CELL REGULATION, Vol. 1, 403–413, April 1990

# Prostaglandin E specifically upregulates the expression of the mannose-receptor on mouse bone marrow–derived macrophages

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**The macrophage mannose receptor (MMR)[1] facilitates the binding and internalization of microorganisms and glycoproteins with terminal mannose residues. The receptor is progressively upregulated as bone marrow precursor cells mature into macrophages and thus may serve as a marker of differentiation. Prostaglandins of the E series (PGE) are known inhibitors of monocyte and macrophage precursor proliferation, an effect often associated with cellular maturation. MMR expression was therefore assessed after exposure of bone marrow macrophage precursor (BMMP) cells to these prostanoids. Receptor expression was determined by ligand binding and via immunoprecipitation of newly synthesized receptor molecules. $PGE_1$ and $PGE_2$ at $10^{-9}$–$10^{-6}$ M upregulated MMR surface expression and biosynthesis four- to sixfold in a dose-dependent manner. BMMPs responsive to prostaglandins were characterized by plastic adherence, F4/80 antigen expression, and nonspecific esterase activity. Prostaglandins accelerated the expression of the MMR in cells by 48–72 h, with maximal levels of receptor expression being identical in control or treated cells. Thus, prostaglandins enhanced mannose receptor expression in adherent but not fully differentiated macrophage precursors. This effect is specific for PGE and is mimicked by dibutyrl cyclic AMP. These results indicate that prostaglandins accelerate MMR expression and hence the differentiation of macrophage precursor cells. Cells resident in the bone marrow secrete abundant prostaglandins, suggesting that a paracrine mechanism may exist to regulate MMR expression and function.**

## Introduction

Bone marrow macrophage precursors (BMMPs), as they differentiate, progressively acquire some phenotypic features important in host defense. One such function is the capacity to internalize bacteria and other particulate matter through nonopsonin, receptor-mediated phagocytosis. It is likely that recognition of terminal mannose residues on targeted particles by cell surface macrophage mannose receptors (MMRs) plays an important role in this process (Stahl *et al.*, 1980). This protein (MMR) is a 172-kDa species unique to members of the monocyte-macrophage family (Wileman *et al.*, 1986), the expression of which is dampened upon activation by bacteria or directly by $\gamma$-interferon (Ezekowitz and Gordon, 1982). Alternatively, the number of MMRs appearing on the surface of BMMPs increases with time in culture or with exposure to vitamin D (Bar-Shavit *et al.*, 1983; Shepard and Stahl, 1984; Clohisy *et al.*, 1987). As both duration of culture and vitamin D treatment prompt macrophage maturation, rapid appearance of cell surface MMR is believed to be a marker of accelerated differentiation (Ezekowitz and Gordon, 1982; Shepherd *et al.*, 1982; Clohisy *et al.*, 1987, 1989).

Eicosanoids are molecules generally found in states such as inflammation, in which they

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[1] Abbreviations: $\alpha$-mem, $\alpha$-modification of Eagle's Medium; AP, aminophylline; BMMP, bone marrow macrophage precursor; BSA, bovine serum albumin; cAMP, cyclic AMP; CSF-1, colony stimulating factor-1; DMSO, dimethylsulfoxide; FCS, fetal calf serum; HBSS, Hanks Balanced Salt Solution; HHBG, HBSS with glucose and BSA; IBMX, 3-isobutyl-l-methyl-xanthine; $^{125}$I-ManBSA, $^{125}$I-mannosylated BSA; $LTB_4$, leukotriene $B_4$; Man-BSA, mannosylated bovine serum albumin; MMR, macrophage mannose receptor; PBS, phosphate-buffered saline; PGE, prostaglandin E; $PGI_2$, prostacyclin; $TxB_2$, thromboxane $B_2$.

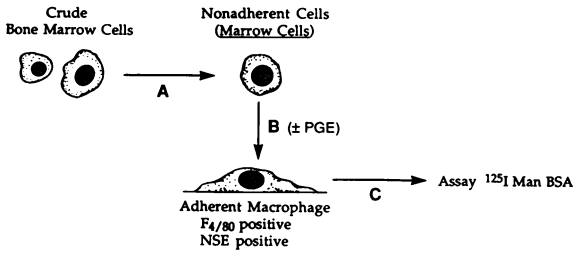


***Diagram 1.* In vitro maturation assay of nonadherent marrow cells.** (A) Bone marrow mononuclear cells are plated for 24 h and adherent cells discarded. These initially adherent cells consist of mature macrophages, fibroblasts, and endothelial cells. (B) The remaining nonadherent *marrow cells,* which contain macrophage precursors and other marrow resident mononuclear cells, are plated in 24-well dishes and maintained in culture for 3, 4, 5, 7 or 10 d. $PGE_1$ is added 48 h (unless otherwise specified) after plating of marrow cells into the 24-well plates. (C) For assessment of surface MMR expression (on days 2–10), adherent cells are washed and labeled with $^{125}$I-ManBSA.

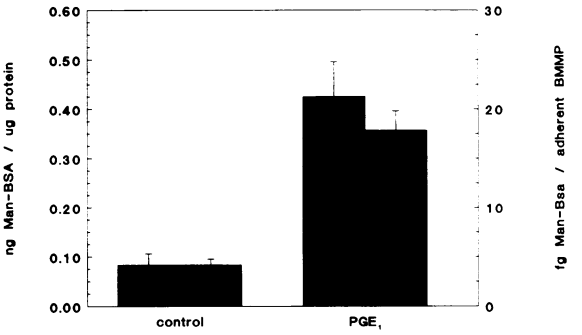


***Figure 1.* Effect of $PGE_1$ on $^{125}$I-Man BSA binding site expression.** $PGE_1$ ($1 \times 10^{-7}$ M) was added to marrow cells that had been cultured for 48 h in the presence of 500 U/ml CSF-1 (stage I). After an additional 24 h, the number of surface $^{125}$I-ManBSA binding sites was determined. The number of $^{125}$I-ManBSA binding sites is upregulated by $PGE_1$ compared with controls both when related to protein (black bars, left ordinate) or to the number of adherent macrophages (hatched bars, right ordinate).

might modulate a multitude of host defense mechanisms (Willis, 1970). Moreover, prostanoids are known to reduce macrophage activation and replication, an event typically associated with cell differentiation (Gentile and Pelus, 1987; Meerpohl and Bauknecht, 1987). These observations led us to hypothesize that prostaglandins also promote expression of the MMR. We herein report that prostaglandins of the E series (PGE) are, in fact, the most potent inducers of the MMR presently known, promoting its expression via enhanced synthesis. Moreover, the process appears adenylate cyclase–mediated and dependent on the stage of macrophage maturation. Most importantly, we document PGE production by resident bone marrow cells consistent with a paracrine regulation of MMR expression in vivo.

## Results

### *Effect of $PGE_1$ on $^{125}$I-mannosylated bovine serum albumin ($^{125}$I-ManBSA) binding*

$PGE_1$ added at a concentration of $1 \times 10^{-7}$M 48 h after plating of marrow cells (diagram 1) causes a 3.5- to 6-fold increase in surface binding of $^{125}$I-ManBSA, whether calculated on a cell- or protein-associated basis (Figure 1). The enhanced binding is dose dependent, with effects seen at nanomolar concentrations, and is therefore evocable by physiological levels of PGE (Figure 2).

