).

  Unfortunately, the above 3 samples did not show any killer activity against 8 pathogenic bacteria, such as:
  - *Pseudomonas aeruginosa* DSM 1117,
  - *Escherichia coli* DSM 1103,
  - *Escherichia coli* DSM 2430,
  - *Staphylococcus aureus* DSM 2569 (for test),
  - *Salmonella goldcoast* PA^{1+},
  - *Enterococcus faecalis* DSM 2570,
  - *Streptococcus agalactiae* DSM 2134 (for CAMP test),
  - *Paseurella multocida* 4c (a strain from the Collection of the Institute of Animal Hygiene, University of Munich, Germany).
- In each case, 100 µl of the toxin solution was given to the first well, and to further wells were given the same volume at successive dilutions: as 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64.

Microtitration plates with killer toxins:

- The following controls were made:
  - w/o cells, for each sample of killer toxin (wells at the edge of the plate),
  - for growing of cells (wells w/o toxins), containing 0.5 M NaCl, buffered by 0.01 M citric (Na^+) buffer, as used in IEX (ion exchange) chromatography for elution of killer proteins.

In order to be able to compare measurements from various microtitration plates (with 5 various killer proteins) with each other, the extinction value of the cell control from each microtitration plate was set to 100% and the measured values of the individual wells of the dilution series adjusted in relationship to it.

Cytotoxic effects of the killer toxins on cells could be recognised when the MTT-ring-opening activity was significantly less than 100%. Within a dilution series, the cytotoxic threshold concentration was taken to be that which gave <80% of the formazan production of the cell control. A formazan production of > 120% was taken to indicate a stimulatory activity.

For each plate, two additional controls were set up which contained the mammalian cell medium only. The results from this trial were included in the final evaluation.

4. RESULTS AND DISCUSSION

It was found that killer toxins from the yeasts *Williopsis mrakii* NCYC 500, *Pichia anomala* UCSC 25F, *Pichia subpelliculosa* NCYC 16 and *Hanseniaspora valbyensis* 13cs/ρ^- do not have inhibitory effects on mammalian cells (Fig.1-10). However, some emphatic inhibition of mammalian cells activity (except human liver cells, Hep-2 and monkey kidney cells, Vero-79) has been observed when 100-times concentrated killer toxin from *Saccharomyces globosus* BKM y 438 was used.

The most sensitive cells towards that toxin were HeLa - human uterus cancer cells, and even then only at 100-times concentration. Further dilution of killer toxin from *Saccharomyces globosus*, i.e. to relative concentrations of 50, 25, 12.5, 6.25 and 3.12-times, showed some stimulation of activity in the case of all tested mammalian as well as embryo bovine lung cells (EBL). A similar tendency has been demonstrated in the case of other killer toxins. The strength of this stimulation is not clear.

The proof of yeast killer toxins cytotoxicity (or their absence) may require more wide-ranging investigation including full particulars of changes in metabolism, viability or in cell-cell signalling such as might give rise to an inflammatory or allergic responses.

The cytotoxicity assay (the level of activity/viability of cells) employed here for preliminary investigation was chosen because it is relative cheap, easily quantified, and reproducible. However, it has become increasingly apparent that, generally, it is not enough for following the effects of drugs, where greater emphasis on changes in metabolic regulation is required. Nevertheless, gross tests of cytotoxicity are still necessary, but there is a growing need for them to be supplemented with more subtle measurements of metabolic perturbation. Perhaps, the most obvious of these is the induction of an inflammatory or allergic response, which need not imply cytotoxicity of the allergen or inflammatory agent, but which is still one of the hardest endpoints to demonstrate *in vitro.*

5. CONCLUSION

Killer toxins, which are used for examinations, display a lethal effect to a wide spectrum of pathogenic microorganisms. From preliminary experiments is clear that the results with 5 different yeast killer proteins, presented on Fig. 1 - 10, did not show emphatic cytotoxicity (especially toxin from *Williopsis mrakii*) or any adverse effect in any mammalian and embryo-cells. In other words they are likely to be harmless to animals and humans tissues cells. Therefore, they could be used for explain pre-therapeutic effect on animal cells in the case of EHEC infections.

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Dr. habil. Anna Teresa Salek
MILAN, Institute of Microbiology
Passau, Germany

INFLUENCE OF YEAST KILLER TOXINS ON CYTOTOXICITY OF SHIGA-LIKE TOXINS. II. BINDING TO SHIGA-LIKE-TOXIN RECEPTORS

1. INTRODUCTION

The allocation of mammalian cell receptors, specific for different drug-proteins and hormones as well as other transmitters, into groups or types, is sufficient known (but not for killer toxins). The need to know how killer toxins work, both in physiology and in pathology, has driven the process of investigating receptors through which they act. This is not only academic interest but also has practical consequences.

Differences in receptors are potentially exploitable to develop specific mimics of their natural ligands (agonists) or to find receptor blockers (antagonists), which antagonize the action of agonists. Many of important drugs (perhaps also killer toxins) have arisen from the knowledge that agonists can act through multiple receptors. The great advantage of using their, selective for one receptor against the other, is that potent medicines with little or no side-effects can be developed, e.g. against action of toxins from the **e**ntero**h**aemorrhagic ***E****scherichia* ***c****oli* (EHEC), pathogenic strains.

Eschrichia coli is a bacterium that is a common inhabitant of the gut of warm-blooded animals, including humans, ingested orally, usually from contaminated food (foodborne pathogen) or by person-to-person transmission. The EHEC are a group of serious pathogenes which includes a most important serotype O157:H7. Humans are more often exposed to non-O157 serotype than to O157 strains.

Enterohaemorrhagic *Escherichia coli* produces protein cytotoxins that are related to Shiga toxins of *Shigella dysenteriae* type 1, called Verocytotoxins: VT_1 and VT_2 or named also Shiga-Like Toxins: SLT_1 and SLT_2. Illnesses caused by *Escherichia coli* O157:H7 infections can range from self-limited watery diarrhoea (haemorrhagic colitis) to life-threatening manifestations such as **h**aemolytic **u**remic **s**yndrome (HUS) or thrombotic thrombocytopenic purpura. *E. coli* that produces a cytotoxic variant of SLT_2 (i.e. SLT_2v) has been associated with diarrhoea and oedema disease of pigs.

The gene for SLT_1 is essentially identical to the gene coding for Shiga toxins. This is the reason why *E. coli* that can produce those toxins are also called STEC (Shiga-toxin producing *E. coli*) or VTEC (Verocytotoxin-producing *E. coli*, because the cytotoxin are active on vero, i.e. kidney cells). These toxins are *N*-glycosidases that leave a specific adenine residue from the 28S subunit of eucaryotic rRNA, which results in the inhibition of protein synthesis. These double toxins (holotoxins) consist of A subunit, which is responsible for the enzymatic activity, non-covalently linked to multiple B subunits that are responsible for binding to cellular receptors on the plasma-membrane of susceptible target cells.

The first step of the toxic mechanism is the binding to receptors on the surface of eucaryotic cells and enter them by endocytic uptake. Shiga-Like-Toxins are specific for glycosphingolipids possessing a terminal Gal-α1-4-Gal - sequence. In the case of Shigatoxins

VT_1 and VT_2, the specific receptor of sensitive cells is globotriosylceramide (Gal-α1-4-Gal - β1-4-Glc-ceramide, Gb_3) (19). Cell lines that are receptive to Verocytotoxins (VT_1 and VT_2), carry Gb_3 - receptors on their cell membranes. High concentrations of receptor Gb_3 have been found for example in human kidney as well as in human endothelial cells, but not in the kidney of other mammals. In rabbits, the target cells are found in the brain and colon. The concentration of Gb_3, which also is found in the ileum of rabbits, increases with the age of the animal.

