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Research paper

Simultaneous metal chelate affinity purification and endotoxin clearance of recombinant antibody fragments

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Abstract

Endotoxins are frequent contaminants of recombinant proteins produced in *Escherichia coli*. Due to their adverse effects, endotoxins have to be removed from recombinant proteins prior their use in cell-based assays or parenteral application. Reduction of endotoxin to less than 10 EU mg^{-1} is, however, one of the most problematic steps during protein purification from *E. coli* and often associated with substantial loss of biological materials. The present paper describes the use of a single step procedure enabling metal chelate affinity purification and endotoxin clearance from antibody fragments produced in *E. coli* using a non-ionic detergent. Endotoxin content was as low as 5 to 9 EU mg^{-1} with a recovery of antibody fragments of over 90%. Non-ionic detergent treatment did not compromise integrity and functionality of these multimeric molecules. Furthermore, recombinant antibody fragments did not stimulate endotoxin-sensitive cell lines confirming the low endotoxin content. In conclusion, this one-step protocol is a rapid, cost effective and automation-compatible procedure suitable for recombinant antibody fragments.

Keywords: Recombinant protein expression; Antibody fragments; Endotoxin removal; Metal chelate affinity chromatography

1. Introduction

The ability to express functional antibody fragments in *Escherichia coli* (Better et al., 1988; Skerra and Pluckthun, 1988) enables the production of significant quantities of material for in vitro experiments, cell-based assays and in vivo applications. Unfortunately, *E. coli* derived products are prone to contamination with endotoxins. Endotoxins are toxic components of the outer membrane of gram-negative bacteria. Exposure to these soluble substances may affect the membrane structure of mammalian cells (Jacobs, 1984; Portoles et al., 1987), inhibit cell growth (Dudley et al., 2003) and decrease tissue culture cell viability (Cotten et al., 1994). Endotoxin induces the activation of monocytes, macrophages (Gao and Tsan, 2003b) and endothelial cells (Munshi et al., 2002). LPS-activated cells release mediators such as pro-inflammatory cytokines, TNF-α and interleukin-1 (Raetz and Whitfield, 2002). Finally, in humans and animals, exposure to endotoxin causes fever and may result in septic shock (Fiuza and Suffredini, 2001; Martich et al., 1993).

The incidence of contaminating endotoxin can complicate the interpretation of experiments or lead to the misinterpretation of results (Dudley et al., 2003; Gao and Tsan, 2003a), highlighting the benefits to eliminate

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endotoxin from *E. coli* derived products. Achieving a low level of endotoxin content together with an excellent recovery rate of the target protein is, however, a difficult task and often dependent on the physico-chemical properties of the target molecule (Petsch and Anspach, 2000).

Several methods have been applied to remove endotoxin from biological products (Petsch and Anspach, 2000). Phase separation using non-ionic detergent takes advantage of the hydrophobic properties of endotoxin trapping them in the detergent phase, whereas the hydrophobic proteins remain in the aqueous phase (Aida and Pabst, 1990). It is, however, time consuming and can lead to protein denaturation. Ultrafiltration is an efficient method to eliminate endotoxin from protein solution. It can, however, lead to a substantial loss of the target molecule (Petsch and Anspach, 2000). Anion exchange resins and selective affinity sorbents, which are based on the differential interaction of endotoxin and proteins with functional groups immobilized on the resin, often lead to a loss of biological material as multiple rounds of binding are needed to eliminate substantial amounts of endotoxins (Anspach and Hilbeck, 1995). A method for endotoxin removal from his-tagged monomer recombinant proteins using affinity chromatography in combination with the non-ionic detergent Triton X-114 has been described previously (Reichelt et al., 2006). However, non-ionic detergent can cause change in protein conformation and impair binding properties (Hsu and Youle, 1997; Hsu and Youle, 1998; Tan and Ting, 2000).

We tested whether this one-step protocol using non-ionic detergent could be applied to antibody fragments composed of two non-covalently linked polypeptide chains (Rauchenberger et al., 2003) and to bivalent antibody fragments containing a small homodimerization domain (dHLX) (Pack and Pluckthun, 1992). As case study, we selected a fully human monoclonal antibody MOR102 (#5) targeting the intercellular adhesion molecule 1 (ICAM-1) derived from the Human Combinatorial Antibody Library (HuCAL®) (Boehncke et al., 2005; Knappik et al., 2000). The antibody MOR102 (#5) was expressed in two different antibody formats: (i) non-covalently linked Fab fragments and (ii) bivalent Fab-dHLX fragments as histidine-tagged protein.

