A cohort study representing a general population (Minorca Island, birth year 1997–1998) showed that in utero transfer of organochlorine compounds (OCs) in children was strongly correlated with the age of the mother and, in the case of hexachlorobenzene (HCB), 4,4′-DDE, and 4,4′-DDT, with the maternal body mass index. Some of these correlations remained significant for the serum concentrations collected in these children at four years. No significant correlations with length of gestation were observed. Breastfeeding and age of lactation were strong determinants of most OC concentrations at four years of age. At this age, the body burden of these compounds was higher than at birth irrespective of maternal or formula feeding, but they accumulated at higher extent in the former case involving concentration increments in blood that surpassed the growth dilution effects, with the only exceptions being pentachlorobenzene (PeCB), HCB, and 4,4′-DDT. 4,4′-DDE exhibited the highest increase in association with breastfeeding, pointing to a specific accumulation pathway via this mode. Compounds with low K_{oa} values such as β-hexachlorocyclohexane showed significant accumulation in four-year-old children but with small differences between the groups that had been raised on either breast milk or formula. Compounds having low K_{oa} values such as HCB showed decreases in concentration and small body burden variation between birth and four years of age, which points to their preferential elimination in these initial periods of infant growth.

Introduction

Several studies in children have documented neurodevelopmental delays and cognition impairment due to prenatal and infant exposure to baseline levels of 4,4′-DDE (1, 2) or polychlorinated biphenyls (PCBs) (1, 3–5). In other cases, the association between exposure to these compounds and detrimental neurodevelopmental effects has not been observed (6, 7).

These pollutants are incorporated into children in utero and through diet, mainly through breastfeeding (2, 8–11). Exposure through breastfeeding is important due to the widespread occurrence of these compounds, their lipophilicity, and their high stability to chemical degradation, which favors their accumulation in human fat. These properties are those characteristic of persistent organic pollutants (POPs), a group that encompasses the aforementioned compounds as well as HCB, hexachlorocyclohexanes (HCHs), and 4,4′-DDT among others, involving up to 12 types of OCs that have been banned by the Stockholm agreement (12).

Some studies have found that OC blood concentrations are higher in breastfed than formula fed children even some years after discontinuation of breastfeeding [e.g., 3.5 years (13), 4 years (14), or 7 years afterward (9)]. These results outline the need to improve our knowledge of the real intake of OCs through maternal and formula feeding within the first years of child development. Full appraisal of the relevance of these ingestion modes also requires accurate comparative estimates with the amounts incorporated in utero. This information is important in view of the reports of the World Health Organization (15) and the American Academy of Pediatrics (16).

In addition, the compound-specific patterns of accumulation through breastfeeding may provide significant information on the physical-chemical properties that enhance the incorporation of pollutants into children through this mode. This information may be relevant from the perspective of implementing new management policies to evaluate the environmental impact of newly synthesized chemicals, such as the European Union regulations for Registration, Evaluation, and Authorization of Chemicals (REACH) (17).

To gain insight into these questions, a detailed comparison between OC in cord blood and in sera collected at four years of age in a cohort of children from Minorca (Balearic Islands, Mediterranean Sea) was undertaken. The island does not have factories producing OCs, but DDT had been used for agriculture in the past. The subjects had therefore been exposed to baseline POP levels and could thus be taken to be representative of the regular exposure to these pollutants in western countries. The cohort recruited all women presenting for antenatal care over 12 months starting in mid 1997 (18). 482 children were enrolled, and 470 (97.5%) provided complete outcome data up to the fourth year visit. Among these, 410 (85%) had OCs measured in cord blood and 285 (59%) in sera collected at four years.

The results provide insight into the maternal determinants and compound properties that influence the accumulation of OCs through maternal and formula feeding, in comparison to in utero intake.

Materials and Methods

Materials. Standards of tetrabromobenzene (TBB), PeCB, HCB, α-, β-, γ-, and δ-HCH, PCBs, 4,4′-DDT, and 4,4′-DDE were purchased from Dr. Ehrenstoffer (Augsburg, Germany). Analytical grade concentrated sulfuric acid (concentrated H$_2$SO$_4$), acetonitrile (CH$_3$CN), isooctane, and n-hexane were purchased from Merck (Darmstadt, Germany).

