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## New ways to optimize breast cancer treatment

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An in vitro model for purging of epithelial tumor cells  
from cryopreserved ovarian tissue of women with  
impending fertility loss due to cancer treatment

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**Submitted**

## Abstract

**Introduction:** Cancer treatment with chemotherapy and/or radiotherapy for the treatment of cancer can lead to impaired fertility in female patients. Cryopreservation and autografting of ovarian tissue is a promising new method for conserving their fertility. However, tumor cell contamination of the autograft may form a problem. Epithelial tumor cell lysis can be obtained with cytotoxic T cell retargeting through bispecific antibody BIS-1, which has a combined affinity for the T cell receptor and epithelial glycoprotein-2 (EGP-2). The aim of this study was to evaluate tumor cell kill (purging) and morphological follicle survival in an *in vitro* model with activated lymphocytes, BIS-1 and EGP-2 positive tumor cells, in the presence or absence of a thawed ovarian tissue. **Methods:** Thawed human ovarian tissue was carefully rendered into suspension by mechanical and enzymatical treatment. Cells of the MCF-7 breast cancer cell line and activated human lymphocytes were co-incubated for 4 h with/without 0.1 µg/mL BIS-1, in the presence or absence of ovarian suspension. After the purging procedure, MCF-7 cell kill was evaluated directly by means of fluorescent detection of remaining MCF-7 cells. Depletion of growing MCF-7 cells was assessed with an MTT assay, after 5 days. The morphology of ovarian tissue was evaluated immunohistochemically. **Results:** MCF-7 cell kill and depletion of cell growth increased with increasing ratio's of lymphocytes to MCF-7 cells, and the addition of BIS-1 further augmented this increase (to a maximum depletion of growing MCF-7 cells of 89%, SD 11%;  $p < 0.001$  compared to depletion without BIS-1). Follicles remained morphologically intact. **Conclusions:** These results show that purging of epithelial tumor cells from ovarian grafts with BIS-1 is possible *in vitro*. Morphologically, follicles remain intact after this procedure. This method may contribute to the safe replacement of ovarian tissue in female cancer survivors.

## Introduction

In females, chemotherapy and/or radiotherapy for the treatment of cancer can cause a reduction of the follicle population within the ovaries, which can lead to a premature menopause (1-6). The cryopreservation of ovarian tissue obtained before cancer therapy is a promising new method for conserving the own fertility of these cancer patients prior to therapy (7). In animal studies the transplantation of frozen-thawed ovarian autografts has led to a resumption of endocrine function and the restoration of fertility (8-14). In one case-report, the successful re-transplantation of cryopreserved ovarian tissue into a previously oophorectomized woman with a non-malignant disease was described (15). In cancer patients however, there is concern that autografting of ovarian tissue can possibly reintroduce tumor cells (16).

Purging of minor quantities of tumor cells has been described in the hematopoietic stem cell transplantation setting (17, 18), but not for solid (tumor) tissue. The epithelial related membrane antigen (EGP-2) with a molecular weight of 38 kDa is known to be widely expressed on breast and ovarian carcinomas (19, 20). The bispecific antibody BIS-1, directed against EGP-2 on tumor cells and CD3 on T lymphocytes, creates functional cross-linking of T cells and tumor cells allowing the delivery of a tumor cell specific lethal hit inducing specific epithelial tumor cell kill in vitro and in vivo (21, 22). In peripheral blood stem cells, this approach resulted in a >3 log tumor cell depletion without affecting clonogenic potential of the hematopoietic stem cells (23). This study was conducted to evaluate whether solid ovarian tissue, rendered into suspension, can be purged in a similar way as hematopoietic stem cell material. Therefore, tumor cell kill and morphological follicle survival were studied in an *in vitro* model in which activated lymphocytes and BIS-1 were added

to the breast cancer cell line MCF-7, in the presence or absence of a suspension of human frozen-thawed ovarian tissue.

