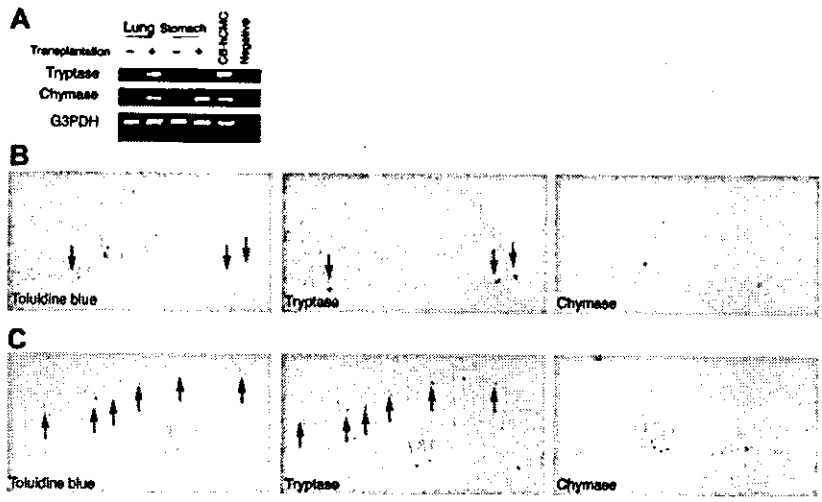

Figure 6. Human mast cell development in the mouse lung and gastric stomach. (A) RT-PCR analysis for tryptase and chymase mRNA expression. The lung and gastric stomach of NOG mice after the transplantation of human CD34$^+$ cells expressed human mast cell-specific tryptase and chymase mRNA. CB-hCMC indicates cord blood-derived human cultured mast cells. (B-C) Histologic findings for lung (B) and gastric stomach (C). Very small numbers of formalin-resistant toluidine blue$^+$ cells appeared in the lung 20 weeks after the transplantation and, in sequential sections, were almost identical to tryptase$^+$ cells (arrows). In gastric stomach, formalin-resistant toluidine blue$^+$ cells were identified in both the mucosa and submucosa. In the acetone-fixed frozen thin sections stained with antichymase mAb, chymase$^+$ cells (white arrowheads) were located only in submucosal lesions. Magnification, ⁄ 200 (toluidine blue and tryptase) and × 100 (chymase).

Peritoneal cavity and mesentery

The 2.1 ± 0.5 × 10^6 cells collected from the NOG mouse peritoneal cavity 20 weeks after the transplantation contained 3.9% ± 1.2% safranin$^+$ CTMCs (n = 4). On the cytospin preparation from the collected peritoneal cells, we failed to detect chymase$^+$ human mast cells (data not shown). Flow cytometry showed that less than 0.9% of the collected peritoneal cells expressed human CD45 (Figure 7C). Among these human CD45$^+$ cells, 2.1% expressed CD203c (0.02% of all peritoneal cells), which were human Kit$^+$ mast cells.

In the mesentery of NOG mice 20 weeks after the transplantation, we recognized a small number of human mast cell clusters consisting of 2 to 6 chymase$^+$ cells (Figure 8A).

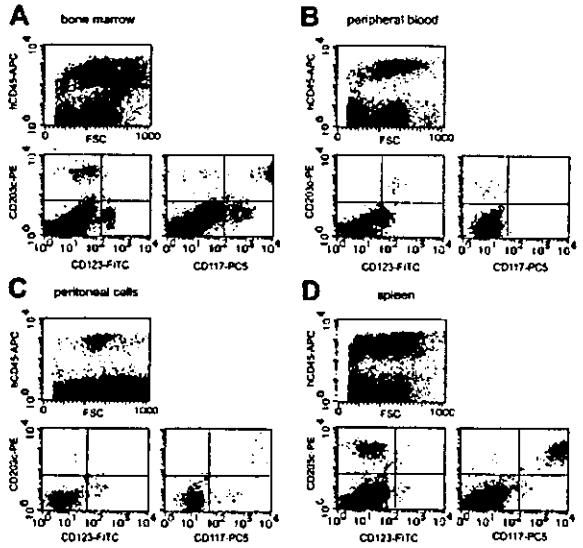

Figure 7. Representative flow cytometric analysis of bone marrow, peripheral blood, peritoneal cells, and spleen from NOG mice after HSC transplantation. Human mast cell and basophil development from the transplanted human CD34$^+$ cells were identified as CD203c/Kit (CD117) double-positive cells and CD203c/IL-3 receptor α-chain (CD123) double-positive cells, respectively, in human CD45$^+$ cells. The percentage of human CD45$^+$ cells differed depending on the transplanted cord blood-derived cells, but the proportion of CD203c/Kit double-positive cells and CD203c/CD123 double-positive cells in reconstituted human CD45$^+$ cells was similar in 4 independent experiments.

Lymph nodes and spleen

Twenty weeks after the transplantation, when human lymphocytes had already been reconstituted, only a small number of human mast cells (0 to 4 cells in the frontal section, n = 4) was identified in the lymph nodes. They were located at the trabecula and comprised human chymase$^+$ connective tissue type mast cells (Figure 8B).

Abundant human chymase$^+$ cells could be identified extensively in the red pulp but not in the white pulp of the spleen of NOG mice (n = 4) by immunochemistry (Figure 8C). These cells did not form clusters in the spleen. As shown in Figure 7D, flow cytometry confirmed the abundant presence of human mast cells as CD203c and Kit double-positive cells (9.8% in human CD45$^+$ cells and 3.3% in all spleen cells).

Discussion

Most of our studies concerning mast cells have used rodent cells. However, rodent and human mast cells show lots of heterogeneities.[34] Their dependence on growth factors is different, and the contents of secretory granules are also different. In particular, both types of human mast cells located in the mucosa and submucosa contain heparin, whereas murine MMCs lack heparin, resulting in their sensitivity to formalin. Thus, rodent mast cells are not always a suitable model for studying mast cells under the physiologic and pathologic condition in humans. The established SCF-dependent human mast cell cultures from HSCs in cord blood,[29,30,35] fetal liver,[31,36] peripheral blood, and bone marrow[37] provide new opportunities for mast cell research. By using in vitro–cultured human mast cells, we have been able to identify several new aspects of human mast cells, for example, the effect of IL-4 on human mast cells.[38-40] We also hypothesized that human mast cells participate in tissue remodeling by releasing fibrosis-induced mediators and cytokines[41] as well as produce enzymes which degenerate the extracellular matrix, known as metalloproteases.[42] However, to study the functional roles and developmental mechanism of mast cells in humans, the establishment of an appropriate in vivo model was needed.

In the study presented here we show for the first time human mast cell development in mice after xenotransplantation. Twenty weeks after xenotransplantation, we noticed the cluster formation of human mast cells, in which sometimes more than 100 human

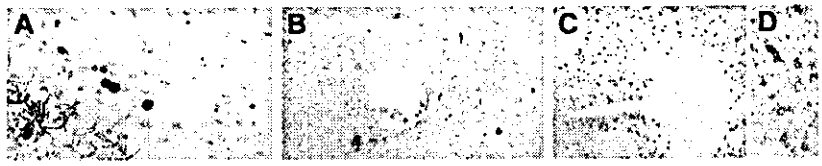

Figure 8. Immunochemical staining for human mast cell-specific chymase in mesentery, lymph nodes, and spleen. (A) In the mesentery, human mast cells formed clusters consisting of 2 to 6 chymase$^+$ cells. (B) In the lymph nodes, chymase$^+$ cells localized around vessels in the trabecula. (C-D) Abundant chymase$^+$ cells were extensively distributed in the red pulp but not in the white pulp of the spleen. Magnification, × 400 (A-B,D) and · 100 (C).

chymase$^+$ mast cells were reconstituted in the dermis of NOG mice. The question arises why human mast cells developed in NOG mice, although those derived from transplanted human HSCs have never been found in other mice. In our opinion, NOG mice have, first of all, a strong capacity for human cell engraftment, as various kinds of blood cells, including T cells, were reconstituted after xenotransplantaion.[21] Second, NOG mice may provide a suitable environment for mast cells, as dramatic mast cell proliferation was observed in the dermis of 20-week-old mice. Mast cell hyperplasia was recently reported in γδ T-cell–deficient mice with NOD background,[43] which means that mast cells in NOG mice may proliferate by being liberated from γδ T-cell regulation. The importance of the environment, which tightly regulates the number of proliferated mast cells, may also be supported by the observation that the number of mast cells in the same size area in NOG mouse skin was almost identical regardless of the origin of the proliferated mast cells.

RT-PCR analysis revealed that murine SCF made a major contribution to human mast cell development in NOG mice. Because we did not administer any human cytokines, the development of human mast cells in NOG mice indicates that SCF alone may be sufficient for human MC_{TC} development. It is also possible that some factors produced from human cells developed in mice from transplanted HSCs are synergistically involved with SCF, because human blood cells, including T cells, were reconstituted in NOG mice. Yet another possibility is that recipient mice supply some factors favorable to human mast cell development, which seems to be supported by the previous finding of an increase of human chymase$^+$ dermal mast cells after healthy human skin plantation onto SCID mice.[44]

In the spleen of NOG mice, we unexpectedly identified abundant human chymase$^+$ mast cell development, indicating that mouse spleen may provide a suitable condition for the development of human connective-tissue-type mast cells from the HSCs or committed precursors. A detailed analysis of the molecular-based mechanisms of the environment provided in the spleen should be very helpful for a better understanding of the requirement for human connective-tissue-type mast cell development.

The development of MMCs in NOG mice is of potential interest, because T-cell–derived cytokines are reportedly important for murine MMC development. Although NOG mice lack murine T cells, we found safranin-negative, Alcian blue$^+$ murine MMCs at almost normal concentrations in gastric mucosa, suggesting that factors supporting murine MMC development are produced not only by T cells.[45] Similarly, the factors required for human MC_T development remain unclear. Although some factors produced by reconstituted human T cells or other human progenies may support MC_T development in NOG mice, another possibility is that SCF is sufficient for mucosal-type mast cell development in humans. In mice, parasite infection induces reactive MMC proliferation depending on the action of IL-3,[46,47] so that parasite infection of NOG mice which have undergone xenotransplantation may provide more information about human MC_T development.

The absence of phenotypically identified mast cells on the smear preparations and CD203c/Kit double-positive cells analyzed by flow cytometry in peripheral blood indicates that human mast cells develop from immature cells without a characteristic phenotype and that their complete differentiation into mature mast cells takes place only after they have migrated to peripheral tissues. This hypothesis seems to be supported by cluster formation of human mast cells in NOG mice. Human mast cell development from contaminated precursors is unlikely, because the time course of human mast cell appearance in NOG mice that received transplants of $lin^-/CD34^+$ cells was not different from mice that received transplants of whole CD34$^+$ cells. Whether immature cells circulating in the peripheral blood are either already committed to becoming mast cells or maintain their capability to differentiate into other lineages than mast cells is an interesting subject for further studies.

In this study, we established for the first time an in vivo model for human mast cell development. Not only human connective tissue-type MC_{TC} but also human mucosal-type MC_T can develop in NOG mice. Our model, thus, provides useful information about human mast cell development from HSCs in vivo. In addition, this model may also pave the way to a potential tool for the in vivo investigation of human mast cell functions.

