QUANTITATIVE ANALYSIS OF TOTAL MACROPHAGE CONTENT IN ADULT MOUSE TISSUES

Immunochemical Studies With Monoclonal Antibody F4/80

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Because of the widespread dispersion of mononuclear phagocytes throughout the body, there is little quantitative information on their total mass, relative numbers in different sites, or mobilization and redistribution in normal individuals or disease states. It is difficult to obtain such information by direct isolation of macrophages (Mϕ)[1] from all sites where they are known to occur and from tissues in which they are deeply embedded. The pool size of Mϕ and their precursors in different compartments has been estimated by van Furth and his colleagues (1) by single-cell analysis after [^{3}H]thymidine labeling, but this method favors enumeration of cells that turn over relatively rapidly, rather than more quiescent resident cells. In principle, antibodies that are specific for Mϕ could be used to quantitate the content of antigen (Ag) in tissues directly, without cell separation. F4/80, a rat monoclonal antibody (Ab) directed against a plasma membrane glycoprotein of apparent M_r 160,000, is a specific and sensitive marker for mature mouse Mϕ after isolation (2) and in situ (3). In this study we have assayed F4/80 Ag content in various tissues of normal adult mice by adapting an absorption immunoassay developed by Williams and his colleagues (4) to measure lymphoid differentiation Ag in tissue lysates. We estimated Mϕ number by calibrating F4/80 Ag content in a murine Mϕ tumor-derived cell line, J774.2. Our results are in good agreement with immunohistochemical findings, and establish that relatively large amounts of F4/80 Ag are found not only in hemopoietic and lymphoid tissues, but also in other sites such as the gastrointestinal tract and normal kidney. This experimental approach provides a basis for further studies on the response of the mononuclear phagocyte system to inflammation and infection.

Materials and Methods

Animals. Tissues, organs, and peritoneal cells were obtained from 8–10-wk-old male mice of the C57BL/6 strain weighing 23 ± 2 g. Swiss mice, Pathology Oxford (PO), were used to prepare Mϕ target cells for binding assays.

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[1] *Abbreviations used in this paper:* Ab, antibody; Ag, antigen; BSA, bovine serum albumin; DME, Dulbecco's modification of Eagle's minimal essential medium; FCS, fetal calf serum; Mϕ, macrophage; PBS, phosphate-buffered saline; RPC, resident peritoneal cells; RPM, resident peritoneal Mϕ; TPC, thioglycollate-elicited peritoneal cells; TPM, thioglycollate-elicited peritoneal Mϕ.

Preparation of Mϕ Target Cells. PO mice aged 8–10 wk were injected with 1.0 ml of thioglycollate broth intraperitoneally and peritoneal cells (TPC) harvested 4–6 d later. Cells were cultivated at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum (FCS) in 96-well plates (Sterilin Ltd., Middlesex, United Kingdom [UK]), at a density of 1×10^5 Mϕ per well, for 2 d. They were then washed free of nonadherent cells, fixed with 0.125% vol/vol glutaraldehyde, and excess glutaraldehyde removed by washing and further incubation with 10% FCS in phosphate-buffered saline (PBS). Plates were stored in PBS with 10 mM sodium azide at 4°C for up to 12 wk.

Preparation of Tissue and Cell Extracts. Organs: Animals were freshly killed by ether and organs removed into ice-cold extraction buffer (PBS containing 3 mM iodoacetic acid, 1.5 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA at pH 7.3). In some experiments animals were first extensively perfused by left ventricular catheterization with PBS containing 10 IU/ml heparin. Organs were weighed, cut into small fragments (~1 mm^3), mixed with an equal weight of extraction buffer, and kept on ice. The tissue was disrupted with an electrically driven homogenizer (Polytron, Kinematica GmbH, Lucerne, Switzerland) for two 30-s bursts with a 20 s interval. Detergent was then added to the required concentration and the homogenate left on ice for 20 min. Extracts were assayed immediately or were frozen at −20°C in aliquots. In some experiments, whole organs were harvested in extraction buffer and stored frozen at −20°C before thawing, homogenization, and detergent treatment, as detailed below. Frozen homogenates showed no loss of Ag during storage for up to 2 mo. Although most tissues could be dissolved by this method, the more viscous, such as thyroid, could not be satisfactorily dispersed.

Gut tissues and bladders were removed whole in extraction buffer, slit open, and the contents washed out with PBS before weighing and homogenization. Lymphoid nodules and Peyer's patches were dissected from the gut wall. Bone marrow cells were obtained by flushing femurs with PBS. The inner surfaces of bone marrow shafts were gently scraped with a 23 gauge needle to recover adherent cells. Testes were removed with the surrounding tunica albuginea intact. Blood was drawn by cardiac puncture into heparinized syringes. In some experiments whole blood was allowed to clot and was separated into serum and cell fractions by centrifugation at 400 *g* for 10 min. Plasma was obtained by centrifugation of heparinized blood at 400 *g* for 10 min. Urine was obtained by bladder puncture with a 23 gauge needle and was separated into pellet and supernatant by centrifugation at 400 *g* for 10 min. Other organs (liver, spleen, kidneys, lungs, brain, heart, adrenals, and thymus) were harvested whole into extraction buffer, weighed, and extracted as described above.

Cells: J774.2 (5) was grown in suspension in Iscove's medium supplemented with 8% FCS and 20 μg/ml gentamicin. Cell density was maintained at $0.5–1.0 \times 10^6$ cells/ml. Cells, >98% viable by trypan blue exclusion, were harvested by centrifugation at 350 *g* for 10 min, resuspended in extraction buffer, homogenized as for organ extracts, and Triton X-100 (Sigma Chemical Co., Poole, UK) added to a final concentration of 1% for 20 min on ice. Aliquots of 5×10^7 cells/ml were stored at −70°C and provided the calibration standard for absorption analysis. The L929 fibroblast line (6) was grown in DME with 3% FCS in tissue culture flasks (Nunclon; Gibco Europe Ltd., Uxbridge, UK); extracts were made and stored as with J774.2. Resident peritoneal cells (RPC) and TPC for binding assays and absorption analysis were harvested from C57BL/6 mice by intraperitoneal lavage, washed once in PBS, and resuspended in PBS with 0.5% bovine serum albumin (BSA) and 10 mM sodium azide.

Antibodies. The following monoclonal Ab were used: (*a*) F4/80, a noncytotoxic rat IgG2b Ab, was used as a concentrated tissue culture supernatant or purified by affinity chromatography on rabbit IgG directed against rat IgG coupled to Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The Ab was judged pure by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. (*b*) MRC OX12, a mouse anti–rat kappa chain Ab (7), was used as purified $F(ab')_2$ fragments, and was a generous gift from Dr. Simon Hunt (Sir William Dunn School of Pathology, Oxford). Antibodies were radiolabeled with ^{125}I using chloramine T (8).

