Three distinctive steps in Peyer’s patch formation of murine embryo

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Abstract
Investigation of the process of Peyer’s patch (PP) formation has been hampered by difficulties in identifying its initial step in the embryo. In this study, we overcame this problem by means of whole-mount immunohistochemistry using mAb against the molecules which were expressed in the cells accumulated at the site of PP development. This method was sensitive enough to distinguish a minute cell cluster in the developing gastrointestinal tract. We analyzed the time course of the expression of various surface markers and found that PP formation proceeds through three successive steps. The first is the appearance of a VCAM-1⁺ cell cluster at 15.5 days postcoitus (d.p.c.). Histological examination of the VCAM-1⁺ clusters suggested that VCAM-1⁺ cells represent a stromal component. The second step is characterized by the accumulation of round cells expressing Ia, IL-7R or CD4 at 17.5 d.p.c. Lymphocytes expressing CD3 or B220 were detected only in the final step which started at 18.5 d.p.c. Using any of these markers, the aggregation was initially detected on the upper jejunum and it extended to the colon as the number of clusters increased. At the neonatal stage, the number reached up to eight or nine, irrespective of the antibodies used for the detection. In the aly/aly mutant mouse, where no lymph nodes or PP are found in the adult, none of these three steps was detected. On the other hand, in the SCID mouse that is defective in the formation of mature lymphocytes, the first and second step proceeded, whereas the third step was undetectable. These findings suggest that the progression of each step is indeed regulated by different mechanisms.

Introduction
Peyer’s patches (PP) are important peripheral lymphoid tissues at the fore front of gastrointestinal (GI) defense. Like other peripheral lymphoid tissues, PP consist of macrophages, lymphocytes and supporting stromal components including follicular dendritic cells (1–3). Moreover, this tissue is covered with unique epithelial cells that constitute the follicle-associated epithelium (FAE). M cells, which are components of FAE, can uptake luminal antigens and pass them to the antigen-presenting cells (4,5). In the sheep, ileal PP undergo development to provide a microenvironment that supports B lymphopoiesis (6,7). Except for this, it has been supposed that the major function of the PP is to facilitate the immune reaction against antigenic stimulation in the GI tract. Moreover, it was suggested that PP is the site of preferential class switching to IgA and thereby recruiting IgA-secreting plasma cells to the lamina propria (8,9), the secreted IgA is carried to the GI lumen by the epithelial transporting system. Indeed, mutant mice that have defect in the formation of PP and lymph nodes have impaired IgA production (10–12).

In addition to PP, lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) constitute gut-associated lymphoid tissue (GALT). The aly/aly mutant mouse that lacks lymph nodes and PP still has IEL, indicating the different origins of these lymphocytes in the GALT (13). Consistent with this, it has been demonstrated that distinctive adhesion molecules are involved in lymphocyte homing to PP and intestinal epithelium (14).

Despite increasing understanding of the molecular and cellular mechanisms regulating lymphocyte homing in the adult mouse, little is known how the lymphoid tissues in the GI tract develops. Whereas studies have demonstrated that human PP develop at ~11 weeks of gestation (15,16), PP formation in the rodent is believed to start after birth (17,18). However, Wilders et al. (19) and Mayrhofer et al. (20) reported
the presence of Ia+ cell clusters in the GI tract of 20 and 18 days postcoitus (d.p.c.) rat embryos respectively, suggesting the earlier onset of PP formation. An obvious barrier to study the early development of GALT, particularly PP, is the difficulty in identifying the site of development at the very initial stage, because developing PP occupy only small regions distributed along the entire gut.

To overcome the problem described above, we used whole-mount immunostaining of the entire gut. The results showed that a number of mAb can be used for whole-mount immunostaining of the embryonic gut and that anti-IL-7R or anti-CD4 mAb identified lymphoid cells clustering at a stage when only a single aggregate is present in the entire gut. Based on a series of staining studies, we propose that PP formation proceeds through at least three distinct stages starting with VCAM-1 expression in the stroma at 15.5 d.p.c., followed by the accumulation of IL-7R+ and/or CD4 null-like lymphoid cells and the formation of mature T and B cells.

