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Regulation of Macrophage Differentiation by Products of Con A-activated Spleen Cells

By

NOBUHIRO NORO*

Department of Zoology, Faculty of Science, Kyoto University, Kitashirakawa, Kyoto 606, Japan

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Abstract. The culture supernatant of Con A-activated murine spleen cells (Con A-sup) stimulated synergistically M_I^- cells with conditioned medium (CM) obtained from murine embryonic fibroblast cultures (M_I^+); resulting in enhancement of phagocytic activity for EA and slight increase of Ia antigens. M_I^{++} cells, thus cultured with Con A-sup and CM, also exhibited stronger accessory cell (A-cell) activity than M_I^+ cells in the system of IL2 production by T cells, and their A-cell activity was further enhanced in the presence of indomethacin while that of M_I^+ cells did not change. These findings suggest that M_I^- cells probably acquire potentiating, as well as inhibitory activity at the same time when cultured with CM and Con A-sup.

The functional maturation caused by Con A-sup seemed to be associated with the expression of a receptor for lymphokine, phagocytosis-augmenting factor (PAF), which is contained in the Con A-sup. Such a receptor appeared to be common to macrophage lineage, since PAF in Con A-sup was absorbed out with splenic adherent cells, peritoneal exudate cells and M_I^+ cells, but not with M_I^- cells, nonadherent splenic lymphocytes and lymphoid cell line cells. PAF is not identical to differentiation factor (DF) in CM and interferon (IFN) which are known to modulate the function of lymphocytes because of bindabilty to Con A-Sepharose, to which DF cannot bind and no bindabilty to Blue-Sepharose, to which IFN can do. The molecular weight of PAF is approximately $2–3 \times 10^4$ daltons by gel filtration analysis.

Thus, the present studies suggest the requirement of at least two signals for the full maturation of macrophages.

Introduction

Macrophages have been found to be important in several different immunological responses. Thus, they are required for induction of primary immune response to heterologous red blood cells in vitro (Mosier & Coppleson 1968) and for a proliferative response induced in vitro to alloantigens (Alter & Bach 1970, Rode & Gordon 1970, Twormey et al. 1970), as well as for the development of cytotoxic T cells (Wagner et al. 1972, McDonald et al. 1973) and besides, the differentiation and function of macrophages is regulated by various soluble factors, i.e. migration inhibitory factor/macrophage activating factor (MIF/MAF) (David & Remold 1979, Pick 1979), interferon (IFN) (Epstein 1979, Hamburg et al. 1980, Steeg et al. 1980), colony stimulating factor (CSF) (Metcalf 1979, Kurland et al. 1979, Moore et al. 1980) and other lymphokines (Scher et al. 1980, Steinman et al. 1980, Guyre et al. 1981). Recently, it has

* Present address: Department of Microbiology, Tokyo Metropolitan Institute for Neurosciences, 2–6 Musashidai, Fuchu, Tokyo 183, Japan.

been reported that a lymphokine obtained from either Listeria-activated or Con A-activated T cells preferentially induced Ia antigens on the surfaces of peritoneal exudate cells (PEC) (Scher et al. 1980, Steeg et al. 1980), and that an conditioned media from mixed lymphocyte reactions (MLC) stimulated the expression of Fc receptors (FcR) on macrophages (Guyre et al. 1981).

These findings led me to examine how and when lymphokines produced by T cells modulate the expression of Ia antigens and FcR of macrophages during the differentiation. To clarify their problems, I used M_1 cells as a model of macrophages.

M_1 cells, which were established from spontaneous myeloid leukemia of an SL/Am strain mouse by Y. Ichikawa at Chest Disease Institute, Kyoto University, are able to differentiate from blastoid cells (M_1^-) to macrophage-like cells (M_1^+), when cultured with conditioned medium (CM) containing a kind of cytokine, differentiation factor (DF), produced by murine embryonic fibroblasts (Ichikawa 1969, Ichikawa 1970). I have shown that they acquire phagocytic activity for immune complexes in addition to latex beads, transglutaminase activity and accessory cell (A-cell) activity cooperating with lymphocytes (Yodoi et al. 1978, Kannagi et al. 1982). These observations suggested that M_1^+ cells are perhaps arrested at a certain stage of the differentiation pathway of macrophages, which are known to exhibit a variety of functions in association with the expression of various markers during their maturation process.

The present paper demonstrates that M_1^- cells when cultured with CM for 24 to 48 hrs express Ia antigens and concomitantly acquire receptors for a lymphokine produced by Con A-stimulated splenic T cells. Enhanced A-cell activity as well as phagocytic activity for EA is induced on M_1^+ cells by coculturing with this lymphokine; suggesting the requirement of at least two signals, a cytokine (DF) and a lymphokine termed phagocytosis-augmenting factor (PAF), for the full maturation of macrophage and the full expression of their function.

Materials and Methods

Mice: Both male and female SL/Am (H-2^q), B10 (H-2^b) and BALB/c (H-2^d) mice were maintained in our laboratory and used at age of 6 to 12 weeks in all experiments.

Antisera: Anti-Ia antisera employed in these studies (kindly provided by Dr. J. G. Ray, NIH, Bethesda) are as follows; A.TH anti-A.TL (anti-Ia^k, directed to Ia-1, 2, 3 and 7), $(A/J \times B10)F_1$ anti-B10.D2 (anti-Ia.11, 16, directed to Ia-11 and 16), $(B10 \times B10.HTI)F_1$ anti-B10.A(3R) (anti-Ia.7, directed to Ia-7), $(A/J \times B10.AQR)F_1$ anti-B10.T(6R) (anti-Ia^q, directed to Ia-13), and $(B10.LG \times A.TFR4)F_1$ anti-B10.D2 (anti-Ia^d, directed to Ia-7, 8, 11 and 16). Immunoglobulin G fraction of rabbit anti-sheep red blood cells (SRBC) antiserum was prepared as described previously. (Miyama et al. 1978).

Cells: M_1^- cells were maintained as myeloblastoid cells in Eagle's minimum essential medium (MEM) with double concentration of amino acids and vitamins (Nissui Seiyaku Co., Ltd., Tokyo), and with 10% of heat-inactivated horse serum (Pel-Freez Biologicals, Rogers, Arkansas). EL4 cells derived from C57BL/6 T-lymphoma were maintained in Joklik-modified MEM (Flow Laboratory Inc., Maclean, Virginia) with 10% fetal calf serum (FCS). L1210 cells derived from DBA/2 B-lymphoma were maintained in RPMI 1640 medium (GIBCO lab., Grand Island, N.Y.) containing 10% FCS and antibiotics. These cells were cultured in

a humidified 5% CO_2 atmosphere. αRVB cells, which are interleukin 2 (IL2)-dependent cell line cells, were maintained with RPMI 1640 medium with 10% FCS, 20% rat IL2 and antibiotics.

Preparation of conditioned medium: Conditioned medium was recovered from secondary cultures of embryonic fibroblasts of SL/Am or BALB/c strain mice as described elsewhere (Maeda et al. 1977).

