FATE OF HEMOGLOBIN PINCYTOSED BY MACROPHAGES IN VITRO

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Cultivated mouse peritoneal macrophages provide a convenient in vitro system for the study of pinocytosis. Homogeneous cell populations are easily collected and can be cultivated in a manner suitable for obtaining morphological or biochemical data. Recent studies of peritoneal macrophages in vitro have clarified many aspects of the physiology of pinocytosis, particularly its relationship to the formation of lysosomes (3–8). When cultivated in the presence of serum, macrophages pinocytose continually. Pinocytic vesicles migrate to the perinuclear region of the cell, where they acquire acid hydrolases to become secondary lysosomes. Both endogenous hydrolases and exogenous macromolecules can be demonstrated in macrophage lysosomes, which are undoubtedly the major site of intracellular digestion. This study and an earlier one were undertaken for a determination of the extent of intracellular digestion of pinocytosed proteins and the fate of the products of digestion.

In the previous study we employed an external label, radioactive iodine, to follow the fate of pinocytosed human serum albumin (HSA) (9). When cultivated in the presence of HSA-^{125}I, macrophages accumulated isotope from the medium. There is considerable evidence that pinocytosis was the mechanism of uptake in these experiments: (*a*) the amount of isotope taken up under various conditions correlated well with the cells' level of pinocytic activity, and (*b*) radioautography showed that intracellular isotope was localized in perinuclear granules, or lysosomes. After a pulse of HSA-^{125}I and transfer to isotope-free medium, macrophages lost intracellular label which appeared as acid-soluble isotope in

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the medium. The radioactive material excreted by pulsed macrophages was identified as iodotyrosine. Recently Gabathuler and Ryser have reported that cultured sarcoma cells also degrade ingested ^{131}I-labeled albumin to iodotyrosine (10).

These results suggested that macrophages digest pinocytosed HSA-^{125}I to the level of amino acids. However, the results actually concerned only the fate of the labeled portions of HSA-^{125}I, peptides containing iodotyrosine. It was possible that only the labeled peptides had undergone complete hydrolysis and excretion, particularly since iodotyrosine is an unnatural amino acid and cannot be utilized by cells (12). It seemed worthwhile, then, to study the fate of a protein containing a different radioactive amino acid, preferably one which would be chemically indistinguishable from a normal cellular metabolite. Hemoglobin was chosen for study since it can be labeled "internally" with leucine-^{3}H or "externally" with ^{125}I.

Mouse peritoneal macrophages were harvested, cultivated and prepared for radioactivity measurements as described previously (9). For all experiments the cells were cultivated as monolayers in 30-cm^2 T flasks in medium consisting of newborn calf serum (nbcs) and medium No. 199. 50% nbcs medium containing the labeled protein was used for studies of protein uptake. In all experiments involving leucine-^{3}H–labeled hemoglobin (leu-^{3}H–Hb), cold leucine was added to the medium at a concentration of 2 mg/ml. This concentration of leucine was adequate to prevent utilization of any leucine-^{3}H which was present in the leu-^{3}H–Hb preparation or which arose from leu-^{3}H–Hb degradation. Labeled protein solutions were sterilized by Millipore filtration and used within a few days of preparation.

Hemoglobin for iodination was obtained from lysates of washed rabbit erythrocytes after removal of the stromata by centrifugation. The hemoglobin solution was dialyzed overnight and iodinated, according to the method of Helmkamp et al. (11), to a specific activity of about 0.005 mc/mg. We made no attempt to remove any possible nonheme proteins from the Hb-^{125}I preparations. To prepare internally labeled hemoglobin, we obtained reticulocytes from phenylhydrazine-treated rabbits according to the procedure of Borsook et al. (1). The procedure for incubation of the reticulocytes was adapted from that described by Burka and Marks (2). Washed reticulocytes were suspended in 6 volumes of medium containing 1 mc leucine-^{3}H (5 c/mmole) and 1 volume rabbit plasma. After an hour's incubation the cells were washed five times and

