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Cell-dislodging methods under serum-free conditions

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Abstract In this work, a BHK21 clone producing a fusion protein consisting of a recombinant human IgG molecule with a cytokine tail, growing in a protein-free medium, was used to test several alternatives to avoid the use of serum for trypsin inactivation, currently used in cell dislodging. These included (1) trypsin inactivated with soybean trypsin inhibitor (STI); (2) cell dissociation solution instead of trypsin; (3) dispase instead of trypsin; (4) trypsin inactivated with fetal calf serum (positive control); (5) non-inactivated trypsin (negative control). Use of a centrifugation step was also tested for each alternative. Results indicate that the best method regarding cell growth, viability and adherent fraction is to use trypsin inactivated with STI followed by a centrifugation step. For all methods tested, the utilization of a centrifugation step always led to improved results. The optimal proportion for total trypsin inactivation is 1:1 trypsin (0.2% w/v) to STI (1 mg ml^{-1}), equivalent to 2 mg trypsin to 1 mg STI. No toxic effect was observed for STI at the concentrations used. Long-term subculturing with this new, alternative dislodging method did not affect cell growth, viability and productivity.

Introduction

If serum-free or protein-free medium is used as an alternative to serum-containing medium, more controllable and reproducible cultures are obtained, downstream processing is made easier, the cost is often lower and contamination risks are minimized. This last factor is extremely relevant in producing human therapeutic products.

Trypsin has been widely used for cell dislodging in primary cell culture to obtain single cells from tissues and organs, e.g. endothelial cells from veins (Stewart et al. 1995) or neuronal cells from nervous systems (Manthorpe et al. 1979). Trypsin has also been widely used for the subcultivation and scale-up of several cell lines, to detach cells from either static or carrier surfaces. The use of trypsin for cell dislodging requires its subsequent inactivation, as over-exposure to trypsin leads to cell damage (Tarone et al. 1982). When cell growth takes place in serum-containing media, trypsin inactivation is done by the serum, but under serum-free conditions, alternatives must be found. Alternatives might consist of another inactivation substance (other than the serum) or another dislodging agent (other than trypsin) (Barlian et al. 1993).

As a trypsin-inactivation substance, soybean trypsin inhibitor (STI) has been commonly used in cell subculturing when serum is absent or present in low amounts in the culture medium. In these circumstances it is necessary to remove any trypsin and STI activity in order to prevent a decrease in plating efficiency and viability (McKeehan 1977); this removal is usually done by centrifugation.

Non-enzymatic cell dissociation solution (CDS) is designed for gentle removal of cells from growing surfaces. It contains EDTA and citrate but no protein and acts on calcium links between cell glucosaminoglycans and similar compounds present in the extracellular matrix adsorbed on the growing surface. As it contains no enzymes, it is very useful in studies dependent upon recognition of cell-surface proteins.

Dispase is a neutral metalloenzyme requiring calcium for activity. It dissociates fibroblast-like cells more efficiently than epithelial-like cells, thus allowing separation of these two cell types from tissues by differential dissociation. Epithelial cells treated with dispase detach in the form of a continuous cell sheet, potentiating the

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generation of epidermal cell sheets for the regeneration of burned skin (Green et al. 1979).

The method of choice should avoid the use of serum, even if it could be almost totally eliminated by a centrifugation step. It should be non-toxic and allow a high cell growth, viability and adherent fraction to be maintained after long-term subculturing.

The purpose of this work is to compare methods for cell dislodging in serum-free conditions, using BHK cells as a model system.

Materials and methods

Cell lines

BHK 21A cells, genetically modified to produce a fusion protein consisting of a recombinant human IgG molecule with a cytokine tail, were obtained from Dr. Hansjörg Hauser (GBF, Braunschweig, Germany).

