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# GROWTH REGULATION BY MACROPHAGES

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## INTRODUCTION

Macrophages play an important role in several aspects of immune regulation, as verified by the articles within this volume. In recent years it has become clear that macrophages play a pivotal regulatory role by influencing the rates of proliferation of multiple cell types. This article is a review focusing on the products secreted by macrophages which alter cell growth.

## COLONY STIMULATING ACTIVITIES

Colony stimulating activities (CSA) which cause hematopoietic progenitor cells to form colonies in vitro have been obtained from a multitude of sources. The most extensively used demonstration system for these activities employs a soft agar culture system incorporating either human bone marrow or, more typically, murine bone marrow as the target cells. Table 1 lists several sources of human and murine colony stimulating activities and the predominant colony type which forms (1-20). Since different types of colonies form depending on the source of CSA, most investigators favor the notion that distinct colony stimulating factors exist which specifically stimulate the proliferation of either neutrophil, eosinophil, macrophage, megakaryocyte or erythrocyte progenitor cells. It is clear that either some types of CSA are either produced by macrophages or that macrophages can induce other cells to produce CSA.

Of particular importance is the observation by Schreier and Iscove (21) that elaboration of some classes of CSA's are dependent on T-lymphocytes (T-cells) and syngeneic accessory cells. They showed that macrophage, granulocyte and erythroid CSA was present in culture supernatants of

Interleukin-2 (IL-2) dependent cloned T-cells only when the specific antigen and accessory cells of the correct haplotype were also present. Little activity was found in medium conditioned constitutively.

When pokeweed mitogen (PWM) (14,15) or endotoxin (LPS) (16,22) was added to fractioned spleen cells, both T-cells and adherent cells were absolutely required for production of CSA; PWM stimulated spleen cells from nude mice did not produce activity unless normal T-cells were also added. It cannot be determined whether macrophages or T-cells were actually producing the CSA's even though both were required.

When LPS was added directly to macrophages and the one to four day conditioned medium was then added to nude mouse spleen cells, CSA was produced (16). Maximal macrophage derived activity was obtained after 24 hours of culture. When macrophages were derived from LPS-unresponsive $C_3H/HeJ$ mice and cultured with LPS, the conditioned medium failed to induce nude mouse spleen cells to produce CSA. But when macrophages were derived from the responsive $C_3H/eB$ mouse, CSA was produced. This data suggested that the T-cells which secrete IL-2 are different from those secreting CSA since nude mice cannot produce IL-2 (23).

Staber, et al., (24) tested pure preparations of the bacterial cell-wall components Lipid A, LPS and murein in cultures of spleen and lymph node cells. They also found that adherent cells were absolutely required for CSA release; mitogenicity, however, was not a prerequisite for release. Muramyldipeptide and LPS degradation products also induced release of CSA. These data suggest that LPS added directly to cultures of adherent peritoneal cells induced CSA, i.e. activity was directly elaborated by macrophages. However, adherent lymphocytes which contaminate the adherent fraction may have been stimulated by an LPS induced macrophage factor.

Pelus, et al, (25) have shown that resident murine peritoneal "monocytoid" cells are capable of constitutive release of colony stimulating activity. Using velocity sedimentation, one population of secretory cells sedimented over a range of 5-8mm/hr and the other had a mean velocity of 4mm/hr. Lactoferrin inhibited the release from the 5-8mm/hr cells, although this inhibitory effect was reversed by addition of LPS. In addition, when LPS was added, a new cell population sedimenting at 1-3mm/hr began releasing CSA.

These apparent discrepancies between constitutive and stimulated release of CSA's need to be resolved. Quantitative differences in the isolation of target bone marrow cells, cell contamination differences in the semi-purified populations under test, and LPS contamination by the culture medium constituents are clearly intangible differences among laboratories which could lead to the conflicting interpretations. Even so it seems very clear that several different colony stimulating factors exist and that macrophages are involved in the production of some of them. Indeed, perhaps the only one they do not produce, or are not involved in producing, is the 70,000 dalton macrophage growth factor (CSF-1) derived from L-929 cells and embryo fibroblasts (17,18). Whether they produce the factors themselves or produce factors which act on lymphocytes which in turn secrete these colony stimulating activities is still unresolved. Until absolutely pure populations of each cell type can be obtained and co-cultured in a controlled manner, the resolution of this problem is likely to evade us.

