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ROLE OF ADHERENT MONONUCLEAR CELLS DERIVED FROM ADULT PIGS AND SUCKLING PIGLETS IN RESPONSE TO POKEWEED MITOGEN-INDUCED IMMUNOGLOBULIN PRODUCTION

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The effect of monocytes as an activator of a function of lymphocytes in pigs was studied using an in vitro Ig production system with pokeweed mitogen (PWM).

When mononuclear cells (lymphocytes) from adult pigs were stimulated by PWM, optimal number of adherent cells (monocytes) collected from serum coated plastic Petri dishes were required for the generation of Ig producing cells, whereas the production was suppressed by the addition of excessive adherent cells.

In neonatal and suckling piglets up to 6 weeks of age, PWM induced B lymphocyte differentiation was enhanced by addition of adherent cells from peripheral blood of adult pigs.

On the other hand, adherent cells derived from piglets did not have the effect of increasing Ig production in the *in vitro* Ig production system with lymphocytes (non-adherent) derived from adult pigs.

Key words: Adherent mononuclear cells, immunoglobulin production, suckling piglet, pokeweed mitogen

INTRODUCTION

Newborn piglets have limited immune responsiveness (ALLEN & PORTER, 1977; KIM, 1975; YABIKI et al., 1974). Recently SUGANUMA et al. (1986) reported that this immunodeficiency might be due to activation of suppressor T lymphocytes.

Such activity with suppressor T lymphocytes in the neonatal period has also been reported (DURANDY et al., 1979; MIYAWAKI et al., 1979; OLDING & OLDSTONE, 1976) in human babies.

Both macrophages and peripheral blood monocytes were shown to play an impor-

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tant role in immunodeficiency in the neonatal stage (DURANDY et al., 1982; FISHER et al., 1981). It is therefore considered that the immunosuppressive activity in the neonatal and suckling stage might be modulated by negative macrophages. However, there have been few studies on the functions of macrophages and monocytes in piglets in relation to immunoresponsiveness.

This study was carried out to elucidate the effect of monocytes as an activator of a function of lymphocytes in suckling piglets using an *in vitro* Ig production system with PWM.

MATERIALS AND METHODS

Animals

Three specific-pathogen-free (SPF) pigs (3 to 8 months old) and three piglets from a SPF sow were used. They were maintained in a minimal disease condition.

Isolation of mononuclear cells from peripheral blood

Blood samples were collected from the jugular vein and mononuclear cells were isolated by Ficoll-conray gradients (NAMIOKA et al., 1983). Briefly, blood was diluted with an equal volume of phosphate buffered saline (PBS, pH 7.2), the diluted blood (10 ml) was layered on a 3 ml of Ficoll-conray solution (10 parts of 33.4% conray with 24 parts of 9% Ficoll, specific gravity 1.084 ± 0.001) and centrifuged for 30 min at 400xG at 4°C. The mononuclear cells recovered from the interface were washed three times in PBS at 4°C. Contaminating erythrocytes were removed by lysing with Tris-buffered isotonic 0.83% NH_4Cl, and the mononuclear cells were washed again twice in PBS at 4°C and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) and HEPES (20mM/ml).

Preparation of the dishes

Four kinds of dishes were prepared as follows: glass Petri dishes (10 cm in diameter), gelatin-serum coated glass Petri dishes, plastic Petri dishes (9 cm in diameter) and serum coated plastic Petri dishes, respectively, and then used for isolation of adherent cells (monocytes). Gelatin-serum coated glass Petri dishes were prepared as previously described (BEVILACQUA et al., 1981). Ten milliliters of gelatin solution were added to the glass Petri dishes and then incubated at 37°C 2h. The gelatin solutions were removed by aspiration and the dishes were dried. FBS or porcine serum heat inactivated at 56°C for 30 min were added in 6 ml portions to the gelatin-coated dishes. After incubation overnight at 4°C, the dishes were washed twice with PBS. The serum coated plastic Petri dishes were prepared as previously described (KUMAGAI et al., 1979). Six milliliters of FBS or porcine serum heat-inactivated at 56 C for 30 min were incorporated into the dishes. These dishes were incubated overnight at 4°C and then rinsed twice with PBS.

