Detection of Clostridium novyi type B α toxin by cell culture systems

Erika Borrmann *, Frank Schulze

Federal Institute for Health Protection of Consumers and Veterinary Medicine, Division 4, Jena, Germany

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Abstract

Ten permanent cell lines were examined for their reaction to the Clostridium novyi α toxin. The action of the toxin was determined after 3 days by microscopic examination and the MTT assay. The α toxin exhibited the strongest effect on ESH-L cells rather than other cell lines. Vero and SFT-R cells reacted in a comparable way, but less sensitively. We were able to show that the cytopathic effect on the three types of cells was neutralised by the international standard for gas gangrene antitoxin (C. novyi) but in no case by heterologous antisera. Our results have shown that the three cell lines were specific indicators for the detection of the cytopathic effect of α toxin. The cytopathic effect can be measured reproducibly by the cell culture assay used. These results are suitable as the starting point for the development of the neutralisation test using cell cultures. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Clostridium novyi is classified into four types designated A, B, C and D based on the production of eight different soluble antigens and, to some extent, on the diseases which they produce in men and animals. Type B is the causative agent of infectious necrotic hepatitis (black disease, deutscher Bradsot), especially in sheep but seldom in other animals. The major pathological determinant is the α toxin which is an oedema-inducing and lethal exotoxin [1]. Although not precisely measured, financial losses to the sheep farms are high when vaccination is not carried out. Economic losses result from deaths and depreciation of infected stock and from the costs of preventive programmes and sanitary disposal of dead sheep affected with the disease [2]. An early prophylactic immunisation with C. novyi toxoid vaccines is recommended in endangered areas, because the disease can be effectively controlled by vaccination of animals [3].

According to the European Pharmacopoeia [4] the potency testing of C. novyi (type B) vaccines for veterinary use requires vaccination of laboratory rabbits followed by quantitative determination of the antibodies against C. novyi α toxin in the rabbit sera by the toxin neutralisation test in mice (TNT). Since this test is cumbersome, time-consuming and causes severe distress and suffering to animals, an in vitro
method using cell cultures should be developed as a possible alternative to the TNT.

As antibody titres in sera determined by neutralisation are based on the detection of the non-neutralised toxins, the first step taken was to test the cytotoxic/cytopathic effect of α toxin on cell cultures and to examine the specificity of the reaction.

Several reports have described the cytopathic effect of the K toxin on different cell cultures: Vero, CHO, HT 29 [5], L-929, PC-12 [2], WI-38 [6] and endothelial cells [7]. The quantitative determination of the antibodies against α toxin, however, has not yet been performed successfully.

2. Materials and methods

2.1. Toxin

C. novyi type B toxin (IRP 425), used as standard for the C. novyi α TNT in mice, was kindly provided by Dr Hauer (Animal and Plant Health Inspection Service, Centre for Veterinary Biologics, Ames, IA, USA). A volume of 0.8 ml of this toxin and 0.2 ml diluent is equivalent to 0.1 l dose [8]. In order to remove any glycerol the K toxin was dialyzed overnight at 4°C against phosphate buffer, pH 7.2. The toxin was characterised by means of SDS-PAGE and immunoblotting. A monoclonal antibody kindly presented by Dr Pietrzykowski was taken as primary antibody for the detection of the toxin pattern in immunoblotting.

2.2. Cell lines

For the detection of cytotoxicity/cytopathicity, the following cell lines were used: embryonal bovine lung cells (EBL), foetal bovine tracheal cells (FBTR), kidney cells of the African green monkey (Vero), rat intestinal cells from the small intestine (IEC-6), hamster ovary cells (CHO-K1), embryonal kidney cells of the rhesus monkey (MA 104), dog kidney cells (MDCK), embryonal skin cells of sheep (ESH-L), Syrian hamster kidney cells (BHK-21) and thymus cells of sheep (SFT-R).

