

# EXPRESSION OF THE MACROPHAGE-SPECIFIC ANTIGEN F4/80 DURING DIFFERENTIATION OF MOUSE BONE MARROW CELLS IN CULTURE\*

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Cells of the mononuclear phagocyte system develop from precursors in the bone marrow (1), but little is known about their pathway of differentiation or its control *in vivo*. The development of morphology (2), cellular enzymes (esterases [3], peroxidase [4]), surface receptors (FcR [3, 5], C3b [6]), or enzyme secretion (lysozyme [3], plasminogen activator:PA<sup>1</sup> [7]) has been studied as macrophages ( $m\phi$ ) mature in the presence of various colony-stimulating factors (CSF) in culture. Surface antigens (Ag), such as F4/80 (8), which are found on mouse  $m\phi$  but not on other hemopoietic or unrelated cells, provide new markers to define stages in the differentiation of  $m\phi$  and to characterize and isolate their precursors.

In this paper, we analyze the expression of Ag F4/80 on cells of the  $m\phi$  lineage from progenitors in bone marrow to mature cells obtained after culture in the presence of L cell-conditioned medium (LCM). F4/80 is also used in a clonal approach to examine  $m\phi$  subset heterogeneity.

## Materials and Methods

*Animals.* Swiss mice and CBA T6T6 mice of both sexes were bred in this department. Similar results were obtained using either sex or strain.

*Cells.* Bone marrow (BM) and resident peritoneal cells were obtained as described (7, 8). Mass liquid cultures of BM were prepared in a BM culture medium (BMCM) consisting of Dulbecco's modification of minimal essential medium (DMEM) (high glucose), 15% heat-inactivated (HI) horse serum (Flow Laboratories, Inc., Irvine, Scotland), 1% HI fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 100  $\mu$ g/ml kanamycin, and 10–20% LCM. BM cells were cultured in 75- or 175-cm<sup>2</sup> tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif., and Nunclon, Gibco, Middlesex, England.) 25 or 50 ml, respectively, at  $8 \times 10^5$  nucleated cells/ml. Flasks were coated with gelatin as described (7).

Cultures were divided into a nonadherent cell (NAC) fraction, usually after 3 d, and adherent

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<sup>1</sup> *Abbreviations used in this paper:* Ab, antibody; AC, adherent cells; Ag, antigen; AR, autoradiography; BM, bone marrow; BMCM, BM culture medium; CFC, cluster-forming cell; CFU-c, colony-forming unit in culture; CSF, colony-stimulating factor; DMEM, Dulbecco's modification of Eagle's minimal essential medium; FACS, fluorescence-activated cell sorter; FITC, fluorescein-isothiocyanate; HI, heat-inactivated; IBA, indirect binding assay; L-15, Leibovitz's medium; LCM, L cell-conditioned medium;  $m\phi$ , macrophage; MPS, mononuclear phagocyte system; NAC, nonadherent cells; NSE 1, nonspecific esterase 1; NSE 2, nonspecific esterase 2; PA, plasminogen activator; PBA, PBS containing 0.1% bovine serum albumin and 10 mM azide; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; RAR, rabbit F(ab')<sub>2</sub> anti-rat Fab.

cells (AC). NAC were harvested by aspiration of medium. AC were recovered with high yield (>90%, viability >90%) from gelatin-coated flasks by treatment for 5 min at room temperature with 0.25% trypsin in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) and 1.5 mM EDTA.

Low density liquid culture was performed in 24-well Linbro plates (76-033-05; Linbro Chemical Co., Hamden, Conn.) with 11-mm Diam glass cover slips.  $2 \times 10^4$  cells in 1 ml BMCM per well were incubated in a humidified  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ .

*Preparation of LCM.* LCM was prepared as described (7), except that L cells were grown in medium with HI horse serum. Batches were assayed by a colony-forming unit in culture (CFU-c) assay and then used at concentrations producing a plateau level of colonies.

*Percoll Gradients.* Discontinuous Percoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden) density gradients were used to enrich preparations for mononuclear cells. Isosmolar Percoll was diluted to a final concentration of 49.5% in PBS and layered over a 90% Percoll cushion. NAC in Leibovitz's medium (L-15; Gibco, Paisley, Scotland) were layered over the gradient and centrifuged for 35 min at 900 *g* (MSE Mistral 2L centrifuge; MSE Scientific Instruments, Sussex, England). All procedures were at  $4^\circ\text{C}$ . The Percoll-medium interface contained the mononuclear cells that were >80% pure, and the interface separating the two Percoll layers contained >95% polymorphonuclear leukocytes (PMN).

*Antibodies.* Five monoclonal antibodies (Ab) were used. (a) F4/80 binds to mouse  $\text{m}\phi$  but not to PMN or lymphocytes (8). (b) 7/4 is a rat monoclonal Ab that binds to murine PMN but not to resident peritoneal  $\text{m}\phi$ , spleen lymphocytes, or thymocytes (S. Hirsch and S. Gordon, unpublished data). (c) 2.4G2 recognizes the IgG2b Fc receptor (9). The purified Fab fragment of this rat Ab was a gift of Dr. J. Unkeless (The Rockefeller University, New York). (d) Mac 1/70 binds to  $\text{m}\phi$  and PMN from mouse spleen, peritoneal exudates, and BM (10). The hybridoma cell line secreting this reagent was the gift of Dr. T. Springer (Harvard Medical School, Boston, Mass.). (e) The purified  $\text{F(ab)'}_2$  fragment of MRC OX12 mouse anti-rat kappa chain was a gift from Dr. S. V. Hunt (Sir William Dunn School of Pathology, Oxford, England), who also provided the same reagent conjugated with fluorescein isothiocyanate (FITC).