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Limited Ability of Antigen-Specific Th1 Responses to Inhibit Th2 Cell Development In Vivo[1]

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Th1 and Th2 cells mutually antagonize each other's differentiation. Consequently, allergen-specific Th1 cells are believed to be able to suppress the development of Th2 cells and to prevent the development of atopic disorders. To determine whether a pre-existing Ag-specific Th1 response can affect the development of Th2 cells in vivo, we used an immunization model of Ag-pulsed murine dendritic cell (DC) transfer to induce distinct Th responses. When transferred into naive mice, Ag-pulsed CD8α^+ DCs induced a Th1 response and the production of IgG2a, whereas CD8α^- DCs primed a Th2 response and the production of IgE. In the presence of a pre-existing Ag-specific Th2 environment due to Ag-pulsed CD8α^- DC transfer, CD8α^+ DCs failed to prime Th1 cells. In contrast, CD8α^- DCs could prime a Th2 response in the presence of a pre-existing Ag-specific Th1 environment. Moreover, exogenous IL-4 abolished the Th1-inducing potential of CD8α^+ DCs in vitro, but the addition of IFN-γ did not effectively inhibit the potential of CD8α^- DCs to prime IL-4-producing cells. Thus, Th1 and Th2 cells differ in their potential to inhibit the development of the other. This suggests that the early induction of allergen-specific Th1 cells before allergy sensitization will not prevent the development of atopic disorders. ***The Journal of Immunology,*** **2005, 174: 1325–1331.**

Immune responses to Ags are heterogeneous with respect to the cytokines produced by Ag-specific Th lymphocytes and the class of Abs secreted by B cells. Th1 cells secrete IL-2 and IFN-γ and promote cellular immunity and the production of IgG2a. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and thereby induce the production of IgE and promote eosinophil-mediated inflammation (1, 2). Once the deviation toward either a Th1 or a Th2 response begins, additional polarization is promoted by the cytokines that are produced by Th cells. This is because the Th1 cytokine IFN-γ suppresses Th2 cell proliferation and promotes Th1 cell differentiation, whereas the Th2 cytokines promote additional Th2 differentiation and inhibit the development of Th1 cells (3, 4). Th1 and Th2 responses are thus considered to be mutually exclusive and reciprocally regulated.

Atopy is an immune disorder characterized by hypersensitivity to common, usually innocuous environmental Ags, and its increasing incidence is a major concern of Western and developed societies. Atopic disorders have in common the elevation of allergen-specific IgE Abs and chronic inflammation typified by the predominant recruitment of eosinophils. These characteristics suggest that an inappropriate Th2-biased immune response to allergens is central to the pathogenesis of atopic disorders (2, 5). This together with the mutual antagonism between Th1 and Th2 cells have led to the hypothesis that the increase in the incidence of atopic disorders is linked to a decrease in the prevalence of infections early in life that induce Th1 responses. It is believed that these early infections may remold the normally Th2-biased neonatal immune system into a Th1-biased one that is less susceptible to allergens (6). Supporting this idea are several epidemiological studies that show an inverse relationship between the development of atopy and the incidence of early infections (7, 8). In addition, atopic infants exhibit a slower acquisition of an IFN-γ-producing capacity and a persistent Th2 phenotype compared with nonatopic infants (9). It has also been suggested that the latter are protected from allergic disorders because they have allergen-specific Th1 responses. IFN-γ that is produced by these Th1 cells during an encounter with allergens is believed to be sufficient to suppress the development of allergen-specific Th2 cells (10).

However, there are observations that do not conform to the view that the stimulation of Th1 responses, at least in early childhood, counteracts the development of atopic disorders. First, the incidence of Th1-mediated autoimmune disorders is increasing in parallel with that of Th2-mediated allergic pathologies (11–13). Second, there is a lower prevalence of atopy among persons infected with helminths, which induce strong Th2 responses (14). Third, passive transfer of Ag-specific Th1 cells could not prevent the development of Th2 cells in a murine model of asthma (15).

These contradictions prompted us to investigate the effect of a pre-existing Ag-specific Th1 response on the development of Th2 response in vivo. Several studies have previously shown that the presence of Th1-inducing stimuli and Th1 cytokines inhibit the development of allergen-specific Th2 cells (10, 16). However, these studies were performed using exogenous cytokines and artificial adjuvants; hence, their observations may not reflect what really happens under physiological conditions.

Dendritic cells (DCs)[3] are APCs that initiate primary immune responses by activating naive Th cells (17, 18). In addition to presenting

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[3] Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand.

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antigenic peptides to TCRs and activating naive Th cells, DCs provide an additional signal that induces Th cell polarization and thereby determines the nature of the immune response (19, 20). The in vivo transfer of DCs that have been pulsed in vitro with Ag efficiently primes Ag-specific Th cells (21) and induces strong Ab responses (22). Thus, in vivo DC transfer can be a useful, nonadjuvant-based method to induce Ag-specific immune responses.

Two distinct DC subsets in murine spleen have been characterized with regard to their expression of the CD8α homodimer and the type of Th response they induce. In vivo studies have shown that the adoptive transfer of Ag-pulsed CD8α^+ DCs triggers the development of Th1 cells, whereas CD8α^- DC transfer induces a Th2-type response to soluble protein Ags (23, 24). We also recently reported that each DC subset induces a distinct Ab profile that reflects its ability to prime specific Th responses (25).

In the present work we used the Ag-pulsed DC transfer system to determine whether Ag-specific Th1 cells can be primed in the presence of a Th2 response and whether this can reverse the overall Th phenotype of the immune system. Whether the pre-existence of an Ag-specific Th1 response prevents subsequent Th2 priming was also examined.

Materials and Methods

Animals

BALB/c mice were purchased from Seac Yoshitomi. DO11.10 mice on the BALB/c background, which are transgenic for a TCR recognizing chicken OVA peptide ($OVA_{323-339}$) in the context of the MHC class II molecule I-A^d (26), were a gift from Dr. S. Sakaguchi (Kyoto University, Kyoto, Japan). All mice were maintained in our pathogen-free facility and cared for in accordance with the institutional guidelines for animal welfare.

Reagents

Murine rGM-CSF and rIL-2 were provided by Kirin Brewery and Shionogi Pharmaceutical, respectively. Murine rIFN-γ and rIL-4 were purchased from R&D Systems. OVA and keyhole limpet hemocyanin (KLH) preparations that contain minimum levels of endotoxin were purchased from Seikagaku Kogyo and Calbiochem, respectively.

Preparation of DCs

DCs were prepared as described, but with a minor modification (23). Briefly, spleens of 8- to 12-wk-old BALB/c mice were digested with collagenase D (Roche), filtered through a nylon sieve, and further dissociated in Ca^{2+}-free HBSS containing 10 mM EDTA. The cells were resuspended in HistoDenz solution (Sigma-Aldrich) and separated into low and high density fractions by centrifugation at 1700 × *g* for 15 min. The low density cells were collected and incubated for 90 min in X-VIVO 15 (BioWhittaker) supplemented with 0.5% mouse plasma, 50 μM 2-ME, and 20 ng/ml rGM-CSF. The nonadherent cells were washed off, and the remaining cells were cultured overnight in fresh medium containing 1 mg/ml OVA or KLH. The floating cells were collected, and the CD8α^+ DCs were positively selected using anti-CD8 MicroBeads and autoMACS (Miltenyi Biotec). From the unselected fraction, CD8α^+ DCs were further depleted, and the CD8α^- DCs were positively enriched with anti-CD11c MicroBeads (Miltenyi Biotec). Flow cytometric analysis revealed that the purified fractions contained >96% CD8α^+CD11c$^+$ and CD8α^-CD11c$^+$ cells (data not shown).

Preparation of naive T cells from DO11.10 transgenic mice

CD4$^+$ cells were positively selected from spleen cells of DO11.10 transgenic mice using Dynabeads mouse CD4 and DETACHaBEAD mouse CD4 (Dynal Biotech) according to the manufacturer's instructions. From the enriched CD4$^+$ cells, naive cells were positively selected with anti-CD62 ligand (CD62L) MicroBeads and autoMACS (Miltenyi Biotec).

Immunization protocols

On day 0, 2 × 10^5 OVA-pulsed DCs in 200 μl of PBS were transferred i.v. into 8-wk-old BALB/c mice. A boost of 100 μg of soluble OVA was given i.v. 7 days later, and the mice were killed on day 14. Sera were collected for the measurement of OVA-specific Ab titers, and spleens were removed for secondary stimulation. Alternatively, KLH-pulsed DCs were administered, and the mice were killed on day 14 for secondary stimulation of splenocytes. To assess the influence of pre-existing Th1 or Th2 responses on the subsequent priming of the other Th subset, mice were injected on day −14 with 2 × 10^5 Ag-pulsed DCs or PBS alone, and the other DC subset was given on day 0.

Spleen cell stimulation

On day 14 spleens were removed, and single-cell suspensions were prepared. Cells (2 × 10^6) were cultured with or without the Ag (100 μg/ml) in 48-well plates in X-VIVO 20 (BioWhittaker) supplemented with 0.5% mouse plasma and 50 μM 2-ME. Supernatants were harvested 72 h later and stored at −40°C for subsequent cytokine analysis by ELISA.

In vivo proliferation of naive Th cells induced by each DC subset

Naive DO11.10 Th cells (2 × 10^6) were transferred i.v. into BALB/c mice 1 day before the administration of OVA-pulsed DCs, and the mice were killed on day 14. The total number of splenocytes was counted, and the phenotype of the transferred DO11.10 Th cells was evaluated by labeling the cells with biotin-conjugated anti-clonotype Ab (KJ1-26; Caltag Laboratories), PE-conjugated anti-mouse CD62L (MEL-14; eBioscience), or FITC-conjugated anti-mouse CD44 (IM7; eBioscience), followed by incubation with allophycocyanin-streptavidin (BD Pharmingen). Anti-mouse CD16/CD32 mAb (2.4G2; BD Pharmingen) was used to block nonspecific binding to the FcRs before staining. The samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The numbers of naive and activated DO11.10 Th cells within a spleen were calculated by multiplying the total number of splenocytes by the percentage of KJ1-266-positive cells, then by the percentage of either CD62L^{high} CD44^{low} or CD62L^{low} CD44^{high} cells within the KJ1-26 positive fraction, respectively.

In vitro priming of naive DO11.10 T cells with DC subsets

OVA-pulsed DCs (1 × 10^4 cells/well) and naive DO11.10 T cells (2 × 10^5 cells/well) were suspended in X-VIVO 15 supplemented with 0.5% mouse plasma, 50 μM 2-ME, and 20 ng/ml rGM-CSF, then cultured in 96-well, U-bottom plates (Falcon; BD Biosciences). rIFN-γ or rIL-4 was added in graded concentrations. On days 3, 4, and 6, the cultures were split and expanded in the presence of rIL-2 (35 U/ml). The cells were harvested on day 9, washed extensively, and counted, and the viable cells were tested for cytokine production. T cells (2 × 10^5 cells/well) were restimulated in a 96-well, U-bottom plate with 30-Gy irradiated BALB/c splenocytes (2 × 10^5 cells/well) with or without OVA (100 μg/ml). Forty-eight hours after restimulation, supernatants were collected and stored at −40°C for subsequent analysis.

