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Enhancement of cytotoxicity of ribosome-inactivating-protein type I by saponinum album is not based on stimulation of phagocytosis

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Abstract

Saponinum album, a mixture of triterpenoic saponins derived from *Gypsophila* species, led to an increased internalization of agrostin, a ribosome-inactivating-protein (RIP) type I in U-937 cells differentiated with interferon-γ or phorbol myristate acetate. Treatment with agrostin only showed no cytotoxicity. It was hypothesized that saponinum album stimulated phagocytosis and by that the uptake of agrostin. For this purpose phagocytosis experiments with Alexa-Fluor-488-labelled 1-μm amino-latex beads and FITC-labelled *Escherichia coli* (K-12 strain) were performed. The results indicated no stimulation of phagocytosis by treatment with saponinum album.

Introduction

Agrostin is a 27 kDa ribosome-inactivating-protein type 1 (RIP-1) which is synthesized by *Agrostemma githago* L. It cleaves an essential adenine residue from the rRNA, leading to inhibition of protein synthesis (Nielsen & Boston 2001). Despite its enzymatic activity, agrostin has no cytotoxicity in ECV-304 cells. Only the combination with special triterpenoic saponins, like saponinum album isolated from *Gypsophila* species with a formyl-function attached to position 4 of the aglycon, was cytotoxic in ECV-304 cells (Hebestreit & Melzig 2003).

There have been several reports on saponins showing improved cell membrane passage of different drugs. Accumulation of cisplatin in human colon carcinoma cells was achieved by combination with triterpenoic saponins, which were glycosides of quillaic acid (Gaidi et al 2002). Increased accumulation of heparin was observed upon treatment with triterpenoic saponins (Cho et al 2003). Similar effects were shown for amino glycosides, insulin (Chao et al 1998) and 4´-O-tetrahydropyranyldoxorubicin (Sasaki et al 1994). Uptake of β-lactoglobulin (a milk allergen) was also significantly higher from the gastrointestinal tract when administered together with *Gypsophila* saponins (Gee et al 1997). In addition to these effects, phagocytic activity could be stimulated in the macrophage cell line RAW 264.7 upon treatment with steroid saponins (Zhang et al 2007) and phagocytosis of β-amyloid peptide was enhanced after treatment of microglial cells with triterpenoic saponins (Joo & Lee 2005). However, the exact mechanism of the saponin effect, leading to an increased uptake of drugs or the enhancement of phagocytosis, remains unclear.

Former studies postulated the formation of micelles of the saponins with cholesterol, leading to an increased permeability through the cell membrane (Gögelein & Hübny 1984) or the creation of pores in the cell membrane (Francis et al 2002) at higher saponin concentrations.

Bachran et al (2006) showed that saponinum album from *Gypsophila* species increased cytotoxicity of a chimeric toxin composed of saporin, also a ribosome-inactivating toxin, and human epidermal growth factor (EGF) by more than 13000-fold at a lower non-permeabilizing concentration (1.5 μg mL^{-1}). This effect was strongly receptor specific, indicating a special mechanism triggered by saponinum album, which led to the internalization of the chimeric toxin.

In this study, experiments with mixtures of agrostin and saponinum album were performed and we tried to correlate cytotoxicity of these mixtures with potential immune

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modulating properties of saponinum album. For this purpose U-937 cells were differentiated with interferon-γ (IFN-γ) or phorbol myristate acetate (PMA) to macrophage-like cells, and we investigated the influence of saponinum album on phagocytosis of Alexa-Fluor-488-labelled 1-μm amino-latex beads and FITC-labelled *Escherichia coli* (*E. coli*, K-12 strain), both either opsonized with or without IgG.

In considering the therapeutic use of saponinum album as an immune modulating drug, time course experiments were especially performed for IFN-γ-treated U-937 cells. Fluorescence was measured by fluorescence-activated cell sorting (FACS) and phagocytosis was examined by epifluorescence microscopy.

Materials and Methods

Agrostin, PMA, human immunoglobulin G (IgG), phenazine methosulfate (PMS), and (sodium 3,3′-([(phenylamino)carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene sulfonate (XTT) were obtained from Sigma. Human interferon-γ (hIFN-γ) was purchased from Roche; Alexa-Fluor-488-2,3,5,6-tetrafluorophenylester (5-TFP), fluorescein-labelled *E. coli*- BioParticles, BioParticles opsonizing reagent (polyclonal IgG) were obtained from Molecular Probes. Polybead amino microspheres 1-μm were purchased from Polyscience, Germany, whilst fibronectin was obtained from Biochrom, Germany. Saponinum album (Erg.B.G, Art. No. 7695) was supplied by Merck, Germany.