Absorption Indirect Binding Assay. F4/80 concentrated tissue culture supernatant at a dilution of 1:80 (unless stated otherwise) in PBS with 0.1% BSA and 10 mM sodium azide, was absorbed with doubling dilutions of cell or tissue extract in 1.5-ml plastic tubes (Beckman microfuge; Beckman Instruments International SA, Geneva) at 4°C for 2 h. At this dilution F4/80 supernatant gives 50% of maximum binding detected by ^{125}I-labeled F(ab')$_2$ OX12 to 1×10^5 glutaraldehyde-fixed Mϕ targets. Cells and tissue extracts were also diluted in PBS with 0.1% BSA and 10 mM azide and detergent at the same concentration as in the undiluted extract (usually 1% Triton X-100). Extracts were then centrifuged at 8,000 *g* for 2 min. 40-μl aliquots of supernatant were transferred onto fixed target cells in 96-well plates on ice and incubated at 4°C for exactly 1 h. The cells were washed three times with cold PBS containing 0.1% BSA and trace amounts of ^{125}I–F(ab')$_2$ OX12 added (3.0×10^5 cpm/well) for 1.5–2 h. The plates were then washed three times with cold PBS containing 0.1% BSA, and 0.5 N sodium hydroxide was added for 10 min. Aliquots of solubilized cells were counted on an Autogamma counter (Packard Instrument Co., Downers Grove, IL). Nonspecific binding of ^{125}I-OX12 to Mϕ targets was determined by assaying the undiluted extracts in the absence of F4/80 Ab, and was usually <10% of the maximum count in the presence of 1% Triton X-100. Absorption of F4/80 Ab with extracts for >2 h at 4°C did not affect the amount of Ab absorbed. All second incubations were carried out for exactly 1 h at 4°C. OX12 binding to F4/80 Ab on Mϕ targets reached equilibrium after 1.5 h at 4°C. Binding of F4/80 Ab to glutaraldehyde-fixed Mϕ targets was slightly increased (20%) in the presence of 1% Triton X-100.

Absorption curves were plotted as a function of ^{125}I–F(ab')$_2$ OX12 bound to Mϕ targets at increasing dilutions of cell or tissue extract. All experiments included a J774.2 extract at 5×10^7/ml as a standard and controls either without extract or without F4/80 Ab to represent, respectively, maximum and minimum binding of second Ab to Mϕ targets. When assayed in the same concentration of detergent, these curves were approximately parallel, with similar levels of maximum binding of second Ab. We estimated the amount of F4/80 Ag in extracts by comparing the dilutions at which extracts absorbed 50% of the F4/80 Ab used in the first incubation. When extracts did not completely absorb the F4/80 Ab added, we determined the 50% level of F4/80 absorption by averaging the maximum and minimum binding of second Ab to Mϕ targets in the absence of tissue extract and F4/80 Ab, respectively. Tissue extracts that showed absorption, but bound <50% of F4/80 Ab were considered to contain detectable but unquantifiable amounts of F4/80 Ag.

Immunohistochemical Localization of F4/80 in Isolated Cells and Tissue Sections. RPC and TPC were cytocentrifuged onto glass slides coated with 0.1 mg/ml poly-L-lysine (Sigma Chemical Co.) and then fixed with 0.125% vol/vol glutaraldehyde for 10 min. Tissue sections were prepared after perfusion fixation through the heart with glutaraldehyde as described (9). Immunoperoxidase labeling of cells and tissue sections with F4/80 was then carried out by the avidin-biotin complex method of Hsu et al. (10) as described (9).

Determination of the Number of F4/80-binding Sites on Intact J774.2, RPM, and TPM. This was determined (*a*) directly, using radiolabeled, purified F4/80 at saturation in a single-step binding assay and (*b*) indirectly, using concentrated F4/80 tissue culture supernatant, followed by ^{125}I–F(ab')$_2$ OX12 in a two-step indirect binding assay with both Ab at saturation. All cells and Ab were in PBS with 0.5% BSA and 10 mM sodium azide at 4°C. All cell washes were carried out with PBS containing 0.1% BSA and 10 mM sodium azide at 4°C.

(*a*) Direct binding assay for F4/80 Ag was carried out in 12.7 × 75 mm plastic tubes containing 2×10^6 freshly harvested cells per tube. Cell numbers were counted on a hemocytometer at the beginning and end of the assay, and showed no significant losses (<5%). Cells remained viable throughout the assay. The percentage of F4/80$^+$ cells in the same cell populations was determined by immunoperoxidase labeling of cytocentrifuged cell preparations. Saturating concentrations of F4/80 Ab were determined by competing ^{125}I-F4/80 Ab with unlabeled F4/80 Ab at different concentrations. Purified F4/80 Ab at saturating concentration (50 μg/ml or 2.0 μg/tube), together with ^{125}I-F4/80 at trace

concentration (1×10^6 cpm/tube) was added to cell pellets for 1 h at 4°C. Specific labeling was inhibited by excess unlabeled F4/80 Ab. Cells were then washed three times, resuspended, and an aliquot removed for cell counting on a hemocytometer. Bound radioactivity was measured in a gamma counter. Nonspecific binding was determined by using L929 fibroblasts instead of cells. The number of molecules of F4/80 Ab bound was calculated assuming an IgG M_r of 1.5×10^5.

(*b*) Saturation indirect binding assay was also carried out in 12.7 × 75 mm tubes containing 2×10^6 freshly harvested cells per tube. The cell number was determined at the beginning and end of assay. Cell pellets were incubated with F4/80 concentrated tissue culture supernatant containing 10 mM sodium azide for 1 h at 4°C. After three washes, pellets were incubated with unlabeled $F(ab')_2$ OX12 at a saturating concentration of 50 μg/ml (2.0 μg/tube) together with ^{125}I–$F(ab')_2$ OX12 at trace concentration (1×10^6 cpm/tube) for 1 h at 4°C. After a further three washes, cell numbers and bound radioactivity were determined. Controls, with PBS containing 0.1% BSA and 10 mM sodium azide instead of F4/80 Ab, were included in all assays. The number of molecules of OX12 Ab bound was calculated assuming an $F(ab')_2$ M_r of 1.0×10^5.

