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Comparison of protein-synthesis rate of alveolar macrophages *in vivo* and *in vitro*

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This paper describes and validates a novel method for measuring rates of protein synthesis of rabbit alveolar macrophages *in vivo*. A rate of 9.3%/day was obtained, compared with 48.9%/day measured *in vitro*. This study suggests that the procedures involved in the isolation of alveolar macrophages for study *in vitro* may themselves activate the cell.

Alveolar macrophages (AMs) can now be readily obtained from the lungs of both animals and humans by bronchoalveolar lavage (Hunninghake *et al.*, 1979). These cells are increasingly the subject of study in normal and in diseased animals and humans. Although the steps involved in the isolation of these cells are relatively simple, AMs are known to be 'activated' by surface stimuli, and it seems possible that the manipulations involved in lavage and washing the cells prior to study *in vitro* might be sufficient to activate the cells. There are many measures *in vitro* of macrophage activation, but most cannot be applied to AMs *in vivo*. The measurement of protein-synthesis rate of AMs is, as far as we are aware, the only measure of activation of AMs accessible both *in vitro* and *in vivo*.

In the present study we have developed methods of measuring the rate of protein synthesis of normal rabbit AMs *in vivo* by using L-[5-^{3}H]proline as a labelled precursor, and compared this with a rate measured *in vitro* by standard techniques. We obtain a rate *in vitro* that is 5-fold higher than *in vivo*. It is probable that AMs obtained by lavage and subsequent centrifugation are already partially activated.

Experimental

Alveolar macrophages

New Zealand White male rabbits (body wt. 1.7–2.3kg), supplied by HOP Laboratories, Cork Farm, Chilham, Kent, U.K., were killed with intravenous sodium pentobarbitone and intubated via a tracheotomy. The lungs were lavaged with three 30ml aliquots of 0.15M-NaCl at room temperature, and the returns collected in a silicone-treated glass bottle precooled in ice. Lavage cells were maintained at 4°C thereafter until either culture or addition of trichloroacetic acid. Blood was obtained by cardiac puncture and collected into heparinized tubes.

After lavage, cells were separated from lavage fluid by centrifugation (100*g*, 10min) and then washed three times by resuspension in phosphate-buffered saline and re-centrifugation. Some lavage returns were slightly contaminated by blood; if so, the cells were suspended in phosphate-buffered saline, layered on to Ficoll/sodium metrizoate ($d = 1.077$) (Lymphoprep; Nyegaard Ltd., Oslo, Norway) and centrifuged at 400*g* for 10min. The nucleated cells were collected from the interface with a silicone-treated Pasteur pipette and washed twice in phosphate-buffered saline. Macrophages, identified by May–Grunewald–Giemsa and non-specific esterase stains, represented at least 96% of nucleated cells in all experiments.

Protein synthesis in vivo

Animals, in groups of four, received intravenous injections of L-[5-^{3}H]proline (Amersham International) (dose: 0.6–2.4mCi/kg body wt.), mixed with unlabelled L-proline (7mmol/kg body wt.) at intervals of 30, 60 and 120min before they were killed. The time of incorporation was taken from the start of the injection of proline until the instillation of the first aliquot of lavage saline.

At the end of the washing steps detailed above, the cell pellet was mixed with 5% (w/v) trichloroacetic acid. The resulting precipitate was washed

Abbreviations used: AM(s), alveolar macrophage(s); phosphate-buffered saline, 137mM-NaCl/2.6mM-KCl/8.1mM-Na_2HPO_4/1.5mM-KH_2PO_4; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

three times in 5% (w/v) trichloroacetic acid by centrifugation, and then successively in acetone/12M-HCl (400:1, v/v), ethanol/diethyl ether (2:1, v/v) and diethyl ether, before drying and hydrolysis in 6M-HCl (110°C, 16h).