HPLC

Saponinum album was separated by HPLC with a Wellchrome instrument (Knauer, Berlin, Germany) using UltraSep ES PHARM RP18E (7 μM) from SepServ (Berlin, Germany). Elution was performed with methanol (A) and trifluoracetic acid in water (0.01%) (B), starting with 20% A for 30 min, 70% A for an additional 40 min and finally 85% A after 70 min. The fractions collected from 39 to 45 min were used for the experiments.

XTT reduction assay

Long-term cell viability was determined using the XTT reduction test with PMS as mediator. First of all 96-well plates were coated with 35 μL fibronectin solution (75 μg mL^{-1}) per well for 30 min at room temperature. After washing with phosphate-buffered saline (PBS, 100 μL), U-937 cells were plated at a density of 50000 cells in 100 μL RPMI 1640 with 10% FBS without phenol red. PMA solution 20 μL (final concentration 20 nM) was added and cells were incubated for 72 h. Alternatively, 20 μL hIFN-γ solution was added (final concentration 1000 U) and cells were incubated for 48 h.

After differentiation with IFN-γ or PMA, cells were washed with PBS (100 μL) and non-differentiated U-937 cells were washed away. Thereafter 100 μL RPMI 1640 with 10% fetal bovine serum (FBS) per well was added. After the addition of 10 μL agrostin/saponinum mixture to each well (final concn 10 pM for agrostin and 4 μg mL^{-1} for saponinum album), cells were incubated for a further 48 h at 37°C and 5% CO_2 in a humidified atmosphere. Finally, 50 μL XTT solution (1 mg mL^{-1}) including 8 μg mL^{-1} PMS was added to each well and cells were incubated for 3 h at 37°C in the dark. All dilutions were made with RPMI 1640 (10% FBS) and absorbance of the coloured formazan was measured at 580 nm in a microplate reader (Tecan Spectra Fluor, Germany).

Cell culture

U-937 cells (ATCC-No. CRL-1593.2) were purchased from DSMZ Braunschweig, Germany. Cells were cultured in RPMI 1640 medium without phenol red supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Fluorescence labelling of amino microspheres and preparation of *E. coli* BioParticles

Polybead-amino microsphere solution 160 μL (~7.3×10^9 microspheres) was added to 450 μL distilled water, then 100 μL $NaHCO_3$ solution (1 M, pH 9) and 40 μL Alexa-Fluor-488 5-TFP solution (0.2 mg) were added. The whole mixture was swirled for 3 h in the dark, centrifuged at 13000 *g*, washed three times (1 mL distilled water), and the microsphere pellet was finally taken up in 1 mL distilled water. Labelling was examined by fluorescence microscopy. A 0.5-mL sample was incubated with 0.5 mL (5 mg mL^{-1}) of an IgG solution (PBS). The mixture was swirled for 1 h in the dark, washed three times with 1 mL distilled water and centrifuged each time at 13000 *g*. Before the phagocytosis assay, particles were vortexed and sonicated at 50 kHz for 1 min.

FITC-labelled *E. coli* (10 mg) were suspended in 0.5 mL PBS. The number of *E. coli* bacteria was determined using a haemocytometer (~4×10^9 mL^{-1}). The opsonization procedure with the opsonizing reagent (polyclonal IgG) was done as described in the user manual (Molecular Probes).

Phagocytosis assay

The 24-well-plates were covered with a fibronectin solution (PBS, 75 μg mL^{-1}) for 30 min at room temperature. After washing with PBS, U-937 were plated at a density of 10^6 in 450 μL RPMI 1640 per well. After addition of 50 μL hIFN-γ solution (PBS, final concentration 1000 U), cells were incubated for 48 h. Alternatively cells were treated with 50 μL PMA solution (PBS) per well (final concn 20 nM) and incubated for 72 h. Medium was removed and cells were gently washed with prewarmed PBS (0.5 mL). Medium (450 μL) supplemented with 4 μg mL^{-1} saponinum album was added to each well. Control cells were incubated in 450 μL medium without saponinum album. After addition of 50 μL of a diluted (PBS) particle suspension (FITC-*E. coli* or Alexa-Fluor-488-microspheres) either opsonized or not, IFN-γ cells were further incubated for 1, 2, 3, or 6 (IFN-γ-treated cells) or for PMA-treated cells only 3 h. (The cell/particle ratio was as follows: *E. coli* and IgG-*E. coli* 1/12, amino-latex and IgG-amino-latex particles 1/100). The particle-containing medium was removed and cells were trypsinized (200 μL/well). PBS 200 μL supplemented with 10% FBS was added to each well to stop trypsination. The mixture was aspirated and each well was rinsed off by

