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Variants of Hamster Fibroblasts Resistant to *Ricinus communis* Toxin (Ricin)

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1. Variant baby-hamster kidney (BHK) cell lines were isolated that grow in the presence of high concentrations of ricin, the toxic lectin of castor beans (*Ricinus communis*). The variant lines were independently derived from several cultures of normal BHK cells which had been exposed to the mutagen, methyl-*N*-nitro-*N*-nitrosoguanidine, before selection by ricin. 2. The cell lines maintain a high degree of resistance to ricin after growth in lectin-free medium for prolonged periods and therefore exhibit stable phenotypes that are different from normal BHK cells. 3. A preliminary classification of the phenotypes was made. Several cell lines bind normal amounts of ^{125}I-labelled ricin, whereas others bind the lectin poorly. 4. A loss of surface receptors for two other lectins, *R. communis* RCA_1 and *Axinella polyploides*, which have specificities similar to ricin, was also found in some but not all of the cell lines showing decreased surface concentrations of ricin receptors. 5. The binding to the ricin-resistant cells of lectins of different sugar specificity, namely *Lens culinaris* lectin and concanavalin A, was similar to, or higher than, to normal BHK cells. 6. Several of the ricin-resistant cell lines were shown to be cross-resistant to the weak cytotoxicity of *Phaseolus vulgaris* lectin. By contrast, some cell lines were more sensitive to concanavalin A than were normal BHK cells.

Surface carbohydrates have been implicated in several important biological properties of cells, such as intercellular adhesion and aggregation. Much of the evidence for these roles is indirect and relies, for example, on the effects of glycosidases on cellular interactions (Roth, 1973; Chipowski *et al.*, 1973). Thus the specific adhesive properties of certain cell lines, for example baby-hamster kidney (BHK) cells, is decreased by removal of surface β-galactosyl residues. In order to study further the biological roles of cell-surface carbohydrates, especially galactose, it would be useful to have available cell variants that exhibit stable alterations in surface glycoprotein structure. These variants would be expected to be produced by selection in the presence of a galactose-binding lectin, such as ricin from castor beans (*Ricinus communis*). The cytotoxic effect of ricin on whole cells first necessitates binding to cell-surface galactose or *N*-acetylgalactosamine residues, and this step is inhibited by lactose or glycoproteins containing galactose (Hughes *et al.*, 1973; Nicolson, 1973, 1974*a*,*b*). Subsequent steps require entry of the bound lectin molecules into the cells, followed by release of ricin intracellularly and inhibition of protein synthesis and cell death (Olsnes *et al.*, 1974; Refsnes *et al.*, 1974; Nicolson, 1974*b*). Therefore cells that are resistant to ordinarily lethal doses of the lectin would be expected in some cases to possess alterations in the plasma membrane and a loss of ricin receptors. Such alterations could be produced, for example, by the defective assembly of the carbohydrate chains of cell-surface glycoproteins containing galactose or *N*-acetylgalactosamine units.

In this paper we describe the isolation and some of the properties of ricin-resistant cell lines of baby-hamster kidney cells. The cells retain resistance to large doses of ricin after growth for long periods in the absence of the lectin and appear to exhibit stable phenotypic changes. Gottlieb *et al.* (1974) and Nicolson and his colleagues (Hyman *et al.*, 1974; Nicolson *et al.*, 1975*c*) have each reported the isolation of cell variants of respectively a murine lymphoma and Chinese-hamster ovary cells that are resistant to relatively low concentrations of ricin (0.1 μg/ml). In our work 22 resistant cell clones, which were selected by ricin concentrations of up to 1.0 μg/ml, have been isolated. All resistant cell clones grew out to give cell lines which exhibited stable properties over the period of investigation. Some of these show a decreased ability to bind ricin and other lectins of similar sugar specificity. Other resistant cell lines bind as much ricin as the normal sensitive cells or more. The possible biochemical bases of resistance in the variant cell lines and their relation to cell variants isolated by other workers are discussed.

Materials and Methods

Cell cultures

Baby-hamster kidney fibroblasts (BHK21 C13) were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and grown at 32.5° or

39°C in Glasgow modified minimal essential medium (Flow Laboratories) supplemented with 10% (v/v) foetal bovine serum, 10% (v/v) tryptose phosphate broth, $NaHCO_3$ (2.0g/litre) and gentamicin (50μg/ml). Cells were transferred every 4 to 6 days at an inoculation density of approx. 10^3 cells/cm^2 in Falcon plastic culture bottles. The cells were removed for subculturing by treatment for 1–2min at 37°C with a trypsin/EDTA solution. The stock solution, containing twice-crystallized trypsin [4mg/ml; type III; Sigma (London) Chemical Co., London S.W.6, U.K.], EDTA (4mg/ml), NaCl (7.6mg/ml), KCl (224μg/ml), glucose (1.8mg/ml) and $Na_2HPO_4,7H_2O$ (268μg/ml), was adjusted to pH7 with NaOH and diluted 1:200 with phosphate-buffered saline (see below) containing no calcium or magnesium salts.

All cell lines were tested as a routine for mycoplasma by the method of Fogh & Fogh (1964) and were negative throughout the course of this study.

Buffer solutions

Phosphate-buffered saline contained NaCl (8.0g), KCl (0.29g), Na_2HPO_4 (1.159g), KH_2PO_4 (0.29g), $CaCl_2$ (0.19g) and $MgCl_2$ (0.19g), diluted to 1 litre with water, pH7.04. Phosphate-buffered iodide contained the same salts, except that KI (0.50g) was substituted for KCl.

Lectins

The two major lectins of castor beans (*R. communis*) were extracted and separated as described by Nicolson & Blaustein (1972) and Nicolson *et al.* (1974). The agglutinin RCA_1, of mol.wt. 120000, was separated from the more toxic lectin RCA_2, hereafter called ricin, of mol.wt. 60000, by gel filtration on a column (2.5cm×90cm) of polyacrylamide (Bio-Gel P150; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Elution of the column with phosphate-buffered saline at 2°C gave two protein fractions, the fraction eluted earlier containing lectin RCA_1. Both fractions were adjusted to approx. 6–10mg of protein/ml (Lowry *et al.*, 1951) and stored as precipitates in saturated $(NH_4)_2SO_4$ solution. We thank Mr. P. Gillett and Dr. M. J. Crumpton for advice on the separation and gifts of purified lectins.

Purified *Phaseolus vulgaris* phytohaemagglutinin was obtained from Wellcome Research Laboratories, Beckenham, Kent, U.K. *Axinella polyploides* agglutinin, extracted and purified by affinity chromatography as described by Maino *et al.* (1975), was kindly provided by Dr. M. J. Crumpton. Concanavalin A was obtained as a three-times-crystallized freeze-dried powder from Miles–Yeda Ltd., Kankakee, Ill., U.S.A. Purified *Lens culinaris* (lentil) phytohaemagglutinin (Howard & Sage, 1969) was kindly provided by Dr. M. J. Crumpton.