We demonstrate that Triton X-114 is effective at eliminating endotoxin using Ni-NTA affinity chromatography from antibody fragments. Protein recovery was at least 90% with endotoxin content as low as 5 to 9 EU mg^{-1} for the two antibody formats tested. Binding activity and functionality were not compromised as assessed in binding and cell-based assays, respectively. Furthermore, antibody fragments purified using Triton-X114 did not activate endothelial cells, which corroborates with low endotoxin content. Therefore, including Triton X-114 washing step in the purification protocol is a straightforward methodology for the removal of endotoxin from recombinant antibody produced in bacteria.

2. Materials and methods

2.1. Strains and plasmids

E. coli TG1 strain was used to express histidine-tagged antibody fragments. Antibody fragments were expressed either as non-covalently linked Fab or as Fab-dHLX format using the pMORPH®×9 plasmid described previously (Rauchenberger et al., 2003).

2.2. Expression

4 ml of overnight culture was used to inoculate 750 ml of 2×YT containing 34 μg/ml chloramphenicol. Cultures were agitated at 200 rpm until the A_{600nm} reached 0.5 and expression was induced by addition of 0.75 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 h at 30 °C. Cells were harvested by centrifugation at 5000 rpm for 30 min.

2.3. Purification and endotoxin removal

Cell pellets containing his-tagged proteins were resuspended in 40 ml of IMAC buffer (500 mM NaCl, 200 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.3). Following addition of 0.1% lysozyme and 10 U/ml benzonase, cells were agitated at room temperature for 30 min. Lysates were centrifuged at 20,000×*g*, 4 °C for 30 min and filtered through a 0.45 μm filter (Millipore, MA). A suspension of 1 ml Ni-NTA agarose (Qiagen, Germany) was poured into columns. Cell extracts were split into two equal aliquots and each aliquot was loaded onto one column that was previously equilibrated with 15 column volumes of IMAC buffer. One column was washed with 50 column volumes of respective buffer containing 0.1% (v/v) of TritonX-114 (Sigma-Aldrich, Germany) followed by 20 column volumes of buffer without detergent at 4 °C. The other column was washed with equivalent column volumes of the same buffer but lacking detergent. Elution was achieved using 200 mM imidazole (Sigma-Aldrich, Germany). Elution fractions containing Fab or Fab-dHLX were loaded onto PD10 gel filtration columns (Amersham Bioscience, Sweden)

and eluted with either 1 × PBS or 3 × PBS, respectively, in order to eliminate the imidazole from samples.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Maxisorp microtiter plates (Nunc, Denmark) were coated with 0.5 μg/ml of recombinant human ICAM-1-Fc (R&D system, MN) in phosphate buffer, PBS, pH 8, for 16 h at 4 °C. Wells were blocked with 0.1 ml of 2% (w/v) bovine serum albumin in PBS, pH 8, containing 0.05% (v/v) of Tween-20 for 1 h at 22 °C. A dilution series of either purified Fab or Fab-dHLX was added to the pre-coated microtiter plates beginning with a concentration of 25 nM and incubated for 1 h at 22 °C. After extensive washing with PBS, Fab and Fab-dHLX were detected with a peroxidase conjugated anti-human Fab antibody (Dianova, Germany) at 2 μg /ml. The signal developed with Quanta Blue fluorescence reporting system (Pierce, IL) was monitored with an ELISA plate reader (Tecan Instruments, Germany) at a wavelength of 430 nm.

2.5. Cell-protein adhesion assay

This assay has been previously described (Boehncke et al., 2005). Briefly, recombinant ICAM-1-Fc (R&D system, MN) was immobilized on Maxisorp microtiter plates (Nunc, Denmark) at a concentration of 5 μg/ml in PBS containing 2 mM $MgCl_2$ (PBS/Mg). Following blocking with 2% bovine serum albumin, wells were washed with PBS/Mg. Serial dilutions of antibody fragments in 50 μl of PBS/Mg were added to each well and incubated for 30 min. SKW-3 cells (T-CLL cell line) labelled with Calcein AM (Molecular Probes, OR) were transferred to each well at 10^5 cells in 50 μl serum-free RPMI 1640 containing 2 mM $MgCl_2$ and incubated for 1 h at 37 °C. The fluorescence was monitored with an ELISA plate reader (TECAN GmbH, Germany) at a wavelength of 530 nm before and after washing steps in a static cell adhesion wash chamber (Glycotech, MD). The percentage of adherent cells was calculated as (fluorescence after wash/fluorescence before wash) × 100.