pipette (200 μL) and samples (400 + 200 μL) were subsequently combined. Finally three wells were mixed (final volume 1.8 mL) and loaded to a FACS tube.

Flow cytometry and fluorescence microscopy

Flow cytometry was performed using a FACScalibur (Becton Dickenson), equipped with an air-cooled 15 mW argon laser. Excitation was at 488 nm and a band-pass 530/30 (BP) filter was used to separate green fluorescence of FITC-labelled *E. coli* and Alex-Fluor-488-labelled microspheres. A minimum of 10000 cells per sample was acquired and data was collected by CellQuest. Analysis of the data was performed using WinMDI 2.8 software. Pictures were collected with an epifluorescence microscope (BX 41, Olympus, Japan), equipped with a digital camera (DXM1200; Nikon, Japan).

Statistical methods

Data from the cytotoxic experiments were analysed by the Mann–Whitney U-test. Data obtained from flow cytometry were analysed by the Wilcoxon-rank-sum test.

Results

Characterization of the saponinum album

Saponinum album is a mixture of different triterpenoic saponins isolated from *Gypsophila* species according to the former German Pharmacopoeia DAB7 monograph Saponinum. The main saponin fraction used in our experiments was separated by HPLC (39–45 min; Figure 1). It was analysed by ^{1}H NMR according to Frechet et al (1991) with similar results. The saponins are characterized by a formyl-function attached to position 4 of the aglycon, an acidic sugar chain bound at position 3 and a second sugar chain in position 28.

Cytotoxicity

Figure 2 represents cytotoxicity of saponin/agrostin mixtures in U-937 cells, which were differentiated (IFN-γ, PMA) before the cytotoxic experiments. Cytotoxicity of agrostin alone (hatched columns) and saponinum album alone (open columns) could be excluded for differentiated U-937 cells. The combination of saponinum album with agrostin was strongly cytotoxic in IFN-γ- and PMA-treated U-937 cells (Figure 2, solid columns), indicating a cytotoxic synergism.

Particle labelling with Alexa-Fluor-488

Figure 3A represents the fluorescence histogram of amino-latex particles which were labelled with Alexa-Fluor-488-5-TFP. Figure 3B shows the appropriate photograph of the labelled particles.

Stainability of the particles was uniform as demonstrated by the Gaussian distribution of the particles in the fluorescence histogram. This was a prerequisite for the measurement of the phagocytosis because an irregular staining pattern of particles would lead to the division of the cell population when measured by FACS.

Phagocytosis assay

Figure 4A shows the flow histogram of IFN-γ-treated cells, which were incubated with IgG-amino-latex particles for either 2 (dotted overlay) or 3 h (black overlay, see arrow). The histogram represents auto-fluorescence of IFN-γ-treated U-937 cells. Comparing the auto-fluorescence, the longer the incubation time with particles the stronger the shift towards higher fluorescence values due to increased phagocytosis. For evaluation, a marker (M1) was set and median fluorescence with the appropriate coefficient of variation (CV) of this region (M1) was determined. As seen in Figure 4B median fluorescence increased over 6 h.

