

Separation and characterization of potential allergens from cultured baby hamster kidney cells

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Summary

Residual cellular material present in foot-and-mouth disease (FMD) vaccines has been implicated as a possible cause of hypersensitivity in cattle. This study was designed to identify and characterize the potentially allergenic components involved. After fractionating baby hamster kidney (BHK) cell lysate, by preparative electrofocusing and gel filtration chromatography, the eluates which were potentially allergenic for cattle were identified by passive cutaneous anaphylaxis (PCA) and PCA-inhibition methods. The potential allergen identified had a molecular weight of approximately 43 000 daltons, sedimentation coefficient of 3.65 S_{20} and isoelectric points between pH 5.37 and 6.25. Based on these results an immunogenic FMD vaccine was produced which failed to provoke clinical sensitization after repeated inoculations. This information should enable manufacturers to identify, monitor and subsequently control the levels of any such potentially allergenic material present in current vaccines produced using BHK cells.

Introduction

Previous results have shown that foot-and-mouth disease (FMD) vaccination sensitizes cattle against baby hamster kidney (BHK) cell lysate, and anaphylaxis often developed when this lysate was injected intravenously (i.v.) into vaccinated animals [1, 2]. The vaccine was also capable of stimulating reaginic antibodies against BHK cell lysate in cattle [3, 4] and of reacting with such antibodies in passive cutaneous anaphylaxis (PCA) tests [5].

The BHK cell allergen in question shared immunological specificity with hamster serum although passive cutaneous anaphylaxis inhibition (PCA-I) tests showed that the cross reactions were approximately 80 times weaker than reactions with homologous antigen [6]. The object of the present study was to fractionate the BHK cell lysate and to identify and characterize the component/s which were potentially allergenic for cattle.

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Materials and methods

Baby hamster kidney cell lysate

Two litres of BHK 21, Clone 13, suspension cells [7] grown in Eagles' medium with 8% normal bovine serum, containing 10^6 cells per ml was centrifuged ($500 \times g$ for 10 min) and washed three times in phosphate buffered saline (PBS). The cells were resuspended in 50 ml of distilled water, frozen and thawed three times by immersing in liquid nitrogen and centrifuged at $1000 \times g$ for 10 min to remove gross debris. Finally the lysate was clarified by spinning at $75\,000 \times g$ for 1 h at 37°C . Dialysis, when required, was carried out against isotonic saline for 24–36 h with at least one change of dialysis fluid.

Gel filtration

Two ml samples of BHK cell lysate, both dialysed and undialysed, were passed down a 100×2.5 cm column of Bio-gel A-5M (Bio Rad Laboratories) equilibrated with 0.1 M tris-HCl buffer, pH 7.2, containing 0.3 M NaCl and 0.02% Na-azide. For elution the same buffer was used at a flow rate of 25 ml/h and 200×2.7 ml fractions were collected.

The fractions were pooled according to the 280 nm optical density (OD) profile, as shown in Fig. 1, and examined for protein concentration, sedimentation coefficient and ability to react with a known reaginic anti-BHK bovine serum in both PCA and PCA-I tests. Marker substances with known molecular weights (blue dextran, pig IgM, thyroglobulin, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A) were also passed down the column and their filtration rates were used to estimate the molecular weight (MW) of the eluted fractions (Fig. 2).

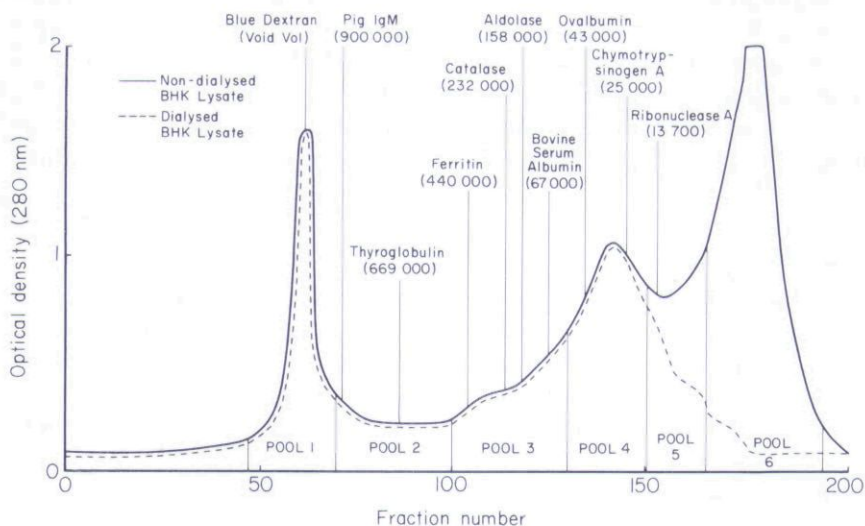


Fig. 1. Elution profile of BHK cell extract on Bio-Gel A-5M gel filtration medium with molecular weight markers.