Isolation of adherent cells

Porcine peripheral blood mononuclear cells in RPMI 1640 medium (containing 10% FBS) were adjusted to cell concentrations of 5×10^5/ml, and 6 ml of the cell suspensions were added to the prepared dishes. They were incubated for 1 or 1.5h at 37°C in a 5% CO_2 atmosphere. After incubation, medium containing non-adherent cells was decanted and the dishes were rinsed twice with warm media. Five milliliters of cold PBS (containing 0.2% EDTA and 5% FBS) were added and the dishes were incubated at 4°C for 15 min. Adherent cells were then gently dislodged by rinsing, washed three times and resuspended in RPMI 1640 medium. The adherent cells were identified as monocytes by staining for peroxidase activity. B lymphocytes were identified by indirect immunofluorescent staining for cell surface IgG. T lymphocytes were identified by E-rosette assay (BUSHMANN & PAWLS, 1980).

Culture of mononuclear cells, adherent cells and non-adherent cells

Each cell preparation was suspended at a concentration of 5×10^5/ml in RPMI 1640 medium supplemented with 10% FBS, antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml) and HEPES (20mM/ml), respectively. Two milliliters of cells were placed in a culture tube (#2063, Falcon USA) with 5 μl/ml of PWM and then incubated at 37°C for 3 days in a humidified atmosphere of 5% CO_2 and 95% air.

Addition of adherent cells from adult pigs to mononuclear cells or non-adherent cells from adult pigs

Mononuclear cells and non-adherent cells from adult pigs were adjusted to cell concentrations of 10^6/ml in RPMI 1640 medium, and 1 ml of the cell suspensions were mixed with an equal volume of adherent cells ($1\times10^5-1\times10^6$). The mixtures were incubated with 5 μl/ml of PWM at 37°C for 3 days in a 5% CO_2 atmosphere.

Addition of the adherent cells from adult pigs to mononuclear cells from suckling piglets, and addition of the adherent cells from suckling piglets to non-adherent cells from adult pigs

Peripheral blood mononuclear cells from suckling piglets were suspended at a concentration of 10^6/ml in RPMI 1640 medium, and 1 ml of the cell suspension was mixed with an equal volume of adherent cells from adult pigs at a density of 1 to 7×10^5.

On the other hand, non-adherent cells from adult pigs were adjusted to cell concentrations of 10^6/ml, and 1 ml of the cell suspension was added to an equal volume of adherent cells from suckling piglets at a density of 1×10^5 to 1×10^7. The mixtures were incubated with 5 μl/ml of PWM at 37°C for 3 days in a 5% CO_2 atmosphere.

Determination of the number of immunoglobulin producing cells

The plaque assay method was used (SUGANUMA et al., 1986). After incubation, cells were washed twice in Hanks' balanced salt solution (HBSS) and resuspended at a concentration of 10^6/ml in HBSS. One hundred μl of the cell suspensions were added

with 20 μl of Protein A coupled sheep red blood cells (SRBC) diluted 1 : 2.5, 20 μl of the antiserum diluted 1 : 20, (rabbit anti-porcine IgG, Miles Scientific, USA) and 20 μl of SRBC-absorbed guinea pig complement (diluted 1 : 4) to 300 μl of HBSS containing 0.6% agarose. This mixture was placed in a 9 cm plastic Petri dish and incubated at 37°C for 12h in a humidified incubator under 5% CO_2. Plaques were counted under indirect light.

RESULTS

Properties of adherent cells in dishes

Mononuclear cells from adult pigs attached to the four kinds of dishes. The properties of the adherent cells in the different dishes are shown in table 1. Almost all the adherent cells in the glass Petri dishes were surface IgG positive (Ig^+)cells except for a few peroxidase positive (PO^+) cells. In the gelatin-serum coated glass dishes, the adherent cells still contained more than 50% Ig^+ cells but only 26.3% PO^+ cells on the average.