## Materials and methods

### **In vitro model**

#### ***Ovarian tissue***

##### Freezing procedure

Human ovarian tissue, obtained with laparoscopy, was frozen from eligible patients since 1998. The freezing procedure of ovarian tissue for eventual transplantation purposes, was considered part of the regular patient care by the Medical Ethical Committee of our institution; the usage of ovarian tissue for the in vitro purging procedure (as described below), in case of the death of the patient prior to possible transplantation, was approved by the Medical Ethical Committee. All patients gave informed consent. The freezing- and thawing procedure was performed as described by Gosden's group (24). Briefly, after collection in sterile, buffered medium the ovary was cut into two parts under sterile conditions. One part was fixed in buffered formalin and embedded in paraffin after which sections for standard hematoxylin-eosin (HE) staining were cut; the other part was used for preparation of the ovarian cortex. Pieces of the cortex of approximately 3 x 3 mm, about 1 mm thickness were incubated for 30' in Leibovitz L15 medium (Life Technologies, Paisley, Scotland) containing 10% autologous patient serum and cryoprotecting agents (1M dimethyl sulfoxide (DMSO) and 0.1M sucrose). Thereafter, they were cooled to -140°C in a programmable freezer (Planer Kryo 10, series II; cooling with -2°C/min. up to -9°C, manual seeding at -9°C; cooling with 0.3°C up to -40°C followed by cooling with -10°C up to -140°C). Finally, the pieces were stored in liquid nitrogen.



### Thawing and suspension procedure

Ovarian tissue was thawed in a water bath at 37°C for maximally 2 min, and washed in a diminishing sequence of DMSO in Leibovitz medium with 10% fetal calf serum (FCS, Life Technologies, Paisley, Scotland). First, the tissue was mechanically rendered into suspension with 27 G needles under sterile conditions. Then, enzymatical treatment was performed in medium containing 10 U/mL DNase I (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 300 U/mL collagenase IA (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 2 h at 37°C. The obtained cell suspension was transferred into RPMI medium (Life Technologies, Paisley, Scotland) with 10% FCS. Next, into each well of a 96 well microtiter plate (Nunclon, Roskilde, Denmark), 100 µL of the cell suspension was distributed. After an overnight culture the wells were inspected with an inverted phase-contrast microscope. In 5 independent experiments, the wells in which microscopically intact follicles were observed were counted and the total suspension in these wells was used for purging experiments as described below.

### ***Effector- and target cells; bispecific antibody***

#### Target cells: fluorescent detection system

The MCF-7 breast cancer cell line was used as EGP-2 positive tumor model. Cells were plated in microtiter plates (Nunclon, Roskilde, Denmark) and cultured overnight for optimal adhesion. Cells were labeled with the fluorescent dye chloromethyl fluorescein diacetate (CMFDA, Molecular probes Europe BV, Leiden, the Netherlands) for 30 min. CMFDA toxicity was established by the percentage spontaneous cell detachment 24 h after labeling. MCF-7 cells (200, 500 or 1000 cells per well) were incubated with increasing concentrations of CMFDA (0.5, 1, 1.5, 5, 10, and 15 µM). Cell detection was adequate at 1.5 µM, without signs of toxicity, and therefore this concentration was used in subsequent experiments.

### Effector cells

Lymphocytes were obtained from peripheral blood of healthy volunteers by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) isolation, washed and incubated *in vitro* with anti-CD3 antibody (at 0.5  $\mu$ g IgG /ml RPMI medium with 10% FCS) followed after 72 h by washing and a subsequent culture in the presence of recombinant human interleukin-2 (rh IL-2, Aldesleukin, Chiron, Amsterdam, The Netherlands) at 100 IU/ml RPMI medium with 10% FCS for 48 h. Thereafter, cells were washed, counted and resuspended in RPMI medium with 10% FCS. This sequence of adding activating agents was shown earlier to induce T lymphocyte activation (23).

### BIS-1

The BIS-1-producing quadroma was produced in our department by fusion of the hybridomas RIV-9 and MOC-31, producing anti-CD3 (IgG3) and anti-EGP-2 (IgG1) antibodies respectively, according to De Lau et al (24). Preparation and purification was performed as described earlier (20). Briefly, BIS-1 was produced on large scale by means of a hollow fiber culture system (Endotronics, Minneapolis, MN). Purification of the hybrid antibodies (IgG3/IgG1) from parental-type antibodies, also produced by the quadroma, was performed by protein A column chromatography. BIS-1 F(ab')<sub>2</sub> was then produced by means of digestion by pepsin followed by G150 Sephadex gel filtration, and added to a 0.9% sodium chloride solution to obtain a final concentration of 0.2 mg/mL.