Cell growth systems

Cells were grown in Scharfenberg's modification 6 (SMIF6) protein-free medium, developed by Drs. Klaus Scharfenberg and Roland Wagner (GBF, Braunschweig, Germany), containing 4 g l^{-1} glucose (final concentration) (Wolf et al. 1993; Scharfenberg and Wagner 1995; Neermann and Wagner 1996). Studies were performed in 25-cm^2 static flasks (Greiner, Frickenhausen, Germany) containing 10 ml medium and 2×10^4 cells ml^{-1} as inoculum. All cultures were performed at 37 °C in a humidified atmosphere containing 7% CO_2 and all media contained puromycin at 5 mg l^{-1} (Sigma, St. Louis, USA).

Cell-dislodging methods

Five cell-dislodging methods were tested, with and without a subsequent centrifugation step; the protocols used are summarized in Table 1. All results were obtained from procedures repeated at least three times and in triplicate with cells growing in protein-free medium SMIF6.

1. Trypsin inactivated with STI

A 1-ml sample of a 0.2% (w/v) trypsin/EDTA solution (GibCo, Glasgow, UK) was used to dislodge a confluent static culture. A washing step before trypsin treatment was not necessary under protein-free conditions. After cell dislodging, trypsin was inactivated with 1 ml STI (Sigma). The optimal trypsin to STI ratio for total trypsin inactivation was achieved after testing several different ratios of STI to trypsin (see *Trypsin inactivation tests*). Single cells were then resuspended with another 8 ml SMIF6 medium to make a total volume of 10 ml. Cells were then counted and seeded at a concentration of 2×10^4 cells ml^{-1} for a total of 10 ml in a 25-cm^2 flask. Using the same inoculum cells, a centrifugation step (10 min at 400 *g*) was performed to remove STI. The pellet was resuspended in 10 ml SMIF6 medium and cells were then seeded at the same concentration previously used without an extra step.

2. CDS instead of trypsin

In this case, cells were washed with 1 ml Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) solution prior to the addition of 1.7 ml (5 ml/75 cm^2) 1× CDS in PBS (Sigma). Cells were incubated for 5–10 min at 37 °C. Single cells were then resuspended with 8.3 ml SMIF6 medium to make 10 ml total volume. All subsequent steps were identical to those described in 1.

3. Dispase instead of trypsin

In this case, 1 ml dispase grade II (1×; 2.4 U ml^{-1}; Boehringer Mannheim) was used instead of trypsin to dislodge a confluent static culture. After incubating for 5 min at 37 °C this solution was removed and the static flask was incubated again for another 10 min at 37 °C. Single cells were then resuspended with 10 ml SMIF6 medium. All subsequent steps were identical to those described in 1.

4. Trypsin inactivated with fetal calf serum (FCS)

This procedure was identical to that used in 1 but, after cell dislodging with trypsin, single cells were resuspended with 9 ml Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% FCS (Sigma).

Table 1 Summary of protocols used for the different cell-dislodging methods. For counting, 2×10^4 cells ml^{-1} were seeded in SMIF6 medium in 25-cm^2 static flasks with 10 ml culture volume. *STI* soybean trypsin inhibitor, *CDS* cell-dissociation solution, *FCS* fetal calf serum, *DMEM* Dulbecco's modified Eagle's medium

Method	Trypsin + STI		CDS		Dispase		Trypsin + FCS		Trypsin	
Cell wash	No		Ca^{2+} and Mg^{+2} free PBS (1 ml)		No		No		No	
Dislodging agent	Trypsin/EDTA (1 ml)		CDS (1.7 ml)		Dispase (1 ml)		Trypsin/EDTA (1 ml)		Trypsin/EDTA (1 ml)	
Incubation time (min)	1–2		5–10		10–15		1–2		1–2	
Inactivation	STI (1 ml)		No		No		FCS (1 ml)		No	
Resuspension vol. of SMIF6 medium (ml)	8		8.3		9		8 (DMEM + 10%FCS)		9	
Total vol. (ml)	10		10		10		10		10	
Centrifugation	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Resuspension vol. of SMIF6 medium (ml)	–	10	–	10	–	10	–	10	–	10

5. Non-inactivated trypsin

After cell dislodging with trypsin, single cells were resuspended with an extra 9 ml SMIF6 medium. All subsequent steps were identical to those described in 1.

