THE QUANTITATIVE ESTIMATION OF PINOCYTOSIS USING RADIOACTIVE COLLOIDAL GOLD

PHILIP DAVIES, ANTHONY C. ALLISON and A. DAVID HASWELL

Division of Cell Pathology, Medical Research Council
Clinical Research Centre, Watford Road, Harrow,
Middlesex HA1 3UJ, England.

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SUMMARY

Radioactive colloidal gold has been used as a quantitative indicator of the pinocytic activity of mouse peritoneal macrophages. The uptake of label is both time and temperature dependent, accumulation being linearly related to time for several hours. Uptake is also dependent on the number of cells and concentration of the radioactive label. Increasing concentrations of newborn calf serum in the culture medium cause dramatic increases in pinocytosis without corresponding increases in protein levels in the cells.

INTRODUCTION

Increased endocytic activity has been shown to accompany a variety of cellular activation processes. These include lymphocyte transformation induced by plant lectins (1), the synthesis of acid hydrolases by murine macrophages cultured in media containing high concentrations of newborn calf serum (NBCS) (2) and the hydrolysis of colloid by thyroid cells after stimulation with thyroid-stimulating hormone (3). It has been established that the presence of a variety of positively charged molecules causes marked enhancement of endocytosis by tumour cells grown in tissue culture (4). On the other hand Cohn and Parks (5,6) have shown that macrophages show increased rates of endocytosis in the presence of various polyanions, adenosine and other adenine nucleotides. Since the rate of pinocytosis by the macrophage is so readily influenced by its extracellular environment, it is of interest to determine whether the activation of this cell type to kill parasites (7) and tumour cells by various agents (8) or to synthesize DNA and divide (9) induces changes in its pinocytic activity.

Colloidal gold has been shown to accumulate in macrophages from various

species (10,11), in L cells (12) and in lymphocytes (13). Electron microscopy shows that the gold is located in structures resembling secondary lysosomes (12,14) in a form which is not readily releasable (10,11). We now wish to show that mouse macrophages accumulate radioactive colloidal gold (^{198}Au) in a time- and temperature-dependent fashion, thus allowing the accurate quantitation of pinocytosis under a variety of conditions.

MATERIALS AND METHODS

Materials. Outbred Swiss mice were used in all experiments. These were either Tyler's Original strain from the Clinical Research Centre's breeding colony or LACA strain from SACI, London Road, Braintree, Essex. Medium 199 was obtained in powder form from Grand Island Biological Co., Grand Island, N.Y. NBCS was from Tissue Culture Services Ltd., Slough, Bucks. The serum was inactivated at 56°C for 30 minutes before use. Radioactive colloidal gold (particle size up to 20 nm, specific activity 4-12 mCi/mg Au) was obtained from The Radiochemical Centre, Amersham, Bucks.

Methods. Mice were killed with ether and cells were collected by irrigation of the peritoneal cavity with 3-5 ml M199 containing 10% NBCS. 5 ml aliquots of the cells were plated in 48 mm plastic Petri dishes (Nunc, Jobling Laboratories Division, Stone, Staffs.) at approximately 1 x 10^6 cells per ml. After removal of non-adherent cells the cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

^{198}Au was diluted to required activity with culture medium. Additions to the cultures were made in one of two ways (a) 50 µl of the radioactive label was added to cultures and vigorously agitated to ensure good mixing, or (b) the culture medium was aspirated and replaced with 5 ml medium containing the appropriate amount of radioisotope. All experiments were carried out with amounts of radioactivity ranging from 0.1 to 1.0 µC/ml of culture medium. Cultures were subsequently incubated for varying times at 37°C in a CO_2-air atmosphere. Controls were included where cultures were placed in sandwich boxes seated on ice and thoroughly flushed with 5% CO_2 in air prior to being sealed and placed in the cold room at 4°C.

At the end of a given incubation period the culture medium, which still contained the bulk of the radioisotope, was removed and the cell sheet washed four times with 4 ml of warm phosphate-buffered saline. Repeated observation showed that the fourth wash contained radioactive counts which were close to background levels. The cell sheet containing incorporated label was removed by the addition of 2.5 ml 0.9% w/v NaCl containing 0.1% v/v Triton X-100 followed by rubbing with silicone bungs. The released cells were transferred to test tubes for counting in a Wallac gamma counter.

