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# An inhibitor of lymphocyte proliferation and lymphokine production released by unstimulated foetal monocytes

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SUMMARY

Supernatants obtained from 3-day cultures of cord blood monocytes inhibited normal lymphocyte activation by either PHA or in a two-way MLC. Both lymphocyte transformation and lymphokine production was significantly inhibited by these supernatants but not by those derived from adult mononuclear cell cultures. The inhibitory material produced by foetal monocytes was dialysable and was non-cytotoxic to target cells. It is postulated that this factor contributes to the depressed maternal cell-mediated immune response observed in pregnancy.

## INTRODUCTION

Of the many theories postulated as to why the foetus is not rejected by the mother, the most plausible would appear to relate to depressed immunological competence of maternal lymphocytes. (Finn *et al.*, 1972; Jones & Curzen, 1973; Purtilo, Hallgren & Yunis, 1972). Numerous workers have described factors in maternal serum or plasma which depress the reactivity of these lymphocytes (Ayoub & Kasakura, 1971; Rabson *et al.*, 1976; Yu *et al.*, 1975) and $\alpha_2$-globulins in maternal serum have been incriminated (Stimson, 1972; Than, Csaba & Sazlo, 1974). Olding & Oldstone (1974) demonstrated suppression of mitosis of maternal cells when mixed with neonatal cells in the presence of mitotic agents and Lawler, Ukaejiofo & Reeves (1975) in similar experiments suggested that the mechanism of this inhibition may depend upon contact between viable cells or on the liberation of inhibitory factors by the neonatal cells.

In this report the effect of supernatants derived from cultured neonatal mononuclear cells (MN) on the ability of normal adult activated lymphocytes to produce a lymphokine, leucocyte inhibitory factor (LIF) (Rocklin, 1974) and to incorporate [$^3$H]thymidine was assessed. The results indicate the presence of a dialysable inhibitory factor produced by neonatal monocytes.

## MATERIALS AND METHODS

*Production of inhibitory supernatants.* Cord blood (CB) from normal placentae and venous blood from normal adult volunteers was collected and mixed with preservative-free heparin (Panheparin, Abbot, 10 u/ml). MN cells were obtained be separating the blood on a Ficoll–Hypaque gradient. The MN cell fraction at the medium–Ficoll interphase was collected, the cells were washed three times in minimal essential medium (MEM) and the MN cell count was adjusted to $2\times10^6$/ml in hepes buffered MEM, pH 7·4 containing 10% FCS (Burroughs Wellcome), 2 mM L-glutamine (Flow Laboratories), 100 u/ml of penicillin and 100 $\mu$g/ml of streptomycin (Complete MEM). Cord blood suspensions were comprised of 85–90% MN cells whereas suspensions from normal venous blood contained fewer contaminating neutrophils. Cell viability as assessed by trypan blue exclusion was greater than 95%.

Unstimulated MN cells were cultured in plastic tissue culture flasks (Falcon) at 37°C in an atmosphere of 5% $CO_2$ in air for 48 hr after which cell free supernatants were obtained by centrifugation at 250 *g* for 10 min and tested for inhibitory activity.

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In a series of experiments supernatants were produced by monocyte—or lymphocyte-rich populations obtained from cord blood. In these experiments MN cells obtained as described above were initially cultured in tissue culture flasks for 18 hr. After this time nonadherent cells were removed and the flasks rinsed vigorously three times with fresh MEM. Of the remaining adherent cells (monocyte-rich) 98% were capable of ingesting live *Candida albicans* in the presence of normal AB serum. The original volume of complete MEM was added to the flasks which were then incubated for a further 48 hr.

Non-adherent cells (lymphocyte-rich) were washed three times in fresh MEM, resuspended to their original volume and reincubated for 48 hr after which the supernatants were collected and tested for inhibitory activity. In some experiments supernatants from CB monocyte-rich cultures were dialysed for 24 hr against 50 vol. of MEM. Dialysed and undialysed supernatants were then tested for inhibitory activity.

*Tests for inhibitory activity.* Supernatants from CB or adult MN cells were tested for their ability to inhibit lymphocyte activation by either allogeneic cells in a two-way mixed lymphocyte culture (MLC) or by PHA. Activation was measured by both [$^3$H]thymidine uptake and by assaying leucocyte inhibitory factor (LIF) production by the activated lymphocytes.