In all cases, cells were observed under a light microscope (Olympus CK-2, Tokyo, Japan) after 4 days of cultivation; photomicrographs were taken for cell morphology comparison (Olympus PM-20, Tokyo, Japan). Suspended and adherent cells were counted in order to determine total cell concentration, viability and adherent fraction.

Cell counting

Cells were counted using a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany). Viable cells were determined using the trypan blue dye-exclusion method. Adherent cells were determined after cell release from the static surface (Moreira et al. 1994).

Trypsin inactivation tests

Several different ratios of STI to trypsin were tested to determine the optimal ratio for total inactivation of trypsin; different volumes of STI at 1 mg ml^{-1} (0, 0.5, 1, 1.5, 2.0 and 3.0 ml) were added to cells suspended with 1 ml trypsin at 0.2% (w/v). This suspension was not centrifuged, in order to test whether the possibly non-inactivated trypsin would lead to cell death. After 4 days of incubation in SMIF6 medium, total cells and viability were quantified.

STI toxicity assay

For the chosen method, using trypsin inactivated with STI followed by a centrifugation step, toxicity assays were conducted. The single cells obtained by this method were resuspended in SMIF6 medium containing several concentrations of STI: 0, 5, 10, 15, 20 and 40 μg ml^{-1}. Cells were then inoculated in SMIF6 medium containing the different STI concentrations and, after 4 days of culturing, the cells were quantified in order to determine total cell concentration and viability. The STI concentration range used was chosen to include concentrations that could remain in culture if STI was not removed by centrifugation.

Results

Total cell concentration

As depicted in Fig. 1, the use of trypsin without inactivation (negative control) led to low cell growth; absence of centrifugation led to still lower cell growth. In the positive control experiments, inactivation with FCS led to much higher cell growth, once trypsin was inactivated and thus was no longer harmful to the cells.

Trypsin inactivation with STI yielded the highest cell growth; centrifugation to remove both compounds (trypsin and STI) led to even higher growth, similar to that obtained with the positive control: FCS inactivation followed by removal by centrifugation. CDS showed the lowest performance of all tested methods, even if compared with the use of trypsin without inactivation (negative control). This solution showed a high toxic effect and, without its removal by centrifugation, results obtained were the lowest of all; it also needed the longest period for cell dislodging, thus increasing the overall length of the procedure. Dispase showed a good performance regarding cell growth, if removed by centrifugation; without this step cells did grow at a much slower rate, thus showing that dispase had some toxic effect. Dispase for cell dislodging proved to be as fast as trypsin and had the advantage of not requiring inactivation, just its subsequent removal by centrifugation. However, the cell growth achieved was lower than that obtained with trypsin plus STI.

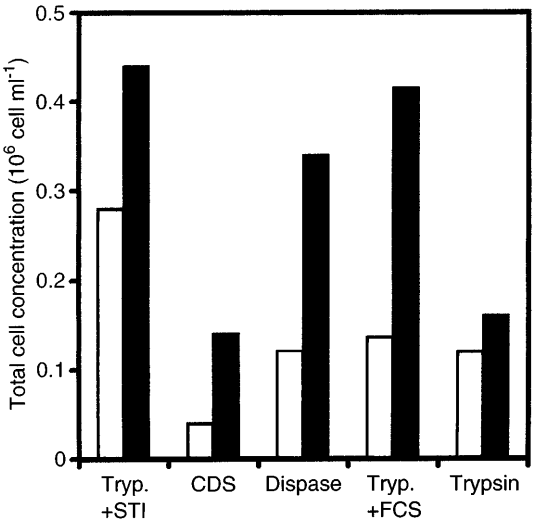

Fig. 1 Comparison of different cell-dislodging methods regarding total cell concentration (i.e. viable suspended + non-viable suspended + viable adherent + non-viable adherent cells); cell concentration was always related to 10 ml culture. These results were obtained after 4 days of static cell culture in protein-free medium SMIF6. *Light bars* without centrifugation; *dark bars* with centrifugation. *STI* soybean trypsin inhibitor, *CDS* cell dissociation solution, *FCS* fetal calf serum

Cell viability

In combination with a high cell growth, the method of choice should also allow high cell viability. As can be seen in Fig. 2, trypsin without inactivation did not reduce cell viability although it led to slow cell growth. However, cells did show unhealthy, rounded morphology with cell membrane blebbing (Fig. 3i). Although not leading to a greater improvement in viability, centrifugation did lead to a better cell morphology (Fig. 3j).

