Reduced Thymic Size and Numbers of Splenic CD4+ and CD8+ Cells in Artificially Reared Mouse Pups

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The effect of early nutrition on the development of the immune tissue and T cells of mouse pups was examined. Newborn mice were divided into three experimental groups: mother-reared (MR) pups, pups that were fed on a milk substitute from the first day (AR-0), and the third day (AR-2), using a hand-feeding system. The average thymic size of the AR-2 pups was respectively significantly larger and smaller than that of the AR-0 and MR pups. In contrast, the splenic sizes of the AR-0 and AR-2 pups were greater than that of the MR pups. The numbers of CD4+CD8− and CD4−CD8+ cells in the spleen of the MR pups were significantly higher than those in the AR-0 pups. These results indicate that early nutrition affected the sizes of the thymus and spleen and the composition of CD4+CD8− or CD4−CD8+ T cells in the spleen.

Key words: nipple rearing; milk substitute; T cell; mouse pup

A newborn infant is first exposed to bacterial and viral antigens immediately during and after birth; this has an effect on its prognostic immune responses. In addition, the size and components of the immune cells are affected by early nutrition. Hasselbalch et al. have reported a decreased thymic size in formula-fed (FF) infants.1) Meanwhile, Yekeler et al. have reported that no significant difference was found between the mean values of the thymic dimensions in the FF and breast-fed (BF) groups.2) Similar studies have been conducted to assess the thymic stress in FF infants, and it has been reported that the thymic index in both BF and FF infants was affected by upper respiratory tract infections.3) Babies in a neonatal intensive care unit who consume formula milk are at a greater risk of morbidity due to septicemia than those who consume breast milk.4) An infant’s intake of breast milk is often disturbed by hospitalization. In addition, some antibiotics are often administered to almost all premature babies, who often consume infant formula as their early nutrition, to protect them against infection. Ellis et al. have shown in their review5) about how milk-borne cytokines and hormones influence the neonatal immune cell function that the neonatal immune system may be influenced by immuno-modulatory agents in maternal milk. It is also known that many immunologically active substances such as prolactin, immunoglobulins, transforming growth factor, lactoferrin, and cytokines are secreted in breast milk and are abundant in colostrum.6–8) In relation to this, it has been reported that, during the lactation period, human milk has the ability to protect against numerous infections such as otitis media, upper and lower respiratory tract infections, diarrhea, urinary tract infections, neonatal septicemia, and necrotizing enterocolitis.9–11) Thus, it is very important to investigate the effects of the type of early nutrition on newborn infants, particularly with regard to immune development. Different forms of early nutrition result in different patterns of intestinal microflora12) which may result in different innate and acquired immune responses in newborn infants.

Many recent studies on innate and acquired immune mechanisms have been reported by using gene knockout and gene transgenic mice. In those experiments, techniques for the artificial rearing of mouse pups are a very useful tool to evaluate nutritional and immunological effects on the development of pups. Gastrostomy and gastric tube feeding have been used in many nutritional

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Abbreviations: AR-0, artificially reared from the first day; AR-2, artificially reared from the third day; BF, breast-fed; FF, formula-fed; MR, mother-reared

investigations in order to facilitate accurate and planned volume administration. However, gastric tube feeding after gastrostomy may involve certain risks of inflammation, because the surgical stress might evoke unnecessary immunological stress in artificially reared pups by bacterial translocation, etc.\textsuperscript{13) }“A surrogate nipple equipped with a nursing bottle” for hand-feeding mice has recently been developed by Hoshiba,\textsuperscript{14) }who is one of the authors of this current study. The design of the nursing system allows mice to suckle milk voluntarily without sustaining injury. A simple, chemically defined formula for the optimum growth of mouse pups is crucial for examining the functional effects of a particular nutrient that is included in the milk. The authors described the simple preparation of a chemically derived artificial formula for mouse pups that was based on the analysis of milk from three strains of mice.\textsuperscript{15) }

The spleen, along with the thymus, is an important organ for immune development. However, the difference in its size and/or T cell pattern when different forms of early nutrition are used has not yet been clarified. The purpose of the study presented here was to investigate the immunological and physiological differences in mouse pups reared by using different forms of early nutrition with or without the ingestion of colostrum.

\section*{Materials and Methods}

Meiji Dairies Corporation’s Committee for Research on Experimental Animals approved the experimental protocol. The experiments were conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals (NRC, 1985).

