Changes in the Sensitivity of Chick Fibroblasts to *Ricinus* Lectin (RCA I) Toxicity in Relation to the Stage of Embryo Development

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The toxic effect of *Ricinus* lectin RCA I, as estimated by the inhibition of [3 H]leucine incorporation, was investigated on chick-embryo fibroblasts at different stages of development. There appeared to be a differential susceptibility of chick-embryo fibroblasts to lectin RCA I. Fibroblasts from 16-day embryos were the most sensitive to its toxic effect in terms of both concentration and time, and cells from 8-day embryos were the least sensitive. This differential sensitivity to the toxic effect of lectin RCA I was closely related to the binding of the lectin: fibroblasts from 16-day embryos had more binding sites (1.5×10^7 /cell) with a high affinity than did 12-day (0.45×10^7 /cell) or 8-day embryos (0.2×10^7 /cell). Studies on the specificity and the removal of bound lectin RCA I by D-galactose indicated that the lectin binding was necessary but not sufficient in itself to cause the toxic effect and that the lectin needed to enter the cells in order to be toxic. The amount of lectin RCA I needed to induce a 50–60% toxicity enters fibroblasts of 16-day embryos more rapidly than those of 12- and 8-day embryos.

Lectins, which are plant agglutinins, have various effects on embryo cells at different stages of differentiation, especially with regard to cell agglutination (Kleinschuster & Moscona, 1972; Krach *et al.*, 1973) and cell growth (Aubery & Bourrillon, 1975, 1976; Kaplowitz & Moscona, 1976). For these effects to take place, the lectins need to bind to cell-surface glycoproteins (Nicolson, 1974). The mechanism of lectin action is not yet clear. Moreover, certain lectins isolated from *Abrus precatorius* (abrin) and *Ricinus communis* (ricin) induce a toxic effect in animal cells (Nicolson, 1974; Olnes, 1972; Olnes & Pihl, 1973). Two types of lectin can be extracted from *Ricinus communis*. Lectin RCA II (mol.wt. 60000), which has been extensively studied (Nicolson *et al.*, 1975), has a weak agglutinating effect, but is highly toxic. Lectin RCA I (mol.wt. 120000) has a powerful agglutinating capacity, but is less toxic than lectin RCA II (Kornfeld *et al.*, 1974). The toxic effects of these lectins on embryo cells have, however, not been investigated.

In the present paper, we report the results of our studies on the toxic effects of lectin RCA I, as estimated by [3 H]leucine incorporation, on chick-embryo fibroblasts at various stages of development.

Materials and Methods

Cells

The fibroblasts were obtained from 8-, 12- and 16-day chick embryos by the method of Rein &

Rubin (1968) modified as previously described (Aubery & Bourrillon, 1975).

Ricinus communis lectin

The tetrameric form (RCA I, mol.wt. 120000) of *Ricinus communis* (castor bean) lectin was purified by the procedure of Nicolson & Blaustein (1972). The preparation of lectin RCA I was homogeneous, as determined by disc gel electrophoresis and analytical centrifugation. Solutions of the lectin were prepared at a final concentration of 100 μ g/ml in 0.15M-NaCl and added to cell cultures at final concentrations ranging from 0.001 to 10 μ g/well.

Labelling of lectin RCA I

The lectin was labelled by the method of Miller & Great (1972) by using [14 C]acetic anhydride (specific radioactivity 10–30 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Labelled lectin was purified by gel filtration on a Sephadex G-25 column (2 cm $\times$ 40 cm), the eluting buffer being 0.005M-NaHCO₃/0.15M-NaCl, pH 7 (buffer A). The specific radioactivity of lectin RCA I was 5.6×10^6 d.p.m./mg. The labelled lectin behaved exactly like native lectin in the erythroagglutination test. The labelled lectin solution was used at a final concentration of 1 mg/ml in buffer A.