Free proline was extracted from both the lavage fluid, after removal of cells as described above, and the plasma. The lavage fluid was freeze-dried, and was redissolved in 3ml of water before mixing with 3ml of 10% (w/v) trichloroacetic acid. For both samples the acid-soluble fraction was obtained by centrifugation (600*g*, 10min).

For each animal the specific radioactivities of proline in AM protein hydrolysate, and free in lavage fluid and plasma, were measured by a chloramine-T oxidation method (Stegemann, 1958) with recently described modifications (Laurent *et al.*, 1982).

The protein-synthesis rate was calculated as described previously (McNurlan *et al.*, 1979; Laurent, 1982) from the equation:

$$\text{Fractional protein-synthesis rate (\%/day)} = \frac{\text{specific radioactivity of amino acid incorporated in protein} \times 100}{\text{specific radioactivity of amino acid in precursor pool} \times \text{time (days)}}$$

The precursor-pool specific radioactivity was taken to be that of the free proline in the cell-free lavage fluid.

Protein synthesis in vitro

AMs were obtained from three rabbits, as described above, using sterile equipment throughout. Cell viability was assessed by Trypan Blue exclusion, and was 97% prior to, and 86% at the end of, the culture period. The cells were pooled and incubated in suspension in silicone-treated 5ml glass bijou bottles with loose-fitting caps while being shaken at 80 strokes/min in a water bath at 37°C in CO_2/air (1:19). Each culture contained 1.6×10^6 cells in 700μl of Krebs–Henseleit (1932) bicarbonate buffer with the following additions: glucose, 10mM; benzylpenicillin, 100 units/ml; normal rabbit plasma concentrations of 18 amino acids (Block & Hubbard, 1962); phenylalanine, 345μM (this is five times the physiological concentration) with L-[4-^{3}H]phenylalanine (Amersham) to a specific radioactivity of 100Ci/mol; L-[5-^{3}H]proline to a specific radioactivity of 100Ci/mol and various proline concentrations (detailed below). In addition, some cultures contained bovine serum albumin (fraction V; Sigma). Each set of cultures was set up in quadruplicate flasks.

At the end of a 2h incorporation period, metabolic activity was stopped by rapid cooling to 0°C, the AMs separated by centrifugation (100*g*, 10min) and washed by centrifugation three times with phosphate-buffered saline, before addition of 5% (w/v) trichloroacetic acid. Washing of the resulting precipitate and acid hydrolysis was as described for experiments *in vitro*. The specific radioactivities of [^{3}H]proline and [^{3}H]phenylalanine in the protein hydrolysate and the culture medium was measured by using a [^{14}C]dansyl chloride double-isotope method exactly as described by Airhart *et al.* (1979). The higher sensitivity of this method as compared with chloramine-T-oxidation assay was required because of the small number of cells in each culture *in vitro*. The specific radioactivities of the [^{14}C]dansyl chloride allowed to react with the amino acid samples were 12200d.p.m./nmol (culture medium) and 1030d.p.m./mol (macrophage AM protein hydrolysate). The quantity of [^{14}C]dansyl amino acid derived from AM protein spotted on to the polyamide sheets contained approx. 4nmol of dansyl-proline and 1.5nmol of dansyl-phenylalanine. After chromatography the spots of these two dansyl amino acids were readily identified, well separated from the other dansyl amino acids (although, at this high loading, resolution of some dansyl amino acids was not complete). The spots were cut out with scissors, as were corresponding regions of the polyamide sheets from reagent blanks put through the assay. Each 'spot' was placed in a glass scintillation vial containing 0.5ml of NCS tissue solubilizer (Amersham International). After 1h, 17μl of 16M-acetic acid, followed by 10ml of FisoFluor scintillation cocktail (Fisons) were added to each vial, and radioactivity was measured in a Packard liquid-scintillation spectrometer, model 3380, with external standardization and automatic correction for spill-over from the ^{14}C channel to the ^{3}H channel. After subtraction of the radioactivity in the spots obtained from the reagent blank ([^{3}H]phenylalanine, 90d.p.m.; [^{3}H]proline, 100d.p.m.; and [^{14}C]dansyl chloride, 20d.p.m.), the ratio of ^{3}H to ^{14}C radioactivity was calculated for each spot, and from this and the known [^{14}C]dansyl chloride specific radioactivity, the corresponding [^{3}H]proline or [^{3}H]phenylalanine specific radioactivity could be obtained. The protein-synthesis rate was calculated, as described above, by assuming the specific radioactivity of the amino acids in the culture medium to be that of the precursor pool.