\textit{Animals}. Ten pregnant, time-dated C57BL/6N mice were purchased from CLEA Japan (Tokyo, Japan). All animals were housed individually under environmentally controlled temperature (25 ± 2°C), humidity (50 ± 5% relative humidity), and light (12-hour light-dark cycle) conditions. They had free access to water and feed (CA-1; CLEA Japan, Tokyo, Japan) and were allowed to deliver spontaneously.

\textit{Artificial rearing}. Within 16 hrs of birth, the pups from 6 litters born on the same day were weighed and randomly divided into groups to eliminate the influence of inter-litter variation. In the MR group, 10 pups were reared by one of their mothers throughout the study. In the AR-0 group, 7 pups were separated from their mothers on the first day of life and were fed the formula described in Table 1 through the Hoshiba nipple or Hoshiba nipple-yajima style equipped with a nursing bottle (that had been developed and manufactured by Meiji Dairies Corporation, Tokyo, Japan). In the AR-2 group, 10 pups were reared by one of the mothers for the first 2 days; thereafter, 7 of these pups were separated from their nursing mother and fed on the formula. In both groups of artificially reared pups, the pups were fed on the milk formula\textsuperscript{15) }5 times per day from 8 a.m. to 11 p.m. The nipple systems were sterilized in an autoclave at 121°C for 15 min before being used. The nursing bottle

\begin{table}
\centering
\caption{Milk Substitute for Mouse Pups}
\begin{tabular}{|l|c|}
\hline
Nutrient (weight/100 ml) & Value by analysis  \\
\hline
Protein (g) & 11.1  \\
Casein & 3.46  \\
Whey & 3.64  \\
Whey protein hydrolysate & 4  \\
Carbohydrate (g) &  \\
Lactose & 2.6  \\
Dextran & 0.45  \\
Fat (g) & 16  \\
Palm oil & 4.79  \\
Coconut oil & 3.99  \\
Soya oil & 3.19  \\
MCT oil & 2.39  \\
Corn oil & 1.6  \\
Cholesterol & 0.04  \\
Minerals (mg) &  \\
Calcium & 366  \\
Phosphorus & 174  \\
Sodium & 95  \\
Potassium & 117  \\
Chloride & 151  \\
Magnesium & 24  \\
Zinc & 1.37  \\
Copper & 0.41  \\
Fluorine & 0.029  \\
Iodine & 0.139  \\
Iron & 4.62  \\
Vitamins (mg) &  \\
Cyanocobalamin & 0.00119  \\
Biotin & 0.018  \\
Folic acid & 0.079  \\
Thiamin hydrochloride & 0.881  \\
Pyridoxin hydrochloride & 1.25  \\
Riboflavin sodium phosphate & 1.415  \\
Calcium pantothenic acid & 2.643  \\
P-amino benzoic acid & 4.404  \\
Nicotinic acid & 4.988  \\
Sodium ascorbate & 67.4  \\
4-Insitol & 49.725  \\
Choline citrate & 0.14  \\
Vit.E: \(\alpha\)-tocopherol & 2.346  \\
Vit.K & 1.982  \\
Vit.A (I.U.) & 42.8  \\
Vit.D (I.U.) & 10  \\
Others (mg) &  \\
Carnitine & 3.5  \\
Picolinate & 2  \\
Ethanolamine & 3.5  \\
Taurine & 15  \\
Cysteine & 22.5  \\
Serine & 27.5  \\
Tryptophan & 27.5  \\
\hline
Energy contents (Kj/100 ml of milk)* & 829.5  \\
Osmolarity (mOsm/kg H\textsubscript{2}O)** & 490  \\
\textit{pH}** & 6.5  \\
\hline
\end{tabular}
\end{table}

*Conversion factors: 1 g protein = 17 kJ, 1 g carbohydrate = 16 kJ, 1 g fat = 37 kJ

**Value by analysis

from their nursing mother and fed on the formula. In both groups of artificially reared pups, the pups were fed on the milk formula\textsuperscript{15) }5 times per day from 8 a.m. to 11 p.m. The nipple systems were sterilized in an autoclave at 121°C for 15 min before being used. The nursing bottle
was filled with the milk substitute that was thawed daily and warmed in an incubator for 5–6 min at 43 °C immediately before feeding time. The pups in the AR-0 and AR-2 groups were housed in a temperature-controlled and humidified nursing box at 33–34 °C.