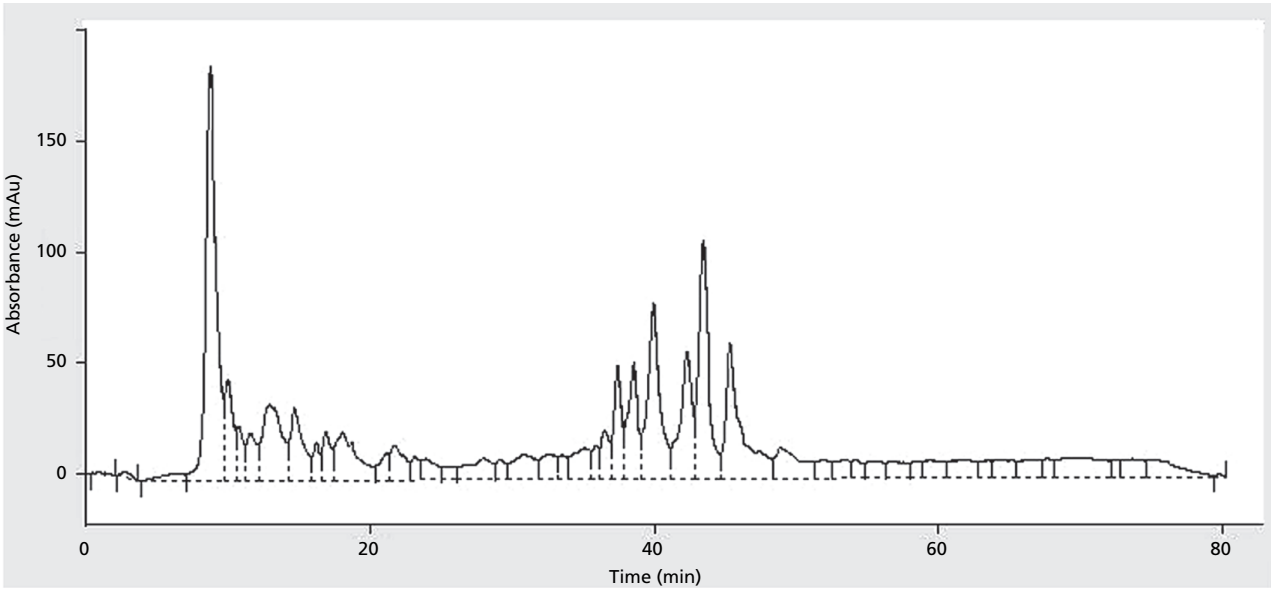

Figure 1 HPLC diagram of saponinum album. For the experiments the fractions collected from 39 to 45 min were used.

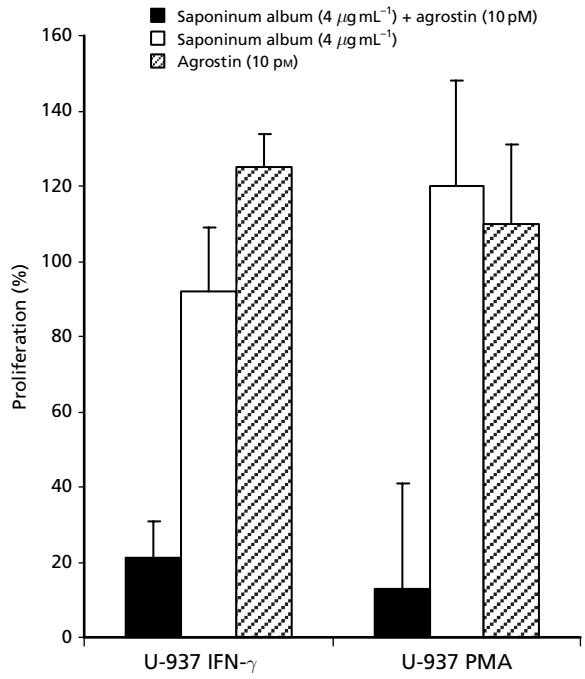

Figure 2 U-937 cells were differentiated either with IFN-γ (1000 U, 48 h) or with PMA (20 nM, 72 h). After washing (PBS, 100 μL), 100 μL RPMI with 10% FBS was added to each well. After addition of 10 μL agrostin/saponin mixture (final concn 10 pM; 4 μg mL^{-1}) to each well, cells were further incubated for 48 h at 37°C and 5% CO_2. XTT solution 50 μL (1 mg mL^{-1}) including 8 μg mL^{-1} PMS was added to each well and cells were incubated for 3 h in the dark. Absorbance was measured at 580 nm in a microplate reader. All values refer to those values (100% values) obtained from cells which were only treated with IFN-γ or PMA. U-Test, $P = 0.05$.