## LYMPHOCYTE STIMULATING ACTIVITIES

In addition to colony stimulating activities, cells of the mononuclear phagocyte compartment also release lymphostimulatory molecules which modulate and profoundly influence the function of T and B lymphocytes (reviewed in

26-28). Principle among these activities is the soluble factor, lymphocyte activating factor (LAF), more recently referred to as interleukin 1(IL-1). The first experimental description of this molecular activity came from the observations by Gery and Waksman (29,30) using adherent cells from human peripheral blood. In this early study and in subsequent work with IL-1, the discriminating activity ascribed to this factor is the ability to stimulate thymocyte proliferation after 72-96 hours in culture. IL-1 acts either alone or synergistically with phytohemagglutinin (PHA) or Concanavalin A (Con A) to trigger thymocyte proliferation. Recent work has confirmed the fact that the mononuclear phagocytes are the cellular source of IL-1. Thus, in experiments using murine peritoneal exudate cells, murine macrophage cell lines, and human peripheral blood monocytes (31-35), IL-1 was generated using a variety of induction protocols. These studies employed LPS, latex particles, viable lysteria organisms, immune complexes, dimethyl sulfoxide, and phorbol myristate acetate (PMA) to induce IL-1 secretion. Co-culture of murine peritoneal macrophages or the murine macrophage tumor cell line P388.D1 with activated T lymphocytes (36,37) also stimulated IL-1 secretion by the macrophage effector cells. Thus IL-1 secretion may be dependent upon the macrophage receiving an activating signal mediated by T lymphocytes.

IL-1 from a variety of sources has been partially purified and characterized (reviewed in 26-28). Human derived IL-1 has a molecular weight of 12,000 to 14,000 daltons and an isoelectric point of pH 6.8 to 7.0. Peaks of activity with larger molecular weight characteristics have been described but not yet fully characterized (32,38,39). Studies using murine IL-1 show it to be a molecule of approximately 15,000 daltons (40), with an isoelectric point of pH 4.8 (41). Both human and murine IL-1 display charge heterogeneity when further separated by DEAE chromatography or by isoelectric focusing

(39,41,42-44).

A recent report by Mizel describes the purification to homogeneity of IL-1 generated by the murine macrophage tumor cell line P388.D1 under a "superinduction" protocol using PMA, cyclohexamide and actinomyacin D (39). However, the results of this study can now be questioned by virture of the fact that recent unpublished data of S. Shimizie, R. T. Smith, and V. C. Maino, University of Florida School of Medicine, shows that PMA binds both to some undefined element in fetal calf serum, and to purified soluble BSA such that a PMA-protein complex shows potent stimulation of thymocyte proliferation by itself and acts synergistically with either Con A or PHA to induce even greater $^{3}$H-Tdr incorporation by stimulated thymocytes. This PMA-protein complex ("SLAF", synthetic lymphocyte activating factor) migrates to a pI value of 4.5-5.0 after isoelectric focusing separation.

Although the effects of IL-1 on thymocytes are well documented, the direct effect of this lymphoproliferative factor on B lymphocyte effector target cells is controversial. Studies by several different laboratories have shown that both human and murine macrophages produce a soluble factor of approximately 13,000-15,000 daltons which augments in vitro plaque-forming responses (PFC) by splenic lymphocytes from nude mice or from B cell enriched (T cell depleted) cultures (31,42,43). This enhancement of the B cell response (PFC response) is produced by conditions exactly comparable to those required for the generation of IL-1 activity. Thus, the use of LPS induction protocols or stimulated T cells would trigger macrophage production of the B cell differentiating factor function as well as IL-1 activity (33,36,42). This seemingly B cell-specific activity has been referred to as B cell activating factor (BAF) by at least one group of workers (45). There have been conflicting reports from different laboratories suggesting that the two

factor activities, IL-1 and BAF, can be separated from each other biochemically (38), and, conversely, by others that the two activities co-purify and are probably properties of a single molecular species which cannot be chemically resolved from one another (42,43). To date there is no definitive analysis of the issue of how the macrophage derived activities variously described as BAF or IL-1 might act on B cells directly or how they might affect B cell maturation by a synergestic interaction with T helper cell activity. Experimental analysis of this question remains a current area of intensive activity. Nevertheless, the data is clear that macrophages produce activities which are involved in the regulation of lymphocyte proliferation.