The sensitivity of various cell lines depends on the presence or absence of the specific toxin receptors. Within the spectrum of receptive cells there are differences in the degree of sensitivity. The degree of sensitivity of a particular cell-line has been demonstrated to depend on the number of binding sites. HeLa - cells should be the most sensitive. They show the strongest effect in response to increasing toxin concentration, whilst Vero cells (kidney cell-line from Rhesus monkeys), as also other kidney cells react sluggishly to low toxin concentrations. The HeLa - cell line with 1.3×10^6 sites per cell is sensitive to Shigatoxins, whilst another with only 2.5×10^5 sites per cell is not sensitive. The binding of Shigatoxins/Shiga-Like-Toxins to its receptor is inhibited by the lectin wheat-germ-agglutinin.

The cellular effect of Verotoxins (Shiga-Like-Toxins) consists of a cessation of protein synthesis and occurs via internalisation of the toxin by means of ***r**eceptor **m**ediated **e**ndocytosis* (RME). The concept is of a sequence of steps involving endocytosis of the material and its storage in a lysosome. The selectivity of RME is based upon the specificity of ligand-receptor-interactions and takes place at particular sites on the cell membrane. These sites contain the protein clathrin and constitute less than 1% of the cell surface area. After fusion of the toxin-containing, clathrin-coated vesical with lysosomes, the A - subunit is split enzymatically and disulfide bridges are reduced, following which the A - fragments (the toxic agent) is set free. This process explains the initial lag before the onset of the toxic effect.

Large amounts of Shiga-Like Toxin have been observed to be produced from those strains that were isolated from patients or animals suffering from the consequent diarrhoea, and also from strains isolated from food that was believed to have caused the illness.

Smaller amounts of toxin have also been found from strains associated with haemorrhagic colitis. That is, not all verotoxic *Escherichia coli* are pathogenic, either because they do not produce the necessary binding factors or else because the quantity of toxin that is produced is too small.

The first pathogenic result of enteric toxin resorption is toxaemia. In rabbits, it has been found that SLT (VT_1) disappears from the blood within minutes. After this, damage to endothelial cells in small vessels and to local intravascular coagulation with deposition of fibrin and thromboses in the brain, intestine and kidneys. The results are ischaemia and necroses, which manifest themselves, for example, as disturbances to the CNS (Central Nervous System).

The effect of Verocytotoxins on cell cultures is cytotoxic. The cells round up, shrink, free themselves from the tissue and become free-swimming in the medium before eventually undergoing lysis. The effect is irreversible and worsens with time with a maximum on the 3rd day. Undiluted culture-medium filtrates show their effect as early as after 24 hours. The toxicity can often be observed even at high dilutions (10^{-5}).

There is no direct effect on the cells' metabolic energy supply. The intracellular K^+ - concentration stays constant for 120 minutes after full inhibition of protein synthesis. A lack of ATP due to cessation of oxidative phosphorylation or an increased ATP - consumption are therefore not involved.

Since pharmacology has been for a long time, and still is, concerned with description of drug effects on living systems (usually in relationship to physiology or pathophysiological

consequences), our interest, important for therapeutic reasons, was to know effects of eventual competitions between two different toxins, SLT and killer toxin, about binding of receptors, for instance, on standard mammalian cells, HeLa, or in some organs, i.e. kidney (e.g. Vero-cells, e.g. PK-15- and V-79), liver (Hep 2) as well as lung (EBL – embryo cells).

As a primary receptors blocker (antagonist) was used yeast killer toxin from *Williopsis mrakii* AS/15ρ$^-$, which is glycoprotein (like lectin with oligosaccharide sequences). Shiga-Like-Toxins from *E. coli* DSM 2403 and *E. coli* DSM 2430 were tested for eventual secondary binding of cell receptors.

The aim of the work was to resolve the therapeutic problem as to how the pathogenicity of Verocytotoxins towards some mammalian cells could be influenced by yeast killer toxins:

- Whether the yeast killer toxin from *Williopsis mrakii* AS/15ρ$^-$ binds to the Verocytotoxin-specific receptors on mammalian cells, and for how long does it bind?
- Is there any evidence for such receptors´s memory?
- Whether these responses can be reversed by washing the yeast killer toxin out?
- Whether yeast killer toxins play protective role against Shiga-Like-Toxins?

2. MATERIALS AND METHODS

We wished to investigate the possible competitions between the two different toxins, which might be due to binding of a common receptor. For this investigation, the yeast killer toxin from *Williopsis mrakii* AS/15ρ$^-$, which is a glycoprotein (similar to a lectin with oligosaccharide sequences) was used as a primary receptor-blocker (antagonist) . Verotoxins from *E. coli* DSM 2403, *E. coli* DSM 2430, *E. coli* 933*j* and *E. coli* 933*w* were tested for possible secondary binding of receptors.

Yeast killer toxins from *Williopsis mrakii* AS/15ρ$^-$ were purified by:

- protein separation using a technique similar to gel filtration on a Sephadex column;
- ion exchange chromatography (IEX) with a strong cation exchanger;
- gel filtration chromatography (GF) using material for separating proteins in the range of 0.5-60 kD.

Yeast killer toxin from *Saccharomyces globosus* y 438 BKM, used for testing of receptor binding was purified by ion exchange chromatography (strong ion exchanger).

Verotoxins from strains of *Escherichia coli* DSM 2403 (VT_1), *Escherichia coli* DSM 933*j* (VT_1) and *Escherichia coli* DSM 933*w* (VT_2) were prepared by overnight cultivation in Caye medium at 37°C. In order to measurement the cytotoxicity towards HeLa cells (MTT test), the supernatant of the culture (after filtration through filter cut-off a 0.22 μm) was used. The sterile-filtered supernatants from the reference bacterial strains were diluted into E´MEM supplemented with 5% FCS (maintenance medium) in such a way as to give a concentration series of 10^{-1}, 10^{-2}, 2×10^{-2}, 5×10^{-2}, 8×10^{-2} and 10^{-3}.

Verocytotoxin assay. The cell-culture medium was replaced with the same volume of toxin-containing maintenance medium and the cells were further incubated. Over the next 3 days the percentage cell death and the percentage of rounded up cells was estimated by microscopic examination of the cells (hemocytometer Thoma).

Vero-cells are well-suited for the detection of Verotoxin despite the fact that even higher concentrations of toxin require 48 hours incubation to demonstrate their full effect. In order to measure the CD_{50} (50% of the **c**ytotoxic **d**ose), 72 h incubation is necessary. The cells of the control grew to a very tight monolayer and could be maintained for over 72 hours. The effect of the toxin was to give cytopathological changes in the form of rounding up,

which then led to cell death. Depending on concentration, the final result was a growth of lytic plaques in, or a complete destruction of, the cell layer.

HeLa-cells. The toxic effects of higher concentrations of toxin are clearly visible after 24 h, although for certain detection the more diluted toxin preparations require, as with Vero cells, 72 h incubation. With HeLa cells, it is difficult to produce a tight monolayer, or to maintain it over 72 hours, and there is also a high spontaneous rate of rounding-up. The appearance of the cells under influence of the toxin is similar to that of Vero cells.

Cultivation of He La cells and measurement of cellular activity were performed as above described (Part I - *Cytotoxicity of yeast killer toxins*). The strain used was from the Collection of the Institute of Animal Hygiene (University of Munich, Germany). Cells were grown in E´MEM supplemented with 5% FCS (growth medium) and sub-cultured every 2-3 days as necessary.