### ***Purging procedure***

#### **Tumor cell kill, direct detection**

Lymphocytes (effector cells) were co-incubated in a 96-well plate (Nunclon, Roskilde, Denmark), in the presence of 0.1 µg/mL BIS-1, at 37°C in a humidified, 5% CO<sub>2</sub>-containing atmosphere, with 200, 500 and 1,000 MCF-7 tumor cells (target cells) labeled with CMFDA as described above. Ratio's of effector to target cells were 0:1, 100:1, 500:1 and 1,000:1, in a total volume of 200 µL RPMI medium with 10% FCS. After 4 h of co-incubation the amount of remaining tumor cells was counted directly by means of an inverted fluorescence microscope (Olympus IMT, Tokyo, Japan), at emission wave length 516 nm, and extinction wave length 492 nm. Tumor cell kill was assessed in 5 independent experiments.

The effect of the presence of the ovarian cortex suspension on tumor cell kill efficiency was evaluated comparing tumor cell kill as described above to tumor cell kill in wells to which also 100 µL of ovarian cortex suspension (prepared as described above, after overnight culture) was added, in a total volume of 200 µL of RPMI medium with 10% FCS. The prior fluorescent labeling of tumor cells allowed assessment of tumor cell kill also in the presence of the ovarian cortex suspension. The effect of adding ovarian cortex suspension on tumor cell kill efficiency was assessed with ovarian tissue of 3 patients.

The percentage tumor cell kill was calculated as the amount of untreated tumor cells (control) minus the amount of remaining tumor cells after treatment, divided by control amount of tumor cells, times 100%.

#### **Tumor cell kill, indirect detection**

To evaluate longer-term effects of the purging procedure on the growing potential of tumor cells, lymphocytes (effector cells) were co-incubated in a 96-well plate (Nunclon, Roskilde, Denmark), in the presence of 0.1 µg/mL BIS-1, at 37°C in a

humidified, 5% CO<sub>2</sub>-containing atmosphere, with 2,000 or 5,000 MCF7 tumor cells (target cells). Ratio's of effector to target cells were 0:1, 10:1, 20:1 or 50:1, in a total volume of 200 µL RPMI medium with 10% FCS (similar as described in experiment described above). After 4 h of co-incubation, the supernatant was removed. Cells were washed with fresh RPMI medium with 10% FCS and the supernatant was removed. Fresh medium (200 µL) was added and cells were cultured for 5 days (during which medium was refreshed one additional time) at 37°C in a humidified, 5% CO<sub>2</sub>-containing atmosphere. To establish tumor cell survival/growth after 5 days, the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to a blue formazan product was evaluated in a standard microculture tetrazolium assay (25). DMSO (100%, 200 µL) was used to dissolve the formazan chrystals, and the plate was read in an ELISA reader (Thermo Max, Molecular Devices, Sunnyvale, CA) at wavelength 490 nm. Comparisons were made with wells containing tumor cells and lymphocytes without BIS-1, tumor cells and BIS-1 without lymphocytes, or lymphocytes alone. In this experiment, no ovarian cortex suspension was added, as the described detection method does not allow discrimination between viable tumor cells or viable ovarian cells. Depletion of growing tumor cells was assessed in 4 independent experiments.

The percentage depletion of growing cells was calculated as the number of untreated tumor cells (control) minus the number of remaining tumor cells after treatment, divided by control number of tumor cells, times 100%. Data were corrected for background lymphocyte formazan production.