Trypsin inactivation with FCS allowed high cell viability, as expected; similar results were observed for trypsin inactivated with STI; for the methods mentioned, centrifugation did not change this parameter but, regarding cell morphology, although no differences were found for trypsin + FCS (Fig. 3g cf. h), great differences were found in the trypsin + STI method, with centrifugation leading to a dramatic improvement in cell morphology (Fig. 3a cf. b). Centrifugation strongly influenced cell viability if CDS was used; however, cell morphology improved only slightly with centrifugation (Fig. 3c, d). With CDS remaining in culture, cell viability

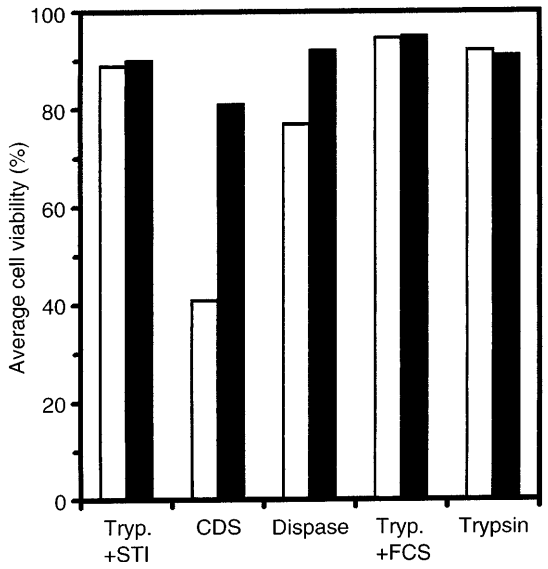

Fig. 2 Comparison of different cell-dislodging methods regarding average cell viability. These results were obtained after 4 days of static cell culture in protein-free medium SMIF6. *Light bars* without centrifugation; *dark bars* with centrifugation

was very low, showing that it is toxic for these cells. This effect was also present, although at a lower level, if dispase was used; dispase removal by centrifugation allowed cell viability to reach similar values to those obtained with trypsin. Cell morphology was also greatly improved by using a centrifugation step (Fig. 3e cf. f), as in the case of the trypsin + STI method.

Average adherent fraction

The adherent fraction is another important parameter to be considered when choosing a cell-dislodging method, since anchorage-dependent cells need to be adherent to static or carrier surfaces or to form aggregates in order to grow in static cultures and bioreactors. Figure 4 shows that trypsin without inactivation led to low adherence if not removed by centrifugation. The same occurred with trypsin with FCS inactivation without removal by centrifugation. Trypsin with STI showed a high adherent fraction even if STI was not removed by centrifugation. CDS led to the lowest adherent fractions, although its removal by centrifugation led to higher adherent fractions. For dispase the average adherent fraction was highly dependent on its removal by centrifugation.

In summary, it can be said that, for all methods tested, increased performance was obtained when the dislodging agent was removed by centrifugation. For the conditions under test, the use of trypsin with STI or with FCS inactivation, or dispase alone, in this order, proved to be the best method. Furthermore, as serum is to be totally avoided and dispase leads to lower cell growth, trypsin inactivated with STI was considered the method of choice. CDS showed the lowest performance of all methods, even if compared with the negative control (trypsin without inactivation).

Trypsin inactivation tests and STI toxicity assay

Several tests were done to evaluate cell growth and viability under different levels of trypsin inactivation. As can be seen in Fig. 5, results show that cell growth and viability increased significantly with increasing STI to trypsin ratios from 0:1 to 0.5:1 to 1:1 ml, showing that trypsin was increasingly being inactivated. However, when this ratio increased from 1:1 to 3:1, no further improvement in either growth or viability was observed.