F4/80, 7/4, and Mac 1 were used as culture supernates. MRC OX12 was used as the second detecting reagent in most indirect radioimmune binding assays (IBA) and indirect immunofluorescence experiments, and affinity-purified rabbit  $\text{F(ab)'}_2$  anti-rat Fab (RAR) (11) was used in others.

*Radioimmunoassays.* Chloramine T was used to label MRC OX12 and RAR with  $^{125}\text{I}$  (11). IBA were performed with saturating concentrations of first Ab and either trace or saturating levels of second Ab (8).

*Esterase Staining and Autoradiography (AR).* Cytochrome preparations were made as described (8). For esterase staining, cells were fixed for 1 min in formol vapor followed by a 30-min incubation with  $\alpha$ -naphthyl butyrate (Sigma Chemical Co. Poole, Dorset) for nonspecific esterase 1 (NSE 1) or *N*-acetyl-DL-alanyl-1-naphthyl ester for nonspecific esterase 2 (NSE 2) (Sigma Chemical Co.) (3). Esterase-stained slides to be used for AR were postfixed in methanol for 5 min. AR was performed with K-2 emulsion (Ilford Nuclear Research, Cheshire, England), the slides were developed in D19 (Eastman Kodak Co. Ltd., England) after 3-6 d, and were washed and stained with Giemsa.

*Fluorescence-activated Cell Sorting and Analysis.* A Becton Dickinson fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Mountain View, Calif.) was used. FITC-conjugated OX12 or RAR was centrifuged (35,000 rpm for 30 min) before use. Both first and second Ab were used at saturation. Negative control cells were first incubated either with PBS containing 0.1% bovine serum albumin and 10 mM azide (PBA) or DE52-purified normal rat IgG, followed by FITC-conjugated reagents. For sorter analysis, cell aliquots were routinely  $10^7$  in 1 ml PBA. For cell sorting,  $\sim 3 \times 10^8$  nucleated cells were labeled in 10-ml siliconized tubes. Erythrocytes were lysed before labeling with Tris-buffered  $\text{NH}_4\text{Cl}$  (12). This treatment did not affect the cloning efficiency of CFU-c. Cells were collected from the sorter into siliconized tubes containing 2 ml L-15. FACS settings were: laser, 100 mW, 488 nm; photo multiplier tube, 680 V; low-angle scatter gain, 2/1; the fluorescence gain was as described below.

*Assays for CFU-c.* The number of CFU-c and cluster-forming cells (CFC) was determined as described (13, 14). Cloning medium contained 20% double-strength  $\alpha$ -MEM (Flow Laborato-

ries, Inc., Rockville, Md.), 15% Iscove's modified DMEM (as supplied by Flow Laboratories, Inc.), 15% HI fetal bovine serum, 10% HI horse serum, 20% LCM, and 20% Bacto-agar (Difco Laboratories, Detroit, Mich.) solution (1.65% in distilled water). Colonies (>50 cells) and clusters (between 5 and 50 cells) were scored at 7 d using a low-power inverted microscope. Colony morphology was determined by staining with Giemsa and counterstaining with hematoxylin after agar plugs had been dried onto glass slides and fixed with methanol (A. Burgess, personal communication).

*Adherence Assays.* (a) To test for the ability of F4/80 to block the acquisition of adherence to glass or gelatin-coated glass,  $2 \times 10^4$  3-d-cultured NAC were plated in each well of an 8-well Multitest microscope slide (Flow Laboratories, Inc.) with 20  $\mu$ l of complete medium and 20  $\mu$ l of saturating Ab or control medium. The slides were incubated for 24 h in a humidified CO<sub>2</sub> incubator. Remaining NAC were washed away with PBS. Slides were fixed with methanol, stained with Giemsa/May Grunwald, and adherent cells were scored.

(b) To test for the ability of F4/80 to block readherence, 4-d cultured m $\phi$  were removed from gelatin-coated flasks (as above) and incubated with Ab or control medium for 1 h at 4°C. Cells were then washed and plated on multitest slides in culture medium at  $1 \times 10^4$  per well. 2 h later, the slides were washed and adherent cells were counted.

*LCM Dependence of Ag Expression.* Adherent m $\phi$  were recovered from gelatin with trypsin, washed, and then replated with or without LCM. Control cultures were refed but not replated at this time. 6 and 24 h later, the cells were trypsinized, counted, and Ag F4/80 was determined by saturation IBA performed in suspension.

## Results

BM cells undergo a marked population shift when cultivated in the presence of LCM (5, 7). Most cells of the erythroid and lymphoid lineage disappear, and, during the first 2–3 d, cultures are predominantly granulocytic and nonadherent. Total cell numbers decrease initially as a large number of myeloid cells die. During this phase, progenitors for PMN and m $\phi$  (CFU-c) and other immature cells of the m $\phi$  lineage can be detected in the NAC fraction. A population of adherent m $\phi$  develops gradually and forms a confluent monolayer after 4–7 d. This system, from uncultured BM to late-stage adherent m $\phi$ , was used to characterize the life history of Ag F4/80.