### Follicle morphology

Morphology and viability of follicles were assessed before freezing or after freezing-thawing of tissue and before and after the purging procedure. Before and after freezing-thawing, pieces of tissue were fixed in buffered formalin, dehydrated through an alcohol series, and paraffin embedded. Before and after the purging procedure, ovarian cortex suspension was spotted on a glass slide. Slides of all material were stained with standard Giemsa staining as well as Periodic Acid Schiff method (PAS, 27), Papanicolau method (PAP, 27), and the MOC31 antibody, directed against EGP-2, for tumor cell presence. Evaluation criteria for morphology on paraffin sections were eosinophilia of ooplasm, clumping of chromatin and wrinkling of the oocyte nuclear membrane as signs of atresia (28).

### **Statistics**

Tumor cell kill, assessed by means of direct (fluorescent) detection or indirect (MTT) detection, was analyzed by means of a two-sided Students' t-test for independent samples. Also the effect of adding ovarian cortex suspension on tumor cell kill was analyzed by means of this test. All analyses were performed with the statistical software program SPSS. A  $p < 0.05$  was considered statistically significant.

## Results

### ***Ovarian tissue***

Ovarian tissue was used from 3 patients. The first two patients were 13 and 17 years old at the time of cryopreservation, and they suffered from acute lymphatic leukemia and an ovarian germ cell tumor of the contralateral ovary, respectively. From the third patient, aged 35 years, cryopreservation of ovarian tissue was performed after prophylactic ovariectomy because of a BRCA1 mutation; this patient gave consent for cryopreservation of ovarian tissue for the purging procedure described here. In the ovarian tissue of the latter patient, no sign of tumor contamination was present.

### ***Purging efficiency***

#### Tumor cell kill, direct detection

Figure 1A reflects the percentage kill of CMFDA labeled tumor cells after lymphocyte induced tumor cell kill in the presence or absence of BIS-1 for 4 h. In the absence of BIS-1, increased tumor cell kill is obtained with effector to target ratio's 500 and 1,000, compared to the control. This kill is further augmented by adding BIS-1, to a maximum at effector to target ratio 1,000 (kill 75.5 %, SD 10.5,  $p=0.002$  compared to kill without BIS-1: 40%, SD 19). Tumor cell kill is similar as described earlier in hematopoietic stem cell harvests (23), with these effector to target ratio's.

The effect of the addition of ovarian tissue on tumor cell kill efficiency is reflected in figure 1B. Tumor cell kill in the presence of BIS-1 is increased with effector to target ratio's 500 and 1,000; there is no difference between tumor cell kill with or without added ovarian tissue.

### Tumor cell kill, indirect detection

In figure 2, the percentage depletion of growing MCF-7 tumor cells is reflected, after a 4 h co-incubation with activated lymphocytes in the presence or absence of BIS-1 and subsequent culture for 5 days. Depletion of growing tumor cells is clearly increased after treatment with activated lymphocytes in the presence of BIS-1, compared to the absence BIS-1. A maximum tumor cell depletion of 89% (SD 11%,  $p < 0.001$  compared to depletion without BIS-1) at effector to target ratio 10 is seen.

### ***Follicle morphology***

#### Effect of freezing

No differences were observed between the tissue that had been passed through the freezing procedure and fresh tissue that was directly embedded in paraffin, when scored with the morphological criteria described in the materials and methods section.

#### Effect of thawing and suspension procedure

The effect of the mechanical and enzymatical suspension procedure after thawing, was evaluated in the ovarian cortex suspension after overnight culture, as described above. The number of wells (in the 96-wells plate) microscopically containing one or more follicles was mean 62, SD  $\pm 30$ . The suspensions in these wells that were not used for purging experiments, were paraffin embedded and scored with the morphological criteria described above. Intact follicles were detected in these suspensions, and a representative sample of frozen-thawed ovarian suspension is shown in figure 3.

Effect of purging

Morphological evaluation of the suspension including follicles, lymphocytes and tumor cells after the purging procedure, scored with the morphological criteria described above, revealed intact follicles remaining. A representative picture is shown in figure 4. No MOC31 positive tumor cells were detected after the purging procedure.

## Discussion

Improvement in anticancer therapies has resulted in more long-term survivors. This has increased the awareness of long-term effects, such as gonadal failure (1-6). As there are only few possibilities to limit the toxic effect of chemotherapy and radiotherapy on ovarian function (29-31), there is a growing need to study the possibilities of ovarian protection and preservation.

