Inhibition of an early stage of erythropoiesis in cultures of young chick blastodisc cells by *Ricinus communis* agglutinin 120[1]

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Summary. An early pre-erythroid phase of erythropoiesis in cultures of dispersed cell suspensions prepared from primitive streak and head-fold chick blastodiscs was very sensitive to inhibition by *Ricinus communis* agglutinin 120.

We have previously reported the preparation of cell suspensions from primitive streak and head-fold chick blastodiscs which yielded mature erythrocytes upon subsequent culture[2,3]. However, the ability of these cell suspensions to yield erythrocytes in vitro proved extremely labile on further manipulation. We have therefore developed improved procedures for preparation of chick blastodisc cell suspensions for which the capacity to differentiate in vitro is considerably more stable[4]. During the course of this study we observed that an early phase of erythropoiesis in cultures of cells prepared by the improved procedures was very sensitive to inhibition by *Ricinus communis* agglutinin-120.

Material and methods. The basic culture medium (BCM) consisted of 7 parts Eagle's minimum essential medium with Earle's salts[5] and with Hepes buffer (Gibco, Grand Island, New York, No. 236) and 2 parts heat-inactivated fetal calf serum (Gibco Lot No. H954620), supplemented with 40 mM methyl-α-mannoside, 25 Ki units/ml aprotinin (Sigma Chemical Co.), 1% of a 29.2 mg/ml solution of L-glutamine (fresh weekly) and antibiotics[4].

Blastodiscs of the Shaver Starcross No. 288 line of White Leghorn fowl were explanted onto a solid minimum medium[6] and those at the primitive streak and head-fold stages of development were selected. They were detached from their supporting vitelline membranes and suspensions containing $4–10 \times 10^6$ single cells/ml were prepared in prewarmed BCM supplemented with 2.5% (v/v) of egg yolk homogenate[4]. Cell suspensions were distributed in 0.8 ml volumes in 35×10 mm tissue culture dishes (Falcon plastics no. 3001) and to each was added a further 0.2 ml of BCM containing any additions. The cultures were then incubated under air – 5% CO_2 and concentrations of erythroid cells present at 4 days were determined as previously[2,3]. *Ricinus communis* agglutinin 120 was obtained from Miles Laboratories, Inc., Elkhart, Ind.

Results and discussion. When added at zero time *Ricinus communis* agglutinin 120 totally suppressed erythropoiesis at a concentration of 0.1 µg/ml and was markedly inhibitory at 0.05 µg/ml (tables 1 and 2, a). However sensitivity to inhibition of erythropoiesis by these concentrations of lectin was decreased markedly when addition to the culture was deferred 18 h. A further decrease in sensitivity was observed after 42 h in culture, although erythropoiesis remained sensitive to inhibition by as little as 2 µg/ml of the agglutinin (table 1).

Table 1. Sensitivity of erythropoiesis to inhibition by *Ricinus communis* agglutinin 120

Experiment	Inoculum (total cells $\times 10^{-6}$/ml)	Agglutinin added at h	Erythroid cells $\times 10^{-6}$/ml at agglutinin µg/ml 0	0.1	0.5	2
1	3.8	0	1.6	0	0	0
		18	1.2	0.9	0	0
2	4.9	0	1.5	0	0	0
		18	–	1.3	0.3	0
		42	–	1.8	1.2	0.9
3	7.3	0	2.2	0	0	0
		18	–	1.9	0.8	0
		42	2.3	2.4	1.9	1.0

Table 2. Inhibition of an early stage of erythropoiesis

Experiment	Inoculum (total cells $\times 10^{-6}$/ml)	Erythroid cells $\times 10^{-6}$/ml at agglutinin µg/ml 0	0.05	0.1	0.2
a) Agglutinin added at zero time					
1	4.7	1.1	0.3	0	0
2	5.3	0.8	0.08	0	0
3	4.9	1.9	<0.1	0	0
b) Agglutinin added at 18 h to attached cells of control cultures					
1		0.6	0.6	0.7	0.4
2		0.3	–	0.2	0.2
3		0.7	0.7	0.6	0.7
c) Agglutinin plus unattached cells of treated cultures added at 18 h to attached cells of control cultures					
1		1.1	1.2	1.3	0.9
2		0.8	0.7	–	0.5
d) Attached cells at 18 h from cultures with agglutinin at zero time plus unattached cells at 18 h from control cultures					
1		1.1	0.4	0	0
2		0.8	0	0	0
3		1.5	0.5	0.2	0

For details of these 3 experiments see the text.

Early hemoglobin-containing precursors of mature erythrocytes are first seen in control cultures after about 42 h of incubation. Prior stages of erythropoiesis appear to occur largely, or even entirely, in cells which attach to the substratum within 18 h of culture, but are stimulated by non-erythroid cells free in suspension (unpublished experiments and below). We therefore examined the effects of pre-treatment with *Ricinus communis* agglutinin upon erythropoiesis in the cells which attached to the substratum within 18 h by 2 approaches (table 2).

