The accumulation of $[^{35}\text{S}]$methimazole by monocytes and macrophages

A. P. Weetman, Catherine Gunn, R. Hall and A. M. McGregor

Department of Medicine, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales, UK

Abstract. We have used an automatic cell harvester and micro culture techniques to examine the accumulation of $[^{35}\text{S}]$methimazole by monocytes, macrophages and lymphocytes. Significant temperature-dependent accumulation of the drug was found in resting monocytes and macrophages; this was increased up to 4-fold by phagocytosis. Lymphocytes accumulated little or no drug and myeloma and leukaemic cell lines accumulated none. These results show that two interrelated cells with endogenous peroxidatic activity take up the antithyroid drug methimazole providing further support for the concept that immunosuppression by this drug in Graves' disease is mediated via an action on antigen-presenting cells.

There is increasing evidence that the immunosuppressive action of methimazole (MMI), the active metabolite of the antithyroid drug carbimazole, is important in determining the outcome of Graves' disease (Weetman et al. 1984a). MMI is concentrated by cells in the thyroid (Marchant et al. 1972) and salivary glands (Connell et al. 1983) as well as by polymorphonuclear leucocytes (Lam & Lindsay 1979). The presence of intracellular peroxidase seems to be important in MMI accumulation (Connell et al. 1983) and lymphocytes, which do not contain peroxidase, do not accumulate MMI (Shewring & Lazarus 1983). We have shown that the immunosuppressive effect of MMI is probably mediated by an action of antigen-presenting cells, such as the monocyte and macrophage, which have a primary role in triggering lymphocyte activation by antigen; in monocytes the drug inhibits oxygen radical generation (Weetman et al. 1983a, 1984b). To support these findings, this study was undertaken to determine whether monocytes and macrophages accumulate MMI.

Materials and Methods

Cell sources and preparation

Peripheral blood mononuclear cells (PBM) were prepared from normal subjects by Ficoll-Hypaque density gradient centrifugation. Monocytes were separated from lymphocytes by adherence to plastic or by Percoll density gradient centrifugation. PBM at a concentration of $2 \times 10^8$/ml in culture medium (RPMI 1640 supplemented with 10% foetal calf serum, 4 mM glutamine, 2 mM pyruvate and 40 mg/l gentamicin), were added in 100 µl aliquots to 96 well flat-bottomed culture plates (Nunclon Delta SL, Nunc, Kamstrup, Denmark). These were incubated for 90 min at 37°C at 5% CO$_2$ in air, then the non-adherent lymphocytes removed. The plates were washed three times with warm medium and the adherent cells which remained used directly for accumulation studies. The cells were greater than 95% pure monocytes as judged by latex bead phagocytosis. Percoll density gradient centrifugation was performed as described elsewhere (Weetman et al. 1983a). This procedure yields greater than 90% pure monocytes and greater than 95% pure lymphocytes. Neutrophils were prepared by mixing 20 ml heparinised blood with 5 ml of 6% Dextran 150 in saline (Fisons, Loughborough, UK) and removing theuffy coat from the tube after allowing the cells to sediment on the bench for 60 min. Neutrophils were recovered from the pellet of a Ficoll-Hypaque gradient. Thyroid cell monolayers were prepared as described by Hinds et al. (1981) from Graves' thyroid tissue. When the monolayer was confluent the cells were removed with trypsin, frozen in a mixture of foetal calf serum and dimethyl sulfoxide (9:1 v/v) and stored in liquid nitrogen. For use the cells were rapidly thawed, resuspended in culture medium, and added to 96 well plates. Human splenic lymphocytes and macrophages were obtained from the spleen of a normal male renal donor by gentle disruption and adherence purification.
Rat macrophages were obtained by collection from the peritoneal cavities of peptone treated normal Wistar rats followed by adherence purification as previously described (Weetman et al. 1983b). K562 is a cell line derived from human leukaemic cells and SPI-USA is a murine myeloma cell line. In all cases the cells were plated out into 96 well plates at a final cell concentration per well of $2 \times 10^5$ and a final well volume (including the addition of phagocytic stimuli and $[^{35}\text{S}]$MMI) of 200 µl.

**Uptake of $[^{35}\text{S}]$MMI**

The method used was to add 1 µCi $[^{35}\text{S}]$MMI (specific activity 25.2 mCi/mmol, Amersham International, UK) in 25 µl culture medium per well to the cells under the conditions described below and incubate for 60 min at 37°C in 5% CO$_2$ in air. Adherent cell populations (monocytes, macrophages and thyroid cells) were dislodged by washing the wells with Earle’s balanced salt solution, Ca$^{2+}$ and Mg$^{2+}$ free (EBSS) and then adding 100 µl of 0.1% trypsin, 0.05% EDTA in EBSS to the cells and incubating at 37°C for 15 min. These previously adherent cells, and all non-adherent populations were harvested onto glass fibre filters using an automatic cell harvester (Skatron, Lier, Norway). $[^{35}\text{S}]$MMI uptake was measured by liquid scintillation counting using an LKB 1215 Rackbeta II counter. Results were expressed as counts per min (CPM) per well from six replicates since all wells contained the same number of cells. In one experiment rat macrophages were incubated in 1 ml volumes ($1 \times 10^6$ cells/ml) without adherence by use of siliconised glass vessels (Repelcoat, H + W Labs, Essex, UK). After 30 min, the cells were added to 96 well plates and immediately harvested onto glass fibre filters. Phagocytosis was initiated in certain experiments by the addition of zymosan or Candida albicans. Zymosan (Sigma, St. Louis, USA) was opsonised as previously described (Weetman et al. 1984b) and used at a final concentration of 0.05%. Candida albicans from stock cultures grown on agar was

