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Development of hematological and immunological characteristics in neonatal rats

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ABSTRACT

As major immunological and hematological parameters evolve during the early period of life, laboratory data must be interpreted in relation to developmental changes. Wistar (WU) rats were sacrificed on PND2, 4, 7, 10, 14, 17 and 21. Peripheral blood, bone marrow, thymus samples and spleen cells were collected and a bronchoalveolar lavage (BAL) performed. Parameters of blood counts changed considerably between time points. IgM and IgG levels steadily increased. Spontaneous spleen cell proliferation was low before PND21, although mitogens had stimulatory effects above baseline. In the spleen, T-lymphocyte counts tripled by PND17 (mainly attributed to $CD8^+$ cytotoxic T-cells and $CD4^+$ T-helper cells). In peripheral blood an increase in B-lymphocytes to about 60% of the cell number was observed. In BAL fluid, macrophages represented 95–98% of the cells. In thymus architecture, lymphoblast migration was seen and epithelial structures appeared. The data presented will help to distinguish between maturational changes and treatment-related effects.

1. Introduction

All living organisms are in constant contact with their changing external environment. The maintenance of physiological homeostasis despite those external influences is a challenging task for the organism in which the immune system in concordance with the hematopoietic system plays a fundamental role.

Adults and very young children display marked differences in the immune system leading to increased susceptibility of the young organism. This means that common infectious diseases may occur more frequently and manifest more severely in young children [1]. There are many intrinsic factors that influence susceptibility. In recent years concerns have been raised that there are many environmental agents such as xenobiotics, that may perturb during development and exacerbate some diseases in early childhood [2–6]. In particular pharmaceuticals that interfere with the immune system may lead to perturbations during growth and development [6]. This increased health burden could adversely impact children's health, by e.g. (1) reduced resistance to bacterial, viral, or parasitic infection diseases leading to increased infection rates in children; (2) increased susceptibility to allergens leading to the development of asthma among children; and (3) increased incidence of childhood cancer as reported for example for acute lymphoblastic leukemia or brain cancer [4,5,7–10]. Furthermore, the incidence of autoimmune diseases is rising and juvenile immune-inflammatory insults or dysfunction are increasingly implicated in neurological tissue disorders [11,12]. The protection of children against toxic agents in the environment is a major challenge to modern society [13]. Consequently, there is a raising awareness of the importance of risk assessment for children's health. This triggers a need for developing experimental approaches that consider the distinctive exposures and special sensitivities of infants and children, such as the developing immune system [4]. Various methods and protocols are available for immunotoxicity screening according to recommendations and guidelines [14,15]. However, these typically include immunotoxicity testing in adult laboratory animals only [5]. Contrary to this, testing in developing organisms is limited and early postnatal immune alterations are poorly understood. In particular standard exposures in developmental and reproductive toxicity are primarily based on adult characteristics and traditionally do not account for the heterogeneity of exposures and potential differential sensitivity of the immune system at various life stages [4]. The range of adverse effects in the developing organism is much wider than in the adult immunotoxicity studies, which are mainly focused on immunosuppression after exposure to toxic agents [5]. Moreover, transient immune effects in adults may be permanent

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in children [16]. The sensitivity of mature and immature individuals should be studied systematically to expand the current limited database [4].

Only recently, regulatory requirements for testing new agents for adverse developmental immunotoxic effects have been adopted, with issue of the OECD technical guidance 443 (Extended One-Generation Reproduction Toxicity Study) [17]. With the EU regulation 1901/2006 on medicinal products for pediatric use, the need for detailed investigation in young populations was emphasized, but still, the decision for the most valuable model in the right age group has to be made case-by-case [18].

Therefore, data sets for juvenile animal studies are still a fundamental research need. It is well known that hematopoietic and immunological parameters evolve during the first days to weeks of life, therefore any regulatory testing of xenobiotics and any laboratory data must be interpreted in relation to those developmental changes.

The combined development of the hematopoietic and immune systems starts very early in the embryonic stage and continues during postnatal maturation. In the early rodent embryo, mesoderm-derived hemangioblasts establish the earliest progenitors of all blood cells [19]. These pluripotent hematopoietic stem cells originate in the yolk sac around gestational day (GD)7 in mice. This "primitive" hematopoiesis allows survival of the embryo until "definitive" hematopoiesis develops from hematopoietic stem cells originating from the aorta/gonad/mesonephros (AGM) region [20]. AGM region hematopoietic progenitors migrate to the fetal liver and spleen around GD9–10 before establishing residence in the bone marrow beginning on GD11 [6,21,22]. The liver, and to a lesser extent the spleen, are the main sites of hematopoiesis of the developing rodent until shortly after birth [19]. Differentiation of hematopoietic stem cells into stem cells for the lymphoid and myeloid lineages has not been demonstrated in mice before GD10. Currently, the mouse model is better understood as data on the chronology of development in the rat are sparse and often derived from in utero hematopoietic stem cell transplantation experiments. In the mouse, the first mature immuno-competent lymphocytes can be found in the developing liver around GD18.5 and in the spleen, hematopoietic stem cells and lineage restricted progenitor cells appear around GD13 [6,23]. Establishment of bone marrow hematopoiesis as the primary hematopoietic organ starts in mice with immigration of hematopoietic cells on GD17.5 [6]. The bone marrow then rapidly assumes primary hematopoietic functions, which persist after birth.