Preparative electrofocusing in granulated gel (PEGG)

A 100 ml slurry of Ultradex (LKB Laboratories) containing carrier ampholytes (Ampholine, LKB Laboratories), pH 3.5–10, and 50 ml of clarified BHK cell lysate was

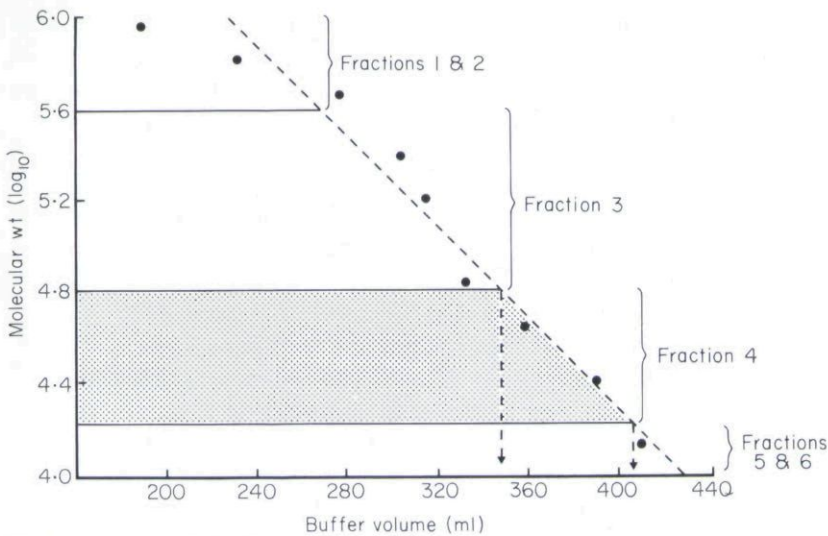


Fig. 2. Calibration of Bio-Gel A-5M column according to elution of molecular weight markers. The elution areas of the pooled BHK fraction are also shown. Pool 4 (shaded) had the greatest activity in PCA and PCA-I tests.

fan-dried to give a stable gel. The gel was placed in a LKB Multiphore apparatus and absorbent paper strips soaked in electrolyte solutions (1 M H_3PO_4 and 1 M NaOH) were used to connect the gel slab to the anode and cathode terminals respectively. Constant power of 8 W was applied to the gel for 16.5 h at 4°C. When the separation was complete an impression print was made by resting a layer of filter paper on the gel surface until saturated. The impression was air dried, fixed with 10% trichloroacetic acid, stained for 5 min in 0.2% Coomassie brilliant blue and destained with methanol/water/acetic acid (5:5:1) mixture. A fractionating grid was pressed into the gel and the contents from each of the fifteen compartments was transferred to 8.5 × 1.2 cm elution columns. After measuring the pH the gel in each column was eluted with 10 ml of PBS. The eluates were pooled in consecutive lots of three and the five samples which resulted were examined for ability to provoke PCA reactions with a bovine serum known to contain anti-BHK reaginic antibodies. Having identified samples 4–12 as allergenic (see Results section) a second PEGG run was carried out on a fresh aliquot of BHK cell lysate. On this occasion the material eluted in samples 4–12 was subjected to an additional PEGG separation using ampholytes with range restricted to pH between 5 and 8. The fifteen eluted samples were again pooled and examined for allergenicity by the PCA-I method using the reaginic antiserum at both 1/32 and 1/16 (final) dilutions.

Analysis of BHK fractions

Protein concentration. Total protein was estimated by means of dye-binding assay [8] using Bio-Rad reagent (Bio-Rad Laboratories).

Sedimentation coefficient. Eluted samples were extensively dialysed against isotonic saline and subjected to analytical ultra-centrifugation in a MSE Centriscan 75 at 40 000 rpm and 20°C. Schlieren scans were taken at 10-min intervals using a 75° knife angle and 550 nm wavelength. The results, calculated as described by Svedberg & Pedersen [9], were corrected by reference to water at 20°C.