Selection procedures

Cells were grown at 32.5°C on 60mm Falcon plastic culture dishes and treated at a low density (approx. 2×10^5 cells per dish) with the mutagen, methyl-*N*-nitro-*N*-nitrosoguanidine (0.5μg/ml of culture medium). After 24h at 32.5°C the medium was removed and replaced with fresh medium containing no mutagen. The cells surviving the mutagen treatment (approx. 28% of the total) were grown for 4 days at 32.5°C to establish mutant phenotypes and then the culture dishes were transferred to 39°C. At this time the medium was changed again to one containing 2% (v/v) foetal bovine serum instead of 10%. Since foetal bovine serum contains glycoproteins that bind ricin strongly, the effective cytotoxicity of ricin solutions is increased in cultures containing lower (2%, v/v) amounts of foetal bovine serum. The cells were incubated at 39°C in the fresh medium for a further 2 days, trypsin-treated and replated on 60mm or 100mm Falcon plastic culture dishes (5×10^5 cells per dish). The cells were grown to a density of approx. 3×10^6 per dish, and then treated with various concentrations of ricin. Ricin (10–20μg/ml) dissolved in complete medium was sterilized by ultrafiltration and added to the culture dishes at the required concentration (0.2 or 1μg/ml). The cultures were incubated at 39°C in the presence of ricin for 7–10 days, with changes of medium every 3 days. In some experiments the concentration of ricin in the medium was increased from 0.2μg/ml after the first 3 days to 0.5 or 1μg/ml. In other experiments the initial concentration (0.2 or 1μg/ml) was maintained throughout. During the time of exposure of cells to ricin, dying cells became detached from the plastic surface and were easily removed by renewal of the medium. However, a few colonies grew up from surviving cells even in the presence of the highest concentration of ricin (1μg/ml). Growth of these colonies was often encouraged by changing the medium after 5–10 days to one containing 10% foetal bovine serum and the appropriate concentration of ricin.

Surviving colonies, growing in well-separated areas of the plate, obtained after this selection procedure were removed by trypsin treatment under sterile glass cylinders and transferred to culture dishes containing fresh medium. Initially growth of the selected cell clones was carried out in Ham's F10 medium (Flow Laboratories) containing 10% (v/v) foetal bovine serum, 10% (v/v) tryptose phosphate and gentamicin (50μg/ml). This medium contained no ricin. As a routine the cells were grown thereafter in Glasgow modified minimal essential medium supplemented as described previously and without ricin.

Growth curves of cell variants

Cells (approx. 5×10^4) were seeded on to 35mm Falcon plastic culture dishes in the usual growth

medium in the absence of ricin. The dishes were incubated at 39°C. At various times duplicate dishes were removed from the incubator, the medium was decanted and the cells were quickly washed twice with trypsin/EDTA solution at 35°C. The washed cells were then detached by incubation for 1–2min at 37°C in trypsin/EDTA solution, and counted with a haemocytometer.

Saturation was considered to have been reached if three successive counts showed no more than a 10% increase in cell density.

Colony growth

The effect of ricin on the ability of normal and variant cells to form colonies was measured as follows. Cells from monolayer cultures were trypsin-treated, counted and diluted to an appropriate cell density in complete medium containing Ham's F10 medium, 10% (v/v) foetal bovine serum, 10% (v/v) tryptose phosphate broth and gentamicin (50μg/ml). Usually 1000 cells were then added to 60mm Falcon plastic culture dishes containing 6ml of complete medium. The dishes were incubated at 39°C, and after 5–7 days visible colonies had formed. These were fixed with 16% (v/v) formaldehyde in 0.9% NaCl and stained with 1% (w/v) Gentian Violet dissolved in 20% (v/v) ethanol. In the absence of ricin the absolute plating efficiency of normal BHK cells, expressed as the number of visible colonies formed compared with the total number of cells plated, was 20–30%.

In parallel experiments ricin was added at known concentrations to the culture dishes. In early experiments ricin was usually added to the culture dishes at the required concentration 1–2h after the addition of the cells. In later experiments the cells were allowed to establish for 2 days after plating before addition of ricin.

The effect of other lectins, *P. vulgaris* phytohaem-agglutinin and concanavalin A, on the plating efficiency of normal and ricin-resistant cells was tested in the same way.

Light-microscopy

For light-microscopy, cells were grown in monolayer culture on Falcon plastic culture dishes, fixed with 70% (v/v) methanol and stained with 10% (v/v) Giemsa (G. T. Gurr, London S.W.6, U.K.) in sodium phosphate buffer, pH6.8.

Iodination of lectins

Lectins were labelled with ^{125}I by lactoperoxidase-catalysed iodination in the presence of an appropriate sugar hapten of the lectin, and purified by affinity chromatography. The iodination of ricin is typical of the procedure. Ricin (approx. 1mg) was dissolved in phosphate-buffered saline (2ml) containing 2.5mM-glucose, 110mM-galactose, 20μg of lactoperoxidase (Sigma)/ml, 0.2 unit of glucose oxidase (Sigma, type V)/ml and 500–1000μCi of carrier-free Na^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.). After incubation at room temperature (18°C) for 15min, the reaction mixture was diluted with ice-cold phosphate-buffered iodide (3ml) and dialysed extensively against repeated changes (each 2–3 litres) of phosphate-buffered saline at 4°C for 2–3 days. The dialysed mixture (approx. 5ml) was then applied to a column (1cm×10cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with phosphate-buffered saline. The column was washed at 4°C with phosphate-buffered saline to remove enzyme reagents and free iodide, and the lectin, which binds specifically to agarose, was eluted with 0.1M-galactose. Two 5ml fractions were collected and these contained the majority of the ^{125}I-labelled ricin. These fractions were dialysed separately against phosphate-buffered saline at 4°C, and diluted with unlabelled ricin to give a solution of 100–200μg of protein/ml and 1×10^5–2×10^5c.p.m./ml. Finally, bovine serum albumin (Sigma) was added to a final concentration of 50μg/ml and the solution was kept frozen until used for the binding assays.

The iodination and purification of *R. communis* agglutinin RCA_1 and *A. polyploides* agglutinin followed exactly the same procedure as described for ricin. Concanavalin A and lentil agglutinin were iodinated in the same way except that galactose was omitted from the reaction mixture and methyl α-D-mannoside was used instead. The last two iodinated lectins were purified by adsorption on columns (approx. 5ml) of Sephadex G-50 and elution with 0.1M-glucose or 0.1M-methyl α-D-mannoside.