Methods

Mice
Pregnant mice of C57BL/6 strain and C.B17/Scid Jcl were purchased from Japan SLC (Shizuoka, Japan) and Japan CLEA (Tokyo, Japan) respectively. The aly/aly mutant mice (10) were a kind gift of Drs R. Shinkura and T. Honjo. Female and male mice were mated overnight, and those with vaginal plug were judged pregnant. The noon of the day when the vaginal plug was found was calculated as 0.5 d.p.c.

mAb
mAb against CD45 (30F11.1; PharMingen, San Diego, CA), VCAM-1 (429 MVCAM.A; PharMingen) and CD3 (Y65.372; Seikagaku, Tokyo, Japan) were purchased. mAb against IL-7R (A7R34) (21), CD4 (GK1.5) (22), CD8α (53-6.7) (23), B220 (RA3-6B2) (24), Ia (M5/114.15.2) (25) and Flk-1 (AVAS12α; Kataoka et al., submitted) were purified from hybridoma culture supernatant as described (26).

Immunohistochemistry
Whole-mount immunostaining was performed as previously described (27) with slight modifications. Appropriate paraformaldehyde concentrations and fixation times were verified for each antibody.

Some whole-mount specimens were embedded in polyester wax, sectioned and further stained by other antibodies (28).

Results

IL-7R+ cells populate the developing gut at the early phase of embryogenesis
We first determined the optimal staining conditions for each mAb individually, using whole-mount preparations of neonatal thymus. Even using the same organ, conditions for optimal fixation varied among the mAb tested in this study. The fixation time, concentration of the fixative and dilution of antibodies were determined.

We applied these mAb to whole-mount immunostaining of the gut at various stages of the embryo (Figs 1 and 2). When the 15.5 d.p.c. gut was whole-mount immunostained by anti-CD45, numerous round cells and dendritic cells were detected throughout (Fig. 1A). In the gut of the same stage, IL-7R+ round cells were also detected, although the number was far
Fig. 2. Surface marker expression during PP formation. Whole-mount specimens of intestine were prepared from embryos at various stages and new born mice, and immunostained by mAb indicated in the figure. All pictures were taken at different magnifications.

Fig. 3. Morphological difference between IL-7R$^+$ and VCAM-1$^+$ cells. Intestines from 18.5 d.p.c. embryos were whole-mount stained by mAb against either IL-7R (A) or VCAM-1 (B). Both pictures are with same magnification. IL-7R$^+$ cells are round and small cells, whereas VCAM-1$^+$ cells are large and with an irregular contour.
less than that of CD45\(^+\) cells (Fig. 1B). On the other hand, cells expressing other lymphocyte markers such as CD4, CD8 or CD3 were not detectable. This staining profile, i.e. the presence of IL-7R\(^+\), but not CD4\(^+\), CD8\(^+\) or CD3\(^+\) cells, was found in the GI tract throughout embryogenesis except in the developing PP. Immunostaining of the sectioned 18.5 d.p.c. intestine (Fig. 1C) demonstrated IL-7R\(^+\) cells in the connective tissue below the gut epithelium. No IL-7R\(^+\) cells were found within the epithelial layer. No cluster formation of IL-7R\(^+\) nor Ia\(^+\) cells was found in the 15.5 d.p.c. embryo.

**PP formation in the embryo**

Next, we investigated when cell clustering in the prospective PP region starts in the embryo. As PP are regions of lymphocyte accumulation, appropriate mAb against lymphocyte surface markers should detect cell clustering in the GI tract. Indeed, whole-mount immunostaining of neonatal gut by anti-IL-7R, -CD3, -CD4 or -B220 detected five to nine cell clusters (Fig. 2).