Determination of Ab titers and cytokine levels by ELISA

Serum levels of OVA-specific Abs were determined as previously described (27). To measure OVA-specific IgE, serum IgE was absorbed to 96-well EIA/RIA plates (Corning Glass) coated with 2 μg/ml anti-mouse IgE (R35-72; BD Pharmingen), and the bound Ab was detected by biotinylated OVA, followed by streptavidin-HRP (BD Pharmingen). To detect OVA-specific IgG1 and IgG2a, the sera were incubated in 96-well plates coated with OVA (50 μg/ml), and the bound Abs were detected by biotin-conjugated anti-mouse IgG1 (A85-1; BD Pharmingen) and IgG2a (R19-15; BD Pharmingen), followed by streptavidin-HRP. The Ab titers were calculated by comparison with internal standards run in each assay. The anti-OVA IgE and IgG2a serum standards were obtained by pooling sera from mice immunized i.p. with OVA and Imject Alum (Pierce). Anti-OVA IgG1 mAb (OVA-14) purchased from Sigma-Aldrich was used as the IgG1 standard. Quantitative ELISAs for IFN-γ and IL-4 in culture supernatants were conducted using OptEIA mouse cytokine sets (BD Pharmingen).

Statistical analysis

Differences between two groups were examined for statistical significance using Student's *t* test for cytokine concentration and the Mann-Whitney *U* test for Ab titers. A value of $p < 0.05$ was considered significant.

Results

Induction of distinct Th and Ab responses by CD8α^+ and CD8α^- DCs

We first evaluated the cytokine and Ab production profiles induced by the transfer of CD8α^+ and CD8α^- DCs. Thus, OVA-pulsed splenic CD8α^+ or CD8α^- DCs were adoptively transferred into

syngeneic BALB/c mice on day 0, and a boost of soluble OVA was given i.v. 7 days later. Mice were killed on day 14, and OVA-specific Ab titers in serum and the cytokine production profile of splenocytes were determined. The transfer of CD8α^+ DCs resulted in a much higher ratio of IFN-γ:IL-4 production by the splenocytes, whereas CD8α^- DC transfer induced Th2 responses with a low IFN-γ:IL-4 ratio (Fig. 1*A*). Moreover, OVA-specific IgG2a was only detected in the mice receiving CD8α^+ DCs, whereas OVA-specific IgE was only detected in mice given CD8α^- DCs (Fig. 1*B*). OVA-specific IgG1 was detected in mice that received either DC subset, although slightly higher levels were detected in mice that were given CD8α^- DCs. These Ab responses were Ag specific, because the injection of KLH-pulsed DCs, followed by a boost of soluble OVA, failed to induce the production of OVA-specific Abs (data not shown). OVA-specific Abs were not detected when mice were injected with OVA-pulsed DCs but did not receive the boost of soluble OVA (data not shown). The induction of distinct Th responses through the transfer of CD8α^+ and CD8α^- DCs was confirmed using KLH as the Ag (Fig. 1*C*).

CD8α^+ DCs fail to prime a Th1 response in an Ag-specific Th2 environment

We next asked whether Ag-specific Th1 cells could be primed in the presence of an Ag-specific Th2 response, thereby skewing the overall Th phenotype of the immune response. Thus, we first introduced OVA-pulsed CD8α^- DCs, then 14 days later we administered OVA-pulsed CD8α^+ DCs. This was followed by a boost with soluble OVA 7 days later, and mice were killed 7 days after this. When splenocytes from these mice were incubated with OVA, they failed to produce IFN-γ and, instead, secreted a substantial amount of IL-4 (Fig. 2*A*). With regard to the serological response, OVA-specific IgG2a were not present in the sera of these

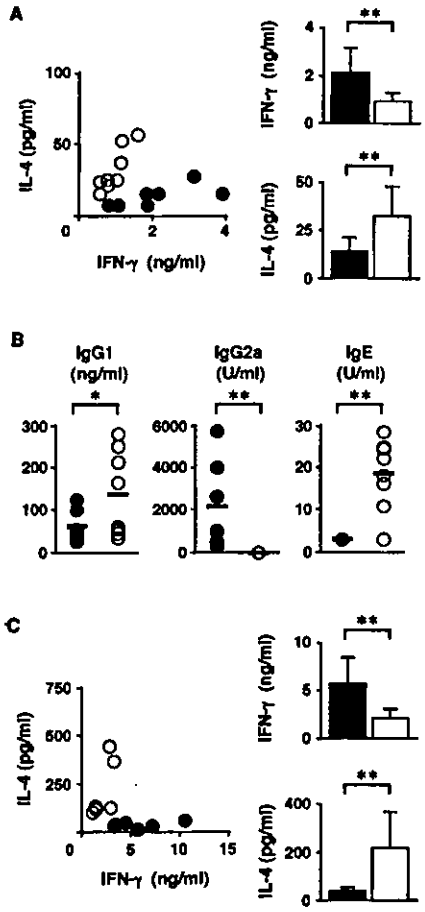

FIGURE 1. The transfer of Ag-pulsed CD8α^+ and CD8α^- DCs induces distinct types of Th and Ab responses. *A* and *B*, Groups of mice were given OVA-pulsed CD8α^+ (n = 7; ●) or CD8α^- (n = 8; ○) DCs and a boost of soluble OVA 7 days later. The mice were killed on day 14, and their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (*A*) and the OVA-specific Ab titers (*B*). *C*, Groups of mice (n = 6) were injected with KLH-pulsed CD8α^+ DCs (●) or CD8α^- DCs (○), and the cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean ± SD of each group. *, $p < 0.05$; **, $p < 0.01$ (by Student's t test for cytokine concentration and Mann-Whitney U test for Ab titers).

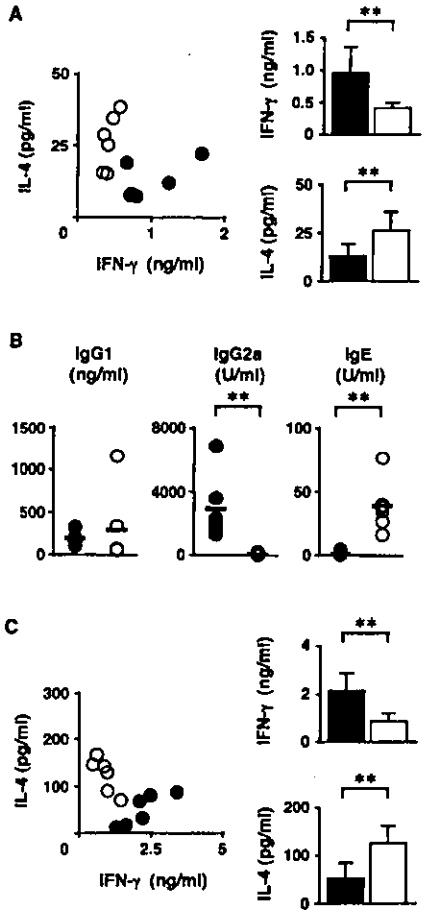

FIGURE 2. CD8α^+ DCs fail to prime Th1 responses in an Ag-specific Th2 environment. *A* and *B*, Groups of mice (n = 6) were injected with 2 × 10^5 OVA-pulsed CD8α^- DCs (○) or PBS (●) on day −14, and 2 × 10^5 OVA-pulsed CD8α^+ DCs were given on day 0. A boost of soluble OVA was given 7 days later, and the mice were killed on day 14. Their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (*A*) and the OVA-specific Ab titers (*B*). *C*, Groups of mice (n = 6) were injected with 2 × 10^5 KLH-pulsed CD8α^- DCs (○) or PBS (●) on day −14, and 2 × 10^5 KLH-pulsed CD8α^+ DCs were given on day 0. The cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean ± SD of each group. *, $p < 0.05$; **, $p < 0.01$ (by Student's t test for cytokine concentration and Mann-Whitney U test for Ab titers).

mice; rather, high levels of OVA-specific IgE were detected (Fig. 2*B*). A similar phenomenon was observed when KLH was used as the Ag in place of OVA; namely, immunization with first KLH-pulsed CD8α⁻ DCs and then KLH-pulsed CD8α⁺ DCs resulted in splenocytes that secreted high levels of IL-4, rather than IFN-γ (Fig. 2*C*).

CD8α⁻ DCs can prime Th2 cells in the presence of an Ag-specific Th1 response

We then assessed whether a pre-existing, Ag-specific Th1 response would inhibit the subsequent development of Th2 cells specific for the same Ag. Thus, OVA-pulsed CD8α⁺ DCs were administered before transfer of OVA-pulsed CD8α⁻ DCs. This resulted in splenocytes that produced IL-4 along with IFN-γ (Fig. 3*A*), namely, a mixed Th1/Th2 response. Moreover, both OVA-specific IgG2a and IgE were generated by this immunization regimen (Fig. 3*B*). When KLH was used as the Ag, both IL-4 and IFN-γ were again produced when the mice had been pretreated with CD8α⁺ DCs (Fig. 3*C*).

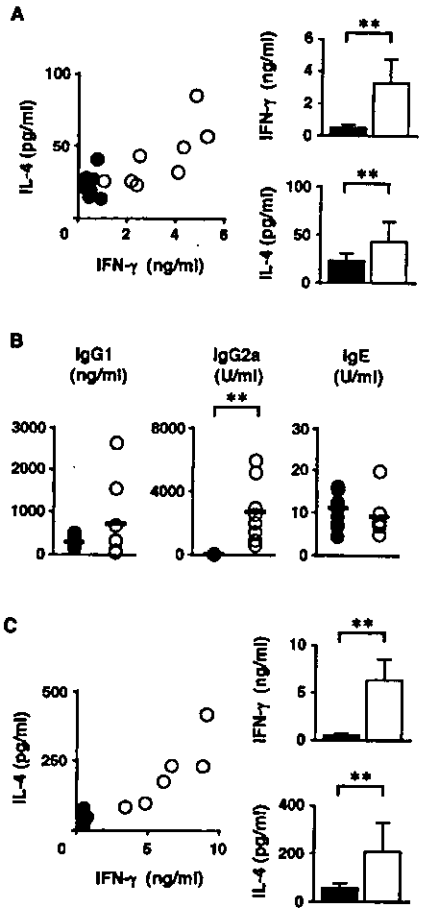

FIGURE 3. CD8α⁻ DCs can prime Th2 cells in the presence of Ag-specific Th1 cells. *A* and *B*, Groups of mice were injected with 2×10^5 OVA-pulsed CD8α⁺ DCs ($n = 8$; ○) or PBS ($n = 11$; ●) on day −14, and 2×10^5 OVA-pulsed CD8α⁻ DCs were given on day 0. A boost of soluble OVA was given 7 days later, and the mice were killed on day 14. Their sera and spleens were harvested and used to determine the cytokine production profile of the splenocytes (*A*) and the OVA-specific Ab titers (*B*). *C*, Groups of mice ($n = 6$) were injected with 2×10^5 KLH-pulsed CD8α⁺ DCs (○) or PBS (●) on day −14, and 2×10^5 KLH-pulsed CD8α⁻ DCs were given on day 0. The cytokine production profile of the splenocytes was determined 14 days later. Each circle represents a single mouse, and the bars show the mean ± SD of each group. *, $p < 0.05$; **, $p < 0.01$ (by Student's *t* test for cytokine concentration and Mann-Whitney *U* test for Ab titers).