As regards STI toxicity, several studies were done with concentrations of STI in the culture medium of 0, 5, 10, 15, 20 and 40 μg ml^{-1}. As can be seen in the results shown in Fig. 6, both cell growth and viability were maintained with STI concentrations up to 40 μg ml^{-1}.

Discussion

As depicted in Fig. 1, the use of trypsin without inactivation (negative control) led to low cell growth, because of the remaining trypsin activity; since trypsin activity was higher in the absence of centrifugation, this led to still lower cell growth; these results confirm those obtained by Tarone et al. (1982). In the positive control experiments, inactivation with FCS led to much higher cell growth, once trypsin was inactivated and thus was no longer harmful to the cells; however, the absence of centrifugation left some serum in the culture medium which, in conjugation with some components of the protein-free medium, may have led to a relative growth-arrest effect. Removing FCS by centrifugation overcame this effect. This behaviour was observed when cells were adapted to protein-free medium: even a small amount of serum led to a decrease in cell growth (data not shown).

As can be seen in Fig. 2, trypsin without inactivation did not reduce cell viability; however, cells did show unhealthy rounded morphology with cell membrane blebbing (Fig. 3i). Although not leading to a greater improvement in viability, centrifugation did lead to a better cell morphology (Fig. 3j). Trypsin inactivation with FCS or STI allowed high cell viability, as expected; for the methods mentioned, centrifugation did not change this parameter, but, although no differences in cell morphology were found for trypsin + FCS (Fig. 3g compared to 3H), great differences were found for the trypsin + STI method, with centrifugation leading to a dramatic improvement in cell morphology (Fig. 3a cf. b). Centrifugation strongly influenced cell viability if CDS was used; however, cell morphology improved only slightly with centrifugation (Fig. 3c, d). With CDS remaining in culture, cell viability was very low, showing that it is toxic for these cells. This effect was also present, although at a lower level, if dispase was used; dispase

