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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

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Lymphocyte transformation with mitogen, phytohemagglitinin (PHA) and concanavalin A (conA) was tested in peripheral blood lymphocytes from suckling piglets aged 1, 2, 3, 4 and 5 weeks old respectively. A study was also made to observe plaque forming cells with sheep red blood cells (SRBC) in the spleen and nucleated cells of the mesenteric lymph nodes (MLN) of piglets aged 2, 3 and 4 weeks respectively. Reratively high levels of lymphocyte responses were seen in the 1 week-old piglets. Differences of stimulation indexes (SI) of lymphocyte responses of the piglets were not significant in the animals aged from 1 to 5 weeks. The effect of administration of peptidoglycan and chemical composites of cultural supernates derived from *Streptomyces olivaceogriseus* sp. *nov* (FR41565) on the lymphocyte responses was investigated. The value of SI among the piglets treated with and without peptidoglycan and FR41565 was not significant. On the other hand, the count of plaque forming cells (PFC) of the splenic and MLN's cells with SRBC in the piglets aged 2 to 4 weeks was low in spite of the SRBC injections, and the count of PFC of MLN was fewer than that of the spleen of any age. The count of PFC was the highest in the piglets treated with the rapid acting immunopotentiator (FR41565) at 2 weeks of age while the highest count was seen in the piglets administered the slow acting substance (peptidoglycan) at 4 weeks of age. These results indicated that differentiation of B lymphocyte in suckling age piglets seems to be restrained by a certain suppressive activity which leads to poor production of immunoglobulin.

Key words: suckling piglet, lymphocyte transformation, plaque forming cell, immunopotentiator, immunodeficiency.

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INTRODUCTION

A slight production of immunoglobulin (Ig) in suckling piglets is detectable initially from 10 to 30 days of age[15,18,19] and colostrum-deprived piglets have been shown to possess transient agammaglobulinemia in this period. This fact reveals the poor differentiation of lymphocytes into antibody-forming cells, *i. e.*, plasma cells (Kim 1975). This immunodeficiency resembles the condition of variable hypogammaglobulinemia with B lymphocytes commonly found in human infants[3]. Previously NAMIOKA et al. (1982) reported on the effect of the administration of certain immunopotentiator (peptidoglycan) on the immunoresponse and the incidence of diarrhoea among suckling piglets: they observed a larger number of IgA-bearing cells in the lamina propria of the mucosa of the small intestines in the peptidoglycan-treated groups aged four weeks than that of the control groups, while no clear difference of serum IgG levels was seen between the treated and the control groups. However, lower incidences of diarrhoea in the piglets treated with peptidoglycan were seen ($P<0.0025$) compared to those of the control group. These results stimulated interest in more concrete studies to elucidate any additional mechanisms involved in the activities of T and B lymphocytes in the neonatal stage.

The purpose of the present report was to study lymphocyte transformation of peripheral blood lymphocytes with mitogens and plaque forming cells (PFC) to SRBC in the spleen and in the mesenteric lymph node of suckling piglets. Furthermore, the authors examined whether or not the immunopotentiator had any influence on the results of this study.

MATERIALS AND METHODS

I *Lymphocyte transformation*

Animals

A total of 19 piglets consisting of 2 litters from 2 specific pathogen free (SPF) sows was used for this study. Nine piglets were from dam No. 1 and the other 10 from dam No. 2 respectively. The piglets were born and reared under SPF conditions. Four piglets from dam No. 1 were administered peptidoglycan (Esai Pharm. Co. Ltd., Japan) extracted from *Bifidobacterium thermophilum* strain P2–91 (NAMIOKA et al, 1982) orally at the dose of 0.25ml, which was derived from 10^{12} of native cells, at birth, 2, 3, 4, and 5 days of age respectively, and the remaining 5 piglets of the same litter were left untreated as controls.