Extraction. Serum and cord blood samples (0.5 mL) were introduced into 10 mL centrifuge tubes, and TBB and PCB209 were added as recovery standards. Concentrated H$_2$SO$_4$ (2
mL) and n-hexane (3 mL) were then added, and the sample was mixed in a vortex (ca. 1500 rpm, 30 s) and then centrifuged (ca. 1500 rpm, 10 min). The supernatant n-hexane layer was aspirated into a second centrifuge tube using a Pasteur pipet. Additional n-hexane (2 mL) was added to the first tube, stirred (vortex ca. 1500 rpm, 30 s), and then centrifuged (ca. 1500 rpm, 10 min). This last step was repeated, yielding 7 mL of combined n-hexane extracts, to which 2 mL of concentrated H2SO4 was added. The sample was then mixed (vortex mixer, ca. 1500 rpm, 90 s), centrifuged as before, and the supernatant was transferred to a conical bottom, graduated tube. The combined extracts were then reduced to near dryness under a gentle stream of nitrogen, at which point PCBI42 in isooctane (10 μL) was added as an injection standard. The sample was then quantitatively transferred to GC vials using four 25μL rinses of isooctane. If an emulsion had been formed at any stage of the extraction, 10–15 drops of MilliQ water were added before sample centrifugation.

**Instrumental Analysis**. A gas chromatograph with electron capture detection (Hewlett-Packard 6890N GC-ECD) was used to quantify PeCB and HCB, PCB congeners #28, #52, #101, #118, #138, #153, #180, p,p′-DDT, and p,p′-DDE. α-, β-, γ-, and δ-HCH were quantified by GC–MS (HP 5973 MSD) in negative chemical ionization mode using ammonia as reagent gas (1.0 mL/min). In both instruments, samples were injected (2 μL) in splitless mode onto a 60 m, DB-5 column with a retention gap (both from J&W/Agilent) using helium as carrier gas (1.5 mL/min). The temperature program was 90 °C for 2 min, 20 °C/min to 140 °C, 4 °C/min to 200 °C held for 13 min, and finally 4 °C/min to 310 °C held for 10 min.

In both instruments, quantification was performed using external standards, with the PCBI42 injection standard used to correct for volume. Recoveries of TBB and PCB209 (75–115%) were used to correct results. Limits of detection (LOD) and quantification (LOQ) ranged between 0.02–0.09 and 0.03–0.13 ng/mL, respectively. These values were calculated from blanks (i.e., the mean of all blanks plus the product of the standard deviation times three LOD or five LOQ). When the compound was absent from the blanks, the LOD was calculated from instrumental data using diluted standards. This method performed satisfactorily in repeated international intercalibration exercises within the Arctic Monitoring and Assessment Program (23).

In all cases, when the concentrations were below the LOD, zero values were introduced. Substitution of these values by half of the LOQ did not change the results or the significance of the statistical tests or the correlation analyses discussed below.

This study was approved by the ethics committee of the Institut Municipal d’Investigació Médica, and all mothers provided a signed consent form.

**Results and Discussion**

**Characteristics of the Population Studied.** Age and body mass index (BMI) of the participant mothers at delivery are given in Table 1. The ages represented nearly the whole range of reproductive activity. BMI encompassed a large spectrum of cases from underweight (15.3) to obesity (48.5). Time of gestation is also indicated. Some cases involved short gestation periods. For children, sex, feeding mode, and length of lactation are also indicated. 83% of children were breastfed. Time of lactation ranged from very short (2 months or less) to very long (more than 1 year). No significant biases between the group of participants at birth (n = 410) and four years later (n = 285) were observed.

**OC Levels.** Among the entire cohort, 4,4′-DDE was the most abundant OC both in cord blood and sera collected at four years (average 1.6 ng/mL in both cases; Table 2). The concentrations of this compound were lower than those reported in Norway (3.0 ng/mL; 20) and higher than in Canada (0.4 ng/mL; 10) or Catalonia (0.83 ng/mL; 21). Lower concentrations of 4,4′-DDT (0.18 and 0.073 ng/mL, respectively) than 4,4′-DDE were observed, likely reflecting that the whole mixture of DDT metabolites correspond to old inputs because a substantial amount of the 4,4′-DDT initially introduced into the environment had already been transformed into 4,4′-DDE (22, 23).