Scatchard analysis (Scatchard, 1949) of $^{125}$I-ManBSA binding by $PGE_1$ ($1 \times 10^{-7}$M)-treated cells was compared with control and with those exposed to 1,25-dihydroxy-vitamin $D_3$. We repeated previously described experiments with 1,25-dihydroxy-vitamin $D_3$ (50 nM added at the time of plating) to demonstrate that our system yields similar results despite changes in cell density (Clohisy *et al.,* 1987). The binding constant was the same in all circumstances ($K_D$ = 3–5 $\times$ $10^{-9}$/ml) (Figure 3), establishing that the differences in cell-associated ligand reflect differences in MMR density. As seen in Figure 3, the prostanoid is a much more potent inducer of MMR expression than is vitamin D.

The specificity of PGE-mediated upregulation of MMR was examined by comparison with $^{125}$I-$\alpha$-thrombin binding (Clohisy *et al.,* 1987), pre-

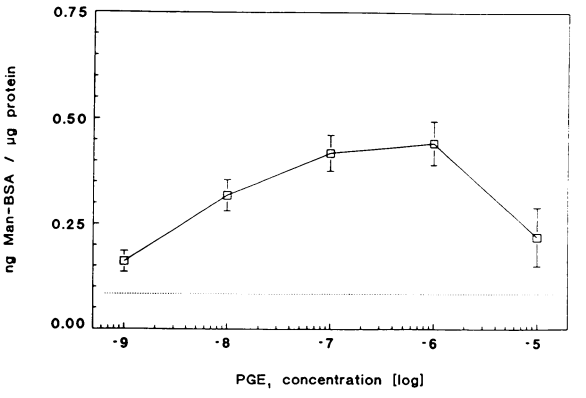


***Figure 2.* The upregulation of $^{125}$I-ManBSA binding site expression is dose dependent over a wide range.** $PGE_1$ was added to marrow cultures at 48 h at the concentrations indicated and $^{125}$I-ManBSA binding assessed at 72 h.

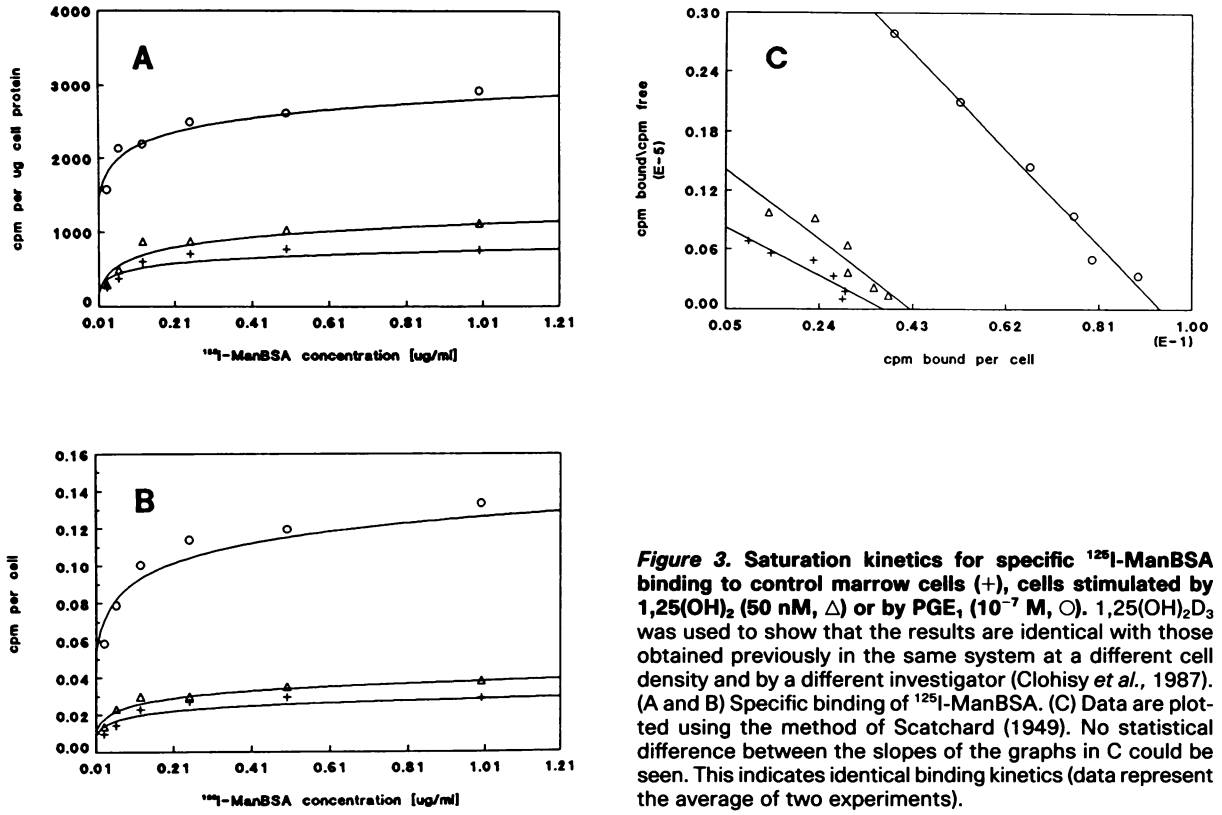


***Figure 3.*** **Saturation kinetics for specific $^{125}$I-ManBSA binding to control marrow cells (+), cells stimulated by 1,25(OH)$_2$ (50 nM, △) or by PGE$_1$ ($10^{-7}$ M, ○).** 1,25(OH)$_2$D$_3$ was used to show that the results are identical with those obtained previously in the same system at a different cell density and by a different investigator (Clohisy *et al.*, 1987). (A and B) Specific binding of $^{125}$I-ManBSA. (C) Data are plotted using the method of Scatchard (1949). No statistical difference between the slopes of the graphs in C could be seen. This indicates identical binding kinetics (data represent the average of two experiments).

viously shown to be unaffected by 1,25(OH)$_2$D$_3$. Similar to vitamin D exposure, no alteration in $\alpha$-thrombin binding occurs with PGE$_1$ treatment, as treated cells express a mean of 378 000 binding sites and control, 353 000.

### *Effect of PGE$_1$ on MMR synthesis*

Our next experiments were aimed at determining if the PGE$_1$ effect on MMR expression reflects enhanced receptor synthesis. Thus, the cells were metabolically labeled with Trans $^{35}$S (methionine/cysteine) and the MMR immunoprecipitated with time (Lennartz *et al.*, 1989).

Figure 4 shows the double bands at 157 kDa and 172 kDa reflecting sequential glycosylation of the MMR protein core (Blum *et al.*, 1989), the appearance of which parallels surface expression of $^{125}$I-ManBSA binding sites assessed using the same batch of cells (Figure 4). The fact that cathepsin D synthesis is unaffected by PGE$_1$ establishes the specificity of the latter on the MMR. Day zero cells do not synthesize detectable receptor protein.

### *MMR expression in response to various eicosanoids*

PGE$_1$ and PGE$_2$ are structurally related, but the latter is the biologically synthesized compound. Both are thought to bind, although with probably slightly different affinities, to the same receptor (Siegl, 1982; Virgolini *et al.*, 1988). As shown in Table 1, MMR upregulation by PGE$_1$ or PGE$_2$ is virtually identical. Despite the fact that prostacyclin (PGI$_2$) also has been reported to bind to the PGE$_1$ receptor (Virgolini *et al.*, 1988; Siegal, 1982), in contrast to PGE$_2$, neither it, leukotriene B$_4$ (LTB$_4$), nor thromboxane B$_2$ (TxB$_2$) affect MMR expression on bone marrow macrophages.