2.6. Cell surface fluorescence (FACS)

Human umbilical vein endothelial cells (HUVECs, Cambrex, MD) were cultured with endothelial growth medium (EGM, Cambrex, MD) containing 2% heat-inactivated fetal calf serum. 100 μl of cells were added at $1.2 \cdot 10^6$ cells/ml to a serial dilution of either antibody fragments or defined amounts of purified lipopolysaccharide 026:B6 from *E. coli* as a control in a 48-well plate and incubated at 37 °C for 16 h. Endothelial monolayers were detached with Accutase (PAA laboratories, Austria) and transferred to a 48-well round bottom plate (Nunc, Denmark). After two consecutive washes with FACS buffer (PBS containing 3% FCS), cells were incubated with 10 μg/ml of BIRR-1 murine anti-ICAM antibody RO.5 (ATCC: R6.5D6) for 40 min at 4 °C. Cells were washed twice with FACS buffer and incubated for a further 30 min at 4 °C with 100 μl of phycoerythrin conjugated goat anti mouse IgG (Jackson Immunoresearch, USA) at a final concentration of 2 μg/ml. Cells were washed twice with FACS buffer and ICAM-1 was quantified using a FACScalibur (Becton Dickinson, NJ).

2.7. Endotoxin and protein concentration

The endotoxin content of different fractions was determined by the Limulus amoebocyte lysate kinetic-QCL assay according to the manufacturer's instructions, (Cambrex Bioscience, MD). In order to determine whether components of the different fractions interfered with the assay, defined amounts of endotoxin (Cambrex Bioscience, MD) were spiked into the samples. Protein concentrations were determined by absorption at 280 nm using NanoDrop Spectrophotometer ND1000 device (Kisker, Germany).

3. Results and discussion

3.1. Endotoxin clearance from recombinant antibody fragments

Antibody fragments derived from the HuCAL® library lack the C-terminal cysteines responsible for a intermolecular disulfide bridge (Fig. 1), since it has been shown that the expression yield of the non-covalently linked Fab fragments was higher than the covalently disulfide-linked Fab fragments (Rauchenberger et al., 2003). Furthermore, mini-antibodies have been developed using self-associating peptides such as a helix-coil-helix peptide (Fig. 1). These dimeric Fab-dHLX antibodies have the same bivalent binding capacity as an immunoglobulin. We tested whether chromatography purification using Triton washing steps (Reichelt et al., 2006) could be used for these molecules without interfering with protein–protein interactions. His-tagged MOR102 (#5) was produced in *E. coli* either in Fab or in Fab-dHLX format and purified according to the protocols described above.

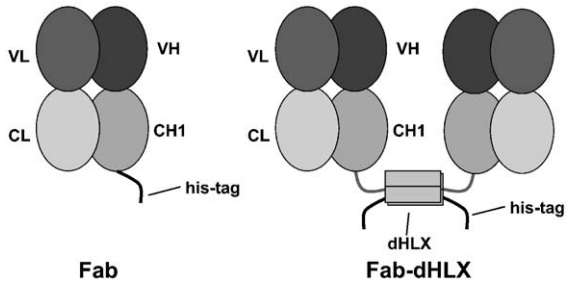

Fig. 1. Antibody MOR102 (#5) was expressed in *E. coli* in either Fab or Fab-dHLX format. Fabs derived from the HuCAL® library lack the cysteines residues responsible for the intermolecular bridge and carry a his-tag in the C-terminal part of the CH1 domain of the heavy chain. In addition to the features of Fab fragments, Fab-dHLX fragments contain a small helix-coil-helix dimerization domain at the C-terminal part of the CH1 domain.