Morphometric Estimation of the Volume Fraction Occupied by F4/80$^+$ Areas in Liver Tissue. F4/80$^+$ cells are fairly uniformly distributed throughout the liver parenchyma and are labeled in high contrast relative to background and cell nuclei (11); thus it is possible to quantitate the volume fraction of liver tissue occupied by F4/80$^+$ areas by morphometry. Consecutive 200X magnification, black and white photographs of 5-μm liver tissue sections, which had been immunoperoxidase labeled with F4/80 Ab, were taken through a blue filter. A square lattice of period 15 mm was superimposed on photographs and intersections were scored for F4/80 positivity, F4/80 negativity, and sinusoidal spaces. We analyzed a total of 4,000 points, comprising consecutive intersections across the whole span of two different planes of liver tissue. We then calculated the numerical density of F4/80$^+$ areas as a proportion of liver tissue to obtain the volume fraction of liver occupied by F4/80$^+$ cells. The limits of confidence for such an estimate depend on the number of intersections sampled and the percentage of volume occupied by the scored component relative to the total volume of the organ (12, 13).

Results

Quantitation of Ag F4/80 by Absorption Assay: Studies With J774.2 Cells. The J774.2 Mϕ-like cell line expresses readily detectable F4/80 Ag (2) and has been used as a source of Ag for purification (P. M. Starkey, unpublished results). We therefore chose it as a homogeneous standard for assays of F4/80 Ag content in tissue and other cell extracts. J774.2 were lysed in detergent under similar conditions to those used for tissue extracts and serial dilutions were incubated with a nonsaturating concentration of F4/80 Ab. Residual free Ab was assayed on glutaraldehyde-fixed TPM target cells. Initial studies were carried out to optimize detergent concentration and other variables, establish specificity for F4/80 Ag, and assess the limits of sensitivity of the assay. Other studies (P. M. Starkey, unpublished results) have shown that F4/80 Ag is stable in, and readily solubilized by, Triton X-100, which was chosen for all experiments reported here. Fig. 1 *a* shows that the absorption assay detected F4/80 Ag in extracts of J774.2, but not of L929 fibroblasts, which do not bind F4/80 Ab (2). Extracts derived from serial dilutions of J774.2 yielded a series of parallel absorption curves.

For J774.2, the relationship between cell number and the dilution of extract giving 50% absorption of Ab was linear, and extrapolated close to the origin (Fig. 1 *b*). The use of a different amount of F4/80 Ab in the first incubation altered the gradient of the calibration plot but not the relationship between cell

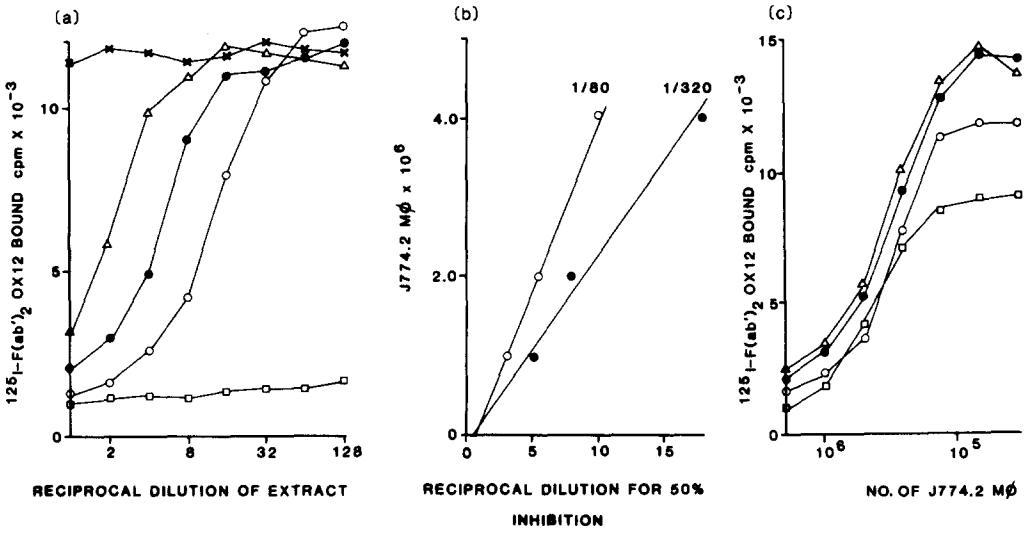

FIGURE 1. Calibration of F4/80 Ag quantitation by absorption analysis. (*a*) J774.2 grown in suspension culture were harvested and adjusted to concentrations of 1.25 × 10^7 cells/ml (△), 2.5 × 10^7 cells/ml (●), and 5.0 × 10^7 cells/ml (○), in PBS with 0.1% BSA and 10 mM sodium azide. L929 fibroblasts (×) were adjusted to a concentration of 5 × 10^7 cells/ml in the same buffer. Aliquots were homogenized and Triton X-100 added to a final concentration of 1% for 20 min on ice. Twofold serial dilutions of 80-μl aliquots of extracts were then absorbed with 20 μl of F4/80 tissue culture supernatant at a final dilution of 1:80 in the incubation mix. Exactly the same experiment was carried out with 5.0 × 10^7 J774.2 cells/ml but without F4/80 Ab (□). (*b*) The numbers of J774.2 cells in the extracts in *a* are plotted against the reciprocal of the dilutions of the extracts giving 50% absorption of F4/80 Ab (○). A similar experiment was carried out with 20 μl of F4/80 tissue culture supernatant at a final dilution of 1:320 in the incubation mix (●). (*c*) Quantitation of F4/80 Ag in J774.2 at different detergent concentrations. J774.2 from suspension culture were harvested and adjusted to a concentration of 5.0 × 10^7 cells/ml in PBS with 0.1% BSA and 10 mM sodium azide. Aliquots were homogenized and Triton X-100 added to final concentrations of 3% (△), 2% (●), and 1% (○) for 20 min on ice. An aliquot of intact cells in the same buffer without homogenization or detergent treatment was also assayed (□). Serial twofold dilutions of 80-μl aliquots of 5 × 10^7 cells/ml were made in the same detergent concentrations as the original extracts, and absorbed with 20 μl of F4/80 tissue culture supernatant at a final concentration of 1:80 in the incubation mix.

number and dilution of extract giving 50% absorption. A 1:80 dilution of F4/80 Ab was used for all assays reported here, which made it possible to measure F4/80 Ag in ~4 × 10^5 J774.2 cells at a concentration of ~5 × 10^6 J774. 2 cells per gram or milliliter of undiluted extract. The effect of detergent is shown in Fig. 1*c*. The J774.2 extract solubilized with 1% Triton X-100 showed a small increase in the amount of F4/80 Ag detected compared with intact cells. The presence of detergent increased both the nonspecific (not shown) and specific levels of Ab binding to target cells, but the 50% F4/80 Ab absorption point controlled for such differences. Concentrations of 2 and 3% Triton X-100 did not increase the amount of Ag detected at 50% absorption. The intracellular pool of F4/80 Ag that can be released by lysis with detergent treatment is small, ~35% of surface F4/80 Ag. We therefore used J774.2 lysed in 1% Triton X-100 to standardize the Ag content of tissue lysates.

