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Letter

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Process-related impurities in the ChAdOx1 nCov-19 vaccine

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Abstract
Sinus venous thrombosis has been linked to immunization with the ChAdOx1 nCov-19 vaccine. The initial trigger for this serious adverse event has not been determined. We analyzed the ChAdOx1 nCov-19 vaccine by biochemical and proteomic methods. We found that the vaccine, in addition to the adenovirus vector, contains substantial amounts of both human and non-structural viral proteins. Among the human proteins, heat-shock proteins and cytoskeletal proteins were particularly abundant. The often-observed strong clinical reaction one or two days after vaccination is likely associated with the detected protein impurities. A linkage to later immune-related adverse events is also conceivable. The here reported identification of specific classes of protein impurities should guide and accelerate efforts to increase the purity of the vaccine and increase its safety and efficacy.
Rare cases of thrombotic thrombocytopenia mediated by platelet-activating antibodies against platelet factor 4 (PF4) have recently been linked to intramuscular (i.m.) vaccination with ChAdOx1 nCov-19. While adenovirus-associated thrombocytopenia has been noted in preclinical and clinical studies, this was observed at an early time point after immunization, only after intravascular injection and only with doses about 1,000-fold higher than those given intramuscularly in case of the ChAdOx1 nCov-19 vaccine. Since we repeatedly could not detect binding of the ChAdOx1 virus to platelets (unpublished) and the thrombotic events mostly occur 7-14 days after immunization, we felt that it was rather unlikely that a direct interaction of the virus with platelets caused the adverse events.

We analyzed 3 different lots of the ChAdOx1 nCov-19 vaccine by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining and compared the staining pattern of the separated proteins with those of HAdV-C5-EGFP, an adenovirus vector purified by CsCl ultracentrifugation. ChAdOx1 nCov-19 is produced in the T-REx-293 cell system to prevent expression of the SARS-CoV-2 spike protein during vector production. The vaccine is purified by a combination of filtration steps and Anion-Exchange Chromatography (AEX). Previously, it has been shown, that simian adenovirus vectors including ChAdOx1 can be purified at high yield and purity using such technology for purification. Although in our experiment the same number of particles was loaded, the staining patterns looked very different (Fig. 1). While staining of HAdV-C5-EGFP proteins resulted in the expected pattern, representing distinct major capsid proteins of HAdV-C5 including Hexon, Penton Base, IIIa and Fiber, silver staining of separated proteins from three different lots of ChAdOx1 nCov-19 showed a large number of bands with different intensities, many more bands than could be explained by proteins from viral particles.
Fig. 1 Protein staining of HAdV-C5 and three ChAdOx1 nCoV-19 lots

3 \times 10^9 \text{ adenoviral vector particles} were separated by SDS-PAGE under denaturing and reducing conditions. Proteins were visualized by silver staining. Known HAdV-C5 proteins are labeled in the figure. Three different vaccine lots (ABV4678, ABV5811, ABV7764) of ChAdOx1 nCoV-19, produced by the manufacturer, were analyzed. kDa: kilodalton

Additionally, we analyzed the ChAdOx1 nCoV-19 lots at the DNA level by quantitative polymerase chain reaction (PCR). Results confirmed the exclusive presence of viral DNA, while genomic DNA of the host cell was not detected (data not shown).

We analyzed all three vaccine charges in detail at the proteomic level. The protein content of a single vaccine dose of 0.5 ml of lot ABV5811, containing 5 \times 10^{10} \text{ viral particles} according to the specification, was determined to be 32 \mu g. This was considerably more than the 12.5 \mu g protein
that would be expected in one dose based on the known 150 MDa molecular weight (MW) of adenovirus. To determine the protein composition of the vaccine, we performed mass-spectrometry analyses from either tryptic in-solution digests directly from the vaccine or from tryptic in-gel-digests from the ChAdOx1 nCov-19 vaccine after size separation by PAGE, with similar results (Suppl. Tabl. S1). Based on intensity comparisons of LC/MS signals, we estimate that in lot ABV5811 about 2/3 of the detected protein amounts were of human and 1/3 of virus origin, while the two other lots consisted of rather equal amounts of human and viral proteins (Fig. 2 and Fig. S1).