Cryopreservation of ovarian tissue is a potential method to maintain fertility in females. In recent years, the procedure for freezing and thawing of ovarian tissue seems to be established (32). Primordial follicles, abundantly present in ovarian tissue of young women, were shown to survive the cryopreservation procedure relatively well (32). Restoration of fertility and endocrine function after the transplantation of cryopreserved ovarian tissue was shown in animals (8-14), and recently in a young woman with a non-malignant disease (15). In cancer patients however, the concern that autografting of ovarian tissue can possibly reintroduce tumor cells appears justified (16, 29). This issue would be resolved if primordial follicle isolation and subsequent *in vitro* maturation were possible. However, this technique is still in its infancy (33). Alternatively, one of the procedures for autografting of cryopreserved ovarian tissue involves reinsertion of a primordial follicle suspension in plasma clots. In the sheep model, this procedure already induced restored estrogenic activity and fertility (32). The preparation of a suspension of isolated follicles introduces the potential to clear, or purge the suspension from possible tumor cells. The same method for purging tumor cells as designed for peripheral blood stem cell harvests (23), may be applied to a suspension of follicle material. With this method, using bispecific antibody BIS-1 to retarget activated lymphocytes, specific tumor cell kill of > 3 logs was obtained while hematopoietic stem cell function remained intact. Since we demonstrated the

effectiveness of tumor cell purging in a breast cancer cell model, this was applied to the cryopreserved ovarian tissue setting also in this study. Moreover, this purging concept may very well be applicable to other tumor types such as B-cell lymphoma, which is sensitive to immunological treatment with monoclonal antibody rituximab (34), and for which a bispecific antibody was also developed in our institution (35).

The cryopreservation and thawing of ovarian tissue in this study was performed according to protocols described by the pioneering group of Gosden (24). The integrity of frozen-thawed follicles after enzymatic isolation, was confirmed by electron microscopy evaluation previously (24). Our results, showing morphologically intact follicles after thawing by light microscopy, are in line with this. For the purging procedure, subsequent to the thawing, a fluorescent detection system was developed to evaluate tumor cell depletion. As our ultimate aim is to culture the suspension of ovarian tissue after the purging procedure, the Chromium<sup>51</sup> release assay, commonly used to evaluate tumor cell depletion, was considered inadequate. With the fluorescent detection system, highly efficient tumor cell kill by activated lymphocytes in the presence of BIS-1 was demonstrated. No (adverse) effect of the presence of ovarian tissue on tumor cell kill was observed, and morphologically intact follicles were detected following the purging procedure.

This study supports the concept that solid tissue, rendered into suspension, can be purged in a similar way as hematopoietic stem cell material. In this in vitro setting to provide proof of principle, no adverse effect of the purging procedure on the morphological aspect of ovarian follicles was found. Future studies will address the important issue of the quantitative and functional survival of the follicles, according to studies performed by Hovatta et al. (28, 36, 37). Also, the potential of the above described method to clear tumor cells from a suspension of ovarian tissue with endogenous tumor cell infiltration will be investigated. As the enzymatic isolation of follicles most likely renders the endogenous tumor cells accessible for



lymphocyte cell kill, similar results are expected as in the described setting with the addition of exogenous tumor cells. To avoid potential aspecific lymphocyte activity directed against the ovarian tissue, the use of autologous patient lymphocytes will probably be preferred in a future patient related setting, although no such activity was observed in this study. In reference to the cell model chosen in this study, one might argue that restoring endocrine function and fertility is undesirable in breast cancer patients, because of possible hormonal growth stimulation of residual disease. However, there is no evidence so far that a pregnancy after breast cancer treatment increases the risk of poor prognosis (6, 38). With regards to the relevance of this study with a breast cancer cell model, it should be noted that a considerable number of breast cancer patients is diagnosed in childbearing years. In the Netherlands, this amounts to  $\pm 1000$  patients per annum: approximately 10% of women yearly diagnosed with breast cancer (39). Together with the trend towards postponed childbearing (40), preservation of fertility for these young cancer patients may become an issue of increasing importance.