HCB was the second major OC with average values of 0.75 and 0.42 ng/mL in cord blood and four years sera, respectively (Table 2). These concentrations were lower than those reported in Norway (1.0 ng/mL; 20), Germany between 1984 and 1985 (2.0 ng/mL; 24), or Catalonia (1.2 ng/mL; 21), but higher than those found in Germany between 1994 and 1995 (0.61 ng/mL; 24) or Canada (0.04 ng/mL; 10). PeCB was the OC found in lowest concentration, 0.081 and 0.023 ng/mL in cord blood and sera collected at four years, respectively. Total concentrations of the ICES 7 PCB congeners were 0.70 and 1 ng/mL, respectively. The PCB distributions were dominated by congener PCB153 in both types of samples. These concentrations were lower than those found in studies from Norway (3.0 ng/mL; 20), USA (2.5 ng/mL; 25), Faroe Islands (1.1 ng/mL; 26), and Germany (0.96 or 1.4 ng/mL; 24), but higher than those reported in The Netherlands (0.38 ng/mL; 28), Canada (0.50 ng/mL; 10), or Catalonia (0.36 ng/mL; 21). Comparison of these data must be done with caution because different PCB congeners were used for the calculation of total PCBs in each study. However, the reported figures may vary by a factor of 2 at the most. In contrast to other studies, PCB congeners of higher volatility were also considered for quantification in this cohort.

The α-, γ-, and δ-HCH isomers were only found above quantification limit in less than 5% of total samples. These compounds were therefore not included in the study. β-HCH was found at concentrations of 0.21 and 0.28 ng/mL in cord blood and sera collected at four years, respectively.

**Maternal Determinants of OC Concentrations in Children.** Cord blood OC concentrations showed significant

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of the Study Population</th>
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<td>participants at birth (at four years)</td>
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<tr>
<td>sex</td>
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<tr>
<td>male</td>
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<td>female</td>
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<td>feeding mode</td>
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<td>maternal milk</td>
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<td>formula milk</td>
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<tr>
<td>time of lactation (weeks)</td>
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<td>0.3–10 (0.3–12)</td>
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<td>10–20 (12–21.5)</td>
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<td>20–28 (21.5–28)</td>
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<tr>
<td>28–100 (28–96)</td>
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<tr>
<td>time of gestation (weeks)</td>
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<td>27–39</td>
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<td>39–40</td>
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<td>40–44</td>
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<td>maternal body mass index</td>
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<tr>
<td>15.3–20.6 (15.3–20.4)</td>
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<td>20.6–22.0 (20.4–22.0)</td>
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<td>22.0–24.3 (22.0–24.2)</td>
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<tr>
<td>24.3–48.5 (24.2–48.5)</td>
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<td>maternal age</td>
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<td>29–32 (29–32)</td>
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<td>32–42 (32–41)</td>
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*a Subset of the same individuals participating in the study at four years old.*
correlations with the age of the mother at delivery for HCB, \(\beta\)-HCH, 4,4'-DDE, 4,4'-DDT, PCB118, PCB153, PCB138, and total PCBs (Table 3). According to these results, older mothers transferred higher OC concentrations into newborns. The compounds exhibiting these correlations were those found in higher concentration in cord blood (Table 2). In fact, with the exception of PCB180, all measured individual OC at average concentrations higher than 0.1 ng/mL exhibited positive correlations with very high significance \((p < 0.0001, \text{***})\). These two compounds were those present in the highest average concentration in the population of newborns (Table 2). The concentrations in sera of four year old children only showed significant correlation of newborns (Table 2). The concentrations in sera of four year old children only showed significant correlation with maternal BMI for HCB \((p < 0.05)\) (Table 3). The same significant correlations for cord blood and sera collected at four years were observed when the OC concentrations were compared to the weight of the mother.