![Fig. 2 Distribution of proteins in three ChAdOx1 nCov-19 vaccine lots](image)

The protein composition of three lots of ChAdOx1 nCov-19 (ABV4678, ABV5811, ABV7764) was analyzed by mass spectrometry following in-solution protein digest. Spectral data was aligned via search engine with human and viral databases. Intensities associated with proteins from the respective organism were subsequently summed.
Beside the expected viral proteins that form the virion (hexon, penton base, IIIa, fiber, V, VI, VII, VIII, IX and others), also several non-structural viral proteins were detected at high abundancy (Fig. 3 and Figs. S2 and S3), although they are not part of the mature viral particle. To the identified non-structural proteins belong, for example, the 100K protein, a multifunctional scaffolding protein involved in the trimerization of hexon, and the DNA-binding protein (DBP), which plays an essential role in the replication of the viral genome. Since in the assembly process during virus propagation only a part of the available viral capsid proteins is used for particle formation, we assume that in the vaccine product also significant amounts of structural proteins were present as monomers, oligomers or incomplete viral capsid assemblies.

Peptides from more than 1000 different human proteins were detected being derived from the human vector production cell line. We note that only a low number of bona-fide platelet proteins were detected and these were only of low abundancies. The detected proteins were derived from different cellular compartments including cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus and others. Relative amounts of human versus viral proteins differed somewhat between the lots (Fig. 2), as was already assumed based on the silver-stained gels (Fig. 1).
Fig. 3. Intensity distribution of the top 20 proteins of the ChAdOx1 nCov-19 vaccine.

Intensities of the most abundant proteins as observed by proteomic characterization following in-solution protein digests of Lot ABV5811 are shown.

MS Data of the 3 different lots indicated that intensity distributions of the proteins between the lots were comparable (Figs. S4, S5 and S6).

Intriguingly, from the human proteins found in the vaccine and beside several cytoskeletal proteins including Vimentin, Tubulin, Actin and Actinin, the group of heat shock proteins (HSPs) and chaperones stood out in abundancy. Among the top abundant proteins (including viral proteins) HSP 90-beta and HSP-90-alpha as cytosolic HSPs (with 9.5 % and 4.3 % of the total protein) and 3 chaperones of the endoplasmic reticulum (transitional endoplasmic reticulum ATPase,
Endoplasmin and Calreticulin) were present (Fig. 3 and Suppl. Table S2). Of note, also the abundant adenoviral non-structural 100K protein has chaperone function. Like many viruses, adenovirus has been reported to induce HSPs during amplification in production cells, likely to accommodate for a need in help by HSPs in the folding and production of large amounts of structural and non-structural viral proteins and in virion assembly. Thus, virus infection-mediated induction of HSPs could contribute to the abundancy of HSPs in the vaccine product in case of insufficient purification.

Proteins from Bos taurus (likely from fetal calf serum, used for growth of T-REx-293 cells), Spike protein of SARS-CoV-2, E1B proteins from HAdV-C5 (from T-REx-293 cells) and Tetracycline-Repressor TetR (from the T-REx-293 producer cells) were detected at low or negligible levels (Data S1).