Binding of ^{125}I-labelled lectins to normal and variant cells

The binding assays were performed on confluent or nearly confluent cell monolayers growing on 35mm Falcon plastic tissue-culture dishes. Duplicate cultures were washed four times with phosphate-buffered saline at room temperature, and 1ml portions of each dilution of a ^{125}I-labelled lectin solution (100–200μg of protein; 1×10^5–2×10^5c.p.m.) were added. To estimate the number of cells present in each binding experiment, duplicate cultures were treated with trypsin, and the cells counted as described above. It was necessary to carry out such determinations for each cell line examined, since these cell lines vary considerably in size and protein content per cell. The dishes containing lectins were kept, with occasional swirling, at room temperature for usually 1h, when the lectin solution was removed and the cells were washed four times with phosphate-buffered saline. The dishes were then inverted and air-

dried overnight. The dry sheets of cells were dissolved at 37°C in 0.5M-NaOH (1ml per dish) and counted for ^{125}I radioactivity in an LKB Wallac gamma spectrometer. Portions (0.1–0.2ml) were removed for measurement of protein by the procedure of Lowry *et al.* (1951) by using bovine serum albumin as standard. In many experiments the dishes were washed twice with 0.5M-NaOH, and the second wash contained negligible radioactivity or protein.

In control experiments the dishes were incubated at 4°C instead of at room temperature (approx. 18°C). The details of experiments in which a suitable sugar inhibitor of the lectin was used, for example, galactose or lactose for *R. communis* lectins (RCA_1 and ricin) or *A. polyploides* lectin and α-methyl mannoside for *L. culinaris* lectin and concanavalin A, are described in the Results section. Specific binding to the cells was calculated from the known specific radioactivity of the ^{125}I-labelled lectin, and expressed on the basis of ng of lectin bound either per mg of cellular protein or per 10^7 cells. Corrections were made for the non-specific binding of lectins to the cells obtained when the appropriate inhibitor of each lectin was present at 10mM final concentration.

Agglutination tests

Cells were removed from culture vessels by incubation in 0.02% (w/v) disodium EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at room temperature. The suspended cells were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and diluted to give aprox. 2×10^6–4×10^6 cells/ml. Agglutination assays were carried out as described by Inbar & Sachs (1969). The highest lectin concentration was usually 500μg/ml and cell agglutination was estimated after 30min incubation at room temperature by direct microscopic examination.

Results

Cytotoxicity of R. communis lectins towards BHK cells

The effects of *R. communis* components RCA_1 and ricin on the growth of normal BHK cells in culture was examined by inhibition of colony formation (Fig. 1). The cells were plated out at a low density of about 1000 cells per culture dish in Ham's F10 medium containing 10% foetal calf serum. After 2 days the cells had attached to the substratum, and growth commenced. When either lectin RCA_1 or ricin was then added to the cultures at various concentrations up to 1–2μg/ml, there was a decrease in the numbers of colonies visible after continued incubation for another 5–8 days. Fig. 1 shows that the concentrations of lectin RCA_1 and ricin required to decrease the number of viable colonies by 50% were 0.5 and 0.06μg/ml respectively. In subsequent

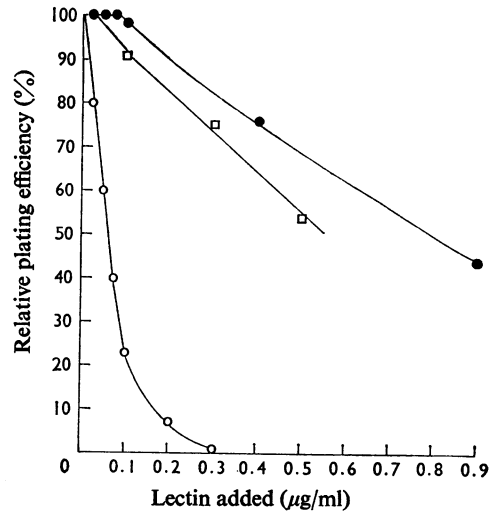


Fig. 1. *Effects of R. communis lectins on BHK cells*

Trypsin-treated cells in Ham's F10 medium plus 10% foetal calf serum were plated on 60mm plastic culture dishes (approx. 10^3 cells per dish). After 1–2 days at 39°C, *R. communis* lectins RCA_1 (□) or RCA_2 (ricin) (○) were added at the stated final concentrations. After further incubation for 5–8 days, viable cell colonies were stained and counted as described in the Materials and Methods section. The relative plating efficiency is the number of colonies developing in the presence of the lectins as a percentage of the number of colonies developed in the absence of lectins. Control dishes contained ricin+5mM-lactose (●).

experiments ricin has been used exclusively to isolate resistant cells.

The minimum concentration of ricin needed to prevent growth of BHK cells under the above conditions was approx. 0.2μg/ml, at which concentration the plating efficiency of the cells was decreased by at least 95%. At concentrations greater than 0.4μg/ml no colonies formed at all (Fig. 1).

Fig. 1 also shows the protective effect of lactose, an inhibitor of ricin binding (Hughes *et al.*, 1973; Nicolson, 1973), on cells incubated with the lectin. When lactose was added (final concentration 5mM) to the cells 4h before addition of ricin, the cytotoxicity of ricin was decreased at least tenfold. On prolonged incubation, cells grew out to confluent monolayers in the presence of 0.4μg of ricin/ml when the inhibitor was present.

Isolation of ricin-resistant cells

In order to increase the number of cells forming colonies in the presence of high ricin concentrations (0.2μg/ml or greater), BHK cells were exposed to the

potent chemical mutagen methyl-*N*-nitro-*N*-nitrosoguanidine for 24h. When mutagenized cells were grown out to high density (approx. 3×10^6 cells per culture dish) and then incubated in the presence of 0.2 μg of ricin/ml, the numbers of colonies growing up were 5–20 times greater than the numbers arising from control culture dishes containing unmutagenized cells and the same concentration of ricin. The effect of mutagenesis was more marked at higher concentrations of ricin (0.5–1 μg/ml). At these concentrations no colonies formed from unmutagenized cells, whereas colonies formed consistently from the mutagenized populations at a frequency of approx. 10^{-6}.

In four separate experiments using different concentrations of ricin, a total of 22 independent colonies were isolated, plated out in fresh medium lacking ricin and grown to high density. Ricin-resistant cell clones Ric^R 1, 2, 3, 4 and 5 were isolated from selection medium containing 0.2 μg of ricin/ml; clones Ric^R 6, 7, 8, 9, 10, 11, 12 and 13 were isolated from cultures containing 1 μg of ricin/ml. The remaining clones were isolated as colonies surviving an initial ricin concentration of 0.2 μg/ml, followed by selection in 1 μg of ricin/ml (Ric^R 14, 15, 16, 17, 18, 19, 20 and 21) or 0.5 μg of ricin/ml (Ric^R 22). These cells were subcultured in ricin-free medium. After five to ten subculturings the cell isolates were replated at low density (1000 cells per culture dish) in the presence of ricin (0.1 μg/ml) to test their sensitivity to the lectin.

In these sensitivity tests ricin (0.1 μg/ml) was added to the growth medium 2h after the addition of cells. Under these conditions normal BHK cells had a relative plating efficiency, defined as the number of colonies developed in the presence of ricin compared with the number of colonies developed in the absence of ricin, of 1–2%, which was some 20% less than when ricin (0.1 μg/ml) was added 2 days after incubation of cells in growth medium (Fig. 1). Of the cell isolates, one clone, Ric^R 3, showed a low resistance to ricin, giving a relative plating efficiency of 2.5%. Nine clones (Ric^R 1, 2, 3, 4, 5, 10, 12, 14, 17 and 22) showed a substantial resistance to ricin, and when plated out in the presence of ricin produced 25–49% of the colonies formed in the absence of ricin. Four clones (Ric^R 6, 9, 16 and 20) showed intermediate relative plating efficiencies of between 50 and 75%. Eight clones (Ric^R 7, 8, 11, 13, 15, 18, 19 and 21) showed high resistance to ricin cytotoxicity, giving relative plating efficiencies of between 75 and 100%.