**Preparation of the formula for the mice.** The composition of the milk substitute for the mice is shown in Table 1. Casein, whey protein, and whey protein hydrolysate from bovine milk were used as the protein sources at proportions of 4:4.5:1. The first step for preparing the formula was to make up the casein mixture. Three amino acids—serine (1.15 g), cystine (0.9 g), and tryptophan (1.08 g)—were added to an alkaline solution (800 ml) containing 1 g of NaOH and 6 g of KOH while continuously agitating with a motor-driven stirrer (three-one motor; Heidon, Tokyo, Japan) at 60–70 °C. Next, casein (160.0 g) was slowly dissolved in the alkaline solution. The casein mixture was sterilized for 30 min in a boiling water bath. In the second step, calcium and magnesium were added to the casein mixture to obtain micelles of casein salts. CaCl$_2$·2H$_2$O (6.8 g), the calcium salt of glycerophosphoric acid (32 g), and MgCl$_2$·6H$_2$O (7.6 g) were mixed in 200 ml of distilled and deionized water (DW) which was autoclaved (at 121 °C for 10 min) and then homogenized with a Polytron-like mixer (Nihonseiki Kaisha, Tokyo, Japan). This mixture was then slowly added to the casein mixture while continuously mixing with an Ultra-Turrax homogenizer (IKA Labotechnik, Germany). Subsequently, CaCO$_3$·2H$_2$O (10 g) and Ca-citrate (4.8 g) were mixed in 100 ml of DW, and this mixture was slowly added to the casein mixture after autoclaving and homogenizing. A mixture of Na$_2$HPO$_4$ (3.2 g) and KH$_2$PO$_4$ (0.32 g) in 50 ml of DW, which had been sterilized in an autoclave in advance, was next added to the casein mixture. A lactose solution (75.64 g of lactose in 220 ml of DW) which had been sterilized in an autoclave in advance was then slowly added to prevent the casein mixture from foaming. Finally, to the casein mixture were added 10 ml of a solution in which FeSO$_4$·7H$_2$O (1.92 g) and citrate·H$_2$O (0.04 g) had been dissolved in 20 ml of DW, 5 ml of a solution in which ZnSO$_4$·7H$_2$O (1.2 g), CuSO$_4$·5H$_2$O (0.3 g), and MnSO$_4$·5H$_2$O (0.05 g) had been dissolved in 25 ml of DW, and 5 ml of a solution in which NaF (0.031 g) and KI (0.05 g) had been dissolved in 25 ml of DW; this was done while continuously stirring after sterilization by Millipore filtration (0.45 μm).

We dissolved the whey protein isolate (160 g) and whey protein hydrolysate (200 g), which had been sterilized by gamma-irradiation (30 KGY) in advance, in 1000 ml of sterilized DW; this solution was then added to the casein mixture after being cooled to below 40 °C while continuously stirring. Next, the casein mixture was added 10 ml of a mixed solution in which carnitine (0.32 g), picolinate (0.16 g), ethanolamine (0.272 g), and taurine (1.2 g) had been dissolved in 20 ml of DW. We subsequently added to this mixture 70 ml of a solution in which a water-soluble vitamin mixture (27.3 g) had been dissolved in a neutralized solution (neutralized to pH 7.0 by 5 N NaOH) of choline dihydrogen citrate (5.88 g) in 73.5 ml of DW on a water bath at 65 °C. Finally, oily vitamins (79.3 mg of vitamin K$_3$, 93.8 mg of vitamin E, and 5.7 mg of a mixture containing vitamins A and D) and six kinds of edible oil (191.5 g of palm oil, 159.6 g of coconut oil, 63.84 g of corn oil, 95.76 g of medium-chain triglyceride (MCT) oil, 127.68 g of soybean oil, and 1.6 g of cholesterol) were sterilized in boiling water for 30 min. The entire oily solution was added to the casein mixture while continuously stirring after being cooled to 40–50 °C.

The final mixture of the formula was homogenized 3 times under high pressure (180 kg/cm$^2$) with a homogenizer (Sanwa Machine Co., Numazu, Japan) that had been sterilized with a hot alkaline solution by steam bubbling at 85 °C for 20 min and then rinsed and cooled with sterilized water in advance. After this homogenization, the artificial milk was aseptically distributed into 50-ml sterilized polypropylene bottles and stored frozen at −40 °C. The osmotic pressure and pH of the formula were 492 mOsm and 6.33, respectively. No enterobacteria were detected in the milk. The frozen formula was then sterilized by gamma-ray irradiation (30 KGY) and stored at −40 °C until needed.

