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Functional heterogeneity of macrophages

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Summary. The antibody-binding activities of rabbit peritoneal macrophages separated on discontinuous gradients of Ficoll were investigated. The antibody was rabbit anti-ovalbumin IgG labelled with ^{125}I. Of the five fractions obtained, one macrophage fraction was found to bind substantially more antibody than the others. These macrophages possessed more Fc receptor sites than the others and the number of Fc receptors (n) and the association constant (K) of these cells was calculated. By electron microscopy, the phagocytic activity of the subpopulation with most Fc receptors was less than that of the others.

INTRODUCTION

Rabbit peritoneal macrophages can be separated into fractions on discontinuous gradients of Ficoll (Walker, 1974) or albumin (Rice & Fishman, 1974). The function of each fraction is different, e.g. the subpopulation of slowly sedimenting cells fixes most antigen and has the highest phagocytic activity, whereas the quickly sedimenting macrophages possess the characteristic Fc receptor function.

The aim of the present study was to investigate the numbers of receptor sites for IgG on macrophages sedimented on discontinuous gradients of Ficoll.

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MATERIALS AND METHODS

Reagents
Solution B, a phosphate-buffered (pH 7·8) saline, and solution F (pH 7·4) were prepared, as previously described (Walker, 1974), but without foetal calf serum. Culture medium 199 contained 1% crystalline BSA (Serva).

Peritoneal exudate cells (PEC)
Rabbits of either sex (1·0–1·5 kg body weight) were injected intraperitoneally with 30 ml of sterile paraffin oil. Four days later, they were killed and their peritoneal cavities were washed with 1·0 litre of sterile solution B containing 5·0 iu/ml crystalline heparin. The exudates were collected, centrifuged at 700 ***g*** for 10 min and the cells were washed twice in solution B and once in culture medium.

Separation of macrophages
Ten ml of cell suspension (PEC) were plated in glass culture plates and incubated at 37° for 120 min in a humidified 7% CO_2–air incubator. The adherent cells were washed with solution F to remove the non-adherent ones. The adherent cells were then removed with a rubber policeman, washed once in solution F and counted. More than 90% of these cells were found to

be macrophages, of which 85–95% were viable, as estimated by the trypan blue dye exclusion test.

Anti-ovalbumin rabbit IgG and labelling with ^{125}I
Rabbits were immunized with four times recrystallized ovalbumin (OA) in Freund's complete adjuvant (Difco). The antibodies were isolated by immunospecific precipitation, by dissociating the precipitate and, at the last stage, by separating antibody from antigen on Sephadex G-200 gel using our earlier method (Kávai, Jusupova & Csaba, 1966), with the slight modification of Aftab & Salahuddin (1973). The IgG antibody did not contain contaminating proteins as judged by immune electrophoresis and analytical ultracentrifugation. The antibody was labelled with ^{125}I using chloramine-T by the method of McConahey & Dixon (1966). The aggregated IgG was removed by centrifuging at 45,000 ***g*** overnight and discarded. The protein content of the labelled monomer rabbit antibody (IgG) was measured at 280 nm in an Unicam SP 500 spectrophotometer and calculated as previously described (Pollack and Kochesky, 1970).

Fixation of ^{125}I-labelled IgG to macrophages
Peritoneal macrophages ($1{\cdot}4$ to $2{\cdot}0 \times 10^7$) were incubated with 150–600 μg of rabbit IgG at 37° for 30 min with constant shaking to keep the cells in suspension. The final volume was 1·0 ml. The cells were then centrifuged at 200 ***g*** for 10 min at 4° and washed twice in culture medium and once in solution F.

Preparation of Ficoll gradient
Using the method of Walker (1974), discontinuous gradients were prepared in 13 ml centrifuge tubes. The layers of Ficoll solution (Pharmacia) were 10, 9, 8 and 5%. The concentrations were checked by measurement of refractive indices.

Separation of macrophages on Ficoll gradient
Macrophages ($1{\cdot}4$ to $2{\cdot}0 \times 10^7$ per ml) incubated with ^{125}I-labelled IgG were washed and then put onto the Ficoll gradient. Each experiment was duplicated. The gradients were centrifuged at 800 ***g*** for 5 min at 4°. The cells at the interfaces of the Ficoll layers were aspirated, counted and the smears stained with Giemsa. The radioactivity adherent to the cells was counted in an Automata Counter (Model 3320, Packard Instrument Co.). The radioactivity of each subclass of cells was referred to 10^5 cells. Parallel to this, cells not incubated with rabbit IgG were also separated on Ficoll gradient. The separated cells of the subclasses were investigated electron microscopically.