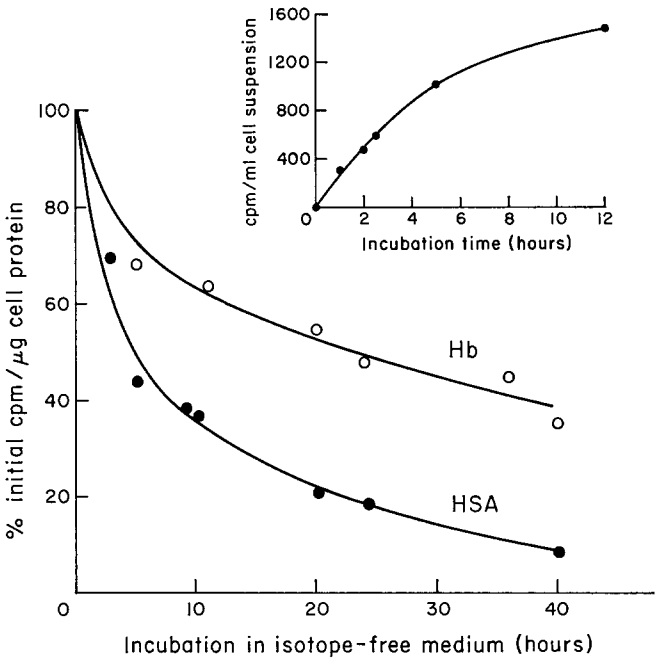

FIGURE 1 The loss of isotope from cells pulsed with HSA-^{125}I and Hb-^{125}I. Prior to counting, the cells were washed six times with saline, then detached from the glass surface by repeated freezing and thawing in 3 ml of distilled water. 1 ml aliquots of the resulting cell suspensions were counted in a well counter. The insert shows the uptake of Hb-^{125}I by cells cultured in 50% serum containing 10^6 cpm/ml Hb-^{125}I.

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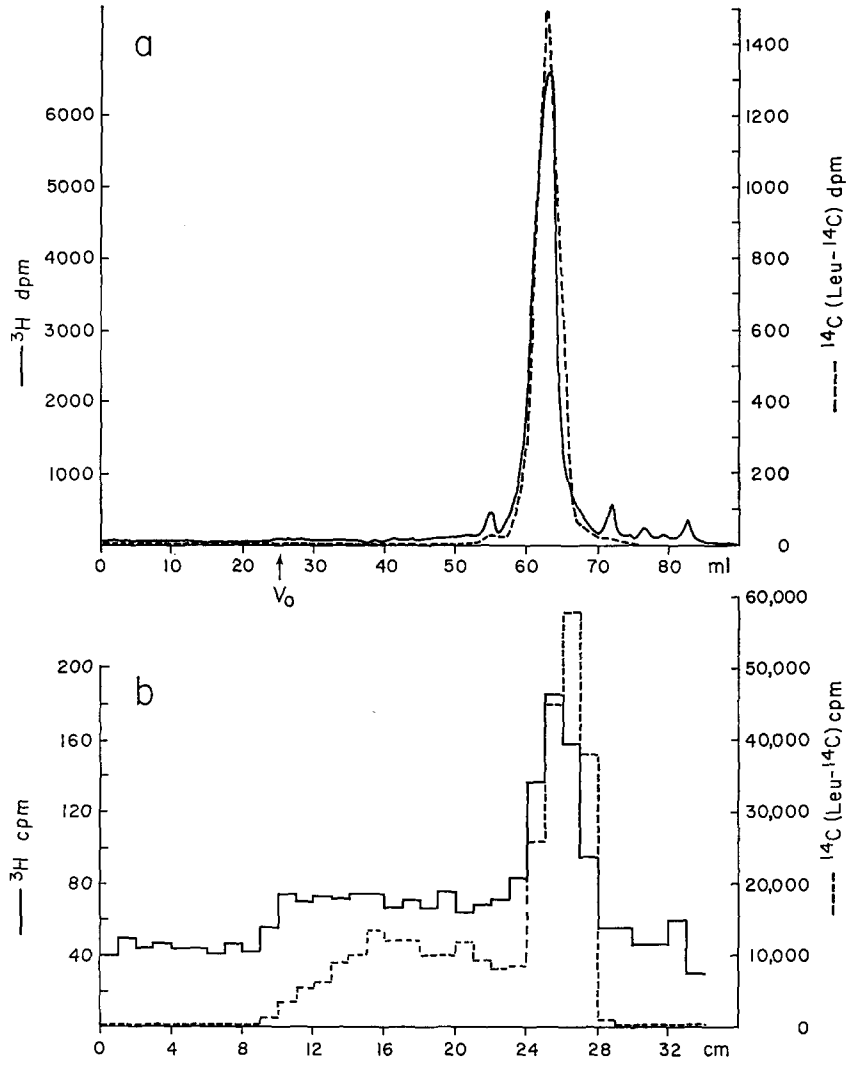