A tightly-grown monolayer of cells in a NUNC-flask was trypsinised with STV, centrifuged at 1200 U/min for 5 minutes and then the pellet was resuspended in fresh E´MEM, supplemented with 5% FCS and adjusted to a cell concentration of 0.5×10^5 cells/ml. Afterwards the flasks were shake-coated with 2 ml cell suspension and incubated at 37°C until a tight monolayer had developed (24 hours).

Toxin assay in microtitration plates using ELISA method (see Part I). Additionally were performed MTT test (show an activity of cells) for control samples:

- HeLa/Vero cells native, w/o toxins treatment,
- Above cells after 3 hours treatment with killer toxin from *W. mrakii* AS/ρ^-,
- Action of Shiga-Like Toxin from *E. coli,* strain DSM 2403, 2430, 933*j* and 933*w* on HeLa/Vero cells,
- An influence of medium Caye on HeLa/Vero cells (72 h treatment),
- An influence of buffers (3 mM citrate-phosphate pH 5 or 5 mM NaCl in 3mM phosphate buffer pH 5 for yeast killer toxin) on HeLa/Vero cells.

The draft of measurements is follow:

- 1st day - a cultivation of HeLa or Vero cells (0.5×10^5/ml) in microtitration plate together with yeast killer toxin (a procedure like in Part I) for 3 hours and next, killer toxin was wash-out and Shiga-Like-Toxin was given for 72 h cultivation into the same well. After that the MTT test was performed.
- 3rd day - performance of MTT test. Influence of yeast killer toxin on HeLa or Vero cells was also controlled. In this case 3 hours before end of experiment (before MTT filling) to wells contained only HeLa/Vero cells was given killer toxin. Next, after 3 hours all wells were filled with MTT and than plates were 4 hours incubated. After that activity (%) of HeLa/Vero cells was measurement.

3. RESULTS AND DISCUSSION

At first it is important to consider some effect of yeast killer toxins from *Williopsis mrakii* AS/15ρ^- and *Saccharomyces globosus* y 438 BKM, as well as Shiga-Like-Toxins from *Escherichia coli* DSM 2403, DSM 2430, 933*j* and 933*w* strains on activity (MTT test) of HeLa and Vero cells (especially HeLa cells). These toxins showed an inhibition of growing cells at dilution of 1:2:

- 100-times concentrated yeast killer toxin (dilution 1:2) from *Williopsis mrakii*

  AS/15ρ^- reduced the activity of PK-15, Hep-2 and V-79 cells only by 10-20%. See Figs. 1 - 6;

- 100- and 50-times concentrated yeast killer toxin (1:2 and 1:4 dilution) from *Saccharomyces globosus* y 438 BKM reduced the activity of EBL, PK-15 cells by 80% or more (for HeLa cells). See Fig. 1, 2, 3;
- 2-fold diluted Shiga-Like-Toxin-containing culture-supernatant of *E. coli* DSM 2403 or 933*j* and 933*w* - culture gave about 20% activity of HeLa cells. See Fig. 11.

Activities (MTT test) of HeLa cells (Fig. 12 and14) and Vero cells (Fig. 13 and 15), which had been treated for the first 24 hours of incubation with yeast killer toxins (*W. mrakii* or *Saccharomyces globosus*) and for the next 3 days with Shiga-Like-Toxins (from *E. coli* DSM 933*j*, 933*w* and 2403), did not show any significant decrease of activity. They were compared with samples treated with Verocytotoxins only (w/o pre-treatment with yeast killer toxins) or with samples treated only by killer toxins from *W. mrakii* or *Saccharomyces globosus*.

In preliminary experiment cytotoxicity of above-mentioned Shiga-Like-Toxins was very small. At first *Escherichia coli* strains (especially from *Escherichia coli* DSM 2430) used in these experiments did not produce enough active toxins. Therefore, results about binding of HeLa or Vero cell receptors by yeast killer toxins (*Williopsis mrakii* AS/15ρ^- and *Saccharomyces glubosus* y 438 BKM) suggested that this binding was not strong enough and probably reversible by wash-out (just before treatment with Shiga-Like-Toxins). In that time the activity of Shiga-Like Toxins (SLT_1 and SLT_2) from *E. coli* DSM 2430 was lower than from *E. coli* DSM 2403 (e.g. Fig. 11).

In order to explain the action of the yeast killer toxin the experiments on receptor binding were repeated with new prepared toxins from *Escherichia coli* DSM 933*j*, 933*w* and 2403 strains. Fig. 11 shows that obtained Shiga-Like-Toxins in this case were very active and toxic for HeLa cells. Therefore, experiments about receptor binding for HeLa and Vero cells were again performed.

Results are presented in Fig. 12, 13, 14 and 15. In these figures we can see a distinct effect of yeast killer toxin from *Williopsis mrakii* and *Saccharomyces glubosus*, which appears to be **protection of mammalian cells against the cytotoxicity of Shiga-Like-Toxins**. A some dilution of yeast killer toxin from *Williopsis mrakii* reduces slowly protective effect for HeLa cells (Fig. 12), however, at lower concentration of killer protein (17 μg/ml, i.e. 5 Units killer activity) that effect is still observed.

Yeast killer proteins form *Williopsis mrakii* and *Saccharomyces glubosus* play evident protective role for mammalian cells, especially for HeLa cells, which are most sensitive in the case of intoxication by Shiga-Like-Toxins (Fig. 12 and 14). Vero cells are more resistant, therefore, the protective effect is not so big (Fig. 13 and 15).

4. CONCLUSION

It was found that yeast killer toxin from *Williopsis mrakii* can protect mammalian cells such HeLa and Vero cells against challenge by Shiga-Like-Toxins (derived from cultures of pathogenic strains of *Escherichia coli*, called EHEC), probably by occupation of intestinal receptor during competitions with infected microorganisms.

The final activities of tested mammalian cells are better when they are pre-treated by killer protein, i.e. before challenge with Shiga-Like-Toxins. It appears that this prophylactic effect could be very interesting for veterinary what has been proof, by <u>orally</u> treatment a big population (about 2000) of healthy and ill (with diarrhoea) pigs (manuscript – confidential data).

We can concluded that yeast killer strains are <u>probiotic</u>, i.e. could eliminate fecal shedding of EHEC strains in pigs when animals prior are treated with developed yeast toxins.

Treatment with yeast killer toxin in pigs following challenge of EHEC can reduce the fecal shedding of SLT when compared with the control animals.

One way to accomplish some objectives to use native yeast killer toxins (large and sensitive glycoproteins) intravenous, would be to generate an immunologically acceptable molecule such anti-idiotypic antibodies against the same receptors in the cell wall of the pathogen which is found to be sensitive to killer toxins. At the molecular level, the relationship between idiotypic and anti-idiotypic antibodies is similar to the complementarity of cell receptors for their ligands. Moreover, anti-idiotypic antibodies induced by idiotypic vaccination (monoclonal antibodies neutralizing *in vitro* anti-microbial activity of a yeast toxin), structurally express an "internal image" of the killer toxin and may have significant physiological implications, as well as some practical applications.

These yeast killer toxin **anti-idiotypic antibodies** are much more stable than killer protein in native form and also not antigenic (20).

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S u m m a r y

It is evident that the results of preliminary experiments with 5 different yeast killer proteins did not show emphatic cytotoxicity or any adverse effect in any mammalian and embryo-cells. Moreover, they are likely to be harmless to animals and humans tissues cells. Therefore, could be used for explain pre-therapeutic effect on mammalian cells (mostly animals) in the case of infections by strains *Escherichia coli*, called EHEC.