The clones showing the highest degree of stable resistance were selected in the experiments using the higher concentrations of ricin (1 μg/ml). In general, clones growing out in the presence of 0.2 μg of ricin/ml retained significantly less resistance when retested after growth for prolonged periods in the absence of the lectin. However, the fidelity of the selection technique is high, since only one clone out of 22 showed a substantial reversion and a large decrease, greater than tenfold, in resistance to ricin after growth for several generations in the absence of the lectin.

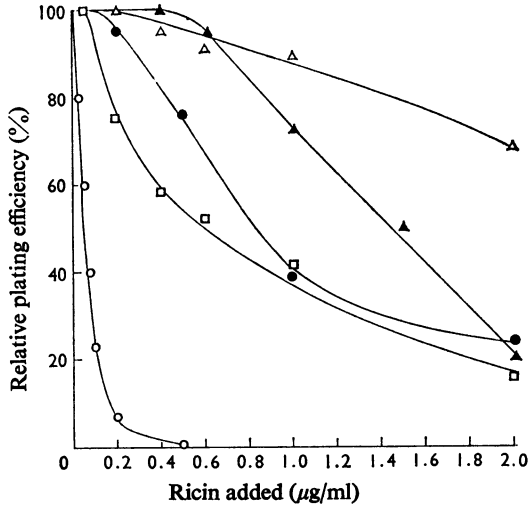


Fig. 2. *Comparison of the sensitivity of BHK cells to ricin cytotoxicity*

The isolation of ricin-resistant cell lines, including Ric^R 14 (●), Ric^R 15 (▲), Ric^R 16 (□) and Ric^R 21 (△), used in these experiments is described in the Materials and Methods section. The cells were cultured in the absence of ricin for several months before retesting resistance to ricin. Estimation of the inhibition of colony formation by ricin was carried out as described in Fig. 1. The sensitivity of normal BHK cells (○) is shown for comparison.

After serial propagation of the ricin-resistant clones in the absence of ricin for about 6 months, several were retested for sensitivity to increasing concentrations of ricin up to 2 μg/ml. In this instance ricin was added 2 days after cells (10^3) were seeded into growth medium. All of the clones shown in Fig. 2 were considerably more resistant to ricin than normal BHK cells at all concentrations of the lectin. It is also noteworthy that the relative degrees of resistance of the various clones appeared to be maintained after this long period. Thus the two clones Ric^R 15 and 21, which when first isolated showed high resistance, maintained high resistance and were unaffected by 0.7 μg of ricin/ml, whereas the plating efficiency of clones Ric^R 14 and 16, showing initially intermediate resistance, was decreased by about half at this concentration of ricin (Fig. 2).

We conclude therefore that the cell clones exhibit stable variations, which confer high degrees of resistance on the cells to the cytotoxicity of ricin.

Growth properties of resistant cells

In general, the various cell clones isolated from media containing ricin grow at similar rates at 33°,

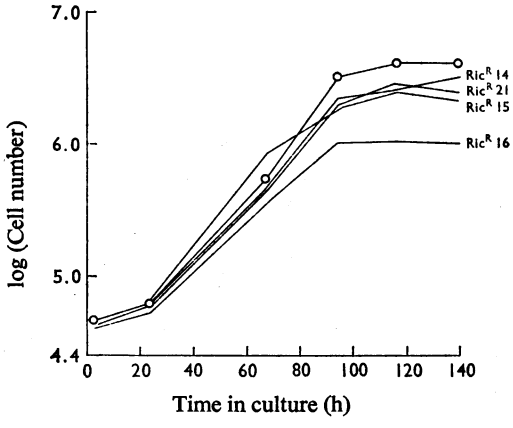


Fig. 3. *Growth of normal and variant BHK cell lines*

Cells were plated out at approx. 5×10^4 cells per 35mm culture dish and incubated at 39°C. At times the cells were removed with trypsin/EDTA solution from selected dishes in duplicate and counted. ○, Normal cells.

37° or 39°C as normal BHK cells. Some typical growth curves for cells growing in monolayer culture are shown in Fig. 3. In exponential growth phase at 39°C normal BHK cells divide every 12–15h and the rates for the four resistant clones were within 20% of this value. Certain differences in growth properties between the resistant clones and normal cells were detected, however. Many resistant clones grow out at confluence to significantly lower cell densities (Fig. 3). This is particularly noticeable for clone Ric^R 20, which grows to a saturation density of approx. 1.2×10^5 cells/cm^2, compared with a value of 6.7×10^5 cells/cm^2 for normal BHK cells. Part of the explanation for the lower cell density of clone Ric^R 20 at saturation probably is the larger size of this cell (approx. twofold) compared with the normal cell.

Secondly, the ricin-resistant clones adhere poorly to the surface of culture vessels and some, particularly clones Ric^R 9 and 17, shed many cells into the medium during monolayer culturing. Somewhat similar behaviour has been reported for clones of Chinese-hamster ovary cells resistant to *P. vulgaris* phytohaemagglutinin (Stanley *et al.*, 1975).

Morphology

The ricin-resistant cells in monolayer culture appear to be morphologically similar to, although somewhat larger than, normal sensitive BHK cells (Plate 1). However, at confluence resistant cell lines exhibit poorly the typical parallel alignment that is characteristic of the parent BHK cell line. The resistant Ric^R 21 is least patterned at confluency. These differences between normal and ricin-resistant clones are observed reproducibly, although such behaviour in culture is difficult to reproduce photographically.