Binding studies

Monolayer cultures reaching confluency were washed three times in Dulbecco's phosphate-buffered

saline, pH 7.4 (Dulbecco & Vogt, 1954). The monolayer was overlaid with 0.5 ml of Dulbecco's phosphate-buffered saline containing ^{14}C -labelled lectin RCA I; the cells were then washed three times and dissolved in 0.5 ml of 0.1 M-NaOH, before being added to 10 ml of scintillation fluid (PCS; Amersham/Searle, Arlington Heights, IL, U.S.A.). To measure non-specific binding of ^{14}C -labelled lectin RCA I, cultures were incubated with the labelled lectin in the presence of 0.1 M-D-galactose, a competitive inhibitor of lectin RCA I binding (Nicolson & Blaustein, 1972). Non-specific binding, which amounted to 10% of the total binding, was subtracted from the total bound radioactivity in order to determine the specific binding of lectin RCA I. The radioactivity of each sample was counted in a liquid-scintillation spectrometer (Inter-technique SL 300). ^{14}C -labelled lectin RCA I was used at final concentrations ranging from 1 to 80 $\mu\text{g}/\text{ml}$. Experimental data were plotted by the method of Scatchard (1949), taking into account the molecular weight of the lectin (120000).

Cell cultures

Primary monolayer cultures were made in 16 mm-diameter wells in 0.5 ml of Eagle's minimal medium (Flow Laboratories, Irvine, Scotland U.K.) supplemented with 1% glutamine, 1% antibiotics (penicillin, streptomycin) and 10% foetal calf serum. The initial seeding concentration was 10^6 cells/ml (0.5×10^6 cells/well). Cultures were grown in humidified air containing 5% CO_2 . A sample of cells was counted in a haemocytometer. Each measurement refers only to viable cells and represents the mean of four samples. The protein content of the samples was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

[^3H]Leucine incorporation

Cell cultures were incubated with 0.5 μCi of [^3H]leucine (The Radiochemical Centre; specific radioactivity 55 Ci/mmol)/well for a 1 h pulse at 37°C. The cells were removed from the wells by trypsin treatment [2.5% (w/v) trypsin in 0.15 M-NaCl; Eurobio, Paris, France]. The labelled cell material was then allowed to precipitate overnight in 2 ml of 10% (w/v) trichloroacetic acid. The precipitate was washed twice with 5% trichloroacetic acid, dissolved in 10 ml of PCS scintillator and then counted for radioactivity in a liquid-scintillation spectrometer (Inter-technique SL 300).

Results

Quantitative interaction between RCA I and chick-embryo fibroblasts at various stages of development

The binding of lectin RCA I was investigated on chick-embryo fibroblast cultures approaching con-

fluency. The fibroblasts from 8-, 12- and 16-day embryos rapidly bound the lectin to plateau values within 30 min at 4°C (incubations were done at this temperature to prevent endocytosis). Confluent fibroblasts from 8-, 12- and 16-day embryos were saturated with lectin at 40 $\mu\text{g}/\text{ml}$. Both quantitative and qualitative differences were noted between the number of lectin-binding sites in relation to the stage of development of the embryos. The Scatchard (1949) plots gave biphasic curves for fibroblasts from 8- and 12-day embryos, suggesting that two classes of binding sites were available for lectin RCA I. There was a 10-fold difference between the affinity constants of the two classes of sites. However, fibroblasts from

Fig. 1. Binding of lectin RCA I: Scatchard plots B represents the amount of bound lectin and F represents the amount of free lectin (both $\mu\text{g}/10^6$ cells), for fibroblasts from: ●, 8-day embryos; ○, 12-day embryos; △, 16-day embryos. Experimental procedures are described in the Materials and Methods section. Incubation was at 4°C to prevent endocytosis. The range of lectin concentrations used was 1–80 $\mu\text{g}/10^6$ cells.