**Analytical experiments.** Ten days after birth, the pups were anesthetized with ethyl-ether. Blood samples were obtained from the abdominal aorta for an immunoglobulin analysis, and the internal organs were collected and individually weighed. The spleen was immersed in an optimal cutting temperature compound (Sakura Finetechical Co., Tokyo, Japan), frozen in liquid nitrogen, and then stored at −80 °C until being sectioned for an immunohistochemical staining analysis.

**Confocal microscopy for immunohistochemical studies.** Tissue slides were prepared by BML (Tokyo, Japan). The frozen sections were cut into 5-μm slices by a cryostat microtome and placed on glass slides. The sections were fixed in acetone, air-dried, and stored at −80 °C until needed for staining. The rat anti-mouse biotin-CD4 antibody and rat anti-mouse FITC-CD8 antibody were kindly provided by Dr. S Ikegami (Food Science Institute, Meiji Dairies Corporation). Streptavidin-rhodamine RedFl-X conjugate was purchased from Molecular Probes, USA. After being incubated in PBS, each tissue slide was incubated in 1% normal rat serum containing PBS for 60 min at 4 °C for blocking. The tissue slides were kept in the dark during immunostaining and microscopy. The number of CD4$^+$ or CD8$^+$ cells was randomly counted from 8 to 10 of the microscopic fields per tissue slide and calculated by image processing, using Scientific Imaging software for Macintosh IP Lab$^TM$/Mac (version 3.5; Scanalytics, Fairfax, VA, USA).
**IgA, IgG, and GPT concentrations in the plasma.** The blood samples were centrifuged at 3000 × g for 20 min at 4 °C after being left at room temperature for 30 min. The supernatants were frozen at −80 °C for analysis. The concentration of immunoglobulin A (IgA) and immunoglobulin G (IgG) in the plasma was analyzed with a mouse IgA or IgG ELISA Quantitation Kit (Bethyl Lab., Montgomery, TX, USA). The glutamate pyruvate transaminase (GPT) activity in the plasma was measured to monitor hepatic inflammation with a kit from WAKO Pure Chemical Industries. (Osaka, Japan).

**Statistical analysis.** Differences in the relative tissue sizes and IgA or IgG concentrations among the three groups were compared by using one-way ANOVA and applying Fisher’s post hoc test (p < 0.05). All statistical analyses were performed with Stat View 4.1 software (Abacus Concepts, Berkeley, CA, USA).

**Results**

**Growth of the pups**

The growth rates of the pups in both of the AR groups were not as high as that of the MR pups during the first few days (Fig. 1A). Thereafter, the AR-2 pups grew to weigh the same as the MR pups, while the AR-0 pups grew at a much slower rate than the MR pups. The mean volume fed to the AR-2 pups was not significantly higher than that fed to the AR-0 pups after 3 days of age (Fig. 1B). The AR-2 pups gained more body weight than did the AR-0 pups on almost all the experimental days. Photographs of the 3-day-old AR-0 pups and 10-day-old AR-2 pups who suckled the formula from the Hoshiba nipple system are shown in Fig. 2a and b, respectively. The AR-2 pups weighed the same as the MR pups after 10 days, as shown in Fig. 2c.

**Comparison of the IgG, IgA, and GPT levels in the plasma**

The IgG levels (Fig. 3A) in the AR-0 and AR-2 pups were significantly lower than that in the MR pups (p < 0.01), although the IgA (Fig. 3B) concentration and GPT (Fig. 3C) activity in the plasma were not significantly different among the groups.

**Comparison of organ sizes**

The percentage of organ weight relative to body weight in the 10-day-old pups is shown in Fig. 4 and Table 2. The relative thymus weight of the AR-2 pups was respectively significantly larger and smaller than that of the AR-0 pups (p = 0.011) and MR pups (p = 0.001) (Fig. 4A). However, the relative spleen weight of the AR-0 pups was significantly smaller than that of the...
than in the MR pups (heart and lung were significantly smaller in the AR pups groups than in the MR pups), whereas the relative organ weights of the liver and kidney were greater in the pups of both the AR respectively), whereas the relative organ weights of the liver and kidney were greater in the pups of both the AR groups than in the MR pups (p < 0.05 for each organ), as shown in Table 2.