Results and discussion

Alveolar-macrophage protein-synthesis rates

Rates of macrophage protein synthesis were calculated both *in vivo* and *in vitro* on the basis of the incorporation of [^{3}H]proline over 2h. The rate obtained *in vivo* after injection of radioactivity with a large amount (7mmol/kg body wt.) of unlabelled proline was 9.3 ± 1.5 (S.E.M., $n = 4$) %/day, compared with a rate of 48.9 ± 0.5 ($n = 4$) %/day obtained *in vitro*, again in the presence of high levels of unlabelled proline (concn. in medium, 4mM). This fivefold difference between the rates *in vivo* and *in vitro* suggests that AMs may be activated by the steps involved in their isolation for studies *in vitro*. However, the validity of this conclusion is dependent on the accuracy of the determined rates and thus on various assumptions associated with their calculation. The experimental basis for the assumptions involved for the study *in vivo* will be discussed fully, as this approach has not been used previously.

Assumptions implicit in the calculation of protein-synthesis rate in vivo

The assumptions are (1) that the specific radioactivity of proline in plasma is constant during the period of observation, and that equilibration between the plasma and alveolar fluid, and hence into the AM, is rapid, (2) that high proline concentrations do not affect protein synthesis, and (3) that it is reasonable to equate alveolar fluid proline specific radioactivity with that of the precursor pool for AM protein synthesis, i.e. prolyl-tRNA in the AM.

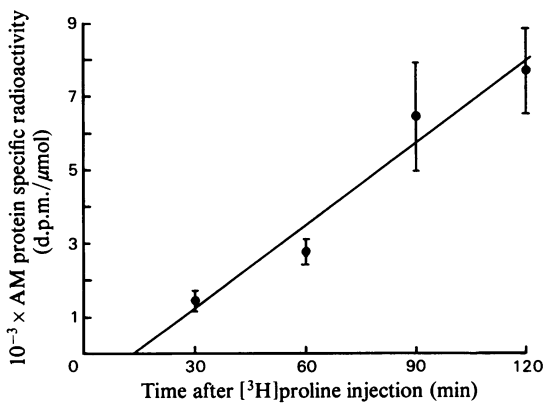

Fig. 1. *Time course of incorporation in vivo of [^{3}H]proline into alveolar-macrophage protein*

The specific radioactivity of macrophage-protein proline is normalized to an infused proline specific radioactivity of 10^6 d.p.m./μmol. Error bars represent ±S.E.M. for groups of four animals, and the line was drawn by least-squares regression analysis.

(1) The use of a large 'flooding' dose of injected proline ensures that assumption (1) holds. This method has been validated in previous studies of protein synthesis in various rabbit tissues, including lung (Laurent, 1982; Laurent & McAnulty, 1983). In the present study, the proline must in additio pass rapidly into alveolar fluid (which bathes the AM), achieving 'flooding' levels of proline to ensure a constant precursor-pool specific radioactivity in the AM.