Table 1. Quantitative interactions between lectin RCA I and chick-embryo fibroblasts at various stages of embryo development
Experimental procedures are described in the Materials and Methods section. The number and the affinity constant of the lectin RCA I-binding sites are obtained by the method of Scatchard (1949). The range of lectin concentrations used was 1–80 µg/ml. Each result gives the range of values obtained from four separate experiments. Fibroblasts from 8- and 12-day embryos exhibit two classes of sites, whereas those from 16-day embryos have only one class.

Age of embryos (days)	Binding sites (number/cell)	Affinity constant, K_A (M ⁻¹)
8	$0.2 \times 10^7 \pm 0.001 \times 10^7$	$3.8 \times 10^7 \pm 0.6 \times 10^7$
	$1.13 \times 10^7 \pm 0.3 \times 10^7$	$3.3 \times 10^6 \pm 0.8 \times 10^6$
12	$0.45 \times 10^7 \pm 0.11 \times 10^7$	$4.1 \times 10^7 \pm 1.5 \times 10^7$
	$4 \times 10^7 \pm 0.4 \times 10^7$	$2.3 \times 10^6 \pm 0.6 \times 10^6$
16	$1.5 \times 10^7 \pm 0.3 \times 10^7$	$1.3 \times 10^7 \pm 0.8 \times 10^7$

Fig. 2. Dose-response of the lectin RCA I effect on [3H]leucine incorporation

Each point gives the range of values obtained from four separate experiments. Embryo age: ●, 8 days; ○, 12 days; △, 16 days. Experimental procedures are described in the Materials and Methods section. The dose-response was studied at 37°C after a 4 h incubation in the presence of lectin RCA I at various concentrations ranging from 0.001 to 10 µg/ml.

16-day embryos exhibited a monophasic curve, suggesting only one class of binding site (Fig. 1). The number of binding sites with a high affinity constant increased with the age of the fibroblast (8 days, 0.2×10^7 /cell; 12 days, 0.45×10^7 /cell; and 16 days, 1.5×10^7 /cell). Thus fibroblasts from 16-day embryos exhibited the highest number of lectin-binding sites with a high affinity constant (Table 1). The number of binding sites with a low affinity constant was 1.13×10^7 /cell and 4×10^7 /cell for 8- and 12-day embryos respectively (Table 1).

Differential cytotoxic effect of lectin RCA I

Lectin RCA I was added to the culture after the latter had reached confluency, which in fibroblasts from 8-, 12- and 16-day embryos occurred after 48,

72 and 96 h respectively. The effects on [3H]leucine incorporation were observed with different lectin concentrations and after various incubation times, namely 1, 2, 4 and 8 h. During these times, the cell number ($0.95 \times 10^6 \pm 0.07 \times 10^6$ cells/ml) and cell protein content ($147.5 \pm 25 \mu\text{g}/10^6$ cells), as measured by the method of Lowry *et al.* (1951), remained constant in the controls as well as in the lectin-treated cultures.

(a) Dose-response. The dose-response was studied after a 4 h incubation in the presence of lectin at various concentrations ranging from 0.001 to 10 µg/ml (Fig. 2). Lectin RCA I at a final concentration of 0.001 µg/ml caused a weak inhibition, namely 18, 15 and 12% of [3H]leucine incorporation in fibroblasts from 8-, 12- and 16-day embryos respectively.

At a final concentration of 0.01 µg/ml, lectin RCA I inhibited [3H]leucine incorporation by 30% in fibroblasts from 16-day embryos, and only by 10–12% in fibroblasts from 12- and 8-day embryos.

At lectin concentrations from 0.1 µg/ml, the toxic effect became more marked. [3H]Leucine incorporation was inhibited by 62% in fibroblasts from 16-day embryos, and by 28 and 12% in fibroblasts from 12- and 8-day embryos respectively. Thus fibroblasts from 8-day embryos appeared to be the least sensitive to the toxic effect of lectin RCA I, and fibroblasts from 16-day embryos the most sensitive. The differences in chick-embryo sensitivity to the toxic effect of the lectin as a function of the stage of development remained similar when a concentration of 0.2 µg/ml was used, and became less marked with a further increase in lectin concentration. At 1 µg/ml there was no significant difference, as the lectin inhibited [3H]leucine incorporation by at least 80% in all the cells, irrespective of the stage of development.