### *Effect of PGE$_1$ on the time course of MMR expression*

These experiments involved exposure of marrow macrophage precursors 48 h after plating to PGE$_1$ ($10^{-7}$M) or carrier dimethylsulfoxide (DMSO). MMR expression was then followed with time. As seen in Figure 5, whereas the MMR appears spontaneously in culture, PGE$_1$ accelerates its expression by 48–72 h. Thus, by day 7, binding by both sets of cells is equivalent. Although PGE$_1$ accelerates MMR expression, unexposed cells ultimately "catch up." Furthermore, we found that exposure of cells to PGE$_1$ on days 7 or 10 does not enhance MMR expression (data not shown).

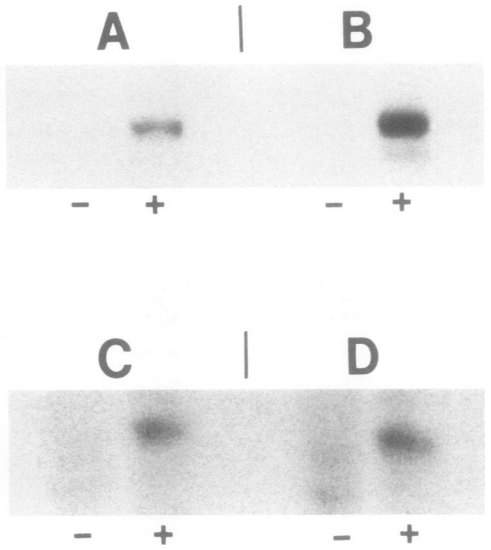


***Figure 4.* Biosynthesis of the mouse MMR is stimulated by treatment of cells with $PGE_1$.** Marrow cells were cultured in the presence or absence of $PGE_1$ added at 48 h followed by radiolabeling at 72 h. Precipitated proteins isolated from control cells are shown in A and C and from $PGE_1$-treated cells in B and D. Samples were immunoprecipitated with normal rabbit serum (−) or specific antiserum (+) directed against the MMR (A and B) or cathepsin D (C and D). Densitometric quantification of each protein showed that the MMR synthesis was induced 2.5-fold by $PGE_1$, whereas cathepsin D synthesis was unchanged. Surface binding studies with $^{125}$I-ManBSA using the same preparation of marrow cells showed a 3.4-fold increase induced by $PGE_1$.

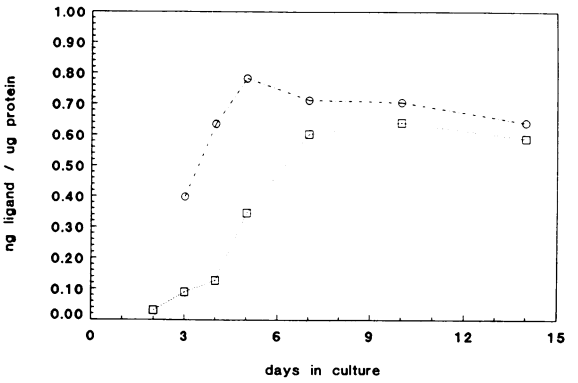


***Figure 5.* The acceleration of $^{125}$I-ManBSA binding site expression on marrow cells by $PGE_1$ (○) with time.** Control cells (□) reach comparable levels at later time in culture. This means that $PGE_1$ accelerates the time course of "spontaneous" MMR expression during in vitro differentiation. However, control cells "catch up," thus finally resulting in an identical phenoptype in regard to MMR expression (n = 3).

### *Effect of macrophage maturation on $PGE_1$-regulated MMR expression*

The findings just described indicated that the responsivity of marrow macrophage precursors to $PGE_1$ is lost with cell differentiation. Thus, to explore the impact of maturation on $PGE_1$-induced MMR expression, our attention turned to the earlier period of culture. First, we added $PGE_1$ at initiation of marrow cell cultures or at various times thereafter and measured $^{125}$I-ManBSA binding in all circumstances 72 h after plating. As shown in Figure 6A, despite only 24 h of exposure, PGE added at 48 h had the most profound effect on MMR expression.

We next determined whether, similar to 2-d cells, 24-h exposure to PGE would enhance MMR expression by freshly plated marrow macrophage precursors. Thus cells, at the time of plating, were exposed to $PGE_1$ ($10^{-7}$M) or carrier for 1 d, after which time the medium was changed without removal of nonadherent cells. As seen in Figure 6B (hatched bars), such treatment does not impact on $^{125}$I-ManBSA binding assessed 48 h later, indicating that MMR expression by early BMMPs is not regulated by the prostanoid. In contrast, exposure of 2-d cells to $PGE_1$ for 24 h, whether nonadherent cells are removed (solid bars) or not (open bars), upregulates the receptor 8- to 10-fold. Thus, $PGE_1$ appears to enhance MMR expression by targeting to adherent but not fully differentiated macrophage precursors, which are >95% positive for F4/80 antigen expression. Moreover, PGE-induced enhancement of MMR expression does

**Table 1.** MMR surface expression in response to eicosanoids

| | Control | $PGE_1$ | $PGE_2$ | $PGI_2$ | $TxB_2$ | $LTB_4$ |
|---|---|---|---|---|---|---|
| 72 h | 100% | 452 + 32% | 491 + 41% | 104 + 11% | 98 + 9% | 101 + 4% |
| 96 h | 100% | 474 + 27% | 532 + 52% | 112 + 14% | 122 + 17% | 105 + 8% |

Eicosanoid compounds ($1 \times 10^{-7}$ M) were added to marrow cells 48 h after plating. MMR surface expression of adherent cells was assessed after 72 h (n = 4) or 96 h (n = 3) by $^{125}$I-ManBSA binding. Binding data are normalized for cell-associated proteins and are expressed as a percentage of DMSO control.