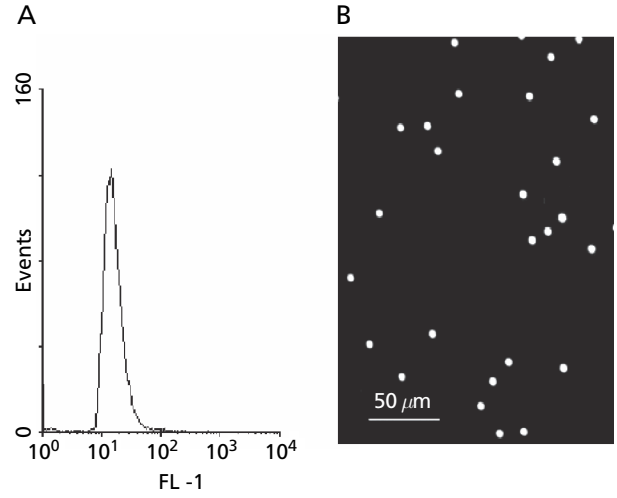

Figure 3 (A and B) Polybead-amino microspheres (7.3×10^9) were suspended in 450 μL distilled water, and 100 μL $NaHCO_3$ solution (1 M, pH 9) and 40 μL (0.2 mg) Alexa-Fluor-488-5-TFP were added. The mixture was swirled for 3 h in the dark, spun down at 13000 *g* and washed three times with distilled water (1 mL). Particles were sonicated (50 kHz) and a sample was loaded to a FACS tube. Green fluorescence was measured using FACScalibur and labelling was examined by fluorescence microscopy (B). Note the Gaussian staining pattern in the flow histogram (A).

There was no significant difference in phagocytosis between saponinum album (4 μg mL^{-1})-treated and control cells (Figure 4B). Non-opsonized particles were not phagocytized (data not shown). Cells were examined by fluorescence microscopy (Figure 4C).

Figure 5 represents phagocytosis of *E. coli* over 6 h in IFN-γ-treated U-937 cells. Evaluation was as described above (flow histograms not shown). There were no differences between saponinum album (4 μg mL^{-1})-treated and control cells. Similar results were obtained for IgG-coated *E. coli* (Figure 5). No differences in phagocytosis could be observed between saponinum album-treated and control cells. Phagocytosis of *E. coli* was also proved by fluorescence microscopy (upper right in Figure 5).

We investigated the phagocytosis of PMA-treated cells, which were incubated with or without saponinum album (4 μg mL^{-1}) (Figure 6). Since there was no stimulation effect of saponinum album on phagocytosis over 6 h (see above), we incubated PMA-treated cells for 3 h with the indicated particles. Evaluation was as described above. However, as seen in Figure 6 no differences between saponinum album-treated and control cells could be detected. Non-opsonized particles were not phagocytized (data not shown).

Discussion

The effect of saponinum album, a mixture of triterpenoic saponins present in *Gypsophila* species, on phagocytosis in U-937 cells, which had been differentiated with either hIFN-γ or PMA to macrophage-like cells (Harris et al 1985) was investigated. There was a strong cytotoxicity of the agrostin/saponinum album mixture indicating a special mechanism which led to the internalization of agrostin molecules. Toxicity of saponinum album and agrostin was negligible (Figure 2). However, because undifferentiated U-937 cells were not susceptible against the indicated agrostin/saponin mixtures (data not shown) we hypothesized that maybe the increase in agrostin/ saponin susceptibility was paralleled by an increase in phagocytic activity triggered by saponinum album. The impairment of latrunculin, an inhibitor of phagocytosis (De Oliveira & Mantovani 1988) on the cytotoxicity of agrostin/saponin mixtures (Hebestreit et al 2006) especially pointed to the involvement of phagocytic processes. Similar effects were observed for bafilomycin A1, an inhibitor of vacuolar ATPase. Bafilomycin A1 inhibited phagocytosis in macrophages (Bidani & Heming 1995) and also hampered cytotoxicity of agrostin/saponinum album mixtures (Hebestreit et al 2006). To investigate the immune modulating properties of saponinum album more precisely we performed a phagocytosis assay.

The phagocytosis assay was performed without quenching fluorescence of adherent particles on the cell surface with trypan blue and we did not work at 4°C to stop phagocytosis at the end of the assay. For the absolute measurement of phagocytosis the assay would be therefore defective. However, our purpose was to detect differences between two cell