TABLE 1 *The properties of adherent cells from porcine peripheral blood mononuclear cells shown by four kinds of dishes*

DISHES	CELL TYPE		
	peroxidase positive cells (%)	surface IgG positive cells (%)	E-rosette forming cells (%)
glass	6.35±2.65	82.1	2.2
gelatin-serum coated glass	26.3±3.15	53.7±6.35	n.d.**
plastic	1	n.d.	n.d.
serum coated plastic	35.3±0.87	2.85±0.08	11.7±0.87
non-adherent*	5.23±1.37	17.7±3.20	35.8±1.05
mononuclear cells	13.4±1.55	16.4±2.67	35.9±2.07

*serum coated plastic dishes, **not done

On the other hand, the adherent cells in the serum coated plastic dishes contained only 2.85% of Ig^+ cells and 35.3% PO^+ cells on the average, whereas PO^+ cells hardly adhered to the non-coated plastic dishes. Following these results, serum coated plastic dishes were employed throughout the study to obtain adherent cells (monocytes). Non-adherent cells in serum coated plastic dishes contained only 5.23% of PO^+ cells on the average. However, there was no difference between non-

adherent cells and mononuclear cells in the ratio of Ig^+ cells and E-rosette forming cells.

Generation of Ig-producing cells in mononuclear cells, non-adherent cells and adherent cells derived from adult pigs

Adherent cells from adult pigs generated only a few Ig-producing cells in the presence of PWM (fig 1). The number of Ig-producing cells in non-adherent cells was less than 20% of that of mononuclear cells.

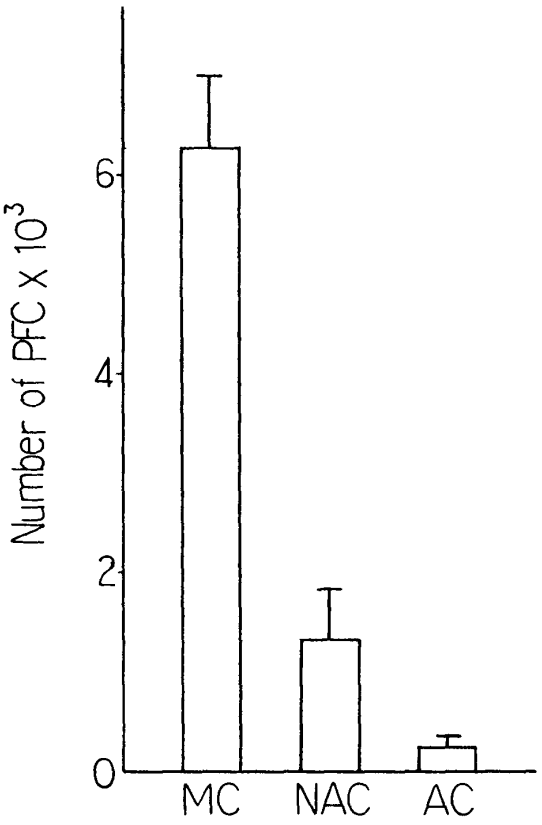

FIGURE 1 Generation of PWM-induced plaque forming cells (PFC) of mononuclear cells (MC), non-adherent cells (NAC) and adherent cells (AC) from adult pigs. 10^6 cells of MC, NAC and AC from adult pigs were cultured for three days in the presence of PWM. Data represent the mean ±S. E.

Addition of adherent cells to non-adherent cells or mononuclear cells from adult pigs

When the adherent cells(AC) from adult pigs added to non-adherent cells (NAC) in a culture tube (●——●), the number of Ig-producing cells increased and was dose-dependent (fig. 2). With the addition of AC at $4x10^5$, the number of Ig-producing cells in cultures reached the level of that of mononuclear cells from adult pigs. However, with the addition of $5x10^5$ AC, the number of Ig-producing cells decreased.