FR41565 [n-Heptanoyl-γ-D-glutamyl-(L)meso-α, ε-diaminopimelyl(L)-D-alanine: molecular weight 502.58, a chemical composite of the supernates derived from the culture of *Streptomyces olivaceogriseus* sp. *nov* (Fujisawa Pharm. Co. Ltd., Japan)] was injected into the 5 piglets from dam No. 2, 3 times at the dose of 50γ/ml subcutaneously at birth, 1 week and 2 weeks of age respectively, and the remaining 5 piglets were left untreated. Of the treated piglets, one died at 13 days of age due to

unknown causes. For comparison of the results, 2 adult pigs aged 7 and 12 months old were employed.

The peptidoglycan used in this study is a slow acting substance[10] while, according to AOKI (1981), FR41565 is a rapid acting one whose efficacy ceases within a week as confirmed by a mouse challenge test with *Salmonella typhimurium.*

Separation of lymphocytes from peripheral blood

Blood (5ml) was collected from the cranial vena cava using a 5ml disposable syringe rinsed with heparine (0.1% in saline solution) when the piglets were 1, 2, 3, 4 and 5 weeks of age respectively. The blood was then transferred to a 15ml sterile plastic tube and diluted 1 : 1 in a phosphate-buffered saline solution (PBS) of pH 7.4. Solutions for density-gradient centrifugation were prepared by mixing 10 parts of 33.4% Conray 400 solution (sodium 5 acetamido-2, 4, 6-triiodo-N-methylisophthalamate ; Daiichi Pharm. Co. Ltd., Japan) and 9% Ficoll aqueous solution (Pharmacia, Sweden) and diluting them with distilled water using a gravitometer to obtain a final specific gravity of 1.085 ± 0.001 at 20°C. The Ficoll-Conray 400 mixture with 1.085 ± 0.001 was used for separation of lymphocytes from the peripheral blood. With this method, good lymphocyte recovery and pure lymphocyte rates were obtained.

Next, the Ficoll-Conray 400 solution (4ml) was pipetted into a 15ml sterile plastic tube, and the diluted blood (10ml) was carefully layered over the solution. After centrifugation at 400 x g for 30 min at room temperature, the lymphocyte rich middle fraction was withdrawn, diluted in the PBS and then centrifuged at 20 x g for 10 min at 4°C. The cells were washed again by centrifugation of 80 x g for 7 min at 4°C with RPMI 1640 medium. After that, the cells were suspended at a concentration of 10^6/ml in RPMI 1640 medium supplemented with 10% bovine fetal serum, antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml, fungizon 2.5 μg/ml) and HERPES (20 mM/ml). The use of this method yielded a recovery rate of more than 60% lymphocytes, that is 90% pure lymphocytes, and the viability of the lymphocytes was more than 95%.

Lymphocyte culture

Phytohemagglutinin (PHA) and concanavalin A (conA) were reconstituted with sterile PBS and tested at concentrations of from 1 to 15 μg/ml in preliminary experiments to determine the optimal dose. Finally, PHA was used at a concentration of 10 μg/ml and conA at a concentration of 2.5 μg/ml.

Two hundred μl of cells (10^6/ml) in triplicate cultures were incubated in 96 flat bottom well microtiter plates with 20 μl of mitogen in PBS alone for 54 h at 37°C in 5% humidified CO_2 in the air.

The cultures were subsequently pulsed with 0.25 μCi of ^{3}H-thymidine (specific activity 21.5 Ci/mmole) in 20 μl PBS for an additional 18 h before harvested by an

automatic cell harvester on glass fiber filters using distilled water. The filters were then transferred to a scintillation solution (PPO 5g plus 0.1g/l of toruene). All samples were counted for 1 min on a scintillation spectrometer. Results were recorded as count per minute (cpm), and stimulation indexes (SI) were calculated as follows: SI=cpm in mitogen stimulated cultures/cpm in unstimulated cultures.

II *Plaque forming cells*

Animals

A total 24 piglets consisting of 3 litters from 3 SPF sows were used for this experiment. The piglets employed were in SPF condition throughout the experiment.