*Expression of Ag F4/80 in Uncultured BM.* Radioimmune IBA and FACS analysis showed that only 6% of uncultured BM were labeled by Ab F4/80, whereas 35% of the cells were labeled by Ab 7/4 (Table I). The F4/80-labeled cells could not be

TABLE I  
*Ag F4/80 and 7/4 on Cells from Mouse BM and BM Cultures*

| Cell source                           | Percent nucleated cells labeled by |                 |                  |     |
|---------------------------------------|------------------------------------|-----------------|------------------|-----|
|                                       | FACS*                              |                 | AR‡              |     |
|                                       | F4/80                              | 7/4             | F4/80            | 7/4 |
| Uncultured BM                         | 5.7 ( $\pm$ 1)                     | 35.0 ( $\pm$ 3) | 5.6 ( $\pm$ 2)   | ND  |
| 3-day cultured BM NAC                 | 11.2 ( $\pm$ 4)                    | 82.0 ( $\pm$ 4) | 11.2 ( $\pm$ 2)  | ND  |
| Percoll mononuclear fraction from NAC | ND                                 | ND              | 31.3 ( $\pm$ 4)§ | ND  |
| 3-day cultured BM AC                  | NQ¶                                | ND              | 97.0 ( $\pm$ 2)  | 0.0 |
| 7-day cultured BM AC                  | NQ                                 | ND              | 98.0 ( $\pm$ 2)  | 0.0 |

\* Mean  $\pm$  SD of three experiments.

‡ Mean  $\pm$  range of two experiments: 1,500 cells counted in each experiment.

§ In IBA, specific binding of F4/80 to this fraction was 10,300 cpm and to the denser fraction (96% PMN, 4% mononuclear) 1,600 cpm. Binding of 7/4 to these fractions was 14,800 and 28,300 cpm, respectively.

|| ND, not done.

¶ NQ, not quantitated due to autofluorescence.

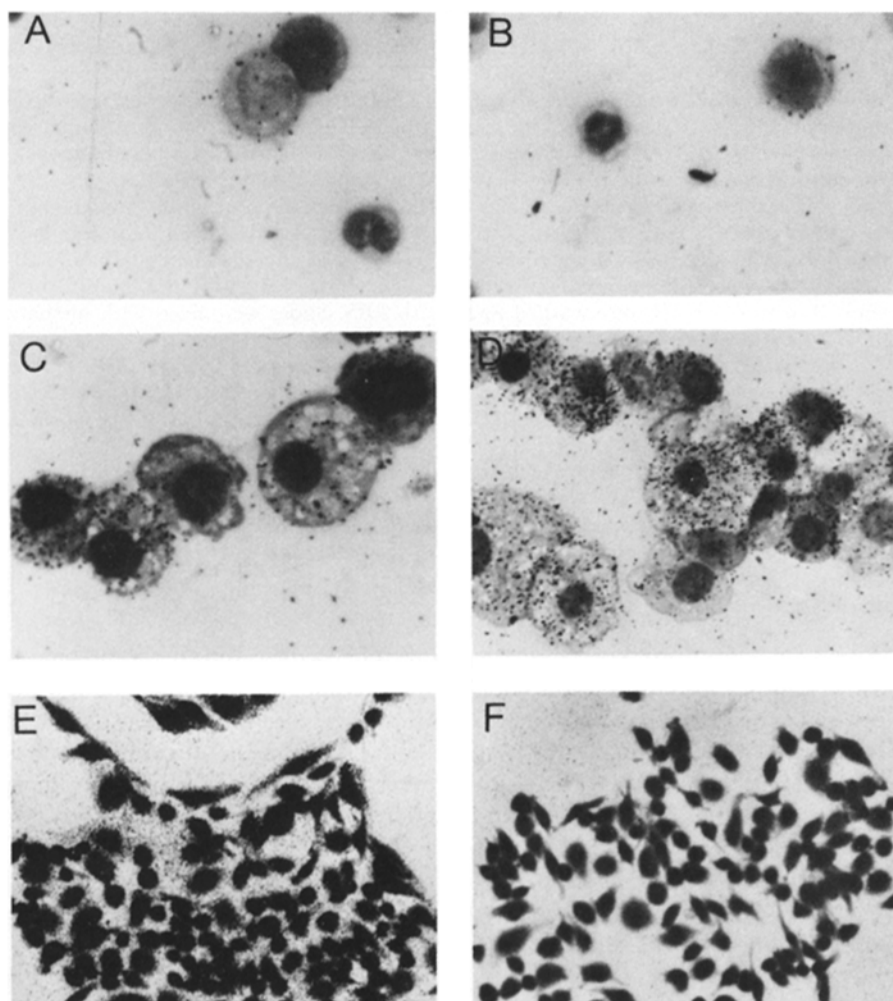


FIG. 1. AR of BM cells. A-D cytochrome preparations labeled with F4/80 and  $^{125}\text{I}$ -MRC OX12. (A) Uncultured BM. A labeled "promonocyte" lies adjacent to a cell with a doughnut nucleus similar to those described by Guilbert and Stanley (15). A mature PMN (lower right) is clearly unlabeled. (B) 3-d nonadherent BM. An immature promonocyte is clearly labeled, a mature PMN is negative. (C) 3-d adherent BM. All cells show moderately heavy labeling. (D) 7-d adherent BM. All cells are heavily labeled. (E, F) Ag expression on independent BM colonies (day 12) labeled with either F4/80 (E) or 7/4 (F). All cells in E are heavily labeled, and none is labeled in F.

depleted by 3-h culture in the presence of serum on either gelatin-coated or tissue culture plastic surfaces. F4/80-positive cells were heterogeneous and consisted of immature cells (10–16- $\mu\text{m}$  Diam nucleus:cytoplasm >1, nucleus deeply basophilic) to monocytes (Fig. 1 A). Less than 1% of recognizable PMN forms showed any significant labeling with F4/80. Approximately 61% of the labeled cells were stained by NSE 1, and almost all NSE 1-positive cells were F4/80 positive.