To examine this question we calculated the ratio of proline specific radioactivity free in lavage fluid (which has sampled alveolar fluid) and in plasma, for the animals 30, 60, 90 and 120min after [^{3}H]proline injection. The means (±S.E.M.) of these ratios for each group of animals were respectively 0.84 ± 0.08, 0.92 ± 0.08, 0.86 ± 0.06 and 0.97 ± 0.06. There is no significant difference between these ratios (one-way analysis of variance, $P > 0.05$), confirming rapid passage of proline into the alveolar fluid. Fig. 1 shows that the incorporation of [^{3}H]proline into AM protein is linear with time, confirming that the proline precursor pool for protein synthesis is of constant specific radioactivity. The intercept of the least-squares regression line suggests that incorporation of [^{3}H]proline starts at 13min. This apparent delay in incorporation may be due to a finite time for equilibration of proline between the various free pools. However, accepting the observed delay of 13min, this period is still short compared with the total incorporation time, and correction by this amount does not substantially alter the rates obtained (10.4%/day versus 9.5%/day). We were not able to measure alveolar fluid proline concentration directly because the 90ml of lavage saline diluted an unknown volume of alveolar fluid. This was also made difficult because in controls (which did not receive injected proline), the lavage returns contained amounts of proline which were at the

Table 1. *Effect of various extracellular proline and albumin concentrations on [^{3}H]proline and [^{3}H]phenylalanine incorporation into proteins of cultured macrophages*

The specific radioactivity of both amino acids was constant under all conditions used (see the Experimental section). Values are means ±S.E.M. for four determinations.

[Proline] (μM)	[Albumin] (g/litre)	Protein specific radioactivity (d.p.m./nmol) [^{3}H]Proline	[^{3}H]Phenylalanine
100	0	3616±48	8997±186
1500	0	8216±138	8797±88
4000	0	8361±89	8444±143
1500	6	8476±91	9141±58
1500	20	7594±105	8859±166

lower limits of the assay employed. However, the total proline content of the lavage fluid returns of controls was less than 40nmol, compared with a mean of 1800 ± 640 (S.E.M., $n = 4$) nmol in the group of animals killed at 30min after injection. This, together with results discussed above, is further evidence supporting our contention that passage of proline into alveolar fluid was rapid and sufficient to achieve high 'flooding' levels of the amino acid for the duration of the experiment.

(2) The second assumption, that high proline concentrations do not affect protein synthesis, has been discussed previously (Laurent, 1982). We have further examined this question in the present study by using techniques *in vitro*. To do this, [^{3}H]phenylalanine was included in the culture medium at constant concentration and specific radioactivity to provide a measure of protein synthesis independent of variations in intracellular proline pool concentrations and specific radioactivities. Table 1 shows that [^{3}H]phenylalanine incorporation was unaffected by proline concentrations over the range 100–4000μM (one-way analysis of variance, $P > 0.05$). This range includes the proline concentration in plasma for normal rabbits (about 200μM) and the concentration 2h after injection of a flooding dose of proline (about 3000μM; Laurent 1982).

(3) Our assumption that when flooding doses of proline are used the specific radioactivities of extracellular (i.e. alveolar fluid) proline and AM prolyl-tRNA *in vivo* are equal is supported by recent work. Studies carried out *in vivo* on heart (Everett *et al.*, 1981), and on perfused lung (Watkins & Rannels, 1980) and perfused liver (Khairallah & Mortimore, 1976) have indicated that, when high doses of amino acid are used, the specific radioactivities of extracellular amino acid and aminoacyl-tRNA become equal. In addition, one of us (Laurent, 1982) used the same protocol of [^{3}H]proline administration *in vivo* and demonstrated that the specific radioactivity of proline in skin procollagen (which has a half-life of less than 30min) equalled that of the tissue free pool, a result implying that the prolyl-tRNA of skin fibroblasts under these conditions also achieved a specific radioactivity equal to that of the tissue free pool. Unfortunately, direct measurement of the prolyl-tRNA specific radioactivity of AMs *in vivo* is precluded by the rapid turnover of the rRNA pool when compared with the time required to isolate and process the AMs and also by the need to use prohibitively large amounts of [^{3}H]proline to achieve a measurable prolyl-tRNA specific radioactivity.

