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0014-4754/91/010070-06$1.50 + 0.20/0

Colloidal gold as a permanent marker of cells

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Received 28 February 1990; accepted 15 May 1990

Summary. We have demonstrated that colloidal gold-labelled serum proteins are taken up by a number of cells in cultures established from the postnatal rodent neopallium. The colloidal gold enters and remains within secondary lysosomes over extended periods of time and, as well, persists after the subculture of these cells. The cell types that readily take up the label in our culture system are type-1 astrocytes, glial precursor cells and macrophages, whereas, only a small number of oligodendrocytes take up the label. The use of serum proteins to introduce colloidal gold into cells therefore seems to be a convenient and easy way to permanently mark cells.
Key words. Astrocytes; colloidal gold; culture; label; lysosomes.

In certain experimental situations it is often desirable to be able to recognize a particular cell or its progeny and then follow its differentiation and development to its final location in the body. One way of doing this is to take advantage of naturally occurring differences between intracellular components such as difference in nuclear size and then follow the fate of these 'labelled' cells in chimeric embryos. This technique has been performed for several decades [1-3]. A recent twist to the use of stable tracers in the study of the development of the nervous system is to generate one's own label by introducing retroviruses into single cells and detecting such cells cytochemically [4]; the major drawback to this latter technique is that one has very little control over which particular cell will take up the virus.

Other labelling techniques have also been employed to tag cells to follow their developmental fate. More commonly, these cells are marked with labels that are diluted as the cells proliferate. The first of such labels used were low signal dyes such as Nile blue sulfate that were taken up by the cells of interest [5]. More recently enzymes or enzyme-linked conjugates which then can be detected cytochemically [6] have been used, as have chemicals with stronger signals such as a variety of fluorescent dyes [7]. One setback in using such markers is that they can be degraded and thus have a limited life within cells. More permanent markers are obtained by incorporating tritiated thymidine [8] or bromodeoxyuridine [9] into cells during the S phase of the cell cycle. The marked cells are identified through the use of autoradiography and immunocytochemistry respectively. The use of these labels, however, requires that the cell population of interest be a proliferative one at the time of labelling.

Other marking methods exist that do not require the cells of interest to be proliferative but only requires that they be capable of phagocytosis. An example of this method is the uptake of polystyrene microspheres into the lysosomes of cultured astrocytes as demonstrated by Emmet and co-workers [10].

We have recently demonstrated that astrocytes in culture readily phagocytose colloidal gold-labelled serum proteins [11] and that the gold ultimately is sequestered within lysosomes. Therefore, it seemed to us that such an accumulation of gold within cells would also serve as a marker of such cells. The objective of this study was firstly to determine whether such a colloidal gold-labelled cell is 'permanently' marked and secondly to determine whether other cell types could be labelled in this fashion.

Materials and methods

Culture preparation and treatment. Cultures consisting mainly of astrocytes were prepared from newborn Swiss mouse neopallium [12] whereas cultures consisting mainly of oligodendrocytes and oligodendrocyte precursor cells were prepared from two-week-old Swiss mouse neopallium [13]. The growth medium consisted of a modified Eagle's Minimum Essential Medium containing 5% (v/v) horse serum [12]. After two weeks of culture the cells were fed with a medium containing colloidal gold-labelled horse serum proteins for a period of four hours. The

cultures were then fed and maintained on standard growth medium. One week later some of the cells were subcultured. After a total of five weeks in vitro the cultures were fixed and prepared for electron microscopy.