Adult MN cells were separated from peripheral blood on a Ficoll–Hypaque gradient as described above and were suspended in complete MEM to a concentration of $2\times10^6$ cells/ml. They were then cultured for 2 hr with or without 10 $\mu$g/ml PHA (Reagent grade, Burroughs Wellcome) in tissue culture flasks (Falcon) after which the pulsed cells were washed three times in MEM, resuspended to their original volume with cord or adult MN supernatant and cultured for a further 72 hr at 37°C in 5% of $CO_2$ in air. For MLC equal volumes of MN cell suspensions from 2 adults each containing $1\times10^6$ cells/ml were mixed, centrifuged, and the cell pellet resuspended in cord or adult blood MN supernatant to a final concentration of $2\times10^6$ MN/ml. Cells were cultured for 72 hr in 5% of $CO_2$ in air at 37°C in tissue culture flasks.

Cell-free supernatants were obtained from unstimulated or PHA pulsed cultures and from MLC by centrifugation at 250 *g* for 10 min, and were assessed for LIF activity. The remaining cell pellets were washed twice in fresh MEM and were resuspended in complete MEM to a concentration of $1\times10^6$ cells/ml. 0·2 ml of each of these suspensions was cultured in triplicate in round-bottomed microtitre plates in the presence of tritiated thymidine (1 $\mu$ci/well methyl-[$^3$H]thymidine sp. act. 17 ci/mol, Radiochemical Centre, Amersham) for a further 18 hr, after which they were harvested by a multiple harvester (Mash II). Each filter was washed successively with phosphate-buffered saline, 5% trichloroacetic acid in water, methanol and allowed to dry. Insta-Gel (Packard) was then added (10 ml) and thymidine incorporation measured in a Packard tri-carb liquid scintillation counter. The stimulation ratio was calculated for each group as follows:

$$SR = \frac{\text{mean ct/min of stimulated cultures}}{\text{mean ct/min of control cultures}}.$$

*LIF assay.* Human PMN leucocytes were obtained by separating whole blood diluted 1:1 in MEM on a Hypaque–Ficoll gradient. The pellet containing PMN and erythrocytes was treated with 0·83% ammonium chloride at 4°C for 10 min to lyse the erythrocytes. The remaining PMN were centrifuged at 200 *g* for 5 min and washed three times in MEM. The resultant suspension contained 98–99% PMN. Siliconized capillary tubes (50 Lambda Yankee disposable micropet Clay Adams) were filled with cell-suspension containing $3\times10^7$ cells/ml. The capillary tubes were then sealed with seal-ease (Clay Adams) and centrifuged for 5 min at 300 *g*. The capillary tubes were cut at the cell–fluid interphase and the capillary stumps were then placed on the floor of plastic disposable migration chamber plates (Sterilin Ltd., Middlesex, England), and held in place by a drop of silicon grease. The chambers were filled with the supernatants to be tested and sealed with a cover slip. They were then incubated for 18 hr at 37°C in 5% $CO_2$ in air. The migration pattern was projected and traced and the area of migration was measured by planimetry. (Each supernatant was assayed in triplicate.)

Percentage inhibition (PI) was calculated as follows:

$$PI = \left(1 - \frac{\text{average area migration in presence of PHA pulsed MLC MN supernatant}}{\text{average area migration in presence of control supernatant}}\right) \times 100.$$

## RESULTS

Supernatants produced by CB MN cells but not those obtained from adult MN cell cultures markedly inhibited both lymphocyte transformation and LIF production by PHA pulsed normal lymphocytes. ($P<0·005$ for both assays). Similar inhibitory activity was demonstrated in the supernatant derived from monocyte-rich CB cell populations but not in those obtained from lymphocyte-rich cultures (Fig. 1).

Similarly in two-way MLC, supernatants obtained from CB MN cells and CB adherent cells, but not normal adult MN cells or CB lymphocytes, significantly inhibited both [$^3$H]thymidine uptake and LIF production ($P<0·001$ for CB MN supernatants and CB monocyte supernatants in transformation studies and $<0·0005$ in LIF experiments) (Fig. 2). In all experiments, viability of the cultures as judged by trypan blue staining, was not significantly affected by the presence of CB or adult supernatants.

Dialysis of CB MN supernatants resulted in loss of the previously detected inhibitory activity (Fig. 3).

