

Cord blood proliferative responses to inhaled allergens: Is there a phenomenon?

Lymphocytes develop in the human fetus during the first trimester, and the fetus is capable of making immune responses from about 20 weeks' gestation onward. These responses have been demonstrated against infectious organisms, such as cytomegalovirus and toxoplasmosis, but also against parasites, such as microfilaria, and antigens derived from food.^{1,2} Establishing that the fetus has made a response to an infection is normally based on measurement of IgM antibodies in the cord blood because IgM is not passed from the mother to the fetus. However, evidence for fetus infection with the parasite filaria has been obtained by measuring IgE antibodies (ie, another isotype that does not cross the placenta).³ For other antigens (eg, food antigens), evidence has been obtained from antigen-specific proliferative responses of cord blood mononuclear cells in vitro.^{4,5} In each case, the presence of a positive response is taken as evidence that the fetus was exposed in utero.²⁻⁵ Recently, several groups have reported proliferative responses of cord blood mononuclear cells to inhalant allergens.⁶⁻⁸ Before considering the data, it is important to distinguish between food allergens and inhalants. The mother may consume as much as 100 g of cow's milk protein per day, and in keeping with this, there are clear reports of infants who have symptoms of milk allergy because of cow's milk protein passing into breast milk.⁹ There is also good evidence that infants can be symptomatically allergic to milk shortly after birth, and cord blood mononuclear cell proliferative responses to cow's milk with stimulation indices of greater than 5 have been reported.^{2,4,5} By contrast, the daily exposure to pollen or dust mite allergens has been estimated to be approximately 10 ng per day.^{10,11} There is no published evidence about infants being born allergic to inhalant allergens. Furthermore, there is no serologic evidence to support the idea that the fetus has made an immune response to allergens inhaled by the mother.²

Abbreviation used

cpm: Counts per minute

SI: Stimulation index

In this issue of the *Journal*, Szepefalusi et al¹² present a very impressive body of data testing the proliferation of mononuclear cells from preterm and term cord blood. The antigens they used were recombinant proteins derived from the pollen of Timothy grass (Phl p 1) and birch trees (Bet v 1). The study had two major objectives: (1) to identify at what stage in pregnancy transplacental priming to these antigens could be identified and (2) to investigate the relationship between cord blood responses and maternal exposure to the relevant antigens. The results provide an opportunity to consider two aspects of the literature on cord blood responses. First, how should in vitro proliferation data be evaluated to reach conclusions about development of an immune response or priming? Second, how convincing is the evidence that cord blood responses are related to exposure of the mother?

When lymphocytes are isolated and cultured ex vivo, there are many factors that can influence proliferation. These include the density of cells in the culture well, antibiotics and other chemicals in the medium, and the serum added.^{13,14} (Cultures can be carried out with FCS, autologous serum, human AB serum, or serum-free media.) There is no doubt that cord blood cells can divide rapidly as judged by stimulation indices obtained with agents such as PHA or IL-2 (see Table II in Szepefalusi et al¹²). Clearly, any factor that induces production of cytokines such as IL-2 within the cultures would stimulate responses nonspecifically, and any antigen with mitogenic activity would act in a similar manner. When evaluating antigen-specific proliferative responses of cord blood cells, it is important to consider the nature of the immature T-cell repertoire. The precursor frequency of a given antigen-specific T cell in the mature repertoire has been estimated to be 1 per 10⁵ cells, although this value may vary considerably.¹⁵ Thus when plating cells at relatively low cell numbers (1 × 10⁵ per well), some wells may contain no antigen-specific cells purely by chance, even if the individual is immune. Assuming an immune response is mounted in utero, the lower prevalence of CD45RO memory T cells in human cord blood compared with adult cells (approximately 12% vs approximately 55%¹⁶) makes it less likely that proliferative responses would be consis-

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tently present in all culture wells. Under these circumstances, evaluating responses on an individual basis depends on having an adequate number of replicates for both the antigen and control wells.^{17,18} Experiments with allergenic peptides derived from the cat allergen Fel d 1 and the dermatophyte antigen Tri r 2 have established the importance of using high numbers of replicates (ie, approximately 12) in standard proliferation assays. This is important for statistical evaluation both for individual responses and for comparing different experimental groups. It could be argued that this approach is not necessary for assays with intact antigen (containing multiple T-cell epitopes), which are capable of stimulating polyclonal cellular responses. Nevertheless, our experience with whole antigen has demonstrated large variations in counts per minute (cpm) among replicate wells, supporting the argument that increased numbers of replicates are required for assessing the significance of proliferative responses.¹⁹ By using stimulation indices (SI) to evaluate responses, the negative results from some individuals are often expressed as less than 2, implying that there is no negative response.^{20,21} With high variation between replicates, SI values of 2 will occur by chance, and their significance can only be assessed by comparing them with both the positive and negative responses seen in cultures from other individuals studied. An alternative approach to establishing the significance of a response is to repeat cultures over a period of time. Obviously, handling cord blood places severe limitations on either replicates or repeat cultures; however, repeat cultures have been reported under two circumstances. Miles et al²² in Southampton repeated cultures at 6 months of age, at which time the responses to mites were negative. In the present report from Vienna, in two cases cord blood was sampled twice in utero, and the cultures were negative on each occasion (Fig 2 in Szepefalusi et al¹²). An important feature of antigen-specific lymphocyte proliferation in vitro is that there are consistent dose responses. Although decreased responses at higher doses can occur, they are typically seen when the response to the lower dose of antigen is strongly positive. The data shown for samples obtained by chordocentesis do not show convincing dose responses to either of the antigens tested. In many studies T-cell proliferation data are presented as change in counts per minute (or change in disintegrations per minute) above background. At first glance, these data may look convincing; however, in at least one study, background counts of up to 20,000 cpm for cord blood cultures were obtained.¹³ Thus even when relatively high changes in counts per minute (ie, approximately 10,000) are reported, the results cannot be interpreted without including background data. Furthermore, the results for background counts reinforce the importance of incorporating sufficient replicates of the antigen and control cultures.