As relatively immature cells were labeled by F4/80, we considered the possibility that Ag F4/80 might be expressed on m $\phi$  progenitors.

BM cells were labeled with F4/80 and sorted on the FACS. F4/80-positive and

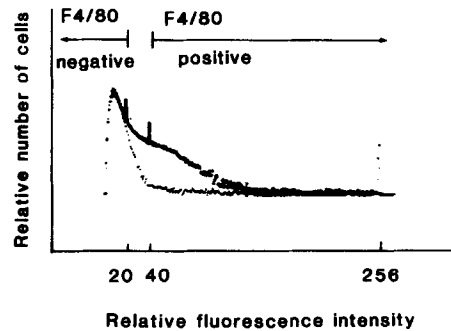


FIG. 2. FACS analysis of F4/80-labeled cells in uncultured BM. Relative fluorescence intensity of cells labeled with F4/80 and FITC-MRC OX12 (heavy trace) is compared with control cells without F4/80 (light trace). The vertical threshold markers indicate the parameters chosen for sorting F4/80-negative and F4/80-positive fractions that comprised 79.7 and 8.5% of the total nucleated cells, respectively. Fluorescence gain, 16/1.

negative populations were collected and, together with control populations, were assayed for the presence of CFU-c and CFC. A typical fluorescence profile obtained in such experiments can be seen in Fig. 2. Labeled, unsorted cells, or labeled machine-passed cells were used as controls. Values for progenitors obtained with these populations were almost identical, so the former control was usually used.

In six experiments, total cell yields were between 30 and 35%. The recovery of CFU-c was >85% of theoretical yield. The recovery of CFC was poorer, varying between 45 and 88%. Results from these experiments were similar, and Table II shows detailed data of two experiments in which >90% of the CFU-c and CFC were recovered in the F4/80-negative fraction. (In a seventh experiment, only 13% of CFU-c and 44% of CFC were recovered. Inexplicably, 54% of the CFU-c and 15% of the CFC were found in the F4/80-positive fraction in this experiment.) Almost all colonies (>95%) displayed m $\phi$  morphology, 4% were undifferentiated, and 1% were mixed (m $\phi$  and granulocyte).

Ag F4/80 expression thus begins relatively early in the m $\phi$  lineage in that NAC with immature morphology can be labeled with F4/80, but Ag appears at a stage beyond the CFU-c and CFC progenitors.

*Expression of Ag F4/80 in Cultured BM.* (a) Nonadherent cells were used after 3 d because it is then possible to obtain nonadherent precursors and adherent m $\phi$  from the same cultures. We were able to recover  $1-2 \times 10^7$  AC and  $3-6 \times 10^6$  NAC from a 175-cm<sup>2</sup> culture flask on day 3 (original inoculum  $3 \times 10^7$  cells). The NAC fraction contained 80–85% of PMN at various stages of maturity, as shown by morphologic or FACS analysis after labeling with Ab 7/4, whereas F4/80 labeled 11% of the NAC (Table I).

We attempted to separate cells of the m $\phi$  and myeloid series on a Percoll density gradient. The recovery of mononuclear cells was usually >80%, and that of mature PMN >55%. With IBA it could be shown that almost all the F4/80-binding cells segregated with the mononuclear fraction, of which 31% could be labeled with F4/80 (Table I). It is likely that less dense immature PMN cofractionated with the F4/80-positive cells because a substantial amount of 7/4 binding to this fraction was detected in IBA.

F4/80-positive cells in the unfractionated or the mononuclear-enriched fraction

TABLE II  
Ag F4/80 on m $\phi$  CFU-c and CFC

| Experiment | Fraction        | Percent of total cells* | CFU-c/10 <sup>5</sup> cells‡ | Total CFU-c recovery  | Percent of expected recovery§ | Percent of total recovery |
|------------|-----------------|-------------------------|------------------------------|-----------------------|-------------------------------|---------------------------|
| A1         | Labeled control |                         | 244.0 ± 65                   |                       |                               |                           |
|            | F4/80 negative  | 76.1                    | 249.0 ± 43                   | 3.7 × 10 <sup>4</sup> | 98.7                          | 99.1                      |
|            | F4/80 positive  | 5.7                     | 69.0 ± 16                    | 2.7 × 10 <sup>2</sup> | 0.9                           | 0.9                       |
| A2         | Labeled control |                         | 120.0 ± 24                   |                       |                               |                           |
|            | F4/80 negative  | 38.0                    | 118.0 ± 25                   | 7.1 × 10 <sup>3</sup> | 91.1                          | 98.4                      |
|            | F4/80 positive  | 4.7                     | 25.0 ± 7                     | 1.2 × 10 <sup>2</sup> | 1.5                           | 1.6                       |
|            |                 |                         | CFC/10 <sup>5</sup> cells‡   | Total CFC recovery    |                               |                           |
| B1         | Labeled control |                         | 541.0 ± 129                  |                       |                               |                           |
|            | F4/80 negative  | 76.1                    | 475.0 ± 46                   | 5.9 × 10 <sup>4</sup> | 86.2                          | 99.0                      |
|            | F4/80 positive  | 5.7                     | 159.0 ± 47                   | 6.2 × 10 <sup>2</sup> | 0.9                           | 1.0                       |
| B2         | Labeled control |                         | 366.0 ± 95                   |                       |                               |                           |
|            | F4/80 negative  | 38.0                    | 181.0 ± 53                   | 1.1 × 10 <sup>4</sup> | 46.0                          | 94.6                      |
|            | F4/80 positive  | 4.7                     | 131.0 ± 37                   | 6.3 × 10 <sup>2</sup> | 2.0                           | 5.4                       |

Sorted and control fractions were cloned in agar and scored as described in Materials and Methods. A1 and B1 constitute the same experiments as do A2 and B2. More restrictive sorting parameters used for A2/B2 account for lower percentage of cells collected.