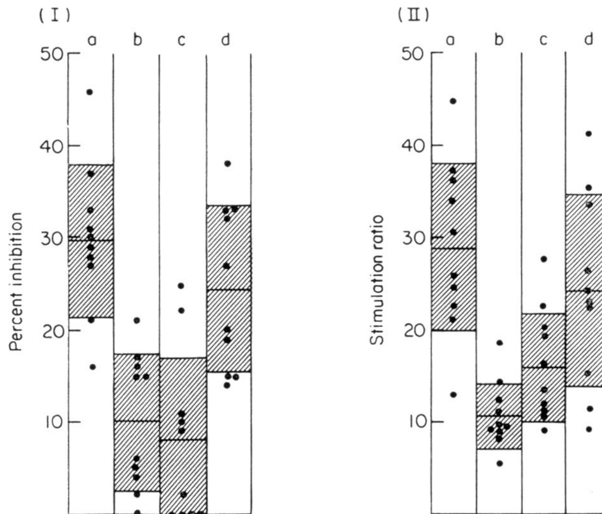


FIG. 1. The effects of supernatants from (a) adult MN cells; (b) CB mononuclear cells; (c) CB-enriched monocytes; (d) CB-enriched lymphocytes, on normal PHA-induced MN cell activation as assessed by (I) LIF production and (II) thymidine uptake. The mean ± s.d. are shown by the shaded areas.

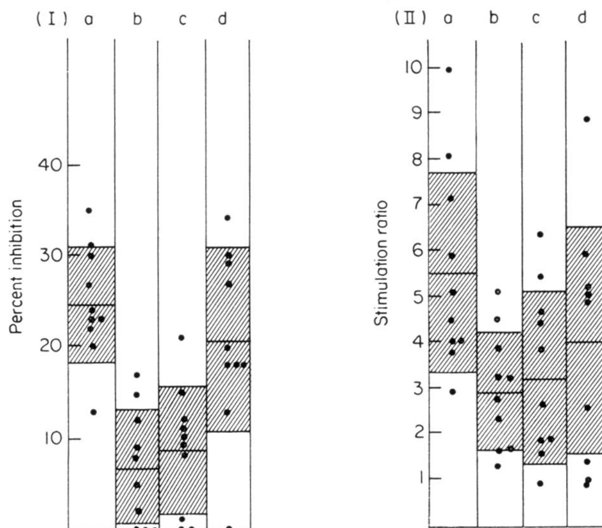


FIG. 2. The effects of supernatants from (a) adult MN cells; (b) CB mononuclear cells; (c) CB-enriched monocytes; (d) CB-enriched lymphocytes on normal two-way MLC as assessed by (I) LIF production and (II) thymidine uptake.

## DISCUSSION

The present study has revealed the production of a factor, by unstimulated foetal monocytes, which is capable of inhibiting normal adult mononuclear cell responses to both PHA activation and to MLC. MN cells obtained from normal adults on the other hand failed to produce this material. Trypan blue exclusion indicated that this inhibition of lymphocyte activation is not due to cytotoxicity. Olding & Oldstone (1976) have previously shown that populations of foetal T-enriched lymphocytes, but not macrophages, inhibit the division of maternal lymphocytes activated by PHA. They have considered these inhibitory cells as T suppressor cells, a population known to be increased in number in foetal life

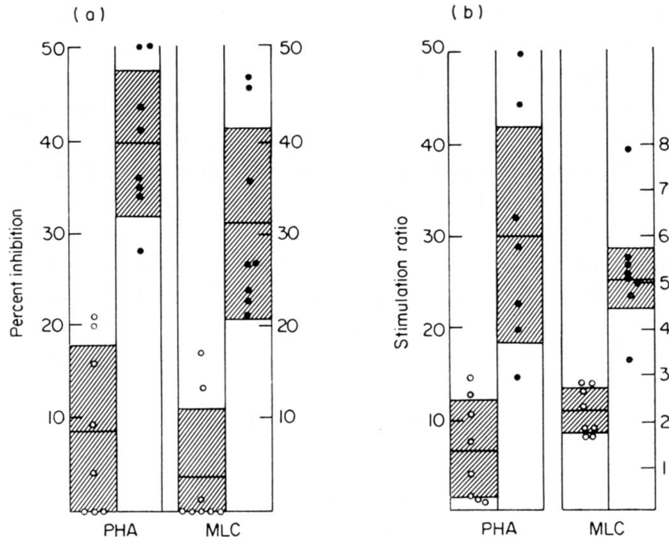


FIG. 3. The effects of dialysed (●) and undialysed (○) CB MN supernatants on (a) LIF production and (b) stimulation ratios of normal adult MN cells stimulated by PHA and in two-way MLC experiments.