Comparison of Absorption and Other Immunoassays of F4/80 Ag: Studies With Intact RPM, TPM, and J774.2. We next compared an absorption assay in the absence of detergent with two other methods, to measure F4/80 Ag on intact cells, and compared surface Ag expression by J774.2 and primary Mϕ populations. Freshly isolated RPC and TPC were chosen since they are readily obtained in suspension by lavage and are known to express different levels of F4/80 Ag (2). Total peritoneal cell populations containing Mϕ and F4/80$^-$ lymphocytes were assayed to avoid possible modulation of F4/80 Ag during adherence and cultivation of Mϕ (2).

All cells were assayed for surface F4/80 Ag in suspension in the presence of sodium azide at 4°C to prevent internalization of Ag or Ab. We estimated the number of F4/80$^+$ cells in RPC and TPC by immunoperoxidase labeling of cytocentrifuge preparations. Surface Ag was measured by (*a*) direct binding assay at saturation, (*b*) indirect binding assay at saturation, and (*c*) absorption analysis, as described. Fig. 2*a* shows an absorption assay with live peritoneal cells and J774.2 without detergent. Table I shows that results obtained by the different assays were in agreement and that the average number of F4/80-binding sites per F4/80$^+$ cell was approximately as follows: J774.2, 61,000–71,000; RPM, 59,000–88,000; and TPM, 13,000–20,000.

These data indicate that similar results were obtained by absorption and other assays, that Mϕ F4/80 could be specifically measured in the presence of F4/80$^-$ cells, and that J774.2 provided a suitable standard to estimate F4/80 Ag in resident Mϕ. Freshly isolated TPM expressed only ~20% of the F4/80 Ag levels of RPM, but subsequent cultivation for 2 d resulted in a marked increase of F4/80 Ag (2), as a result of new synthesis (P. M. Starkey, unpublished results). They were thus suitable for use as Mϕ targets in absorption assays.

F4/80 Ag Content in Tissue Extracts by Absorption Assay. Further experiments were undertaken to extend the absorption assay to tissue extracts. Control experiments were done with murine liver, which contains substantial levels of F4/80 Ag by absorption assay (Fig. 2*b*) and by immunohistochemistry (11) in which Kupffer cells are specifically labeled. Extracts were routinely prepared in 1% Triton X-100. Addition of 0.2% deoxycholate did not significantly increase detectable F4/80 Ag (<10%). Centrifugation (6,000 *g* for 30 min at 4°C) after homogenization in 1% Triton X-100 resulted in incomplete release of F4/80 Ag into the supernatant and total tissue extracts were therefore analyzed without subfractionation. Fig. 2, *b* and *c* illustrate inhibition assays with a number of tissues that vary in F4/80 Ag content. Inhibition was readily detectable with extracts of liver, spleen, bone marrow, small and large bowel, mesenteric lymph nodes, and kidney. Small amounts of F4/80 Ag could be detected in whole blood and none in heart (not shown) or brain. Thus, F4/80 Ag detection by immunoassay correlated well with previous immunohistochemical findings (11, 14, 15). Further evidence was sought for specificity of binding of F4/80 Ab by tissue extracts. The rat monoclonal Ab F4/80 used here does not bind to Mϕ of other species (2). A rabbit liver extract prepared in the same way as mouse tissues showed no nonspecific binding of F4/80 Ab. It should be noted that rat liver contains large amounts of rat Ig, which competes with F4/80 Ag in the second

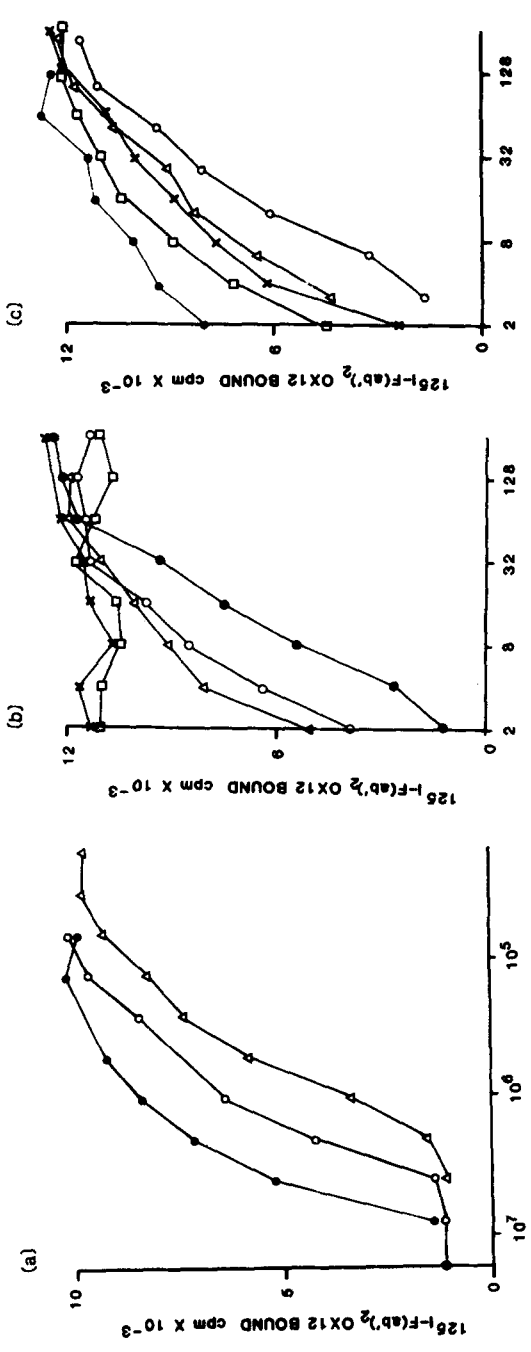

FIGURE 2. (*a*) Absorption assay of F4/80 Ag on intact RPC, TPC, and J774.2. RPC (○), TPC (●), and J774.2 (△) were suspended in PBS with 0.1% BSA and 10 mM sodium azide at 4°C, without homogenization or detergent treatment. Doubling dilutions of 80-μl aliquots were made in the same buffer and absorbed with 20 μl of F4/80 tissue culture supernatant at a final concentration of 1:80 in the incubation mix. (*b* and *c*) Quantitation of F4/80 Ag in different tissue extracts. Tissue extracts were mixed with an equal weight of extraction buffer, homogenized, and Triton X-100 added to a final concentration of 1% for 20 min on ice. Doubling dilutions of 80-μl aliquots of tissue extracts in the same detergent concentration were incubated with 20 μl of F4/80 tissue culture supernatant at a final concentration of 1:80. (*b*) Absorption curves for extracts of kidney (△), spleen (●), liver (○), brain (□), and a xenogeneic tissue extract from rabbit liver (×). (*c*) Absorption curves for extracts of femoral bone marrow (○), mesenteric lymph nodes (△), blood (●), small bowel (□), and large bowel (×).

