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Detection of host-derived contaminants in products of recombinant DNA technology in *E. coli*: a comparison of silver-staining and immunoblotting

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Contamination of medicines produced in *E. coli* by recombinant DNA methodology with host-cell proteins is considered a potential problem with this type of method. In this report techniques for the detection of trace quantities of host-cell proteins in SDS-gel electrophoretograms were examined. Detection of *E. coli* proteins by immunoblotting, using antisera raised in rabbits to lysates of *E. coli*, was compared with detection using the ultrasensitive silver stain. Silver staining detected a larger number of *E. coli* proteins in a one-dimensional electrophoresis system than did immunoblotting. Proteins that were markedly antigenic in the rabbit were detected at a greater sensitivity by the immunoblotting approach. Both techniques detected contaminant proteins in a preparation of methionyl human growth hormone produced in *E. coli* known to be contaminated with host-cell proteins. No contaminating proteins were seen by either technique in more rigorously purified preparations of growth hormone. A combination of these two approaches would provide useful evidence of purity of medicines produced by recombinant DNA technology, and is potentially applicable to a wide range of host-vector systems.

Technology for the production of pharmacologically active peptides and proteins by recombinant DNA methodology, for therapeutic use in man, is now well established. Recombinant DNA (r-DNA) human insulin, produced in *Escherichia coli* K12 (Chance et al 1981) has been available for some years. Methionyl human growth hormone, also produced in *E. coli* (Olsen et al 1981) is under clinical evaluation in a number of countries. Other products made by recombinant DNA technology that are being used clinically include interferons, tissue plasminogen activator, interleukins, growth factors and certain vaccine antigens. Recombinant DNA techniques are also being used to produce proteins from non-bacterial host systems, particularly yeasts and mammalian cell lines.

Assessment of the safety of such products is the subject of an advisory document produced by the World Health Organization (WHO 1983) and is of general concern to national regulatory authorities. Of particular concern has been the role of residual host-cell proteins in eliciting an immune response in patients treated with recombinant DNA derived products. Bloom et al (1979) have shown that contamination of animal insulins with traces (100–200 ppm) of gut peptides resulted in the production of antibodies in patients receiving these preparations. More recently, Fryklund (1984) has presented data suggesting that contamination of methionyl human growth hormone with proteins derived from the *E. coli* host can generate antibodies to the growth hormone molecule, possibly via an adjuvant effect of the contaminating proteins. Manufacturers and control authorities are thus concerned with developing techniques which will detect very small quantities of residual cell proteins.

Detection of a few ppm of *E. coli* polypeptides in recombinant DNA derived insulin has been reported using a solid phase radioimmunoassay (Baker et al 1981) which used as antigen a partially purified preparation of *E. coli* peptides derived from an intermediate step in the purification of the product and an antiserum raised in rabbits to this preparation. Although the assay system described was sensitive and specific, the procedure offered little qualitative information on the nature of the antigen detected. In addition, such antisera generally recognize a relatively limited number of components, are not generally available to control authorities and would have to be separately prepared for each new product to be tested. Jones & O'Connor (1982) reported the analysis of human growth hormone produced by recombinant DNA technology by SDS-gel electrophoresis in which host-derived proteins were detected by a combination of immunoblotting using an anti-*E. coli* antiserum and ultrasensitive silver staining. The use of such techniques in quality

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control of recombinant DNA biologicals has attracted the interest of regulatory authorities since they do not require access to in-house antisera raised to specific contaminants. In this report we compare the use of ultrasensitive silver staining with immunoblotting using broad specificity anti-*E. coli* antisera in the detection of *E. coli* K12 derived proteins following SDS-gel electrophoresis of lysates of *E. coli*, and of samples of r-DNA methionyl human growth hormone at different degrees of purity.

MATERIALS AND METHODS

Materials

E. coli K12 was supplied by Dr E. Griffiths. Donkey anti-rabbit precipitating antiserum, obtained from Mr S. Sufi, Chelsea Hospital for Women, London, was further purified to give the IgG fraction by precipitation with 45% ammonium sulphate and ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman Biochemicals Ltd, Maidstone, UK) as described by Johnstone & Thorpe (1982). Following radioiodination using the chloramine-T method of Hunter & Greenwood (1962), ^{125}I-Donkey anti-rabbit IgG was purified by Dowex chromatography (Johnstone & Thorpe 1982). Sodium ^{125}-iodide was purchased from Amersham International plc, Amersham, UK. The rabbit anti-human growth hormone antiserum used was the NIBSC reagent code no. 66/250. All other reagents were analytical grade where obtainable.