Concluding, this study provides a first step into the direction of purging cryopreserved ovarian tissue from tumor cells. This would imply that patients with an increased risk of tumor cell contamination of the ovary, do not have to be excluded from gonadal cryopreservation beforehand. The safe replacement of ovarian tissue in female cancer survivors to restore their endocrine function and fertility, would be a major step forward in the improvement of the quality of life for these women.

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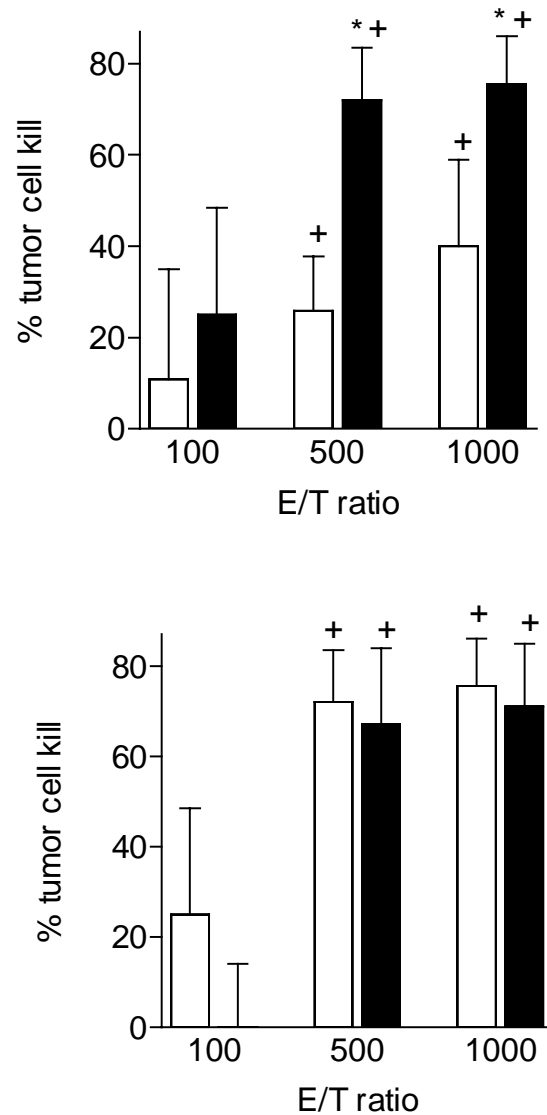
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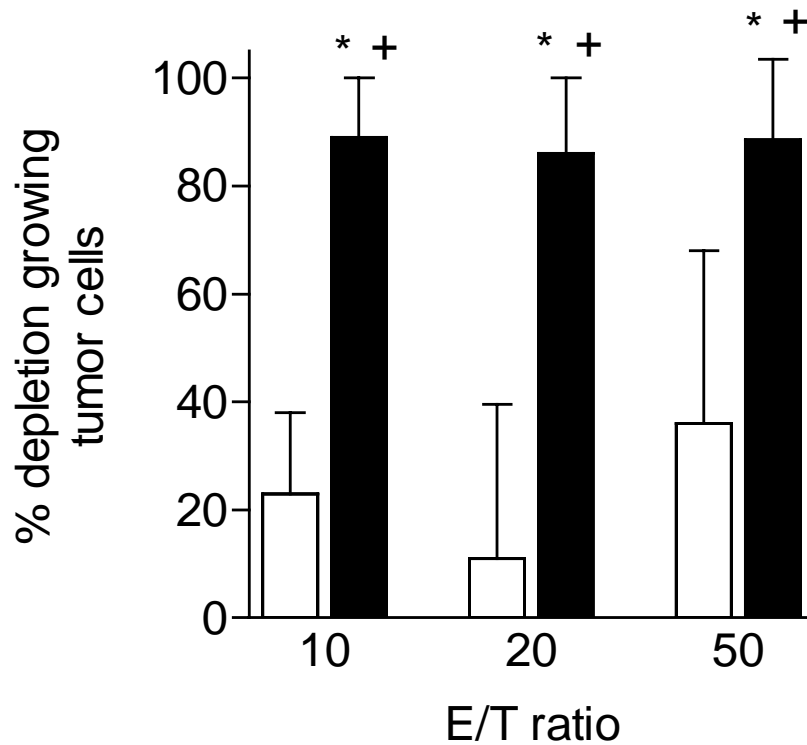
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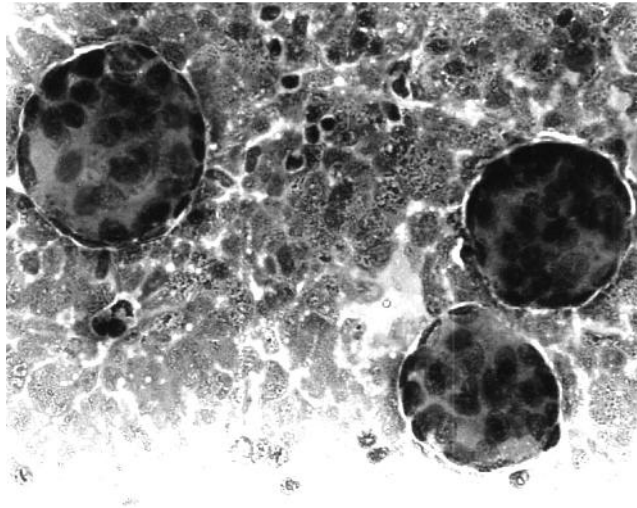
**Figure 1:** Direct assessment of tumor cell kill by fluorescent cell detection