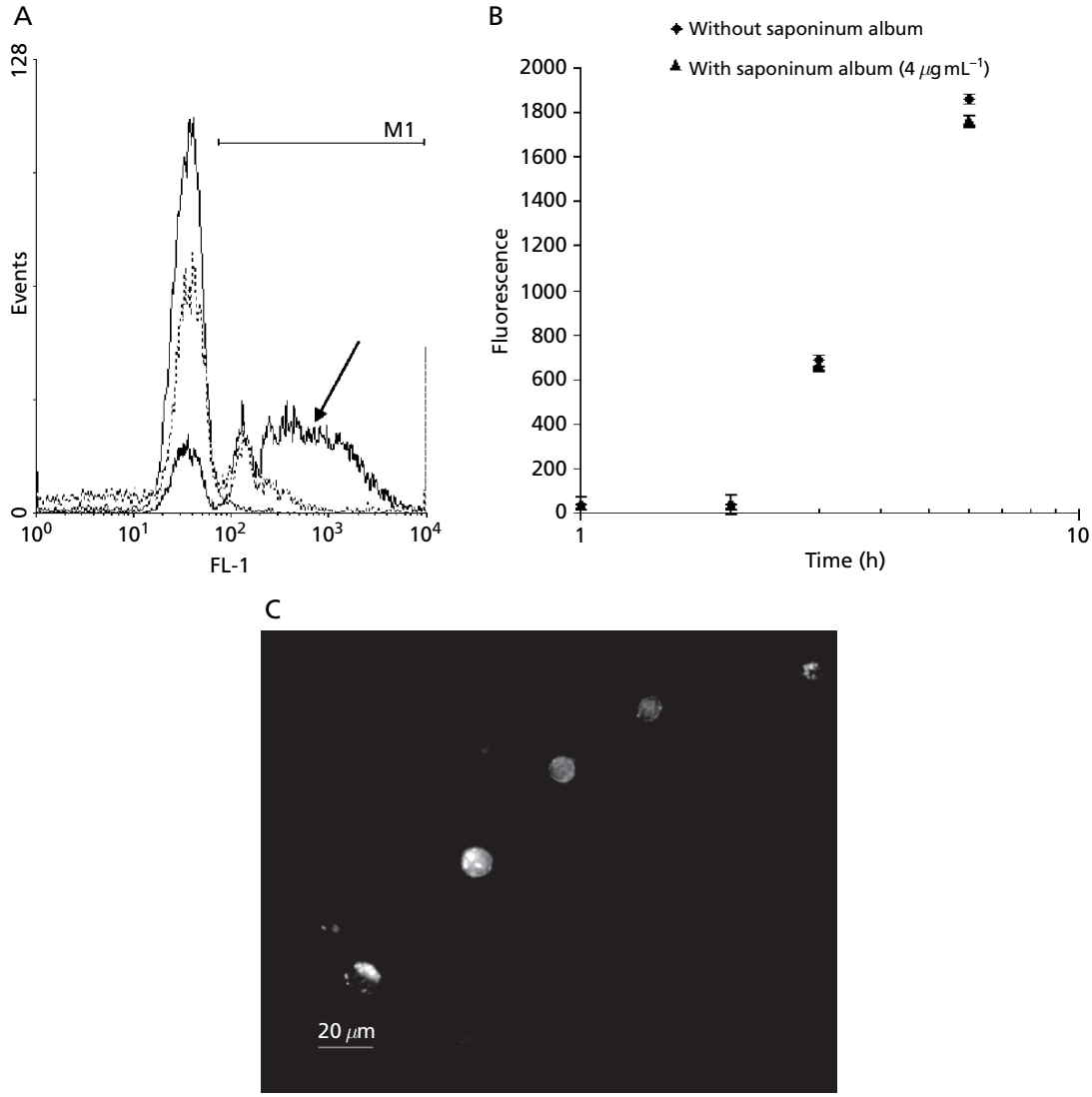

Figure 4 (A–C) U-937 cells were differentiated with IFN-γ (48 h) (see Materials and Methods) and incubated without (control cells) or with 4 μg mL^{-1} saponinum album. After addition of the IgG-amino-latex particles, cells were incubated for 1, 2, 3 or 6 h (cell/particles = 1/100). A. Flow histogram obtained from U-937 cells incubated for 2 (dotted overlay) or 3 h (black overlay, see arrow). The histogram represents auto-fluorescence of U-937. The longer the incubation time with particles the stronger was the shift towards higher fluorescence values. For evaluation a marker was set (M1) and median fluorescence of this region M1 was determined. B. The increase of the median fluorescence over 6 h. There were no significant differences in phagocytic activity between control and saponinum album-treated U-937 cells (rank sum test, $P = 0.05$). The phagocytosis was examined by fluorescence microscopy (C).

populations of the same origin. We assumed that the treatment of samples would be the same, the ratio of errors between control and saponinum album-treated cells would be constant. Differences in phagocytic activity should be therefore detectable and an examination of phagocytosis by fluorescence microscopy would be sufficient.