Preparation of spleen cell supernatants: Spleen cell suspensions of BALB/c mice were prepared by passing through sterile stainless mesh with MEM. After washing with MEM, the cells were suspended in RPMI 1640 medium with 5% FCS and antibiotics. Ten million of spleen cells were incubated in the presence or absence of 10 μg/ml of concanavalin A (Con A, Pharmacia Fine Chemicals, Uppsala, Sweden) in 2 ml of media in Linbro tissue culture plates (Flow Lab., Maclean, Virginia) for 48 hrs at 37°C in a humidified 5% CO_2 atmosphere. Con A was added to the control cultures at a final concentration of 10 μg/ml immediately before the termination of the culture. The supernatants of these cultures collected by centrifugation, were used as Con A-sup or Control-sup respectively after filtrating through 0.45 μm Millipore filters. These sups were stored at −80°C until use.

Depletion of splenic adherent cells: Details are described elsewhere (Ly & Mishell 1973). Briefly, spleen cells were suspended in MEM with 5% FCS and passed twice through Sephadex G10 (Pharmacia Fine Chemicals, Uppsala, Sweden) columns to remove adherent cells. About 30% of the applied cells were recovered from the column.

Depletion of T cells from spleen cells: Spleen cell suspension at a concentration of 2×10^7 cells/ml were incubated with a 1: 2500 dilution of monoclonal anti-Thy1.2 antibodies (F7D5, Olac Ltd., Bicester, England) for 30 min at room temperature. The cells were then washed, resuspended in medium, and incubated with 1: 10 dilution of normal rabbit serum as complement source for 40 min at 37°C, followed by washing three times with MEM. By this procedure, approximately 40% of spleen cells were lysed.

Differentiation of M_1 cells with CM and Con A-sup: Unless otherwise stated, the following culture system was employed. M_1^- cells were cultured at a final concentration of 2.5×10^4 cells/ml in 1 ml of medium used for maintaining M_1^- cells in Linbro tissue culture plates, in the presence of 10 to 30% of CM. After incubation in a CO_2 incubator for 24 hrs, various dose of Con A-sup or Control-sup were added to the culture. After incubation for further 24 hrs, cells were harvested and tested their binding and phagocytic activities for EA. The biological activity of Con A-sup for M_1^- cells was usually determined by measuring its augmenting activity for EA phagocytosis, in comparison with M_1^- cells cultured with CM alone or CM plus Control-sup. In some experiments, Con A-sup concentrated to 10-fold by ultrafiltration using Diaflo YM5 membranes (Amicon Corp., Lexington, Ma) was used instead of unconcentrated Con A-sup. Hereafter, M_1^- cells cultured with CM alone or CM plus Control-sup named M_1^+, and the cells with CM plus Con A-sup, M_1^{+*} cells.

IL2 production by spleen cells: Spleen cells passed through Sephadex G10 columns for depleting of adherent cells, were cocultured with various number of M_1^-, M_1^+ or M_1^{+*} cells in the presence of 5 μg/ml of Con A for 24 hrs at 37°C. IL2 activity in the culture supernatants was measured by proliferative response of IL2-dependent cell line cells, αRVB. 0.1 ml each of serially diluted supernatants was mixed with an equal volume of 10^4 αRVB cells in 96-wells culture plates.

After incubation for 24 hrs at 37°C, 0.5 μCi/well of tritiated thymidine (^{3}HTdR) was added to each well. Cells were harvested on glass-filters after 4 hrs' incubation and the radioactivity in the filter was counted by a Packard scintillating counter. The dilution factor on half value of the maximum uptake of ^{3}HTdR in the control supernatants which were obtained from αRVB cells cultured with serially diluted rat IL2, was determined as 50 units. IL2 unit in each culture was determined as the 50-fold value of the dilution factor obtained from the test supernatants, divided by that in control supernatant.

Detection of Ia antigens: Ia antigens on M_1 cells were detected by two different methods. One was immunofluorescence staining. Cells were stained by the sandwitch technique for Ia antigens under conditions that maintained viable cells. The first antibody was anti-Ia^q antiserum, and the second was FITC (fluorescein isothiocyanate, BBL, Cockeysvill, MD)-conjugated rabbit anti-mouse Ig preabsorbed with SL/Am thymocytes. Details of the staining procedure are described elsewhere (Kyoizumi et al. 1982). Cells, thus stained, were examined under a fluorescence microscope and by a FACS IV in some experiments.

Another system was the complement-dependent microcytotoxic test. One million of cells were admixed with optimal diluted anti-Ia alloantisera and 1 : 10 diluted guinea pig complement in 1 ml of MEM. The mixtures were incubated at 37°C for 1 hr, followed by washing with the medium, the proportion of viable cells was determined by adding equal volume of 0.2% trypan blue to each tube.

Detection of Fc receptor for IgG (FcR): FcR was detected by rosette formation at 37°C with SRBC sensitized with rabbit anti-SRBC IgG antibodies (EA). The details of EA preparation and EA-rosette formation are described elsewhere (Miyama et al. 1978).

Phagocyic activity for EA: Cells to be tested were suspended at the concentration of 10^5 cells/ml in 0.2 ml of MEM with 5% FCS and were mixed with an equal volume of 0.5% EA. The cell mixtures were centrifuged at 500 r.p.m. for 5 min, and incubated at 37°C for 3 hrs. After gently pipetting, the cell suspensions were treated with hemolytic Gey's solution to lyse free and membrane attached EA. After washing, the proportion of cells which ingested more than one EA was determined by counting at least 200 cells under a light microscope.

Absorption of Con A-sup: Cells to be used for absorption of Con A-sup were washed three times with MEM in minisoap tubes (NUNC, Roskilde, Denmark) and mixed with Con A-sup at the concentration of either 3×10^7 or 10^8 cells/ml in the case of M_1^-, M_1^+, EL4, L1210 and PEC, and either 10^8 or 2×10^8 cells/ml in the case of normal or G10-passed BALB/c spleen cells. The mixtures were kept at 4°C for 1 hr with shaking and the cells were removed by centrifugation before testing the biological activity of these supernatants.

Interferon (IFN) assay: Viral CPE (cytopathic effect) reduction method was carried out by using L cells and VSV (vesicular stomatitis virus). The results were expressed as IU (international unit). The details are described elsewhere (Yamamoto & Kawade 1980).

Fractionation of Con A-sup: Three kinds of methods were chosen for the fractionation of Con A-sup to analyze the biological properties of the active component(s) in the sup. First, either 2 or 3 ml of 10-fold concentrated materials were applied on a column (2×90 cm) of Sephacryl S–200 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with phosphate-buffered saline. Three ml each fraction was collected and protein concentration was determined by absorbance at 280 nm. Each fraction was then dialyzed against RPMI 1640 medium and the

biological activity of each aliquot was tested for the standard method described above. Calibration of the column was performed by blue dextran (mol. wt. 2,000,000), ovalbumin (OVA, mol. wt. 43,000) and cytochrome c (Cyt. c, mol. wt. 12,384).

Second, Con A-coupled Sepharose CL-4B (Con A-Sepharose) was prepared by the method of Cuatrecasas et al (Cuatrecasas 1970). Two ml of the 10-fold concentrated meterial was absorbed with 2 ml of Con A-Sepharose at room temperature for 60 min with gently shaking. After removing the effluent, beads were packed in column, washed with Hanks balanced salt solution (HBSS), and eluted with HBSS containing 0.2 M α-methyl-D-mannoside (αMM). Effluents, washings and eluates were adjusted to 2 ml vol, and dialyzed against RPMI 1640 before testing their biological activity.

