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Interdependence of *in vitro* responsiveness of cord and maternal blood lymphocytes to antigens from oral bacteria

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SUMMARY

A group of thirty-five mothers and their babies at parturition were examined by the *in vitro* lymphocyte transformation test to determine sensitization by oral bacterial antigens, B-cell mitogens and dental plaque. Lymphocytes from babies of sensitized mothers with gingival or periodontal disease gave the highest frequency (70 and 63%) and magnitude (mean stimulation index of 3·4 and 3·3) of response in cultures stimulated by *Actinomyces viscosus* and *Veillonella alcalescens*. However, IgM antibodies to *V. alcalescens* antigen were absent from cord sera. With one exception, stimulation of lymphocytes from babies of unsensitized mothers with clinically healthy gingiva was not found with these antigens. The response of cord lymphocytes from mothers with gingival or periodontal disease to antigens from oral bacteria, as compared with the response of cord lymphocytes from mothers with clinically healthy gingiva, seemed specific, since a corresponding difference in response to unrelated antigen PPD was not found.

The response of cord and maternal lymphocytes to B-cell mitogens was also determined. Maternal lymphocytes responded in the following decreasing order of effectiveness: dextran sulphate, levan, lipopolysaccharide and dextran B1355; whereas cord lymphocytes were stimulated in the reverse order of effectiveness.

INTRODUCTION

It has been reported that some bacterial and viral antigens from infected pregnant mothers can cross the placental barrier and sensitize the foetal lymphoid system. Brody, Oski & Wallach (1968) demonstrated that cord blood lymphocytes from mothers with *Escherichia coli* baciluria respond by *in vitro* proliferation to this antigen. Horton *et al.* (1976) found a direct correlation between the *in vitro* response of cord blood lymphocytes to pooled dental bacterial plaque and the severity of periodontal disease in mothers. They suggested that pregnant women with chronic periodontal disease seed plaque antigens across the placenta to sensitize foetal lymphocytes.

Dental bacterial plaque consists of a large variety of micro-organisms and their products such as lipopolysaccharides, levans and dextrans. Thus plaque is a focus, where T cell-dependent bacterial antigens coexist with B-cell mitogens (Ivanyi & Lehner, 1974). The aims of this investigation were to examine mothers and their babies at parturition for evidence of sensitization to oral bacterial antigens and plaque. Furthermore, the *in vitro* response to lipopolysaccharide, levan and dextran was investigated, as there is no report so far on stimulation of human cord blood lymphocytes by B-cell mitogens.

MATERIALS AND METHODS

Patients. A group of thirty-five mothers between the ages of 17–30 years and their babies were investigated at parturition. Following delivery, the periodontal condition of mothers was assessed by the gingival index (GI; Löe & Silness, 1963) and by Russell's periodontal index (PI; Russell, 1956). On these occasions bacterial dental plaque was collected as described

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previously (Ivanyi & Lehner, 1971). Mothers were further classified according to the presence or absence of gingival (G) or periodontal disease (PD) into two groups: twenty seven G–PD patients (GI > 0·4, PI > 0·4) and eight controls (C) with clinically healthy gingiva (GI < 0·4, PI < 0·4). Foetal cord blood was obtained immediately *post-partum* in a heparinized tube, care being taken to exclude contamination by maternal blood. The maternal blood specimens were obtained 4–5 days *post-partum*.

Stimulants. Veillonella alcalescens and *Actinomyces viscosus* sonicates were prepared as described (Ivanyi & Lehner, 1970) and used at the optimal concentration of 20 μg of protein per ml of culture for *V. alcalescens* and of 10 μg of protein per ml of culture for *A. viscosus*. The sonicates of plaque were prepared as described previously (Ivanyi & Lehner, 1971) and 0·1 ml of the optimal dilution (0·5 mg/ml wet weight; corresponding to approximately 300 μg of protein per ml) of plaque was added to lymphocyte cultures. Preservative-free PPD was used at a concentration of 10 μg/ml of culture (Ivanyi & Lehner, 1974). Lipopolysaccharide (LPS) from *V. alcalescens* was prepared as described (Ivanyi, Lehner & Burry, 1973) and used at an optimal concentration of 10 μg/ml of culture. Levan from *Corynebacterium levaniformis* and dextran B1355 from *Leuconostoc mesenteroides* were used at optimal concentrations of 500 μg of levan and 50 μg of dextran per ml of culture (Ivanyi & Lehner, 1974). Sulphated dextran (DS) was obtained from Pharmacia (Uppsala) and used at an optimal concentration of 50 μg/ml of culture (Ivanyi, 1977).