As shown in Table 1, the use of 0.1% Triton X-114 in washing steps was successful at reducing endotoxin to a concentration of 5 to 9 EU mg^{-1}, whereas washing step lacking detergents were ineffective at eliminating endotoxin. Endotoxin content in purified protein fraction using the standard protocol was higher than 30,000 EU mg^{-1} in the final product. In contrast to this, endotoxin content of biological materials was as low as 5 EU mg^{-1} for Fab-dHLX and below 10 EU mg^{-1} for Fab (Table 1) when Triton X-114 was implemented. Protein recovery was at least 90% compared to samples purified using the standard protocol. No noticeable differences in endotoxin removal efficiency were observed between the two different antibody formats used (Table 1). To appreciate the efficiency of Triton X-114 washing steps to remove endotoxin, a purified Fab antibody fragment spiked with 15,000 EU mg^{-1} was loaded onto Ni-NTA and washed with Triton X-114 containing buffer. A final endotoxin concentration in the eluted Fab was found to be below 2 EU mg^{-1} (data not shown). Thus, the application of Triton X-114 washing steps removed more than 99% of the initial endotoxin content. To test whether Triton X-114 treatment could dissociate the non-covalently linked VL-CL polypeptide from the his-tagged VH-CH1 polypeptide during the purification procedure, antibody fragments were analyzed by SDS-PAGE. As shown in Fig. 2, Triton X-114 treatment did not lead to the loss of the VL-CL polypeptide. An additional band of 25 kDa was observed in dHLX-Fab samples. While we are currently analyzing this by-product, it was, however, found independently of the purification protocol being used. Thus, affinity chromatography purifications combined with Triton X-114 washing steps enable endotoxin clearance with an excellent recovery rate of antibody fragments, while preserving the integrity of these heterodimers.

Table 1
Endotoxin levels of purified antibody fragments

Format	Triton	Protein recovery	EU (ml^{-1})	EU (mg^{-1})
Fab	−	100%	>24,000	>16,000
	+	92%±5	11.5±5	9.5±4
Fab-dHLX	−	100%	>30,000	>30,000
	+	96%±6	7.5±5	5.0±3

Endotoxin content following various antibody purification schemes. Each antibody format was purified using Ni-NTA agarose in the presence (+) or in the absence (−) of Triton X-114 as described in Materials and methods. Three independent preparations of each antibody fragment were subjected to endotoxin removal using Triton X-114 and tested for endotoxin content (standard deviations are indicated).

3.2. Antibody binding activity

To test whether protein functionality was affected by the Triton X-114 treatment, antibodies in Fab and Fab-dHLX formats purified either under standard or Triton protocols were analyzed for their binding capacity to equal an amount of immobilized ICAM-1 molecules in ELISA. As shown in Fig. 3A,B, no obvious difference in binding activity could be detected between antibody fragments purified under the standard protocol and the Triton-X114 protocol. Slightly better EC_{50} values were measured for Fab antibody fragments purified using Triton X-114 treatment in comparison to the standard protocol and were 0.18 nM±0.07 and 0.23 nM±0.1, respectively (Fig. 3A). These differences were within a standard deviation and consequently not considered as significant. The EC_{50} of antibody fragments in Fab-dHLX format purified under either Triton X-114 or the standard protocol were very similar and determined to

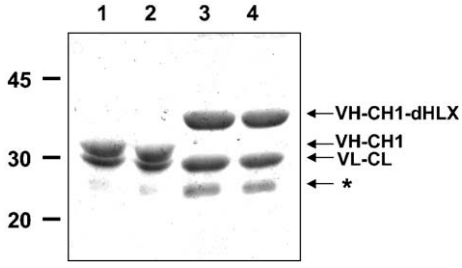

Fig. 2. Antibody fragments were analysis by SDS-PAGE. 5 μg of each recombinant antibody were loaded onto a 15% SDS polyacrylamide gel and visualized by Coommasie Blue staining. Lanes 1 and 2: his-tagged Fab, and lanes 3 and 4: his-tagged Fab-dHLX. Lanes 1 and 3: purified proteins using the standard protocol; lanes 2 and 4: proteins purified with Triton X-114. Molecular weights and corresponding polypeptides are indicated as well as the by-product (*).