CD8α⁺ and CD8α⁻ DCs prime similar numbers of naive Th cells

We speculated that the opposite effects of CD8α⁺ and CD8α⁻ DCs preimmunization on subsequent Th priming by the other DC subset may reflect differences in their potential to prime absolute numbers of naive Th cells. In other words, should CD8α⁻ DCs prime many more Ag-specific Th cells than CD8α⁺ DCs, this might leave fewer Ag-specific naive Th cells after the transfer of CD8α⁻ DCs, which might limit the subsequent Th1 priming induced by CD8α⁺ DC transfer. To evaluate the potential of each DC subset to prime naive Th cells, naive DO11.10 Th cells were transferred into BALB/c mice 1 day before the injection of OVA-pulsed DC subsets, and the phenotype of the transferred Th cells in spleen was analyzed 14 days later. As shown in Fig. 4*A*, most DO11.10 Th cells remained $CD62L^{high}$ $CD44^{low}$ in the absence of DC transfer. However, after the transfer of either type of OVA-pulsed DC, about half the DO11.10 Th cells were activated and had acquired the $CD62L^{low}$ $CD44^{high}$ phenotype (Fig. 4*A*). When the absolute numbers of naive and activated Th cells in spleens were calculated, it became clear that the transfer of either DC subset left similar numbers of DO11.10 Th cells unprimed, and that these cells constituted the majority of the transferred DO11.10 Th cells (Fig. 4*B*). However, the transfer of CD8α⁻ DCs did generate more activated Th cells (Fig. 4*C*). Thus, CD8α⁺ and CD8α⁻ DCs do not differ in their potential to prime naive Th cells, and similar numbers of Ag-specific naive Th cells remain after the transfer of either DC subset. However, CD8α⁻ DC-primed Th2 cells do proliferate more vigorously than CD8α⁺ DC-primed Th1 cells.

Exogenous IL-4 abolishes the CD8α⁺ DC-induced in vitro development of Th1 cells, but IFN-γ fails to suppress the development of Th2 cells induced by CD8α⁻ DCs

It is generally accepted that IFN-γ from Th1 cells inhibits the development of Th2 cells, whereas IL-4 secreted from Th2 cells

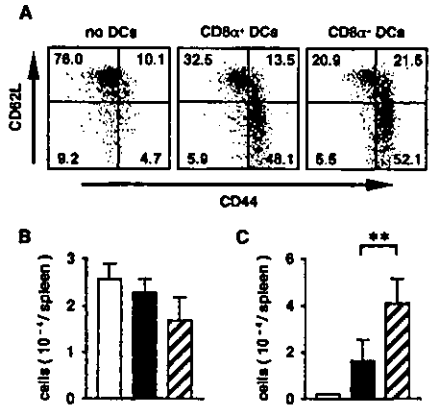

FIGURE 4. CD8α⁺ and CD8α⁻ DCs prime similar numbers of naive Th cells. Naive DO11.10 Th cells were transferred i.v. into BALB/c mice 1 day before the administration of OVA-pulsed CD8α⁺ DCs ($n = 4$; ■), CD8α⁻ DCs ($n = 6$; ▨), or PBS ($n = 3$; □). The mice were killed on day 14, and the phenotype of the $KJ1\text{-}26^+$ cells (*A*) and the absolute numbers of naive (*B*) or activated (*C*) $KJ1\text{-}26^+$ cells in spleens were determined. **, $p < 0.01$ (by Mann-Whitney *U* test).

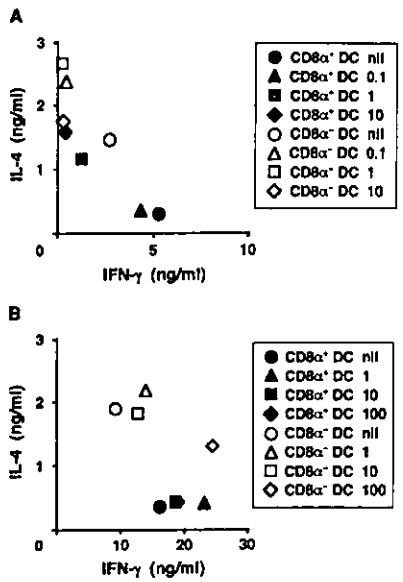

FIGURE 5. Effects of exogenous IL-4 and IFN-γ on in vitro DC-induced Th cell differentiation. Naive DO11.10 T cells were primed with OVA-pulsed CD8α$^+$ (●, ▲, ■, and ◆) or CD8α$^-$ (○, △, □, and ◇) DCs. Graded doses of rIL-4 (*A*) or rIFN-γ (*B*; nanograms per milliliter) were added at the same time. The primed T cells were restimulated 9 days later with 30-Gy irradiated BALB/c splenocytes and OVA, and the culture supernatants were obtained for assaying cytokine concentrations by ELISA. The data shown are representative of three independent experiments, all with similar results.

inhibits the priming of Th1 cells. However, our in vivo data indicate that whereas pre-existing Ag-specific Th2 cells do indeed effectively abolish the subsequent development of Th1 responses (Fig. 2), preprimed Ag-specific Th1 responses cannot suppress the subsequent priming of naive Th cells into the Th2 type (Fig. 3). We next evaluated the effect of adding exogenous IFN-γ and IL-4 to cultures of naive DO11.10 cells during their in vitro priming by CD8α$^+$ and CD8α$^-$ DCs. In the absence of these exogenous cytokines, CD8α$^+$ DCs induced the development of Th1 cells, whereas CD8α$^-$ DCs induced the development of Th2 cells (Fig. 5). The addition of 1 ng/ml rIl-4 to the priming culture abolished the priming of Th1 cells by CD8α$^+$ DCs and induced the development of Th2 cells (Fig. 5*A*). In contrast, as much as 100 ng/ml rIFN-γ added to the priming culture only partially suppressed IL-4 production by CD8α$^-$ DC-primed Th cells, although the production of IFN-γ was increased (Fig. 5*B*). These results suggest that the suppressive effects of IFN-γ on the induction of Th2 cells by CD8α$^-$ DCs are limited, whereas IL-4 can overcome the Th1 priming signal provided by CD8α$^+$ DCs and effectively induce the development of Th2 cells.

Discussion

Many current therapeutic approaches to allergic disorders focus exclusively on the amelioration of symptoms, and few primary measures to prevent the development or overcome the activity of allergen-specific Th2 cells have been proposed. However, advances in our understanding of the Th1/Th2 paradigm have raised the possibility that allergic disorders may be treated or prevented by inducing allergen-specific Th1 responses (10). To elucidate how Ag-specific Th1 and Th2 responses interact in vivo, we used a system of well-defined murine DC subset transfer to induce distinct Th responses. Two distinct DC subsets in murine spleen have been characterized with regard to the expression of the CD8α homodimer. There are about twice as many CD8α$^-$ DCs as CD8α$^+$ DCs (28, 29). The functional properties of each DC subset are not immutably fixed, and many additional factors contribute to their ability to regulate T cell priming (20). In particular, neither CD8α$^+$ nor CD8α$^-$ DCs can induce optimal T cell responses in their immature state, and their maturation is a prerequisite for both DCs to become potent activators of naive T cells. Moreover, although both DC subsets are in an immature state under steady state conditions, they become activated in response to pathogen-derived signals; overnight in vitro culture also activates these cells (30). Using ex vivo Ag-pulsed DCs for immunization eliminates the in vivo contribution of these factors and thus is not a physiological means to induce Ag-specific T cell responses; nevertheless, this method is useful because it obviates the need to administer artificial adjuvants and exogenous cytokines in vivo.

We found that the adoptive transfer of Ag-pulsed CD8α$^+$ DCs induced the development of Th1 responses and the production of IgG2a, whereas CD8α$^-$ DCs primed a Th2 response and induced the production of IgE (Fig. 1). Because IFN-γ induces Ig class switching to IgG2a, and IL-4 enhances the production of IgE (31), our results indicate that in vivo transfer of each DC subset induces a distinct type of Th response, which then leads to a distinct Ab profile after a boost with soluble Ag. This is supported by the fact that OVA-specific Abs were not detected when mice were injected with OVA-pulsed DCs but did not receive a boost of soluble OVA (data not shown). Although the administration of soluble Ag is associated with the induction of tolerance in some circumstances, this is unlikely to have occurred in our experimental system, because boosting with soluble OVA resulted in increased levels of cytokine production by the in vitro splenocyte cultures (data not shown). Notably, substantial amounts of IgG1 Ab, which are generally considered to be associated with a Th2 response, were also produced after the transfer of CD8α$^+$ DCs, although a slightly higher level was induced by CD8α$^-$ DCs (Fig. 1*B*). With regard to this, one study has suggested that the production of IgG1 is not entirely Th2 dependent (32), and both Th1 and Th2 cells have been observed to induce similar levels of IgG1 (33). There was no significant correlation between levels of particular Ab isotypes in the sera and levels of the various cytokines that were produced by the in vitro splenocyte culture, which suggests that the way the Th cytokines function is complex. The ability of each DC subset to induce distinct Th responses was also confirmed in vitro (Fig. 5). Because this culture system was comprised of DCs and Th cells alone, this observation indicates that the two DC subsets issue distinct signals that lead to the differentiation of a particular Th cell phenotype. These observations together indicate that CD8α$^+$ and CD8α$^-$ DCs induce distinct types of Th responses both in vivo and in vitro that effectively support the production of specific Ab isotypes in vivo.

We found that in the presence of an Ag-specific Th2 environment due to the transfer of Ag-pulsed CD8α$^-$ DCs, CD8α$^+$ DCs cannot induce Th1 responses (Fig. 2). However, the reverse is not true, because the Ag-specific Th1 response induced by the transfer of Ag-pulsed CD8α$^+$ DCs cannot effectively suppress subsequent priming of the Th2 response by CD8α$^-$ DCs. Rather, in this situation the production of IL-4 increased along with that of IFN-γ, indicating a mixed Th1/Th2 response (Fig. 3). We showed that these discrepant abilities to block subsequent priming do not arise from the fact that CD8α$^-$ DCs prime so many T cells that too few are left for CD8α$^+$ DCs to prime a sufficient response, because CD8α$^+$ and CD8α$^-$ DCs did not differ significantly in their potential to prime naive Th cells. In other words, similar numbers of Ag-specific naive Th cells remained after the transfer of each DC

subset (Fig. 4*B*). However, there were twice as many CD8α^- DC-primed Th2 cells compared with CD8α^+ DC-primed Th1 cells (Fig. 4*C*), which means that CD8α^- DC-primed Th2 cells proliferate more vigorously than CD8α^+ DC-primed Th1 cells. These findings are in accordance with the observation of an in vitro study that shows that CD8α^+ DCs prime naive Th cells as well as CD8α^- DCs, but then induce the Th cells they activate to undergo Fas-mediated apoptosis (34). One conclusion of these latter observations may be that the failure of CD8α^+ DCs to inhibit subsequent Th2 priming by CD8α^- DCs could be due in part to the inability of CD8α^+ DCs to leave sufficient numbers of Th1 cells in vivo. However, we also found that exogenous IFN-γ did not efficiently inhibit the potential of CD8α^- DCs to prime IL-4-producing cells (Fig. 5*B*), whereas the addition of IL-4 abolished the Th1 priming potential of CD8α^+ DCs in vitro (Fig. 5*A*). Thus, our results indicate that there is a genuine discrepancy between Th1 and Th2 cells in their potential to inhibit the development of the other, because Th2 cells can effectively suppress Th1 development, but not vice versa.