Lymphoid cell suspensions and DNA synthesis. Maternal and cord lymphocytes were isolated from heparinized blood by density gradient centrifugation on Ficoll–Triosil (Ivanyi *et al.*, 1973). Lymphocytes were cultured at a concentration of 10^6 cells per ml of culture, in medium RPMI 1640 enriched with added L-glutamine (2 mmol/ml), penicillin (100 u/ml), streptomycin (100 μg/ml) and 10% human AB serum.

The cultures were maintained, harvested and assayed as described in detail previously (Ivanyi & Lehner, 1970). The results were expressed as counts per minute (ct/min) per 10^6 viable lymphocytes and as stimulation indices (SI), representing the ratio of [^{14}C]thymidine uptake between antigen (mitogen) and saline-stimulated cultures. Analysis of the unstimulated cultures showed that a stimulation ratio of 1·8 for maternal and 2·2 for cord lymphocytes included three standard deviations above the mean and represented a significant degree of stimulation ($P = 0·01$).

Immunofluorescent technique for surface-bound immunoglobulin. The fluorescence technique was carried out on cord and maternal blood lymphocytes according to the method of Fröland & Natvig (1972) as described previously (Ivanyi *et al.*, 1973).

Serum immunoglobulins. IgM serum levels were estimated according to the immunochemical method of of Mancini *et al.* (1963) as used by Lehner (1969).

Haemagglutinating antibodies. These were estimated by a modification of the micromethod of Sever (1962) as described previously (Ivanyi, Challacombe & Lehner, 1973).

Immunofluorescent antibodies. Doubling dilutions of sera were added to air-dried smears of bacterial cells, washed three times with PBS and then overlaid with anti-human IgM or IgG fluorescein-isothiocyanate conjugates (Wellcome), as described previously (Lehner, 1970).

RESULTS

Comparison of in vitro responses of lymphocytes from neonatal cords and mothers with gingival or periodontal disease with those from neonatal cords and mothers with clinically healthy gingiva

In the group of patients with G–PD, both maternal and cord lymphocytes responded by increased DNA synthesis to all three related antigens (mean SI of 3·9 and 2·5 for plaque, mean SI of 5·8 and 3·3 for *V. alcalescens*, mean SI of 5·5 and 3·4 for *A. viscosus*) and to unrelated antigen PPD (mean SI of 8·6 and 2·4) (Fig. 1). The *in vitro* response of maternal lymphocytes to these antigens was significantly higher than that of cord lymphocytes ($P < 0·01$). In contrast, in the control group (C) no significant *in vitro* response of maternal or cord lymphocytes was observed in the presence of plaque (mean SI of 1·2 and 1·0), *V. alcalescens* (mean SI of 1·6 and 1·3) and *A. viscosus* (mean SI of 1·5 and 1·4). However, maternal and cord lymphocytes responded by increased DNA synthesis to the unrelated antigen PPD (mean SI of 9·0 and 2·3). Furthermore, there was no significant difference between the response of the two groups of cord blood cultures and maternal cultures to PPD. These results show that babies from sensitized mothers respond by *in vitro* lymphocyte proliferation to antigens from oral bacteria, while babies from unsensitized mothers do not respond.