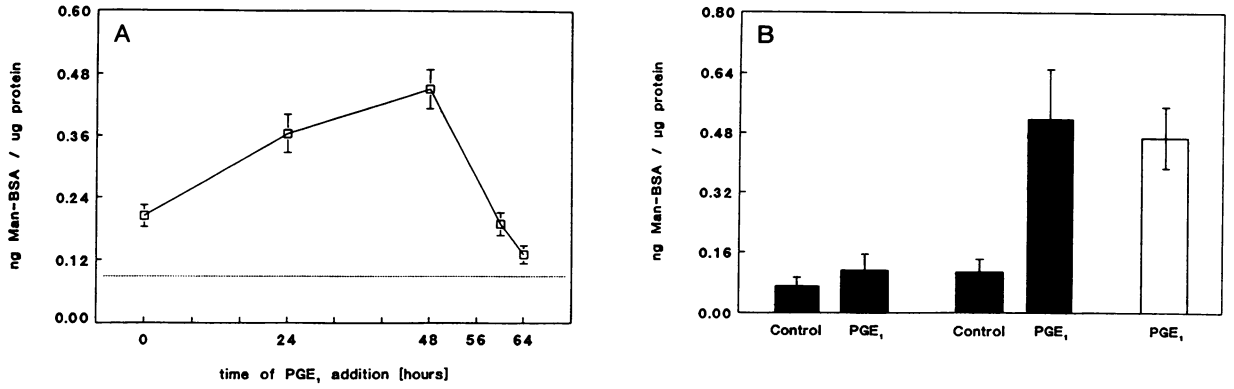


***Figure 6.*** **(A) Different responsivity to $PGE_1$ ($10^{-7}$ M) in regard to the expression of $^{125}$I-ManBSA binding sites with time in culture.** Marrow cells were cultured for 72 h under all circumstances with addition of $PGE_1$ at different time points. Maximum response could be elicited when $PGE_1$ was added at 48 h. This means that, despite a shorter time for the response to the agent, MMR upregulation was distinctly higher (n = 3). (B) $^{125}$I-ManBSA binding results obtained from "wash off" experiments. Marrow cells, at the time of plating, were exposed to $PGE_1$ ($10^{-7}$ M) or DMSO carrier. After 24 h media were changed and the stimuli withdrawn without removal of nonadherent cells. $^{125}$I-ManBSA binding site expression was assessed at 72 h. The filled bars represent results from cells that were first cultured for 48 h in $\alpha$-MEM (containing CSF-1 and FCS). The nonadherent cells were washed off at 48 h and $PGE_1$ ($10^{-7}$ M) or DMSO added to the remaining, adherent macrophages. $^{125}$I-ManBSA binding sites were assessed also at 72 h (hatched bars). The open bars show cells that received $PGE_1$ ($10^{-7}$ M) at 48 h by addition to the medium without any further changes in the culture conditions and without removal of the nonadherent cells (n = 4).

not require the presence of the nonadherent cells in the culture.

### *Effect of cell cycle and colony stimulating factor-1 (CSF-1) on PGE-regulated MMR expression*

Gene expression may be cell-cycle dependent, and we therefore examined the effects of $PGE_1$ on DNA synthesis. Figure 7 shows the prostanoid to be distinctly antiproliferative in the first 2 d of culture but only minimally so when added at 48 h. Because the $PGE_1$ antiproliferative effect appeared inversely related to its effect on MMR expression, we were obligated to explore further the impact of DNA synthesis on receptor appearance. To this end, we exploited our previous findings that the replication rate of bone marrow precursors can be modulated by ambient CSF-1 levels. Specifically, 4 h after exposure to 50 U/ml of the growth factor, <5% of the cells are in S-phase of the cell cycle, compared with 40%–50% exposed to 1000 U/ml (Clohisy *et al.*, 1989). Thus, cells were plated in media containing either 50 (hatched bars), 500 (black bars), or 1000 (crosshatched bars) U/ml CSF-1 and $PGE_1$ ($10^{-7}$M) or DMSO added at 48 h (Figure 8A). Adherent cells were analyzed at 72 h. As expected, reduction of CSF-1 to nonmitogenic levels (50 U/ml) completely ablated the $PGE_1$-induced upregulation of the MMR, whereas cells cultured with 500 or 1000 U/ml increased MMR expression in response to $PGE_1$. Similar results were seen at 96 h (data not shown).

Because withdrawal of CSF-1 may be antidifferentiative as well as antiproliferative, we con-

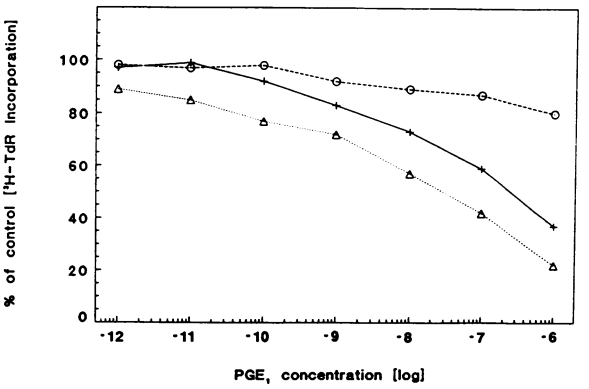


***Figure 7.*** **Antiproliferative properties of $PGE_1$ are dose dependent.** $PGE_1$ was either added at 0 h and mitogenesis read at 24 h (+) or read at 48 h (△). Alternatively, $PGE_1$ was added at 48 h and mitogenesis read at 72 h (○). The amount of $PGE_1$ that was used for the MMR regulation studies resulted in <15% inhibition of growth when added at 48 h. As expected, the antiproliferative action of $PGE_1$ on marrow cells was maturation dependent: when added at plating the inhibition was much greater than when added later to more mature cells (representative of three experiments). Data obtained with $PGE_2$ are very similar and are therefore not shown.

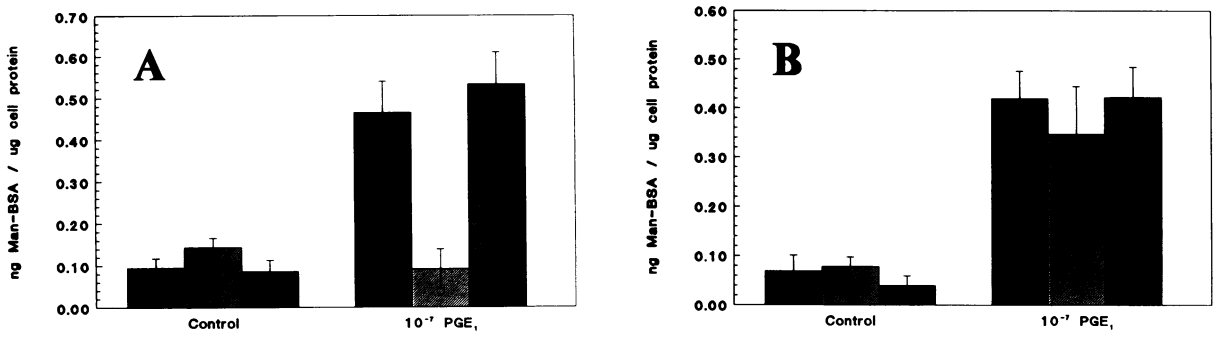


***Figure 8.* (A) Results from marrow cells that were cultured for 72 h in media containing different concentrations of CSF-1 (50 U/ml, hatched bars; 500 U/ml, filled bars; 1000 U/ml, crosshatched bars).** After 48 h they received $PGE_1$ ($1 \times 10^{-7}$ M) or appropriate amounts of DMSO. CSF-1 (50 U/ml) sustained cell survival; however, the response to $PGE_1$ was greatly diminished. The level of "spontaneous" MMR synthesis (control) was uninfluenced. (B) Cells that were cultured in 500 U/ml of CSF-1 and then underwent a change of media at 44 h. They were exposed at this time to varying amounts of CSF-1 (50 U/ml, hatched bars; 500 U/ml, filled bars; 1000 U/ml, crosshatched bars) for the remaining 28 h. At 48 h they received either $PGE_1$ ($10^{-7}$ M) or DMSO. The different proliferational states dependent on the CSF-1 concentration did not influence $PGE_1$-induced upregulation of MMR (n = 3).

ducted a second set of experiments. Marrow cells were grown from 0 h to 44 h in the presence of 500 U/ml CSF-1. At 44 h the medium was replaced and the CSF-1 content was either lowered to 50 U/ml, maintained at 500 U/ml, or increased to 1000 U/ml. Four hours after this change, $PGE_1$ or DMSO was added. Regardless of CSF-1 content (and the fact that >90% of cells exposed to 50 U/ml of the growth factor were in $G_0/G_1$ phase), a 2.5- to 3.5-fold increase in the expression of $^{125}$I-ManBSA binding sites was obtained in each circumstance whether measured at 72 (Figure 8B) or 96 h (data not shown). These experiments suggest that BMMPs require physiological amounts of CSF-1 during initial in vitro culture to allow MMR expression in response to $PGE_1$. Having matured to the point of adherence, they are fully responsive to $PGE_1$-induced upregulation of $^{125}$I-ManBSA binding, regardless of whether ambient CSF-1 is above maintenance levels.