Finally, absorption of Con A-sup with Cibacron Blue F3G-A dye-coupled Sepharose CL-6B (Blue-Sepharose, Pharmacia Fine Chemicals, Uppsala, Sweden) was performed by the method of Thompson et al. (Thompson et al. 1975). Briefly, 3 ml of 10-fold concentrated culture filtrate which was dialyzed against 10 mM Tris-HCl buffer pH 7.5, was absorbed with 2 ml of Blue-Sepharose at room temperature for 60 min with gently shaking. The beads were washed and eluted by the same procedure as described for Con A-Sepharose, except the application of 10 mM Tris-HCl buffer pH 7.5 for washing and the same buffer containing 1 M NaCl for elution, instead of αMM.

Results

Augmentation of phagocytic activity in M_I^+ cells by Con A-sup

To see the modulating effect of Con A-sup on the differentiation of M_I^- cells, the cells were cultured with CM, Con A-sup or their mixtures for 48 hrs and the phagocytic activity for EA was examined. As shown in Table 1, addition of both Con A-sup with CM resulted in drastic augmentation of the phagocytic activity: approximately 3 folds as high as those of cells cultured with Con A-sup alone or CM plus Control-sup for 48 hrs. Since such a synergistic activity was not detected in the Con A-sup from T-depleted spleen cell culture (Fig. 1), the active component(s), termed phagocytosis-augmenting factor (PAF), seemed to be a T cell product. Usually, most of phagocytic M_I^+ cells cultured with CM alone for 48 hrs ingested 1 or 2 EA particles. However, the culture with CM plus Con A-sup resulted in increase of

Table 1. Augmentation of phagocytic activity on M_I^+ cells by Con A-sup.

Cultured with	Phagocytosis of EA (%)
Control-sup	0
Con A-sup	14.4
CM+Control-sup	12.6
CM+Con A-sup	34.8

M_I^- cells were cultured with 10% of Con A-sup or Control-sup (10-fold concentrated) for 48 hrs in the presence or absence of 10% CM. Phagocytic activity for EA in those cells were assayed after 48 hrs' culture. The results are expressed as the percentage of phagocytic cells ingesting 1 or more EA.

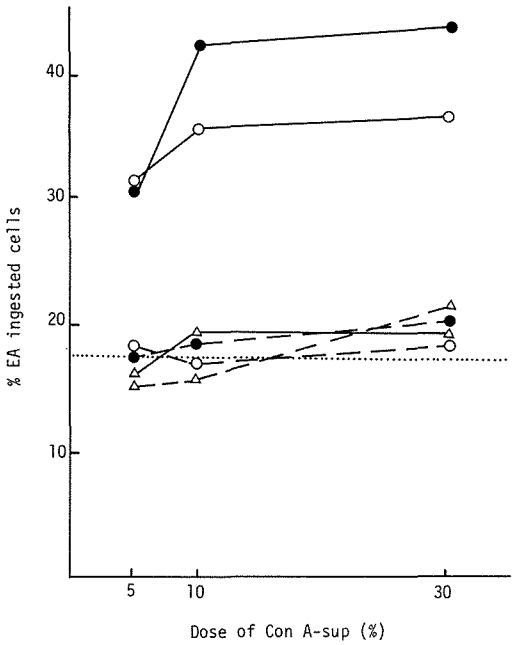

Fig. 1. Requirement of T lymphocytes for production of PAF in Con A-sup. Spleen cells were treated with monoclonal anti-Thy 1.2 antibody or normal mouse serum (NMS) at 37°C for 30 min. Cells were extensively washed and further incubated with rabbit complement at 37°C for 40 min. Residual cells were washed and cultured in the presence of 10 μg/ml Con A in order to obtain Con A-sup for 48 hrs. M_I^- cells were cultured with both 10% CM and various doses of each Con A-sup and their phagocytic activity was assayed. Data are represented as the percentage of phagocytic cells ingesting 1 or more EA. Dotted lines express the percentage of phagocytic cells when M_I^- cells were cultured with 10% CM alone. Open circle (○), closed circle (●) and triangle (△) represent the percentage of phagocytic cells when M_I^- cells were cultured with Con A-sup obtained from untreated, NMS and anti-Thy 1.2 treated spleen cell cultures respectively, in the presence (real line) or absence (broken line) of 10% CM.

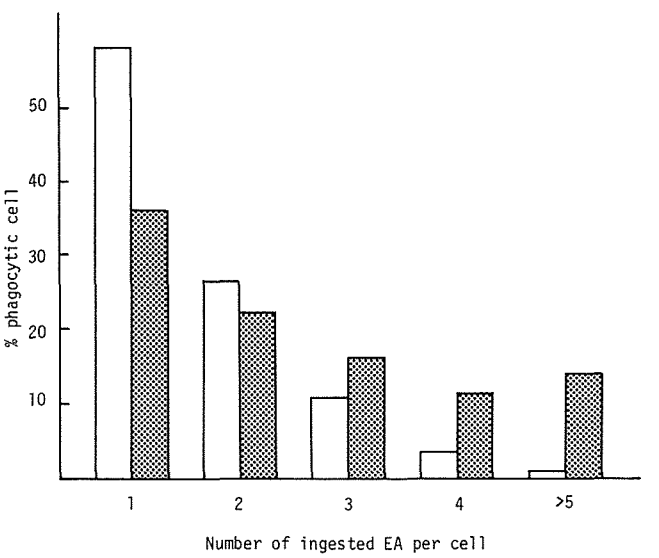

Fig. 2. Increase of phagocytic capacity per cell in M_I^+ cells co-cultured with Con A-sup. M_I^+ cells, which were precultured with 10% CM for 24 hrs, were cultured with 10% unconcentrated Con A-sup. After further 24 hrs' culture, they were stained with May-Grüenwald-Giemsa technique and the number of the ingested EA per phagocytic cells were counted under a microscope. Open (▭) and dotted (▩) column represents the percentage of the cells ingested each number of EA against total phagocytic cells in the culture with CM alone and CM plus Con A-sup, respectively. Mean number of the ingested EA per cell were 1.6 and 2.5, respectively.

Table 2. Effect of residual Con A in Con A-sup on enhancement of phagocytic activity in M_I^+ cells.

CM	Con A-sup	Treatment of Con A-sup	Phagocytosis of EA (%)
−	−	—	0
+	−	—	10.4
−	+	—	14.7
−	+	Sephadex G50	14.9
−	+	100 mM αMM	N.D.
+	+	—	32.0
+	+	100 mM αMM	35.3
+	+	Sephadex G50	32.1

Residual Con A in Con A-sup were inactivated by the following procedures. First, Con A-sup (10-fold concentrated) were incubated with equal volume of Sephadex G50 beads at 4°C for 1 hr. M_I^- cells were cultured with 10% CM and 10% of each sup for 48 hrs and their phagocytic activity for EA was assayed. Second, M_I^- cells were cultured with 10% CM and original Con A-sup (10-fold concentrated) in the presence of 100 mM αMM for 48 hrs and their phagocytic activity was assayed. Data are represented as the percentage of phagocytic cells ingesting 1 or more EA.

phagocytic cells ingesting 3 or more EA (Fig. 2); suggesting that Con A-sup increased not only the number of phagocytic M_I^+ cells, but also enhanced phagocytic capacity of each phagocytic M_I^+ cell. These cells were termed M_I^{++} hereafter.