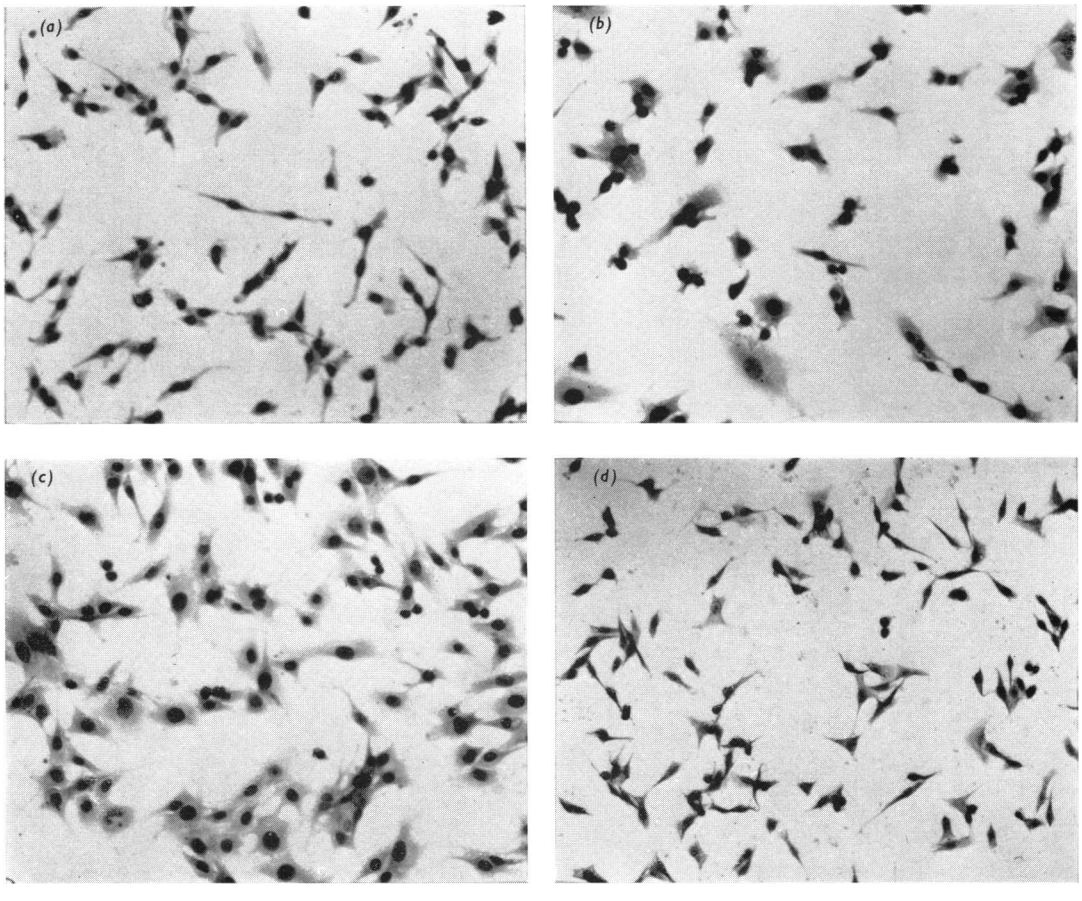
Binding of ricin to normal and variant cells

The most obvious mechanism by which cells may become resistant to ricin is by a decrease in the ability of those cells to bind the lectin at the cell surface. Therefore we have screened our various resistant cell lines for their ability to bind ricin in comparison with normal BHK cells. Incubation of intact viable cells with increasing concentrations (up to 75μg/ml) of [^{125}I]iodinated ricin resulted in binding of the lectin to cell-surface receptors (Fig. 4*a*). The binding was prevented by lactose, an inhibitor of ricin, and a greater than 80–85% decrease in binding was found at lactose concentrations of 10mM or greater (Fig. 5).

The binding data shown in Fig. 4(*a*) were treated by the Scatchard (1949) relation to obtain the total numbers of lectin-binding sites. As shown in Table 1 the binding capacity of the BHK cell lines towards ricin varies extensively. No significant differences were found in ricin binding between normal BHK cells and ricin-resistant cell lines Ric^R 1, 2 and 16, and clones Ric^R 12, 19 and 22 actually bound more ricin than the normal sensitive cells. By contrast, cell lines Ric^R 6, 7, 9, 10, 14, 15, 17, 18, 20 and 21 bound ricin poorly. In many cases only 10–20% of the binding sites present on normal BHK cells were found (Table 1).

The relative binding capacities of several of the various resistant cell lines were also tested against the less toxic *R. communis* lectin RCA_1 (Fig. 4*b*). The relative values were found to be similar to those measured with ricin (Table 1). However, clones Ric^R 6, 10, 17 and 18 appeared to bind normal amounts of lectin RCA_1, but bound decreased amounts of ricin.

The total number of binding sites for lectin RCA_1 or ricin on normal BHK cells (approx. 6×10^6 sites per cell; Table 1) is similar to other estimates (Nicolson *et al.*, 1975*a*,*b*). Control experiments showed that the maximum binding of ^{125}I-labelled lectins was obtained within 30min at room temperature. At 4°C the rate of binding of ^{125}I-labelled lectins to the cells was lower than at room temperature, but the total number of binding sites per cell was unchanged. It is therefore likely that the binding experiments carried out at room temperature are not complicated by endocytosis of surface-bound lectin molecules, which has been reported in other systems (Noonan & Burger, 1973). Fig. 5 shows additional evidence for this conclusion. Thus at least 80% of the ^{125}I-labelled lectin bound to normal BHK cells under our standard conditions is released from the cells when lactose is added at 10mM final concentration. This result would not be expected if the lectin had entered



EXPLANATION OF PLATE 1

Light-micrographs of Giemsa-stained cells

(*a*) Normal BHK cells; (*b*) Ric^R 17 cells; (*c*) Ric^R 18 cells; (*d*) Ric^R 21 cells. Magnification × 100.