\* The value represents the percentage of total nucleated cells sorted into each fraction.

‡ Frequency ± SD of quadruplicate determinations. Frequency in controls varied with LCM batch.

§ The expected recovery of progenitor cells was calculated from the frequency of each progenitor in the labeled control population and the total number of cells recovered from the FACS.

|| The percentage of all colonies recovered in each sorted fraction.

resembled immature m $\phi$  (Fig. 1 B), but more mature forms were also observed. The labeled cells could not be depleted by a further 3-h culture over gelatin or tissue culture plastic. As in uncultured BM, all NSE 1-stained cells were F4/80 positive and ~35% of the F4/80-positive cells were NSE 1 negative. Cells in the NAC fraction gave rise to adherent m $\phi$  within 24 h when transferred to new culture vessels in the presence of LCM.

(b) The adherent cells generated in mass liquid cultures under the present conditions are almost all m $\phi$ , as defined by phase-contrast microscopy (nucleus:cytoplasm  $\ll$  1, ruffled membranes, prominent phase-dense and phase-lucent cytoplasmic vesicles). Approximately 97% of these cells label with F4/80 (Table I), and a similar percentage stain with NSE 1, whereas none is labeled by anti-PMN Ab 7/4. This population is highly autofluorescent when excited at 488 nm, thus severely restricting work on the FACS.

*Quantitation of Ag F4/80 on Cultured Populations.* When the populations described in the preceding sections were analyzed on the FACS, it was evident that the cells labeled by Ab F4/80 showed increasing fluorescence intensity in the following order: uncultured BM < nonadherent cultured BM (day 3) < early adherent cells (day 3) < later adherent cells (day 5-9). The progressive shift of the fluorescence profiles to the right (brighter) and the lower amplification gains needed for detection can be seen in Fig. 3. Because both first and second Ab were used at saturation, these observations suggested that the quantity of Ag per cell increased during maturation.

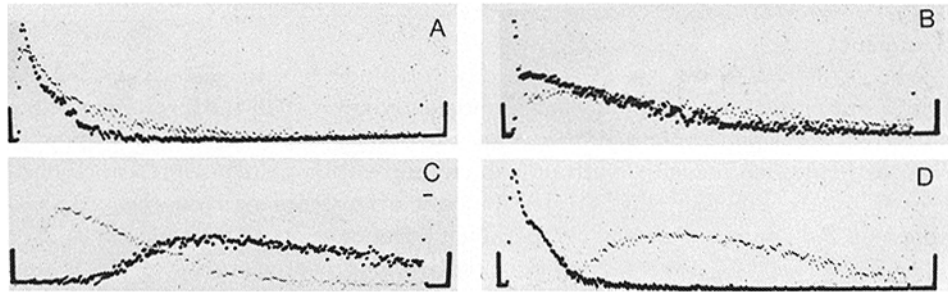


FIG. 3. FACS analysis of F4/80 labeled cells in uncultured and cultured BM. Cells were labeled with F4/80 and FITC-RAR. (A) Relative fluorescence intensity of F4/80-labeled cells in uncultured BM (heavy trace) compared with that of labeled cells in 3-d nonadherent BM (light trace). Labeled cells 6 and 11%, respectively, relative to controls (data not shown). Fluorescence gain, 4/1. (B) Relative fluorescence intensity of 3-d adherent BM (light trace) compared with autofluorescence control (cells incubated without FITC-RAR) (heavy trace). The level of autofluorescence makes quantitation of this population on the FACS impossible. Fluorescence gain, 0.5/1. (C) Relative fluorescence intensity of 9-d adherent cells (heavy trace) compared with autofluorescence control. Even with the large amount of autofluorescence, it is clear that the entire population is labeled. Fluorescence gain, 0.5/1. (D) Relative fluorescence intensity of 3-d nonadherent cells (heavy trace) compared with 9-d adherent cells (light trace). When the same fluorescence gain is used for both populations, the increase in the amount of Ag per cell is evident. Fluorescence gain, 0.5/1.

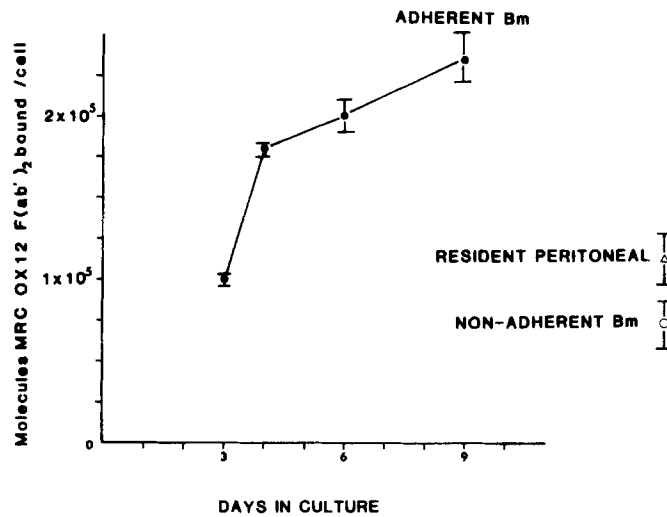


FIG. 4. Expression of Ag F4/80 on BM-cultured  $m\phi$ . All cell populations were assayed in suspension. Each point represents the mean  $\pm$  range of duplicate determinations. The results in four experiments showed an identical trend.