A possibility that the augmenting effect by Con A-sup might be due to the remaining Con A in the sup, was excluded by facts that Con A-sups, either incubated with Sephadex G–50 beads to eliminate or with 100 mM αMM to inactivate the residual Con A, still had the same activity as original sup (Table 2).

Accessory cell function of M_I^{+} cells

To analyze the function of M_I^{+} cells, the A-cell activity of M_I^+ cells to produce IL2 from Con A-activated T cells was compared to that of M_I^- cells.

As shown in Figure 3, M_I^- cells failed to support IL2 production by Sephadex G10-passed spleen cells (A), even in the presence of indomethacin in the culture (B). In contrast, the addition of M_I^{+} cells gave rise to intensive IL2 production by non-adherent spleen cells which was approximately 2 fold as much as by spleen cells supplemented with M_I^+ cells (A). It is noteworthy that the addition of indomethacin results in drastic increase of IL2 production by splenic lymphocytes cocultured with M_I^{+} cells (B). However, the promoting effect of M_I^+ cells was slightly enhanced by culturing with indomethacin (B).

These results imply that Con A-sup enhances the A-cell activity as well as phagocytic activity of M_I^+ and M_I^{+} cells simultaneously exhibit potentiating and inhibitory abilities for lymphocytes since indomethacin is known to block the possible release of inhibitory prostaglandins.

Since Ia antigens are well known to require for the expression of A-cell function in immune responses, I tried to detect Ia antigens on the surface of M_I^+ cells. M_I^+ cells, which were cocultured with CM for 48 hrs, were lyzed in the presence of complement by anti-Ia^k, anti-Ia^q, anti-Ia.11, 16 as well as anti-Ia^d but not by anti-Ia.7. The specificity observed on M_I^+ cells

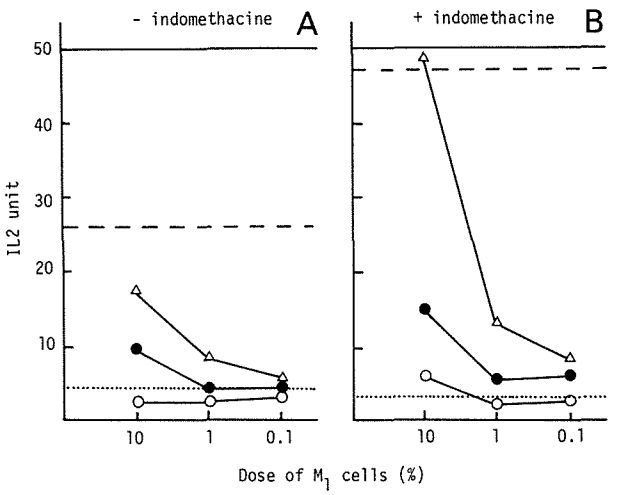

Fig. 3. Accessory cell activity of M_1^{++} cells on IL2 production on Sephadex G10-passed spleen cells. Spleen cells depleted adherent cells by passage through Sephadex G10 columns, were mixed with three types of M_1 cells (M_1^-, M_1^+ and M_1^{++}) and stimulated with 5 μg/ml of Con A in the presence (B) or absence (A) of 5 μg indomethacin. After 24 hrs' culture, IL2 activity in the culture fluid was determined by the uptake of ^{3}HTdR of αRVB cells co-cultured with them. IL2 unit in each fraction is represented as relative number against control supernatant (real line) from rat spleen cells when the rat IL2 unit was 50. Broken and dotted lines express the IL2 unit in the supernatants from murine whole spleen and G10-passed spleen cells without any M_1 cells, respectively. Open circle (○), closed circle (●) and triangle (△) express the activity in the supernatants from G10-passed spleen cells cultured with M_1^-, M_1^+ and M_1^{++} cells, respectively.

are well accordant with those on SL/Am spleen cells. On the other hand, no M_1^- cells were lyzed by these antisera and complement (data not shown). However, there were no significant difference in the proportion of Ia$^+$ cells determined by cytotoxic test between M_1^+ and M_1^{++}

Table 3. Comparison of Ia antigen expression on the surface of M_1^+ cells with on that of M_1^{++} cells.

CM	Con A-sup	Cytotoxic Index (%)
−	−	−19.9
−	+	−6.0
+	−	23.6
+	+	19.2

M_1^- cells were cultured with either 10% CM alone, 10% Con A-sup (10-fold concentrated) alone or their mixtures for 48 hrs. One million of those cells were incubated with 1: 20 diluted anti-Ia^q antibodies and 1: 10 diluted guinea pig complement at 37°C for 1 hr, followed by extensive washing. Control cells were incubated with complement alone. The proportion of viable cells was determined and were as cytotoxic index calculated by the following formula:

$$\text{Cytotoxic Index (\%)} = 100 - \frac{\text{Experimental-Control}}{\text{Control}} \times 100$$

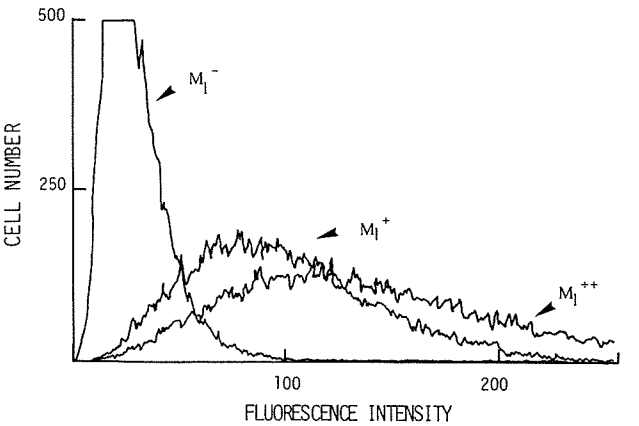

Fig. 4. Comparison of Ia antigen expression on the surface of M_I^+ with on that of M_I^{++} cells. Cells to be tested (M_I^-, M_I^+ and M_I^{++}) were stained for detection of Ia antigens by the indirect immunofluorescence technique using anti-Ia^q antiserum FITC-conjugated rabbit anti-mouse Ig antibody. The stained cells were analyzed by using FACS IV. The results are represented as superimposed profiles.

cells (Table 3). Therefore, Ia antigens on M_I^+ and M_I^{++} cells were semiquantitatively compared to each other by using FACS. As shown in Fig. 4, the superimposed profiles of M_I^+ and M_I^{++} cells stained with anti-Ia^q and FITC-conjugated rabbit anti-mouse Ig antibodies indicate the slight increase of fluorescent intensity in M_I^{++} cells. Taking together, PAF seems to preferentially modulate FcR-dependent function ingesting immune complexes, while little the expression of Ia antigen on M_I^+ cells.