It is generally accepted that IFN-γ produced by Th1 cells and IL-4 produced by Th2 cells act antagonistically in Th cell differentiation (4, 35). IL-4 directly triggers the differentiation of Th2 cells (36, 37) and counteracts Th1 differentiation by down-regulating the expression of the IL-12R β_2-chain on Th cells (38), hence blocking the IL-12 signal transduction pathway. In contrast, IFN-γ suppresses IL-4-induced IL-4R gene expression by mRNA destabilization (39). However, although IL-4 can induce Th2 differentiation even in the presence of IL-12 (40, 41), IFN-γ treatment of early developing Th2 cells does not lead to their reduced IL-4 or increased IFN-γ production (38). Therefore, it is likely that exogenous IFN-γ could not overcome the Th2-inducing signal provided by CD8α^- DCs in the absence of IL-12 (Fig. 5*B*), whereas IL-4 by itself was sufficient to counteract the Th1-inducing signal provided by CD8α^+ DCs (Fig. 5*A*).

Our findings regarding the Th1/Th2 paradigm are important for our understanding of allergy pathogenesis and how we can prevent or treat allergies, because they suggest that allergen-specific Th1 responses cannot be easily primed once allergen-specific Th2 responses have evolved. Moreover, that CD8α^- DCs could induce the production of IL-4 and IgE after Th1 priming with Ag-pulsed CD8α^+ DCs suggests that allergen-specific Th2 responses are likely to be primed even in the presence of allergen-specific Th1 cells, resulting in a mixture of Th1 and Th2 responses. These results are in contrast with studies reporting that a Th1 response inhibits Th2 cell development. Several possibilities may explain this discrepancy. First, we used in vitro Ag-pulsed DCs to induce a distinct type of Th response; these DCs may be different from those involved in infection and allergy. An infection may induce Th1 differentiation more effectively than the administration of CD8α^+ DCs. There is the possibility that a single administration of CD8α^+ DCs could not induce a sufficiently strong Th1 response and that larger numbers or repetitive administration of CD8α^+ DCs may be required to prime enough Th1 cells to suppress the Th2 response induced by CD8α^- DCs. In addition, the administration of DCs at different time points may have altered the outcome. Although these possibilities cannot be excluded, we nevertheless believe that our observations reflect a true facet of the Th1/Th2 paradigm, because CD8α^+ DCs induced a substantial amount of IFN-γ production in splenocyte cultures, which proves the efficacy of Th1 induction to a certain extent. The inhibition of Th1 priming by preimmunization with CD8α^- DCs could be attributed to some type of tolerance induction. Indeed, high levels of IL-10 were produced by the ex vivo splenocyte cultures after the transfer of CD8α^- DCs and by Th cells primed with CD8α^- DCs in vitro (data not shown). Thus, IL-10 produced by Th2 cells after an encounter with CD8α^+ DCs may have inhibited Th1 priming.

It is now believed that the original Th1/Th2 concept regarding allergy pathogenesis was oversimplified. Recent studies report that Th1 as well as Th2 cytokines are up-regulated in atopic patients (42, 43). Although administration of allergen-specific Th1 cells inhibited Th2-cell-mediated asthma in some animal models (44, 45), other studies reported cooperation between allergen-specific Th1 and Th2 cells in the pathogenesis of allergic disorders (46, 47). Moreover, a recent study reported that both Th1 and Th2 type allergen-specific responses were enhanced in atopic children, along with a decreased Th1 response to nonspecific mitogens (48). This suggests the importance of universal Th1 skewing, rather than introducing an allergen-specific Th1 response to prevent allergic disorders. Another report has shown that although the cytokine profiles of allergen-specific Th cells from nonallergic subjects and from subjects who have outgrown the allergy are indeed Th1-skewed, the absolute numbers of these cells are extremely low compared with those in allergic patients (49). Therefore, it seems that the allergen-specific Th1 responses in nonatopic individuals are associated with nonresponsiveness to allergens. Moreover, several studies indicate an association between a Th1-inducing stimulus and the induction of tolerance. For example, intestinal bacterial flora are needed for the induction of oral tolerance (50, 51), and treatment with *Mycobacterium vaccae* generates IL-10-producing regulatory T cells (52). It was also shown that regulatory T cells selectively express TLRs and are activated by LPS (53). These observations indicate that the antiallergic effects of Th1-inducing agents cannot be attributed solely to the priming of allergen-specific Th1 cells; rather, they may involve the induction of tolerance.

In conclusion, when we used a transfer system of well-defined murine DC subsets to induce specific types of Th cells, we found a discrepancy between Th1 and Th2 cells in their potential to inhibit the development of the other. Th1 priming was abolished in the presence of Ag-specific Th2 cells, but Th1 cells could not inhibit subsequent priming of Th2 cells. Our results suggest that the induction of allergen-specific Th1 cells, even before allergy sensitization, will not be able to prevent the development of atopic disorders. Additional research into the complex mechanisms governing the initiation of and protection from allergic disorders is needed before other therapeutic approaches aimed at blocking the development or activity of allergen-specific immune cells can be devised. In particular, it will be important to determine how healthy individuals respond to allergens, because this will, in conjunction with our rapidly expanding understanding of allergy pathogenesis, provide new insights into the immune mechanisms that mediate resistance to allergies.

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第45回日本臨床血液学会総会
教育講演6

先端医療開発に必要なGMP準拠細胞プロセッシング —Institutional GMP構築の必要性—

前 川 平

Key words : Cell therapy, Regenerative medicine, Gene therapy, Institutional GMP

I. はじめに

21世紀に入り爆発的スピードで展開しつつある再生医学の基礎研究と移植治療の進展を高所から鳥瞰すれば，「再生」と「移植」を融合させて構築した先端医療開発のプラットフォームからあたらしい治療法を創成し，その福音を一刻もはやく病める人々に還元することが社会的にも強く要請されていることは自明である。移植治療は臓器移植から細胞移植へと展開しつつあり，再生医学の基礎研究の成果は，少し前までは夢想だにしなかった事実をわれわれに教えてくれている。これらの研究成果を融合させ，わが国で開発したあたらしい治療法を世界に発信するためには，世界的なルールに基づいた細胞プロセッシング・システムを構築することが喫緊の課題である。

臨床試験研究には，科学的，倫理的に高い水準と信頼性が当然要求される。科学性はEvidence-Based Medicineで求められるものであり，倫理性はGCP（Good Clinical Practice：医薬品の臨床試験の実施基準），およびICH（日米欧医薬品規制ハーモナイゼーション国際会議）で担保される。信頼性はこれらを遵守することで保証される。GCPは，ニュールンベルグ綱領，ヘルシンキ宣言，リスボン宣言などを基盤として作り上げられてきたものであり，ICHは優れた医薬品のグローバルな研究開発の促進と承認審査資料の国際的ハーモナイゼーションの必要性を指摘したものである。ICH-GCPを形作るインフラストラクチャーの重要な部分を，GMP（Good Manufacturing Practice：医薬品の製造管理および品質管理に関する基準）およびGLP（Good Laboratory Practice：動物実験での標準操作手順と信頼性保証）が形成している。これらのことから，今後発展して行くと考えられる細胞治療，再生治療，遺伝子治療など，細胞自体を治療に応用しようとする探索的臨床試験研究（トランスレーショナル・リサーチ）にも，GMP準拠の細胞プロセッシングが不可欠であるとされている。もはや，わが国の治療開発研究だけが例外であることは許されないのが世界の趨勢である。細胞をもちいた治療法，すなわち再生治療や多くの遺伝子治療を開発しようとする場合，品質を保証するプロセッシングを受けた細胞を用いることで，探索的臨床試験研究の透明性と説明責任を担保でき，安全性と信頼性が高まり，ひいては患者さんにあたらしい治療法を早く提供できるという意味で医療倫理に直結する。

このように細胞治療（Cell Therapy）の開発には，細胞プロセッシング（Cell Processing）というヒト細胞の調製，培養，加工の工程が不可欠であり，その品質管理についてはGMPを遵守して行わなければならない。したがって，GMP準拠細胞プロセッシングがなければ，あたらしい細胞治療や再生治療の開発はできない。その根底には，安全な輸血療法に通じる考え方が脈々として流れている。

本稿では，第45回日本臨床血液学会総会教育講演で述べた事項を中心に，GMP準拠無菌的細胞プロセッシングについて，とくに先端医療開発，なかでも治療法として確立するかまだ判然としない（＝企業が本格的に参入することがまだ難しい）実験的治療の色彩の濃い，ヒト細胞をもちいた治療開発を行おうとする大学や研究所の目的に特化したGMP，すなわちinstitutional GMP構築の必要性を述べてみたい[1]。なお，具体的なハードに関してはすでに報告しているので参照されたい[2]。

II. わが国におけるGCPの制定と医薬品および治験薬GMP

医薬品GMPは医薬品の製造を厳密な管理のもとで行うための規範であり，米国，EU，日本，その他の国におけるGMPは，ほぼ同じ内容がそれぞれ特有のスタイルによりまとめられている。わが国においては，少なくと

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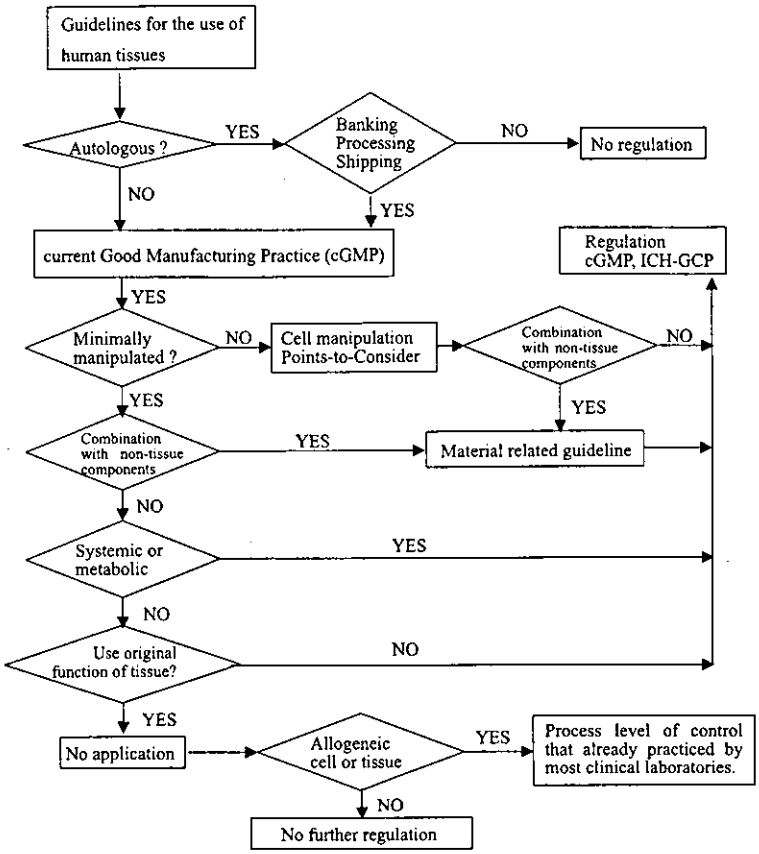

Fig. 1 Regulatory requirements associated with the proposed flow chart of cell and tissue based processing.