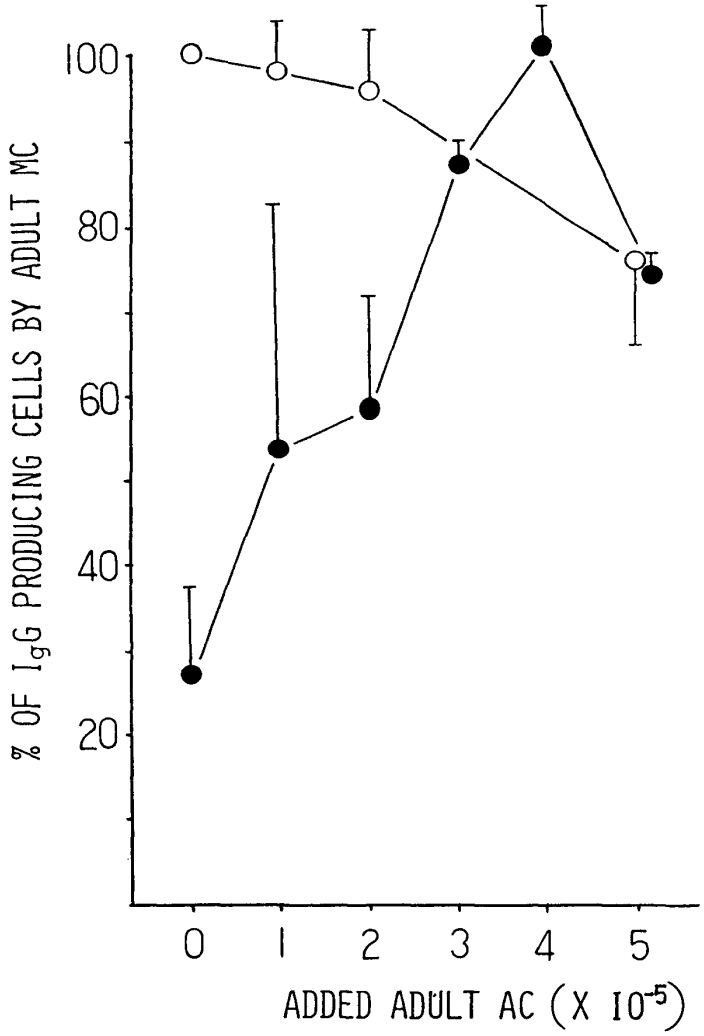

FIGURE 2 The effect of adherent cells(AC) from adult pigs on the generation of Ig-producing cells by mononuclear cells (○——○) and non-adherent cells (●——●) from adult pigs. Results are expressed as the percentage of Ig-producing cells by mononuclear cells (MC) from adult pigs.

With the addition of adult AC at $5x10^5$ to mononuclear cells(○——○), the number of Ig-producing cells decreased. When more than $5x10^5$ adult AC added to mononuclear cells, the number of Ig-producing cells also decreased (data not shown).

Addition of adult AC to mononuclear cells from suckling piglets

When adult AC were added to mononuclear cells from suckling piglets at 1, 3, 5 and 6 weeks of age, the number of Ig-producing cells increased clearly according to increasing additions of the adult AC (fig. 3).