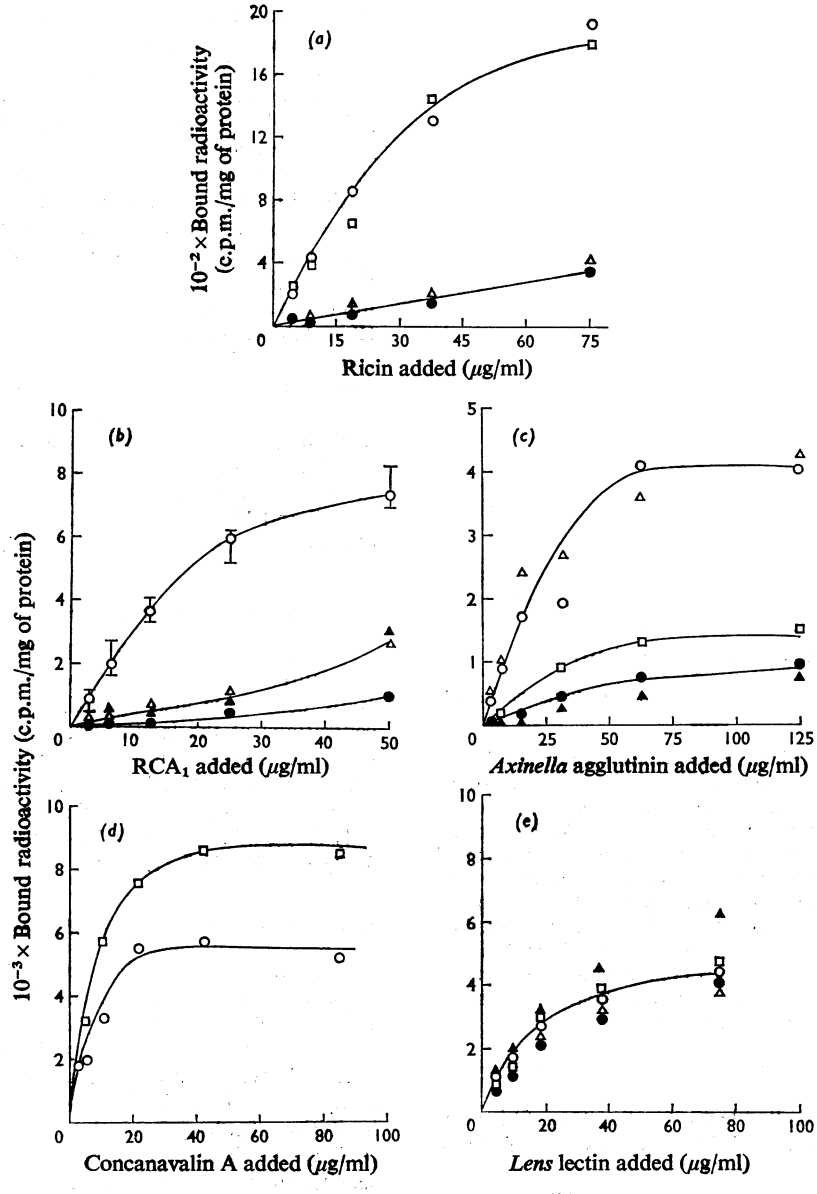


Fig. 4. *Binding of lectins by BHK cell lines*

Cells (approx. 4×10^6) growing in monolayer culture on 35mm-diameter plastic dishes were incubated for 1h at room temperature with ^{125}I-labelled lectins at the concentrations indicated. Bound lectins were estimated as described in the Materials and Methods section. (*a*) *R. communis* lectin RCA_2, ricin; (*b*) *R. communis* lectin RCA_1 (the interval bars represent the ranges found); (*c*) *Axinella polyploides* agglutinin; (*d*) concanavalin A; (*e*) *Lens culinaris* lectin. The curves shown, were obtained by using normal BHK cells (○) and the ricin-resistant cell lines Ric^R 14 (●), Ric^R 15 (▲), Ric^R 16 (□) and Ric^R 21 (△). These data and similar binding curves obtained with the other ricin-resistant cell lines (not shown) were used to calculate the total numbers of binding sites as shown in Table 1.

the cell by endocytosis and suggests that at most only a small proportion (15–20%) of the bound lectin could be present inside endocytotic vesicles. The major part is located on the cell surface, and lactose causes its removal by competition with the surface receptor sites. The effect of lactose concentration on

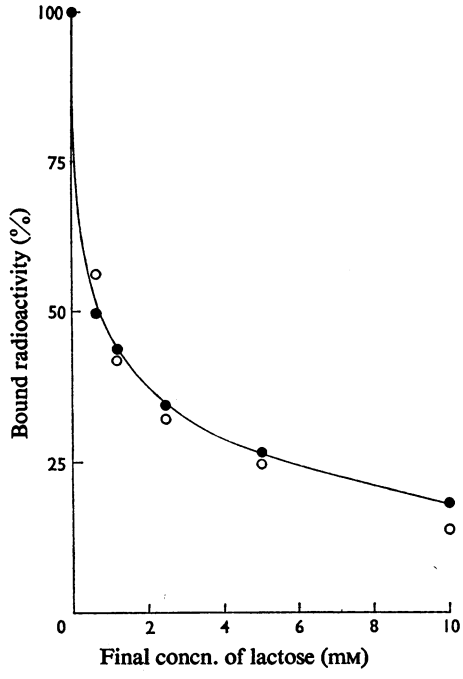


Fig. 5. *Effects of lactose on ricin binding to normal BHK cells*

○, Intact cells were incubated as described in Fig. 4 with ^{125}I-labelled lectin (50μg, 10^5c.p.m.) for 30min at room temperature in the presence of various concentrations of lactose. Bound radioactivity was then determined as described in the text and is expressed as a percentage of that bound to cells in the absence of lactose. ●, Intact cells were incubated with ^{125}I-labelled lectin (50μg, 10^5c.p.m.) for 30min at room temperature. The cells were washed to remove unbound lectin and then reincubated for 30min at room temperature in various concentrations of lactose. The cells were again washed and the radioactivity remaining bound to the cells was determined.

reversal of ^{125}I-labelled lectin binding (Fig. 5) is very similar to the direct inhibition by lactose of lectin binding to the cells (Fig. 5).

Number of binding sites for other lectins

Since there appeared to be some discrepancy between the relative capacities of the BHK cell lines to bind *R. communis* lectins RCA_1 and ricin, which are reported to exhibit similar sugar specificities, we measured (Fig. 4*c*) the affinity of the cell lines for another galactose-binding lectin, from *A. polyploides* (Maino *et al.*, 1975). Normal BHK cells contain approx. 10^7 surface binding sites for *Axinella* lectin (Table 1). Of the seven variant clones tested, Ric^R 7, 9 and 21 carry similar numbers of *Axinella* receptors, although all three clones bind much less ricin than the normal cells, and clone Ric^R 21 also binds very low amounts of *Ricinus* RCA_1 lectin.

In distinction from the results obtained with the galactose-binding lectins of *R. communis* and *A. polyploides*, none of the resistant cell lines tested were found to be deficient in receptor sites for *L. culinaris* lectin (Fig. 4*e*) nor for concanavalin A (Fig. 4*d*). These two lectins show similar sugar specificities and are inhibited by mannosides. For *L. culinaris* agglutinin, *N*-acetylglucosamine also appears to be important in the binding of glycoprotein receptors (Kornfeld *et al.*, 1971). Many of the ricin-resistant cell lines bind substantially more *L. culinaris* agglutinin and concanavalin A than do normal BHK cells (Table 1). There is, for example, a 1.5–3-fold increase in binding of concanavalin A by clones Ric^R 14, 15 and 21. The increased binding of *L. culinaris* lectin is generally less pronounced (Table 1), and in two clones, Ric^R 7 and 14, there appears to be a small decrease (20–40%) in the total numbers of binding sites.

Agglutinin with concanavalin A

The variant cell lines, Ric^R 14, 15 and 21, that bind substantially more concanavalin A than normal BHK cells were also agglutinated at smaller concentrations of the lectin. The minimum concentrations of concanavalin A required to aggregate greater than 90% of the normal BHK cells was 125μg/ml, whereas the variant cell lines required only 7.8μg/ml for Ric^R 14, 15.6μg/ml for Ric^R 15 and 31.2μg/ml for Ric^R 21.

Sensitivity of ricin-resistant cells to other lectins

Concanavalin A and *P. vulgaris* agglutinin are toxic to a variety of animal cells in culture (Ozanne, 1973; Wright, 1973; Stanley *et al.*, 1975). The concentrations required for a significant toxic effect on normal BHK cells are very high (Figs. 6*a* and 6*b*), in comparison with ricin (Fig. 1), as determined by the inhibition of colony formation. The basis of the cytotoxicity of these lectins is unknown. BHK cells sensitive to *P. vulgaris* lectin and concanavalin A are quickly rounded in morphology in the presence of these lectins, and any colonies that form contain predominantly rounded cells. In addition concanavalin A, but not *P. vulgaris* lectin, above a concentration of 25μg/ml causes significant precipitation of foetal bovine serum glycoproteins. This precipitate covers the entire surface area of the plastic culture dishes as a fine uniform layer, and probably has a detrimental effect on cell growth.