In several studies the cord blood responses to dust mite allergens have been qualitatively greater than those reported with pollen allergens. However, many of these studies have used crude dust mite extracts (rather than purified antigens) and the serum-free medium Aim V.^{7,20,21} These culture conditions have produced proliferative responses in a large pro-

portion of tested individuals in several different populations. In Australia, using dust mite extract and serum-free medium, Upham et al²¹ concluded from proliferation data that there was universal sensitization of normal adults to mite antigens.²¹ Using the same culture conditions, lymphocytes from 18 of 19 two- to three-year-old children living in Sweden with very low mite exposure (ie, <0.6 µg/g of dust) responded in vitro.²⁰ Finally, another study showed that under the same experimental conditions, proliferation was equally common in cord blood from low-risk and high-risk neonates.⁷ Use of this serum-free medium in other laboratories has consistently suppressed specific responses, except those to mite antigen.²³ The conclusion from these experiments appears to be that mite extracts can be mitogenic and that this effect is more marked in serum-free conditions.

Using Bet v 1 and Phl p 1 as antigens makes it possible to ask directly whether the proliferative responses observed were influenced by exposure of the mother to the relevant pollen.²² As the authors recognize, the answer is clearly negative; that is, proliferative responses were no more likely to occur if the mother had been exposed. With dust mite allergen, evaluating exposure is more complex because each home is different, and seasonal variations are less well defined than pollen seasons. However, at least 3 groups have reported on the relationship between dust mite exposure at home and cord blood mononuclear cell proliferation in response to mite allergen.²⁴⁻²⁶ In each case the results have been negative; that is, the responses have not been greater or more common with samples coming from infants whose mothers had higher exposure at home. The conclusion at present has to be that neither for pollens nor for dust mites is there convincing evidence that the responses seen in cord blood are related to maternal exposure. It has recently been suggested that the weak responses in cord blood reflect the development of the T-cell repertoire in response to diverse antigen exposure.²⁷ If so, these responses may be biologically interesting, but they have not been shown to be relevant to understanding how infants become allergic.

The very low quantities of allergen inhaled (ie, approximately 10 ng per day) pose serious problems in understanding how the infant receives sufficient antigen to make a response. It is possible that antigen transfer to the fetus could be facilitated (or blocked) by maternal IgG antibody. However, if maternal IgG antibody facilitated transfer of antigen to the infant, the mother would have a greater effect on the development of allergic responses than the father, and this is not so; those studies showing a larger effect of the mother's history on the development of asthma do not extend to the subsequent development of allergic responses.^{28,29} Certainly by the age of 8 to 10 years, when the atopic phenotype is well established, the father's allergic history has the same influence as the mother's allergic history. A simple calculation of the quantity of allergen that could possibly enter the mother's serum per day suggests that this is approximately 1 ng/L, which would be very difficult to measure. It is equally difficult to see how the infant could make an immune response to quantities of this kind.

Objective evidence for an immune response to inhalant allergens is unusual under the age of 2 years. Tests that can be repeated consistently include skin tests and assays of IgG or IgE antibodies in serum.^{30,31} Indeed, the relationship between sensitization in early childhood and exposure to dust mite or cockroach allergen in homes makes it clear that allergen exposure plays a critical role in the development of allergic responses.³² By contrast, the evidence about sensitization or priming of the fetus is unconvincing. The modest proliferation responses reported cannot be analyzed statistically because there are too few replicates and too much random variation in the system. It is always possible that proliferation or cytokine production in vitro reflects some form of weak priming related to the generation of immune diversity in the fetus; however, these events do not appear to be related to allergen exposure. At present, we would conclude, at least for inhalant allergens, that the proliferative responses are not consistent and not related to exposure of the mother, and they do not predict outcome for the infant in terms of either sensitization or disease. Given the very low quantities of allergen and the fact that objective evidence of sensitization is unusual under the age of 2 years, it is very unlikely that infants make a response to inhalant allergens in utero.

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