### *$PGE_1$ effect on CSF-receptor expression*

No significant effects of $PGE_1$ added at various time points on CSF-1 receptor expression could be found, as assessed by ligand-binding studies with $^{125}$-iodinated stage V CSF-1.

### *Cyclic AMP effect on $^{125}$I-ManBSA binding*

Cyclic AMP (cAMP) levels in lysates of adherent bone marrow macrophages (48 h) increase (as determined by immunoassay) more than 100-fold in the $PGE_1$-stimulated ($1 \times 10^{-7}$M) cells compared with DMSO controls (from $5.3 \pm 1.2$ fMol/ml to $552 \pm 32.7$ fMol/ml), suggesting that the cyclic nucleotide may mediate the prostanoid's effect on MMR expression. In fact, $^{125}$I-ManBSA binding is enhanced by dibutyryl-cAMP, particularly in the presence of aminophylline (AP) or IBMX (Figure 9, data from IBMX were similar to AP and are not shown).

### *PGE production by bone marrow cells*

To investigate whether marrow cells might autoregulate differentiation, we assessed endogenous PGE production in vitro and in vivo. Medium conditioned by marrow macrophage precursors cultured for 1 or 5 d does not contain detectable $PGE_2$. Initially adherent cells, namely

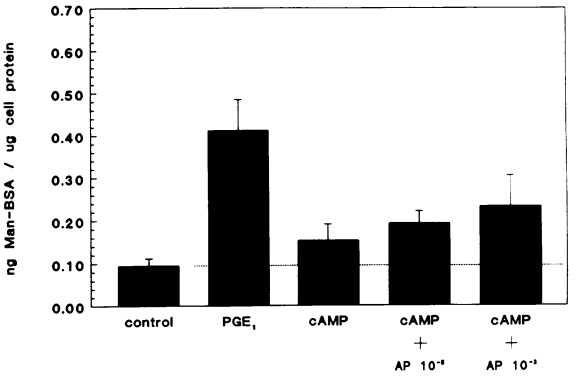


***Figure 9.* $^{125}$I-ManBSA binding after stimulation with $PGE_1$ ($10^{-7}$ M, at 48 h), dibutyryl-cAMP ($10^{-4}$ M, at 48 h), and dibutyryl cAMP together with aminophylline (AP, $10^{-5}$ M or $10^{-3}$ M, respectively, at 48 h).** Exogeneous dibutyryl-cAMP could not stimulate MMR expression to the same height as $PGE_1$; however, the effect could be increased by addition of aminophylline (n = 3).

those generally discarded after the overnight adherence step, do, however, release between 56 and 70 pg/100 μl (1.7–2.2 × $10^{-9}$M) of $PGE_2$ per day. This population contains mature macrophages, fibroblasts, and endothelial cells (diagram 1), all of which are known to secrete $PGE_2$ (Sestini *et al.,* 1984; Schror, 1985; Piquet-Pellorce *et al.,* 1987; Diaz *et al.,* 1989). Moreover, crude, homogenized mouse bone marrow contains measurable amounts of $PGE_2$ (up to 70 pg/100 μl [2.3 × $10^{-9}$M]). Thus, whereas $PGE_2$ does not autoregulate MMR expression in our cultures of macrophage precursors, in vivo the cells are likely to be exposed to substantial amounts of the prostanoid. Moreover, addition of indomethacin ($10^{-6}$M) to marrow cell cultures at different times did not influence MMR expression.

## Discussion

Macrophages ingest a wide variety of particles without the need for opsonization. It appears that the participation of receptors recognizing sugar residues of glycoproteins is important in this process. For example, Speert *et al.* (1988) recently reported that the mannose receptor facilitates phagocytosis of unopsonized *P. aeruginosa* by monocyte-derived macrophages.

Because the phagocytic capacity of macrophages progresses with differentiation, we and others postulated in a previous study that MMR expression would also mirror the maturational process (Ezekowitz and Gordon, 1982; Shepherd *et al.,* 1982; Clohisy *et al.,* 1987, 1989). In fact, we found that $^{125}$I-ManBSA binding by BMMPs progressively increases as the cells spontaneously mature in culture and that the upregulation is enhanced by 1,25-dihydroxyvitamin $D_3$, an agent known to promote macrophage differentiation.

We found in the present study that prostaglandins also enhance MMR expression and are the most potent such agents yet tested. Whereas vitamin D treatment doubles radioligand binding capacity, prostanoids increase it up to sixfold. Moreover, this property appears specific to PGE and the upregulation specific to the MMR.

MMR expression at the cell surface may reflect a host of intracellular events. Mannosylated ligands are bound by the plasma membrane receptor, whereupon the complex is internalized and disassociated in endosomes, and the receptor recycles to the cell surface from a large intracellular pool. Thus, plasma membrane density may reflect alterations in trafficking of the receptor as well as its rate of turnover. We found that freshly isolated BMMPs fail to express or synthesize detectable MMR protein, whereas $PGE_1$ treatment leads to its enhanced synthesis. Moreover, parallel increases in MMR production and $^{125}$I-ManBSA binding suggest that, after receptor synthesis, maturing BMMPs rapidly translocate it to the plasma membrane.

Although data accumulated thus far indicated that prostaglandins enhance MMR expression at a particular point in time, they did not establish whether these agents alter the ultimate phenotype of BMMPs. Our time course experiments indicate, however, that $PGE_1$ accelerates appearance of the membrane protein by 48–72 h, but ultimately, control cells differentiate and bind as much $^{125}$I-ManBSA as do those treated with $PGE_1$. Thus, it appears that prostaglandins accelerate the time course of differentiation of the macrophage—and, as a consequence, MMR expression—but not the cells' ultimate phenotype. We also found that, consistent with its role as a macrophage-differentiating agent, $PGE_1$ loses its upregulating effect on the MMR as BMMPs mature in vitro.

These data indicate that PGE accelerates MMR expression within a specific temporal window appearing shortly after BMMP isolation. We therefore turned to early periods of culture to explore the effect of cell maturation on the prostaglandin-mediated event and found receptor upregulation to be most profound when $PGE_1$ is added 48 h after plating. Moreover, the prostanoid fails to influence MMR expression by more immature (i.e., nonplastic adherent) BMMPs. Taken with the loss of responsivity of these cells with prolonged (>7 d) culture, there appears to be a maturational window in which prostaglandins are capable of impacting on the MMR.

As cells mature, their replicative rate generally declines. Thus, the possibility existed that the PGE-mediated effect reflects changes in cell cycle. In fact, we found that $PGE_1$ suppresses DNA synthesis in early BMMP cultures. Alternatively, prostaglandin-induced MMR expression remains constant regardless of whether or not the majority of cells are undergoing DNA synthesis, establishing that the effect is cell-cycle independent.