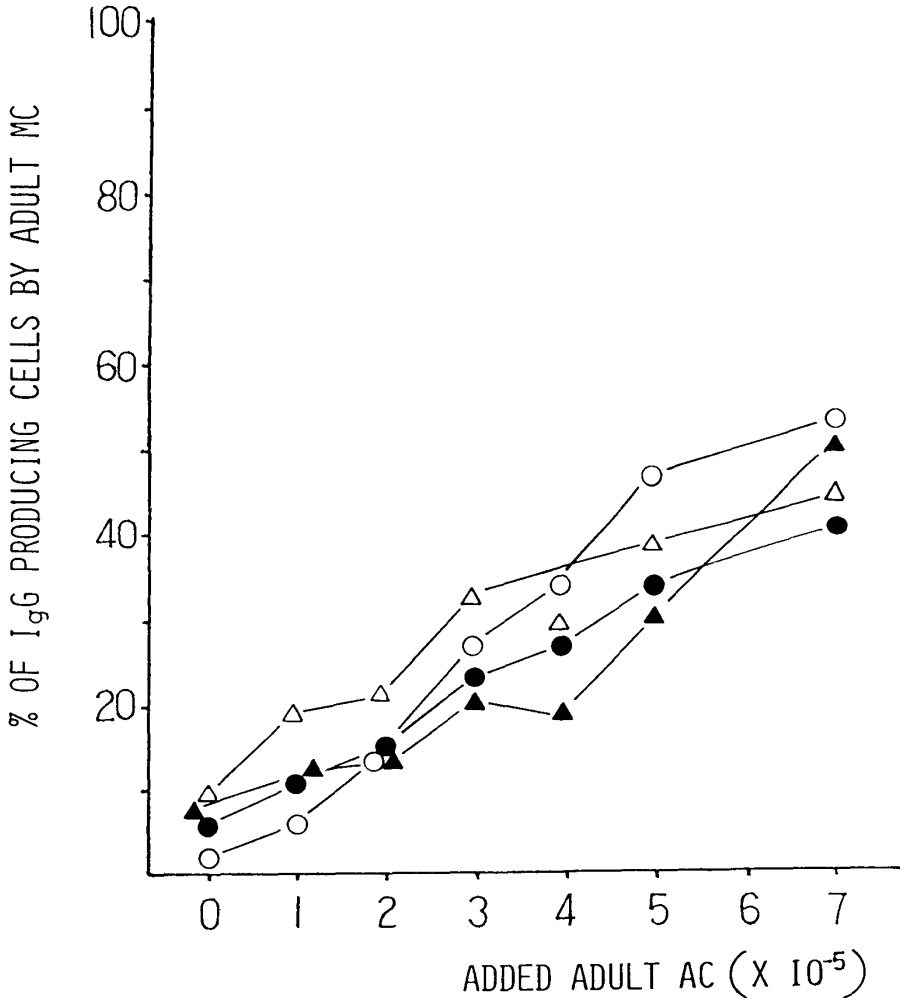

FIGURE 3 The effect adherent cells (AC) from adult pigs on the generation of Ig-producing cells by mononuclear cells from suckling piglets. Results are expressed as the percentage of Ig-producing cells by adult MC. ○——○: 1 week ●——●: 3 weeks △——△: 5 weeks ▲——▲: 6 weeks

Addition of piglet AC to non-adherent cells derived from adult pigs

When AC from piglets of five or six weeks of age were added to non-adherent cells from adult pigs, the number of Ig-producing cells in these cultures remained the same (fig. 4).