Comparison of PAF activity in Con A-sup, CM and IFN

In order to exclude a possibility that PAF in Con A-sup is identical to DF in CM produced by fibroblasts, the affinity of Con A-sup to Con A-Sepharose was compared with that of CM as shown in Figure 5. The DF activity in CM to induce the differentiation of M_I^- to M_I^+ cells was fully recovered in effluents but not in washings and eluates, while PAF activity in Con A-sup in only eluates. These results indicated that PAF activity in Con A-sup is at least different from DF activity in CM with regard to the sugar structure of the molecule.

Since IFN is known to modulate macrophage functions, I tried to clarify whether PAF activity in Con A-sup was due to IFN or not. As shown in Figure 6, PAF in Con A-sup was not absorbed with Blue-Sepharose which is known to bind to IFN; indicating that PAF in Con A-sup is perhaps different from IFN.

In order to confirm this possibility, both IFN and PAF activities were measured before and after absorption of Con A-sup with either whole or G10-passed spleen cells. As shown in Table 4, IFN activity was equally absorbed by whole and G10-passed spleen cells, while PAF was only absorbed by whole spleen cells but not by G10-passed spleen cells. These results also support the possibility that PAF in Con A-sup is different from IFN.

Sequential effect of Con A-sup with CM on differentiation of M_I^- cells

To determine the target cells of PAF in Con A-sup, phagocytic activity of M_I^-, M_I^+ and M_I^{++}

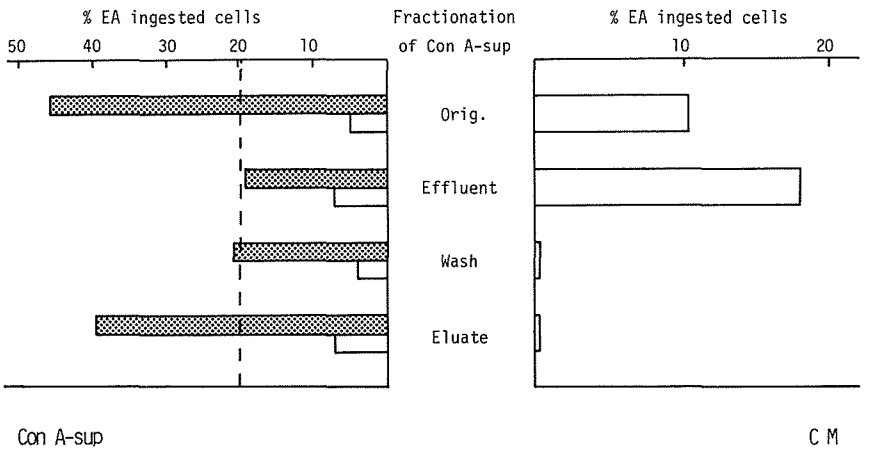

Fig. 5. Difference between DF in CM and PAF in Con A-sup on the bindability to Con A-Sepharose. Con A-sup and CM (10-fold concentrated) were absorbed with Con A-Sepharose at room temperature for 60 min and eluted from the column with αMM. A: M_I^+ cells, which were precultured with (▒) or without (□) 10% unfractionated CM for 24 hrs, were cultured with 10% each fraction of Con A-sup for further 24 hrs and tested for phagocytic activity. B: M_I^- cells were cultured with 5% each fraction of CM for 48 hrs and phagocytic activity was assayed. Broken line in A expresses the percentage of phagocytic cells when M_I^- cells were cultured with 10% CM alone.

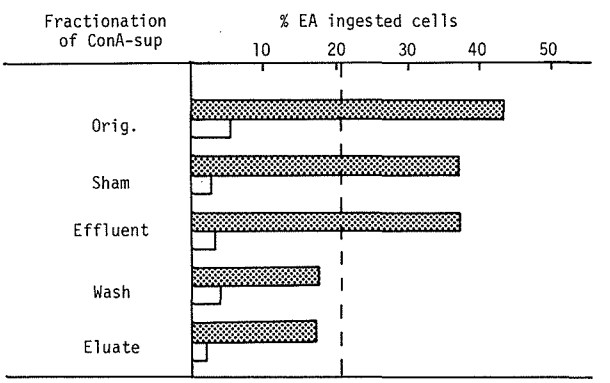

Fig. 6. Difference between IFN and PAF on bindability to Blue-Sepharose. Con A-sup (10-fold concentrated) were absorbed with Blue-Sepharose at room temperature for 60 min and eluted from the column with 1 M NaCl. M_I^- cells, which were cultured with (▒) or without (□) 10% CM for 24 hrs, were cultured for further 24 hrs with each fraction and their phagocytic activity was assayed. Control fractionation was performed without Blue-Sepharose as Sham in the figure. Data are represented as the percentage of phagocytic cells ingesting 1 or more EA. Broken line expresses the percentage of phagocytic cells in cultures with 10% CM alone.

cells was analyzed by sequentially adding of CM and Con A-sup to the cultures. As shown in Table 5, the synergistic effect of Con A-sup with CM was also detected when M_I^- cells were primarily cultured with CM for 24 hrs and then secondarily cultured with Con A-sup for further 24 hrs after extensive washing. However, the effect was not detected when M_I^- cells were

Table 4. Absorption of PAF and IFN activity in Con A-sup with several types of cells.

CM	Con A-sup	Absorbed with	Phagocytosis of EA (%) Exp. 1	Exp. 2	IFN activity in Con A-sup (U/ml)
−	−	—	0	0	—
+	−	—	13.8	9.6	—
+	+	—	26.6	27.9	100
+	+	whole spl. cells	16.9	10.2	60
+	+	G10-passed spl. cells	28.3	N.D.	70
+	+	PEC	17.9	N.D.	N.D.
+	+	L1210	N.D.	28.2	N.D.
+	+	EL4	N.D.	22.4	N.D.
+	+	T_2D_4	N.D.	28.0	N.D.

Unconcentrated Con A-sup was incubated at 4°C for 60 min with 10^8 of PEC, spleen cells with or without adherent cells or 3×10^7 of several types of cell line cells. M_I^- cells were cultured with 30% of each Con A-sup after precultured with 10% CM for 24 hrs. After further 24 hrs' incubation, their phagocytic activity and IFN activity in each Con A-sup were assayed. Phagocytic and IFN activities are expressed as the percentage of phagocytic cells ingesting 1 or more EA and as international unit per ml, respectively.

Table 5. Sequential effect of Con A-sup and CM on differentiation of M_1 cells.

Day 1	Day 2	Phagocytosis of EA (%)
—	—	0
—	CM	0.4
CM	—	13.4
CM	CM	10.3
—	Con A-sup	2.7
Con A-sup	Con A-sup	12.8
CM	Con A-sup	35.4
Con A-sup	CM	20.0

M_I^- cells were cultured with either medium, 10% CM or 10% Con A-sup (10-fold concentrated) for 24 hrs. After extensive washing, those cells were further cultured with them for 24 hrs by the combinations as shown in the table. Cells were harvested and their phagocytic activity was assayed. Data are expressed as the percentage of phagocytic cells ingesting 1 or more EA.

cultured with Con A-sup alone or CM after preculturing with Con A-sup. These results suggest that PAF in Con A-sup preferentially acts on M_I^+ and that a receptor for PAF in Con A-sup perhaps presents on M_I^+ cells.