Three piglets from dam No. 1 and 4 from dam No. 2 were inoculated subcutaneously with FR41565 3 times at the dose of 50 γ/ml at birth, 1 week and 2 weeks of age respectively, and the remaining 3 piglets from dam No. 1 and 4 from dam No. 2 were left untreated. The peptidoglycan described previously was administered orally at the dose of 0.25ml to 5 piglets from dam No. 3 at birth, 2, 3, 4 and 5 days of age respectively. The other 5 piglets from dam No. 3 were left untreated as controls.

Immunity

After the blood was collected aseptically from sheep, an equal volume of sterilized Alsever solution was added and centrifuged at 710 x g for 10 min in 4°C, and the supernatant was discarded. The packed cells were washed 3 times with sterilized saline at 710 x g for 10 min to obtain 40% SRBC saline suspension. The SRBC suspension was inoculated twice into all of the piglets employed: 0.5ml at birth and 1.5ml intraperitonealy at 4th day before carrying out PFC assays respectively.

Medium

4.7g of Eagle's MEM medium (Nissui Co. Ltd., Japan) were dissolved in 500ml of distilled water and then sterilized (at 121°C for 15 min), after which 0.15g of glutamine (Nissui Co. Ltd., Japan) was added. Next, 1ml of penicillin (200,000 u/ml) and 1ml of streptomycin (20.0 mg/ml) were added to 500ml of the medium. Finally, the pH of the medium was adjusted at 7.2 with bicarbonate.

Anti-swine IgG serum

Anti-swine IgG rabbit serum (Sigma, U.S.A. lot No. 90F-8825) was dissolved with 2ml of sterilized distilled water and then kept at −40°C until use. Eagle's MEM medium was added for serial dilution of the IgG serum.

Complement

For the purpose of absorption, packed SRBC were added to pooled fresh sera

derived from 3 guinea pigs, stirred cautiously and kept at room temperature for 30 min, after which the sera were washed once by centrifugation of 400 x g for 7 min. For the serial dilution of sera (complement), Eagle's MEM medium was used.

SRBC suspension

Ovine blood was collected aseptically from the juglar vein and added to Alsever solution at an equal volume and then washed 3 to 4 times by centrifugation of 400 x g for 10 min. After the supernatant was discarded, 15% of the SRBC suspension was made with Eagle's MEM medium.

Cell suspension

Soon after sacrifice by bleeding, the spleen and the MLN (the nearest portion to the duodenum) were removed aseptically and put into a Petri dish filled with cool Eagle's MEM medium (pH 7.2). The organs were cut finely with scissors, and then small pieces of the tissues were filtrated through an 80 mesh cytosiever while pouring Eagle' MEM medium. The filtrated tissue suspension was washed 3 times with Eagle's MEM medium by centrifugation at 300 x g for 10 min in 4°C. For the spleen suspension, 0.83% of NH_4Cl solution was added at an equal volume and kept at room temperature for 10 min to obtain good hemolysis before the centrifuge. The nucleated cells were calculated using a Neubauer count plate, and a living cell count was made by staining with 0.3% of trypan blue. Finally, the cell suspension in Eagle's MEM medium was adjusted to 10^7 nucleated cell/ml. All the procedures were carried out under aseptic conditions.

PFC assays

After the preliminary surveys, the materials mentioned above were mixed as the optimal dose ; the system is outlined as follows :

	Direct	Indirect
Eagle's MEM medium	50 μl	25 μl
Anti-swine IgG serum (×160)	—	25 μl
Complement ×4 for the spleen cells	25 μl	25 μl
×1 for MLN's cells	25 μl	25 μl
Cell suspension (10^7 nucleated living cells/ml)	100 μl	100 μl

The mixed materials were poured into a Cunningham chamber by capillary pipette and then closed by a paraffin-vaseline mixture. The chamber was kept at 37°C for 90 min in a moisturized 5% CO_2 incubator, and the plaque was counted under low power microscope. The final PFC count was made to convert into a count of that per 10^6 nucleated cells.