## FIBROBLAST STIMULATING ACTIVITIES

The growth of nontransformed fibroblasts is strictly dependent on soluble extracellular hormones and growth factors (46), which are usually supplied in tissue culture by the addition of whole blood serum to a chemically defined culture medium. Pledger, et al. (47,48) showed that serum components could be separated into two functionally distinct groups. The initiation of cell cycle traverse was controlled by platelet-derived growth factor (PDGF) which is released from a sequestered state if platelets are present during clot formation. PDGF was said to make quiescent cells "competent" to respond to factors present in platelet-poor plasma (PPP). PPP, which is made by removing the formed elements from whole blood before clotting is allowed to occur, regulated the temporal traverse of competent cells through the cell cycle and was thus said to contain "progression" activity. Based on these data, Stiles et al. (49) proposed a "dualistic" model of <u>in vivo</u> fibroblast proliferation. This model postulates that cells in the body are bathed in an ultrafiltrate of plasma which contains all the factors necessary for proliferation except those

possessing competence activity. Fibroblast growth in any particular tissue is postulated to be regulated by the localized concentration of competence factors.

This model appears to accurately describe the serum-stimulated in vitro growth of fibroblasts as well as the proliferation which is observed in vivo during early stages of wound healing where PDGF is locally released as platelets are lysed during clot formation. There are, however, several examples of in vivo fibroblast proliferation which can be observed in the apparent absence of the accumulation and lysis of platelets. Since several of these examples, such as the proliferation commonly found in site of chronic inflammation or in tumors, are also characterized by a significant accumulation of macrophages, it seems likely that cells in the mononuclear phagocyte series might be a source of competence activity.

Leibovich and Ross (50) reported that in vivo wound healing was impaired in guinea pigs with an experimentally depressed number of macrophages. Later, the same authors reported that peritoneal macrophages cultured in PPP produced a factor(s) that stimulated the proliferation of fibroblasts (Leibovich and Ross, 51). However, in a later publication, Leibovich (52) presented evidence that the mitogenic substance was produced by a macrophage-dependent metabolism of a plasma constituent, casting doubt on the role of this cell type as a primary source of stimulatory activity. More recently Glenn and Ross (53) reported that activated human peripheral monocytes cultured in medium containing PPP and either con-A or endotoxin also produced mitogenic activity for fibroblasts.

Over the past several years there has been a number of papers showing a putative role for macrophages in the control of fibroblast proliferation (54,55,56, and 57). These results were obtained using a multitude of

experimental systems, asking, at least in principle, if macrophages could produce activity which could act in any of several ways to stimulate fibroblast proliferation. Although these data are of potential interest from an *in vitro* point of view, if the model of proliferation developed by Stiles, et al. (49) is correct, any macrophage elaborated mitogens which are important regulators of fibroblast proliferation *in vivo*, should act synergistically with PPP in a manner similar to the PDGF-mediated effects. An important question is whether macrophages elaborate a mitogenic activity which acts as a competence factor.

As shown in Table 2, both resident peritoneal cells and thioglycollate-elicited peritoneal macrophages which were maintained for 3 days in culture medium containing 5% PPP elaborated mitogenic activity for BALB/c-3T3 cells. Because of the limited number of cells available in primary cultures and since there are multiple cell types present in peritoneal washes, the ability of cloned murine macrophage-like tumor cells to elaborate mitogenic activity was also investigated. As shown in Table 3, the macrophage-like tumor line $P388D_1$ also elaborated activity which stimulated the proliferation of fibroblasts. The amount of mitogenic activity at any concentration of conditioned medium was dependent on the length of exposure to the $P388D_1$ cells (data not shown). In addition, the macrophage-like cells produced activity when they were maintained in serum-free medium. Similar results were obtained using several of the common macrophage-like cell lines, and indicated that the metabolism of a plasma factor was not responsible for the production of the activity.

For a mitogen to act as a competence factor, it is necessary that it act synergistically with PPP to stimulate proliferation. As shown in Table 4, this criteria was satisfied for medium conditioned by $P388D_1$ cells. The addition of only plasma-free medium conditioned for 3 days by $P388D_1$ cells did

not stimulate the proliferation of 3T3 cells. The addition of medium containing only PPP was also not effective in stimulating growth. However, when medium containing both the macrophage-elaborate products and PPP were added together, a stimulation of DNA synthesis equivalent to that seen following serum stimulation was observed. The other criteria for a competence factor proposed by Stiles, et al. (49), is that a transient exposure to the mitogen is sufficient to stimulate one round of DNA synthesis and that cells exposed to the factor alone remain 12 hr from the initiation of DNA synthesis. These criteria have also been shown for crude macrophage-conditioned medium (data not shown). The activity which stimulates the proliferation of fibroblasts is not identical to IL-1, since they can be separated on DEAE columns (data not shown).