It was found that yeast killer toxin from *Williopsis mrakii* can protect mammalian cells such HeLa and Vero cells against challenge by Shiga-Like-Toxins (derived from cultures of pathogenic strains of *Escherichia coli).* The final activities of tested mammalian cells are better when they are pre-treated by killer protein, i.e. before challenge with Shiga-Like-Toxins. It appears that this prophylactic effect could be very interesting for veterinary what has been proved on a big population (about 2000) of healthy and ill (with diarrhoea, i.e. haemorrhagic colitis) pigs (manuscript – confidential data).

We can concluded that yeast killer strains are probiotic, i.e. could reduce or eliminate fecal shedding of EHEC strains in pigs prior treated with developed yeast toxins.

Key words:

Cell receptors, yeast killer toxins, Shiga-Like-Toxins Verocytotoxins, EHEC, mammalian cells, diarrhoea, haemorrhagic colitis, uremic syndrome, prophylactic, therapeutic effect.

Address for correspondence:

Anna Teresa Salek
e-mail: Anna.Salek@T-Online.de

Abbreviations:

EHEC-**E**ntero**h**emorrhagic **E**scherichia **c**oli, HUS-**h**aemolytic **u**remic **s**yndrome, RME **r**eceptor **m**ediated **e**ndocytosis, SLT – **S**higa-**L**ike-**T**oxin, VT – **V**erocyto**t**oxin, MTT-test **m**icro**t**i**t**ration test, DMSO – **d**i**m**ethyl **s**ulfo**o**xid, D´MEM - (**D**ulbecco´s **m**odified **E**agle **M**edium), FCS - **F**oetal-**C**alf-**S**erum, STV – **S**aline **T**rypsin **V**ersen Solution, PBS - **p**hosphate **s**aline-**b**uffer, ELISA - **E**nzyme-**L**inked **I**mmuno **S**orbent **A**ssay, YNB-glu - **Y**east-**N**itrogen **B**ase **G**lucose medium, IEX chromatography – **i**on **ex**change chromatography.

Fig. 1. Toxicity of different killer toxin for EBL´ cells.

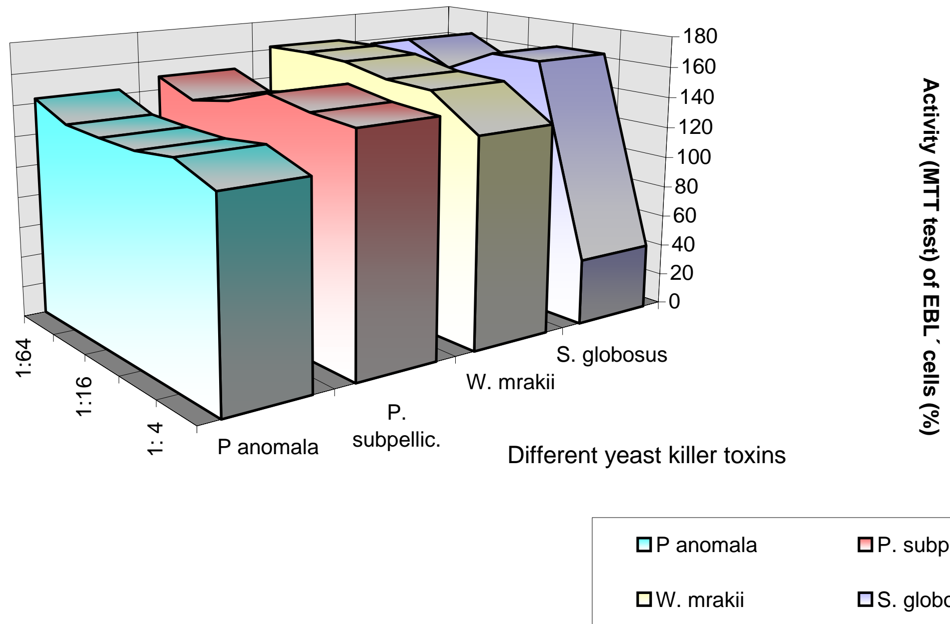
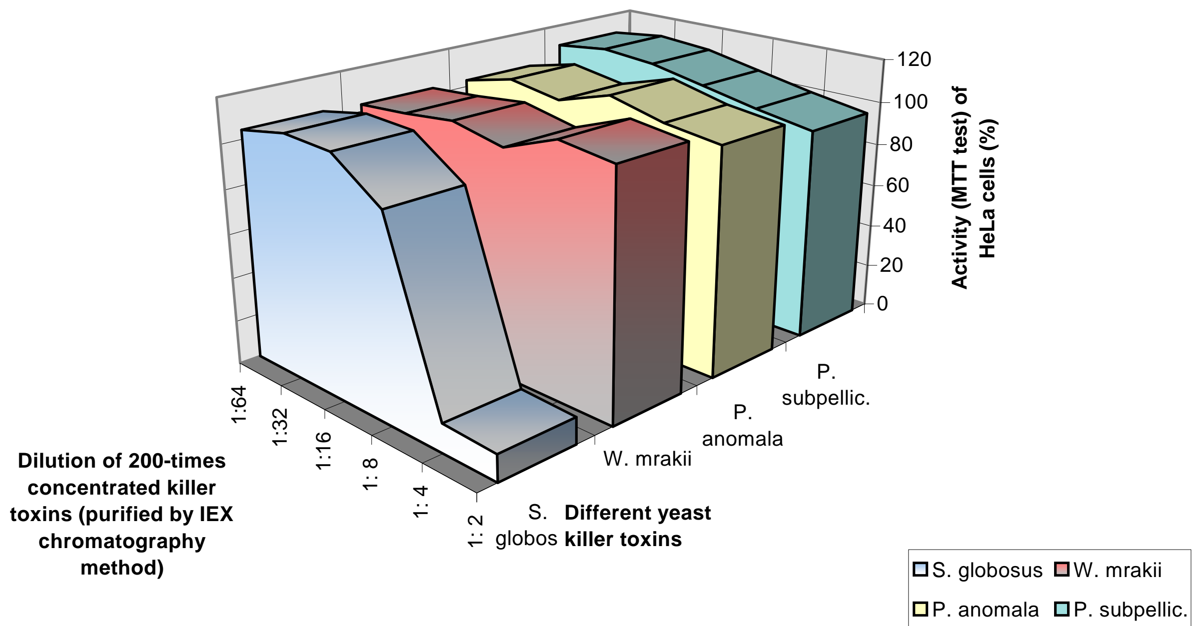


Fig. 2. Toxicity of different killer toxins for HeLa cells.