It is noteworthy that the degree of toxicity of *P. vulgaris* agglutinin on the ricin-resistant cell clones differs from normal BHK cells as regards both relative plating efficiency (Fig. 6) and cell morphology,

Table 1. *Lectin-binding sites of intact BHK cell lines*

The binding data shown in Figs. 4(*a*)–4(*e*) were used to calculate the total number of binding sites for *R. communis* lectins RCA_1 and RCA_2 (Ricin), *Axinella polyploides* agglutinin, *Lens culinaris* agglutinin and concanavalin A (Con A) by the Scatchard method. ND, Not determined.

	10^{-6} × Sites per cell				
Cells	RCA_1	Ricin	*Axinella*	*Lens**	Con A
BHK normal	6.0	5.6	9.9	20.5	9.0
Ric^R 1	ND	6.3	ND	ND	ND
Ric^R 2	6.7	6.3	ND	ND	ND
Ric^R 6	9.7	0.9	ND	ND	ND
Ric^R 7	ND	2.4	9.0	12.2	13.7
Ric^R 9	ND	0.7	15.2	36.0	ND
Ric^R 10	8.2	0.8	ND	ND	ND
Ric^R 12	8.2	8.2	ND	ND	ND
Ric^R 14	0.4	0.7	1.1	17.3	18.7
Ric^R 15	0.6	0.8	1.3	28.9	32.4
Ric^R 16	ND	5.9	0.4	32.8	11.8
Ric^R 17	6.2	1.5	ND	ND	ND
Ric^R 18	7.3	2.6	ND	ND	ND
Ric^R 19	9.7	11.0	ND	ND	ND
Ric^R 20	ND	1.1	ND	ND	ND
Ric^R 21	0.3	0.6	9.9	32.8	13.6
Ric^R 22	6.2	8.4	ND	23.0	12.0

* Calculated for a mol.wt. of 10^5 for the lectin.

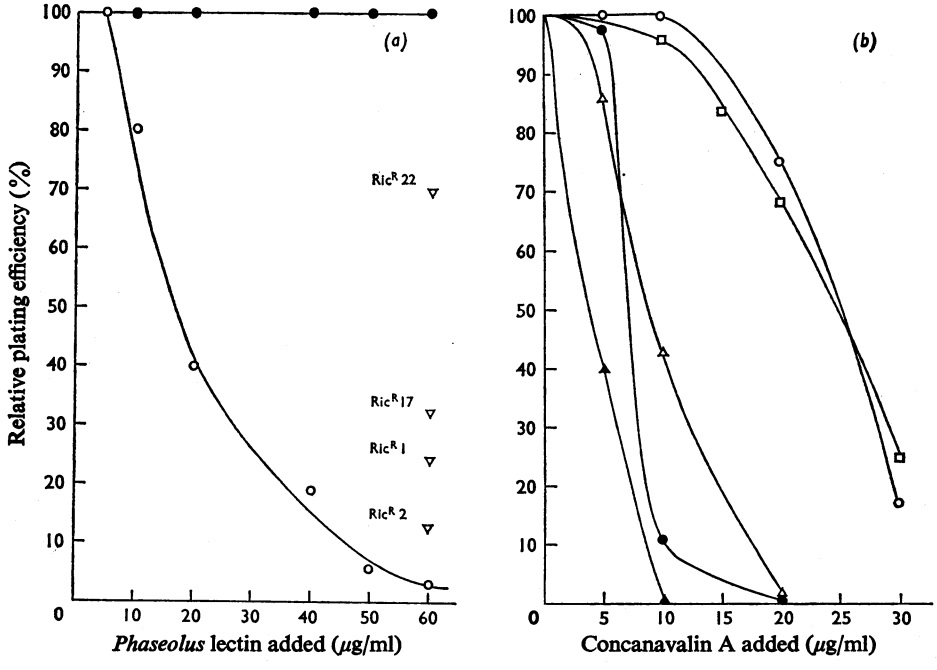


Fig. 6. *Effects of (a) Phaseolus vulgaris lectin and (b) concanavalin A on the colony-forming ability of normal BHK cells* (○) *and ricin-resistant clones* Ric^R 1 *and* Ric^R 16 (□), Ric^R 14 (●), Ric^R 15 (▲) *and* Ric^R 21 (△)

Experimental details are described in Fig. 1 and in the Materials and Methods section. The experimental points obtained for the survival of clones Ric^R 15 and Ric^R 21 in the presence of *P. vulgaris* lectin were identical with those obtained with Ric^R 14 and have therefore not been included in (*a*). The relative plating efficiencies of clones Ric^R 1, 2, 17 and 22 in the presence of 60 μg of lectin/ml are also indicated (▽).

which remained fibroblastic. The concentration of *P. vulgaris* lectin required to inhibit by 50% formation of colonies by normal BHK cells was approx. 15–20 μg/ml (Fig. 6). Three of the ricin-resistant clones, Ric^R 14, 15 and 21, were unaffected in colony tests at lectin concentrations up to at least 60 μg/ml. Although all of the cell lines showed some increase in their resistance to *P. vulgaris* lectin, some of the lines, especially Ric^R 1, 2 and 17 and to a lesser extent Ric^R 22, were still measurably sensitive to the lectin (Fig. 6).

P. vulgaris lectin, like ricin, binds to *N*-acetylgalactosamine and galactose units, and it is therefore not unexpected that mutations affecting the receptor activity of the cells towards ricin might also alter binding of *P. vulgaris* lectin. Since lectin binding presumably is an essential first step in the weak cytotoxicity of *P. vulgaris* lectin, ricin resistance might also be expressed as the increased survival of the cells in medium containing *P. vulgaris* lectin.

In contrast with the results obtained with *P. vulgaris* agglutinin, none of the ricin-resistant cell lines tested appears to have acquired (Fig. 6*b*) any detectable cross-resistance to concanavalin A, a lectin that binds to mannose groups of glycoproteins. Resistant cell lines, for example Ric^R 1 and Ric^R 16, binding as much ricin as normal BHK cells (Table 1), were equally sensitive. However, those cell lines which bind ricin poorly, for example Ric^R 14, 15 and 21, were killed by approx. 3–5-fold lower concentrations of concanavalin A than normal BHK cells. This result is consistent with the higher binding of concanavalin A to these cells and their greater degree of agglutinability by the lectin. However, the relevance of these latter parameters for the increased sensitivity of the cells to concanavalin A cytotoxicity is unknown.