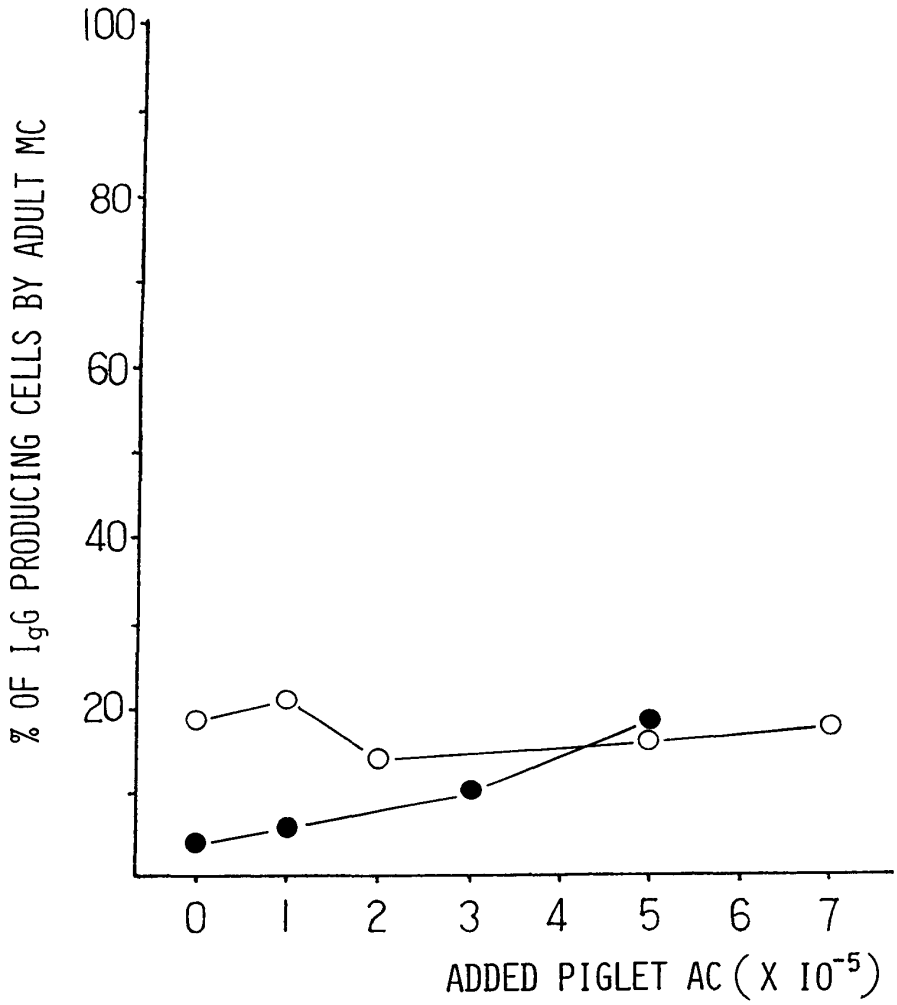

FIGURE 4 The effect of adherent cells (AC) from suckling piglets at five (●——●) or six (○——○) weeks of age on the generation of Ig-producing cells by non-adherent cells from adult pigs. Results are expressed as the percentage of Ig-producing cells by adult MC.

Discussion

Although there have been many reports on the isolation of monocytes from peripheral blood in man, little information has appeared concerning these findings in pigs.

In this study, less than 10% of monocytes in adult pigs attached to a plastic or glass surface, while approximatley 35% of the cells that adhered to serum coated plastic Petri dishes were classified as monocytes. On the other hand, the adherent cells obtained from the serum coated plastic surface contained only 12% of E-rosette forming cells and generated only a few Ig-producing cells in the presence of PWM. Therefore, the optimal procedure for obtaining "adherent cells" as monocytes in pigs was established in this study.

In humans macrophages have been shown to be required for PWM-stimulated lymphocyte proliferation and generation of Ig-producing cells (Rosenberg & Lipsky, 1979). When purified monocytes were added in excess to monocyte-depleted lymphocytes or to unseparated cells, a suppression of Ig production (Meyling & Waldmann, 1981) occurred.

In this study, when adherent cells from adult pigs were removed from peripheral blood mononuclear cells, the Ig-producing ability (non-adherent cells) declined in spite of the fact that no difference in the ratio of T and B lymphocytes between non-adherent cells and mononuclear cells was seen. Furthermore, when adherent cells were added to non-adherent cells, the number of Ig-producing cells reached the level of that obtained with peripheral blood mononuclear cells. However, when over $5x10^5$ adherent cells were added to mononuclear cells derived from adult pigs, a suppression of Ig production occurred.

As was shown in humans, the adherent cells from the present adult pigs had both an enhancing and a suppressive effect on Ig production of mononuclear cells in a dose-dependent manner.

In a previous study on pigs, Suganuma et al. (1986) reported that the suppressor activity of newborn T lymphocytes was still observed at four weeks of age.

In our experiments, PWM induced B lymphocyte differentiation of suckling piglets was enhanced by the addition of adherent cells from adult pigs into the piglets' mononuclear cells. In contrast, adherent cells from piglets had no effect on increase of Ig production when the cells were added to non-adherent cells from adult pigs. From the findings mentioned above, it might be considered that adherent cells (monocytes) derived from piglets possess some modulations for suppressive activity to Ig production, or the cells may be in an immature state to enhance the activity of helper T lymphocytes. The reasons why the generation of Ig-producing cells in peripheral blood mononuclear cells from suckling piglets is low might be because adherent cells, as the negative macrophage, modulate the activity of suppressor T

lymphocytes, which leads to poor Ig-production. It was interesting that the suppressive function of adherent cells was still observed at six weeks old and that the Ig-production reached about half the adult mean, although no suppressor activity of T lymphocytes was found at this age (SUGANUMA et al., 1986).