RESULTS

Results of lymphocyte responses in piglets with and without treatment of immunopotentiators

Results of the cpm and SI of lymphocytes from nontreated piglets at various ages and those of adult pigs were given in table 1.

Although the blastogenesis of the piglets lymphocytes with mitogens varied individually, they showed a good response even at 1 week of age; these results were almost the same as those obtained for the adult pigs. Responses of lymphocytes with mitogen also varied among the piglets from different dams. ^{3}H-thymidine (^{3}H-Tdr) incorporation of stimulated lymphocytes with PHA and conA from the piglets of dam No. 1 showed a tendency to decrease their cpm with increasing age while the SI with PHA were approximately constant throughout the experiment, i. e., from the age of 1 week to 5 weeks. On the other hand, SI with conA had a tendency to decrease in the 3 and 4 week-old piglets. Levels of ^{3}H-Tdr incorporation of the stimulated lymphocytes from the piglets of dam No. 2 were relatively the same throughout the experiment. In this case, however, SI with PHA showed a tendency to increase in the 4 and 5 week-old piglets while the degree of SI with conA remained constant in the animals of the same age. In all piglets employed, the changes of SI with the mitogens were not significant from 1 to 5 weeks of age.

In table 2, the results of the mean SI values of lymphocyte transformation with PHA and conA of the cells from the 1 to 5 week-old piglets administered and those without peptidoglycan and FR41565 treatment were shown. The differences of blastogenesis of lymphocytes between the piglets treated with immunopotentiators and the non-treated ones were not significant.

PFC of the splenic and the nucleated cells of the mesenteric lymph nodes with and without immunopotentiators

Unlike the results of blastogenesis of lymphocytes, in general, the PFC of the spleen and the MLN of the piglets aged 2 weeks to 4 weeks were lower in spite of the fact that SRBC was inoculated as a stimulator; these results are given in tables 3 and 4. Furthermore, there was no clear second immune response as far as the count of PFC was concerned. On the other hand the PFC count of the nucleated cells of MLN was much lower than that of the cells in the spleen. However, the number of PFC in the MLN as well as the spleen gradually increased with ageing i. e., from 2 or 3 weeks to 4 weeks of old.

The immunopotentiating activity between FR41565 and peptidoglycan appeared to be different. In the case of the FR41565 inoculated piglets, the highest PFC was observed at 2 weeks of age after which the count was decreased at 3 and 4 weeks; thus the acting duration of the substance seems to be very short (table 3) as was described previously.

TABLE 1 *Mean cpm and SI value of lymphocyte transformation with PHA and conA in piglets of various ages*

MITOGEN	DAM NO.			WEEK 1	2	3	4	5
PHA	1	(n=5)	cpm	16,048±4,134*	18,984±2,788	15,188±496	10,051±1,267	7,524±1,51
			cpm without PHA	275±75*	329±109	287±73	266±136	149±36
			SI	65.9±13.6*	76.4±15.8	65.6±13.4	76.4±30.6	74.7±29.7
	2	(n=5)	cpm	21,478±864	20,123±855	18,863±736	23,027±1,426	24,020±2,635
			cpm without PHA	227±25	206±26	201±28	231±34	198±33
			SI	101.1±14.4	102.0±11.3	98.4±12.3	111.2±12.3	127.5±13.6
ConA	1	(n=5)	cpm	21,345±3,482	20,309±2,529	14,614±2,735	10,635±1,114	12,492±778
			cpm without conA	275±75	329±109	287±73	266±136	149±36
			SI	92.8±22.8	96.3±28.9	62.5±12.7	76.1±25.8	118.6±35.2
	2	(n=5)	cpm	26,864±1,337	25,806±2,138	23,275±1,031	23,192±903	23,079±1,946
			cpm without conA	227±25	206±26	201±28	231±34	198±33
			SI	123.8±12.3	130.3±15.0	121.9±16.0	113.1±14.2	123.8±14.6