(b) Time-course studies. The effect of lectin RCA I at a final concentration of 0.2 µg/ml on [3H]leucine incorporation was investigated at various times on fibroblasts from 8-, 12- and 16-day embryos (Table 2). After a 1 h incubation, no significant toxic effect was noticed. The [3H]leucine incorporation was inhibited

Table 2. Toxic effect of lectin RCA I (0.2 µg/ml) on [3 H]leucine incorporation at various times in fibroblasts from 8-, 12- and 16-day embryos

Experimental procedures are described in the Materials and Methods section. Each result gives the range of values obtained from three separate experiments. Numbers in parantheses are the percentage inhibition compared with the control. For specificity assay, lectin RCA I was preincubated with 0.1 M-D-galactose (22°C for 30 min) and then the mixture was added to cell cultures.

Time (h)	Treatment	Embryo age	Incorporation (c.p.m./10 ⁶ fibroblasts)		
			8 days	12 days	16 days
1	Control		3025 ± 282	3483 ± 39	1083 ± 44
	RCA I		3261 ± 190 (0)	3431 ± 146 (2)	1087 ± 104 (0)
	RCA I-galactose complex		3106 ± 206 (0)	3359 ± 95 (4)	1050 ± 68 (3)
2	Control		3783 ± 310	5186 ± 484	2187 ± 147
	RCA I		3177 ± 260 (16)	3662 ± 166 (30)	1293 ± 121 (41)
	RCA I-galactose complex		3494 ± 80 (8)	4592 ± 44 (12)	1560 ± 57 (29)
4	Control		5077 ± 470	5569 ± 524	2662 ± 175
	RCA I		3808 ± 290 (25)	3075 ± 140 (45)	671 ± 14 (75)
	RCA I-galactose complex		4615 ± 184 (10)	4154 ± 87 (25)	1321 ± 106 (51)
8	Control		6835 ± 525	6465 ± 462	3010 ± 84
	RCA I		999 ± 25 (85)	965 ± 45 (85)	582 ± 17 (82)
	RCA I-galactose complex		1845 ± 60 (73)	1810 ± 128 (72)	722 ± 56 (76)

Fig. 3. Removal by D-galactose (0.1 M) of labelled lectin RCA I (50 µg/ml) bound to chick-embryo fibroblasts aged: (a) 8 days; (b) 12 days; (c) 16 days

Each point gives the range of values obtained from two separate experiments. Incubation was at 37°C. ●, Total bound lectin RCA I; ○, residual bound lectin RCA I after D-galactose washing. For the total binding of lectin RCA I, the cells were incubated with lectin from 5 to 120 min and then they were washed three times in Dulbecco's buffered saline. For removal assay, the cells were incubated with lectin for various lengths of time (from 5 to 120 min), before the addition of D-galactose. The cells were then washed three times in Dulbecco's buffered saline.

by 16, 30 and 41 % after a 2 h incubation in fibroblasts from 8-, 12- and 16-day embryos, respectively. After a 4 h incubation, the inhibition of [3 H]leucine incorporation reached 25, 45 and 75 %, respectively. Thereafter, the [3 H]leucine incorporation was almost totally inhibited (85 %) at all stages.

These results confirmed the highest sensitivity of fibroblasts from 16-day embryos to the toxic effect of lectin RCA I.