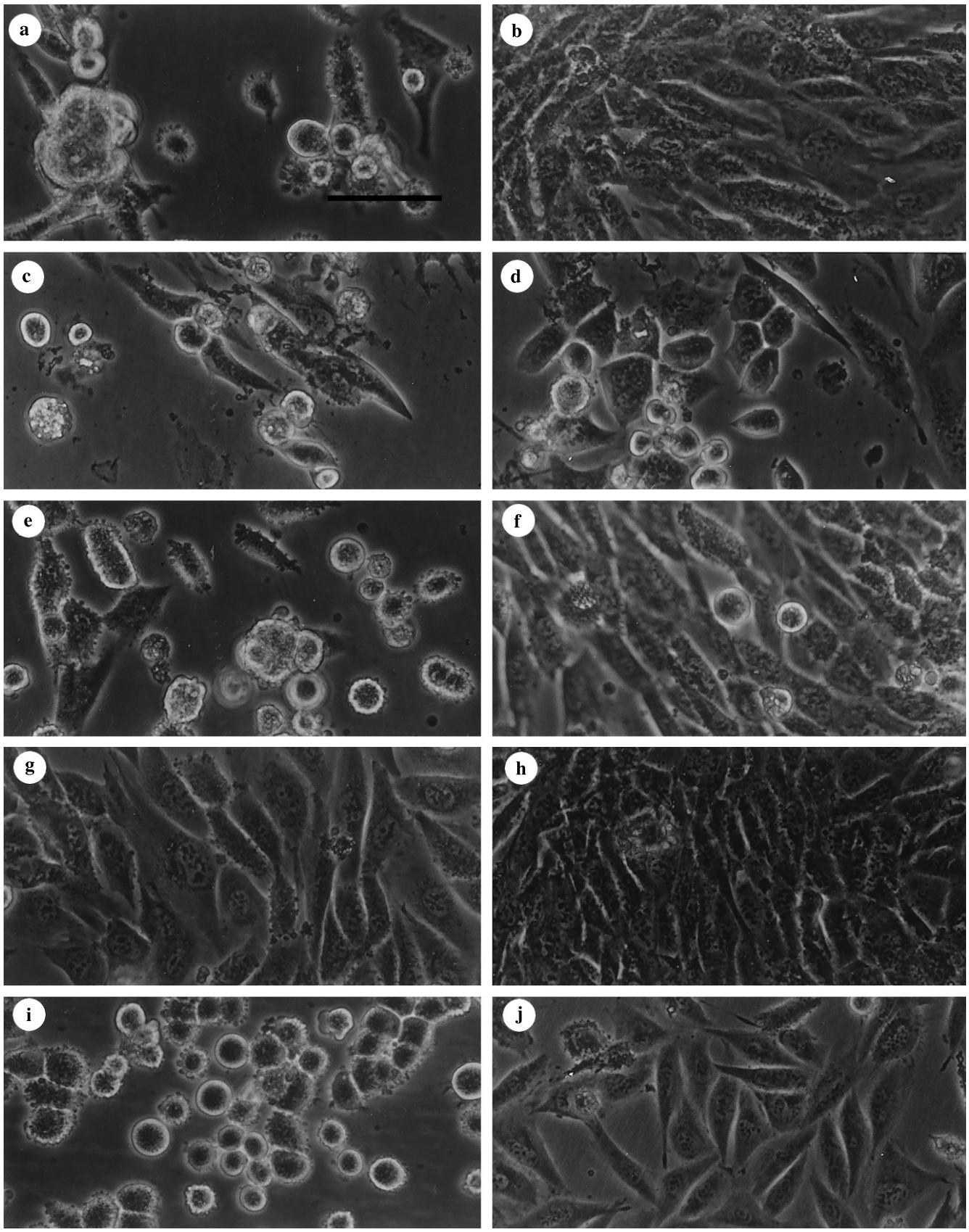

Fig. 3 a-j

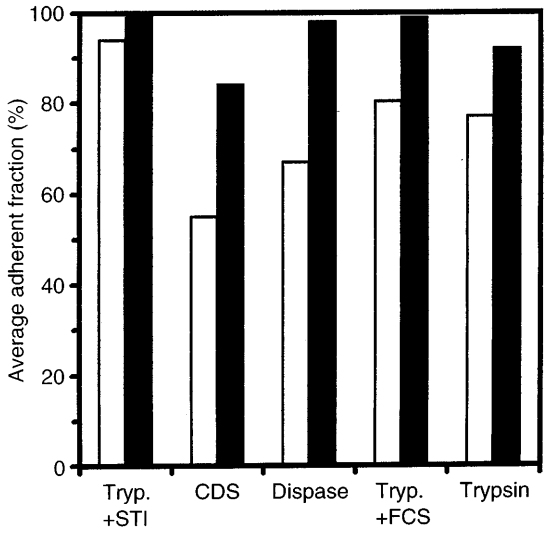

Fig. 4 Comparison of different cell-dislodging methods regarding average adherent fraction, i.e. (viable adherent + non-viable adherent)/total cells. These results were obtained after 4 days of static cell culture in protein-free medium SMIF6. *Light bars* without centrifugation; *dark bars* with centrifugation

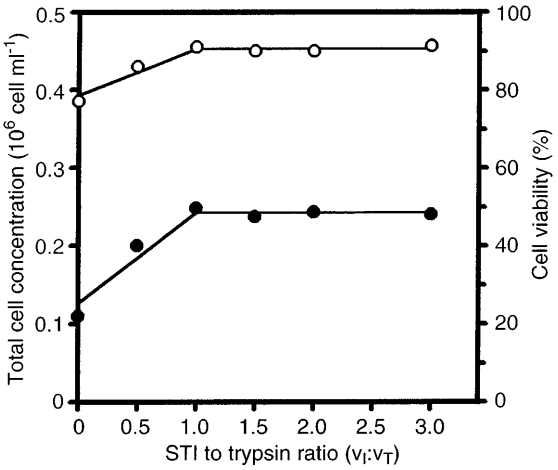

Fig. 5 Trypsin inactivation tests. Different volumes of STI solution (1 mg ml^{-1}) were added to cells suspended with 1 ml trypsin (0.2% w/v). Centrifugation was not used for the non-inactivated trypsin to act. These results were obtained after 4 days of static cell culture in protein-free medium SMIF6. *Dark circles* total cell concentration; *light circles* cell viability

removal by centrifugation allowed cell viability to reach similar values to those obtained with trypsin. Cell morphology is also greatly improved by use of a centrifugation step (Fig. 3e cf. f), as in the case of the trypsin + STI method.