X-axis: effector:target ratio's; Y-axis: percentage tumor cell kill of CMFDA labeled MCF7 tumor cells after 4 h co-incubation with activated lymphocytes, relative to the kill in untreated MCF7 cells. An (\*) reflects a significant difference between the white and black bar; a (+) reflects a significant difference with the untreated control sample (kill 0%). **A:** effect of BIS-1. White bar: absence of BIS-1; black bar: presence of BIS-1. **B:** effect of ovarian cortex suspension in the presence of BIS-1. White bar: absence of ovarian cortex suspension; black bar: presence of ovarian cortex suspension.



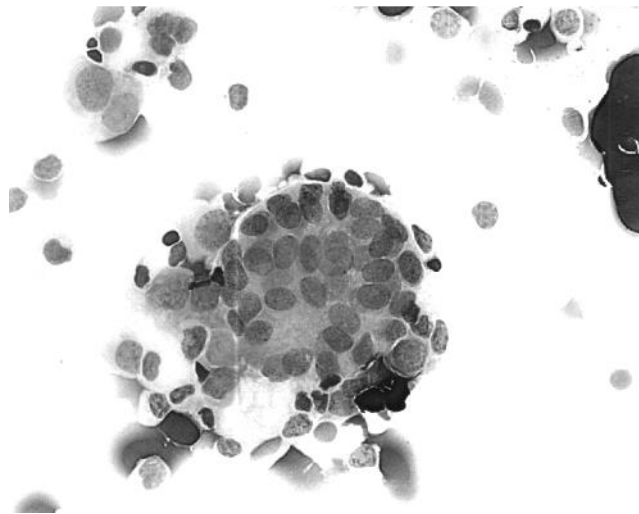
**Figure 2:** Indirect assessment of tumor cell kill by MTT assay

X-axis: effector:target ratio's; Y-axis: percentage depletion of growing MCF7 tumor cells after 4 h co-incubation with activated lymphocytes, and subsequent culture for 5 days, compared to tumor cell depletion in untreated MCF7 cells. White bar: absence of BIS-1, black bar: presence of BIS-1. An (\*) reflects a significant difference between the white and black bar; a (+) reflects a significant difference with the untreated control sample (depletion 0%).



**Figure 3:** Effect of freezing-thawing procedure on follicle morphology

Shown is a representative piece of frozen-thawed ovarian tissue, at 40x10 magnification, with PAS staining. Three intact follicles are shown.



**Figure 4:** Effect of purging procedure on follicle morphology

Shown is a representative part of frozen-thawed ovarian tissue, after the purging procedure including activated lymphocytes and BIS-1, at 40x10 magnification with PAP staining. One intact follicle as well as lymphocytes are shown.





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