A series of cultures with and without lectin were incubated for 18 h and the free cells in suspension were then removed. The attached cells were carefully rinsed with 1 ml of warm BCM, supplemented with 1 ml of warm BCM containing varying concentrations of the agglutinin, and re-incubated. Attached cells from control cultures yielded fewer mature erythrocytes and late reticulocytes (table 2, b) than corresponding undisturbed control cultures (table 2, a). However there was no inhibition of erythropoiesis in the former cultures by 0.1–0.2 µg/ml of *Ricinus communis* agglutinin. In contrast neither the attached cells from treated cultures supplemented with normal BCM medium nor the unattached cells from any 18 h culture yielded any erythroid cells (data not shown).

In a concurrent series of experiments erythropoiesis by control attached cells supplemented with free cells from cultures containing up to 0.1 µg/ml of agglutinin was equal to that in undisturbed control cultures (table 2, c). Erythropoiesis in cultures containing the converse combination of cells was markedly impaired (table 2, d).

The mode of action of the lectin in inhibiting erythropoiesis in our cultures is not yet known. However our data clearly indicate that cell surface receptors with specificity for *Ricinus communis* agglutinin 120 are involved in an early reaction essential for erythropoiesis by our chick blastodisc cells.

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Biochemical and autoradiographical distribution of hyaluronic acid in calf rib cartilage[1]

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Summary. In calf rib cartilage, about one half of total hyaluronate is soluble with guanidinium hydrochloride, the other half only after collagenase treatment. Evidence is presented for its pericellular and intracellular distribution.

In hyaline cartilage, small amounts of hyaluronic acid (0.4–0.8%, w/w) interact with proteoglycans and link proteins to form large salt-soluble aggregates[2,3] in the extracellular matrix. In calf rib cartilage, hyaluronic acid represents 1.0–1.5% of total uronate[4,5]; however most hyaluronate is found in the salt-insoluble residue[4,6]. Moreover, small concentrations of hyaluronate inhibit proteoglycan biosynthesis of cultured chondrocytes[7–10], whereas exogenous proteoglycans in the medium stimulate it[7]. Thus, hyaluronic acid appears to have important biological properties for regulating proteoglycan biosynthesis. Its localization in vivo needs further clarification in hyaline cartilage.

Materials and methods. For the investigation of biochemical distribution of hyaluronate, 2 g of fresh cartilage slices (50–100 µm thick) from calf ribs were successively extracted with 0.4 M and 4.0 M guanidinium hydrochloride (GuHCl) in 0.05 M sodium-acetate buffer, pH 5.8[11]. The insoluble residue, dialyzed free of salt, was treated twice with proteinase-free collagenase (EC 3.4.4.19, 20 U/mg, Calbiochem A grade)[12] yielding collagenase extracts I and II and cartilage cells which appeared bare and well-shaped in the light microscope. The 4 extracts, as well as the cells, were digested exhaustively by papain[11] before hyaluronate fractions were isolated from them by applying Ecteola cellulose chromatography[4]. In these fractions, hyaluronate was degraded specifically with leech hyaluronidase (EC 3.2.1.36) and analyzed[4,13]. No hyaluronate-degrading activities could be observed with cartilage slices.

For autoradiographical investigations, cartilage slices (100 µm thick) were incubated for 300 min anaerobically with D-glucosamine -6-^3H (N)[5] and washed with a solution containing formaldehyde (40 g/l) and cetylpyridinium chloride (5 g/l). Labelled slices were embedded in paraffin and cut to 7 µm thick slices at right angles to their surface. After removing paraffin with xylene from the mounted slices, the tissue sections were treated with leech hyaluronidase[4] followed by the stripping film procedure with Kodak AR 10 film[14]. Silver grains were counted over those cartilage cells, which show clear-cut boundaries, no unspecific background in their surroundings and their nuclei situated in the plane of section.

Results and discussion. In hyaline cartilage of calf ribs, about 50% of total hyaluronate was solubilized with 0.4 M and 4.0 M GuHCl, the rest could be dissolved only after

Table 1. Biochemical localization of hyaluronate in calf rib cartilage in vivo

Hyaluronate content (µmoles hexosamine*/mg DNA**)					
Total	0.4 M guanidine extract	4.0 M guanidine extract	Collagenase extract I	Collagenase extract II	Cartilage cells
8.7	2.2	1.7	2.7	0.7	0.3
100%	25%	20%	31%	8%	3%

* Estimated with modified Elson-Morgan reaction[5]. ** Isolated and measured acc. to Kleine[13].