Figure 4 shows that trypsin without inactivation or with FCS inactivation led to low adherence if not removed by centrifugation; however, trypsin with STI led to a high adherent fraction even if centrifugation was not used. CDS led to the lowest adherent fractions, although its removal by centrifugation led to higher adherent fractions. For dispase the average adherent fraction was highly dependent on its removal by centrifugation. When trypsin inactivated by FCS was used, adherent fractions were not affected by its presence, but serum in conjugation with some medium components was harmful, as observed earlier in the growth studies. Except for this case, lower adherent fractions were due to the presence of dislodging-agent activity; centrifugation mostly overcame this effect.

Cell-dislodging-agent activity must always be removed by centrifugation; even if inactivated trypsin is used (with STI or FCS), better results are obtained with a subsequent centrifugation step. Under the culture conditions used, trypsin inactivated with STI or FCS gave the best results; dispase was also appropriate, but lower growth was achieved. CDS has proved not to be useful in this case, leading to the lowest overall performance, lower even than non-inactivated trypsin, which was considered as the negative control. As methods using serum are to be avoided and were used only as controls, trypsin inactivated with STI followed by a centrifugation step proved to be the best dislodging method for cell subculture under the protein-free conditions used.

Fig. 3a–j Influence of the different dislodging methods on cell morphology after 4 days of static cell culture in protein-free medium SMIF6. Trypsin inactivated with soybean trypsin inhibitor (STI) without (**a**) and with (**b**) a centrifugation step; CDS instead of trypsin without (**c**) and with (**d**) a centrifugation step; dispase instead of trypsin without (**e**) and with (**f**) a centrifugation step; trypsin inactivated with FCS (positive control) without (**g**) and with (**h**) a centrifugation step; non-inactivated trypsin (negative control) without (**i**) and with (**j**) a centrifugation step. Photographs were taken with an inverted phase-contrast microscope at 200× magnification. *Scale bar* (**a**) 100 μm

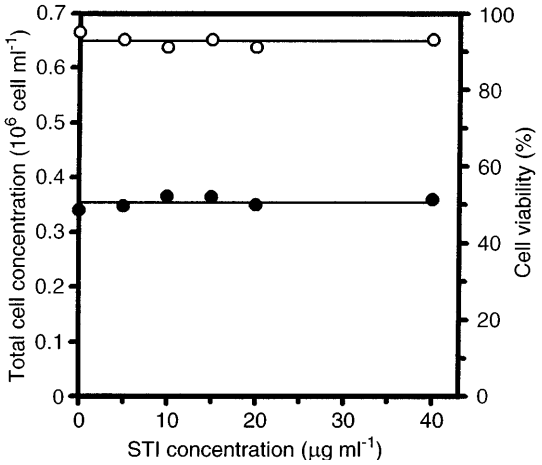

Fig. 6 STI toxicity assay. After single cells had been obtained by using trypsin inactivated with soybean inhibitor followed by a centrifugation step (chosen method) they were resuspended in SMIF6 medium containing several different concentrations of soybean trypsin inhibitor: 0, 5, 10, 15, 20 and 40 μg ml^{-1}. Cells were then inoculated in SMIF6 medium and, after 4 days static culturing, cells were quantified in order to obtain total cell concentration and viability. *Dark circles* total cell concentration; *light circles* cell viability

Table 2 Summary of costs involved in the different cell-dislodging methods

Dislodging agent	Amount used[a] (ml)	Cost (%)
Trypsin + STI	1.0	100
Dispase II	1.7	78
CDS	1.0	85
Trypsin + FCS	1.0	40

[a] To dislodge a confluent monolayer of cells in a 25-cm^2 static flask

From Fig. 5 it can be concluded that the optimal volume ratio of STI (0.1 mg ml^{-1}) to inactivate trypsin (0.2% w/v) totally is 1:1; this ratio indicates that 1 mg STI inactivates 2 mg trypsin, in accordance with reported values ranging from 1 mg to 3 mg trypsin that can be inactivated with 1 mg STI (Morris 1995).