Vero, ESH-L, EBL and FBTR cells were cultivated in minimal essential medium (Eagle) containing Earle’s salts (MEM). MA 104 cells grew in Dulbecco’s minimal essential medium (DMEM) and the IEC-6 cells grew in DMEM with the addition of HEPES and insulin. MDCK cells were cultivated in MEM containing non-essential amino acids (NEAA). MEM containing NEAA and 120 mg l⁻¹ sodium pyruvate was used for the cultivation of BHK-21 and SFT-R cells. CHO-K1 cells were cultivated in MEM containing 150 μg ml⁻¹ proline. Foetal calf serum (FCS, 10%), 2 mM glutamine and 50 μg ml⁻¹ gentamicin were added to the media. The use of inactivated FCS was necessary for the cultivation of CHO-K1. All cells were maintained at 37°C in a 5% CO₂ atmosphere.

2.3. Antisera

The following antisera were used:

- International standard for gas gangrene antitoxin (C. novyi, equine, 3rd standard, 1966, Statens Seruminstitut, Copenhagen, Denmark), dissolved in 1 ml phosphate buffer, pH 7.2, containing 1100 international units.
- International C. perfringens α antitoxin (5th standard, 1963, Central Veterinary Laboratory, Weybridge, UK), dissolved in 5 ml phosphate buffer, pH 7.2, containing 270 international units.
- International standard for Clostridium welchii (perfringens) type D antitoxin (1st standard, 1954, Central Veterinary Laboratory, Weybridge, UK), dissolved in 5 ml phosphate buffer, pH 7.2, containing 250 international units.
- International standard for Clostridium welchii (perfringens) type B antitoxin (1st standard, 1954, Central Veterinary Laboratory, Weybridge, UK), dissolved in 5 ml phosphate buffer, pH 7.2, containing 1000 international units.

2.4. Cell culture assay

The action of the α toxin on cells was determined by a cell test which has been described previously [9]. The cell culture assay was performed in 96-well plates (Costar Europe Ltd., Badhoevedorp, The Netherlands). In each well of a 96-well plate already containing 50 μl of the medium used for cell cultivation (5% FCS), 50 μl of the toxin solutions were
added in their respective dilution steps, as well as 50 µl cell suspension. Each of the dilution steps was measured in duplicate. For the cell control (cells without contact of toxin) 50 µl of medium was used instead of toxin solution. On each plate, the cell control was measured six times. The plates were incubated at 37°C under 5% CO₂ for 3 days.

The cell suspensions were prepared by the removal of confluent growth from the cell culture flasks using trypsin, followed by resuspension in the medium (5% FCS). The same media were used for preparation of the toxin dilution series.

The neutralisation test used to obtain evidence of specificity was performed as described earlier [10]. In each well of a 96-well plate, the toxin and the antiserum in serial dilution were mixed and incubated at room temperature for 1 h. The cell suspension was then added. The plates were incubated at 37°C under 5% CO₂ for 3 days. The cell suspension and the toxin solution were used as controls.

2.5. Evaluation

The determination of viable cells was carried out with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test [11] and microscopic examination. The optical density (OD) was measured at 550 nm using an ELISA reader (SLT GmbH, Crailsheim, Germany).

The number of cells which remained viable after exposure to the toxin was calculated by means of the evaluation programme from SLT relating to respective cell controls (% viable cells = (ODtest/ODcell control)×100).

3. Results

The comparison of the cytopathic effect of C. novyi type B α toxin on different cell lines is shown in Fig. 1. The results are means of triplicate determinations. The calculated standard deviations varied between 3% (minimum) and 25% (maximum). The cells which remained viable after toxin contact were determined depending on the toxin concentration.

The α toxin exhibited the strongest effect on the ESH-L cells as compared to the other cell lines. Exposure of ESH-L cells to highly concentrated α toxin resulted in rounded cells (cytopathic effect) beside lysed cells (cytotoxic effect). The proliferation of the cells was clearly reduced. Higher dilution of the toxin resulted in spindle-like beside rounded cells.
Fig. 2. Morphological alterations of the cells exposed to α toxin for 3 days in microtiter plates. Light microscopy, magnification ×90. A: ESH-L cells without toxin contact (cell control), $1.25 \times 10^4$ cells per well, the cells are confluent and show a fibroblast-like morphology. B: ESH-L cells after contact with α toxin (titre 1:100), complete rounding. C: Vero cells without toxin contact (cell control), $1.0 \times 10^4$ cells per well, the cells are confluent and show a fibroblast-like morphology. D: Vero cells after contact with α toxin (titre 1:100), complete rounding. E: SFT-R cells without toxin contact (cell control), $1.0 \times 10^4$ cells per well, the cells are confluent and show an epithelial-like morphology. F: SFT-R cells after contact with α toxin (titre 1:100), rounding and retracting cells.
(Fig. 2A,B). All investigated cells exhibited the same type of rounding effect (Fig. 2C–F), but the ESH-L cells were more sensitive than the other cells. Approximately 50% viable cells were measured at a toxin titre of 1:1000 on ESH-L, 1:2000 on Vero and 1:3000 on SFT-R cells.