Ag levels on different populations were assayed quantitatively using IBA with both first and  $^{125}\text{I}$ -labeled second Ab at saturation. These experiments were combined with AR to correct for the percentage of labeled cells.

Previous experiments had shown that the amount of  $\text{F(ab)'}_2$  second Ab found was much lower on adherent cells than on identical populations assayed in suspension. The reason for this discrepancy is not known. As some of the populations were nonadherent, all quantitative assays were performed in suspension. Adherent cells were recovered from gelatin-coated surfaces either with trypsin or with 3 mM EDTA

in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS in the cold. Ag levels were identical with either of these treatments.

The F4/80 cells in the NAC fraction (day 3) bound  $\sim 6 \times 10^4$  molecules  $^{125}\text{I}$ -MRC OX12 F(ab')<sub>2</sub> per cell. This value was consistently lower than the levels detected on all the adherent populations studied. After the development of an adherent population, Ag expression increased with time in culture, until a plateau level was reached, usually by the 9th day (Fig. 4). The amount of Ag expressed on the surface of adherent, 3-d cultured cells was comparable to that on resident peritoneal  $\text{m}\phi$ .

*Ag F4/80 and Cell Adherence.* One of the defining characteristics of  $\text{m}\phi$  is their ability to adhere to various surfaces. BM  $\text{m}\phi$  appear to become more adherent with time in culture. Because Ag F4/80 is found specifically on  $\text{m}\phi$  and its expression increases with time in culture, we investigated the possible role of Ag F4/80 in cell adhesion.

Nonadherent precursors in 3-d cultures of BM mature into adherent cells in the presence of LCM. F4/80 and two other monoclonal Ab were incubated at saturating levels with such nonadherent cells to test whether they could prevent their subsequent adherence to glass or gelatin surfaces. There was no inhibition of adherence by any of these reagents compared with a control supernate (Table III, experiment 1).

Furthermore, F4/80 preincubated with detached, 4-d cultured BM  $\text{m}\phi$  was unable to prevent the readherence of these cells (Table III, experiment 2). In both types of assay, more cells were found on gelatin than on glass surfaces. Ag F4/80 therefore does not play a part in the development of adherence or the continued expression of adherence by  $\text{m}\phi$  under the present conditions.

*Role of LCM in Ag Expression.* Growth of BM cells (13, 14) and expression of certain differentiated functions, e.g., secretion of PA (7), depend on the presence of CSF in the culture medium. Because LCM is present continuously and Ag F4/80 increases during cultivation, we examined the possible role of LCM in its expression. 3- and 4-d adherent cells were used because the maximum increase in Ag F4/80 occurs during this period. Cells were trypsinized and washed to ensure CSF-depletion and then were replated with or without LCM. The results obtained in such an experiment are shown

TABLE III  
*Monoclonal Ab Do Not Affect Adherence of BM  $\text{m}\phi$*

| Experiment | Ab                | Cells per field adhering to* |        |          |        |
|------------|-------------------|------------------------------|--------|----------|--------|
|            |                   | Glass                        | % PMN‡ | Gelatin  | % PMN‡ |
| 1          | Control supernate | 36 ± 2                       | 4.8    | 59 ± 9   | 0.0    |
|            | F4/80             | 40 ± 9                       | 1.2    | 70 ± 12  | 0.0    |
|            | 7/4               | 33 ± 6                       | 0.0    | 57 ± 6   | 0.0    |
|            | Mac 1             | 43 ± 12                      | 3.5    | 58 ± 13  | 2.6    |
| 2          | F4/80             | 65 ± 9                       | ND§    | 119 ± 35 | ND     |
|            | 7/4               | 72 ± 13                      | ND     | 128 ± 21 | ND     |

Experiment 1 shows an attempt to block acquisition of adherence by 3-d cultured NAC. Experiment 2 was an attempt to prevent adherence of detached 4-d cultured BM  $\text{m}\phi$ .

\* Value represents the mean ± SD of six fields from duplicate wells. Counted at  $\times 400$  magnification.

‡ Remainder of cells were all  $\text{m}\phi$ .

§ ND, not done, as all these cells were  $\text{m}\phi$ . Similar results were obtained in two independent experiments.



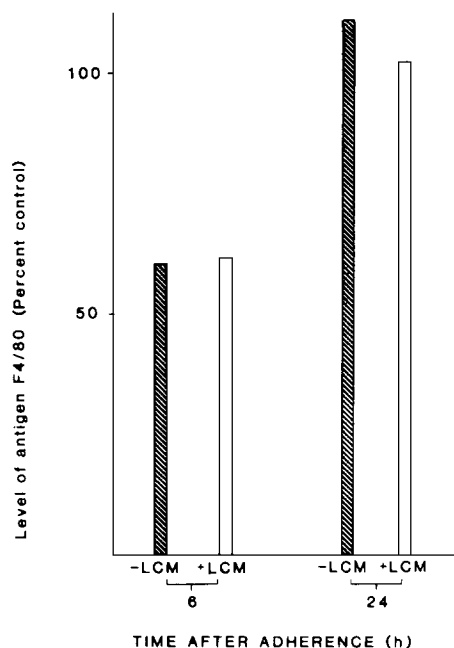


FIG. 5. The effect of LCM and of cell adherence on expression of Ag F4/80 by cultured BM  $m\phi$ . Ag levels are expressed as the amount of MRC OX12 F(ab')<sub>2</sub> bound per cell as a percentage of controls refed but not replated at T<sub>0</sub>. Control populations bound  $1.5 \times 10^5$  molecules of MRC OX12 F(ab')<sub>2</sub> at 6 h and  $2.3 \times 10^5$  molecules at 24 h.

in Fig. 5. 6 h after replating, there was a large decrease in the amount of Ag F4/80 per cell relative to controls that had been refed but not replated. 18 h later, the amount of Ag on cells cultured with or without LCM was the same as that found on control cells, even though Ag on control cells increased by >50% during the 24 h of the assay. Ag F4/80 expression thus appears to be independent of LCM. The process of adherence itself, however, exerts a significant effect on Ag expression, as shown by the large decrease at 6 h.