REFERENCES

1) ALLEN, W. D. & PORTER, P. (1977): The relative frequencies and distribution of immunoglobulin-bearing cells in the intestinal mucosa of neonatal and weaned pigs and their significance in the development of secretory immunity *Immunology.*, **32**, 819–824
2) BEVILACQUA, M. P., AMRANI, D., MOSESSON, M. W. & BIANCO, C. (1981): Receptors for cold-insoluble globulin (Plasma fibronectin) on human monocytes *J. Exp. Med.*, **153**, 42–60
3) BUSCHMANN, H. & PAWLAS, S. (1980): A study of porcine lymphocyte populations; I. Separation of porcine lymphocyte subpopulations *Vet. Immunol. Immunopathol.*, **1**, 215–224
4) DURANDY, A., FISCHER, A. & GRISCELLI, C. (1979): Active suppression of B lymphocyte maturation by two different newborn T lymphocyte subsets *J. Immunol.*, **123**, 2644–2650
5) DURANDY, A., FISCHER, A. & GRISCELLI, C. (1982): Inability of newborns' or pregnant women's monocytes to suppress pokeweed mitogen-induced responses *Ibid.*, **128**, 525–529
6) FISCHER, A., DURANDY, A. & GRISCELLI, C. (1981): Role of prostaglandin E_2 in the induction of nonspecific T lymphocyte suppressor activity *Ibid.*, **126**, 1452–1455
7) KIM, Y. B. (1975): Immunodeficiency in Man and Animals 549–557, Massachusetts: Sinauer Associations
8) KUMAGAI, K., ITOH, K., HINUMA, S. & TADA, M. (1979): Pretreatment of plastic petri dishes with fetal calf serum. A simple method for macrophage isolation *J. Immunol. Methods.*, **29**, 17–25
9) MEYLING, F. G. & WALDMANN, T. A. (1981): Human B cell activation *in vitro*: Augmentation and suppression by monocytes of the immunoglobulin production induced by various B cell stimulants *J. Immunol.*, **126**, 529–537
10) MIYAWAKI, T., SEKI, H., KUBO, M. & TANIGUCHI, N. (1979): Suppressor activity of T lymphocytes from infants assessed by co-culture with unfractionated adult lymphocytes in the pokeweed mitogen system *Ibid.*, **123**, 1092–1096
11) NAMIOKA, S., OHSUGI, T., FUJIMOTO, T., & MAEDE, Y. (1983): Lymphocyte transformation of peripheral blood lymphocytes and plaque forming cells of the spleen and the mesenteric lymph node in suckling piglets with and without immunopotentiators *Jpn. J. Vet. Res.*, **31**, 53–64
12) OLDING, L. B., & OLDSTONE, M. B. A. (1976): Thymus-derived peripheral lymphocytes from human newborns inhibit division of their mothers' lymphocytes *J. Immunol.*, **116**, 682–686
13) ROSENBERG, S. A. & LIPSKY, P. E. (1979): Monocyte dependence of pokeweed

mitogen-induced differentiation of immunoglobulin-secreting cells from human peripheral blood mononuclear cells *Ibid.*, **122**, 926–931

14) SUGANUMA, A., ISHIZUKA, A., SAKIYAMA, Y., MAEDE, Y., & NAMIOKA, S. (1986): B lymphocyte differentiation and suppressor activity by T lymphocytes derived from neonatal and suckling piglets *Res. Vet. Sci.*, **40**, 400–405

15) YABIKI, T., KASHIWAZAKI, M. & NAMIOKA, S. (1974): Quantitative analysis of three classes of immunoglobulins in serum of newborn pigs and milk of sows *Am. J. Vet. Res.*, **35**, 1483–1489