Cell distribution of receptors for PAF

Since the results as described above suggest that PAF may be specific for macrophages, I next confirmed this possibility by absorption test. As shown in Figure 7, absorption of unconcentrated Con A-sup with 10^8 of M_I^+ cells, precultured with 10% CM for 24 hrs, resulted

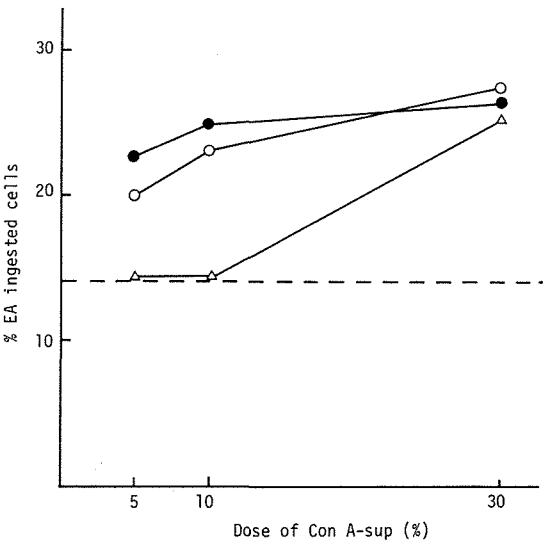

Fig. 7. Expression of a receptor for PAF in Con A-sup on M_I^+ cells. Unconcentrated Con A-sup was absorbed at 4°C for 1 hr with 10^8 of either M_I^- (●) or M_I^+ (△) cells which were cultured with 10% CM for 24 hrs. To see the synergistic effect of original (○) and absorbed each Con A-sup, M_I^+ cells, which were cultured with 10% CM for 24 hrs, were cultured for further 24 hrs with various doses of each Con A-sup and the phagocytic activity was assayed. Data are expressed as the percentage of phagocytic cells ingesting 1 or more EA. Broken line expresses the percentage of phagocytic cells in cultures with 10% CM alone.

in the specific elimination of PAF activity. However, the activity still remained in the Con A-sup absorbed with an equal number of M_I^- cells. These findings indicate that receptors for PAF in Con A-sup are expressed on M_I^+ cells but not on M_I^- cells. Furthermore, it can be seen in Table 4 that the PAF activity in Con A-sup is absorbed by whole spleen cells including adherent cells and PEC, but not by G10-passed spleen cells, T and B lymphoma cells, EL4 and L1210 respectively. Taking together, it seems probable that the receptors for PAF in Con A-sup are expressed not only on M_I^+ cells, but also on tissue macrophages.

Physico-chemical properties of PAF

As shown in Table 6, activity in Con A-sup was completely abrogated by dialysis against at pH 2.0 but was resistant to heating at 56°C for 30 min; indicating again that PAF is different from α-, β-IFN. Gel filtration of Con A-sup using Sephacryl S-200 resulted in the recovery of PAF activity in the fraction between OVA and cytochrome c (Fig. 8); indicating that the

Table 6. pH and temperature sensitivity of PAF.

CM	Con A-sup	Treatment of Con A-sup	Phagocytosis of EA (%) Exp. 1	Exp. 2
−	−	—	0	0
+	−	—	9.3	12.4
+	+	—	27.6	39.7
+	+	pH 2.0	12.8	N.D.
+	+	56°C 30 min	N.D.	30.1

Unconcentrated Con A-sup was heated at 56°C for 30 min or dialyzed against 0.1 M glycine-HCl buffer pH 2.0 overnight. M_I^+ cells, which were cultured with 10% CM for 24 hrs, we cultured with 30% of these Con A-sup for further 24 hrs and phagocytic activity was assayed. Data are represented as the percentage of phagocytic cells ingesting 1 or more EA.

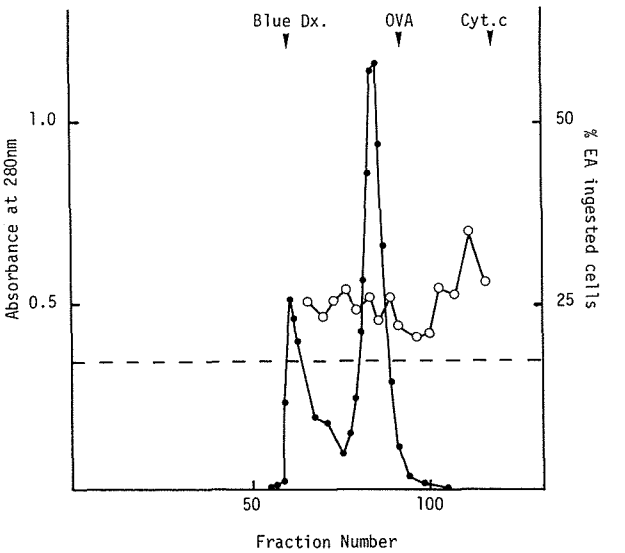

Fig. 8. Gel filtration of Con A-sup by Sephacryl S–200. Con A-sup (10-fold concentrated) was filtered through Sephacryl S–200 and each fraction was dialyzed against RPMI 1640 medium. $M_{\bar{1}}$ cells were cultured with 10% CM for 24 hrs and each fraction from the column were added to the cultures at a final concentration of 50%. After incubation for further 24 hrs, phagocytic activity was assayed. Open circle (○) represents the percentage of phagocytic cells ingesting 1 or more EA in each fraction and closed circle (●) represents absorbance at 280 nm. Broken line represents the percentage of phagocytic cells in cultures with 10% CM alone.

molecular size of PAF is approximately 20,000 to 30,000 daltons; indicating again a difference of PAF from γ-IFN, of which molecular weight is approximately 50,000 daltons (Friedman 1981).

Discussion

Some investigators have also announced the requirement of the interactions between macrophages and T lymphocytes (Gemsa 1981) as shown in the present studies. Vogel and Rosenstreich (1979) reported that defective FcR-mediated phagocytosis in C3H/HeJ macrophages restored by the culture with the supernatants derived from Con A-stimulated spleen cells. Tzehoval et al. (1981) reported that T cell-deficient mice (nu/nu) failed to generate a functional antigen-presenting macrophage subpopulation and restored their function by the transfer of hydrocortisone-resistant thymocyte. Present studies indicated that those interactions between macrophages and T lymphocytes are essential to express their functions and besides, the differentiation of macrophages is regulated by cytokine (DF) as well as lymphokine (PAF) as depicted in Fig. 9.