TABLE I

Comparison of Surface F4/80 Ag Expression by Intact RPM, TPM, and J774.2 Measured by Different Immunoassays

	Surface F4/80 Ag per F4/80$^+$ cell (by absorption analysis)*	Average F4/80 site number per F4/80$^+$ cell	
		Direct binding assay: F4/80 Ab bound × 10^4	Indirect binding assay: F(ab')$_2$ OX12 bound × 10^4
		nmol	
RPC	5.2	8.8 ± 0.8	5.9 ± 0.5
TPC	1.0	2.0 ± 0.2	1.3 ± 0.1
J774.2	3.9	6.1 ± 1.0	7.1 ± 1.6

Average F4/80-binding site numbers per F4/80$^+$ cell were determined on freshly isolated RPC, TPC, and J774.2 in 10 mM sodium azide at 4°C. Total yields per mouse of RPC were 4.8 × 10^6, and of TPC, 1.8 × 10^7, of which 32 and 72%, respectively, were F4/80$^+$. The percentage of F4/80$^+$ cells in each population was determined by 1,000-cell differential counts on immunoperoxidase-labeled cytocentrifuged preparations. Results from direct and indirect binding assays are given as mean ± range of at least three determinations. Data from absorption analysis are obtained from the plots shown in Fig. 2*a*.

* Absorption analysis results are given in arbitrary units of F4/80 Ag adjusted such that the amount of F4/80 Ag per F4/80$^+$ cell on TPC is 1.0.

TABLE II

Extensive Perfusion Does Not Alter F4/80 Ag Content of Mouse Liver and Spleen

		n	Specific activity (× 10^4) of F4/80 Ag J774.2 equivalents per mg tissue	Weight of organ per mouse	Total activity of F4/80 Ag J774.2 equivalents per organ
				mg	
Liver	Unperfused	6	1.6 ± 0.3	1,012 ± 131	1.6 × 10^7
	Perfused	3	1.5 ± 0.3	1,060 ± 82	1.6 × 10^7
Spleen	Unperfused	7	5.0 ± 0.2	111 ± 20	5.5 × 10^6
	Perfused	3	4.6 ± 0.4	108 ± 15	5.0 × 10^6

F4/80 Ag content of unperfused and perfused organs was determined by absorption analysis against J774.2 as calibration standard. F4/80 Ag content is given in units of J774.2 equivalents. The J774.2 standard used was prepared in 1% Triton X-100, as for tissue extracts. Data are mean ± SD of at least three determinations.

stage of the indirect binding assay; thus this tissue cannot be used as a control for Ag specificity.

Finally, we evaluated the contribution of blood to assays for F4/80 Ag in highly vascularized organs such as liver and spleen, since blood contained small, but detectable amounts of Ag. Table II shows that extensively perfused murine liver and spleen contain amounts of F4/80 Ag similar to those of unperfused controls and that the contribution of blood was therefore negligible. These experiments also show the reproducibility of F4/80 Ag assays in organs from different animals. By reference to a standard we estimated that mouse liver and spleen contain ~1.6 × 10^7 and ~5 × 10^6 J774.2 equivalents, respectively.

Distribution of F4/80 Ag in Different Tissues of the Mouse. We next compared F4/80 Ag in extracts from liver, spleen, and other tissues of the mouse. Organ weights were recorded so F4/80 Ag per organ could also be calculated. All tissues extracts were obtained without perfusion and were prepared by homogenization followed by detergent treatment with 1% Triton X-100. Table III shows the weights of each mouse organ or tissue and the specific and total F4/80 Ag activity in these extracts. Table II gives results for liver and spleen, as discussed above. Representative absorption curves are shown in Fig. 2, *b* and *c*. All results are given in J774.2 equivalents. The concentrations of F4/80 Ag/mg tissue are highest in femoral bone marrow, spleen, mesenteric and cervical lymph nodes, and large bowel ($5.5–1.8 \times 10^4$ J774.2 equivalents/mg). Liver, kidney, Peyer's patches, small bowel, stomach, adrenals, testes, thymus, and lungs showed lower concentrations ($1.6–1.0 \times 10^4$ J774.2 equivalents/mg). Detectable but unquantifiable amounts were found in blood, plasma, and urine. The resuspended urine pellet after centrifugation contained no F4/80 Ag. F4/80 Ag was undetectable in brain and heart extracts, which showed little or no immunocytochemical labeling, respectively. Tissues not assayed for F4/80 Ag include skeletal muscle, skin, bone, other endocrine glands, placenta, blood vessels, and body fluids such as cerebrospinal fluid, breast milk, and lymph.

TABLE III

F4/80 Ag Content of Different Tissues and Organs of the Mouse

Tissue or organ	*n*	Specific activity ($\times 10^4$) of F4/80 Ag J774.2 equivalents per mg tissue or organ	Weight of tissue or organ	Total activity of F4/80 Ag J774.2 equivalents per tissue or organ
			mg	
Femoral bone marrow	3	5.5 ± 0.6	11.5 ± 1	6.3×10^5
Lung	3	1.0 ± 0.2	74 ± 20	7.4×10^5
Kidney	3	1.6 ± 0.5	138 ± 25	2.2×10^6
Brain	3	ND	403 ± 40	ND
Heart	2	ND	165 ± 10	ND
Testis	3	1.1 ± 0.2	88 ± 4	9.7×10^5
Bladder	1	<0.5	23 ± 7	—
Adrenals	2	1.3 ± 0.3	2.2 ± 0.3	2.8×10^4
Stomach	3	1.3 ± 0.3	154 ± 26	2.0×10^6
Small bowel	3	1.4 ± 0.3	940 ± 160	1.3×10^7
Large bowel	3	1.8 ± 0.3	785 ± 130	1.4×10^7
Cervical lymph nodes	3	2.3 ± 0.5	48 ± 3	1.1×10^6
Mesenteric lymph nodes	3	2.8 ± 0.6	46 ± 4	1.3×10^6
Peyer's patches	2	1.6 ± 0.4	34 ± 7	5.6×10^5
Thymus	3	1.1 ± 0.3	52 ± 3	5.5×10^5
Blood	3	<0.5	—	—
Urine	3	<0.5	—	—

The F4/80 Ag content of various tissues was determined by absorption analysis against J774.2 as standard. For paired organs, i.e., femurs, lungs, kidneys, testes, and adrenals, F4/80 Ag content is given for one organ. All tissue extracts were from unperfused animals and were prepared by homogenization and detergent treatment with 1% Triton X-100, as for the J774.2 standard. ND, not detectable. Data are given as mean ± range.