Notwithstanding the results discussed above, there is evidence obtained *in vitro* that suggests potential problems which may affect calculations *in vivo*. One is that exogenous protein can act as a source of amino acid for protein synthesis in AMs (Rannels *et al.*, 1982). In the alveolus, macrophages are bathed in a fluid containing protein, predominantly albumin, at an unknown concentration, although very probably much lower than that in plasma. This exogenous protein, because it does not contain labelled amino acid, might lower the precursor-pool specific radioactivity in the cell, leading to an erroneously low measurement of protein-synthesis rate *in vivo*. To assess this problem, cells were incubated in various concentrations of bovine serum albumin and [^{3}H]proline incorporation measured. The results are shown in Table 1. At an albumin concentration of 20g/litre, there was a significant ($0.01 < P < 0.02$, Student's t test) but small fall in [^{3}H]proline incorporation, but this was not apparent with 6g of albumin/litre. This effect is unlikely, therefore, to bias significantly a comparison of protein-synthesis rates *in vivo* and *in vitro*.

A potentially more profound methodological problem in the use of radiolabelled amino acids to measure protein-synthesis rates is illustrated by recent work *in vitro* on lung fibroblasts (Hildebran *et al.*, 1981) and guinea-pig AMs (Airhart *et al.*, 1981). At physiological levels, an increase in extracellular amino acid concentration at constant specific radioactivity will increase ^{3}H-labelled-amino acid incorporation (and therefore the apparent protein-synthesis rate) via an increase in aminoacyl-tRNA specific radioactivity. At high extracellular amino acid concentration, aminoacyl-tRNA specific radioactivity reaches a plateau, where it is unaffected by changes in the extracellular amino acid concentration. This effect is demonstrated in Table 1, where it can be seen that [^{3}H]proline incorporation rises when the proline concentration in the medium is raised from 100mM to 1500mM, but does not change again when the latter increased to 4000mM. Our comparison is made at extracellular proline concentrations in excess of 1500mM, i.e. in the plateau region.

Although aminoacyl-tRNA specific radioactivity does reach a plateau at high extracellular amino acid concentrations *in vitro*, it still may be less than the specific radioactivity of the extracellular amino acid (Hildebran *et al.*, 1981; Hammer & Rannels, (1981). This contrasts with the situation *in vivo* outlined above, where flooding doses of amino acid seem to be successful in equalizing the specific radioactivities of the two pools. Thus rates *in vivo* are likely to be unbiased, whereas rates *in vitro* may be underestimated because of this effect, which would amplify the difference reported in the present study.

The present study is the first to examine

macrophage protein metabolism *in vivo*. We obtained a protein fractional synthesis rate of 9.3%/day. This contrasts with the value of 35%/day for total lung protein measured in rabbits (Laurent, 1982), and suggests that AMs are metabolically relatively inert in the normal lung. When AMs were cultured in suspension *in vitro* we obtained a rate of 48.9%/day. This is very similar to the value of 50.7%/day obtained by Hammer & Rannels (1981) with New Zealand White rabbits and similar experimental conditions, except that phenylalanine was the radioactive precursor amino acid. When corrected for phenylalanine-tRNA specific radioactivity (which was 59.9% of that of culture-medium phenylalanine), their rate became 86%/day. The rate we have obtained *in vitro* should therefore be considered a minimum.

We have found at least a fivefold difference between the fractional synthesis rate obtained *in vitro* and that obtained *in vivo*. It therefore seems likely that, in the lungs of normal animals, the AM is in a quiescent state with a low synthetic rate *in vivo*, and that obtaining the cells by bronchoalveolar lavage and low-speed centrifugation is sufficient to activate them partially to a state with a substantially higher rate of protein synthesis. These results should be borne in mind when extrapolating observations made *in vitro* to the situation in the intact animal.

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