Figure 6 shows that STI did not show any growth inhibition effect even when present in the culture medium at concentrations up to 40 μg ml^{-1}. This STI concentration is considerably higher than those that could remain in culture, if not removed by centrifugation; since the chosen method included STI removal by centrifugation, this eliminates any possible toxic effect of STI at the concentrations used.

As the chosen method is to be used regularly, it can not have any limiting effect on cells, regarding growth, viability or productivity, even in long-term utilization. To test this, extended subculturing was tried and it was concluded that long-term subculturing using the chosen method did not affect cell growth, viability or productivity (data not shown).

Trypsin is easy to work with and dislodges cells rapidly; however, it is of animal origin and is thus a potential source of contamination; it also has the disadvantage referred to above (but only for some specific applications) of destroying membrane proteins. The use of dispase minimizes contamination risks, this being its major advantage in industrial applications, although it is not as easy to work with as trypsin. For a final decision, cost should also be considered. Trypsin inactivated with STI is the method of choice for cell dislodging under serum-free conditions on the laboratory scale, although, as presented in Table 2, it is the most expensive method; however, for industrial purposes the use of dispase looks economically advantageous, since it does not need any inactivating substance, thereby reducing its costs (78% of the final cost of the trypsin + STI method). Thus, dispase is also a method worth considering, but the relative loss in growth performance should always be taken into account.

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References

Barlian A, Ganassin RC, Tom D, Bols NC (1993) A comparison of bovine serum albumin and chicken ovalbumin as supplements for the serum-free growth of chinook salmon cells, SHSE-214. Cell Biol Int 7: 677–684

Green H, Kehinde O, Thomas J (1979) Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci 76: 5665–5668

Manthorpe M, Adler R, Varon S (1979) Development, reactivity and GFA immunofluorescence of astroglia-containing monolayer cultures from rat cerebrum. J Neurocytol 8: 605

McKeehan WL (1977) The effects of temperature during trypsin treatment on viability and multiplication potential of single normal human and chicken fibroblasts. Cell Biol Int Rep 1: 335–343

Moreira JL, Alves PM, Aunins JG, Carrondo MJT (1994) Changes in animal cell natural aggregates in suspended batch cultures. Appl Microbiol Biotechnol 41: 203–209

Morris CB (1995) Routine subculturing – adherent cells. In: Doyle A, Griffiths JB, Newell DG (eds) Cell and tissue culture: laboratory procedures, vol 1. Wiley, Chichester, 4A: 1.4

Neermann J, Wagner R (1996) Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells. J Cell Physiol 166: 152–169

Scharfenberg K, Wagner R (1995) A reliable strategy for the achievement of cell lines growing in protein-free medium. In: Spier RE, Griffiths JB, Beuvery C (eds) Animal cell technology "developments towards the 21st century". Butterworth-Heinemann, Oxford, 619–623

Stewart GJ, Wang Y, Niewiarowsky S (1995) Methylcellulose protects the ability of anchorage dependent cells to adhere following isolation and holding in suspension. Biotechniques 19: 598–604

Tarone G, Galettop G, Pratt M, Comoglio PM (1982) Cell surface molecules and fibronectin-mediated cell adhesion: effect of proteolytic digestion of membrane proteins. J Cell Biol 94: 179–186

Wolf H, Scharfenberg K, Wagner R (1993) Verfahren zur Herstellung von Aktivimpfstoff gegen Epstein-Barr-Virus-Infektionen. German Patent DP 43 13 620.6