*Clonal Analysis of Ag Expression.* The expression of F4/80 and of several other Ag was studied on independent colonies derived from BM in culture to further define heterogeneity in this system. Isolated  $m\phi$  colonies develop when BM cells are plated at low density in liquid culture with LCM. By the 4th day, these colonies consist of 8–12 cells, although some already have >35 cells. These colonies appeared to consist of cells of the  $m\phi$  lineage only because all colonies and cells were stained by NSE 1 and none with NSE 2 (Table IV, experiment C). After 8 d, most colonies contained 50–70 cells, and by the 12th day colony size ranged up to several hundred cells. Morphology at all stages, like colony size, was variable, with colonies of well-spread cells sometimes lying adjacent to colonies containing mainly rounded cells.

At various times, glass cover slips with colonies were incubated with saturating levels of first Ab (40  $\mu$ l/cover slip), washed, and then incubated with <sup>125</sup>I-labeled second Ab. After washing, bound radioactivity was determined on a Packard Gamma Counter (Packard Instrument Co. Inc., Downers Grove, Ill.) and the cover slips were processed for AR. As shown in Table IVa and Fig. 1 E, all colonies and all the cells in these colonies were heavily labeled with Ab F4/80 after 8 d in culture. Similar results

TABLE IV *Clonal Analysis of Ag Expression*

| (a) | Ab             | Day 8              |          | Day 12             |          |
|-----|----------------|--------------------|----------|--------------------|----------|
|     |                | Number of colonies | Positive | Number of colonies | Positive |
|     |                |                    | %        |                    | %        |
|     | Medium control | 28                 | 0        | 140                | 0        |
|     | Mac 1          | 87                 | 100      | 96                 | 99       |
|     | 2.4G2          | ND*                | ND       | 125                | 99       |
|     | 7/4            | 28                 | 0        | 111                | 0        |
|     | F4/80          | 107                | 98       | 73                 | 100      |

Colonies derived from mouse BM after 8 and 12 d in culture.  
Positive colonies contained very few, if any, unlabeled cells. Almost all the cells in negative colonies were unlabeled.  
\* ND, not done.

| (b) | Experiment | Ab or esterase stain | Colonies counted* | Positive colonies | Total cells counted | Positive cells |
|-----|------------|----------------------|-------------------|-------------------|---------------------|----------------|
|     |            |                      |                   | %                 |                     | %              |
|     | A          | Medium               | 39                | 0.0               | ND‡                 | ND             |
|     |            | Mac 1                | 66                | 94.0              | ND                  | ND             |
|     |            | 7/4                  | 34                | 0.0               | ND                  | ND             |
|     |            | F4/80                | 39                | 59.0              | 188                 | 57.0           |
|     | B          | 7/4                  | 21                | 0.0               | 212                 | 1.4            |
|     |            | F4/80                | 37                | 95.0              | 369                 | 86.0           |
|     | C          | NSE 1                | 28                | 100.0             | 89                  | 100.0          |
|     |            | NSE 2                | 25                | 0.0               | 156                 | 0.0            |

Ag expression and esterase staining in early colonies derived from mouse BM after 4 d in culture.  
\* Colonies with <10 cells were scored as positive if >2 cells were labeled. All cells on the cover slips were counted.  
‡ ND, not done.

were obtained with 2.4G2 and Mac 1, whereas the anti-PMN Ab 7/4 did not label any colonies or cells (Fig. 1F).

As shown in Table IVb, the percentage of early colonies (4 d) that could be labeled with F4/80 showed some variability (59–95%). Although 57–86% of all cells in these colonies could be labeled, there was a significant negative population, and positive cells were often labeled less heavily. Labeled and unlabeled cells in these preparations were often immature morphologically and could not be distinguished.

These results indicated that although the level of Ag varied considerably during cell maturation, m $\phi$  in colonies of independent origin were all able to express several antigenic markers.

### Discussion

The BM culture systems used in this study provide different stages in m $\phi$  development and make it possible to define expression of m $\phi$ -specific Ag such as F4/80 on precursors and their progeny. The CFU-c and CFC progenitor cells in freshly isolated BM lack Ag F4/80 but give rise to F4/80 positive m $\phi$  in culture. The Ag is expressed before precursors become adherent, and the level of Ag increases as adherent m $\phi$  proliferate further.

F4/80 binds to 6% of cells in uncultured BM. Because the frequency of CFU-c and

CFC is ~0.1% (13, 14) and these progenitors are F4/80 negative by FACS sorting and cloning, the labeled cells probably represent more mature stages of the  $m\phi$  lineage. Labeled cells could not be depleted by adherence; they resembled immature "mononuclear" cells morphologically, and we have not observed labeling by F4/80 of any recognizable stage of PMN, lymphoid, or erythroid cells in these or previous (8) studies. Guilbert and Stanley (15) have noted a similar population in fresh BM that binds  $^{125}\text{I}$ -CSF, but we do not know if F4/80 and CSF bind to the same cells. These two markers clearly do not overlap completely because the CFU-c must express a receptor for CSF to grow in LCM.