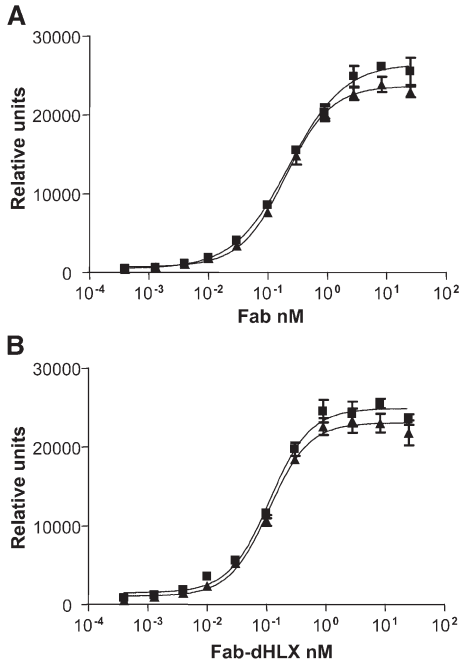

Fig. 3. Antibody fragments were tested for binding activity to ICAM-1 molecule in ELISA. (A) Serial dilutions of the Fab antibody fragment purified using either the standard protocol (■) or Triton X-114 washing steps (▲) were added to plates pre-coated with ICAM-1 molecules. Following 1-h incubation, binding activity was monitored by an ELISA plate reader using a peroxidase conjugated anti-human Fab antibody. (B) The same binding assay was also performed using the purified Fab-dHLX antibody fragment using either the standard protocol (■) or Triton X-114 (▲). Three independent measurements were performed for each antibody fragment (standard deviations are indicated).

be 0.09 nM ± 0.05 and 0.08 nM ± 0.05, respectively (Fig. 3B). Thus, the binding activity of the purified antibody fragments irrespective of the format used was not compromised by the endotoxin removal process.

3.3. Antibody functionality in a cell-based adhesion assay

Antibody MOR102 (#5) in IgG format has been previously reported to inhibit adhesion of the SKW3 cells to ICAM-1 molecule (Boehncke et al., 2005). Functionality of Fab and Fab-dHLX antibody fragments purified by using either standard or Triton X-114 procedure was tested in a cell-based adhesion assay on immobilized ICAM-1. As shown in Fig. 4A,B, antibody fragments purified using either Triton X-114 or standard protocols were able to inhibit adhesion of SKW3 cells to ICAM-1 molecules. Antibody fragments in Fab format purified using the standard protocol or Triton X-114 were found to have a similar IC_{50} of 13.6 nM and 10.2 nM, respectively (Fig. 4A). While inhibition capacities of the purified Fab-dHLX antibody fragments using either the standard protocol or Triton X-114 were alike and the IC_{50} was 6.1 nM and 4.6 nM, respectively, a slight decrease in percentage of cell-adhesion was observed for cells incubated with Fab-dHLX purified using Triton X-114 (Fig. 4B). This observation could be attributed to residual traces of Triton X-114 remaining in the sample. However, a decrease in percentage of cell-adhesion was not observed for Fab samples. In conclusion, no obvious differences in inhibitory activity of antibody fragments were observed applying either the standard or Triton X-114 protocol.

3.4. LPS-dependent cell activation

The presence of endotoxin contaminants in protein preparations can lead to the misinterpretation of results

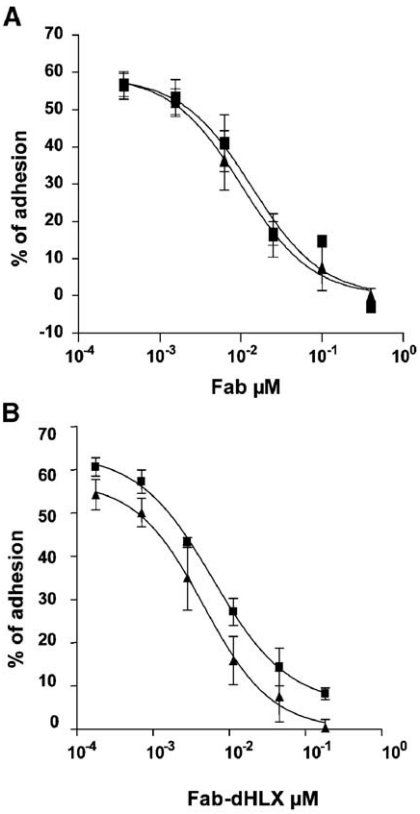

Fig. 4. Cell-based adhesion assay using antibody fragments. Serial dilutions of Fab antibody fragment (A) or Fab-dHLX antibody fragment (B) purified using either the standard protocol (■) or Triton X-114 washing steps (▲) were added to plates pre-coated with ICAM-1 molecules. SKW3 cells labelled with Calcein AM were added to plates. Percentage of cell-adhesion was calculated after 1-h incubation as (fluorescence after wash / fluorescence before wash) × 100.Three independent measurements were performed for each antibody fragment (standard deviations are indicated).

(Osterloh et al., 2004). For example, a recombinant Kringle 5 protein was reported to inhibit endothelial cell growth (Cao et al., 1999). Subsequently, Kringle's anti-endothelial cell activity was found to be related to its contamination with endotoxin (Dudley et al., 2003). This highlights the potential pitfalls of utilizing *E. coli* derived products in cell-based assays using endotoxin-sensitive cell lines.