Methods

Electrophoresis. Sodium dodecyl sulphate (SDS) gel-electrophoresis was performed in 1·5 mm thick slab gels by the method of Laemmli (1970). Gel dimensions were 13 × 13 mm, and electrophoresis was carried out with an initial constant current of 10 mA per gel for 2 h, followed by resolution at 25 mA for 4–6 h.

Staining with Coomassie blue was performed by immersing the gels in a 0·2% solution of Kenacid blue R200 (Sigma Chemicals) in methanol–acetic acid–water (55 : 7 : 43) for 16 h and destaining in methanol–acetic acid–water (4 : 1 : 6). Silver staining was performed as described by Marshall (1984).

Immunoblotting. The procedure used for immunoblotting was essentially that described by Johnstone & Thorpe (1982). Briefly, following electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher & Schill Anderman and Co Ltd, East Molesey, UK) by transverse electrophoresis, using the EC-420 electroblot apparatus (Uniscience Ltd, Cambridge, UK). Unreacted nitrocellulose was blocked by washing in phosphate-buffered saline containing 3% bovine haemoglobin (PBS-Hb). The membrane was then incubated with gentle shaking in a solution of either rabbit-anti *E. coli* antiserum or rabbit anti-growth hormone antiserum (200 µl in 30 ml PBS-Hb) for 16 h at room temperature (20 °C) and, following further washing, with ^{125}I donkey anti-rabbit IgG (10^7 counts min^{-1} in 30 ml PBS-Hb). Bands of bound ^{125}IgG were visualized by autoradiography (1–3 days) using X-S1 film (Kodak, Hemel Hempstead, UK) in a Protex cassette fitted with an intensifying screen (Cuthbert Andrews Ltd, Watford, UK).

Preparation of antigens. Lysates of *E. coli* K12 were prepared according to the method of O'Farrell (1975). Briefly, *E. coli* K12 was grown to log phase, centrifuged and stored as frozen pellets. Samples (0·32 g) were suspended in 1 ml lysis buffer (8·0 M urea, 2% (v/v), nonidet P40, 5% (v/v) 2-mercaptoethanol) and lysed by repeated freezing and thawing. Protein content was determined by the method of Lowry et al (1951).

Sub-fractions of the crude lysate were prepared by SDS-gel electrophoresis as follows: bacterial lysate (200 µl, 4·6 mg protein) was diluted to 1 ml with 0·38 M Tris HCl, 0·2% SDS, pH 8·7 and applied evenly over the entire upper surface of a slab gel. Following electrophoresis (see above), the entire gel was sliced horizontally into 5 equal regions. To recover the separated proteins, each strip was homogenized in 6 ml 1% SDS and, after incubation at room temperature overnight, filtered. The molecular weight range of each of the isolated sub-fractions was determined by analytical SDS-gel electrophoresis and comparison with molecular weight standards.

Production of antisera. Female New Zealand White rabbits (aged 12 weeks) were injected at multiple intradermal sites with either whole *E. coli* lysate (4·6 mg per animal) or with one of the sub-fractions (0·3–0·5 mg per animal) emulsified in Freunds' complete adjuvant. After 8 weeks, boost injections with similar quantities of protein emulsified in Freunds' incomplete adjuvant were given at single intramuscular sites, at three-week intervals. Test bleeds were taken 12 days after each injection. Ten days after the fifth boost the rabbits were bled out. Antisera were freeze-dried and sealed in ampoules using the methods described for international standards (WHO 1978).

RESULTS

Antigens

At least 80 different proteins were visualized in a one-dimensional electrophoretic separation of the crude lysate of *E. coli* (Fig. 2a). The five sub-fractions that were used to immunize rabbits contained proteins in the following molecular weight ranges: sub-fraction 1, 92 000–200 000; sub-fraction 2, 68 000–92 000; sub-fraction 3, 44 000–73 000; sub-fraction 4, 33 000–520 000; sub-fraction 5, 0—33 000 (results not shown).