CD4\(^+\) and/or CD8\(^+\) cells in the spleen
The numbers of splenic CD4\(^+\)CD8\(^-\) and/or CD4\(^-\)CD8\(^+\) cells in the 10-day-old pups are shown in Figs. 5 and 6. The numbers of CD4\(^+\)CD8\(^-\) cells and CD4\(^-\)CD8\(^+\) cells were significantly lower in the AR-0 pups than in the MR pups (p < 0.05). Interestingly, the numbers of CD4\(^+\)CD8\(^-\) cells and CD4\(^-\)CD8\(^+\) cells in the AR-2 pups tended to be higher than those in the AR-0 pups (p = 0.076 for CD4\(^+\)CD8\(^-\) cells; p = 0.052 for CD4\(^-\)CD8\(^+\) cells), while the numbers were not significantly different from those in the MR pups.

Discussion
This is the first report on immune cell development in mouse pups that had been artificially reared immediately after their day of birth by using a chemically refined milk formula. The tissue sizes and/or the CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) T cell patterns of the spleen were affected by early nutrition. A mere 2-day intake of colostrum could promote both an increase in the size of the thymus and T cell development in the spleen, as with the breast-fed pups.

The T cells in a fetus are basically naïve and can be activated by their exposure to exogenous bacterial antigens from the time immediately after birth. How-
ever, it has recently been reported\textsuperscript{16} that immune events in the mother during gestation may modulate the pattern of immune development in the offspring; for these reasons, the newborn littermate pups were divided at random into three experimental groups in our experiments.

The IgG level in the MR pups was significantly higher than that in the 2 groups of AR pups (Fig. 3A), although the concentration of IgA in the plasma did not differ among the groups (Fig. 3B). These data indicate that plasma IgG in the MR pups was transferred to them by the absorption of IgG present in the maternal milk, as is the case with rat pups.\textsuperscript{17} IgA of maternal milk might not be transferred to the plasma of the pups. The production of IgA in the suckling mice at 10 days of age may be about 10 times lower than that in adult mice.\textsuperscript{18} In a study on rats, the development of IgA bearing lamina propria lymphocytes was delayed until 14 days of age,\textsuperscript{19} and the plasma IgA concentration of MR pups at 14 days of age was 2.5 μg/ml,\textsuperscript{20} this being approximately 15% of that at 4 wk of age in Sprague-Dawley rats.\textsuperscript{21}

It is very interesting that the relative thymus weight of the AR-2 pups was respectively significantly larger and smaller than that of the AR-0 and MR pups 10 days after birth (Fig. 4A). The thymus size of artificially reared mouse pups not exposed to any physical injury was smaller than that of the MR pups, and increased after the intake of colostrum for only 2 days. These results indicate that colostrum may contain a stimulating factor that aids the development of the thymus or that the increased weight gain may promote enlargement of the thymus. On the other hand, the relative size of the spleen in the AR-0 and AR-2 pups was greater than that in the MR pups (Fig. 4B). In relation to these findings, Gala and Shevach have reported that an administration of bromocriptine to mothers repressed the growth and both the size of the thymus and the spleen of their pups.\textsuperscript{22} Bromocriptine is a drug that specifically suppresses pituitary prolactin and would decrease the level of prolactin in the milk.\textsuperscript{23} However the size of the spleen in the AR-0 pups had not decreased at 10 days of age in our experiment. Prolactin may be produced in the spleen at 10 days of age in the AR-0 pups, because mRNA of prolactin was expressed in all spleen samples during 0–60 days postpartum.\textsuperscript{24} In addition, the spleen may be more sensitive to an intestinal antigenic stimulus after artificial rearing, because the number of adherent bacteria in the distal colon of the AR-0 pups was significantly higher than that of the MR pups (Data not shown). We have previously shown in a rat study that bacterial translocation easily occurred during the suckling period and that the number of intestinal adherent bacteria was higher in AR pups than that in MR pups.\textsuperscript{20}

The relative sizes of the lung and heart were also significantly smaller in the pups of both the AR groups than in the MR group, as shown in Table 2. It is possible that the breathing capacity of the AR pups did not increase or that they were inactive because they had no rivals competing for the maternal nipple. Appropriate exercise appears to be essential for achieving normal growth of the heart and lung.