**Discussion**

The intramuscular injection of proteins that are not part of the active principle of the vaccine itself might have effects at different levels. Larger amounts of viral proteins, not being incorporated into the viral capsid, might influence the quality of the immune response and potentially negatively affect activity and efficacy of the vaccine. From preclinical studies it is known, that T-cell responses to adenoviral proteins can limit the immunogenicity of adenovirus-vector encoded transgenic antigens. The injection of the vast majority of the more than 1000 different proteins contained in the vaccine is not expected to result in adverse effects. However, some of the detected proteins might be more than inert bystanders. Extracellular HSPs modulate innate and adaptive immune-responses, can exacerbate pre-existing inflammatory condition, have been associated with autoimmunity and can even become target of auto-immune responses themselves. They very efficiently initiate specific immune responses by receptor-mediated uptake of HSP-peptide
complexes in antigen-presenting cells (APCs), mainly via CD91 and scavenger receptors\textsuperscript{12,15}. Since the HSPs present in the vaccine are derived from T-REx-293 cells, in principle they could mediate the transfer to APCs of peptides derived from the 293-cell source, of autologous peptides from vaccinated individuals and also from viral proteins. Several of the HSPs also have ATPase activity and might directly be involved in the activation of platelets by the generation of ADP after intramuscular injection of the vaccine into the ATP-rich skeletal muscle. Among the viral proteins detected, the adenoviral penton base is another candidate for inducing early toxicity via an RGD motive, present in a solvent-exposed loop of penton base, by interacting with integrins on cell membranes including of platelets. Thus, we consider it likely that the here documented protein impurities are involved in the strong clinical reactions with flu-like symptoms, very often observed one or two days after vaccination.

In the biopharmaceutical industry, the removal of host cell proteins (HCPs) from the biological product is a critical quality attribute, since residual HCP might pose a risk to patient safety\textsuperscript{16-18}. According to regulation and regulatory oversight\textsuperscript{19}, standard assays to monitor removal of HCP during and after purification of biopharmaceuticals are Enzyme-Linked Immunosorbent Assays (ELISAs), that are based on polyclonal antibodies isolated from larger animals, preferentially goat or sheep, after immunization with cell lysates or supernatants from the producer cell. In case of the production of secreted recombinant proteins, such assays have been part of standard operating procedures, used together with other methods, to assure and document the absence of HCPs in the final product. The apparent lack of detection of HSPs and cytoskeletal proteins in the ChAdOx1 nCov-19 vaccine with ELISA-based methods can be explained by the extremely high homology of HSPs and cytoskeletal proteins between different species, so that in immunized animals no antibodies against these proteins were generated. When taking some of the most abundant HCPs found in our study, there is 99.59\% identity at the amino acid level between human and sheep HSP
90-beta, 100% identity between human and goat transitional endoplasmic reticulum ATPase and 98% identity between human and sheep vimentin. Almost certainly, these proteins would be missed, if quality control was only based on such an ELISA and not complemented by orthogonal methods such as direct staining of protein gels, capillary electrophoresis or LC-MS. Obviously, when the production process involves lysis of the producer cells, standard HCP ELISAs with authorized use in production processes for secreted recombinant proteins are not suitable for quality control of complex biological products such as adenovirus-based vectors and vaccines. With the many different contaminant proteins detected in the ChAdOx1 nCov-19 vaccine and therefore an immanent uncertainty, whether or not (some of) the impurities might have long-term immune-related side effects, it is necessary to improve the purification process for the vaccine to potentially increase its safety and reduce concerns. This might have the additional benefit of also enhancing the antiviral efficacy of the vaccine.

The establishment of robust assays for detection of HCPs can be complicated and very time-consuming\(^\text{16}\), in particular if processes for new and complex biopharmaceuticals have to been developed, time that maybe is not always available in times of a pandemic. However, the identification of specific process-related impurities in ChAdOx1 nCov-19, as reported here, should guide and accelerate the required next steps.

**Methods**

**Purification of HAdV-C5-EGFP**

HAdV-C5 vector particles used in this study were EGFP-expressing *E1*-deleted replication-incompetent vector particles based on human adenovirus species C type 5 (based on GenBank AY339865.1, sequence from nt 1 to 440 and from nt 3,523 to 35,935). The CMV-promoter controlled EGFP expression cassette was subcloned from a pEGFPN1 plasmid (6085-1; Clontech).
Particles were produced in E1-complementing N52.E6 cells. In brief: 2 x 10^8 cells were transduced with 6 x 10^{10} total vector particles from stock solution. Forty-eight hours after transduction cells were harvested, resuspended in 3 ml 50 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.4) and lysed by three consecutive freeze/thaw cycles. Cell debris was removed by centrifugation at 2,000 x g for 10 min, vector particle-containing supernatants were layered on a CsCl step gradient (density bottom: 1.41 g/ml; density top: 1.27 g/ml, 50 mM HEPES, 150 mM NaCl, pH 7.4) and centrifuged at 176,000 x g for 2 h at 4°C. Vector particles were rescued and further purified by a consecutive continuous CsCl gradient (density: 1.34 g/ml, 50 mM HEPES, 150 mM NaCl, pH 7.4) and centrifuged at 176,000 x g for 20 h at 4°C. Subsequently, vector solutions were desalted by size exclusion chromatography (PD10 columns, 17-0851-01; GE Healthcare). Physical vector titers were determined by optical density measurement at 260 nm as described earlier.