Production of antisera

To assess the responses of rabbits immunized with preparations of *E. coli* proteins, crude lysates of *E. coli*, separated by SDS gel-electrophoresis, were challenged by immunoblotting with test bleeds from the animals taken at different stages during the immunization schedule. The responses of six animals, immunized with crude lysate and sub-fractions 1 to 5, respectively, are shown in Fig. 1. Immunization with the crude lysate produced an antiserum recognizing at least 40 different antigens in a one-dimensional electrophoretic separation. Immunization with specific molecular weight cuts initially produced antibodies recognizing a limited number of antigens in approximately the same molecular weight ranges. By the fourth boost, however, most of the animals had produced complex antisera that were largely similar to that produced by the animal immunized with the whole lysate, presumably reflecting contamination of the sub-fractions with proteins outside the selected molecular weight range. Sera from animals 1–6 were pooled to produce a combined antiserum which was used in all subsequent studies.

Sensitivity of detection

Detection of *E. coli* proteins by immunoblotting and by silver staining following electrophoresis of loads of 0·09–11·5 μg of protein is shown in Fig. 2. At the lowest load used (0·09 μg of total protein), the immunoblot detected at least 13 bands following 2 days autoradiography, although not all are easily visible on the photograph. By comparison, the silver staining technique was somewhat less sensitive. Visualization of all but the major bands was lost below 1·44 μg total protein (Fig. 2a) and no bands at all were seen below 0·18 μg total protein (not shown).

A number of protein bands visualized by silver staining were not visualized in the immunoblot system, indicating that certain *E. coli* proteins were not, or only poorly, antigenic in the rabbits. Protein bands detected by immunoblotting were generally more diffuse than those detected by silver staining, making it difficult to assess the total number of antigens recognized in the gels with higher loadings of proteins. After silver staining, the electrophoretic profile of the whole *E. coli* extract exhibited several

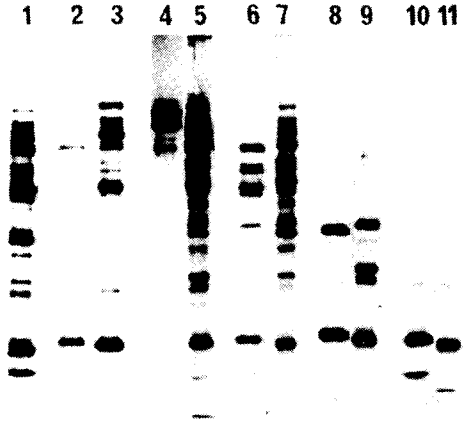

FIG. 1. Production of antisera to whole *E. coli* lysate and sub-fractions of *E. coli* lysate. Rabbits were immunized with whole *E. coli* lysate or sub-fractions as described in Materials and Methods. Test bleeds were taken 12 days after each boost. Whole *E. coli* lysate (11·5 μg total protein per lane) was fractionated by SDS-gel electrophoresis and visualized by immunoblotting using antisera produced by the following immunization schedules: Lane 1, whole lysate, 4th boost, Lanes 2 and 3, sub-fraction 1, 1st and 4th boosts, respectively, Lanes 4 and 5, sub-fraction 2, 1st and 4th boosts, Lanes 6 and 7, sub-fraction 3, 1st and 4th boosts, Lanes 8 and 9, sub-fraction 4, 1st and 4th boosts, Lanes 10 and 11 sub-fraction 5, 1st and 4th boosts. Autoradiographs were developed for 16 h.

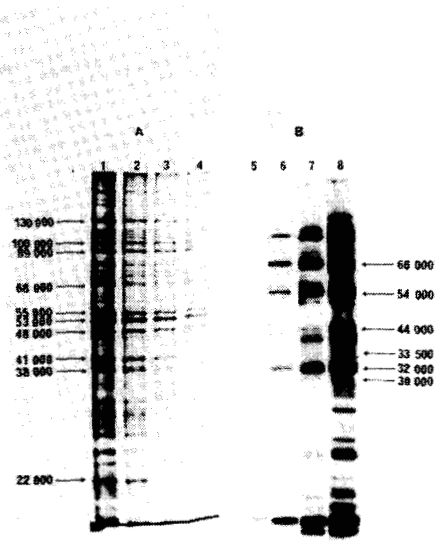

FIG. 2. Detection of *E. coli* proteins by silver staining and by immunoblotting. (A) Whole *E. coli* lysate was fractionated by SDS-gel electrophoresis. Amounts loaded were: Lane 1; 11·5 μg protein, 2; 5·75 μg, 3; 2·88 μg, 4; 1·44 μg. Bands were visualized by silver staining. Molecular weights were determined by comparison with standards. (B) Whole *E. coli* lysate was fractionated by SDS-gel electrophoresis. Loads were: 5; 0·09 μg protein, 6; 0·36 μg, 7; 1·44 μg, 8; 5·75 μg. Visualization was by immunoblotting using combined antiserum. Gels were autoradiographed for 2 days. Molecular weight ranges of proteins were determined by comparison with proteins of known molecular weight, radioiodinated by the method of Hunter & Greenwood (1962) and transferred to nitrocellulose by electroblotting.

