### Essentials for starting a pediatric clinical study (3): Dynamic changes in early development of immune system in macaque monkeys -The significance from standpoint of preclinical toxicity test using nonhuman primates-

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**ABSTRACT** — Macaque monkeys are essential laboratory animals in preclinical safety assessment for human-specific biological products including humanized antibody drug. In most case, investigators are leaving their ages out of consideration, and young individuals aged around 3 years are mainly used because of their small individual differences in biological responses to various stimulations. Since the immune system starts to develop just after birth and remarkable phenotypic and functional changes occur in various kinds of immunocompetent cells during the first few years of life in macaque monkeys, their actual immunological condition must be carefully considered in case of safety assessment of novel drugs which modulate human immune function. The early development of major immune functions of macaque monkeys is summarized as follows. These findings suggest that immunocompetent cells drastically differentiate into activated ones during early development. 1) The serum immunoglobulin contents gradually rise with increasing age up to sexual maturity. 2) The blood group-associated antibodies, anti-A and anti-B antibody, are detected around 40-days of age and antibody levels rapidly increase after one year old. 3) Infant cynomolgus monkeys obviously produce the significant levels of IgG antibody against *Campy*lobactor jejuni within 4 weeks after infection when maternal antibody becomes undetectable (8 weeks of age). 4) The frequency of lymphocyte subpopulations expressing the resting surface phenotypes is much higher than that having activated phenotypes in neonates, and the relative population of lymphocyte subsets with resting phenotype decrease with increasing age, while the subpopulation associated with activation gradually increase with age.

Key words: Macaque monkeys, Early development, Immune function, Major lymphocyte subsets, Phenotype of lymphocyte

The mammalian newborns come into the world under immature state in immune function, and they are exposed to enormous kinds of foreign antigens surrounding them just after birth. The early development of immune functions in infant might reflect the immune responses to the exposed foreign antigens, including activation and clonal expansion of various immunocompetent cells, following phenotypic and functional changes. These drastic changes in immune function are complete when they become 4 to 5 years old, age of sexual maturity of macaque monkeys. In the case of using infant or young monkeys to preclinical safety assessments, the pattern of development must be carefully considered. Here, I summarize the early development of immune functions of macaque monkeys and show one example of difference in signal transduction via surface molecule expressed on lymphocytes between infant and adult monkeys.

## Early development of total immunoglobulin levels and specific antibody levels:

Since immunoglobulin (Ig) detected originally in normal sera is taken to be "natural antibodies" produced by certain immune responses against various antigens, Ig contents might be a useful parameter to evaluate the

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age-related change of humoral immune functions. Fig. 1 summarized the age-related changes in serum immunoglobulin G (IgG), A (IgA) and M (IgM), and blood group-associated anti-A antibody levels in cymonolgus monkeys (Terao, 1981; Fujimoto et al., 1982; Terao et al., 1983). Only a trace of IgA was detected in one-fifth of newborns and the levels gradually rose with increasing age. At 5-9 years of age, IgA level was still lower than the adult level although it continued to rise throughout early adulthood (3-5 years old). IgM could be detected in small amounts in every newborn, indicating intra-uterine synthesis of IgM by fetus. During the first year of life, a rapid increase of IgM was observed, having attained to the adult level at 5 years of age. In contrast to IgA and IgM levels, IgG level on the day of birth was nearly the same as that for the adult level. IgG level decrease to a minimum at 2-3 months of age. Thereafter, IgG level increased and reached the adult level at the age of 3-4 years. The decrease of IgG level during the first half year of life indicates that the large portion of IgG in the newborn's sera is composed of IgG transferred from mother monkey; maternal antibody. In order to know the time of disappearance of maternal IgG in infant sera, hemagglutination inhibition (HI) antibody against measles virus was examined with 70 infants aged 0 day to 18 months. The antibody titer fell rapidly with the development of infants, becoming undetectable in sera in more than 6-month-old infants (Fujimoto *et al.*, 1983).

As regards a specific antibody level, the naturally occurred blood group antibody levels were examined because both anti-A and anti-B antibody must be produced by specific immune responses to antigens which share the same antigenic determinants with A and B antigens. Both anti-A and anti-B antibodies were absent from sera of group-A and B monkeys aged less than 2 weeks. Those antibodies could be detected around the age of 40days, increasing gradually with age and reached a peak at 4 to 5 years of life (Fig. 1, Terao et al., 1983). Regarding the antibody response against infectious agents, we have detected significant levels of IgG antibody to Campylobactor jejuni in infant cynomolgus monkeys at 8-weeks of age when maternal antibody became undetectable, suggesting that infant monkeys are able to produce a significant level of IgG antibody against infectious agents (Kohno et al., 1988).

## Phenotypic changes in surface marker on major lymphocyte subsets:

The peripheral major lymphocytes, T-, B- and natural killer (NK) -subsets express characteristic combinations



Fig. 1. Age-related changes in anti-A blood group-associated antibody levels and total serum IgG, IgA and IgM in cynomolgus monkeys. Each level is expressed as percentage to adult level.