Table 2. *Preliminary classification of ricin-resistant BHK cell lines*

Class	Cells	Ricin concn. for selection (μg/ml)	Properties
I	Ric^R 1 Ric^R 2 Ric^R 22	0.2	Low resistance to ricin, but greater than normal cells. Bind same or higher amounts of ^{125}I-labelled ricin. Sensitive to *P. vulgaris* lectin.
II	Ric^R 12 Ric^R 16 Ric^R 19	1.0	High resistance to ricin. Binds same or higher amounts of ^{125}I-labelled ricin. Insensitive to *P. vulgaris* lectin.
III	Ric^R 17	1.0	High resistance to ricin. Binds less ^{125}I-labelled ricin. Sensitive to *P. vulgaris* lectin.
IV	Ric^R 6 Ric^R 7 Ric^R 9 Ric^R 10 Ric^R 14 Ric^R 15 Ric^R 18 Ric^R 20 Ric^R 21	1.0	Very high resistance to ricin. Binds less ^{125}I-labelled ricin. Insensitive to *P. vulgaris* lectin.

Discussion

The metabolic blocks in the clones described in the present paper and by others (Gottlieb *et al.*, 1974; Hyman *et al.*, 1974; Nicolson *et al.*, 1975*c*) that allow survival of cells incubated in the presence of ricin are unknown. Several classes of resistant cells would be expected, however, from the presently accepted mode of action of ricin on sensitive cells (Refsnes *et al.*, 1974; Olsnes & Pihl, 1972).

A preliminary classification of the cells described in the present paper is shown in Table 2. Among the resistant BHK-cell clones there are several that appear to bind ^{125}I-labelled ricin to similar extents to the normal sensitive cells. It is probable therefore that in these variants resistance is not due to a metabolic defect affecting the structure of the bulk of the specific receptors required for adsorption of ricin. Some of the resistant cells may be altered in their ability to internalize the lectin molecules bound at the cell surface. Other cell lines binding normal amounts of ricin may contain ribosomes that have become insensitive to the toxin, but direct tests of the sensitivity of protein synthesis (Sperti *et al.*, 1973; Montanaro *et al.*, 1973) in extracts of our ricin-resistant BHK cells to decide between these alternative mechanisms have not been carried out. However, it should be noted that only a small proportion of the ricin-binding sites may be involved in endocytosis and cell killing. The great majority of bound lectin molecules may be irrelevant. Although there is as yet no direct evidence for this hypothesis, Nicolson *et al.* (1975*c*) have found that a ricin-resistant lymphoma cell line (Hyman *et al.*, 1974), showing only a slight decrease in ricin-binding sites, contains greatly decreased amounts of two minor surface components, of mol.wt. 50000 and 35000. Nicolson *et al.* (1975*c*) suggest that entry of ricin into sensitive cells depends on binding of the lectin to these glycoproteins, and their loss renders the cells resistant to ricin toxicity.

A preliminary sub-classification of the ricin-resistant BHK cell lines binding normal or nearly

normal amounts of ricin may be made, however, on the basis of their sensitivity to *P. vulgaris* agglutinin. Although the basis of the weak cytotoxicity of this lectin is unknown (Stanley *et al.*, 1975), it is reasonable to assume that this is different from the specific intracellular effects (Sperti *et al.*, 1973; Montanaro *et al.*, 1973) of ricin on protein synthesis. If this is the case then the only common step in cell killing by ricin and *P. vulgaris* lectin may be in the adsorption of the lectins to the same or closely similar surface receptor sites followed by endocytosis. It is known that both lectins bind the monosaccharides, galactose and *N*-acetylgalactosamine, and glycoproteins containing these sugars (Irimura *et al.*, 1975). An alteration affecting the structure of a surface carbohydrate component involved in ricin binding and endocytosis would therefore be likely to affect simultaneously the cytotoxic effects of *P. vulgaris* agglutinin. As indicated in Table 2, clones Ric^R 1, 2 and 22 are resistant to ricin, although there is no decrease in the number of ricin-binding sites. These cells remain sensitive to *P. vulgaris* agglutinin. These clones may therefore be resistant to ricin at a ribosomal site concerned with protein synthesis.

A second class of ricin-resistant clones, represented by clones Ric^R 12, 16 and 19, showing no measurable decrease in ricin-binding sites, may nevertheless be defective in some receptor activity, since these cells are cross-resistant to *P. vulgaris* agglutinin. This reasoning assumes, of course, that the simultaneous acquisition of resistance to ricin and *P. vulgaris* lectin is not due to an accumulation of several mutations in the ricin-resistant cell lines. This assumption seems likely to be true, however, since cell lines, for example Ric^R 3, reverting to ricin sensitivity simultaneously become susceptible to *P. vulgaris* lectin.

All of the remaining ricin-resistant cells (Table 2), for example Ric^R 6, 7, 9, Ric^R 14 and 15, Ric^R 17 and 18, and Ric^R 21, bind appreciably less ricin than do the normal sensitive cells. These cell clones appear to fall into two main classes; cells that bind approx. 20–60% less lectin than the normal cells, for example, clones Ric^R 7, 17 and 18, and secondly cells that show a more drastic decrease of between 80 and 90% in the binding of ricin, for example clones Ric^R 9 and 14. These clones are very cross-resistant to *P. vulgaris* agglutinin and are likely to be defective therefore in receptor sites important in endocytosis of the lectins (Table 2). Clone Ric^R 17 is an exceptional case, however. The number of ricin-binding sites is markedly decreased, indicating a loss of galactose and *N*-acetylgalactosamine units in the cell-surface glycoproteins of these cells. Nevertheless, the cells are sensitive to *P. vulgaris* phytohaemagglutinin. There are two possible conclusions to be made from these results. Thus the *P. vulgaris* agglutinin may bind to only a small proportion of the receptors for ricin. Therefore a structural change which decreases the attachment of ricin to some receptor glycoproteins need not affect to the same extent the binding and subsequent cytotoxicity of *P. vulgaris* lectin.

An alternative explanation may lie in the specificity of binding of *P. vulgaris* lectin and ricin to the carbohydrate chains of receptor glycoproteins. Although both lectins bind strongly to galactose and *N*-acetylgalactosamine it is probable that sugar residues adjacent to these in glycoprotein oligosaccharides considerably influence the extent of binding (Adair & Kornfeld, 1974; Irimura *et al.*, 1975). Thus a change in the sequence of sugars surrounding the primary binding site or a loss of a sugar from the sequence may affect differently the affinity of ricin or *P. vulgaris* lectin towards the new or shortened oligosaccharide sequence. A similar reasoning can be applied to other galactose-binding lectins such as *R. communis* RCA_1 and *Axinella* lectin.

The length of the carbohydrate sequence recognized by galactose-binding lectins, such as ricin or *P. vulgaris* lectin, is unknown. However, concanavalin A binds most tightly to oligosaccharides containing three or more mannose residues (So & Goldstein, 1968). Since none of the resistant cell lines is defective in concanavalin A-binding sites (Table 1) it is very unlikely that there have been any changes to mannose-containing sequences. The increased binding of concanavalin A to those resistant cell lines that show decreased binding of galactose- or *N*-acetylgalactosamine-specific lectins is best rationalized by assuming some loss of peripheral sugars, including galactose and *N*-acetylgalactosamine, resulting in increased exposure of mannose units.

A. M. holds a Beit Fellowship, and A. U. is a sandwich student on secondment from the North East London Polytechnic.

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