Older gestational age has also been related to higher OC levels in cord blood \((10, 27)\). However, this association was not found in the present study \((p > 0.09; \text{Table 3})\) or in a previous study of a cohort from Ribera d’Ebre (Catalonia) \((28)\).

The correlations of the cord blood OC concentrations with maternal age and BMI are consistent with higher OC accumulation in mothers of higher age and BMI \((28)\), whereby the OCs from the mothers are ultimately transferred to child in uterus. To the best of our knowledge, this is the first report of a direct relationship between maternal BMI and the concentration of some OCs in children at birth and at four years old.

**Influence of Milk Feeding.** The average concentrations of HCB, 4,4'-DDE, 4,4'-DDT, PCB congeners #153, #138, and...
and total PCBs in sera collected at four years exhibited significantly higher values in breastfed than artificially fed children (Figure 1). The degree of significance of the differences was very high ($p < 0.0001$) for most of these compounds. Calculation of the average values over the whole dataset of samples collected at four years ($n = 285$) or the subset of children providing samples at four years and cord blood ($n = 244$) showed nearly the same degree of significance (Figure 1). The internal consistency of the database was then confirmed by calculation of the same means using the cord blood data. As expected, no significant difference ($p > 0.05$) was observed between maternally and formula fed children (in either the databases of $n = 285$ or $n = 244$) (Figure 1). The results of Figure 1 demonstrate that breastfeeding is very significant for the concentrations of OC in four-year-old children (i.e., children who stopped breastfeeding 2.3–3.5 years before being tested).

In agreement with this observation, the period of lactation was also correlated with the concentrations of HCB, $\beta$-HCH, 4,4′-DDE, PCB118, PCB153, 4,4′-DDT, PCB138, PCB180, and total PCBs accumulated in four-year-old breastfed children (Table 4). In all cases, longer lactation corresponded to higher concentrations in sera. Examination of the same correlations using cord blood data did not show any significant correlation (Table 4). Again, this result was expected, but, as in the previous case, it was calculated to check for the internal consistency of the database.

The studies showing that breast milk is an important source for the incorporation of OCs into children even several years after breastfeeding had stopped are based on correlations between duration of lactation and serum OC concentrations. Mixtures of 4,4′-DDE (9), 4,4′-DDT (14), $\beta$-HCH (9), HCB (9), and PCB having diverse congeners with more than five chlorine substituents (9, 13, 14) have been found in higher concentration in children fed with maternal milk for...
long periods. The results observed in the population of Minorca are in agreement with these previous results. In this case, the nursing period encompassed a very wide time range (0.3–100 weeks), and nearly all OCs showed significant correlation with this determinant (Table 4). Only PeCB and the PCB congeners having three (e.g., #28), four (e.g., #52), and some having five (e.g., #101) chlorine atoms were not correlated to lactation time.

Further understanding of the relevance of breastfeeding to overall OC intake can be obtained by comparing the average values between maternally and formula fed children. In the Minorca cohort, the average concentrations of HCB, 4,4'-DDE, 4,4'-DDT, PCB congeners #153, #138, #180, and total PCB were significantly higher in subjects that had been breastfed in infancy (Figure 1). These results were in close agreement with the aforementioned correlations with duration of breastfeeding as well as with those of previous studies. However, β-HCH and PCB118 whose concentrations were observed to be directly proportional to duration of breastfeeding (Table 4) had average values that were not significantly different between the two feeding mode groups (p > 0.05) (Figure 1). This contrast indicates that, despite higher incorporation of these compounds with milk ingestion, their background intake due to formula feeding or in utero exposure was high enough to diminish the significance of breastfeeding (e.g., average β-HCH concentrations in maternal and formula feeders were 0.30 and 0.20 ng/mL, respectively). This result shows that, independently of correlations with duration of breastfeeding, statistical comparisons of average OC concentrations for each feeding mode are needed to determine the real contribution of breastfeeding to OC intake in children.

**Changes in OC Concentrations.** In breastfed children, the average concentration differences between sera collected at four years and cord blood showed an increase in the concentrations of all OC, with the only exceptions being PeCB, HCB, and 4,4'-DDT (Figure 2). No change was observed for PCB180. The pattern of these differences was entirely distinct in formula fed children. In this case, lower levels were observed for nearly all OCs. Similar results were found when the calculations were performed over the whole population (n = 410 and 285 for cord blood and sera at four years, respectively) or only over the subjects who provided both cord blood and sera at four years (n = 244).