**Adenoviral vectors used in this study**

According to the manufacturer (AstraZeneca) the ChAdOx1 nCov-19 vaccine (lot numbers ABV4678, ABV5811, and ABV7764) has a physical titer of 1 x 10^{8} VP/µl and is dissolved in 10 mM histidine, 7.5% sucrose (w/v), 35 mM NaCl, 1 mM MgCl\textsubscript{2}, 0.1% polysorbate 80 (w/v), 0.1 mM EDTA and 0.5% EtOH (w/v). Lots were stored at 4 °C. None of the lots was expired at the time experiments were conducted.

HAdV-C5-EGFP vector particles, produced in house, had a physical titer of 2.9 x 10^9 VP/µl, were dissolved in 50 mM HEPES, 150 mM NaCl, 10% glycerol, pH 7.4 and stored at -80°C.
Staining of proteins separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

3 x 10^9 vector particles dissolved in 30 µl were mixed with SDS-loading buffer (30 mM Tris, 1% SDS, 5% glycerol, bromophenol blue, pH 7.5) containing 0.2 M β-mercaptoethanol and heated for 5 min at 96°C. Reduced and denatured proteins were separated by SDS-PAGE and subsequently stained in gel by coomassie or silver staining.

Silver staining: proteins were fixed (50% MeOH, 12% AcOH, 0.05% HCHO) for 30 min and washed for 15 min with 50% EtOH. Subsequently, proteins were equilibrated for 1 min (0.8 mM Na₂S₂O₃), washed with dH₂O, impregnated for 20 min (11.78 mM AgNO₃, 0.05% HCHO) and washed again with H₂O. Protein bands were visualized by the deoxidation of adsorbed silver ions to silver (0.57 M Na₂CO₃, 0.05% HCHO, 15.8 μM Na₂S₂O₃). Signal development was stopped (50% MeOH, 12% AcOH) once protein bands were visible.

Coomassie staining: protein gels were stained by coomassie (3.5 mg/ml Coomassie Brilliant Blue R-250, 30% EtOH, 10% AcOH) for 12 h. Subsequently, the gel was destained (30% EtOH, 10% AcOH, exchanged every 20 min) for 2 h.

Sample preparation

For sample clean-up, 0.5 ml vaccine were precipitated employing Methanol/Chloroform extraction based on well-established protocols. To this end, 2 ml Methanol were added and mixed, another 500 µl chloroform were added and the mixture was thoroughly vortexed. After addition of 1.5 ml of water and mixing, suspension was centrifuged for 1 min at 14,000 x g. The resulting top-layer was removed and another 2 ml of Methanol added. Following centrifugation for 5 min at 14,000 x g, methanol was removed and the pellet collected for further analysis.
**Determination of protein concentration**

For assessment of protein concentration in the ChAdOx1 nCov-19 vaccine, the extracted protein pellet was resuspended in 50 µl water. Homogenization was achieved by employing ultrasonication for 10 min. The samples were analyzed using an NanoDrop OneC (Thermo Fisher) according to the manufacturers protocol. Absorption at 280 nm was used employing the “A280 mg/ml” routine embedded in the instrument (Ver. 1.3, DB version 1) and pure water as a blank reference.