characteristic groups of intensely staining bands, corresponding to molecular weights of 38–41 000, 48–55 000, 68–75 000 and 13 000 (Fig. 2a). In comparison, detection of proteins by immunoblotting (Fig. 2b) revealed similar groups of bands showing intense reaction with the antiserum, in approximately similar molecular weight regions. Strongly visualized groups of bands corresponded to proteins of molecular weights of 66 000, 54 000, 40–44 000 and 30–33 000.

Detection of contaminants in preparations of biosynthetic growth hormone

Three preparations of biosynthetic methionyl human growth hormone (Somatonorm I, II and III), produced in *E. coli* by recombinant DNA technology, were analysed by SDS-gel electrophoresis. Proteins were detected by immunoblotting using the combined anti-*E. coli* protein antiserum (Fig. 3a), by immunoblotting using a specific anti-human growth hormone antiserum (Fig. 2b) and by silver staining (Fig. 3c). Silver staining revealed major bands of molecular weights of 22 000 and 44 000 in all three preparations, corresponding to growth hormone and to growth hormone dimer (Lewis et al 1980). Both of these major bands reacted strongly with the growth hormone antiserum (Fig. 2b). Small quantities of apparently higher molecular weight aggregates of growth hormone were seen in Somatonorm I. In addition, a minor band of molecular weight 24 000 was seen in the less pure preparations. Somatonorms I and II, which also cross-reacted with the growth hormone antiserum. In Somatonorm I, two groups

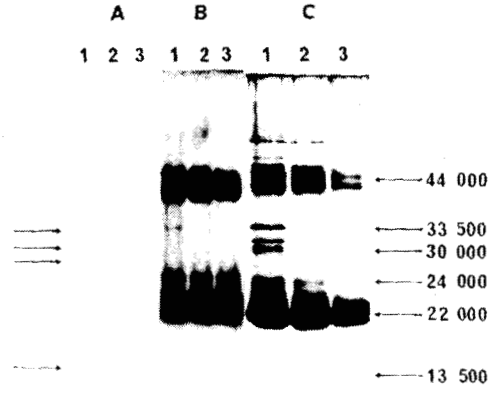

FIG. 3. Analysis of preparations of recombinant DNA methionyl growth hormone. Samples (0·5 mg in 20 μl) of Somatonorm I, II or III, as indicated above each lane, were fractionated by SDS-gel electrophoresis. (A) Bands visualized by blotting with anti-*E. coli* antiserum. The positions of bands visible upon cold, transmitted light illumination in Lane 1 are indicated by arrows. (B) Bands visualized by blotting with anti-growth hormone. (C) Bands visualized by silver staining.

of contaminants were observed which were apparently not related to growth hormone, at molecular weights of 30 000–33 000 and 10 000–13 500. Although not clearly seen in the photograph, at least four of these contaminants exhibited cross-reaction with the anti-*E. coli* protein antiserum, and the 33 500 molecular weight protein also reacted with the growth hormone antiserum. No proteins reacting with the anti-*E. coli* protein antiserum were seen in Somatonorms II and III (Fig. 3a, lanes 2 and 3). The major protein bands present, growth hormone and growth hormone dimer, showed no detectable cross-reaction with the pooled anti-*E. coli* protein antiserum.

DISCUSSION

Depending on the proposed method and frequency of clinical administration of the product, an acceptable level of contamination with host-cell proteins may be as low as 10 ppm. Methods to detect host-cell proteins in products for parenteral administration made by recombinant DNA technology must therefore be able to detect proteins present in much smaller concentrations than those detected by conventional monochromatic staining. Techniques should also be able to detect contaminants having similar electrophoretic properties to the product.

Repeated, long-term immunization of rabbits with preparations of *E. coli* proteins containing urea-denatured material produced complex antisera reacting with many antigens (Fig. 1). There seemed to be little advantage in immunizing the rabbits with specific molecular weight cuts from the *E. coli* protein extract, and antisera with equally complex mixtures of antibodies were obtained by repeated immunization with the unfractionated *E. coli* extract.