The pattern of *in vitro* lymphocyte response to various B-cell mitogens differed in mothers and cords (Fig. 1). In mothers, the highest stimulation was induced by DS (mean SI of 4·3 for G–PD and 3·6 for C) and by levan (mean SI of 3·6 for G–PD and 2·3 for C). LPS and dextran B1355 stimulated maternal lymphocytes to a lesser extent (mean SI of 2·4 for G–PD and 2·0 for C with LPS and a mean SI of 2·0 for G–PD and 1·7 for C with dextran B1355). In contrast, cord lymphocytes responded by increased DNA synthesis to dextran B1355 (mean SI of 4·3 for G–PD and 4·0 for C) and to LPS (mean SI of 4·1 for G–PD and 3·6 for C), whilst no significant stimulation was induced by levan (mean SI of 1·8 for

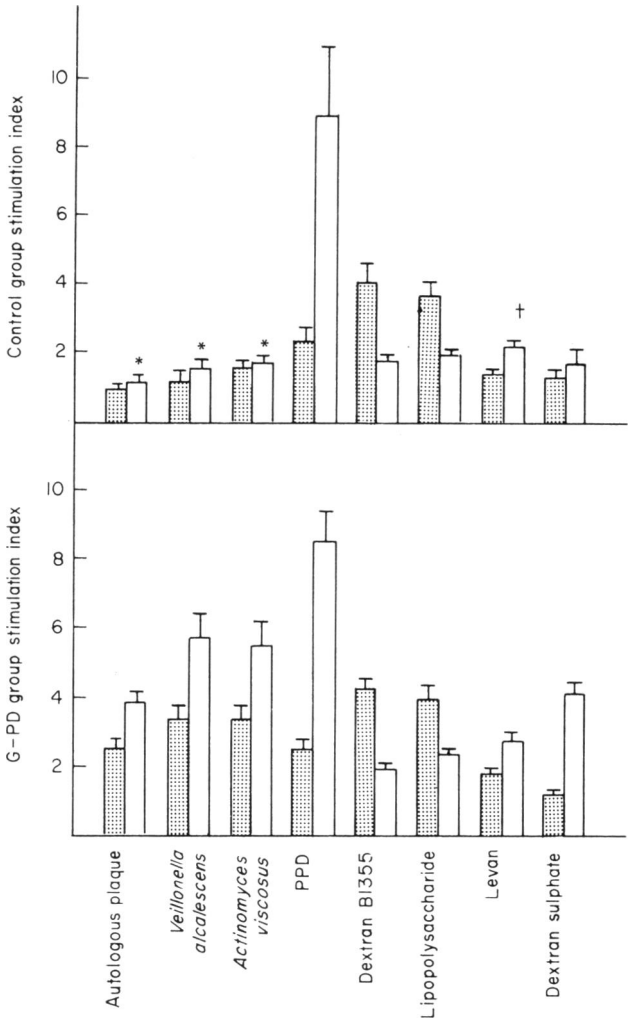

FIG. 1. The *in vitro* lymphocyte response of neonatal cords and mothers with or without gingival or periodontal disease. (▨) Cord lymphocyte response; (□) mother lymphocyte response; all differences were significant ($P < 0{\cdot}01$) except: (*) not significant; and (†) $P < 0{\cdot}05$. Wilcoxon test for pair differences was applied to compare the *in vitro* response of mothers and cords in groups of twenty-seven patients and eight controls. Stimulation index of 1·8 for maternal and 2·2 for cord lymphocytes represents a significant degree of stimulation. Mean ct/min of control cultures with saline were $660 \pm 33{\cdot}5$ for maternal and 2732 ± 257 for cord lymphocytes.

G–PD and 1·4 for C) or DS (mean SI of 1·3 for G–PD and 1·2 for C). These results show that B-cell mitogens stimulated maternal lymphocytes in the following decreasing order of effectiveness: DS, levan, LPS and dextran B1355. The reverse order of mitogenic potency applied to cord blood lymphocytes: dextran B1355, LPS, levan and DS. Furthermore, the percentage of surface immunoglobulin-bearing lymphocytes in cord blood (mean of 41·5%) was more than two-fold higher than in maternal peripheral blood (mean of 16·75%) (Table 1).