Postnatal development significantly differs between species because of differences in the relative degree of fetal maturity at birth. This also applies to the development of the hematopoietic and immune systems. In mice, there is an immediate disappearance of hematopoietic cells from the liver after birth. Leukocytes are then mainly produced in the bone marrow. Splenic hematopoiesis persists for several weeks after birth in rodents. Only small numbers of B- and T-lymphocytes are reported to be found in the spleens of newborn rats [24]. The number of B-cells increases and the T-cell to B-cell ratio decreases between 2 and 3 weeks of age [6].

While the first weeks of postnatal life in rodents are marked rather by morphological maturation of the immunological relevant organs than by gaining full functionality, any insult inflicted at these early stages may have consequences for the fate of the immune system. For example, if the colonization of immune-relevant tissues with precursor cells is disturbed, life-long impairments might result. However, established immune functional tests such as T-cell dependent antigen response (TDAR) and keyhole limpet hemocyanin (KLH) test are normally conducted in the post-weaning rat but this is when full immune-function maturity is reached. This is due largely to the difficulty in performing functional tests in rodents during the juvenile stage because the development of the immune system in rodents is delayed compared with humans. Still, interference with immunological maturation at an earlier stage may result in impaired immune response later in life. Therefore, juvenile rodents are a relevant population for assessment of toxicity on the maturing immune system. Other species used in regulatory testing such as dogs or non-human primates have a much slower rate of development and lower litter sizes resulting in lower statistical power and are also more resource-intensive.

To explore this approach further, a juvenile animal study was conducted and is reported here. It includes the following blood and immune assessments over the first 3 weeks after birth in rats: (1) standard blood and immune parameters (i.e., hematology and histology); (2) additional blood and immune parameters focused on development and maturation (e.g. distribution, composition, and appearance of the various subsets in blood, spleen, and bone marrow) and (3) early functional assessment of humoral and cellular immunity. The protocol and data generated provide a robust dataset that gives detailed information on the hematopoietic system and passive immune status of juvenile rats. The information generated is recommended for incorporation into regulatory toxicity testing to better understand the juvenile origin of adult onset disease in these key biological systems.

2. Materials and methods

2.1. Animal husbandry

All experiments were conducted in juvenile Wistar (WU) rats (Crl:WI(WU)) whose parents were purchased from Charles River (Sulzfeld, Germany). Animals were kept at 22 ± 2 °C, 50 ± 20% relative humidity, and with 12:12 h lighting. Food and tap water were provided ad libitum. Parental animals, except lactating dams, were single-housed. Lactating dams were housed with their litters. Pairing was done over-night and positive mating confirmed the next morning by vaginal smears. Dams were allowed to litter naturally and the date of birth was defined as postnatal day 0 (PND0).

2.2. Ethical approval

All experiments were carried out with the approval of the local animal welfare authorities and the governmental authority (Bezirksregierung Niedersachsen, Germany).

2.3. Necropsy and blood sampling

A total of 21 litters, 2–5 litters per time point, were sacrificed on PND2, 4, 7, 10, 14, 17, or 21 respectively, with pup numbers ranging from 16 to 44 pups per time point. Shortly before necropsy, pups were pre-treated with intra-peritoneal injections of 5 μL heparin (heparin Na, ratiopharm GmbH, Ulm, Germany; 5000 I.E./mL), following CO_2 anesthesia and exsanguination. The thoracic and abdominal cavities were opened and blood samples were drawn. From PND2–4, the blood was collected directly from the thoracic cavity after opening of the heart's left ventricle, starting PND7 the samples were taken from the abdominal *V. cava*. Two blood smears per animal for differential blood count were prepared immediately.

2.4. Preparation of samples for immune testing

For assessment of immunological parameters, part of the blood samples was converted to serum. Peripheral blood cells were isolated from heparinized blood collected on PND2–21. The obtained cells were suspended in PBS. Cell numbers were determined using a Coulter Counter and adjusted for flow cytometry. Starting on PND7, blood samples were used for characterization of cells by flow cytometry. On PND10 bone marrow samples were collected