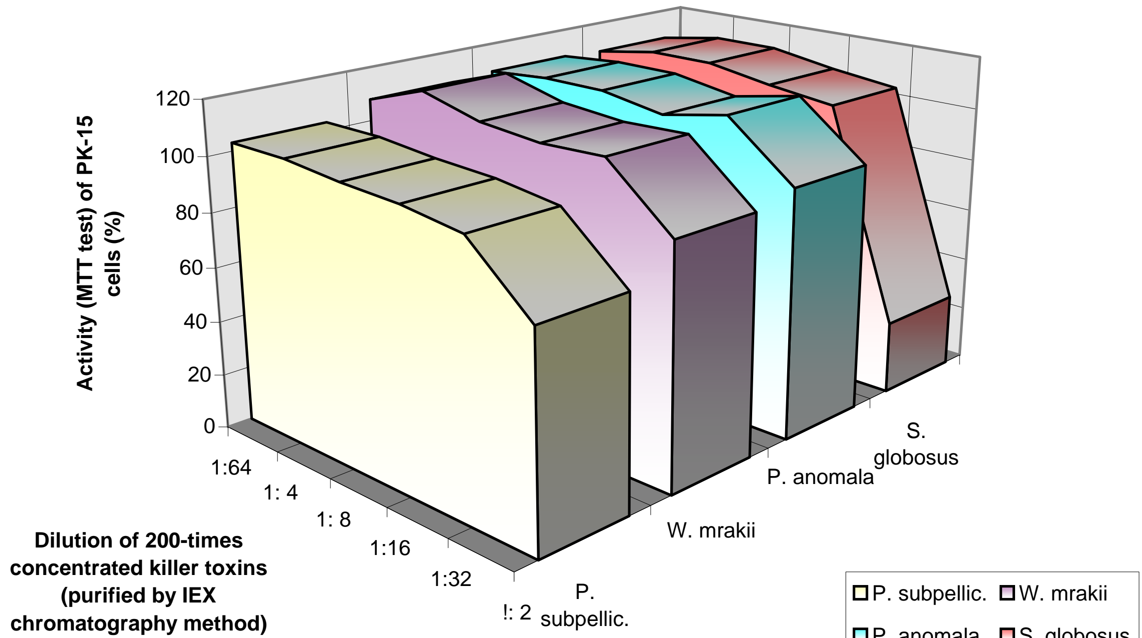


Fig. 3. Toxicity of different killer toxins for PK-15 cells.
Activity (MTT test) of PK-15 cells (%)
120
100
80
60
40
20
0
1:64
1: 4
1: 8
1:16
1:32
!: 2
Dilution of 200-times concentrated killer toxins (purified by IEX chromatography method)
P. subpellic.
W. mrakii
P. anomala
S. globosus
P. subpellic.
W. mrakii
P. anomala
S. globosus

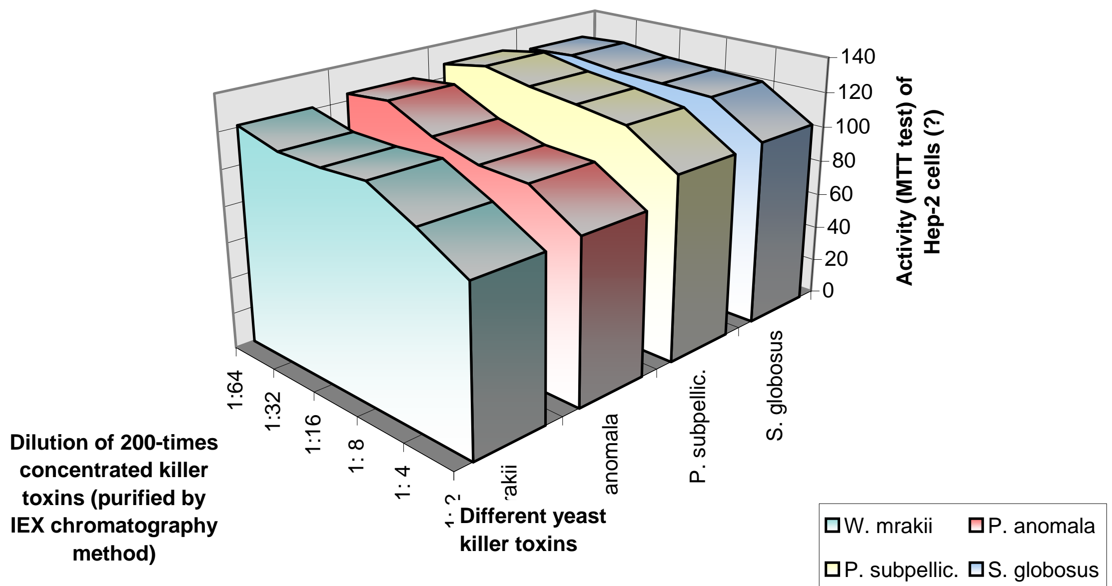


Fig. 4. Toxicity of different killer toxins for HEP-2 cells.
140
120
100
80
60
40
20
0
Activity (MTT test) of Hep-2 cells (?)
1:64
1:32
1:16
1: 8
1: 4
anomala
P. subpellic.
S. globosus
Dilution of 200-times concentrated killer toxins (purified by IEX chromatography method)
Different yeast killer toxins
W. mrakii
P. anomala
P. subpellic.
S. globosus

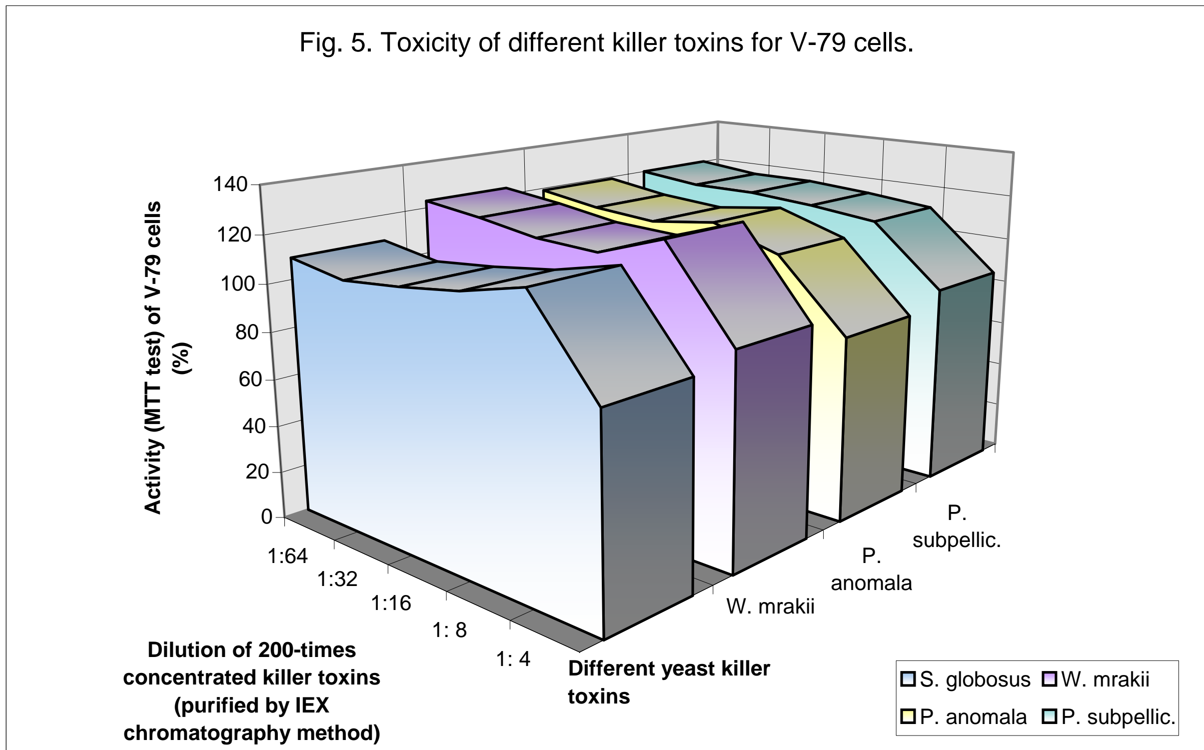


Fig. 5. Toxicity of different killer toxins for V-79 cells.
Activity (MTT test) of V-79 cells (%)
140
120
100
80
60
40
20
0
1:64
1:32
1:16
1: 8
1: 4
Dilution of 200-times concentrated killer toxins (purified by IEX chromatography method)
Different yeast killer toxins
W. mrakii
P. anomala
P. subpellic.
S. globosus
W. mrakii
P. anomala
P. subpellic.