	(n=2)						
ConA	Adult pig cpm	27,978[†]	21,737[‡]	PHA	Adult pig cpm	23,011[†]	18,507[‡]
	control	279	294		control	279	294
	SI	100.3	73.9		SI	82.5	62.9

*Mean value ±SD (values are expressed as count per minutes), [†] 7 months old, [‡] 12 months old

TABLE 2 *Mean SI value of lymphocyte transformation in piglets treated with and without immunopotentiator*

MITOGEN	DAM NO.		IMMUNOPOTENTIATOR	WEEK				
				1	2	3	4	5
PHA	1	(n=4)	Peptidoglycan	88.5±29.2*	57.5±10.6	74.9±2.6	98.5±32.0	98.9±30.4
		(n=5)	Nontreated	65.9±13.6*	76.4±15.8	65.6±13.4	76.4±30.6	74.7±29.7
	2	(n=5)	FR 41565	112.6±12.6	111.5±9.4	110.7±15.2	98.2±13.3	133.8±54.8
		(n=5)	Nontreated	101.1±14.4	102.0±11.3	98.4±12.3	111.2±12.3	118.6±35.2
ConA	1		Peptidoglycan	132.2±47.0	71.3±20.9	58.2†	104.1±21.3	133.8±54.8
			Nontreated	92.8±22.8	96.3±28.9	62.5±12.7	76.1±25.8	118.6±35.2
	2		FR 41565	129.9±19.6	143.8±5.7	114.2±11.4	95.2±7.0	115.3±17.3
			Nontreated	123.8±12.3	130.3±15.0	121.9±16.0	113.1±14.2	123.8±14.6

*Mean SI value ±SEM, † SI value of 1 piglet was expressed

TABLE 3 *PFC of the spleen and MLN treated with and without FR41565*

		WEEK					
		2		3		4	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
Spleen	FR41565 treated	31* (2/0–63)†	314 (2/80–547)	37 (2/0–91)	100 (2/0–211)	39 (3/11–91)	108 (3/23–177)
	Nontreated	29 (2/0–57)	90 (2/46–120)	607 (2/154–1112)	1272 (2/963–1721)	56 (3/11–160)	140 (3/17–325)
MLN	FR41565 treated	11 (2/0–17)	181 (2/131–279)	7 (2/0–17)	27 (2/0–63)	10 (3/0–23)	39 (3/11–57)
	Nontreated	3 (2/0–6)	25 (2/11–29)	11 (2/0–29)	98 (2/46–143)	11 (3/0–34)	82 (3/0–262)

* Mean PFC/10^6 nucleated living cells

† ( ): Number of piglets employed/range of PFC

The 8 piglets sacrificed at 2 and 3 weeks were derived from dam No. 1, and the remaining 6 sacrificed at 4 weeks were from dam No. 2

TABLE 4 *PFC of the spleen and MLN treated with and without peptidoglycan*

		WEEK			
		3		4	
		Direct	Indirect	Direct	Indirect
Spleen	Peptidoglycan treated	370* (2/268–530)†	337 (2/234–439)	773 (3/257–1186)	871 (3/296–1436)
	Nontreated	621 (2/348–929)	608 (2/428–809)	477 (3/103–906)	583 (3/91–935)
MLN	Peptidoglycan treated	5 (2/0–11)	9 (2/6–17)	44 (3/11–74)	77 (3/34–177)
	Nontreated	9 (2/0–17)	38 (2/17–63)	27 (3/0–63)	61 (3/0–177)

* Mean PFC/10^6 nucleated living cells

† ( ): Number of piglets employed/range of PFC

The piglet represented in this table were derived from dam No. 3

The PFC of the splenic cells non-treated with FR41565 in 3 weeks of age was exclusively high either in direct or indirect methods i. e., mean PFC count of direct was 607 and that of indirect was 1272 (table 3). The definition on this results has been unclear. However, these exception had also been reported by Kim (1975) as far as PFC of the nucleated cells in the swine spleen was concerned. On the other hand, the splenic and MLN PFC counts of the piglets administered peptidoglycan were the highest in number at 4 weeks of age compared to those of the non-treated controls (table 4). However, the number of indirect PFC (respond to IgG) was rather higher than that of direct PFC in the 2 week-old piglets treated with FR41565. Correspondingly, a higher number of indirect PFC was observed in the 4 week-old piglets treated with peptidoglycan as compared to that of the non-treated control of the same aged piglets.