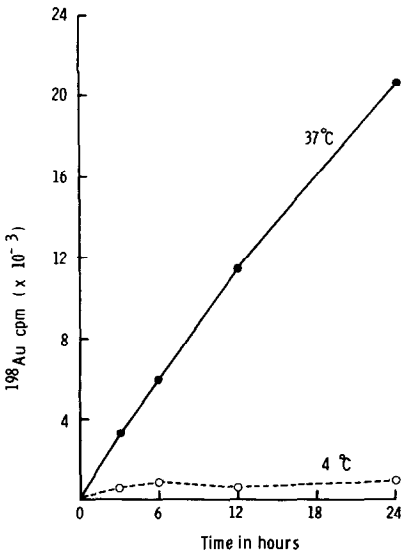
Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Cells were counted optically after fixation in 2.5% v/v glutaraldehyde Selected areas were counted using an eyepiece graticule and stage micrometer.

RESULTS AND DISCUSSION

Fig. 1 shows that adherent mouse peritoneal cells take up ^{198}Au in a time- and temperature-dependent manner over a period of 24 hours. The cells used in this experiment had been in culture for five days before adding the radioactive label, so that they were fully adapted to their culture conditions. In other experiments where cells had been in culture for only 36 hours before the addition of label ^{198}Au uptake was linearly related to time for less than 10 hours. When this technique is used for quantitation of pinocytosis (see below) it is therefore important to establish that uptake of label is linearly related to time during the duration of the experiment.

The uptake of ^{198}Au is also linearly related to the number of cells present in the culture (Fig. 2). It is noticeable that the counts obtained at $4^{\circ}C$ do not increase in relation to cell number. This is explained by the fact that counts obtained at $4^{\circ}C$ are derived from at least three possible sources (a) gold attached to the thin layer of protein that covers the culture dishes as described for horseradish peroxidase by Steinman and Cohn (16), (b) gold attached to the surface of macrophages, (c) gold taken up by the cells by pinocytic mechanisms operative at low temperatures as described by Casley-Smith (17).



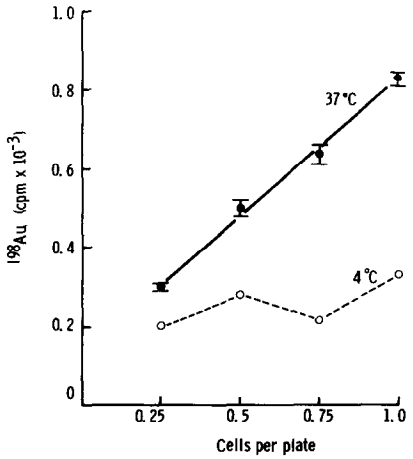


Fig. 1. The dependence of ^{198}Au uptake on time and temperature. Macrophages were cultured for 5 days in M199 + 50% NBCS prior to addition of 0.5 µC ^{198}Au per plate.

Fig. 2. The dependence of ^{198}Au uptake on cell number. Cells were collected at approximately 1 x 10^6/ml and diluted appropriately with medium prior to plating. After initial washing they were maintained in culture for 36 hours when the medium was removed and replaced with M199 + 50% NBCS containing 0.2 µC/ml ^{198}Au. Uptake was measured over a period of 3 hours. Each point represents the mean $\pm$ standard deviation of 4 values.

Increasing the concentration of radioactive label resulted in a proportionate increase in radioactivity incorporated into cells at 37°C (Table 1).