If the organ or tissue weight in a mouse is taken into consideration, the total amounts of F4/80 Ag in different tissues or organs can be compared (Table III and Fig. 3). Since femoral bone marrow contained the highest concentration of F4/80 Ag, it was important to obtain an estimate of the proportion of femoral to total bone marrow cells in a mouse. Such estimates have been obtained by others using 59-Fe labeling, and assuming that the rate of hemoglobin synthesis is the same in all parts of the marrow. Femurs were found to comprise 12% of the total marrow in male (C3H × C57BL) F_1 mice (16) and ~12.8% of the total mouse marrow (R. Schofield and T. M. Dexter, personal communication). This was taken into account in estimating total bone marrow F4/80 Ag content. The largest amounts of F4/80 Ag were found, in descending order, in liver, large bowel, small bowel, bone marrow, spleen, and kidney (1.6×10^7 to 2.2×10^6 J774.2 equivalents). Smaller amounts (2×10^6 to 5×10^4 J774.2 equivalents) are found in stomach, testes, lungs, mesenteric and cervical lymph nodes, thymus,

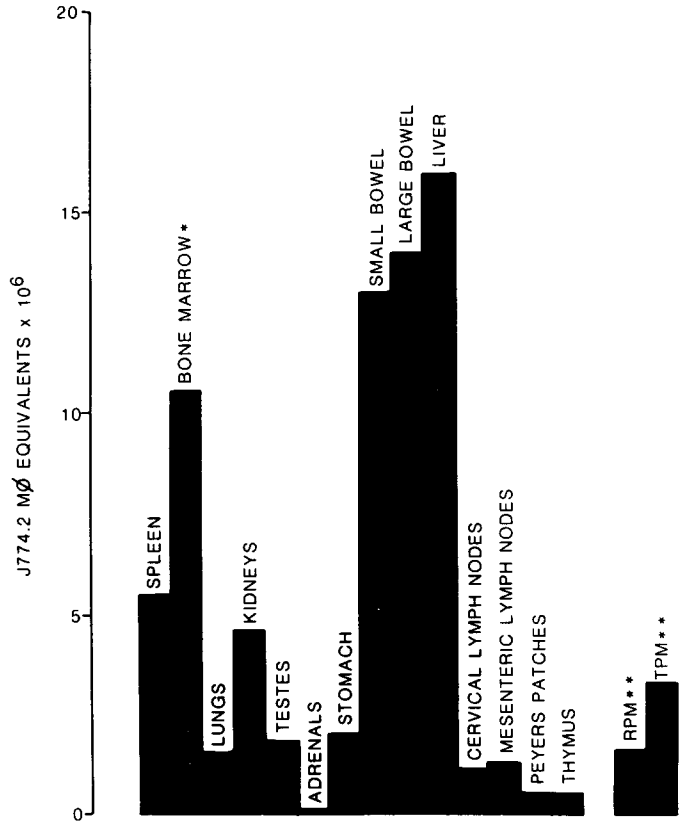

FIGURE 3. F4/80 Ag content of various tissues and organs of the C57BL/6 mouse. F4/80 Ag-specific activity was multiplied by total tissue or organ weight per mouse. For paired organs, the total weight of both organs has been taken into account. All values shown for tissues and organs are for unperfused animals. (*) The size of the total bone marrow compartment relative to the bone marrow content of two femurs has been estimated from ^{59}Fe-labeling data (see text). (**) Surface F4/80 Ag present on 4.8×10^6 RPC and 1.8×10^7 TPC, of which 32 and 72%, respectively, were F4/80$^+$ by immunoperoxidase labeling (see Table I).

Peyer's patches, and adrenals. For comparison, freshly harvested total RPM (1.5×10^6 Mϕ/mouse) and TPM (1.3×10^7 Mϕ/mouse) expressed 1.8×10^6 and 3.3×10^6 J774.2 equivalents of F4/80 Ag, respectively, on their surface.

Morphometric Analysis of F4/80$^+$ Areas in Mouse Liver. To try to relate F4/80 Ag levels in liver as estimated by absorption assay to those detected by immunohistochemistry, we analyzed by morphometry the amount of F4/80 labeling in sections of liver. The volume fraction of total liver tissue occupied by F4/80$^+$ areas was 3.0 ± 0.33%. Sinusoidal spaces, biliary capillaries, portal triads, and hepatic and central veins occupied 16%, while F4/80$^-$ areas occupied the remaining 81%.

Discussion

We have described a method to quantitate F4/80 Ag and estimate the Mϕ content of different tissues in the normal adult mouse. F4/80 Ag offers several advantages for direct immunochemical assay of Mϕ in the presence of other cells. It is highly specific for Mϕ localized by immunocytochemistry in a variety of sites and physiologic states (3), and is expressed by virtually all mature Mϕ analyzed by several methods in vitro (2, 17). Moreover, the antigenic determinant detected by monoclonal Ab F4/80 is remarkably stable to proteolysis, even after limited cleavage of the plasma membrane glycoprotein (P. M. Starkey, unpublished results). The Ag is not released by Mϕ in large amounts for transfer to other sites, as shown by discrete Mϕ-specific labeling in tissues and by detection of very low levels in plasma and urine. Specificity controls in the present study provided evidence that F4/80 Ag activity was absent from cells and tissues known to carry little or no Ag. The present assay is able to detect small amounts of F4/80 Ag, but is quantitative only at Ag levels $>4 \times 10^5$ J774.2 equivalents or 5×10^6 J774.2 equivalents per gram or milliliter extract.

To test the validity of the inhibition assay, we compared the amount of cell surface F4/80 Ag on three Mϕ populations, by absorption, and direct and indirect binding assays. The ratio of surface F4/80-binding sites on different cells obtained by absorption analysis agreed with ratios obtained by the other methods. Comparison of intact and detergent-lysed J774.2 indicated that the F4/80 intracellular pool is small (~25% of the total cellular content). In tissues, total cellular F4/80 Ag was measured after lysis and detergent treatment and results expressed in units of similarly treated J774.2 cells. The amount of F4/80 Ag present per tissue Mϕ is unknown, but an idea can be gained from comparison of F4/80 site numbers on intact RPM and J774.2, or approximately 59,000–88,000 and 61,000–71,000 sites, respectively.