Antisera used in this study were raised to preparations of *E. coli* protein that had been denatured by urea and SDS. The possibility exists that antisera of different specificities would be produced to non-denatured bacterial proteins. Foreign proteins expressed in high yield in recombinant *E. coli* are generally insolubilized in intracellular granules and purification procedures involve the use of harsh denaturing conditions to solubilize the product. It seems appropriate, therefore, in the case of *E. coli*-derived products, to raise antisera to denatured cellular proteins. For quality control of products secreted in soluble form, as might be expected in recombinant yeast, antisera to more gently extracted cellular proteins would be more appropriate.

Fewer proteins were recognized in the crude extract by the anti-*E. coli* proteins antiserum by immunoblotting than were revealed by silver staining (Fig. 2), as would be expected. The immunoblotting method, however, resulted in significant broadening of the bands of protein visualized, and it was not possible to discriminate all of the antigens that the antiserum recognized in a one-dimensional electrophoretic system. It was not possible to be certain that bands recognized in the immunoblot system are the same as those seen by the silver stain. The two patterns were, however, qualitatively similar, and the molecular weights of proteins that appeared to be common to the two patterns, when independently determined, were also similar. It seems reasonable to assume therefore that at least several of the major *E. coli* proteins seen in the silver-stained gels and in the immunoblots were the same. The working ranges for either technique were similar. Sensitivity of detection would therefore be expected to be roughly the same for the two techniques, with silver staining offering the advantage of resolving a greater number of components in the lysate.

A combination of the techniques of immunoblotting and silver staining in the analysis of biosynthetic human growth hormone is shown in Fig. 3. In the relatively impure Somatonorm I, *E. coli* derived protein contaminants were detected by both silver staining and immunoblotting. Again, silver staining appeared to be the more sensitive technique and immunoblotting with anti-*E. coli* antisera offered no advantage in detecting contaminants. The use of product-specific and contaminant-specific antisera in immunoblotting did, however, permit discrimination between host-derived contaminants and electrophoretic variants of the product, which had been reported in purified biosynthetic growth hormone (Jonsdottir et al 1984). Such interpretation must however be cautious, since at least one contaminant reacted with both anti-*E. coli* and anti-growth hormone antisera. Somatonorm III, the currently available product, is a highly purified preparation whose only apparent contaminant is a trace of aggregate of hGH. With the amounts loaded on the gels shown in Fig. 3, the density of the silver stain did not show a linear relation with increasing quantity of hGH, and the extent of aggregation in Somatonorm I was thus overestimated. Staining of smaller amounts loaded (not shown) suggested that such aggregates were present in trace quantities only. The earlier preparations, Somatonorm I and II were not rejected directly on the grounds of purity since at that time silver staining was not widely applied (Fryklund, personal communication).

Staining of protein by silver is a sensitive detection technique that does not rely on the use of specific antisera. It has the disadvantage that the gel generally needs to be overloaded with respect to the product, and a contaminant of similar electrophoretic properties would therefore not be detected. The use of broad specificity antisera in immunoblotting allows the detection of antigens at very low concentrations, and could detect antigens that were not separated from the product by electrophoresis, but non-antigenic proteins would not be detected. In this study the two detection techniques have been compared using a single SDS gel electrophoresis system separating on the basis of molecular size. Both techniques would be applicable to non-denaturing electrophoresis or isoelectric focusing systems to allow resolution of similar sized contaminants. It would remain the case however that in gels overloaded with respect to the product, much of the gel is obscured when stained with silver. *E. coli* antigens of similar molecular weight to growth hormone were not seen in this study, and it remains only a theoretical advantage of immunoblotting. The use of an immunoassay based on antisera to selected contaminants (Baker et al 1981) has the advantage of higher sensitivity (down to 4 ppm) and better quantitative information, but has the disadvantages of detecting only a very limited number of contaminants, and of providing no qualitative information on any contaminants. The preparations of Somatonorms I, II and III examined in this study, when tested by immunoradiometric assay for *E. coli* proteins, contained 700, 70 and 2 ppm *E. coli* proteins, respectively (Fryklund, personal communication). A combination of the three techniques would however provide good evidence of the purity of a product made by recombinant DNA procedures using *E. coli*.

Although this study did not demonstrate any clear advantage to be gained from the use of anti-*E. coli* antisera in immunoblotting, it is likely that such techniques will continue to be regarded as useful quality control steps by regulatory authorities. The antisera used in these studies have been prepared in ampoules coded 84/658, and are available on request from the National Institute for Biological Standards and Control.

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