The incidence of maternal–cord pair response patterns was also studied in terms of the percentage of positive ($SI \geqslant 1{\cdot}8$ for maternal and $SI \geqslant 2{\cdot}2$ for cord) or negative response out of the total number of pairs examined (Table 2). The results show that between 44–70% of babies from sensitized mothers responded by *in vitro* lymphocyte transformation to the sensitizing antigen. The highest incidence of the response was found in cord lymphocyte cultures with *A. viscosus* (70% for G–PD and 12·5% for C) and *V. alcalescens* (63% for G–PD and 12·5% for C), followed by plaque (55% for G–PD) and PPD (44% for G–PD and 50% for C). Lymphocytes from babies of unsensitized mothers failed to respond to

TABLE 1. Percentage of immunoglobulin-bearing lymphocytes in cord and maternal blood

Subject	Polyvalent immunoglobulin (%)
M.I.	
Cord	48
Mother	21
C.O.	
Cord	29
Mother	13
N.A.	
Cord	37
Mother	14
L.A.	
Cord	52
Mother	19

these antigens, with the exception of one baby who responded to one antigen (autologous plaque) when the maternal response was negative.

The pattern of response of cord lymphocytes as compared with maternal lymphocytes to B-cell mitogens was the same as described above; while 100% of babies (from both groups) responded to dextran B1355 and LPS, the highest incidence of maternal response was found in lymphocyte cultures with DS (100%) and levan (96% for G–PD and 87·5% for C).

The in vitro response of maternal and cord lymphocytes to autologous and homologous plaque

The effect of autologous and homologous plaque on lymphocytes from eight mothers and their newborn babies was also studied (Fig. 2). The response of lymphocytes from the donor of homologous plaque showed a SI of 3·5 and the cord lymphocytes from her baby a SI of 2·5. No significant difference was found between the response of maternal lymphocytes to autologous (mean SI of $3{\cdot}2 \pm 0{\cdot}47$) and homologous (mean SI of $2{\cdot}9 \pm 0{\cdot}39$) plaque. On the other hand, enhanced DNA synthesis was observed in cord lymphocyte cultures stimulated by homologous plaque (mean SI of $5{\cdot}9 \pm 1{\cdot}28$) as compared with autologous, that is, maternal plaque (mean SI of $2{\cdot}8 \pm 0{\cdot}57$, $P < 0{\cdot}01$ by Wilcoxon test for pair differences).

TABLE 2. Patterns of *in vitro* lymphocyte responses in maternal–cord pairs

	Incidence of maternal–cord pair response patterns (%)							
	G–PD group				Control group			
Stimulants	(+, +)*	(+, −)	(−, +)	(−, −)	(+, +)	(+, −)	(−, +)	(−, −)
Autologous plaque	55	37	4	4	0	12·5	0	87·5
Veillonella alcalescens	63	37	0	0	12·5	12·5	0	75
Actinomyces viscosus	70	26	0	4	12·5	12·5	0	75
PPD	44	52	0	4	50	50	0	0
Dextran B1355	77	0	23	0	37·5	0	62·5	0
LPS	85	0	15	0	62·5	0	37·5	0
Levan	11	85	0	4	12·5	75	0	12·5
Dextran sulphate	7	93	0	0	0	100	0	0

Twenty-seven G–PD and eight control maternal–cord pairs were examined; maternal (+) = stimulation index $\geq 1{\cdot}8$; cord (+) = stimulation index $\geq 2{\cdot}2$.

* In each pair maternal lymphocyte response is given first and cord lymphocyte response second.