も次の3つの要件を満たすことが求められている。第1に「人為的な誤りを最小限にする」こと，第2に「医薬品に対する汚染および品質変化を防止すること」，第3に「高度な品質を保証するシステムを構築する」ことである。これは，現在，医療のリスクマネジメントにも求められていることに相通じるものであり，安全で効率的な輸血治療の実践と同様の事項が要求されているわけである。

医薬品にかかわる治験および市販後臨床試験を科学的かつ適正に実施するための基準として，平成9年3月27日厚生省令第28号で「医薬品の臨床試験の実施に関する省令」，いわゆるGCP省令が公布され，同年4月1日より実施された。GCP省令の第17条第1項において，「治験依頼者は，治験薬の品質の確保のために必要な構造設備を備え，かつ適切な製造管理および品質管理の方法が採られている製造所において製造された治験薬を実施医療期間に交付しなければならない」と定められている。これを受けて，治験薬を製造する際に遵守すべき「適切な製造管理および品質管理の方法の基準」(いわゆる治験薬GMPソフト）および「必要な構造設備の基準」（いわゆる治験薬GMPハード）として定められたものが治験薬GMPである。

A. ヒト細胞・組織医薬品GMPソフト

わが国におけるヒト細胞・組織医薬品GMPソフトは「細胞・組織医薬品等の取り扱いおよび使用に関する基本的考え方（薬務公報第1867号別添1，平成13年2月21日)」および「ヒト由来細胞・組織加工医薬品等の品質および安全性の確保に関する指針（同別添)」として示されている。これらは，平成8年度から10年度にかけて行われた厚生科学研究「組織細胞工学技術をもちいた医療材料・用具の有効性，安全性，品質評価に関する研究」(主任研究者　中村晃忠 国立医薬品食品衛生研究所・療品部長）の報告書[3]および国立医薬品食品衛生研究所報告書に早川堯夫氏が要約している論文[4]などを基に作成されたと思われる。報告の中で示されているフローチャートを改変して，Fig. 1に示す。

では，いったいどのような細胞操作が規制の対象となるのであろうか。米国FDAの考え方では，細胞操作が最低限度（minimally manipulated）である場合は，とくに規制の対象とならないが，それを上回る有意な操作がおこなわれる場合（more than minimally manipulated）は，規制の対象となり承認を必要とするとされている。"Minimally manipulated"の定義は，「操作がその組織の

本来の性質（＝再生や修復の機能をつかさどる能力に関する性質）を損なわない場合」とされている。たとえば，組織の切断，すりつぶし，洗浄，エチレンオキサイドやガンマー線による滅菌，細胞分離，凍結乾燥，凍結保存などは「最低限度の操作」とされている。わが国の実情を考慮して具体的な例をあげると，院内で行う（＝業として行わない）洗浄血小板や洗浄赤血球の作成，骨髄細胞や末梢血幹細胞の分離，凍結操作は「最低限度の操作」と考えられる。しかし，規制の対象にならないからと言って，いい加減で済まされるわけがない。国の承認は必要でなくても，院内で標準作業手順書（SOP: Standard Operating Procedure）を作成し，その操作方法などをしかるべき部署（たとえば輸血部）などにおいて管理するべきである。他方，“more than minimally manipulated”は，細胞を培養したり，骨髄細胞から血管内皮細胞を分離・培養したり，またサイトカインなどをもちいてある細胞分画を増幅させたり，樹状細胞や抗原特異的細胞障害性T細胞の培養，遺伝子導入，膵臓ランゲルハンス島細胞を分離して門脈経由で肝臓に移植したりする場合などがこの範疇に含まれる。当然，将来的にはES細胞をもちいた再生治療も含まれよう。cGMPあるいはcGTPの概念は，c（current）という言葉で表されるように，治療法開発の進展に伴い変わって行くものである。たとえば，CD34陽性細胞を分離して移植を行うことはmore than minimally manipulatedよりもminimally manipulatedとして考えられるようになってきている。

B. 医薬品GMPおよび治験薬GMPからヒト細胞GMPへ

米国では細胞治療，再生治療および遺伝子治療等に用いる治療用細胞の作製は，2001年1月FDAの提言，cGTP（Good Tissue Practice：“Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule”(cGTP) [21 CFR パート 1271][5]に準拠して行われている。わが国では薬務公報第1867号及び厚生労働省告示第101号があり，平成15年7月から発効した改正薬事法・血液新法で“「生物由来製品」については，高度な製造工程管理を必要とすることを踏まえ，製造所における構造設備や製造管理・品質管理の方法（いわゆるGMP）に関し，通常の医薬品・医療機器等における製造基準に加え，厚生労働省令で定める付加的な基準に適合しなければならない”としている。これらに関する下位の法令は平成17年4月に公布予定とされている。その草案をみると，いわゆる錠剤などの医薬品GMPを基にしたもので，生物由来製品と言っても，血漿分画製剤や抗体治療薬などを念頭に置いたものである。錠剤や血漿分画製剤と，治療に用いようとする細胞では，そのプロセッシング方法は大きく異なる。細胞治療の開発にもちいる治療用細胞のプロセッシングに，錠剤などの医薬品GMPの規制を手直ししたとしても，そのまま適応することは難しいと懸念される。繰り返すが，錠剤，液体や蛋白質と，細胞のプロセッシングは当然異なる。

医薬品GMPでは平成11年4月に「薬局等構造設備規則」第8条の2として生物学的製剤などの製造所の製造設備に関する規定が，また平成13年4月には第8条の3としてヒト細胞・組織医薬品の製造所の構造設備に関する規定が追加されている。しかし，最近の細胞治療，再生治療，遺伝子治療などの進展を考慮したヒト細胞治療用GMPのハード面（施設の規格面）を指導する詳細なガイドラインがまだ制定されていないところに大きな問題がある。一方，米国FDAも1996年4月に発表された21 CFR 211のサブパートCのなかで施設のハード面を規定しているが，取り立てて詳細なものではない。“Any such building shall have adequate space for the orderly placement of equipment and materials to prevent mix-up between different components, drug products, and to prevent contamination”と述べるに留まっていたが，2002年9月，米国CBERからpreliminary concept paperとして，“Sterile Drug Products Produced by Aseptic Processing Draft”が発表され，現在パブリックコメントを聴取している最中である。序文には「Poor cGMP conditions at a manufacturing facility can ultimately pose a life threatening health risk to a patient」と明記されている。この中で，無菌的プロセッシング施設が具備すべき機能やその設計基準，および無菌的プロセッシング技術や管理の必要事項が具体的に示されている。これは主に医薬品の無菌的プロセッシングを念頭に置いているものであり，かなり厳しい内容になっているが，多くの事項は無菌的細胞プロセッシングにも適応可能であるように考慮されている。

III. Institutional GMPの必要性

米国の大学や先端医療開発センターで行われている細胞プロセッシングに，FDAはどのようにかかわっているのであろうか？　基礎研究の成果を臨床応用しようとする際，まず研究者や臨床研究医が臨床試験計画書を作成するとともに，それが細胞治療，再生治療，遺伝子治療などにかかわる場合には，細胞プロセッシングに必要な施設の詳細やバリデーション・マスタープラン，品質管理手順書，標準作業手順書（SOP）などGMP準拠細胞プロセッシングに必要な書類をFDAに提出する。FDAはこれらの研究計画に使用される治療用細胞を，IND（Investigational New Drug）として審査を開始するが，同時に施設の査察，およびGMP書類についても指導を行う。この際，FDAの係官は，錠剤などの医薬品GMPと細胞プロセッシングに必要なGMPとの差異を理解して

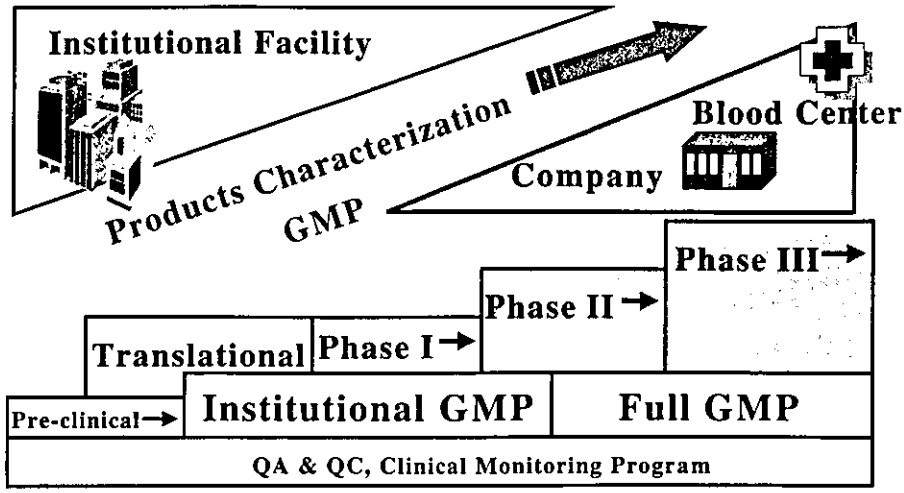

Fig. 2 Stepwise approach: regulatory requirements increase with product development. Institutional GMP should be established to advance translational research in academia.

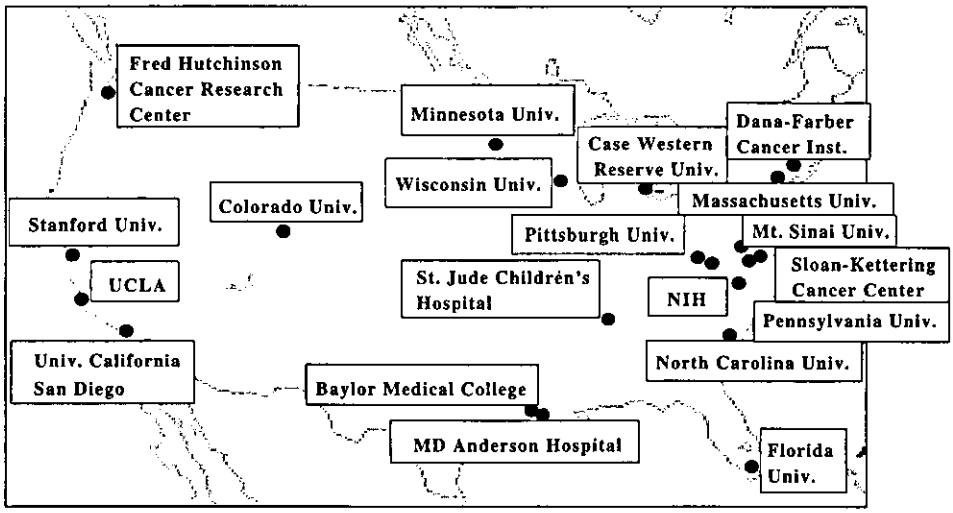

Fig. 3 Cell and molecular therapeutics facilities in USA

おり，企業などに要求される製品を作製するのに必要なGMP（=full GMP）の内容をもとにしつつも，大学などの実情を考慮した実験的治療開発に必要なＧＭＰ（=institutional GMP）の作成に協力を惜しまない。たとえば，膵臓ランゲルハンス島細胞移植に必要な細胞プロセッシングに関しては，FDAの係官が施設を査察するとともに，大学の人的余裕などを考慮し，研究者や臨床研究医とともにGMP構築に必要な書類を点検し，最低限必要な事項を示し，不備な点があれば改善するように指導を行っている。企業などに要求されるfull GMPと大学などに必要とされるinstitutional GMPの大きな違いは，前者では違反や不備があれば製造中止命令が出され罰則を伴うが，後者では改善するように勧告を行う，と言うことである。成文化されたinstitutional GMPがあるわけではないが，このように米国では大学などにおけるあたらしい治療法の開発を積極的に支援すると言うスタンスをとっている。わが国で，大学から厚生労働省にGMP査察を依頼した場合，おそらく人的資源の豊富な製薬企業で錠剤などを商品化するためのfull GMP，しかも細胞プロセッシングとは相容れない事項を要求してくるであろう。FDAが行っているようなinstitutional GMPを考慮した教育的指導が現時点では期待できないわが国では，研究者や臨床研究医自らがそのスタンダードを構築する必要がある。わが国においてinstitutional GMPを構築する必要があると主張する所以である（Fig. 2）。