Cohn and his collaborators (18) have demonstrated that mouse macrophages respond to high concentrations of NBCS in their culture medium by forming more pinocytic vesicles and lysosomal enzymes. Fig. 3 shows that macrophages cultured in medium containing low NBCS concentration take up only small amounts of label. However as NBCS concentrations increase the rate of pinocytosis also increases dramatically. It is seen that the increases in pinocytic activity are not accompanied by corresponding increases in cell protein

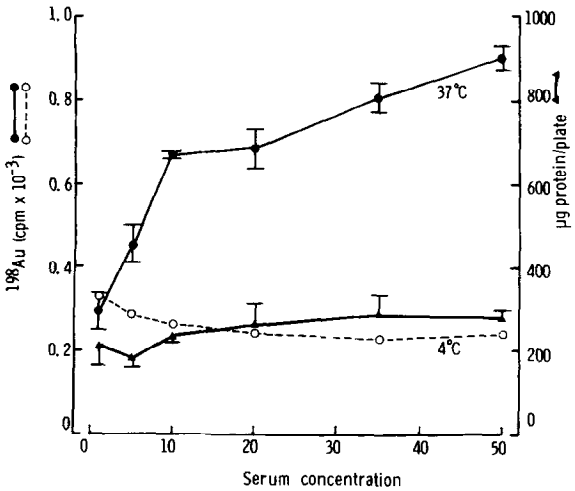


Fig. 3. The influence of the concentration of NBCS in the culture medium on the uptake of ^{198}Au by macrophages. The cells were initially cultured in M199 + 10% NBCS and then changed to M199 containing appropriate amounts of NBCS 18 hours prior to adding ^{198}Au. All cultures were exposed to 1 µC ^{198}Au/ plate for 3 hours. Each point represents the mean ± standard deviation of 4 values.

levels. This is to be expected since Cohn and Benson (2) showed that the response of the macrophage to increased pinocytosis was selective, as reflected by large increases in the specific activity of acid hydrolases.

It has been shown by others (10,11) that colloidal gold is quantitatively retained by macrophages after pinocytosis, and our recent work (19) demonstrated that macrophages preincubated with ^{198}Au retained more than 95% of the incorporated label over a subsequent 72 hour incubation period in a label-free medium. Since we have also found that uptake of label is linearly related to time it should be possible to use ^{198}Au as an indicator of pinocytosis. Table 2 shows the results of an experiment where the rate of pinocytosis by macrophages cultured in M199 containing either 10% or 50% NBCS has been determined. The values obtained were based on the following assumptions (a) the cell sheet occupies a negligible fraction of the total volume of the culture, (b) the label is freely suspended in the culture

Table 1. The effect of increasing ^{198}Au concentration on uptake of label. Cells were cultured in M199 + 50% NBCS. Plates were incubated with increasing concentrations of ^{198}Au for 4 hours at 37°C or 4°C. Each value represents the mean ± standard deviation for five determinations.

Concentration ^{198}Au (μC/plate)	Uptake of label (cpm ^{198}Au)	
	37°C	4°C
0.5	1,027 ± 32	249
1.0	1,860 ± 85	295
2.5	4,939 ± 257	498
5.0	11,727 ± 350	1,197

medium, (c) that the label does not become attached to and concentrated by the membranes which envelop endocytic vacuoles. The last assumption is supported by Table 1 which shows the linear increase in uptake of label associated with increasing ^{198}Au concentrations in the culture medium. Moreover it has been shown by electron microscopy (19) that only occasional gold particles are associated with the plasma membranes of macrophages exposed to colloidal gold.

It is seen that cells cultured in 50% NBCS have approximately double the pinocytic activity of those in 10% NBCS which is in good agreement with the values given in Fig. 3. The values in Table 2 are in excess of those obtained for soluble markers of pinocytosis such as horseradish peroxidase (14), rabbit haemoglobin (20) and human serum albumin (21). The reasons for this is not clear; the colloidal nature of ^{198}Au may in some way influence its rate of uptake, and the amount of natural anti-mouse macrophage macroglobulin in the NBCS can greatly alter the rates of pinocytosis. There is strong evidence that ^{198}Au uptake does occur by micropinocytosis since its accumulation within macrophages is resistant to cytochalasin B (22), which has been shown to inhibit completely macropinocytosis visible by light