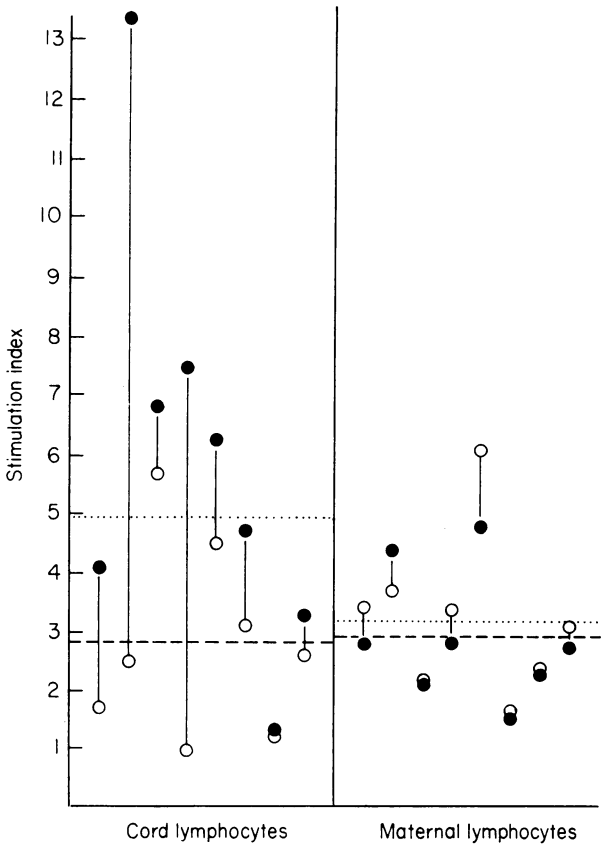

FIG. 2. The *in vitro* response of maternal and cord lymphocytes to autologous and homologous plaque. (○) Autclogous plaque; (●) homologous plaque; (·····) mean stimulation index for homologous plaque; (– – –) mean stimulation index for autologous plaque. Mean ct/min of control cultures with saline were 577 ± 56 for maternal and 2778 ± 521 for cord lymphocytes.

Levels of IgM and antibodies in maternal and cord sera

IgM levels were estimated in sera from eleven cord–maternal pairs, as increased IgM in cord serum indicates a significant foetal antigenic stimulation (Alford *et al.*, 1975). The mean level of cord IgM (12·2 mg/100 ml) and maternal IgM (213 mg/100 ml) was found within normal limits (Table 3). Haemagglutinating antibodies to *V. alcalescens* were assayed in cord and maternal sera from thirty-five cord–maternal pairs (Table 3). These antibodies were shown previously to reside predominantly in the

TABLE 3. Levels of IgM and antibodies to *Veillonella alcalescens* in maternal and cord sera

Sera	Serum IgM, mean (mg/100 ml)	*V. alcalescens* antibody titre (mean $\log_2$)		
			Immunofluorescent	
		Haemagglutinin	IgM	IgG
Mother	213 ± 28·5	5·8 ± 0·39	4·3 ± 0·37	1·4 ± 0·47
Cord	12·2 ± 0·71	0	0	0·7 ± 0·28
(Number of pairs)	11	35	9	9

IgM class (Ivanyi, Challacombe & Lehner, 1973). All maternal sera showed antibodies to *V. alcalescens*, irrespective of the presence or absence of gingival or periodontal disease (mean $\log_2$ titre of 5·8). In contrast, the antibody activity was not detected in any of thirty-five cord sera tested. Nine maternal and cord sera were further tested for the presence of IgM and IgG antibodies to *V. alcalescens* by immunofluorescence. The results show that IgM antibody activity was not detected in any of the nine cord sera tested.

DISCUSSION

We have shown that cord and maternal lymphocytes differed in response to various B-cell mitogens. In mothers, B-cell mitogens stimulated peripheral blood lymphocytes in the same decreasing order of effectiveness; DS, levan, LPS and dextran B1355, as we have found previously (Ivanyi & Lehner, 1974; Ivanyi, 1977). In contrast, cord lymphocytes responded to B-cell mitogens in the reverse order from that of maternal lymphocytes. The highest level and incidence of stimulation was induced by dextran B1355 and by LPS whilst a significant level of stimulation was not induced by levan or by DS. These findings are consistent with the concept that B lymphocytes consist of distinct subpopulations at various stages of differentiation which differ in their proliferative response to various B-cell mitogens (Gronowitz, Coutinho & Möller, 1974). In the mouse, DS activates primitive B cells, LPS a broad population of cells in an intermediate stage of differentiation and PPD only activates mature cells (Gronowitz *et al.*, 1974). The pattern of proliferative response of cord and maternal lymphocytes to various B-cell mitogens may well represent the differentiative pathway of B cells in man; from primitive cells responding to dextran B1355, to cells in intermediate stage of differentiation responding to LPS and finally to mature cells responding to levan and DS.