「そのようなGMPの構築は大学で行うべきことでな

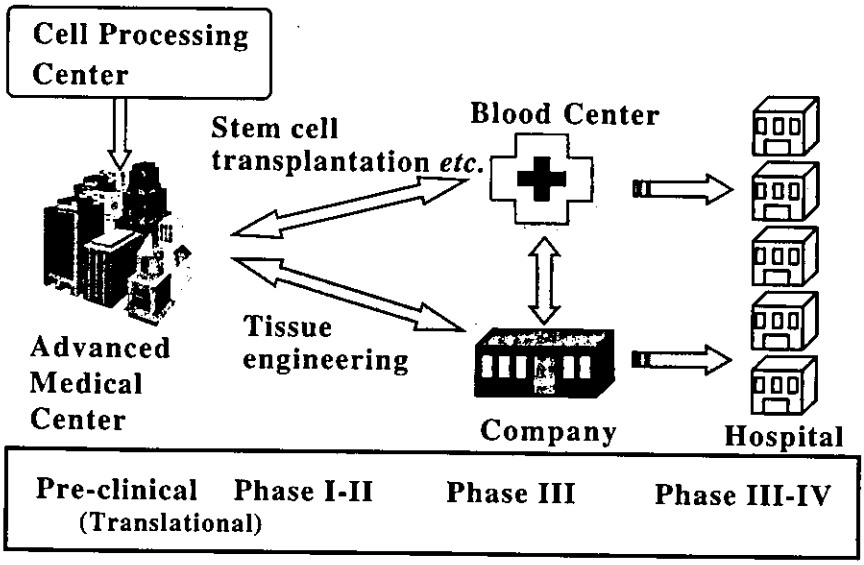

Fig. 4 Future prospects of cell and molecular therapeutic system in Japan

く，企業に任せておけば良い」と言う批判も耳にする。確かに，培養皮膚などすでに臨床応用されているような分野では企業の参入も期待できる。しかし，大学や先端医療センターで開発しようとしているのは，基礎研究の成果をもとにした，まだ実験的医療の段階のものがほとんどである。今後治療法として確立されるかどうか不透明であり，保険適応されるようになるかどうかなどまったく分からないのが現状であろう。このような段階の実験的医療開発の細胞プロセッシングにかかわるGMPの構築に，企業がリスクをとって積極的に参入することはきわめて困難である。ベンチャー企業を育成する土壌が未成熟なわが国においてはなおさらである。加えて，企業の中でも，細胞プロセッシングに必要なGMPのノウハウを確立しているところはきわめて少ない。このような現状では，大学や先端医療センターが主導するかたちで，研究者，臨床研究医，薬剤師，技師，GMPコンサルタント，企業の先端医療開発部門の研究者が一致協力して，世界的ルールにもとづいたものを構築して行く必要がある。

「全国各地の大学に細胞プロセッシングセンターを作る必要があるのか」と言う批判もある。確かに，莫大な費用がかかる細胞プロセッシングセンターを全国のすべての大学に建設する必要はないであろう。米国では，Fig. 3に示すように，先端医療開発を行っている大学や先端医療センターには，細胞プロセッシングセンターを併設しているところが多い。先端医療開発は集約すべきである。臨床応用可能であると言う目処がつけば，企業も参入が容易になるであろうし，また将来的に治療法として確立され，保険適用もされるようになれば，輸血製剤と同様に，全国に78箇所ある血液センターが細胞プロセッシングセンターの役割を担い，各病院へ供給するようになると考えている（Fig. 4）。

「細胞プロセッシングはサイエンスではない。大学や先端医療研究センターで行う必要はない」と言う意見もある。確かに，細胞プロセッシングは基礎研究などのピュアー・サイエンスではない。わが国では，基礎研究の成果を臨床応用しようとしても，そのインフラストラクチャーが不十分であるために欧米の後塵を拝している。基礎研究の成果を社会に還元する「実学」の必要性が認識されるようになり，そのインフラストラクチャーの一部を細胞プロセッシングが占める。科学知を社会に還元することの必要性を主張し，それを行うのが大学や先端医療研究センターであるべきと考えるならば，先端医療開発のための細胞プロセッシングをどこで行うべきかは明白であろう。現在，米国では，この細胞プロセッシングはレギュラトリー・サイエンスとして発展しつつある。

「細胞プロセッシングにGMPは本当に必要なのか」との疑問もよく聞く。すでに述べてきたことであるが，異なった観点からの例をあげる。食品衛生の分野ではHACCP（Hazard Analysis & Critical Control Point）と言う規制があり実施されている。牛乳などのパックには，HACCPに基づいて製品化されている旨が明記されているし，HACCPに基づいて製造されたものでなければ輸出できない。GMPはこのHACCPの根幹を形成している。某学会のランチョンセミナーの弁当で食中毒が発生したのは記憶にあたらしいが，これも衛生管理基準などGMPを形づくるルールに違反があったものと考えられる。

「Full GMPとinstitutional GMPのダブル・スタンダー

Table 1 Necessary requirements for the establishment of GMP cell processing at academic institution (seven elements for institutional GMP)

1. Facility design (completed)
2. Facility management (not yet completed, some parts are on going)
3. GMP documentation (still on going)
4. Educate and train GMP technicians (urgently needed and most important)
5. Close collaboration of clinicians, researchers, technicians, engineers and GMP consultants (necessary condition)
6. Close collaboration of academia, government, and company (necessary condition)
7. Financial support (necessary condition)

ドができることは好ましくない」との意見も聞く。行政側からすれば法令のダブル・スタンダードは好ましくないのは理解できる。米国でもinstitutional GMPが法令化されているわけではない。しかし，上述してきたように大学や先端医療開発センターではinstitutional GMPが必要であり，法令でなく指針として示すべきと考える。

VI. 今，必要なこと

著者が，わが国で最初のGMP準拠細胞プロセッシングセンター計画（東大医科研）にかかわった平成8年には，その建設や設計のノウハウすらなかった。現在では，細胞プロセッシングセンター建設のハード面に関しては，条件が整ってきたと言える。その詳細は厚生労働科学研究費の報告書のなかで述べた[2]。施設建設のハード面はほぼ完成したものの，大学や先端医療センターで行う細胞プロセッシングの構築の中でとくに重要なことは，GMPに関する品質管理などの知識を持った技官の育成である（Table 1）。米国では，これらの技官を育成するためのトレーニング・コースがすでに設けられている。加えて，米国の先端医療開発センターに併設された細胞プロセッシングセンターでは，必ずと言ってよいほど，先端医療開発に関わる臨床研究医（MD）と研究者（PhD），および専門知識をもった技官（PhDであることが多い）が一致協力してその運営にあたっている。

わが国においては，細胞治療・再生治療の先端医療開発に必須の細胞プロセッシングに関する詳細なガイドラインは提示されていない。この原因は，おそらく，実際に先端医療の開発に携わる医師や研究者がその重要性を認識してこなかったことにある。実際，先端医療の開発にかかわる臨床家や臨床研究医の多くは，たとえ細胞プロセッシングの重要性を認識していたとしても，これらは自らの守備範囲や研究範疇ではないし，論文などの業績にはならないとして蔑ろにしてきたと考えられる。あるいは，これらの細胞プロセッシングに関する事項や作業は外部に委託すれば良いと考え，自らこの分野に入ろうとしなかったと考えられる。これらの先端医療開発にかかわる臨床研究医も，今後，細胞治療・再生治療の開発に関する細胞プロセッシングの重要性を十分認識する必要があり，治療技術のひとつとして考えるようにならなければならない。しかも，細胞治療・再生治療の開発に必要な細胞プロセッシング技術の確立は，企業やクリーンルームの建設を請け負う建設会社のエンジニア，GMPやISOの知識を有する専門家や薬剤師，薬学の専門家に任せておけば済むものではない。細胞治療や再生治療開発の細胞プロセッシング技術を我が国において根付かせるためには，これらの専門家とともに，実際の先端医療開発において診療にかかわる臨床研究医が自ら，GMP準拠細胞プロセッシングの必要性を認識し，異分野の研究者と「細胞プロセッシングの重要性」を共通言語として一致協力してルールづくりを行い，そこに厚生労働省の官僚の協力を得ることが必要である。臨床家や臨床研究医は，医学・医療の専門家としての立場から，エンジニアは空調システムなどの設計・建設の専門家としての立場から，薬剤師は薬学の専門家あるいは衛生管理の専門家としての立場から，厚生労働省の官僚は政策などの法制化の専門家としての立場から，すべての人々が「研究成果を社会に還元する」ことの重要性を認識して，同じテーブルについて議論しなければならない。

V. おわりに

わが国でも細胞治療や再生治療開発のためには，細胞プロセッシングセンターの必要性がようやく認識されてきたが，なかには単なる実験室のなかにクリーンベンチを置いて，それで大丈夫だとして同じインキュベーターで何人もの細胞を同時に培養したり，あるいは倫理委員会で承認されたから良しとして，ウシ胎児血清で培養した細胞を投与している施設がある。また，通常の実験室で研究者が作製したリポソームを投与している施設もある。現時点ではこれを規制する法律はわが国にはないが，

だからこそ，安全性や有効性が十分確認されていない先端医療，とくに細胞治療や再生治療で，ヒト細胞を培養したり，遺伝子導入したりといった操作を行う場合は，GMP準拠の規格を有するクリーンルームで，GMPの管理手順に従って行う必要があり，先端医療開発にたずさわるものすべてが遵守しなければならない基本的ルールである。安全性や治療効果もまだわからない実験的探索医療であるからこそ，このような厳格な規制は必要であり，最初からルーズなやり方では取り返しがつかないことになる。規制に従って行い，ここまでは大丈夫だということが明らかになってはじめて徐々に規制を緩和して行く方向に持って行くべきである。

以上，細胞治療，再生治療，遺伝子治療など，先端医療開発のためには，大学や先端医療センターで行う細胞プロセッシングを対象としたinstitutional GMPの具体的なソフトの構築が喫緊の課題であり，厚生労働省，文部科学省の官僚や学識経験者の慧眼に期待したい。

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5) http://www.fda.gov /cber/rules/gtp010801pr.pdf

2．細胞治療・再生治療開発に関するレギュレーションと細胞プロセシング

笠井　泰成・前川　平

細胞治療とは，ヒト細胞を輸注・移植することにより行う治療法の総称である。細胞治療には，細胞プロセシングが必要となるが，これらの工程には医薬品の製造と同じように安全性と高い品質管理が必要である。わが国では，この細胞プロセシングに関する規則の整備が遅れており，細胞治療を含む先端医療の開発を進めるために早急な対応が求められているが，その現状を紹介する。また，細胞プロセシングを行うための施設（細胞プロセシングセンター：CPC）が具備すべき機能やその設計基準，および無菌的プロセシング技術や管理の必要事項などについて述べる。