The toxin test was also used for the determination and standardisation of the neutralisation test conditions. The parameters using the ESH-L cells were the following:

- Test media: all dilutions were made with the cell culture medium (5% FCS);
- Cell number: 1.25×10^4 cells per well;
- Comparison of the cell passages: 13th–33rd passages;
- Age of the cells: 4 days after passage;
- Evaluation of viable cells: MTT test.

The specificity of the ESH-L cell line as well as the Vero and SFT-R cell lines for the detection of the \( \alpha \) toxin was determined in the neutralisation test. The international standard for gas gangrene antitoxin was able to neutralise the action of the toxin up to a 1:10000 dilution of the antiserum. The heterologous antisera were unable to neutralise the cytopathic effect in any case.

4. Discussion

The reproducible and specific detection of the cytopathic effect of the \( C. novyi \) \( \alpha \) toxin on ESH-L cells is a prerequisite for the development of a neutralisation test using cell cultures as an alternative to the TNT. In comparison to other cell lines, the ESH-L cells were found to react most sensitively to the toxin. The cell shape alterations were characterised by retraction and rounding when the toxin was highly concentrated. The use of the toxin at a dilution >1:2000 resulted in spindle-like cells side by side with rounded cells. The effects observed were in agreement with reports in the literature. The \( C. novyi \) \( \alpha \) toxin belongs to the so-called large clostridial cytotoxins (LCT) with reference to the high molecular mass of the single-chain molecules, their in vitro action on the microfilament system of cultured cells and a series of in vivo actions [12]. LCTs added to cells in culture are taken up by receptor-mediated endocytosis into cytosol. The contact of cultured cells with the \( \alpha \) toxin results in irreversible changes in cell shape based on the action on the actin cytoskeleton and by minor effects at the vimentin and tubulin systems [13,14]. The \( \alpha \) toxin has been found to have glycosyltransferase activities that use activated sugars to modify different small GTP-binding proteins [15].

Cell cultures can be effective indicators of free toxin in the TNT, but it is necessary to demonstrate that the cytopathic effects have been caused by the toxin and not by other components such as preservatives, detoxifying agents, non-immunogenic bacterial toxins and other excipients [6]. For this reason, it is necessary to find sensitive cells in order to remove interfering components by dilution. Therefore, the ESH-L cells would be the most suitable from the range of cells investigated. The ESH-L cells, however, were growth-limited cells (so-called finite cell line) and it was not possible to use cells after the 33rd passage because of the morphological alterations of the cells. The Vero and SFT-R cells were less sensitive than the ESH-L cells, but both cell lines are permanently growing cells.

Clostridial vaccines are multicomponent vaccines and beside \( C. novyi \) \( \alpha \) toxoid also contain \( C. perfringens \) \( \epsilon \) and \( \beta \) toxoids. It was very important to demonstrate that the cytopathic effect on the cells had been caused by the \( \alpha \) toxin and not by other components. The neutralisation tests using both homologous antiserum and heterologous antisera demonstrated the specificity of the reaction of \( \alpha \) toxin on the ESH-L, as well as the Vero and the SFT-R cells. In addition, the calculated standard deviations demonstrate that the cytopathic effect on the three cell lines could be measured reproducibly. Our results have shown that the three cell lines are suitable indicators of excess toxicity of \( C. novyi \) \( \alpha \) toxin in the TNT using cell cultures. These results are the starting point for the development of the neutralisation test using cell cultures.

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References