We have also shown that neonates from sensitized mothers with gingival or periodontal disease respond by *in vitro* lymphocyte proliferation to antigens from oral bacteria and dental plaque, while babies from unsensitized mothers do not respond. Only one infant out of twenty-seven responded to one antigen (autologous plaque) when the maternal response was negative. An occasional paradoxical response of cord lymphocytes in the absence of response by maternal lymphocytes was observed in two other cases, without any reasonable explanation (Russell, 1975). We suggest that increased levels of dextran or LPS in that particular plaque could have been responsible for the positive response.

The increased response of cord lymphocytes from the G–PD group of mothers to antigens from oral bacteria seemed specific, since a corresponding difference in response to PPD was not found. The mean level of stimulation of neonatal cord lymphocytes to all tested antigens was, however, lower than that of maternal lymphocytes. The highest stimulation of cord lymphocytes was induced by *V. alcalescens* and *A. viscosus*, followed by plaque and PPD, which incidently was the most potent antigen for maternal lymphocytes. Cord and maternal lymphocytes differed in response to homologous plaque. Whereas maternal lymphocytes showed comparable responses to autologous and homologous plaque, enhanced DNA synthesis was observed in cord lymphocyte cultures in the presence of homologous plaque as compared with autologous plaque.

A large proportion of neonates from sensitized mothers reacted by *in vitro* lymphocyte proliferation; 70% of babies responded to *A. viscosus*, 63% to *V. alcalescens*, 55% to plaque and 44% to PPD. However, some neonates from sensitized mothers did not show an *in vitro* lymphocyte response. Similar results were also reported by other authors (Russell, 1975; Horton *et al.*, 1976). We cannot explain why not all neonates from sensitized mothers manifested the *in vitro* response, but it is possible that factors such as the degree of sensitization, the presence of clinically apparent disease or the permeability of the placenta may play a role. The mode of transmission of the transplacental sensitization is still a matter of controversy. It was suggested that transplacental passage of plaque antigens was responsible for the sensitization of foetal lymphocytes (Horton *et al.*, 1976). If this explanation were correct, one would expect to find IgM antibodies in cord sera, as this has been suggested to be a sensitive measure of foetal antigenic sensitization and has been used in the search for mild or subclinical intrauterine infections (Alford *et al.*, 1975).

As we were unable to detect IgM antibodies to *V. alcalescens* in neonatal sera, foetal antigenic stimulation seems unlikely to be an explanation for our finding. An alternative explanation for the foetal lymphocyte sensitization would be the transplacental passage of maternal lymphocytes, but this seems also unlikely, as it occurs only rarely in humans (Adinolfi, 1974). Finally, it has been reported that lymphocytes from neonates of clinically healthy mothers who showed sensitization to antigens of *Mycobacterium leprae* responded to this antigen *in vitro* (Barnetson, Bjune & Duncan, 1976). As the sensitized mothers showed no signs of leprosy or other mycobacterial disease, the authors suggested that transplacental passage of soluble lymphocyte factor could be responsible for the foetal lymphocyte sensitization. A similar suggestion was made by Stern (1976) from his work in mice. Although our data does not allow a definite conclusion about the mechanism of foetal sensitization, it is tempting to suggest that a lymphocyte factor which can cross the placenta could play a role.

As the duration of neonatal lymphocyte sensitization has not been studied, it is not clear if this might have any effect on the infants' gingival and periodontal condition after the teeth have erupted. We can envisage the situation in which a heightened sensitivity to bacterial plaque antigens might influence the child's periodontal condition and this aspect needs to be further explored. If the neonatal lymphocyte sensitization were to persist and affect the periodontal status of the child, this might be a 'congenital' factor in periodontal disease and might account for its very high frequency. Furthermore, dental plaque control in the mother might then acquire a new significance, in terms of treatment in the mother and prevention in the offspring of periodontal disease.

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