On the other hand, some of these experiments involved withdrawal of CSF-1 to maintenance (i.e., nonproliferative) levels. As the macrophage-specific growth factor also has differentiating properties, its removal may have

effects other than anti-proliferative that might secondarily impact on the MMR. We therefore determined whether PGE-mediated MMR upregulation is CSF-1 dependent. Consistent with a maturational window of responsivity, we noted that early cultures require physiological amounts of CSF-1 for prostanoid-stimulated MMR expression to occur. Once BMMPs have reached the optimal stage of differentiation (i.e., 48 h), however, the PGE effect is independent of CSF-1 levels. Moreover, the data that $PGE_1$ does not influence CSF-1–receptor expression (a receptor that appears early in macrophage differentiation [Bartelmetz and Stanley, 1985]) underscore that the compound is modulating cell development at a relatively late stage in differentiation. It also confirms our findings of the $PGE_1$ effect being CSF-1 independent once precursors reached a responsive developmental stage. We would also point out that similar, although less extensive, studies were performed using highly purified (stage V) CSF-1 as a growth factor for culture. These experiments quantitatively mirrored those just described, revealing progressive spontaneous appearance of the MMR and its upregulation by $PGE_1$ (data not shown), indicating that elements other than CSF-1 in the stage I preparation are irrelevant to PGE regulation of the receptor.

PGE, on interacting with its receptor, generally activates adenylate cyclase, and a number of subsequent signals are known to be cAMP dependent (Yu *et al.,* 1976). We found, as expected, that $PGE_1$ markedly enhances intracellular cAMP levels, and—most importantly—a cAMP analogue, particularly in the presence of phosphodiesterase inhibitors, also prompts appearance of the MMR. The fact that $^{125}$I-ManBSA binding in the presence of dibutyrl cAMP does not reach those levels induced by $PGE_1$ may reflect failure of the exogenous cyclic nucleotide to accumulate maximally in critical cellular compartments.

Finally, we turned to the possibility that marrow cells might, through endogenous production of prostaglandins, autoregulate MMR expression. Thus, we measured $PGE_2$ production by cultured cells and found that our purified BMMP population, maintained for up to 5 d in culture, failed to condition the medium with detectable levels of the eicosanoid. Alternatively, other marrow resident cells do produce $PGE_2$, and the prostanoid is present in freshly extracted mouse marrow. Thus, the potential of paracrine regulation of MMR expression by BMMP exists in vivo.

## Methods

Unless otherwise specified, all chemicals and media were obtained from Sigma (St. Louis, MO). Horse and fetal calf sera (FCS) were purchased from Hazelton Dutchland Research Products (Denver, PA) and GIBCO (Grand Island, NY). The FCS were found to be free of inhibiting factors (Clohisy *et al.,* 1989). Murine L929 cells were a gift of Dr. H.S. Lin (Washington University Medical School, St. Louis, MO) and ManBSA (42 mol of sugar/mol of protein) was purchased from E-Y Laboratories (San Mateo, CA). $\alpha$-Thrombin is a gift of Dr. J. Fenton (New York State Dept. of Health, Albany, NY) and 1,25-dihydroxivitamin $D_3$ was a gift from Dr. Milan Uskovic (Hoffman-LaRoche, Basel, Switzerland).

### *Preparation of stage I CSF-1*

CSF-1 was prepared by modification of the method described by Stanley (1985). Serum-free conditioned medium from L929 cells was chromatographically purified by batch calcium phosphate method. Specifically, 200 ml of calcium phosphate gel (300 ml of 0.4 M $Na_3PO_4$ plus 3 l of 0.057 M $CaCl_2$) was added to 1.5 l of L929 conditioned media, stirred for 30 min, and allowed to settle. The supernatant was discarded and the gel rinsed five times with 3 mM $Na_3PO_4$ (pH 6.5) and three times with 100 mM $Na_3PO_4$. Supernatants were collected from the high molarity phosphate rinses, concentrated, and dialyzed against deionized water. CSF-1 activity was determined by the use of a bioassay of cellular proliferation against known standards, as described previously (Clohisy *et al.,* 1987). Total protein concentration was determined with the use of the method of Lowry *et al.* (1951). Typical final specific activities for stage I CSF-1 were $10^6$ U/mg protein. When necessary, CSF-1 was purified to stage V by the method of Stanley *et al.* (1975).

### *Complete culture medium*

The $\alpha$-modification of Eagle's Medium ($\alpha$-MEM) with 500 U/ml stage I CSF-1 (unless otherwise specified), 15% FCS, and penicillin/streptomycin (100 U/ml and 100 $\mu$g/ml, respectively) was used.

### *Marrow cells*

Nonadherent cells were obtained from bone marrow cultures of 9- to 12-wk-old male A/J mice (Jackson Laboratories, Benton Harbor, MI) or $C_3H$ mice (Jewish Hospital of St. Louis, MO) and prepared as described previously (Tushinski *et al.,* 1982). In brief, the ends of freshly harvested femurs or tibiae were excised, and the cells were collected by flushing the medullary cavity with $\alpha$-MEM (4°C) through an 25-gauge needle. The marrow plug was dispersed by several passages through an 18-gauge needle, and the cells were pelleted ($800 \times g$ for 7 min at 4°C). The pellet was resuspended in $\alpha$-MEM (4°C) and the number of nucleated cells determined by counting an aliquot of the resuspended cells in 4% acetic acid. Cells ($1 \times 10^6$ cells/ml) were then seeded into tissue culture dishes (Falcon Plastics, Lincoln Park, NJ) at a density of $3.4 \times 10^5$ cells/cm$^2$ in the presence of complete medium containing 500 U/ml of stage I CSF-1. After a 24-h incubation, nonadherent cells were collected and the adherent cells discarded. The nonadherent population was pelleted ($800 \times g$ for 7 min at 4°C), resuspended in Pronase solution (1 ml of 0.02% w/v Pronase, B grade, [Calbiochem, La Jolla, CA], 1.5 mM EDTA in phosphate-buffered saline (PBS)/$10^7$ nucleated cells) and incubated for 15 min at 37°C. Pronase treatment was stopped by the addition of horse serum (0.2

ml/10 ml Pronase solution) and the suspension layered onto 15 ml of horse serum (4°C) and incubated on ice for 15 min. The top layer was collected and relayered on a 15 ml horse serum gradient (1200 × *g* for 7 min at 4°C). The pelleted cells were resuspended in complete medium and the number of nucleated cells was determined. This group of nonadherent cells, which is 24-h post–bone marrow cell isolation and freshly recovered from Pronase treatment, is designated "*marrow cells.*" When these cells are cultured in the presence of 500 U/ml CSF-1 for 7 d, >99% of colony forming units [CFU-(c)s] share macrophage morphology and plastic adherence and are positive for the monocyte-specific enzyme α-naphthyl acetate esterase as well as for surface expression of the macrophage lineage specific antigen F4/80 (Austyn and Gordon, 1981).

### Adherent bone marrow–derived mononuclear phagocytes

Marrow cells were cultured at a concentration of 150 000 cells/ml in 24-well plates (NUNC, Naperville, IL) in 2 ml of complete medium/well or in 50 ml of medium in tissue culture dishes (Falcon Plastics). After designated periods, adherent cells were used for binding studies or immunoprecipitation, respectively.

### PGE and MMR expression

At various times, $PGE_1$ or $PGE_2$ was added in the marrow cell cultures at a final concentration of $1 \times 10^{-7}$ M (unless otherwise indicated). $PGE_1$ and $PGE_2$ stock solutions were dissolved in DMSO at a concentration of 1 mg/ml and diluted from there. $PGI_2$ and $LTB_4$ solutions were prepared similarly in DMSO and ethanol, respectively. 1,25-dihydroxy-vitamin $D_3$ (Hoffman-LaRoche) was added at a final concentration of 50 nM from a stock solution in ethanol. The final concentration of ethanol or DMSO added to the cultures was <0.01%. Additionally, appropriate controls included the amount of ethanol or DMSO carrier.