FIGURE 2 Chromatographic analysis of the trichloroacetic acid–soluble fraction of washout media. The cells in six T flasks were pulsed with leu-^{3}H–Hb, washed and reincubated in 1% serum. After 24 hr the media were removed, pooled, and precipitated with cold 10% trichloroacetic acid. The trichloroacetic acid supernatant was extracted with ether for removal of trichloroacetic acid and concentrated prior to chromatography. In both types of chromatography, the ^{14}C counts are from leucine-^{14}C, used as a reference compound, and the ^{3}H counts are from the washout media. *a*, Chromatography on Biogel P-2 (exclusion limit of molecular weight 1600) with 0.05 M phosphate buffer, pH 6.8. The void volume (V_o) is indicated. *b*, Paper chromatogram on Whatman No. 1 paper, in butanol: acetic acid:water (*50:12:50*), run for 16 hr. The paper was dried and cut into strips for counting. The presence in the sample of the acid-soluble components of medium No. 199 accounts for the tailing observed in this chromatogram.

then lysed with distilled water. The lysate was centrifuged for 1 hr at 10,000 *g* and dialyzed overnight. The specific activity of the resulting leu-^{3}H–Hb ranged from 0.005–0.02 mc/mg. In all preparations, less than 1% of the radioactivity was soluble in cold 10% trichloroacetic acid. Chromatography of the leu-^{3}H–Hb on Sephadex G-75 showed that tritium activity and heme absorption were eluted in a single peak. Prior to scintillation counting, leu-^{3}H–Hb was

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decolorized by extracting the heme in acetone containing 5×10^{-4} M HCl. The globin was sedimented, washed twice with acetone, dried, and dissolved in water. The resulting globin solution was counted directly in scintillation fluid.

The uptake and turnover of Hb-^{125}I were first investigated and compared to previous results for HSA-^{125}I (9). As with HSA-^{125}I, maximal labeling of the cells with Hb-^{125}I occurred after about 20 hr of cultivation in 50% nbcs. Then the amount of intracellular isotope corresponded to about 10^{-3} μg Hb per microgram cell protein, as compared to about 3×10^{-4} μg HSA per microgram cell protein, under similar culture conditions. For a study of the fate of ingested Hb, cells were "pulsed" by 24-hr incubation in medium containing Hb-^{125}I (10^6 cpm/ml), washed gently, and transferred to isotope-free medium. During this washout period, intracellular isotope decreased, about 50% being lost in 24 hr. As shown in Fig. 1, the loss of isotope from cells pulsed with Hb-^{125}I was somewhat slower than the loss from cells pulsed with HSA-^{125}I. The isotope lost by cells could be accounted for as trichloroacetic acid–soluble isotope appearing in the medium. In the case of HSA-^{125}I, the trichloroacetic acid–soluble isotope had been shown to arise from intracellular degradation of HSA-^{125}I, as well as inorganic deiodination of residual HSA-^{125}I in the washout medium. It seems reasonable to conclude that Hb-^{125}I is also digested intracellularly to acid-soluble fragments which are excreted by the cells into the medium.

The nature of the acid-soluble products of leu-^{3}H–Hb digestion was next investigated. First, preliminary experiments showed that the measured uptake and rate of digestion of Hb were the same whether leucine-^{3}H or ^{125}I was used as a protein label. The tritium activity lost by leu-^{3}H–Hb–pulsed cells could be accounted for as trichloroacetic acid–soluble isotope appearing in the medium. Control media, which were removed from the cells at the start of the washout period and maintained at 37°C for 24 hr, showed negligible increases in trichloroacetic acid–soluble isotope. Thus the acid-soluble isotope in the washout media did not arise from noncellular degradation of leu-^{3}H–Hb remaining from the pulse. Fig. 2 shows the results of chromatography on paper and on Biogel P-2 of the trichloroacetic acid–soluble fraction of washout media. It is clear that virtually all of the trichloroacetic acid–soluble tritium activity in the washout media was associated with leucine.

The possibility that labeled fragments of leu-^{3}H–Hb other than leucine-^{3}H are excreted cannot be excluded. Peptides of low specific activity or low concentration would not have been detected. Alternatively, labeled peptides released by macrophages may have undergone further digestion by any peptidases present in the 1% nbcs medium. This latter consideration should also apply to events in vivo, where macrophages are exposed to much higher concentrations of serum. In any case, the present results show that by pinocytosis and subsequent digestion processes macrophages are capable of producing utilizable amino acids from ambient proteins.

In conclusion, it appears that pinocytosed Hb is handled much like HSA by macrophages. In both cases the labeled digestion product excreted by cells is the protein's labeled amino acid residue. It is reasonable to infer from these studies that macrophages can, in general, digest pinocytosed proteins to the level of amino acids. If so, pinocytosis could play a role in cellular nutrition. This may be especially important in rapidly maturing macrophages, both in culture and in vivo at sites of inflammation. Finally, pinocytosis by macrophages throughout the reticuloendothelial system may contribute to the catabolism of serum proteins and the formation of amino acids for use by other cells.

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