Although single-cell analysis of peritoneal and bone marrow culture–derived Mϕ shows relatively uniform F4/80 expression within these populations, it is clear that the level of F4/80 Ag per cell varies with cell maturity, inflammatory and immune stimulation, and adhesion (2, 17, 18). We confirmed previous observations (2) that freshly isolated thioglycollate broth–elicited peritoneal Mϕ express less F4/80 on their surface than RPM, as do blood monocytes and immature Mϕ in spleen (S. H. Lee, unpublished results), and bone marrow (17). Unpublished studies (P. M. Starkey) with bacillus Calmette-Guérin–activated Mϕ, in which F4/80 Ag is down-regulated, show that synthesis of F4/80 Ag

decreases in these cells. It is likely that Mϕ populations in vivo display marked heterogeneity in F4/80 Ag content per cell; extrapolation from F4/80 Ag content to Mϕ cell number would thus be inaccurate. Variation in F4/80 Ag expression in resident tissue Mϕ of normal animals is probably due to immaturity. Estimates based on mature resident tissue Mϕ would underestimate the number of Mϕ in organs such as spleen, which contain a significant pool of recently generated Mϕ (19).

Absorption analysis gave figures of 1.6×10^7 and 5.0×10^6 J774.2 equivalents per liver and spleen, respectively. In these highly vascularized organs, the reduction of F4/80 Ag content after perfusion was negligible, consistent with the finding of very low Ag concentrations in blood. It is interesting to compare the Mϕ content of liver and spleen in J774.2 equivalents with yields of Mϕ from these organs after various methods of cell isolation. Other investigators have obtained the following Kupffer cell yields from mouse liver: $8–10 \times 10^6$/liver (D. Lepay and R. Steinman, personal communication), $8.3 \pm 0.3 \times 10^6$/g liver (20), $6.1 \pm 0.6 \times 10^6$/g liver or 9.1×10^6 per liver (21) and 3.6×10^6 per liver (22). By electron microscopy, a morphometric estimate of the volume fraction occupied by Kupffer cells in rat liver using 0.04-μm sections was $2.1 \pm 0.31\%$ (23). We found the volume fraction occupied by $F4/80^+$ positive labeling in the mouse liver to be $3.0 \pm 0.33\%$, using 5-μm sections. So far we know of no morphometric estimates of the absolute number of Kupffer cells in mouse or rat liver, or indeed of Mϕ in any other organs. From the spleen of the C57BL/6 mouse, $\sim 3.0 \times 10^6$ Mϕ can be harvested after collagenase perfusion via the portal vein and digestion (S. H. Lee, unpublished observations); in comparison, 4.0×10^6 Mϕ can be harvested after collagenase digestion from the BALB/c mouse and 2.1×10^6 Mϕ from the CBA mouse (19). Very similar estimates of 2.1×10^6 Mϕ per CBA mouse spleen and 4.5×10^6 Mϕ per BALB/c mouse spleen, after collagenase digestion, have been obtained by other investigators (24, 25).

Thus, the estimated number of J774.2 equivalents in liver and spleen appears compatible with known Mϕ yields after isolation. Mesenteric and cervical lymph nodes, another site of moderately heavy labeling with F4/80 upon immunohistochemistry (14), also showed concentrations of Ag in the expected range. However, we found detectable and quantifiable amounts of F4/80 Ag in Peyer's patches, where immunohistochemical labeling has been shown to be scant. This high value is likely due to mucosal lining attached to apparently isolated Peyer's patches. Gastrointestinal tissues were found to contain large amounts of F4/80 Ag. The gastrointestinal tract is therefore a potentially rich source of Mϕ which has hitherto been relatively neglected (26). Since animals used in this study were raised in isolation, but not on germ-free diets, gut tissues were exposed to foreign antigens and, not surprisingly, the small and large bowel together contain the largest amount of F4/80 Ag found in tissues of normal mice. Immunohistochemical labeling of F4/80 Ag in the gut clearly demonstrates $F4/80^+$ cells in large numbers in the lamina propria throughout the gastrointestinal tract (11).

Femoral bone marrow was the richest source of tissue F4/80 Ag, followed closely by spleen. Immunohistochemical analysis also demonstrated heavy labeling of hemopoietically active marrow and the red pulp of spleen (14). We used

a correction factor derived from ^{59}Fe-labeling to estimate the total F4/80 Ag content of marrow, but further studies are needed to examine the uniformity of Mϕ distribution. Isolation experiments in progress in our laboratory (P. Crocker, unpublished) indicate that femoral marrow contains a substantial population of mature stromal F4/80$^+$ Mϕ, as well as immature mononuclear phagocytes.

Other organs with significant F4/80 Ag content were kidney, testis, and lung. These findings are corroborated by immunohistochemical labeling that shows F4/80$^+$ cells in the renal medullary and cortical interstitium and the juxtaglomerular apparatus of the kidney (15), on 20% of interstitial cells of the testis (27), and on alveolar Mϕ (11). The total Ag content of the testes was surprisingly high, but the weight of both testes comprised 0.83% of the total weight of the adult mouse. Only organs with relatively high Ag content can be assayed by this method. Several tissues with lower F4/80 Ag–specific activity but which do contain Mϕ, fell outside the range of quantitation (e.g., blood) or detection (e.g., brain) (28).

It is interesting to speculate on the total number of Mϕ in the mouse from the data obtained in this study. A necessary assumption would be that resident tissue Mϕ contain on average the same amount of F4/80 Ag as the J774.2 cell. The limitations of such an assumption have already been discussed. A second assumption would be that tissues which were not assayed, e.g., skin and bone, or completely quantitated, e.g., lymphoid tissues, do not contain especially large Mϕ numbers. The tissues that were surveyed yielded 8×10^7 J774.2 equivalents in total (Fig. 3). A conservative estimate for the total number of Mϕ in a normal adult mouse would be $>1 \times 10^8$. From kinetic labeling studies, it is apparent that there is a continuous turnover of tissue Mϕ (1, 21, 29, 30), replenished by recruitment from the bone marrow or by local proliferation in tissues such as the spleen (19). It has been estimated (21) from kinetic radiolabeling studies that >56% of labeled blood monocytes enter the liver in the normal steady state. [^{3}H]thymidine selectively labels the immature Mϕ population, whereas F4/80 preferentially labels mature Mϕ (17). The latter would therefore give a more direct indication of the number of resident Mϕ in the liver compared with other tissues. Our data suggest that liver comprises <20% of the total Mϕ population.

We have presented a method to compare the F4/80 Ag content of different organs; it can also be used to analyze the recruitment of Mϕ to sites of inflammation. Studies to be reported elsewhere indicate that, in murine malaria, for example, total F4/80 Ag content in liver and spleen increase by ~8- and ~13-fold, respectively, at the peak of infection, and that Mϕ in these organs display other antigenic changes associated with cell activation. Antigen markers such as F4/80 therefore provide novel probes to measure the mass of the mononuclear phagocyte system in situ and its regulation within different compartments of the body.