To determine when the first accumulation of lymphoid cells starts, we stained whole-mount specimens of the gut from various embryonic stages (Fig. 2). Among the mAb used for staining, the IL-7R and CD4 are the lymphoid markers that detect cell clustering first, at ~16–17 d.p.c. As Wilders et al. (19) suggested that the accumulation of Ia\(^+\) cells is the earliest step in PP formation of the rat, we stained the embryonic gut with an anti-Ia mAb to examine this notion. We found that Ia\(^+\) cells aggregate almost simultaneously with IL-7R\(^+\) cells at 17.5 d.p.c. The expression of CD3 and B220 in the cell clusters was delayed >24 h.

**VCAM-1 expression characterizes the earliest step of PP formation prior to the appearance of IL-7R\(^+\) clusters**

A histological study of human PP demonstrated the expression of VCAM-1 and ICAM-1 in the stromal and vascular components (16). In fact, Arroyo et al. (29) demonstrated that \(\alpha_\text{v}\) integrin, which binds to VCAM-1 when associated with \(\beta_1\) integrin, is essential for lymphocyte homing to PP. Hence, VCAM-1 should be expressed at the site of prospective PP prior to the accumulation of lymphoid cells and macrophages. As shown in Fig. 2, a positive VCAM-1 cell cluster was detected in the upper intestine at 15.5 d.p.c., >1 day before the clustering of IL-7R\(^+\) cells. To compare the shape of IL-7R\(^+\) and VCAM-1\(^+\) cells, whole-mount stained specimens of 18.5 d.p.c. GI tract were flat-mounted. Virtually all IL-7R\(^+\) cells were small and round (Fig. 3A), while most VCAM-1\(^+\) cells were large and had an irregular contour (Fig. 3B), suggesting that they represent a different cell population. Tissue sections of the anti-VCAM-1 (dark blue) stained gut, which was further immunostained by anti-Flik-1 mAb (brown), showed that a large proportion of cells in the PP region was VCAM-1\(^+\) (Fig. 4A). Consistent with their appearance in the flat-mount specimen, VCAM-1\(^+\) cells were irregular and sometimes elongated. Most, if not all, Flik-1\(^+\) cells in PP as well as other regions that represent vascular endothelium were VCAM-1\(^-\). Intense VCAM-1 expression appeared to be restricted to the stromal component in the PP region, while most vascular endothelial cells were VCAM-1\(^-\) (Fig. 4B). Unlike the well organized array of Flik-1\(^+\) cells in other regions of the gut, the distribution of Flik-1\(^+\) cells within the PP region appeared largely disorganized (Fig. 4A). This suggested that active vascularization is still underway in the PP.

**PP formation progresses from the upper to the lower intestine**

The result in Fig. 2 suggested that PP formation, irrespective of the mAb used for staining, always began as a spot at the upper jejunum near the duodenum. In this section, the earliest PP markers, VCAM-1 and IL-7R, were used to
determine the process of PP extension. As shown in Fig. 5, only two VCAM-1+ spots were detected in the upper intestine of the 16.5 d.p.c. embryo (Fig. 5A), while nine spots were distributed from the upper to the lower intestine on the day of birth (Fig. 5B). In the gut of the 18.5 d.p.c. embryo, IL-7R+ cells were tightly packed in the upper jejunum (Fig. 5C), while those in the ileum and colon were distributed diffusely (Fig. 5D and E).

Analysis of mutant mice
The results in the preceding sections indicated that PP formation proceeds in a stepwise manner, starting from the regional expression of VCAM-1, accumulation of cells marked by the expression of IL-7R, CD4 or Ia and finally appearance of CD3 or B220 positive cells.

To examine whether or not a distinct cell population is responsible for each step, particularly for the second and third steps, we analyzed the expression of each marker in neonatal PP of mutant mice. In the aly/aly mouse which has neither lymph nodes nor PP due to an unknown mutation, no regional cluster formation marked by VCAM-1 or IL-7R was detected (data not shown). This suggested that the aly/aly mouse has a defect from the earliest step of PP formation. Despite the complete absence of VCAM-1+ spots in the GI tract of the aly/aly mouse, however, IL-7R+ cells were widely distributed in the GI tract (data not shown).