(Gershon, 1975). Their system depended upon intimate foetal-maternal cell interaction. The experiments described in this paper, however, utilized supernatants obtained from cultured foetal mononuclear cells and these exerted a similar effect without the benefit of cell-to-cell contact. Since the active supernatant was produced by foetal adherent cell cultures but not by lymphocytes, it may be inferred that a separate mechanism to that described by Olding & Oldstone is operative. It could be hypothesized, that foetal monocytes are capable of spontaneously producing a soluble material which increases the numbers or the activity of suppressor T-lymphocytes. This product by entering the maternal circulation could in addition be responsible for the depressed cell-mediated immune responses observed in pregnancy by numerous workers. Ayoub & Kasakura (1971) showed that adult lymphocytes suspended in foetal plasma reacted significantly less to PHA stimulation than when cultured in adult plasma. Yu *et al.* (1975) observed that maternal as well as foetal serum could inhibit normal adult PHA responsiveness suggesting that this phenomenon may be due to a soluble factor elaborated by the foetus which crosses the placenta. Since the inhibitory material produced by the foetal MN cells was readily dialysable, it could presumably pass the placental barrier.

A number of workers have demonstrated that macrophages may have inhibitory effects upon immune responses when added to responding lymphocytes *in vitro* (Parkhouse & Dutton, 1966; Moore & Schoenberg, 1968; Diener, Shortman & Russel, 1970). More recently humoral factors produced by macrophage cultures have been shown to antagonize lymphocyte activation measured by DNA synthesis or protein synthesis from radioactive precursors. Nelson (1973) has found that 'activated' macrophages from mice produce a potent inhibitor of lymphocyte transformation, whereas the inhibitor described by Waldman & Gottlieb (1973) and Calderon, Williams & Unanue (1974) was produced by unstimulated macrophages. In this respect the inhibitory material produced by foetal macrophages resembles that described by the latter two groups. Moreover it is also dialysable and non-cytotoxic to target cells. It has previously been shown (Fernbach, Kirchner & Herberman, 1976) that an inhibitory effect of macrophage supernatants on [$^3$H]thymidine incorporation of tumour cells was not caused by a true inhibition of cellular proliferation but was due to the presence of lysed cell thymidine which competed with the labelled material. In this study, however, before [$^3$H]-labelled thymidine was added to cell cultures they were washed free of supernatant material. Furthermore, not only did foetal MN cell supernatants inhibit lymphocyte transformation but they also limited the production of the lymphokine LIF after activation by both PHA and MLC.

Although the nature of the inhibitory effect is unknown adherent cells have been shown to have important regulatory function in initiating or diminishing helper or suppressor-T cell function. Erb & Feldman (1975) have indicated the essential role of macrophages in the generation of T-helper cells. Yoshinaga, Yoshinaga & Waksman (1972) have shown that macrophages may suppress DNA synthesis by rat spleen or lymph node cells, and have suggested that the effect may be due not only to a direct effect of macrophages but also to a macrophage-dependent suppressor-T cell effect. Similarly it has been shown that adherent cells taken from the spleens of GVH mice exert a suppressive effect on T-cell helper function (Elie & Lapp, 1975).

It could be postulated, therefore, that the soluble substance produced by foetal monocytes crosses the placenta and either activates maternal T-suppressor cells or inhibits T-helper cell function. This mechanism could be responsible for the suppressed PHA responsiveness of maternal lymphocytes, and may thus be inhibiting the efferent limb of the immune response, aiding foetal graft survival.

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## REFERENCES


AYOUB, J. & KASAKURA, S. (1971) *In vitro* response of foetal lymphocytes to PHA, and a factor plasma which suppresses the PHA response of adult lymphocytes. *Clin. exp. Immunol.* **8**, 427.

CALDERON, J., WILLIAMS, R.T. & UNANUE, E.R. (1974) An inhibitor of cell proliferation released by cultures of macrophages. *Proc. nat. Acad. Sci. (Wash.)*, **71**, 4237.