### $PGE_2$ and $LTB_4$ assay

Mouse femurs or tibiae were harvested as described and the cells spun down. The resulting supernatants as well as supernatants of 24-h cultures from crude bone marrow and supernatants from marrow cell cultures at different times were analyzed for $PGE_2$ and $LTB_4$ by radioimmunoassay (Amersham, Arlington Heights, IL).

### cAMP determination

cAMP levels were determined by radioimmunoassay using a modification of the method of Harper and Brooker (1975). Adherent cells were treated with $PGE_1$ as indicated at 37°C for 25 min. The reaction was stopped by the addition of perchloric acid to a final concentration of 0.6 N. The sample was neutralized with 3 M KOH and centrifuged. A 100-μl aliquot of the supernatant was combined with 100 μl of 20 mM MES (2-[N-morphino]ethanesulfonic acid) buffer. Five microliters of 2:1 triethylamine:acetic anhydride was added to acetylate the cAMP. The mixture was vortexed immediately and brought to 1 ml final volume with MES buffer. Aliquots were incubated with polyclonal anti-cAMP antisera and $^{125}$I-cAMP at 4°C for 16–20 h. Unbound label was separated from bound by the second antibody technique. Tubes were centrifuged and the pellet counted in a gamma counter. This assay has a lower limit of sensitivity of 5 fmol cAMP/ml.

### Adherent cell counts

Plastic adherent macrophages were washed three times with cold PBS, detached from the tissue culture plastic after a 5-min incubation at 20°C in 0.005% Zwittergent (Calbiochem), and counted in a hemocytometer (Bar-Shavit *et al.*, 1983; Clohisy *et al.*, 1989).

### [$^3$H]thymidine incorporation assay

Adherent mononuclear phagocytes were pulsed with 20 μCi of [$^3$H]thymidine (ICN, Costa Mesa, CA) and incubated at 37°C. After a 4-h incubation, the cells were rinsed with PBS and incubated (37°C) for 30 min in 10% trichloroacetic acid. They were then rerinsed with an ethanol:ether (3:1) solution and extracted for counting in 0.1 N NaOH.

### ManBSA iodination

ManBSA (300 μg) was dissolved in 300 μl of PBS and mixed with 2 mCi of $Na^{125}I$ (Amersham) and 600 μg of chloramine T in 60 μl of 0.1 M $Na_3PO_4$ buffer (pH 7.6) (Konish *et al.*, 1983). The reaction was terminated after 10 min on ice by addition of 380 μl of sodium metabisulfate (2.4 mg/ml) and 380 μl of potassium iodine (10 mg/ml). Free iodine was removed by running the sample on a Sephadex G-50 column (1 × 20 cm) buffered in 10 mN tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5). Samples (0.4 ml) were collected and active fractions identified by gamma counting. Protein determination was performed by the method described by Miller (1959), and specific activity was typically 5–8 × $10^6$ cpm/μg ManBSA, with >95% of total counts being trichloroacetic acid precipitable. The ligand was used within 2 wk of iodination.

### $^{125}$I-ManBSA binding assay

The assessment of surface binding sites on adherent bone marrow mononuclear phagocytes by the use of $^{125}$I-ManBSA was modified from techniques previously described (Konish *et al.*, 1983). Binding assays were carried out at 4°C with a nonspecific binding representing <10% of total cell-associated counts.

The cells were washed three times (0.4 ml/well/wash) with Hank's Balanced Salt Solution (HHBG) (10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES], 10 mM Tris, 0.1% glucose, and 10 mg/ml BSA, pH 7.1) and incubated with 0.2 ml of 2 μg/ml $^{125}$I-ManBSA in HHBG (unless otherwise indicated) plus 0.2 ml of HHBG ± 4 mg/ml mannan (final volume 0.4 ml/well). Equilibrium binding was achieved after a 48-h incubation, and the level of cell-associated ligand was determined. The incubation medium was aspirated, and cell monolayers were rinsed quickly six times with Hank's Balanced Salt Solution (HBSS). Cells were dissolved in 0.1 N NaOH (0.5 ml/well), and cell-bound radioactivity of NaOH-solubilized material was measured by gamma counting. Duplicate values were determined for all binding points. The protein concentration was assessed by the method described by Lowry *et al.* (1951).

### $^{125}$Iodination of α-thrombin and α-thrombin–binding assay

These were performed as described previously (Clohisy *et al.*, 1987). Specific activity of α-thrombin was typically at 5–6 × $10^{12}$ cpm/g. All linear and nonlinear graphs were calculated from the measured values by an iteration program. Transformation of binding data to determine dissociation

constants and estimate the number of available binding sites was performed by methods of Scatchard (1949).

### *CSF-1 binding assay*

CSF-1 binding was assayed using a modification of the methods of Stanley *et al.* (1975). Cells were plated as above. Sixteen hours before the assay, cells were washed to remove any CSF-1 and placed in $\alpha$-MEM supplemented with 15% FCS, which did not contain any CSF-1, to upregulate the receptor. All binding assays were carried out at 4°C. Adherent cells were washed three times with 0.5 ml of binding buffer ($\alpha$-MEM supplemented with 0.2% BSA, 0.02% azide and buffered with 25 mM HEPES, pH 7.4). Binding buffer (100 $\mu$l) and 100 $\mu$l of $^{125}$I-stage V-CSF-1 ($2.4 \times 10^7$ cpm/ml) were added and then incubated for 1 h. Nonspecific binding was determined by preincubation of cultures with 2000 U unlabeled CSF-1 in 100 $\mu$l of binding buffer. $^{125}$I-CSF-1, prepared by the methods of Stanley *et al.* (1975), was added as above and incubated for 1 h. At the conclusion of the binding time, cells were washed five times with ice-cold HBSS to remove unbound isotope and solubilized with 0.1 N NaOH for gamma counting.

### *Biosynthetic radiolabeling and immunoprecipitation of MMR*

Marrow cells ($5 \times 10^7$) were incubated for 3 d in tissue culture medium in the presence or absence of $1 \times 10^{-7}$ M $PGE_1$. After this pretreatment, the cells were biosynthetically labeled for 3 h with Trans $^{35}$S (ICN) as previously described (Lennartz *et al.*, 1989). The radiolabeled cells were washed two times with cold PBS and frozen at −20°C. Cells were thawed and lysed by dounce homogenization in 5 mM TRIS (pH 7.4) in the presence of 1 $\mu$M leupeptin and 1 $\mu$m pepstatin. Whole cell membranes were centrifuged at 100 000 $\times$ *g* for 30 min (4°C) and used for immunoprecipitation. The mannose receptor is tightly associated with cell membranes and is localized exclusively in this fraction isolated from cells. The membranes were solubilized in 1% Triton-X-100 (in PBS with leupeptin and pepstatin). The detergent solubilized membranes were precleared with protein A sepharose and then immunoprecipitated with a rabbit antiserum specific for the mouse MMR. This polyclonal antibody was generated against the mouse receptor isolated from J774 macrophages and shown to be specific by Western blotting and immunofluorescence. Membrane fractions were subsequently immunoprecipitated with a rabbit antiserum specific for mouse cathepsin D (Blum *et al.*, 1989). Immunoprecipitated proteins were analyzed on 10% polyacrylamide electrophoresis gels (Laemmli, 1970). Electrophoresis gels with $^{35}$S-radiolabeled proteins were impregnated with Enlightning (New England Nuclear–Dupont, Wilmington, DE) for autoradiography. Radiolabeled proteins were quantified after autoradiography with the use of an E-C transmission densitometer.