The magnitudes of change were different for maternally and formula fed groups. The increments associated with the former were much smaller (0.5 ng/mL at the most) than the decreases observed for the latter (up to 1.5 ng/mL) (Figure 2). As expected, dilution resulting from growth of the children tended to reinforce the decreases and counterbalance the increases. In this cohort, the average growth involved changes from ca. 3.2 kg at birth to ca. 16.2 kg at four years of age corresponding to approximate blood volumes of 0.24 and 1.2 L, respectively.

The distinct concentration values observed for PeCB, HCB, and 4,4'-DDT in breastfeeders can be related to their low log(Kow) values (<7 at 36.5 °C), which were significantly lower than the constants of the other compounds (Table 2). The significance of the Kow constants was more evident when considering that β-HCH, which has the lowest Kow of the...
whole group of compounds but has a log(K_{ow}) > 8, exhibited the same behavior as the other OCs studied. The low extent of accumulation of PeCB and HCB indicates that the more volatile compounds are less retained. Exhalation is a possible efficient route of excretion in these early periods of infant growth (29). 4,4′-DDT does not exhibit K_{ow} and K_{oa} constants that are significantly different from the other OCs that show increments at four years. The distinct behavior of this compound can be explained by transformation into 4,4′-DDE, a phenomenon commonly observed in mammals (23).

**Changes in OC Body Burden.** The weight of each child at birth and at four years of age was compiled for an estimate of the blood volume (assuming 75 ml/kg: 30), which in combination with the aforementioned OC concentrations allowed estimation of differences in body burden among participants. As shown in Figure 2, the average body burdens of all OC increased in both breast milk and formula groups. However, significant contrasts were observed between the two feeding mode groups.

In the breastfeeding group, the major increase was observed for 4,4′-DDE (1.8 μg) and the second for total PCB (1.3 μg). The PCB increase was dominated by increments of the more chlorinated congeners such as #153 (0.5 μg), #138 (0.3 μg), and #180 (0.2 μg) whereas the less chlorinated congeners (e.g., #28 and #52), exhibited small increases (<0.1 μg). These results were consistent with a group of human milk samples analyzed in the context of this work for reference and general studies showing that the more chlorinated congeners are those predominant in breast milk (31). HCB and β-HCH increased by 0.5 and 0.3 μg, respectively. The high levels of 4,4′-DDE incorporation through breastfeeding were consistent with previous observations for other cohorts (11). The high accumulation of this compound could not be explained by specific OC physical-chemical properties. Thus, it may have reflected a specific metabolic mechanism of incorporation into breast milk, which would enhance its intake in relation to other OCs.

In artificial feeding, the major increment was observed for total PCBs (0.6 μg) involving higher increases of the congeners with five and six chlorine atoms, and lower increases of the others. The increase in 4,4′-DDE was very little (0.1 μg) as compared that in breastfeeding. The small increases of PeCB and HCB, 0.06 and 0.04 μg, respectively, are consistent with their low K_{oa} as discussed above. In contrast, β-HCH exhibited an increment of 0.2 μg and was the compound for which changes were most similar in both feeding modes. This OC can be differentiated from the others included in the study by its low log(K_{oa}) values (3.8 at 36.5 °C, Table 2). Thus, this OC was the least hydrophobic of the whole group, suggesting that its incorporation into children via breastmilk is relatively low. However, after intake, this compound accumulates in a fashion similar to the other OC that have log(K_{oa}) > 8, which reinforces the aforementioned results and indicates a preferential elimination of those compounds with low K_{oa}.

**Acknowledgments**

We thank J. Font and M. Gari for their help in sample analysis. M. C. Alvaro is thanked for her useful advice on the interpretation of results. This research was supported by the Instituto de Salud Carlos III, Red de Grupos INMA (G03/176), and project PI0414666.

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Received for review September 17, 2005. Revised manuscript received December 22, 2005. Accepted December 27, 2005. ES0518427