Finally, ^{125}I-labelled IgG was put on Ficoll gradient without macrophages, but the activity remained on the top of the Ficoll gradient.

Calculations for the Scatchard plot
The molecules of IgG adherent to one cell (r), and the molecules of IgG free in the surrounding medium (c) were calculated for each experimental point by using Avogadro's number and a molecular weight of 145,000 for IgG. A Scatchard plot of r/c *vs* r was constructed, with the relationship $r/c = nK - rK$ (Arend & Mannik, 1973). The value n, on the x-intercept, represented the amount of IgG molecules on each cell at saturation, i.e. the number of receptor sites per cell. The slope of the line, K, represented the effective association constant and was expressed in M^{-1}. All curve fitting was done by the least squares method with a computer. The mean value of data for ten separate experiments and the standard deviation were calculated; s.d. $= \pm[\Sigma(x-\bar{x})^2/n-1]^{\frac{1}{2}}$.

RESULTS

Macrophages were separated into five fractions on a Ficoll gradient, following Walker (1974). Figure 1 shows the percentages of macrophages which accumulated at the interface. The quantities of ^{125}I-labelled IgG fixed to different macrophage fractions are shown in Fig. 2.

When low amounts of ^{125}I-labelled IgG were incubated with macrophages, the radioactivity was nearly constant in each fraction (●). With a slightly greater amount the radioactivity showed two peaks, one of them at the upper and the second at the lower part of the gradient (■). With larger amounts of radioactivity, there was a peak near the bottom and another at the top of the gradient (○ and □).

In the middle fractions no difference was observed between the two lines. The most important phenomenon was that cells found near the bottom of the gradient fixed the most ^{125}I-labelled IgG, while those near the top of the tube did not show any dose-related uptake of ^{125}I-labelled IgG.

The radioactivity on the top of the gradient was mainly due to free unbound ^{125}I-labelled IgG. This interpretation was supported by the finding that the cells in these fractions contained little radioactivity after one washing, and the position of radioactivity

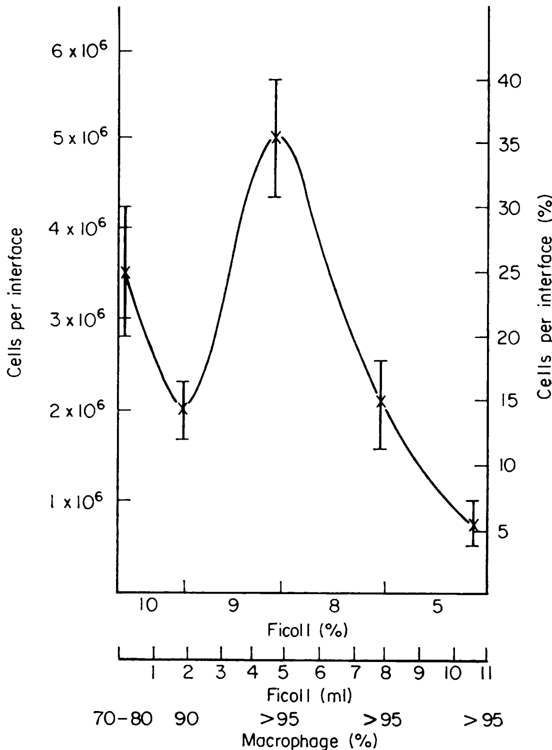

Figure 1. Distribution of rabbit peritoneal macrophages ($1 \cdot 43 \times 10^7$) on Ficoll gradient (for 5 min at 800 *g*).

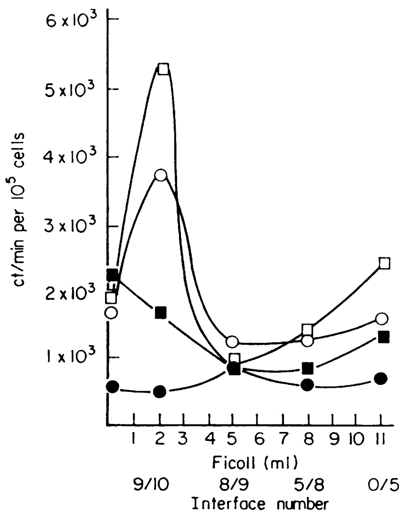

Figure 2. The uptake of ^{125}I-labelled rabbit IgG by macrophage fractions as a function of IgG concentrations. (●) 151 μg IgG per $1 \cdot 43 \times 10^7$ cells; (■) 303 μg IgG per $1 \cdot 43 \times 10^7$ cells; (○) 4·53 μg IgG per $1 \cdot 43 \times 10^7$ cells; (□) 553 μg IgG per $1 \cdot 43 \times 10^7$ cells.

was similar to that of the ^{125}I-labelled IgG put on the Ficoll gradient without macrophages.