It is known that stress hormones such as corticoids are related to immune responses.\textsuperscript{25,26} In relation to the effects on immunological responses of the stress induced by maternal deprivation, Barreau \textit{et al.}\textsuperscript{27} have reported that maternal deprivation for 3 hours per day from 2 to 15 days of age affected the immunological response of mouse pups against inflammatory reagents at 12 weeks of age. Coutinho \textit{et al.}\textsuperscript{28} have also demonstrated that neonatal maternal separation altered stress-induced responses. Therefore, we measured the concentration
of 8-hydroxy-deoxyguanosine (8-OH-DG) in the urine as a stress parameter in another experiment with artificially reared mice. The result revealed that the concentration of 8-OH-DG in the AR and MR pups 10 days after birth was not significantly different (data not shown). In addition, it was often observed during rearing that the pups sucked the fingers of the person who nursed them. These findings led us to think that artificial rearing by using the Hoshiba nipple would induce little stress in the mouse pups when initiated a few days after birth. However, additional studies are required to clarify the causes and effects of the stress induced in AR pups.

The present experiments also showed (as shown in Figs. 5 and 6) that the numbers of CD4+CD8− cells in the spleen tended to be higher in the AR-2 pups than in the AR-0 pups. The numbers of CD4+CD8− cells and CD4−CD8+ cells in the AR-2 pups were almost the same as those in the MR pups. On the other hand, the relative size of the spleen in the AR-0 and AR-2 pups was respectively significantly and not significantly larger than that in the MR pups (Fig. 4B). It may be possible that the total numbers of CD4+CD8− cells and CD4−CD8+ cells in the spleen were higher in the AR-2 pups than in the MR pups. Further studies should be performed to determine the exact T cell numbers by direct counting with a flow cytometric analysis.

Environmental differences in nursing conditions such as bacterial contamination by exposure to the mother’s feces or by the personnel handling the pups could affect the development of acquired immunity. Umesaki et al. have examined the expression of major histocompatibility complex class II molecules on epithelial antigen-presenting cells, the ratio of the number of CD4−CD8+ cells to that of CD4+CD8− cells in aP America in the large intestine, and the number of IgA-producing cells in the colon of germ-free mice and 3 kinds of gnotobiotic mice. They suggested in the results section the occurrence of immunological responses to such indigenous bacteria as segmented filamentous bacteria (SFB). However, it has also been reported that SFB could be detected in the ileum immediately after weaning. It would be important to confirm whether SFB participate in the development of immunity even in suckling pups.

We succeeded in artificially rearing mouse pups from the day immediately after birth by using the Hoshiba nipple. We have already reported a chemically derived milk substitute. In this study, we had to refine the protein source, because the casein and whey protein present in a ratio of 6:4 in the previous milk substitute was insufficiently digested in the stomach of pups younger than 8 days of age (data not shown). Therefore, the revised milk substitute used in this study for newborn mice contained a hydrolysate of whey protein as a partial replacement for casein which promoted digestibility in the newborn mice.

In spite of the refinement of the milk substitute, the growth of the AR pups was slower than that of the MR pups during the first week after birth, as shown in Fig. 1A. The weight of the AR-2 pups was almost the same as that of the MR pups at 10 days, but the AR-0 pups gained significantly less weight than the AR-2 pups. These data indicate not only that the ingestion of colostrum for only the first 2 days promoted the growth of the newborn pups, but also that good catch-up during the newborn period in neonatal mice may influence the subsequent growth rate.

We did not include a hand-fed group in which mouse milk was provided as a control, because it was impractical for the pups to aseptically suckle from the human hand a sufficient volume of fresh mouse milk in order to obtain full growth.

In conclusion, this is the first report on the artificial rearing of mice from the first day after birth by using a chemically derived milk substitute without causing physical injury. A surrogate nipple was used for hand-feeding the neonatal mice to avoid causing any injuries. The manner in which the AR pups suckled and swallowed the formula through the nipple is similar to that by which human infants receive formula from a bottle. Comparative studies of the immune systems of neonatal mice that were artificially reared from the first or third day after birth were also reported. These studies clarified that the intake of colostrum for only the first 2 days could activate T cell development in the spleen of the AR pups at 10 days of age, this being similar to what was observed in the MR pups. A considerable number of experiments should be conducted to clarify the mechanism for T cell activation by immuno-modulating substances like prolactin, TGF, hormones or cytokines in colostrum. The Hoshiba nipple and the chemically derived milk substitute for mice are available for use in investigating the role or function of a milk-borne substance or an external substance that is considered to have potential biological activity in neonatal mice.

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