To test whether antibody fragments purified under Triton X-114 could be applied with endotoxin-sensitive cell lines, Fab and Fab-dHLX were evaluated in a cell-based assay using endothelial cells. Endothelial cells bear receptors inducible by many inflammatory stimuli, including LPS (Springer, 1990). Previous studies have demonstrated the influence of endotoxin on endothelial cells such as alterations in gene expression and induction of specific cell surface proteins. For instance, exposure to endotoxin induces the expression of the ICAM-1 molecule on the surface of endothelial cells (Norata et al., 2005). These findings could be confirmed by our experiments. ICAM-1 expression on the surface of the HUVEC cells was enhanced by purified endotoxin in a concentration dependent manner (Fig. 5A). An induction of ICAM-1 expression could be already detected at endotoxin levels below 10 EU/ml. We next examined whether antibody MOR102 (#5) fragments purified using Triton-X114 enhanced ICAM-1 expression on the surface of the HUVEC cells. Following incubation of HUVEC cells with antibody fragments purified using either the standard protocol or Triton-X114, ICAM-1 molecules were detected with the murine BIRR-1 antibody recognizing a distinct antigenic epitope from MOR102 (#5) by FACS. Fig. 5B demonstrates that surface levels of ICAM-1 were not altered in the presence of the antibody fragment MOR102 (#5) purified using either Triton-X114 or in the presence of an unrelated antibody fragment cleared of endotoxin by ultrafiltration, likewise in the absence of antibody fragments. In contrast, surface levels of ICAM-1 were significantly increased following exposure to antibody fragments purified using the standard protocol.

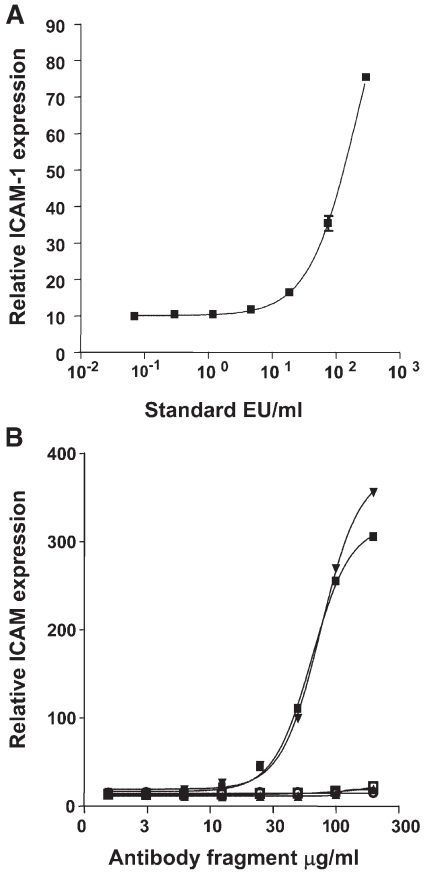

Fig. 5. LPS-dependent ICAM-1 expression on endothelial cells. (A) ICAM-1 expression corresponding to the median fluorescence (relative ICAM-1 expression) was quantified following exposure to a defined amount of purified *E. coli* endotoxin (as indicated) to determine the sensitivity of the assay. (B) Following exposure to an unrelated antibody fragment purified using either the standard protocol (▼) or the standard protocol followed by ultrafiltration to remove endotoxin (□), the MOR102 (#5) purified using the standard protocol (■) or Triton-X114 (▲) or in the absence of antibody fragment (○) endothelial cells expression of ICAM-1 molecules was quantified by FACS using an anti-ICAM-1 antibody directed against an epitope different from the binding site of the MOR102 (#5) (standard deviations are indicated).

4. Conclusion

The presented procedure successfully eliminates endotoxin contaminants from non-covalently linked antibody fragments and from bivalent antibody fragments while preserving integrity and functionality. Endotoxin content was as low as 5 to 9 EU mg^{-1} and protein recovery was more than 90% in comparison to the standard protocol. Binding activity and functionality were not affected by Triton X-114 treatment as demonstrated in ELISA binding and in cell-based adhesion assays. In contrast to conventional methodology, recombinant antibodies purified by Triton X-114 did not stimulate ICAM-1 expression on the surface of endotoxin-sensitive cell lines such as endothelial cells. Thus, this protocol enabling the removal of endotoxin contaminants during affinity purification can be applied for non-covalently linked antibody fragments and for bivalent antibody fragments. In conclusion, this one-step protocol is a rapid, cost effective and automation-compatible procedure suitable for antibody fragment production.

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