The cells in the 90% interface fraction were considered in more detail. Using the method of Arend & Mannik (1973), the number (n) of sites for IgG on macrophages of this fraction and the association constant (K) were calculated (Fig. 3). The data for the

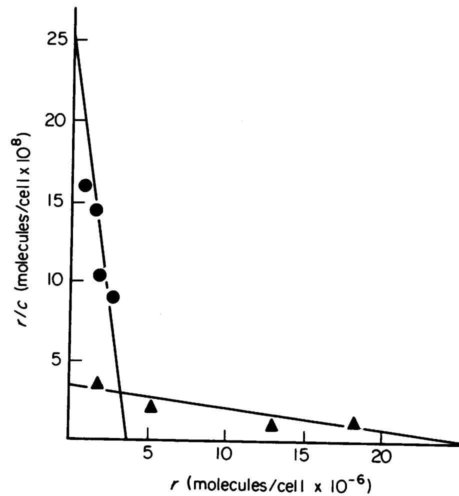

Figure 3. Scatchard plots demonstrating the association constants (K) and the numbers of IgG receptor sites (n). (●) Separated macrophages in 9/10 interface ($2 \cdot 00 \times 10^6$ cells). (▲) Total macrophage population ($1 \cdot 43 \times 10^7$ cells). For (●): $n = 2 \cdot 589 \times 10^7$ sites/cell; $K = 1 \cdot 325 \times 10^4$ M^{-1}. For (▲): $n = 3 \cdot 627 \times 10^6$ sites/cell; $K = 6 \cdot 75 \times 10^5$ M^{-1}.

unseparated whole population of macrophages were also shown. The Fig. represents the result of a single experiment. In ten experiments the number (n) of IgG receptors per macrophages in the 9/10 interface fraction was $2 \cdot 24 \pm 0 \cdot 42 \times 10^7$ sites per cell. The mean association constant between the rabbit IgG and the receptor sites was $3 \cdot 76 \pm 2 \cdot 51 \times 10^4$ M^{-1}. When the fixed ^{125}I-labelled IgG was referred to the total cell count, the value of n was one magnitude ($1 \cdot 82 \pm 0 \cdot 26 \times 10^6$ sites per cell) less while the value of K was one and half magnitudes greater ($5 \cdot 6 \pm 1 \cdot 7 \times 10^5$ M^{-1}).

By electron microscopy, cells with phagocitic activity were mainly at the top and on the bottom of the Ficoll gradient. These macrophages had villus-like processes at the surface and very often large and clear vacuoles were present in the cytoplasm.

DISCUSSION

The results presented above supported the earlier observation of Walker (1971, 1974) and Rice & Fishman (1974), who have established that peritoneal macrophages are functionally heterogenous and that some fractions possessed antigen-binding capacity. Walker (1974) has showed that antigen complexed with its specific antibody fixes to the Fc receptor of different macrophages through an antibody receptor site.

Our results prove that only one fraction of macrophages separated on a Ficoll gradient show significant fixation of monomeric radiolabelled IgG. Walker (1974) found phagocytic macrophages in the fraction at the bottom of the gradient. This makes it probable that denatured ^{125}I-labelled IgG was taken up by these macrophages. As only one fraction of macrophages fixed IgG, the earlier calculations based on total number of macrophages must be revised (Kesztyüs, Kávai & Csaba, 1972).

An important technical problem in these experiments is the removal of unfixed IgG from the cell suspension. This was achieved by washing the cells six times and by the extrapolation of the fixed IgG to zero washing (Arend & Mannik, 1973; Phillips-Quagliatia, Levine & Uhr, 1968; Csaba, Kávai, Jusupova & Kesztyüs, 1976). This is time-consuming work as the radioactivity fixed to the cells must be counted after each washing. Figure 2 suggests that the centrifugation of cells in a Ficoll gradient is a useful way of removing the unfixed labelled IgG from the IgG fixed to the macrophages.

The immunological function of these cells remains to be determined, as does the question of whether the density and functional heterogeneity of macrophages reported here are related to stages of cellular maturation (van Furth, 1976). These experiments could suggest that older macrophages have more phagocytic capacity than younger ones arrived newly after the stimulation with oil. The progressive loss of high avidity Fc receptors could be due to phagocytosis or endocytosis.

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