Summary

We have estimated the macrophage content of different tissues of the normal adult mouse using F4/80, a highly specific antigen marker for mature mouse macrophages. An absorption indirect binding assay was used to quantitate F4/80 antigen against a calibration standard made from the J774.2 macrophage-like

cell line. The richest sources of tissue F4/80 antigen were found to be bone marrow, spleen, cervical and mesenteric lymph nodes, large bowel, liver, kidneys, and small bowel. The organs that have the highest total F4/80 antigen content are the liver, large bowel, small bowel, bone marrow, spleen, cervical and mesenteric lymph nodes, and kidney. We conclude that the mononuclear phagocyte system is mainly distributed in the gastrointestinal tract and liver, followed by hemopoietic and lymphoid tissues.

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References

1. van Furth, R., J. W. M. Van der Meer, A. Blusse van oud Alblas, and W. Sluiter. 1981. Development of mononuclear phagocytes. *In* Self Defense Mechanisms. Role of Macrophages. D. Mizuno, Z. A. Cohn, K. Takeya, and N. Ishida. University of Tokyo Press, Elsevier/North Holland Biomedical Press, Amsterdam. 25–44.
2. Austyn, J. M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11:805.
3. Hume, D. A., and S. Gordon. 1985. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80. *In* Mononuclear Phagocytes. IVth Leiden Conference. R. van Furth, editor. Martinus Nijhoff, The Hague. In press.
4. Williams, A. F. 1977. Differentiation antigens of the lymphocyte cell surface. *Contemp. Top. Mol. Immunol.* 6:83.
5. Ralph, R., J. Prichard, and M. Cohn. 1975. Reticulum cell sarcoma: an effector cell in antibody dependent cell-mediated immunity. *J. Immunol.* 114:898.
6. Sanford, K., W. R. Earle, and G. D. Likely. 1948. The growth in vitro of single isolated tissue cells. *J. Natl. Cancer Inst.* 9:229.
7. Hunt, S. V., and M. H. Fowler. 1980. A repopulation assay for B and T lymphocyte stem cells employing radiation chimaeras. *Cell Tissue Kinet.* 14:445.
8. Jensenius, J. C., and A. F. Williams. 1974. The binding of anti-immunoglobulin antibodies to rat thymocytes and thoracic duct lymphocytes. *Eur. J. Immunol.* 4:91.
9. Gordon, S., P. Starkey, D. Hume, A. Ezekowitz, S. Hirsch, and J. Austyn. 1984. Plasma membrane markers to study differentiation, activation, and localisation of murine macrophages. Ag F4/80 and the mannosyl fucosyl receptor. *In* Handbook of Experimental Immunology. D. M. Weir, C. A. Herzenberg, C. C. Blackwell, and C. A. Herzenberg. Blackwell Scientific Publications, Oxford. Fourth ed. In press.
10. Hsu, S. M., L. Raine, and H. Fanger. 1981. The use of avidine-biotin-peroxidase (ABC) complex in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577.
11. Hume, D. A., V. H. Perry, and S. Gordon. 1984. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80. Macrophages associated with epithelia. *Anat. Rec.* 210:503.
12. Aherne, W. A., and M. S. Dunnill. 1982. Morphometry. Edward Arnold (Publishers) Ltd., London. p. 181.
13. Anderson, J. A., and M. S. Dunnill. 1965. Observations on the estimation of the quantity of emphysema in the lungs by the point sampling method. *Thorax.* 20:642.

14. Hume, D. A., A. P. Robinson, G. G. MacPherson, and S. Gordon. 1983. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs. *J. Exp. Med.* 158:1522.
15. Hume, D. A., and S. Gordon. 1983. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80. Identification of resident macrophages in renal medullary and cortical interstitium and the juxtaglomerular apparatus. *J. Exp. Med.* 157:1704.
16. Briganti, G., V. Covelli, G. Silini, and P. N. Srivastava. 1970. The distribution of erythropoietic bone marrow in the mouse. *Acta Haematol. (Basel).* 44:355.
17. Hirsch, S., J. M. Austyn, and S. Gordon. 1981. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J. Exp. Med.* 154:713.
18. Ezekowitz, R. A. B., J. M. Austyn, P. D. Stahl, and S. Gordon. 1981. Surface properties by bacillus Calmette-Guérin-activated mouse macrophages. Reduced expression of mannose-specific endocytosis, Fc receptors, and antigen F4/80 accompanies induction of Ia. *J. Exp. Med.* 154:60.
19. van Furth, R., and M. M. C. Diesselhoff-den-Dulk. 1984. Dual origin of mouse spleen macrophages. *J. Exp. Med.* In press.
20. Knook, D. L., and E. Ch. Sleyster. 1977. Preparation and characterisation of Kupffer cells from rat and mouse liver. *In* Kupffer Cells and Other Liver Sinusoidal Cells. E. Wisse and D. L. Knook, Editors. Elsevier/North Holland Biomedical Press, Amsterdam. 273–288.
21. Crofton, R. W., M. M. C. Diesselhoff-den-Dulk, and R. Van Furth. 1978. The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. *J. Exp. Med.* 148:1.
22. Richman, L. K., R. J. Klingenstein, J. A. Richman, W. Strober, and J. Berzofsky. 1979. The murine Kupffer cell. I. Characterisation of the cell serving accessory function in antigen-specific T-cell proliferation. *J. Immunol.* 123:2602.
23. Blouin, A. 1977. Morphometry of liver sinusoidal cells. *In* Kupffer Cells and Other Liver Sinusoidal Cells. E. Wisse and D. L. Knook, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 61–71.
24. Humphrey, J. H., and D. Grennan. 1981. Different macrophage populations distinguished by means of fluorescent polysaccharides. Recognition and properties of marginal zone macrophages. *Eur. J. Immunol.* 11:221.
25. Joshua, D. E., J. H. Humphrey, D. Grennan, and G. Brown. 1980. Immunosuppression in BALB/c mice bearing the plasmacytoma TEPC-183: massive increase in red pulp macrophages induced by the tumor. *Immunology.* 40:223.
26. Tanner, A. R., M. J. P. Arthur, and R. Wright. 1984. Macrophage activation, chronic inflammation and gastrointestinal disease. *Gut.* 25:760.
27. Hume, D. A., D. Halpin, H. Charlton, and S. Gordon. 1984. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: macrophages of endocrine organs. *Proc. Natl. Acad. Sci. USA.* 81:4174.
28. Perry, V. H., D. A. Hume, and S. Gordon. 1985. Immuno-histochemical localisation of macrophages and microglia in the adult and developing mouse brain. *Neuroscience.* In press.
29. Blusse van oud Alblas, A., and R. Van Furth. 1979. Origin, kinetics, and characteristics of pulmonary macrophages in the normal steady state. *J. Exp. Med.* 149:1504.
30. Blusse van oud Alblas, A., H. Mattie, and R. Van Furth. 1983. A quantitative evaluation of pulmonary macrophage kinetics. *Cell Tissue Kinet.* 16:211.