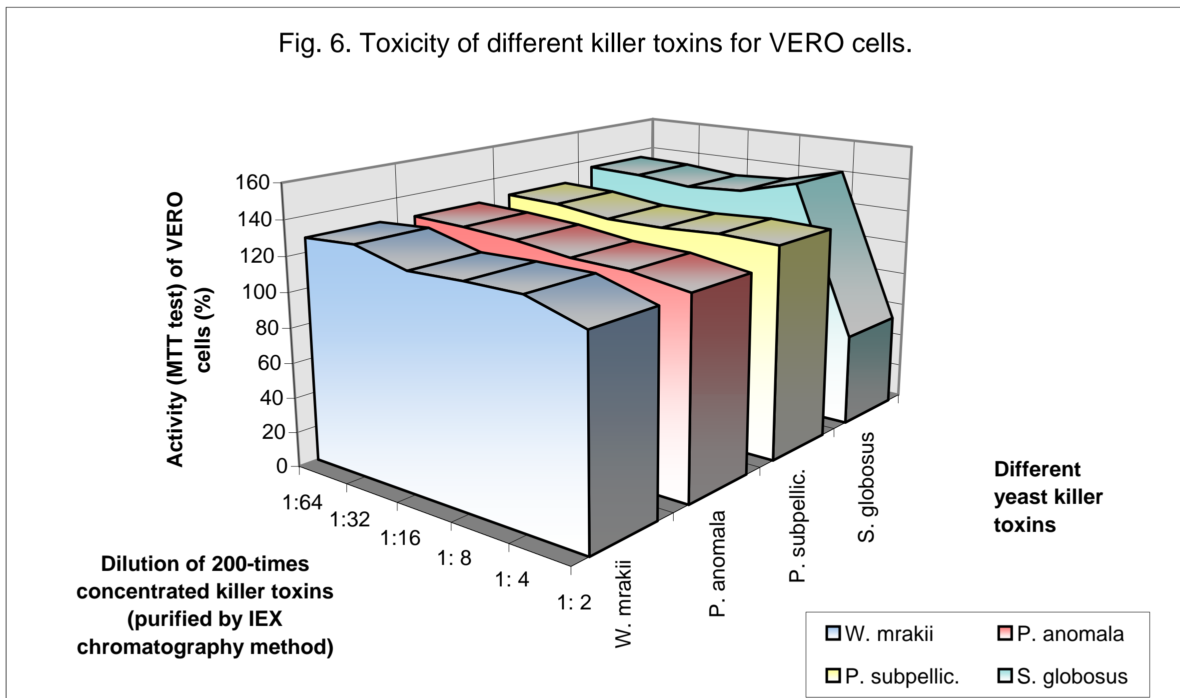


Fig. 6. Toxicity of different killer toxins for VERO cells.
Activity (MTT test) of VERO cells (%)
160
140
120
100
80
60
40
20
0
1:64
1:32
1:16
1: 8
1: 4
1: 2
Dilution of 200-times concentrated killer toxins (purified by IEX chromatography method)
W. mrakii
P. anomala
P. subpellic.
S. globosus
Different yeast killer toxins
W. mrakii
P. anomala
P. subpellic.
S. globosus

Fig. 7. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for HEP-2 cells.

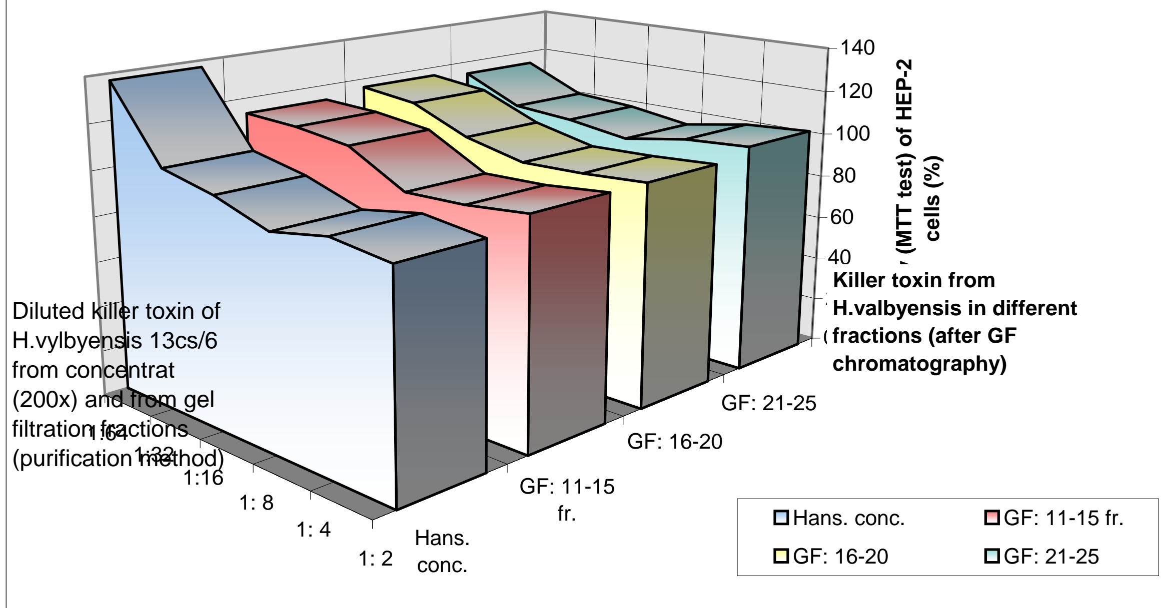
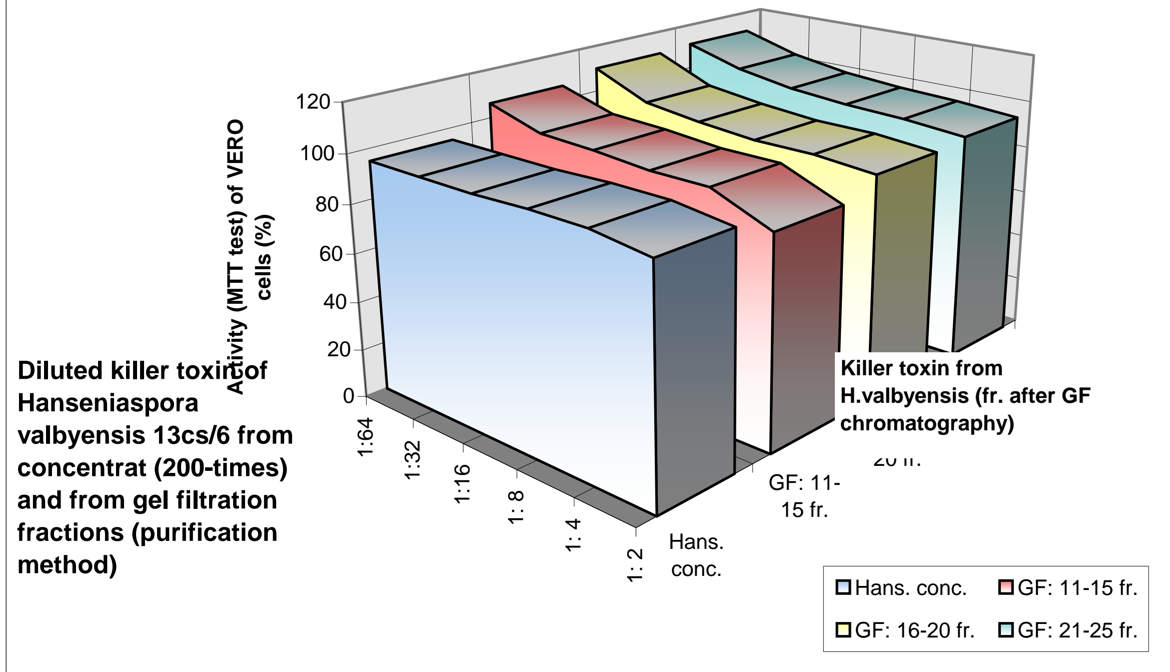


Fig. 8. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for VERO cells.