Allergenicity demonstrated by PCA test [10]. After injecting 0.4 ml xylazine

(Rompun, Bayer) intramuscularly, the chest walls and flanks of white Saanen goats were clipped and the skin marked out in grids containing squares of about 3 cm each side. Cattle serum known to contain reaginic antibodies was prepared as doubling dilutions (1/2–1/128) in saline and injected intradermally in random order, 0.1 ml to a square. Three days later 10 ml of 2% Evans blue dye in isotonic saline was injected intravenously (i.v.) followed by one or more of the BHK eluate pools in 6 ml volumes, allowing an interval of at least 35 min between the eluate injections. Allergenic BHK fractions were identified when blue staining developed at the dermal injection sites shortly after the intravenous injections.

Allergenicity demonstrated by passive cutaneous anaphylaxis inhibition (PCA-I) test [6]. Young white Saanen goats were sedated, clipped and marked as for the PCA test. Doubling dilutions of the test samples (1/1–1/32) were mixed in equal volume with two dilutions (1/8 and 1/16) of the known anti-BHK reaginic serum (using isotonic saline as diluent) and held for 24 hr at 4°C. These mixtures and control dilutions of the reaginic antiserum (1/2–1/256) in isotonic saline were coded and injected intradermally in random order, 0.1 ml per square. Three days later 10 ml of 2% Evans blue and 6 ml of BHK lysate (500 mg % protein) were injected i.v. PCA reactions were indicated by blue stains in the skin at the sites of the control antiserum injections and the inhibition produced by admixture of various dilutions of the test materials was noted.

Results

The samples eluted from the gel filtration column after application of both dialysed and undialysed BHK cell lysate were divided into six fractions according to their 280 nm absorption profiles as shown in Fig. 1. Dialysis influenced the 280 nm absorption characteristics of pools 5 and 6, that is in the area where substances of the lowest molecular weights were eluted, but made no appreciable difference to the absorption profiles of fractions 1–4.

The elution sites of the molecular-weight marker substances are also shown in Fig. 1, and in Fig. 2 their MW ($\log_{10}$) have been plotted against their elution buffer volume. A linear relationship was demonstrable (coefficient of correlation $r=0.986$) in the case of all points other than those of the largest MW substances. This graph was used as a standard slope to estimate the approximate MW limits of the pooled BHK lysate fractions. These are shown in Table 1 together with the protein concentrations, sedimentation coefficients, and PCA and PCA-I results.

Pools 3, 4 and 5 all contained allergenic materials which reacted with the anti-BHK reaginic antibodies in both the PCA and PCA-I tests but pool 4 consistently produced the strongest reactions. The MW of the substances in pool 4 ranged from 61 500 to 16 700 daltons with elution midpoint at 32 000 daltons approximately.

Tables 2 and 3 show the PCA and PCA-I activity and the pH range of each of the eluted fractions from PEGG runs. Fractions 7–9 from the first (pH 3.5–10) PEGG run, corresponding to pI values of 5.9–6.8 contained almost all the allergenic material which produced strong PCA reactions with the reaginic anti-BHK antiserum. The material eluted in samples 4–12 of a repeat run were subjected to a second (pH 5.0–8.0) PEGG separation resulting in the impression print shown in Fig. 3. After fractionation and elution the highest levels of allergenic activity, as indicated by PCA-I were obtained from samples 7–12 corresponding to iso-electric points between pH 5.37 and 6.25. These fractions were subsequently concentrated, dialysed and run down a Bio-Gel A-5M column. This produced four fractions in the 61 500–16 700 dalton region

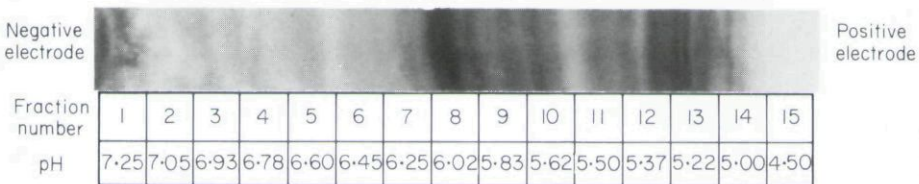
Table 1. Analysis of gel filtration fractions

	Pool no.					
	1	2	3	4	5	6
Molecular weight (approximate range)	unreliable estimate	> 409 500	409 500–61 500	61 500–16 700	16 700–6 300	< 6300
Percentage of total protein (%)	10.07	17.72	34.72	27.78	5.90	3.81
Sedimentation coefficient (S ₂₀)	N.T.	14.3	4.3	3.65	N.T.	N.T.
PCA reaction	—	—	+	+++	+	—
PCA inhibition titres*						
Reagin dilution 1/32	4	4	32	64	32	4
Reagin dilution 1/64	2	4	2	32	6	2

N.T. = Not tested.