はじめに

近年，ヒトの身体を構成している細胞や組織を利用して医療に用いる，すなわち細胞治療や再生治療などの研究が盛んに進められている。細胞治療とはヒトの細胞を輸注・移植することによって行う治療法の総称であり，従来から行われている輸血治療を原型とし，造血器幹細胞移植，細胞移入免疫療法，遺伝子治療，再生医療などがこれに含まれる。

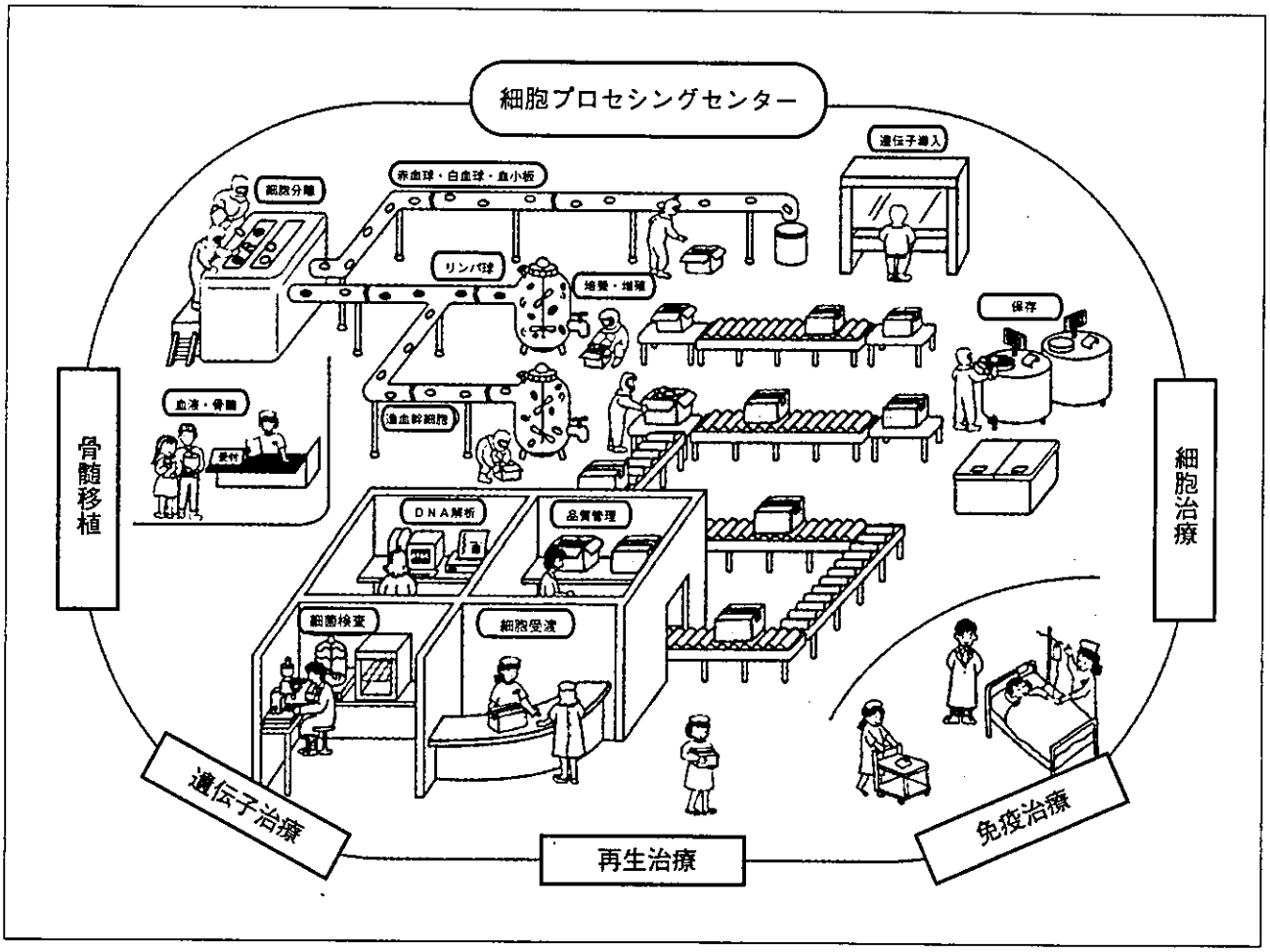
細胞治療には，細胞プロセシング（cell processing）[用語1)]という細胞の調整，培養，加工などの工程が必要となる。細胞プロセシングを受けたヒトの組織や細胞を「細胞医薬品」として治療に応用するためには，これらの作業工程に医薬品や原薬の製造と同等の安全性と高い品質管理が求められていることは容易に理解できる。欧米では，細胞自体を治療に応用しようとする探索的臨床研究（トランスレーショナルリサーチ）にはGMP[用語2)]準拠の細胞プロセシングが必須とされている。細胞治療や再生治療に関する基礎研究の成果を新しい治療法として臨床応用するための臨床研究にはGMPに準拠した細胞プロセシングセンター（CPC：cell processing center）の存在が不可欠である（図❶）。

Ⅰ．GMPと細胞治療

GMPとは医薬品の製造管理および品質管理に関する国際基準である。GMPが世界で初めて施行されたのは1964年で，それまでにスウェーデンや米国などで発生していた医薬品の微生物汚染による事故防止のために医薬品の安全性と品質を確認する規定の検討が1962年から始められていた。わが国では，1980年に法制化され，1997年には「『生物学的製剤等の製造管理及び品質管理基準』及び『生物学的製剤等の製造所の構造設備規準』（生物学的製剤等GMP）について」として薬発第506号が出されている。また，1999年には「医薬品及び医薬部外品の製造管理及び品質管理規則」として

key words
細胞治療（cell therapy），細胞プロセシング（cell processing），探索的臨床研究（translational research），GMP（good manufacturing practice），GTP（good tissue practice），バリデーション（validation）

図❶　GMPに準拠したCPC



厚生省令第16号なども出されている。

GMPは，医薬品の製造を行う際に原料の受け入れから最終製品の出荷に至る全工程について，高い品質を保証するため，一般の品質管理に加え管理組織，製造管理，品質管理，および製造設備の面で種々の方策を定めており，次の3つの基本的な要件を満たさなければならない。第一に「人為的な誤りを最小限とする」こと，第二に「医薬品に対する汚染および品質変化を防止する」こと，第三に「高い品質を保証するシステムを設計する」ことである。設備や技術の両面からの工夫や改善により，これらの3つの要件を遵守することで，医薬品の製造と品質管理のレベルが向上していくと期待できる。

GMPの規制を受ける細胞操作とは，一体どのようなものであろうか。米国FDA（Food and Drug Administration）は，細胞操作が最低限度（minimally manipulated）である場合には特に規制の対象とならないが，それを上まわる有意な操作が行われる場合（more than minimally manipulated）には規制の対象となり，FDAの承認が必要であるとしている。“minimally manipulated”の定義は，「操作がその組織の本来の性質（＝再生や修復の機能をつかさどる能力に関する性能）を損なわない場合」とされている[1]。また，“more than minimally manipulated”は，細胞を培養したり，骨髄細胞から血管内皮細胞を分離・培養したり，またサイトカインなどを用いてある細胞分画を増幅させたり，樹状細胞や抗原特異的細胞傷害性T細胞を培養したり，遺伝子を導入したり，膵臓ランゲルハンス島細胞を分離して門脈経由で肝臓に移植したりする場合などが，この範疇に含まれる。当然，将来的にはES細胞を用いた再生治療も含まれよう。

医薬品に限らず，2003年7月から施行されてい

る改正薬事法[用解3]で定められた「特定生物由来製品」[用解4]や「生物学的製剤等」についても，医薬品と同様の安全性と信頼性が求められるのは当然であろう。輸血製剤はもとより，細胞プロセシングを受ける細胞や組織も，GMPに準拠した製造管理と品質管理が求められるべきである。しかしながら，わが国における規制や関連指針の整備はいまだ不十分である。

Ⅱ．細胞治療に必要なインフラストラクチャー

細胞治療に関する探索的臨床試験研究の開発には，科学的・倫理的に高い水準と信頼性が要求される。こういった細胞治療を行うには，まず細胞プロセシングに適した施設や細胞治療を行うためにGMP基準などのインフラストラクチャーの整備が必要となる。そのうえでGMPに準拠したCPCの構築を進めていく。

米国では治療用ヒト細胞の作製は，2001年1月にFDAが提言したcGTP（Good Tissue Practice : Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products ; Inspection and Enforcement ; Proposed Rule）に準拠して行わねばならないとしている。cGTPは主に細胞治療による感染症の伝播を危惧したものであり，その防止策に関するルールや規制を記載したものである。わが国におけるヒト細胞・組織医薬品GMPは「細胞・組織医薬品等の取り扱いおよび使用に関する基本的考え方（薬務公報第1867号別添1，平成13年2月21日）」および「ヒト由来細胞・組織加工医薬品等の品質および安全性の確保に関する指針（同別添2）」として示されている[2]。さらに，上述した改正薬事法で生物由来製品[用解5]の特性に応じた付加的な基準が設けられ，これらに関連した下位の法令は2005年4月に公布予定とされている。

また，今後わが国の大学や先端医療センターなどで行われる細胞治療や遺伝子治療をはじめとするトランスレーショナルリサーチでは，製薬企業などにおける医薬品製造のためのGMP（Full GMP）とは異なる規制，すなわち大学などの人的余裕などを考慮したInstitutional GMPの構築が必要と思われる。Full GMPとInstitutional GMPの差異を端的に述べるなら，前者ではルール違反があれば製造中止命令が出され罰則を伴うが，後者の場合は改善するように勧告を行い，大学側はこれを受け入れてよりよいシステム作りを行えるよう指導していこうというのが米国FDAの基本的考え方である。米国では先端医療（技術）開発が国家の命運を握ると考えており，基礎研究の成果を積極的に社会へ還元するべきであるというスタンス（国家戦略）をとっている。残念ながら，わが国ではこのようなスタンスをとりたくても，そのためのインフラストラクチャーはまったく未整備といわざるをえない。しかし，日本政府が手をこまねいている間にも欧米では先端医療開発が爆発的なスピードで展開されている。行政側の準備体制が整うのを待っている時間的余裕はない。臨床研究医や研究者自らが一致協力してInstitutional GMPを構築し自主規制を行いつつ，トランスレーショナルリサーチを実施してゆくべきである[3]。

Ⅲ．CPCに求められるもの

米国のCenter for Biologics Evaluation and Research（CBER）からは「Sterile Drug Products Produced by Aseptic Processing」が発表されており，この中で無菌的プロセシング施設が具備すべき機能や設備について必要事項が示されている。この中で要求されている事案も含め，GMPに準拠したCPCに必要な構造とその運用管理について概略を述べる。

1．CPCの設計

作業目的に応じた適切な広さと設備を整えたクリーンルームの配置には，交差汚染や混合防止のための人や物の動線に配慮し設計を行う。特に無菌操作は，明確に区別されたエリア内で行う必要がある。人の移動は逆戻りしない一方向の動線が基本となるが，十分な交差汚染防止の対策がとられていれば利便性を考えた動線計画を考えてもよい。また，物品の移動にはパスボックスの設置が有効だが，室圧が陰圧に設定されているエリアでは逆に清浄度低下の原因ともなる。

作業区域の天井，壁および床の表面は，なめらかでひび割れや隙間がなく，塵や埃がたまりにく