### *Statistics*

Unless otherwise indicated, experiments were carried out three or more times. Values represent the average ± SE. Significance was tested with the Student's *t* test.

## Acknowledgments

The authors express special appreciation to Dr. Donald Kohan (Washington University, Department of Nephrology) for assessing cAMP levels. Additionally, the authors thank Jean C. Chappel and Stephen Kling for their devoted and skillful technical help, as well as Lisa MacConnel and Jane Wodicker for their expert secretarial assistance. In particular, we thank Geoffrey S. Nash (Washington University, Division of Gastroenterology) for very helpful critical comments and discussion. This work was supported by National Institutes of Health Grants DE-05413, AR-08070, DK-21474, DK-33165, and AI-20015 and by grants of the Deutsche Gesellschaft für Verdauungs- und Stoffwechselstörungen and the Pharmacia GmbH to S.S.

## References

Austyn, J.M., and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. *11,* 805–815.

Bar-Shavit, Z., Teitelbaum, S.L., Reitsma, P., Hall, A., Pegg, L.E., Trial, J., and Kahn, A.J. (1983). Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin $D_3$. Proc. Natl. Acad. Sci. USA *80,* 5907–5911.

Bartelmez, S.H., and Stanley, E.R. (1985). Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage specific HGF: assay of hemopoietin-1. J. Cell. Physiol. *122,* 370–377.

Blum, J.S., Diaz, R., Diment, S., Fiani, M., Mayorga, L., Rodman, J.S., and Stahl, P.D. (1989). Proteolytic processing in endosomal vesicles. Cold Spring Harbor Symp. Quant. Biol. *Immunological Recognition:* 54, 287–292.

Clohisy, D.R., Bar-Shavit, Z., Chappel, J.C., and Teitelbaum, S.L. (1987). 1,25-dihydroxyvitamin $D_3$ modulates bone marrow macrophage precursor proliferation and differentiation. J. Biol. Chem. *262,* 15922–15929.

Clohisy, D.R., Chappel, J.C., and Teitelbaum, S.L. (1989). Bone marrow macrophages autoregulate mannose receptor expression. J. Biol. Chem. *264,* 5370–5377.

Diaz, A., Varga, J., and Jimenez, S.A. (1989). Transforming growth factor-beta stimulation of lung fibroblast prostaglandin $E_2$ production. J. Biol. Chem. *264,* 11554–11557.

Ezekowitz, R.A.B., and Gordon, S. (1982). Down-regulation of mannosyl receptor-mediated endocytosis and antigen F4/80 in Bacillus Calmette-Guerin–activated mouse macrophages. J. Exp. Med. *155,* 1623–1637.

Gentile, P.S., and Pelus, L.M. (1987). In vivo modulation of myelopoiesis by prostaglandin $E_2$. II. Inhibition of granulocyte-monocyte progenitor (CFU-GM) cell cycle rate. Exp. Hematol. *15,* 119–126.

Harper, J.F., and Brooker, G. (1975). Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotide Res. *1,* 207–218.

Konish, M., Sheperd, V., Hold, G., and Stahl, P. (1983). Uptake of glycoproteins and glycoconjugates by macrophages. Methods Enzymol. *98,* 301–304.

Laemmli, U.K. (1970). Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature *227,* 680–685.

Lennartz, M.R., Sessions-Cole, F., and Stahl, P.D. (1989). Biosynthesis and processing of the mannose receptor in human macrophages. J. Biol. Chem. *264,* 2385–2390.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurements with the folin phenol reagent. J. Biol. Chem. *193,* 265–275.

Meerpohl, H.G., and Bauknecht, T. (1987). Role of prostaglandins on the regulation of macrophage proliferation and cytotoxic functions. Prostaglandins *31,* 961–972.

Miller, G.L. (1959). Protein determination for large numbers of samples. Anal. Chem. *31,* 964.

Piquet-Pellorce, C., Homo-Delarche, F., and Dy, M. (1987). Mise en evidence d'une synergie entre le facteur stimulant les colonies de granulocytes et de macrophages (GM-CSF) et l'interleukine-I (IL-1) pour la stimulation de la synthese de prostaglandin $E_2$ par des cellules de moelle osseuse. C.R. Acad. Sci. [III] *305,* 345–350.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. NY Acad. Sci. *51,* 660–672.

Schror, K. (1985). Prostaglandins, other eicosanoids and endothelial cells. Basic Res. Cardiol. *80,* 502–514, 1985.

Sestini, P., Tagliabue, A., and Boraschi, D. (1984). Modulation of macrophage suppressive activity and prostaglandin release by lymphokines and interferon: comparison of alveolar, pleural and peritoneal macrophages. Clin. Exp. Immunol. *58,* 573–580.

Shepherd, V.L., Campbell, E., Senior, R.M., and Stahl, P.D. (1982). Characterization of the mannose/fucose receptor on human mononuclear phagocytes. J. Reticuloendothel. Soc. *32,* 423–431.

Shepherd, V.L., and Stahl, P.D. (1984). Macrophage receptors for lysosomal enzymes. In: Lysosomes in Biology and Pathology, Vol. VII, ed. J.T. Dingle, R.T. Dean, and W. Sly, Amsterdam: Elsevier Scientific Publishing, 83–98.

Siegl, A.M. (1982). Receptors for $PGI_2$ and $PGD_2$ on human platelets. Methods Enzymol. *86,* 179–192, 1982.

Speert, D.P., Wright, S.D., Silverstein, S.C., and Mah, B. (1988). Functional characterization of macrophage receptors for in vitro phagocytosis of unopsonized pseudomonas aerugiosa. J. Clin. Invest. *82,* 872–879.

Stahl, P., Schlesinger, P.H., Sigardson, E., Rodman, J.S., and Lee, Y.C. (1980). Receptor mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell *19,* 207–215.

Stanley, E.R., Hansen, G., Woodcock, J., and Metcalf, D. (1975). Colony stimulating factor and the regulation of granulopoiesis and macrophage production. Federation Proc. *34,* 2272–2278.

Stanley, E.R. (1985). The macrophage colony-stimulating factor, CSF-1. Methods Enzymol. *116,* 564–587.

Tushinski, R.J., Oliver, I.T., Guilbert, L.J., Tynan, P.W., Warner, J.R., and Stanley, E.R. (1982). Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell *28,* 71–81.

Virgolini, I., Muller, C., Hermann, M., Schutz, W., and Sinzinger, H. (1988). Evaluation of the prostaglandin-receptors in human and rat liver: interspecies differences at the prostaglandin receptor-level. Prostaglandins *36,* 807–818.

Wileman, T.E., Lennartz, M.L., and Stahl, P. (1986). Identification of the macrophage mannose receptor as a 175 kDa membrane protein. Proc. Natl. Acad. Sci. USA *83,* 2501–2505.

Willis, I.A. (1970). Identification of prostaglandin $E_2$ in rat inflammatory exudate. Pharmacol. Res. Commun. *2,* 297–301.

Yu, J.H., Wells, H., Ryan, W.J., and Lloyd, W.S. (1976). Effects of prostaglandins and other drugs on cyclic AMP content of cultured bone cells. Prostaglandins *12,* 501–503.