A variety of lymphokines are known to be involved in the regulation of macrophage functions. Interferon, which is included in Con A-sup, is known to have strong potential to activate macrophage. Indeed, it is reported that M_1 cells, when cultured with β-IFN in the presence of DF, increase in phagocytic activity for latex beads (Tomida et al. 1980). However,

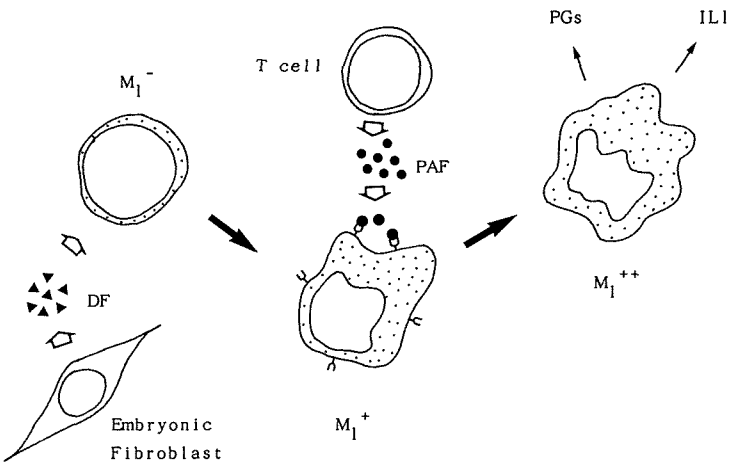

Fig. 9. Two-step differentiation of M_1 cell.

it is clear that there is no identity of PAF in Con A-sup with IFN from the following results: 1) Anti-viral activity in Con A-sup was equally eliminated by the absorption with both whole and G10-passed spleen cells, whereas PAF activity enhancing phagocytosis of M_1^+ cells was only eliminated by whole spleen cells (Table 4). 2) PAF in Con A-sup did not bind to Blue-Sepharose, whereas IFN is known to bind to it (Jankowski et al. 1976). 3) PAF had molecular weight of 20,000 to 30,000 daltons, whereas murine γ-IFN 55,000 daltons (Friedman 1981). 4) PAF was eliminated by dialysis against buffer at pH 2.0, but α-, β-IFN was stable to its treatment.

Leonard et al. (1978) reported that MAF isolated from PPD-stimulated spleen cell culture obtained from BCG-immunized mice had molecular weight of 55,000 daltons and was labile either to heating at 56°C for 30 min or at pH 4.0. Moreover, this factor bound to Blue-Sepharose while not to Con A-Sepharose. Although it is unclear whether MAF is identified with IFN or not, these results suggest that PAF in Con A-sup may be different from MAF like this at least in its physico-chemical properties.

CSF has been shown to have some macrophage stimulatory activity (Kurland et al. 1979, Metcalf 1979, Moore et al. 1980). However, crude CSF preparation, which was obtained from culture supernatant of L929 cells (Worton et al. 1969) failed to enhance phagocytic activity of M_1^+ cells in the presence of CM (N. Noro, unpublished observation); suggesting a difference of PAF from CSF.

PAF is evidently different from DF in CM (Fig. 5), which was obtained from secondary culture of embryonic fibroblasts. Thus, these findings also indicate that PAF is a lymphokine produced by T cells as shown in Fig. 1 and that two signals are required for full maturation of M_1 cells; one is a factor, perhaps cytokine, from non-lymphocytic cells and the other is a lymphokine from activated T cells.

Since PAF is absorbed with PEC and spleen adherent cells as well as M_1^+ cells as shown in Table 4, macrophages having a receptor for PAF seem to be target cells. I indicate that PAF failed to increase Ia-positive populations in M_1^{+} cells (Fig. 4 and Table 3), while could

do phagocytic populations in the cells, by synergistic effect with DF in CM (Table 1). These results suggest that PAF may be effective on Ia^+ cells in M_1^+ cells. Honda et al. (1984) have reported that M_1^+ cells can be separated to two subpopulations on responsibility to three types of chemotactic factors; one, which is a T cell product, preferentially attracts Ia-positive M_1^+ cells and the others, which are components of IgG and C5 respectively, attract Ia-negative M_1^+ cells. These findings support an idea that lymphokines selectively act on Ia^+ macrophages to induce their functional differentiation. On the other hand, it has been reported that a soluble mediator, termed macrophage (Ia^+)-recruiting factor (MIRF), which was elaborated by Listeria monocytogenes-activated T cells, preferentially induced PEC rich in Ia^+ macrophages (Beller et al. 1980, Scher et al. 1980, Beller et al. 1981, Scher et al. 1982). Steeg et al. (1980) have also reported that a considerable proportion of Ia^- PEC became Ia^+ when cultured with γ-IFN in the supernatants of Con A-stimulated spleen cell cultures. Although identification of MIRF with γ-IFN is still unclear, these findings indicate that such a lymphokine as either MIRF or γ-IFN in Con A-sup is effective on Ia^- but not Ia^+ macrophages. These observations suggest again that PAF is perhaps different from such lymphokines.

It is important that PAF activity is abrogated by the absorption of normal macrophages (PEC and spleen adherent cells) as well as M_1^+; implying that PAF or PAF-like mediator act on macrophage differentiation in vivo. PAF activity was also detected in the supernatant of mixed lymphocyte cultures (N. Noro, unpublished observations). This finding suggest that PAF or PAF-like mediator play an important role in various immune responses as well as proliferative response to mitogen.

Prostaglandins are non-specific inhibitor for lymphocyte proliferation and released from macrophage as a most potent producer (Gemsa 1981). Macrophages stimulated with lymphokines, antigens or various pharmacological agents are known to produce higher amount of prostaglandins than unstimulated macrophages (Gordon et al. 1976, Friedman et al. 1979, Gemsa 1981, Morley 1981). I also showed that A-cell activity of M_1^{+} cells was remarkably enhanced by addition of indomethacin as blocker of prostaglandin release (Fig. 3). Usually, addition of an excess number of accessory macrophages to lymphocyte cultures results in suppression of the responses (Niederhuber 1978, Nussenzweig 1980). I have also observed that the addition of excess M_1^+ and its mutant Mk_1 cells (Yodoi et al. 1978, Kyoizumi et al. 1980) promptly brought the anti-SRBC response in vitro to background level. The present observations suggest that prostaglandin perhaps causes suppression. Thus, M_1 cell is strongly useful as a model system for detailed analysis on circuit between macrophages and T lymphocytes.

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References

Alter, B. J. and F. H. Bach (1970) Lymphocyte reactivity in vitro. I. Cellular reconstituion of purified lymphocyte response. Cell. Immunol. 1: 207.

Beller, D. I., J. M. Kiely and E. R. Unanue (1980) Regulation of macrophage populations. I. Preferential induction of Ia-rich peritoneal exudates by immunologic stimuli. J. Immunol. 124: 1426.

Beller, D. I. and E. R. Unanue (1981) Regulation of macrophage populations. II. Synthesis and expression of Ia antigens by peritoneal exudate macrophage is a transient event. J. Immunol. 126: 263.

Cuatrecasas, P. (1970) Agarose derivatives for purification of protein by affinity chromatography. Nature 228: 1327.

David, J. R. and H. G. Remold (1979) The activation of macrophages by lymphokines. *In* Biology of the Lymphokines. Edited by Cohen, S., E. Pick and J. J. Oppenheim. Acad. Press Inc., New York. 121.

Epstein, L. B. (1979) The comparative biology of immune and classical interferons. *In* Biology of the Lymphokines. Edited by Cohen, S., E. Pick and J. J. Oppenheim. Acad. Press Inc., New York. 443.

Friedman, S. A., E. Remold-O'Donnell and W. F. Piessens (1979) Enhanced PGE production by MAF-treated peritoneal exudate macrophages. Cell. Immunol. 42: 213.

Gemsa, D. (1981) Stimulation of prostaglandin E release from macrophages and possible role in the immune response. *In* Lymphokines Vol. 4. Edited by Landy, M. Acad. Press Inc., New York. 335.

Gordon, D., M. A. Bray and J. Morley (1976) Control of lymphokine secretion by prostaglandins. Nature 262: 401.