DISCUSSION

There have been few studies on lympocyte transformation of suckling age piglets from the view point of the influences of immunopotentiators. Recently, however, several blastogenetic studies have been published on lymphocytes of peripheral blood in adult swine. BUSCHMAN and PAWLAS (1980) reported that cpm of lymphocytes with PHA (10 μg/ml) and conA (10 μg/ml) of pigs obtained from an abattoir were 46,631–4,558 and 25,316–4,449 respectively in the cases in which ^{14}C-thymidine 0.1 μCi (specific activity 500–600 mCi/mmol) was used. PETERS and VEERKAMP (1982) observed that the range of the lymphocyte's blastogenesis using PHA of 4 adult swines was cpm 30,000–40,000 with one exception (cpm 245,310). Judging from these results, it might be considered that mitogenic activity for transformation of lymphocytes in peripheral blood of piglets aged 1 to 5 weeks is relatively the same as that for adult lymphocytes.

On the other hand, BINNS (1982) described that during the secondary response *in vitro* up to 3×10^5 anti-mouse RBC plaque-forming cells per 10^8 cells put in cultures and in the primary response, up to 6×10^4, while unstimulated cultures normally have less than 1% of these levels. MUSCOPLAT et al. (1978) reported the number of PFC per 10^7 of the spleen-nucleated cells in adult swine to which no SRBC stimulation had been done to be 291–928. In our experiment, however, PFC were remarkably few both in the splenic and the MLN nucleated cells in spite of the inoculations of SRBC. And moreover, no secondary immune response was seen at 3 or 4 weeks of age as far as the PFC was concerned. This fact might indicate that differentiation of B lymphocytes in piglets of this age is unclear because the lymphocytes were not well developed the majority of them were in a polyclonal state. However, the indirect PFC of 2 week-old piglets treated with FR41565 and that of 4 week-old piglets administered the peptidoglycan relatively increased respectively compared to that of the non-treated piglets. It might be considered, therefore, that the B lymphocytes

obtained certain influences from these substances. This result resembled somewhat that of the increasing of IgA-bearing cells seen in the lamina propria of the intestinal mucosa of 4 week-old piglets treated with the same peptidoglycan used in this study (NAMIOKA et al. 1982).

Some workers have reported that since the population of suppressor-T lymphocytes is dominant in the human fetus and in the newborn, the poor immunoglobulin may be due to inhibition of B lymphocytes to become differentiated at this stage[11,12,13]. On the other hand, it is commonly accepted that cell-mediated suppression of immunoglobulin synthesis occurs in human diseases due to common variable hypogammaglobulinemia with B lymphocyte[3,16,17].

Recently, CARLSSON et al. (1976), INGVARSSON et al. (1978), and other workers have observed that neonatal piglets produce a relatively high amount of α-fetoprotein (1 mg/ml of serum). This fact suggests that some physiological condition of suckling piglets seems to be at a fetal stage. From the point of view mentioned above, it may be considered that immaturity of B lymphocytes in suckling age piglets seems to be due to certain suppressive activities. However, further studies are needed to clarify this speculation.

In the present study, the level of lymphocyte transformation between piglets treated with and without immunopotentiators was not significant. One reason for this may be that although T lymphocytes of piglets possess a helper function, immunopotentiators may not evidently elevate the function of helper-T lymphocytes due to their more restrained activity with suppressor-T lymphocytes. It is hoped that future analyses will produce more concrete evidence to support this view.

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