Table 2. The rate of pinocytosis of ^{198}Au by macrophages grown in M199 containing 10% or 50% NBCS. Plates were incubated for 2 or 4 hours at 37°C or 4°C with 1 μC ^{198}Au/plate. At the end of the experiment the medium and washes were retained for determination of the total amount of radioactivity per plate. Counts in cells represent the mean values obtained on five plates at 37°C corrected for counts obtained at 4°C

	Concentration NBCS			
	10%		50%	
	2 hrs	4 hrs	2 hrs	4 hrs
Counts in cells (cpm)	990	2,017	2,266	3,700
Total counts (cpm)	633,532	630,214	629,958	606,054
Volume of medium (ml) taken up by 10^6 cells/hr	0.0039	0.00400	0.0089	0.0076
% total medium taken up by 10^6 cells/hr	0.0682	0.0698	0.1570	0.1328

microscopy in macrophages (23). It is well known that gold inhibits lysosomal hydrolases (24) and causes cell damage (12). However these effects only occur at levels of gold well in excess of those used in our experiments, in which no cytotoxic effects were detectable.

It is therefore clear that ^{198}Au can be used as a sensitive, quantitative indicator of pinocytosis in macrophages over intervals of several hours. This is facilitated by the retention of ^{198}Au in secondary lysosomes over long periods of time, unlike soluble markers of pinocytosis which are broken down enzymatically and released from the cells. A marker such as gold will be useful for evaluating the role of pinocytosis in the response of the macrophage to physiological and pathological changes in its environment. Moreover, preliminary experiments in our laboratory (Tolnai, S., Davies, P. and Allison, A.C., unpublished work) have demonstrated that ^{198}Au can be used to measure pinocytosis by L cells.

REFERENCES

1. Biberfeld, P., J. Ultrastruc. Res., 37, 41 (1971).
2. Cohn, Z.A. and Benson, B., J. exp. Med., 121, 835 (1965).
3. Wetzel, B.K., Spicer, S.S. and Wollman, S.H., J. Cell Biol., 25, 593 (1965).
4. Ryser, H.J-P., Bull. Swiss Acad. Med. Sci., 24, 363 (1968).
5. Cohn, Z.A. and Parks, E., J. exp. Med., 125, 213 (1967).
6. Cohn, Z.A. and Parks, E., J. exp. Med., 125, 457 (1967).
7. Mackaness, G.B., J. infect. Dis., 123, 439 (1971).
8. Alexander, P. and Evans, R., Nature New Biol., 232, 76 (1971).
9. Mauel, J. and Defendi, V., Exp. Cell Res., 65, 377 (1971).
10. Gosselin, R.E., J. gen. Physiol., 39, 625 (1956).
11. Cohn, Z.A. and Benson, B., J. exp. Med., 122, 455 (1965).
12. Herman, M.M., Bensch, K.G., Marich, K.W. and Glick, D., Exp. Mol. Path., 16, 186 (1972).
13. Cahill, R.N.P., Virch. Archiv. Abt. B. Zellpathol., 7, 174 (1971).
14. Cohn, Z.A. and Fedorko, M.E. in "Lysosomes in Biology and Pathology", J.T. Dingle and H.B. Fell, eds., North Holland Publishing Co. Ltd., 1969, vol. 1, p.43.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., 193, 265 (1951).
16. Steinman, R.M. and Cohn, Z.A., J. Cell Biol., 55, 186 (1972).
17. Casley-Smith, J.R., J. Microscop., 90, 15 (1969).
18. Cohn, Z.A., Adv. Immun., 9, 163 (1968).
19. Davies, P., Allison, A.C. and Haswell, A.D., Biochem. J., In press.
20. Ehrenreich, B.A. and Cohn, Z.A., J. Cell Biol., 38, 244 (1968).
21. Ehrenreich, B.A. and Cohn, Z.A., J. exp. Med., 126, 941 (1967).
22. Wills, E.D., Davies, P., Allison, A.C. and Haswell, A.D., Nature New Biol., 240, 59 (1972).
23. Allison, A.C., Davies, P. and de Petris, S., Nature New Biol., 232, 153 (1971).
24. Ennis, R.S., Granda, J.L. and Posner, A.S., Arth. Rheum., 11, 756 (1968).