## SUMMARY

The evidence reviewed here indicates that macrophages, either acting alone or in concert with other cells, influence the proliferation of multiple types of cells. Most of the data indicate that these effects are mediated by soluble macrophage-elaborated products (probably proteins) although the role of direct cell-to-cell contacts cannot be ruled out in all cases. A degree of success has been achieved on the biochemical characterization of these factors, due mainly to their low specific activity in conditioned medium and the lack of rapid, specific assays. It is our belief that understanding the growth-regulating potential of macrophages is an important and needed area of research.

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Table 1. Sources and Target Cells Responsive to Colony Stimulating Activities

| Source | Colony Types Found |
|---|---|
| human: | |
| peripheral blood leukocytes | granulocytic and macrophagic |
| embryonic kidney cells | granulocytic and macrophagic |
| spleen cells | granulocytic and macrophagic |
| vascular cells | granulocytic and macrophagic |
| lung cells | granulocytic and macrophagic |
| giant tumor cells | granulocytic and macrophagic |
| human placenta | granulocytic and macrophagic |
| human serum | granulocytic and macrophagic |
| murine: | |
| mouse lung conditioned medium | granulocytic and macrophagic |
| endotoxin serum | granulocytic and macrophagic |
| pregnant mouse uterine extract | granulocytic and macrophagic |
| pokeweed mitogen spleen conditioned medium | granulocytic, macrophagic, megakaryocytic and erythoid |
| endotoxin spleen conditioned medium | granulocytic, macrophagic, megakaryocytic and erythoid |
| embryo fibroblast conditioned medium | macrophagic |
| L-cell conditioned medium | macrophagic |
| WEHI-3 conditioned medium | granulocytic and megakaryocytic |

Table 2.

Mitogenic Activity Elaborated by Primary Murine Macrophages

| | Percentage Labeled Nuclei | | | |
|---|---|---|---|---|
| | % Macrophage Medium | | | |
| | 0 | 10 | 30 | 100 |
| Resident Peritoneal Cells | 4 | 6 | 16 | 20 |
| Thioglycollate-Elicited Cells | 6 | 17 | 47 | 67 |

Peritoneal cells were maintained for 72 hr in serum free DME. The conditioned medium was then centrifuged and filtered, and the indicated concentration was added together with 5% PPP and 5 μCi/ml $^{3}$H-thymidine to quiescent cultures of BALB/c-3T3 cells. The number of cells initiating DNA synthesis during a 36 hr period was determined by autoradiography.

Table 3.

Elaboration of Mitogenic Activity by P388D$_1$ Cells

| | Percentage Labeled Nuclei | | | |
|---|---|---|---|---|
| | % Macrophage Medium | | | |
| | 0 | 10 | 30 | 100 |
| Conditioned with PPP | 4 | 41 | 82 | 100 |
| Conditioned without PPP | 5 | 32 | 62 | 84 |

P388D$_1$ cells were maintained for 72 hr in DME either with or without supplementation with 5% PPP. The medium was then harvested, centrifuged and sterile filtered, and added to quiescent BALB/c-3T3 cells with the PPP concentration adjusted to 5%. The number of cells initiating DNA synthesis was determined by autoradiography.

Table 4

Synergism of $P388D_1$ Conditioned Medium with PPP

| | Percentage Labeled Nuclei | | | |
|---|---|---|---|---|
| | % Macrophage Medium | | | |
| | 0 | 10 | 30 | 100 |
| .25% PPP | 2 | 4 | 7 | 5 |
| 1.00% PPP | 4 | 17 | 21 | 26 |
| 2.50% PPP | 4 | 27 | 39 | 58 |
| 5.00% PPP | 3 | 62 | 87 | 98 |

Serum-free DME which had been exposed to $P388D_1$ cells for 72 hrs was added with 5μCi/ml $^3$H-thymidine and the indicated concentrations of PPP to quiescent cultures of BALB/c-3T3 cells. Following a 36 hr incubation the fibroblasts were harvested and the percentage of labeled nuclei was determined by autoradiography.