Specificity of the lectin RCA I effect

In order to test the specificity of the lectin toxicity by using its specific inhibitor, lectin RCA I (0.2 μ g/ml) was incubated with D-galactose (0.1 M) before being added to the cell cultures. After a 2 h incubation, the toxic effect of lectin was partially (50–60 %) inhibited by D-galactose in fibroblasts from 8-, 12- and 16-day embryos. After an 8 h incubation, the toxic effect of the lectin was no longer inhibited (only 15 %) by D-galactose in fibroblasts from 8-, 12- and 16-day embryos (Table 2). The inhibitory effect of D-galactose on lectin toxicity therefore decreased with time.

Removal of bound labelled lectin RCA I

In an attempt to investigate the reversibility of lectin RCA I binding to embryo cells, the cells were incubated with lectin for various lengths of time, before the addition of D-galactose (0.1 M). This incubation was performed at 37°C, so that endocytosis might occur. D-Galactose released 95–98 % of the labelled lectin after a 5 min incubation, 85–88 % after a 30 min incubation, but only 65–70 % after 90 min at all stages of development (Fig. 3), indicating that the removal of bound lectin decreased with time until 90 min. Thereafter, the amount of lectin RCA I that could not be released remained at 30–35 % of the bound labelled lectin. These results suggested that lectin RCA I could enter the cells. From Fig. 3, it was concluded that 0.12 μ g of lectin RCA I remained bound to fibroblasts from 16-day embryos from 15 min of contact between lectin and cells, and this amount was sufficient to induce a 60 % toxicity. During this time a similar amount of lectin RCA I remained bound to fibroblasts from 12- and 8-day embryos, but inducing only 28 and 12 % toxicity respectively. In order to reach a 50–60 % toxicity (as well as in fibroblasts from 16-day embryos), fibroblasts from 12-day and 8-day embryos needed to bind at least 0.2 and 0.6 μ g of lectin RCA I; this was obtained only from 30 and 90 min of contact respectively.

Discussion

A differential cytotoxicity of lectin RCA I was observed in chick-embryo fibroblasts in relation to their stage of development. Fibroblasts from 16-day

embryos were the most sensitive to the toxic effect of the lectin as measured by [3 H]leucine incorporation, and those from 8-day embryos were the least sensitive.

The particular sensitivity of 16-day-embryonic fibroblasts may be related to the fact that they have a larger number of lectin-binding sites with a high affinity constant than 8- and 12-day-embryonic fibroblasts. There would therefore be a direct correlation between the binding of lectin RCA I and its toxic effect.

The most likely explanation for the biphasic curves seen in the Scatchard (1949) plots for 8- and 12-day-embryonic fibroblasts was that there were two classes of binding site and that two different affinities were involved (Adair & Kornfeld, 1974). Another possibility would be that there was only one class of binding site, but that the binding of the lectin exhibited negative co-operativity (Levitzki & Koshland, 1969). The 16-day-embryonic fibroblasts, however, appeared to have only one class of binding site, suggesting that a quantitative and/or qualitative modification may take place in the binding sites during embryo development.

Moreover, under culture conditions (at 37°C), lectin RCA I or a part of it could also be bound to sites other than sites containing galactosyl residues, since the specific inhibition of the lectin toxicity by D-galactose became less marked with time.

It has been observed that ricin (RCA II) needs to enter the cells in order to have a toxic effect (Nicolson *et al.*, 1975). Similarly, lectin RCA I could enter the cells, probably either because of endocytosis or as a result of facilitated permeability. This hypothesis is supported by the results of the experiments on the release of bound lectin RCA I by D-galactose (0.1 M), since the amount of bound labelled lectin not released by D-galactose increased with time under the experimental conditions in which the toxic effect was observed.

Moreover, our results suggest that the amount of lectin RCA I needed to induce a 50–60 % toxicity enters 16-day-embryonic fibroblasts more rapidly than those of 8- and 12-day embryos.

If lectin must enter cells in order to exert its toxic effect, ribosomes could be the primary subcellular sites for the action of lectin RCA I, as has been reported for cycloheximide (O'Brig *et al.*, 1971; Baker & Humphreys, 1972), abrin and ricin (Olness, 1972; Saltvedt, 1976).

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