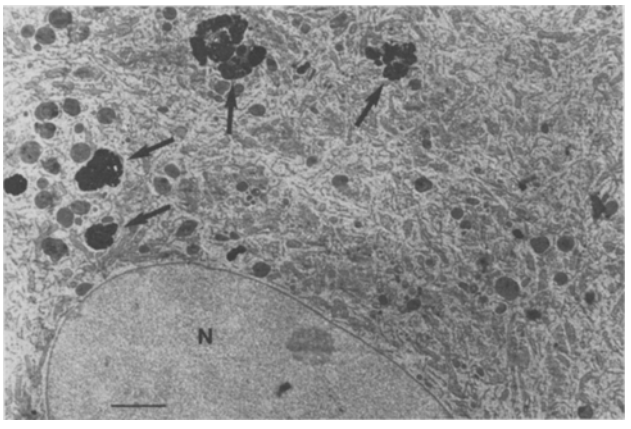
Colloidal gold labelling of serum proteins. Colloidal gold of approximately 50-nm particle size was prepared according to the procedure of Frens[14]. Serum proteins were bound to the colloidal gold according to the procedure of Slot and Geuze[15]. In brief, 400 ml of colloidal gold solution was brought to pH 6.5 by the addition of 5 ml of 0.1 M K_2CO_3; then 1.0 ml of horse serum was slowly added with agitation. This solution was centrifuged at 15,000 g for 20 min and the supernatant discarded. The gold pellet was washed in distilled water and recentrifuged and finally suspended in 20 ml of modified Eagle's Minimum Essential Medium.

Microscopical techniques. Cultures were fixed in the Petri dishes for 40 min with 1% paraformaldehyde / 2.5% glutaraldehyde in 0.08 M cacodylate buffer, postfixed for 1 h with 1% OsO_4/1,8% $K_4Fe(CN)_6$ in 0.08 M cacodylate buffer, dehydrated with ascending concentrations of ethanol and embedded in Epon/Araldite. Small sample areas of the cultures were then cut out and thin sections (~ 60 nm) were cut in a plane horizontal to the culture substratum. The sections were mounted on formvar-coated copper slot grids, stained with uranyl acetate and Reynold's lead citrate and examined with a Philips 400 electron microscope.

Results and discussion

Examination of the astrocyte-enriched cultures by phase microscopy after the four-hour labelling period showed that the majority of cells in the cultures appeared to have taken up the label. Previous studies have demonstrated that confluent astrocytic cultures consist of multiple layers of cellular processes and generally only the most superficial layer of cells in direct contact with the culture medium take up the colloidal gold-labelled proteins[11]. Hence, in these confluent cultures only one quarter of the astrocytic cells contained the label; however, in less dense cultures where all astrocytes make direct contact with the medium, all astrocytes take up the colloidal gold-labelled serum proteins. This label was still present after an additional three weeks in vitro. Subculturing did not appear to affect the distribution of the gold label. In the oligodendroglial-enriched cultures only about one tenth of the cells had appeared to have taken up the gold label.

When both primary and secondary five-week-old cultures were examined electron microscopically, it could be seen that the label was present within secondary lysosomes (fig.). In both the astrocyte-enriched and oligodendrocyte-enriched cultures, the cell types that contained the label were mainly type-1 astrocytes (fig.) while only few mature oligodendrocytes contained label. In addition, macrophages and glial precursor cells contained the gold label. The glial precursor cells are likely the O2A cells described by Raff and Miller[16].



Type-1 astrocyte two weeks after subculturing and a total of five weeks after the establishment of the primary culture from two-week-old mice. Note the colloidal gold containing secondary lysosomes [arrows]. N = nucleus. Bar = 2.0 μm.

We have, thus, demonstrated that it is possible to label in vitro a number of cell types with colloidal gold. The label is retained within the cells for a period of at least three weeks and probably for the life of the cell. More importantly, the label can be readily demonstrated at both the ultrastructural and light microscope levels. In the latter case heavily labelled cells are readily detected in semithin sections and it should be possible to demonstrate lightly labelled cells through the use of silver autometallography[17]. This mode of labelling cells is thus very comparable to that described by Emmet and co-workers[10].

In the present study we have used a mixture of serum proteins bound to colloidal gold particles. A previous study[11] has demonstrated that the gold-labelled serum proteins were taken up mainly via phagocytosis. The initiation of this phagocytic activity seems to be receptor mediated in that simply adding colloidal gold-labelled serum proteins to conditioned medium results in vigorous phagocytic activity in cultured astrocytes. We do not know which of the serum proteins bind to the colloidal gold nor do we know which of these bound serum proteins initiate phagocytosis in astrocytes or glial precursor cells. It may be that different proteins activate phagocytosis in different cell populations and thus it may be possible to use the present colloidal gold method to selectively and permanently label cells in a mixed cell population.