Guyre, P. M., G. R. Crabtree, J. E. Bodwell and A. Munck (1981) MLC-conditioned media stimulate an increase in Fc receptors on human macrophages. J. Immunol. 126: 666.

Hamburg, S. I., H. B. Fleit, J. C. Unkeless and M. Rabinovitch (1980) Mononuclear phagocytes: Responders to and producers of interferon. Ann. N. Y. Acad. Sci. 350: 72.

Honda, M., T. Masuda, T. Yoshimura and H. Hayashi (1984) Chemotactic subpopulation of macrophage cell line cells (M_1 cells) discerned by three macrophage chemotactic factors from delayed hypersensitivity reaction sites. Cell. Immunol. (in press)

Ichikawa, Y. (1969) Differentiation of a cell line of myeloid leukemia. J. Cell. Physiol. 74: 223.

Ichikawa, Y. (1970) Further studies on the differentiation of a cell line of myeloid leukemia. J. Cell. Physiol. 76: 175.

Jankowski, W. J., W. von Muenchhausen and E. Sulkowski (1976) Binding of human interferons to immobilized Cibacron Blue F3GA: the nature of molecular interaction. Biochemistry. 15: 5182.

Kannagi, R., K. Teshigawara, N. Noro and T. Masuda (1982) Transglutaminase activity during the differentiation of macrophages. Biochem. Biophys. Res. Commun. 105: 164.

Kurland, J. I., L. M. Pelus, P. Ralph, R. S. Bockman and M. A. S. Moore (1979) Induction of prostaglandin E synthesis in normal and neoplastic macrophages: role for colony-stimulating factors distict from effects on myeloid progenitor cell proliferation. Proc. Natl. Acad. Sci. U. S. A. 76: 2326.

Kyoizumi, S., N. Noro, K. Teshigawara, S. Sakaguchi and T. Masuda (1982) A cloned cell line, Mk_1, possessing Ia antigens and accessory cell activity. J. Immunol. 128: 2586.

Leonard, E. J., L. P. Ruco and M. S. Meltzer (1978) Characterization of macrophage activation factor, a lymphokine that causes macrophages to become cytotoxic for tumor cells. Cell. Immunol. 41: 347.

Ly, I. A. and R. I. Mishell (1973) Separation of mouse spleen cells by passage through columns of Sephadex G-10. J. Immunol. Methods. 5: 239.

Maeda, M., M. Horiuchi, S. Numa and Y. Ichikawa (1977) Characterization of a differentiation-stimulating factor for mouse myeloid leukemia cells. Gann. 68: 435.

McDonald, H. R., R. A. Phillips and R. G. Miller (1973) Allograft immunity in the mouse. II. Physical studies of the development of cytotoxic effector cells from their immediate progenitors. J. Immunol. 111: 575.

Metcalf, D. (1979) Production of colony-stimulating factors by lymphoid tissues. In Biology of the Lymphokines. Edited by Cohen, S., E. Pick and J. J. Oppenheim. Acad. Press Inc., New York. 515.

Miyama, M., J. Yodoi, K. Kuribayashi, A. Takabayashi and T. Masuda (1978) Immunological properties of Fc receptor on lymphocytes. 1. Functional differences between Fc receptor positive and negative lymphocytes in humoral immune responses. Cell. Immunol. 35: 253.

Moore, R. N., J. J. Oppenheim, J. J. Farrar, C. S. Carter. Jr, A. Waheed and R. K. Shadduck (1980) Production of lymphocyte activating factor (Interleukin 1) by macrophages activated with colony-stimulating factors. J. Immunol. 125: 1302.

Morley, J. (1981) Role of prostaglandins secreted by macrophages in the inflammatory process. In Lymphokines Vol. 4. Edited by Landy, M. Acad. Press Inc., New York. 377.

Mosier, D. E. and L. W. Coppleson (1968) A three-cell interaction required for the induction of the primary immune response in vitro. Proc. Natl. Acad. Sci. U. S. A. 61: 542.

Niederhuber, J. E. (1978) The role of I region gene products in macrophage-T lymphocyte interaction. Immunol. Rev. 40: 28.

Nussenzweig, M. C., R. M. Steinman, B. Gutchinov and Z. A. Cohen (1980) Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. J. Exp. Med. 152: 1070.

Pick, E. (1979) Mechanism of action of migration inhibitory lymphokines. In Biology of the Lymphokines. Edited by Cohen, S., E. Pick and J. J. Oppenheim. Acad. Press Inc., New York. 60.

Rode, H. N. and J. Gordon (1970) The mixed lymphocyte cultre: a three component system. J. Immunol. 104: 1453.

Scher, M. G., D. I. Beller and E. R. Unanue (1980) Demonstration of a soluble mediator that induces exudates rich in Ia-positive macrophages. J. Exp. Med. 152: 1684.

Scher, M. G., E. R. Unanue and D. I. Beller (1982) Regulation of macrophage populations. III. The immunologic induction of exudates rich in Ia-bearing macrophages is a radiosensitive process. J. Immunol. 128: 447.

Steeg, P. S., R. N. Moore and J. J. Oppenheim (1980) Regulation of murine macrophage Ia-antigen expression by products of activated spleen cells. J. Exp. Med. 152: 1734.

Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydings and I. S. Mellman (1980) Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. J. Exp. Med. 152: 1248.

Thompson, S. T., K. H. Cass and E. Stellwagen (1975) Blue Dextran-Sepharose: an affinity column for the dinucleotide fold in proteins. Proc. Natl. Acad. Sci. U. S. A. 72: 669.

Tomida, M., Y. Yamamoto and M. Hozumi (1980) Stimulation by interferon of induction of differentiation of mouse myeloid leukemic cells. Cancer. Res. 40: 2919.

Twormey, J. J., O. Sharkey, J. A. Brown, A. H. Laughter and P. H. Jordan (1970) Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. J. Immunol. 104: 845.

Tzehoval, E., P. De Baetselier, M. Feldman and S. Segal (1981) The peritoneal antigen-presenting macrophage: control and immunogenic properties of distinct subpopulations. Eur. J. Immunol. 11: 323.

Vogel, S. N. and D. L. Rosenstreich (1979) Deffective Fc receptor mediated phagocytosis in C3H/HeJ macrophages. I. Correction by lymphokine-induced stimulation. J. Immunol. 123: 2842.

Wagner, H., M. Feldmann, W. Boyle and J. W. Schrader (1972) Cell-mediated immune response in vitro. III. The requirement for macrophages in cytotoxic reactions against cell-bound and subcellular alloantigens. J. Exp. Med. 136: 331.

Worton, R. G., E. A. McCulloch and J. E. Till (1969) Physical separation of hemopoietic stem cells from cells forming colonies in culture. J. Cell. Physiol. 74: 171.

Yamamoto, Y. and Y. Kawade (1980) Antigenicity of mouse interferons: Distinct antigenicity of the two L cell interferon species. Virology. 103: 80.

Yodoi, J., T. Masuda, M. Miyama, M. Maeda and Y. Ichikawa (1978) Interaction of lymphocytes and macrophage cell line cells (M_1 cells) 1. Functional maturation and appearance of Fc receptor in M_1 cells. Cell. Immunol. 39: 5.