**Proteomic analysis of the ChAdOx1 nCov-19 vaccine**

For the in-solution digest, 6 µg of protein was reduced with 5 mM DTT (AppliChem) for 20 min at RT and subsequently alkylated with iodoacetamide (Sigma-Aldrich) for 20 min at 37°C. Trypsin (Thermo Scientific) was added in a 1:50 enzyme-protein ratio and digested overnight at 37°C. Vaccine samples separated via SDS-Page were prepared as follows: After Coomassie-staining, gel lanes were cut into 20 pieces. Individual pieces were washed by alternating incubation in the respective protease buffer and 50 % buffer /50% Acetonitrile (ACN) thrice for 10 minutes each. Following vacuum drying, samples were reduced with 5 mM DTT (AppliChem) for 20 min at RT and subsequently alkylated with iodoacetamide (SigmaAldrich) for 20 min at 37°C. For protease digests, gel slices were reconstituted with Trypsin solution (0.33 ng/µl Trypsin in 50 mM ammonium bicarbonate) Digest was carried out over night at 37°C. LC/MS and bioinformatical analysis was carried out as described above, with the exception of shortening the LC gradient to 65 min in total.

Employing an LTQ Orbitrap Elite system (Thermo Fisher Scientific) online coupled to an U3000 RSLCnano (Thermo Fisher Scientific), samples were analyzed as described previously, with the following exceptions: Separation was carried out using a binary solvent gradient consisting of
solvent A (0.1% FA) and solvent B (86% ACN, 0.1 % FA). The column was initially equilibrated in 5% B. In a first elution step, the percentage of B was raised from 5% to 15% in 10 min, followed by an increase from 15% to 40% B in 145 min. The column was washed with 95% B for 4 min and re-equilibrated with 5% B for 20 min.

MS analysis was performed using an LTQ Orbitrap Elite system (Thermo Fisher Scientific) with the following settings: MS1 full scans were acquired in profile mode from m/z 370-1700 with the orbitrap detector, resolution was set to 30,000. The 20 most intense ions from the survey scan were picked for CID fragmentation, with collision energy set to 35% and an activation Q of 0.25. Singly charged ions were rejected and m/z of fragmented ions were excluded from fragmentation for 60s. MS2 spectra were acquired employing the LIT at rapid scan speeds.

**MS data analysis and statistics**

Database search was performed using MaxQuant Ver. 1.6.3.4 (www.maxquant.org). Employing the build-in Andromeda search engine, MS/MS spectra were correlated with the UniProt human reference proteome set (www.uniprot.org) and a database containing the expected virus protein sequences for peptide identification. Carbamidomethylated cysteine was considered as a fixed modification along with oxidation (M), and acetylated protein N-termini as variable modifications. False Discovery rates were set on both, peptide and protein level, to 0.01.

**Detection of adenoviral and human genome DNA by qPCR**

DNA of $1 \times 10^{11}$ vector particles of HAdV-C5-EGFP and AstraZeneca ChAdOx1 nCoV-19 was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, G1N350) according to the manufacturer instructions. As a control, DNA of $2 \times 10^6$ HEK293T cells (ATCC CRL-3216) was isolated. DNA was eluted in 10 mM Tris, pH 8.5. Concentration was determined by optical
density measurement at 260 nm. DNA samples were analyzed for their viral and human genome DNA content by quantitative real time PCR. Primers used amplified parts of the adenoviral E4 region, which is present in both adenoviral strains analyzed (forw.: 5’ TAGACGATCCCTACTGTACG 3’; rev.: 5’ GGAAATATGACTACGTCCGG 3’), the human actin gene (forw.: 5’ GCTCCTCCTGAGCGCAAG 3’; rev.: 5’ CATCTGCTGGAAGGTGGACA 3’) and the human ribosomal protein L4 gene (forw.: 5’ ACGATACGCCATCTGTCTGTCGCA 3’; rev.: 5’ GGAGCAAAACAGCTTCTGTGGTC 3’). 20 ng DNA was added to 10 µl SYBR Green (KK4502; Kapa Biosystems), and 0.4 µl 10 pmol/µl of each forward and reverse primer in a total volume of 20 µl. Thermocycles: 1 cycle: 10 min 95 °C; 40 cycles: 30 sec 95 °C, 30 sec 60 °C, 8 sec 72 °C; 1 cycle: 10 min 72 °C.