Acknowledgments. The authors thank Mrs Alison Oles for technical assistance and Mr Osman Kademoglu for photographic assistance.

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0014-4754/91/010075-03$1.50 + 0.20/0

The insect gut: A new source of ecdysiotropic peptides

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Summary. Proctodaea of European corn borer (*Ostrinia nubilalis*) and gypsy moth (*Lymantria dispar*) last instars (larvae) contain prothoracicotropic factors that stimulate the prothoracic glands (PGs) of the gypsy moth to produce both ecdysone and 3-dehydroecdysone (precursors to the insect molting hormone) in a dose-dependent manner. In a separate in vivo assay, injections of proctodaeal extracts into gypsy moth larvae that were head-ligated before the release of the molt-stimulating brain hormone, PTTH, resulted in a pupal molt.
Key words. PTTH; ecdysteroids; ketoreductase; proctodaeum; prothoracic glands.

Prothoracicotropic hormones (PTTHs) are important ecdysiotropic neurohormones that stimulate the insect prothoracic glands (PGs) to produce an ecdysteroid which is a precursor to the molting hormone, 20-hydroxyecdysone[1,2]. The only site of synthesis reported for PTTH is a pair of neurosecretory cells located in either the lateral or median protocerebrum of the brain[3–5]. In response to endogenous and/or exogenous cues, PTTH is released from its neurohemal organ[6] into the hemolymph and transported to the PGs. While it was previously thought that ecdysone was the only ecdysteroid secreted by the PGs[7,8], it is now known that PGs of the tobacco hornworm, *Manduca sexta*[9] and of the gypsy moth, *Lymantria dispar*[10], produce 3-dehydroecdysone, a precursor, which is then converted to ecdysone by a 3β-forming-3-ketoecdysteroid reductase (ketoreductase), and later by peripheral tissues to the physiologically active molting hormone, 20-hydroxyecdysone. Since Beck and his colleagues[11] reported the existence of a factor (proctodone) in the hindgut which stimulated the onset of development in diapausing ('hibernating') European corn borer (*Ostrinia nubilalis*) larvae, it was decided to examine larval proctodaeal extracts for the presence of ecdysiotropic factors. We now report that in addition to the brain, large amounts of ecdysiotropic factor(s) are present in the hindgut of *O. nubilalis*, and in the hindgut of *L. dispar*; and that these factors stimulate the production of both ecdysone and 3-dehydroecdysone by the PGs of *L. dispar*.

Proctodaea minus recta (pylorus and anterior intestine)[12] were dissected from laboratory-reared *O. nubilalis*[13] and *L. dispar* prepupae (last stage larvae in the process of, or having completed gut purge)[14], and placed in Grace's medium (GIBCO, Grand Island, NY) for 20–60 min prior to being frozen at -20 °C. When needed, proctodaea were thawed and homogenized in ice-cold Grace's medium. To remove tissue debris and large proteins, homogenates were boiled for 2.5 min, centrifuged at 16,000 × g and 4 °C (3 min), and the supernatants removed and stored on ice. A triple incubation in vitro PG assay, modified from previously described in vitro assays[15–19], was utilized to measure the ecdysiotropic activity in these extracts (fig. 1).

Results and discussion

The antibody (provided by W. E. Bollenbacher, University of North Carolina, Chapel Hill, and characterized by us [unpublished results]) used here for the RIA, detects ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, and 20,26-dihydroxyecdysone, but not 3-dehydroecdysone. Since the only RIA-detectable ecdysteroid produced by *L. dispar* PGs (with our antibody) is ecdysone[18], detectable ecdysteroid in 'G' or 'Gc' (fig. 1) was due to the production of ecdysone, while the detectable ecdysteroid in 'K' or 'Kc' (fig. 1) was due to the production of both ecdysone and 3-dehydroecdysone, the latter having been converted to ecdysone by the added ketoreductase.