On the other hand, both VCAM-1+ and weak IL-7R+ cluster formation was detected in the SCID mouse (Fig. 6A and B), although CD3+ and B220+ cells in the PP region did not accumulate (Fig. 6C and D). So far, we could not detect mice with defects in both the second and the third steps with an intact first step.

Discussion
This study is the first comprehensive analysis of the development of PP by whole-mount immunostaining. Whole-mount immunostaining provides a powerful tool for analyzing the development of lymphoid tissues, particularly PP that
Fig. 6. Presence of VCAM-1 and IL-7R expression in the PP of new born SCID mouse. Whole-mount preparations of intestines from new born SCID mice were stained by mAb against VCAM-1 (A), IL-7R (B), CD3 (C) or B220 (D). Expression of VCAM-1 and IL-7R was detectable in the PP of SCID mice (arrow heads in A and B), whereas no accumulation of CD3+ or B220+ cells was observed (C and D).

are integrated in the GI tract rather than in an independent organ like the spleen or lymph node. Theoretically, serial sections of the entire gut may be able to provide the same information as whole-mount immunostaining. However, whole-mount immunostaining has advantages, in that many samples can be simultaneously processed and it displays a clear view of positive areas. Although its usefulness has already been demonstrated in embryology, no attempts have been made to apply this technique to analyze the immune system. Technically, the most difficult part is to determine the fixation conditions for each organ and antibody, as they vary considerably according to the nature of the epitope. Moreover, difficulties in multicolor staining constitute an obvious limitation of this technique, as compared with flow cytometry. However, as eventually shown in this study, even a single cluster containing a few positively stained cells can be distinguished from the background. Moreover, this technique is sensitive enough to define the shape of single cells. Thus, only an immunohistochemical examination of whole-mount specimens can detect such minute cell aggregates as those found in the initial stage of PP formation. In conclusion, the present study highlights the importance of whole-mount immunostaining in investigations of the development of lymphoid tissues.

We demonstrated for the first time that VCAM-1 expression in the stromal components characterizes the very initial step of PP formation. As there is no reliable means with which to prospectively determine the site of PP, it is difficult to formally rule out the possibility that VCAM-1 is expressed in regions other than the site where lymphocytes appear at a later phase. However, indirect evidence suggests that the VCAM-1+ region in the 15.5 d.p.c. embryo becomes PP. The mode by which VCAM-1+ regions increase is basically the same as that of IL-7R+ clusters, i.e. both appeared first in the upper intestine and the positive spots increase towards the colon until the number of regions reached about eight at the time of birth. Indeed, Coutinho et al. (30) reported that PP first develop in the duodenum of the lactent opossum. Finally, the number of spots of CD3 or B220 expression at the time of birth is almost similar to that of the VCAM-1+ or IL-7R+ spots. As PP are the most prominent sites of lymphoid cell aggregation in the gut, it is more likely that those markers are expressed in the regions where VCAM-1, IL-7R and Ia are expressed.
Given that the clusters containing VCAM-1+ IL-7R+, CD4+ and Ia+ cells represent the very initial phase of PP formation, it is notable that PP formation during embryogenesis occurs in a stepwise manner. The first step is the expression of VCAM-1 in the stromal components at 15.5 d.p.c. Histological analysis of tissue sections from whole-mount stained samples indicated that VCAM-1+ cells are irregularly shaped and probably include mesenchymal cells (Fig. 4). Because we could not detect clustering of CD45+ cells at the 15.5 d.p.c. of embryo, we believe that VCAM-1 is expressed in the stromal cells prior to the clustering of hematopoietic cells. Nevertheless, it is of interest to understand the molecular basis of such restricted VCAM-1 expression. That no VCAM-1+ spot formed in the aly/aly mouse suggests that PP formation in this mouse is defective from the earliest step. Some studies of the lymphotxin-α (LT-α) null mutant mice suggests that LT-α is an essential factor for PP formation, although how it works during the development is not understood (11,12,31). Our results will help to understand the process in which null mutation of the LT-α gene results in the failure of PP formation. Since LT-α is thought to be produced only by the lymphocyte population, it is possible that lymphocytes play an important role for PP formation. Our results indicated that PP formation in SCID mice was detectable on the expressions of VCAM-1+ or IL-7R+ clusters (Fig. 6). Therefore, mature lymphocytes which are absent in SCID mice do not seem to participate in such early PP formation. It is important to clarify whether LT-α participates in each step of PP formation and whether LT-α is produced by immature lymphocytes.