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**Fig. 1.**

$[^{35}\text{S}]$MMI accumulation by lymphocytes and monocytes from 4 subjects compared with background. Cells from subject 1 were separated by adherence: it was assumed that 10% of the PBM were monocytes and adherent to calculate monocyte numbers per well. Cells from subjects 2–4 were separated by Percoll. Each point in this and subsequent figures is the mean of six replicates; the bar represents the sd. Significance values for comparison with background CPM: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Fig. 2.**

$[^{35}\text{S}]$MMI accumulation by two cell lines, K562 and SPI-USA, thyroid follicular cells and neutrophils compared with backgrounds for each experiment. A and B indicate thyroid cells from two separate donors. * $P < 0.001$. 
suspended in culture medium and used at a final concentration in the wells of $1.2 \times 10^6$ yeast cells/ml with 12.5% fresh AB Rh pos. serum to opsonise the yeast.

Statistics
Analysis was performed using Student's $t$-test.

Results

Background accumulation of $[^{35}S]$MMI
As shown in Fig. 1, monocytes accumulated $[^{35}S]$MMI in the resting state over 1 h at 37°C when separated by adherence (subject 1) or by Percoll (subjects 2—4). Lymphocytes from subjects 2 and 3 gave significantly higher CPM ($P < 0.01$) than background (wells with $[^{35}S]$MMI alone) but this was not found with lymphocytes from subjects 1 and 4. In subject 4 culture was performed without the addition of foetal calf serum to the culture medium and this allowed the addition of trypsin (final concentration 1%) to the lymphocytes 15 min prior to harvesting for comparison with trypsinised monocytes.

The accumulation of $[^{35}S]$MMI by other cells is shown in Fig. 2 confirming that neutrophils and thyroid follicular cells accumulate the drug but myeloma and leukaemia cells do not.

Effect of temperature
Accumulation of $[^{35}S]$MMI by monocytes was dependent on temperature, being optimal at 37°C (Fig. 3). This was also found with thyroid cells and neutrophils but not with lymphocytes.

Effect of phagocytosis
Accumulation in monocytes, neutrophils and splenic macrophages was increased by phagocytic stimuli but there was no effect of such stimuli on lymphocytes or cell lines (Fig. 4).

Rat macrophages
The time course of $[^{35}S]$MMI accumulation was studied using rat macrophages. The mean CPM ± SD (minus background) were 5 min: 1022 ± 111, 15 min: 2131 ± 209, 30 min: 2869 ± 793 and 60 min
3415 ± 657. Significant accumulation also occurred if the cells were kept in siliconised glassware for 60 min: mean CPM ± SD (minus background) 1129 ± 125. A small but significant increase was seen with the addition of a phagocytic stimulus to these cells: mean CPM: 1366 ± 71 (P < 0.01).

Discussion

We have adopted the automatic cell harvester, usually used to assess [3H]thymidine incorporation in experiments of lymphocyte blastogenesis, to collect cells from 96 well plates for the assessment of [35S]MMI accumulation. This method has the advantages of ease of harvesting for liquid scintillation counting and a microculture system which allows many replicates to be performed. Accumulation of the drug was assessed by comparing CPM from wells containing cells with CPM from wells without cells and was found to occur with thyroid cells and neutrophils as previously described using alternative methods (Marchant et al. 1972; Lam & Lindsay 1979). Using the cell harvester [35S]MMI accumulation was found in monocytes even though these cells had to be trypsinised off the wells due to their adherence. Collection of cells in this way is incomplete, but this would tend to lower the true CPM for the monocyte-containing wells. Trypsin itself is unlikely to have had any effect since accumulation was found in rat macrophages kept in siliconised vessels which were not trypsinised. In addition lymphocytes from subject 4 in Fig. 1 were cultured in medium without foetal calf serum to which trypsin was added for 15 min before harvesting and there was no accumulation in these cells. It is also likely that by washing the monocytes three times with EBSS followed by trypsinisation before harvesting, the true accumulation of [35S]MMI will be underestimated compared with wells harvested directly in which the cells were not subjected to washing.

Besides the adherence of monocytes and macrophages to plastic, another problem with the study of these cells is that no method currently exists to produce complete monocyte and lymphocyte separation. Both adherence purification and Percoll density gradient separation gave similar results for monocyte accumulation; it is possible that monocyte contamination may have produced the small accumulation seen in lymphocytes from subjects 2 and 3 (Fig. 1). It was assumed for subject 1 that all monocytes were adherent and that they comprised 10% of the population, thus resulting in each well containing 2 × 10^5 cells. This is probably an over-estimate of true cell number so that MMI accumulation was probably even greater than that shown. Nonetheless in all cases monocytes showed significantly higher accumulation than lymphocytes and leukaemic and lymphoid cell lines did not accumulate MMI. Drug uptake by monocytes was dependent on temperature and was also increased by phagocytic stimuli (as in neutrophils) but no effect was found with such stimuli added to lymphocytes, indicating that this increase was not due to non-specific binding of drug to stimulating agent with retention on the glass fibre filters. Macrophages, the tissue resident derivatives of monocytes, also accumulated MMI at rest and with phagocytosis.

In summary, these results show that the major antigen-presenting cells, namely monocytes and macrophages, accumulate MMI. These cells possess peroxidase enzymes (Daems et al. 1979) which seem to be a prerequisite for thionamide drug uptake (Connell et al. 1983). Thus it seems likely that the antigen-presenting cell is a key target of MMI-induced immunosuppression by virtue of drug uptake. Further studies are now required to identify the existence of any intracellular effects of this since reduction in oxygen radical synthesis by MMI may involve events occurring only at the cell surface or within the phagosome (Weetman et al. 1984b).

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References


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