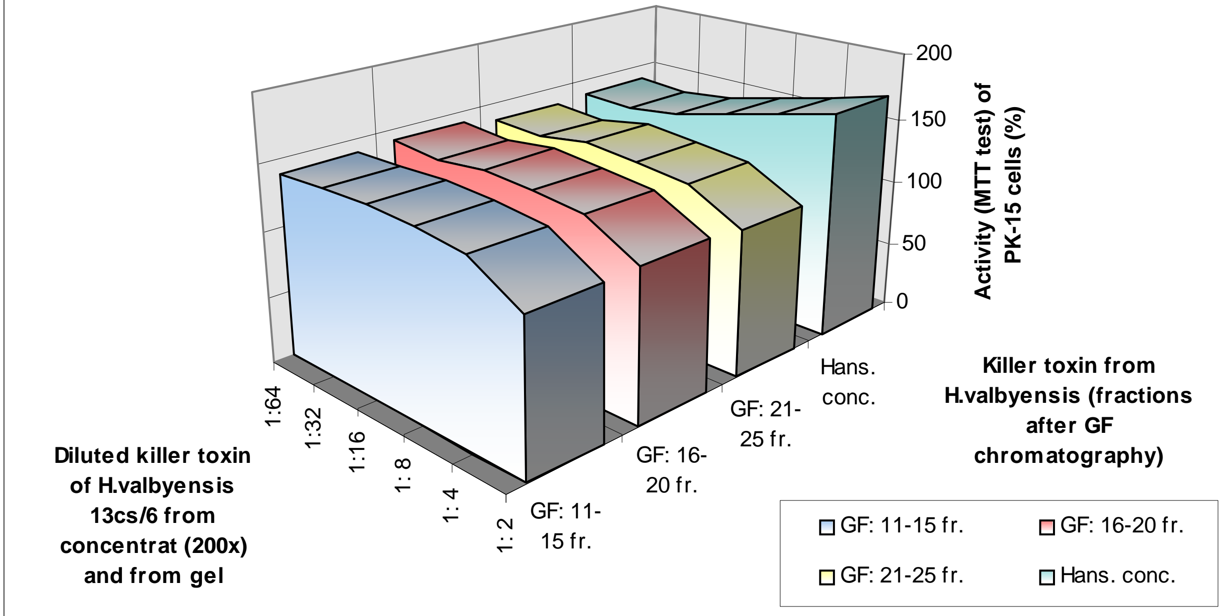


Fig. 9. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for PK-15 cells.

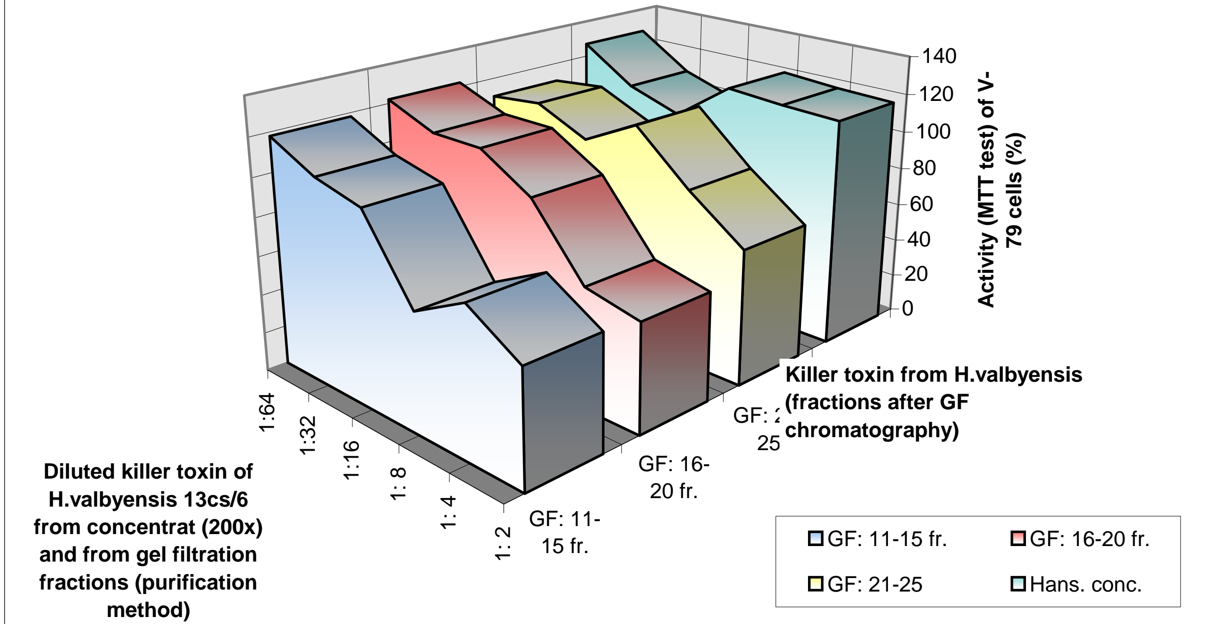


Fig. 10. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for V-79 cells.

Fig. 11. Toxicity of **Shiga-Like-Toxins** from different strains of *Escherichia coli* and yeast killer toxin (fr. 1-10/GF) from *Williopsis mrakii* AS/15.

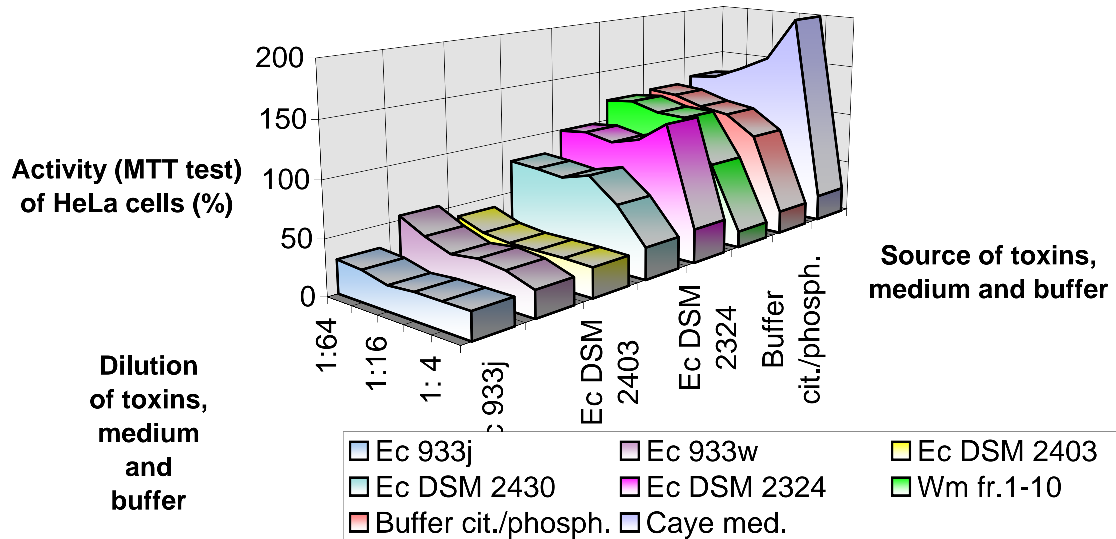


Fig. 12. Receptors binding effect. Pretreatment of **HeLa** cells by yeast killer toxin from *Williopsis mrakii.*