It is interesting that VCAM-1 expression in the stromal cells characterized the first step of PP formation. The ligand of VCAM-1 which is expressed on lymphocytes is α4β7 integrin (VLA-4) (14). Although the null mutation of the α4 integrin gene is embryonic lethal, an injection chimera experiment indicated α4 integrins are essential for T cell homing to PP (29). On the other hand, the β7 null mouse is viable and have lymphocytes in PP, although the number is decreased (32). This suggested that α4β7, which binds to MAdCAM-1 on mucosal high endothelial venules (HEV), may account for only a part of α4-dependent homing to PP, and α4β1 integrin may contribute to the rest of the α4-dependent process of PP formation. Our result collaborates well with these studies and further indicate that the VCAM-1/α4β1-dependent process starts from the earliest step of PP formation.

The second step is the accumulation of blood cell lineages. IL-7R+, CD4+ and Ia+ cells are found in the initial cluster. However, we did not detect CD3+ nor B220+ cells, although they are found in the thymus or fetal liver of the same stage. Thus, it is yet to be determined if those cells expressing IL-7R and CD4 represent lymphocytes or other blood cell lineages. Spencer et al. (15) reported that in humans at 11 weeks of gestation, clusters of cells expressing CD4 are present in the fetal ileum, but they do not express CD3. They suggested that CD4+ cells may represent macrophages, because a portion of human macrophages can express CD4. However, as CD4 expression in murine macrophages has not yet been detected, this possibility is unlikely in the mouse. Thus, these cells might be better referred to as null-like cells at this moment.

The third wave of PP formation is represented by the accumulation of lymphocytes expressing CD3 or B220, which starts immediately prior to birth. The fact that this step was undetectable in the SCID mouse supports the notion that this is due to the entry of mature lymphocytes or maturation of extant cells in the clusters. As mature lymphocytes are already present in the 17 d.p.c. embryo, we prefer the former notion that PP are not ready to accept circulating mature lymphocytes. We found that many Flk-1+ cells in the PP are not yet organized to the vascular lining. Thus, it is likely that formation of the vascular network in PP is completed rather late. Considering that mature lymphocytes home through the HEV, we speculate that development of a functional vascular system that can allow homing of mature lymphocytes is formed after 18 d.p.c. Conversely, this indicates that cell accumulation at the second step is independent of the development of this vascular system.

Taken together, PP formation occurs during the embryonic stage before antigen stimulation, and the entry of lymphoid cells and macrophages into this region proceeds in a stepwise manner. We are considering the sequence in which the regional induction of a stromal cell component initiates the process, by which lymphocytes and macrophages scattered in the mesenchyme of the GI tract accumulate, probably upon development of the lymphatic systems. Subsequently, the specialized vascular system, like HEV, develops to allow the entry of circulating lymphocytes. Consistent with this notion, one investigator has proposed an initial sequence for lymph node formation in the rat. According to this model, condensation of mesenchymal cells around the popliteal vein, that occurs at 17 d.p.c., induces coalescence and subsequent indentation of pre-existing lymph vessels to form lymph capsules (33). In order to dissect the initial step for the formation of lymphatic organs, however, markers that can distinguish the endothelium of lymphatic, venous and arterial vessels are essential. Efforts to develop such markers are currently in progress.

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Abbreviations
d.p.c. days postcoitus
FAE follicle-associated epithelium
GALT gut-associated lymphoid tissue
GI gastrointestinal
HEV high endothelial venules
IEL intraepithelial lymphocytes
LPL lamina propria lymphocytes
LT-α lymphotxin-α
PP Peyer’s patch
SCID severe combined immunodeficient mouse

References
Peyer’s patch formation of embryo