Since treatment of U-937 cells with IFN-γ leads to a strong increase of immune receptors, e.g. Fc-receptor γ (Guyre et al 1983), it seemed reasonable that the increased susceptibility of differentiated U-937 against agrostin/saponin mixtures was due to an up-regulation of immune receptors leading to an increased uptake of agrostin molecules. However, we could not correlate the increased uptake of agrostin molecules with an increased phagocytic activity after treatment of cells with saponinum album. Similar results were obtained for PMA-treated cells.

Regarding the concentration of agrostin (10 pM) and saponinum album (4 μg mL^{-1}) used in this study it was likely that the internalization of agrostin facilitated by saponinum album was due to a specific mechanism. This was shown for the cytotoxicity of constructed immunotoxins, which was increased by more than 10 000-fold when immunotoxins were administered simultaneously with saponinum album (Bachran et al 2006), which was likely due to the induction of endocytosis since phagocytosis was not affected by saponinum album (see above).

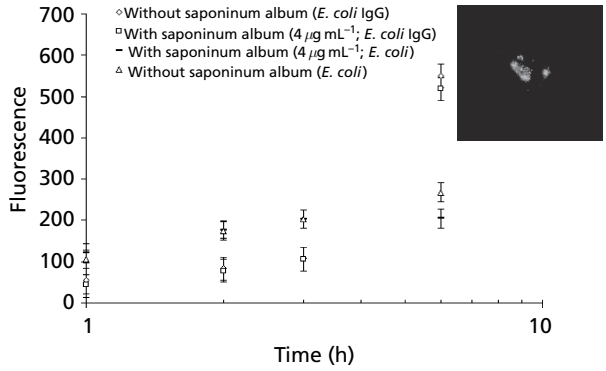

Figure 5 U-937 cells were differentiated with IFN-γ and prepared for flow cytometry (see Materials and Methods) with the exception of adding 50 μL *E. coli* suspension either opsonized (*E. coli* IgG) or not (*E. coli*). Relation cell/particle = 1/12. Evaluation was as described in Figure 4, median fluorescence of region M1 (defined in Figure 4) increased during 6 h. There was no significant difference in phagocytosis of non-opsonized (*E. coli*) or opsonized (*E. coli* IgG) bacteria between control and saponinum album-treated (4 μg mL^{-1}) differentiated U-937 cells (rank sum test, $P = 0.05$).

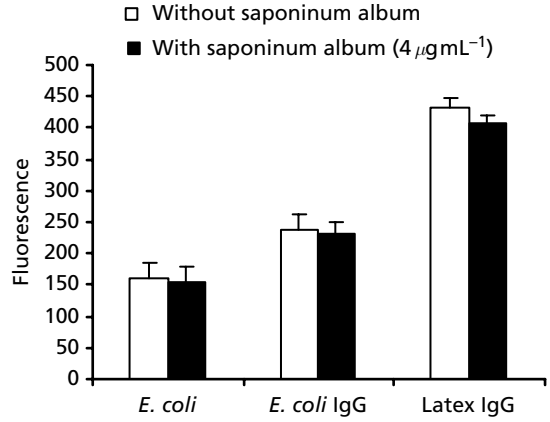

Figure 6 Before the phagocytosis assay, U-937 cells were differentiated with PMA (20 nM, 72 h, see Materials and Methods) and treated with saponinum album (4 μg mL^{-1}). Control cells were only cultured in medium. After addition of the indicated particle suspensions, cells were incubated for 3 h. Relation cell/particle = 1/100 (latex IgG); 1/12 (*E. coli*); 1/12 (*E. coli* IgG). Evaluation was as described in Figure 4. A marker was set and median fluorescence was determined. Columns represent this median fluorescence with the appropriate coefficient of variation. Note that there was no difference in phagocytosis between control and saponinum album-treated U-937 cells (Wilcoxon rank sum test, $P = 0.05$).

Conclusions

Phagocytic activity of IFN-γ- and PMA-stimulated U-937 cells was not enhanced after treatment with saponinum album (4 μg mL^{-1}). This indicated that the internalization of agrostin seemed not to be the result of a phagocytic process induced by saponinum album.

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