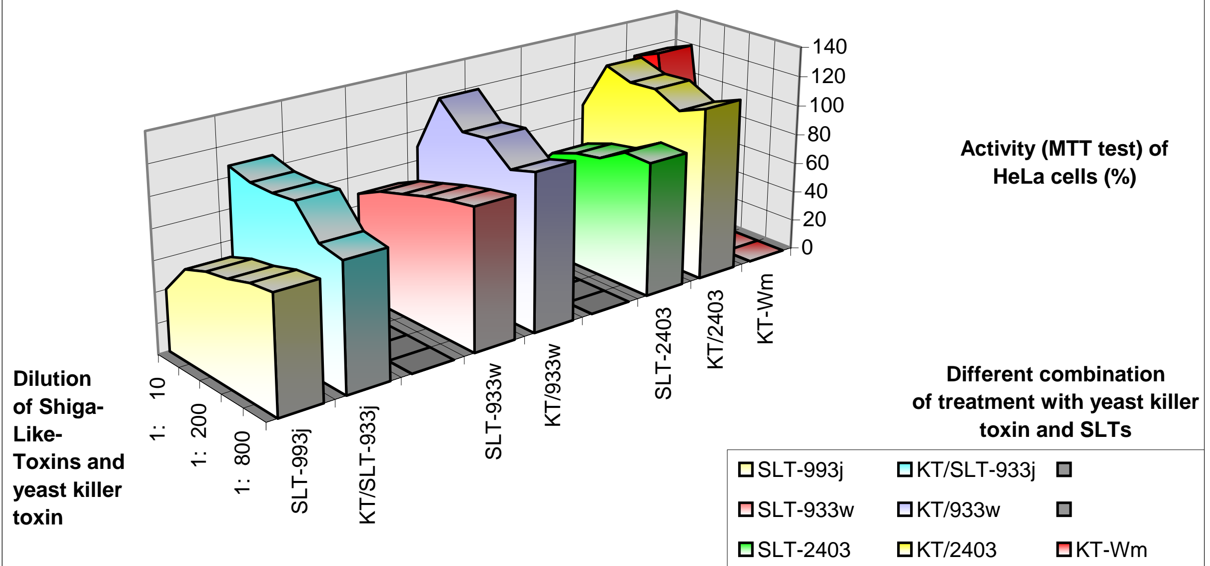


Fig. 13. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Williopsis mrakii*.

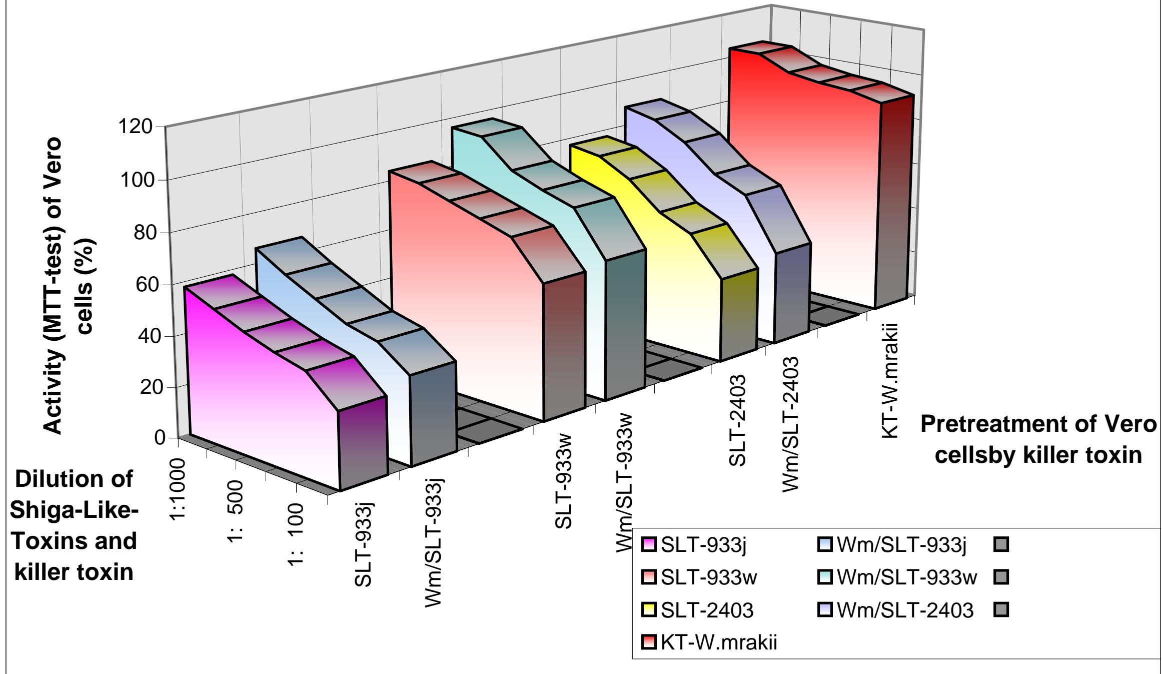


Fig. 14. Receptors binding effect. Pretreatment of **HeLa** cells by killer toxin from *Saccharomyces globosus*.

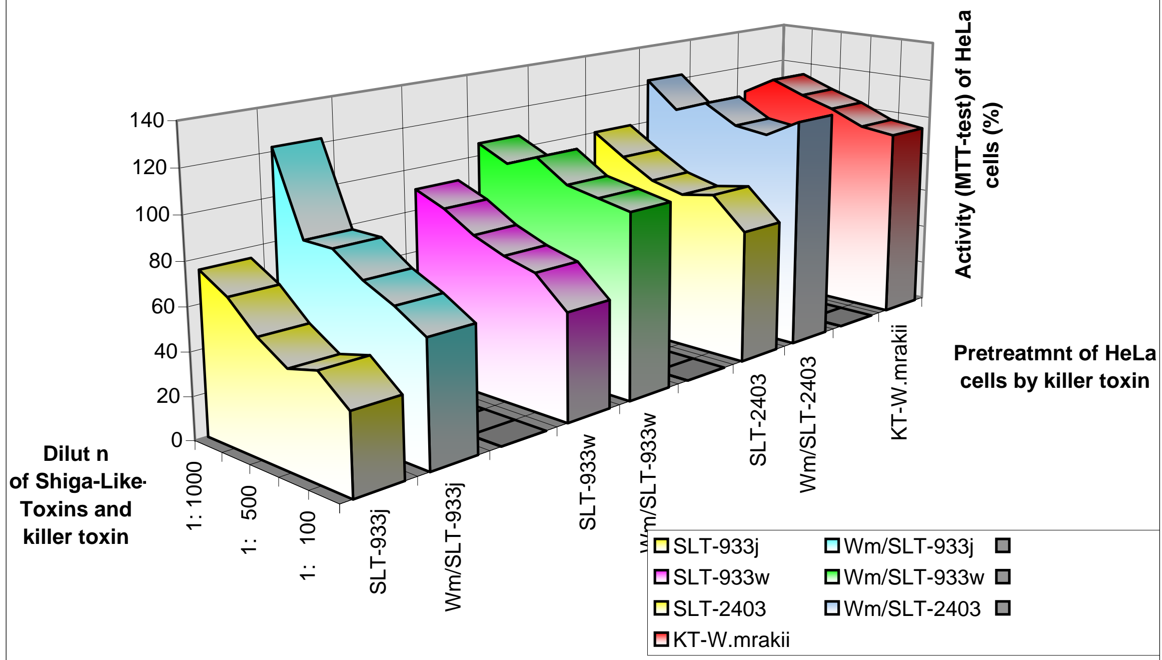


Fig. 15. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Saccharomyces globosus*.