of cell surface molecules detectable with monoclonal antibodies (mAbs). According to the increase in number of mAbs as well as to the progress of multicolor flow cytometory, it became possible to subdivide lymphocytes further into discrete subpopulations. The early development of eight lymphocyte subsets was determined for pigtailed macaque infants from 0 to 800 days of age using two-color flow cytometry (Terao et al., 1988). Four major lymphocyte subsets, CD20+/B, CD4+/T, CD8+/T and CD16+/NK, could be further divided into 2 or 3 subpopulations by using mAbs that detect surface molecules associated with activation. As shown in Fig. 2, the frequency of lymphocyte subpopulations having surface resting or naive phenotypes, IgD+/B cells, CD45RA+/CD4+ Tcells, and CD18dull/CD8+ T-cells, was much higher than subpopulations having activated phenotype, IgD-/B cells, CD45RA-/CD4+ T-cells and two CD18bright(bri)/CD8+ T-cells. There was a complete absence of two CD18bri/ CD8+ subsets, CD18bri/CD8bri and CD18bri/CD8dull, during the first 300 days of life (Data not shown). The relative proportion of lymphocyte subsets with resting phenotype decreased with increasing age, while the subpopulations associated with activation gradually increased with age. These findings suggest that every immunocompetent cell gradually differentiates from resting cell to activated one through the stimulation with various kinds of foreign antigens during early development. This possibility is supported by evidence that few expanded T-cell clones could be detected in peripheral blood mononuclear cell (PBMC) from fetus and neonates, then the number of expanded T-cell clones increase with increasing age up to

5 years old (Nam *et al.*, 2000a). In addition, the telomere length in peripheral lymphocytes significantly decreased in relation to age, suggesting that the clonal expansion needs the repeated replication in lymphocytes during activation process, resulted in accumulation of T-cell clones and shortening of telomere length of lymphocytes (Lee *et al.*, 2002).

# Differences in response to agonistic antibodies to CD3 and CD28 between infant and adult monkeys:

The most remarkable difference in phenotype of peripheral T-cells between human and cynomolgus monkeys is the substantial presence of CD4+/CD8+ (DP) Tcells in periphery (Akari et al., 1997). DP T-cells exhibit a resting memory phenotype and increase in proportion with age and up to 10% of peripheral T-cells expressed CD4 and CD8 simultaneously after 10 years old. We have to recognize that there are 3 different T-cells, CD4+/CD8-, CD4-/CD8+ and CD4+/CD8+ (DP) T-cells, in peripheral blood of adult cynomolgus monkeys. Every peripheral T-cell showed dynamic phenotypic changes in expression of CD28 and CD29 surface antigens in relation to age in cynomolgus monkeys (Nam et al., 1998a, 1998b). Almost CD4+/CD8- T-cells constantly expressed CD28 throughout life. On the other hand, more than 60% of CD4-/ CD8+ T-cells expressed CD28 in infants and the proportion of CD28+/CD8+ T-cells rapidly decrease in relation to age and reached 20% at around 10 years old. The DP T-cells showed the most drastic age-related phenotypic changes in CD28 and CD29 expression. As shown in



Fig. 2. Summary of fluctuation pattern of major lymphocyte subsets during early development in pigtailed monkeys.

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Fig. 3, more than 90% of peripheral DP T-cells expressed CD28 antigen in infants. The proportion of CD28+ T-cells rapidly decrease in relation to age and reached bottom at around 10 years, after that CD28 antigen was constantly expressed on 20 to 25% of peripheral T-cells. In contrast less than 10% of DP T-cells expressed CD29 antigen in infant monkeys, following rapid increase up to 10 years old when almost DP T-cells express CD29. The CD28 molecule provides co-stimulation signals necessary to Tcell activation, and in vitro co-stimulation of T-cells with both anti-CD3 and anti-CD28 antibodies induce activation of T-cells, resulting in proliferation and cytokine-release in cynomolgus monkeys (Nam et al., 2000b). The agerelated changing pattern of CD28+ T-cells might suggest the difference in function (signal transduction) of CD28 molecule between young and adult cynomolgus monkeys. To demonstrate this possibility, peripheral CD3+ Tcells were purified from infant (3 years old) and adult (10 years old) cynomolgus monkeys, and then cultured with plate-fixed anti-CD3 antibody alone or both anti-CD3 and anti-CD28 antibodies. After 4-days culture, the content of interferon gamma (IFN $\gamma$ ) in supernatant were measured by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4, more than 90% of T-cells expressed CD28 in young monkeys, whereas half of T-cells expressed CD28 in adult monkeys, confirming the previous data. Interestingly, only adult monkey T-cells could be activated and released high concentration of IFNy after stimulation with anti-CD28 and/or anti-CD3 antibodies. Young monkey Tcells showed very low responses to stimulation with not only anti-CD3 antibody alone but also co-stimulation with anti-CD3 and anti-CD28 antibodies, although more than 90% of T-cells expressed CD28 antigen. The effect of co-stimulation with anti-CD28 agonistic antibody on T-cell activation was not clear in this experiment because no difference in T-cell response was observed between stimulation with anti-CD3 antibody alone and co-stimulation with anti-CD3 and anti-CD28 antibodies in adult cynomolgus monkeys. Although this result is very preliminary and precise analysis must be needed in future, it can be proposed that the reactivity or sensitivity to agonistic antibody might be low in young monkey T-cells as compared to adult ones. It must be necessary that the actual immunological condition must be carefully considered in case of using young monkeys as surrogate to evaluate the safety and efficacy of new drug that modulates the human immune function.

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Fig. 3. Age-related changes in CD28 and CD29 expression on peripheral CD4+/CD8+ (DP) T-cells in cynomolgus monkeys.

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**Fig. 4.** Expression of CD28 antigen on T-cells and IFNγ production after *in vitro* stimulation of purified T-cells with anti-CD28 and/or anti-CD3 antibody in young and adult cynomolgus monkeys.

Black and gray bar show the results obtained from either monkey in both age groups.

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