PCA reactions: — negative, + mild, ++ severe, +++ very severe.

* Final dilutions.

**Fig. 3.** Impression print from a pH 5.0–8.0 preparative electrofocussing run showing pH values from all the gel fractions collected.

identified previously (see above). Of these fractions only fraction 2 produced inhibition of PCA reactions in goats (data not shown). The peak of this fraction corresponded to a MW value at 43 000 daltons.

Table 2. Analysis of fractions from the first preparative electrofocussing run using carrier ampholytes with pH 3.5–10

Fraction nos	1–3	4–6	7–9	10–12	13–15
pH range of eluted pool	9.1–3.4	9.1–7.3*	6.8–5.9*	5.5–4.9*	4.5–3.85
PCA results	—	+	+++	+	—

* This material was pooled for further fractionation on a pH 5–8 gel.

In an attempt to support this data inactivated FMD virus harvest was fractionated by gel filtration into low (pools 3, 4, 5 and 6 from Fig. 1) and high (pools 1 and 2 from Fig. 1) MW components which were formulated into vaccines. For comparison a vaccine was also formulated from starting material after diluting it to a similar extent to the column eluates. The vaccines were injected into groups of four cattle on four occasions at 2-week intervals. Ten days later the animals were challenged i.v. first with

Table 3. Analysis of fractions from the second preparative electrofocussing run using ampholytes with pH 5-8

Fraction Nos	1-3	4-6	7-9	10-12	13-15
pH range of eluted pools	7.25-6.93	6.78-6.45	6.25-5.83	5.62-5.37	5.22-4.50
PCA inhibition titres:					
RD 1/32	32	32	> 64	> 64	16
RD 1/16	4	8	> 64	> 64	3

RD = Dilution of the reagin-containing serum.

BHK cell lysate and then with whole vaccine and their sera were also tested for neutralizing antibodies. The results showed that vaccine made from harvest devoid of low MW molecules retained FMD immunogenicity but was not allergenic, while vaccine made from harvest containing these molecules was allergenic but devoid of FMD immunogenicity. The vaccine made from the starting material was both potent and allergenic.

Discussion

In this study BHK cell lysate was resolved into fractions by both gel filtration and preparative electrofocusing. The former method separated the lysate according to the weight and shape of the molecules while the latter effected separation according to the isoelectric points.

A combination of the two techniques allowed the potential allergen/s to be characterized in some detail. The PEGG was used twice over. The first run could be regarded as a rough separation to identify the broad band in which most allergenic material was located, while the second run separated the material further and provided an estimate of the isoelectric range of the potential allergen/s with greater precision. This material was subsequently separated by gel filtration and a single allergenic fraction was identified.

The component which was potentially allergenic for cattle, as indicated by its ability to react with bovine reagenic antiserum in PCA and PCA-I tests, was of relatively low MW (approximately 43 000 daltons), had a sedimentation coefficient of 3.65 S₂₀ and an isoelectric point between pH 5.37 and 6.2. Furthermore, the removal of these low MW components from a BHK produced FMD vaccine will prevent clinical sensitization without affecting its immunogenicity. Therefore, in principle FMD antigenicity and BHK allergenicity are separable by filtration procedures.

It is interesting to note that many of the substances previously identified as human allergens have MW and sedimentation characteristics similar to those of the BHK cell components identified as potential allergens in this study. These include components of horse dander [11], ragweed pollen [12], rye grass pollen [13], Cow's milk [14] and egg white [15].

Antiserum raised to this potentially allergenic component of BHK cells in rabbits has been used to develop two *in vitro* assay techniques, single radial immunodiffusion and enzyme linked immunosorbent assay. These techniques will provide valuable *in vitro* methods for monitoring the levels of potential allergens throughout commercial production processes involving BHK cell culture, for example FMD and rabies vaccine production. This in turn should enable vaccine manufacturers to reduce the

risk of any hypersensitivity reactions to vaccines produced in BHK cells by ensuring that the potentially allergenic components are maintained at low/non-sensitizing levels.

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