Data availability

The data supporting the findings of this study are available within this paper.

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Author contributions

L.K., S.W. and S.K. designed experiments. L.K. und R.R. performed biochemical analyses. R.R. performed mass spectrometry analyses. L.K. and S.K. analyzed data of biochemical analyses. R.R. and S.W. analyzed the mass spectrometry data. L.K. and R.R. prepared figures. L.K., S.W. and S.K. prepared the manuscript. All authors commented on the manuscript.

Ethics declarations

Authors declare that they have no competing interests.

Supplementary Information

Supplementary Figs. 1-6.

Supplementary Table 1

Protein List. Comprehensive overview of protein identifications and quantifications based on LC/MS analysis of in-solution digests of Lots ABV7764, ABV4678 and AB5811 (first sheet) and in-gel digest of ABV5811 (second sheet).

Supplementary Table 2

Top 30 proteins, detected in lot ABV5811. List of the most abundant proteins in ABV5811 as identified following LC/MS analysis of the in-solution digested vaccine.
Fig. S1

Distribution of proteins of the ChAdOx1 nCov-19 vaccine following PAGE and in-gel digests

The protein composition of lot ABV5811 was analyzed by mass spectrometry following in-gel protein digests. Spectral data was aligned via search engine with human and viral databases. Intensities associated with proteins from the respective organism were subsequently summed.
Fig. S2

Intensity distribution of the top 20 proteins of the ChAdOx1 nCov-19 vaccine

Intensities of the most abundant proteins as observed by proteomic characterization of lot ABV5811 following in-gel digest are shown.
Fig. S3

Distribution of Structural/Capsid and Non-Structural/Non-Capsid viral proteins of the ChAdOx1 nCov-19 vaccine following in-solution digest

The protein composition of lot ABV5811 was analyzed by mass spectrometry following in-solution protein digests. Spectral data was aligned via search engine viral databases. Intensities associated with Capsid versus Non-Capsid proteins were subsequently summed.
Fig. S4

Quantitative comparison of protein intensities observed for lots ABV5811 and ABV4678. Protein intensities as observed via proteomic characterization of lots ABV5811 and ABV4678 show an overall linear relationship for both viral and human proteins. Top 20 proteins colored as indicated.
**Fig. S5**

**Quantitative comparison of protein intensities observed for lots ABV5811 and ABV7764.**

Protein intensities as observed via proteomic characterization of lots ABV5811 and ABV7764 show an overall linear relationship for both viral and human proteins. Top 20 proteins colored as indicated.
**Fig. S6**

**Quantitative comparison of protein intensities observed for lots ABV7764 and ABV4678.**

Protein intensities as observed via proteomic characterization of lots ABV5811 and ABV4678 show an overall linear relationship for both viral and human proteins. Top 20 proteins colored as indicated.
Protein staining of HAdV-C5 and three ChAdOx1 nCoV-19 lots $3 \times 10^9$ adenoviral vector particles were separated by SDS-PAGE under denaturing and reducing conditions. Proteins were visualized by silver
staining. Known HAdV-C5 proteins are labeled in the figure. Three different vaccine lots (ABV4678, ABV5811, ABV7764) of ChAdOx1 nCoV-19, produced by the manufacturer, were analyzed. kDa: kilodalton

Figure 2

Distribution of proteins in three ChAdOx1 nCov-19 vaccine lots The protein composition of three lots of ChAdOx1 nCov-19 (ABV4678, ABV5811, ABV7764) was analyzed by mass spectrometry following in-solution protein digest. Spectral data was aligned via search engine with human and viral databases. Intensities associated with proteins from the respective organism were subsequently summed.
Figure 3

Intensity distribution of the top 20 proteins of the ChAdOx1 nCov-19 vaccine. Intensities of the most abundant proteins as observed by proteomic characterization following in-solution protein digests of Lot ABV5811 are shown.

Supplementary Files

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