We studied NAC and AC obtained after 3-d culture to enrich for  $m\phi$  precursors at the time when the cells first become adherent. The yield of NAC is low, and up to 85% of these cells are PMN as judged by morphology and labeling with Ab 7/4, but this fraction contains  $m\phi$  precursors that have not yet acquired the ability to adhere to a gelatin-coated surface. The cells remain nonadherent when passed over gelatin a second time and give rise to adherent  $m\phi$  with high plating efficiency when cultured overnight in the presence of LCM. Approximately 11% of the NAC (70% of the non-PMN fraction) express low levels of Ag F4/80, and many, but not all, stain for NSE 1. Because all NSE 1-positive cells label with F4/80, the Ag is probably first detectable at an earlier stage than the esterase product. The remaining F4/80-negative cells, 4% of total, ~30% of the non-PMN fraction, may be earlier stages of  $m\phi$  or remnants of the lymphoid or erythroid series. It is not known if nonadherent  $m\phi$  precursors are related to NK or K cells (16, 17). Because their physical properties do not permit complete separation from PMN, depletion with anti-PMN Ab should make it easier to purify and characterize the  $m\phi$  precursors in these populations.

The AC that accumulate from the 3rd day of culture are almost all labeled by F4/80, and Ag levels increase progressively. These cells show characteristic  $m\phi$  morphology and express other markers such as FcR and NSE 1. Because Ag F4/80 precedes adherence, we asked whether the Ag plays a role in the acquisition of adherence, but no such role could be detected. As in previous studies with peritoneal  $m\phi$ , adherence itself resulted in a transient decrease of detectable Ag, and it is possible that the small proportion of F4/80-negative AC in early clones is due to adherence as well as immaturity and decreased expression during mitosis (S. Hirsch and S. Gordon, unpublished data) in cells with low levels of Ag. Once higher levels of Ag are present, all adherent  $m\phi$  express Ag during proliferation.

Growth in this system depends on the presence of CSF, but LCM did not directly influence Ag expression under conditions in which CSF controls PA secretion and DNA synthesis. Ag F4/80 increases steadily as cells mature in culture, and resident peritoneal  $m\phi$ , which express other markers of the mature  $m\phi$ , also express a relatively high level of this Ag, but a pattern of gradual increase in Ag with maturation may not necessarily occur during differentiation in vivo. For example, bacillus Calmette-Guérin-activated peritoneal  $m\phi$  display increased levels of Ia Ag but markedly reduced levels of Ag F4/80, FcR, and mannose-specific receptors for endocytosis (18). Different patterns of Ag expression during the life history of the  $m\phi$  may emerge for Ia and other Ag than the one described here for Ag F4/80.

A particular advantage of the BM culture system is that large numbers of clones can be derived by selecting only for the ability to grow in the presence of CSF. If the mononuclear phagocyte system consisted of more than one lineage, this could be

reflected in differential expression of Ag by independent colonies. Although colonies did show distinctive morphology, we did not observe heterogeneity of F4/80 or other Ag in these studies.

Another mechanism by which diversity could be generated among peripheral m $\phi$  is by selective recruitment of circulating monocytes into certain tissues or by the acquisition of new markers in their local environment. We have not detected differences within populations of resident, elicited, or bacillus Calmette-Guérin-activated peritoneal m $\phi$ , although the levels of Ag expressed varied markedly among populations (8, 18). Because Ag F4/80 has also been detected on blood monocytes, lung, and spleen m $\phi$ , the present evidence would indicate that variation in expression of Ag F4/80 can be ascribed to the stage of development of the m $\phi$  or its state of activation rather than to independent subsets of mononuclear phagocytes. Nevertheless, further studies with F4/80 and new markers are needed with colonies grown from BM and other sites such as blood, lung, and the peritoneal cavity, as well as after stimulation *in vivo*.

Earlier studies reported that CFU-c are able to give rise to both m $\phi$  and granulocytes (19, 20). In the present studies, cells that expressed Ag F4/80 seem to be committed to the m $\phi$  lineage. However, because the LCM used as the source of CSF favors development of m $\phi$ , it will be necessary to examine this further with a CSF preparation that yields a more balanced growth of PMN and m $\phi$ . Ab F4/80 and other Ab specific for m $\phi$  and PMN provide ideal reagents to study commitment of precursors to each lineage.

### Summary

We have defined the expression of the macrophages (m $\phi$ )-specific antigen (Ag) F4/80 during differentiation in culture. The progenitor cells—the colony-forming unit in culture and cluster-forming cell—lacked Ag F4/80 but gave rise to colonies of F4/80-positive adherent m $\phi$ , as shown by fluorescence-activated cell sorting and clonal assays with L cell-conditioned medium as the source of growth factor.

Ag F4/80 first appeared on a nonadherent precursor found in mass liquid BM cultures after 3 d. Once adherent, m $\phi$  expressed high levels of Ag F4/80 and other markers. The role of L cell-conditioned medium and of adherence on expression of Ag F4/80 was also examined. Clonal analysis of F4/80 and other Ag, Mac-1, and 2.4G2 (FcR) showed that all cells in all independent colonies come to express these markers.

These studies establish that F4/80 is a marker for the more mature stages of m $\phi$  development and that Ag expression increases progressively during maturation *in vitro*. Heterogeneity of Ag expression can be ascribed to variation in development and not to independent subsets of the m $\phi$ .

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