DIENER, E., SHORTMAN, K. & RUSSEL, P. (1970) Induction of immunity and tolerance *in vitro* in the absence of phagocytic cells. *Nature (Lond.)*, **225**, 731.

ELIE, R. & LAPP, W.S. (1975) GVH induced immunosuppression: regulatory function of macrophages in the humoral immune response. *Immune Recognition* (ed. by A. S. Rosenthal), p. 563. Academic Press, New York.

ERB, P. & FELDMANN, M. (1975) Role of macrophages in the *in vitro* induction of T-helper cells. *Nature (Lond.)*, **254**, 352.

FERNBACH, B.R., KIRCHNER, H. & HERBERMAN, R.B. (1976) Inhibition of the mixed lymphocyte culture by peritoneal exudate cells. *Cell. Immunol.* **22**, 399.

FINN, R., ST. HILL, C.A., GOVAN, A.J., RALFS, I.G., GURNEY, F.J. & DENYE, V. (1972) Immunological responses in pregnancy and survival of foetal homograft. *Brit. med. J.* **iii**, 150.

GERSHON, R.K. (1975) *Immune depression and cancer*, (ed. by G. W. Siskind, C. L. Christian and S. A. Litwin), p. 16. Grune and Stratton, New York.

JONES, E. & CURZEN, P. (1973) The immunological reactivity of maternal lymphocytes in pregnancy. *J. Obstets. & Gynaec. Brit. Comm.* **80**, 608.

KASAKURA, S. (1971) A factor in maternal plasma during pregnancy that suppresses the reactivity of mixed leukocyte cultures. *J. Immunol.* **107**, 1296.

LAWLER, S.D., UKAEJIOFO, E.O. & REEVES, B.R. (1975) Interaction of maternal and neonatal cells in mixed lymphocyte cultures. *Lancet*, **ii**, 1185.

MOORE, R.D. & SCHOENBERG, M.D. (1968) Restimulation of antibody synthesis by antigen in cultures of lymphocytes. *Nature (Lond.)*, **219**, 297.

NELSON, D.S. (1973) Production by stimulated macrophages of factors depressing lymphocyte transformation. *Nature (Lond.)*, **246**, 161.

OLDING, L.B. & OLDSTONE, M.B.A. (1974) Lymphocytes from human newborns abrogate mitosis of their mothers' lymphocytes. *Nature (Lond.)*, **249**, 161.

OLDING, L.B. & OLDSTONE, M.B.A. (1976) Thymus-derived peripheral lymphocytes from human newborns inhibit division of their mothers' lymphocytes. *J. Immunol.* **116**, 682.

PARKHOUSE, R.M.E. & DUTTON, R.W. (1966) Inhibition of spleen cell DNA synthesis by autologous macrophages. *J. Immunol.* **97**, 663.

PURTILO, D.T., HALLGREN, H.M. & YUNIS, E.J. (1972) Depressed maternal lymphocyte response to phytohaemagglutinin in human pregnancy. *Lancet*, **i**, 769.

RABSON, A.R., BEY, M.C., KERRICH, J.E. & KOORNHOF, H.J. (1976) The blocking by autologous serum of maternal cell-mediated immune reactions to placental antigen. *South Afr. Med. J.* **50**, 201.

ROCKLIN, R.E. (1974) Products of activated lymphocytes: leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor (MIF). *J. Immunol.* **112**, 1461.

STIMSON, W.H. (1972) Transplantation—nature's success. *Lancet*, **i**, 684.

THAN, G.N., CSABA, I.F. & SZABO, D.G. (1974) An antigen associated with pregnancy. *Lancet*, **ii**, 1578.

WALDMAN, S.R. & GOTTLIEB, A.A. (1973) Macrophage regulation of DNA synthesis in lymphoid cells: effects of a soluble factor from macrophages. *Cell. Immunol.* **9**, 142.

YOSHINAGA, M., YOSHINAGA, A. & WAKSMAN, B.H. (1972) Regulation of lymphocyte responses *in vitro*. *J. exp. Med.* **136**, 956.

YU, V.Y.H., WALLER, C.A., MACLENNAN, I.C.M. & BAUM, J.D. (1975) Lymphocyte reactivity in pregnant women and newborn infants. *Brit. med. J.* **i**, 428.