Chapter

Pathology of Intestinal Transplantation: Rejection and a Case of Tolerance

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Abstract

Small bowel transplants are less common than other organ transplants. Histological criteria for rejection of the transplanted small intestine were proposed at the 8th International Symposium on Small Intestinal Transplantation 2003-2004. The Banff Conference on Transplant Disease Pathology, an international conference on the rejection of small bowel transplants, was held in 2019, and unifying diagnostic criteria were discussed (https://banfffoundation.org/pittsburgh-2019/). These histological criteria are expected to be standardized in the near future. This review outlines new findings such as apoptosis and apoptotic-body phagocytic findings in the lamina propria and behavior of natural killer T (NKT) cells, in addition to previously known crypt Fas-related apoptosis in acute cellular rejection. Furthermore, we review the case of a recipient who has shown no rejection for 5 years after transplantation. In the transplanted small intestine of this patient, the lymphocytes were replaced by those of another male patient.

Keywords: intestinal transplantation, histology, rejection, natural killer T cells, apoptosis, tolerance

1. Introduction

1.1 Current status of small bowel transplantation

Small bowel transplantation (SBT) is one of the standard treatments for patients who are unable to consume a regular diet and have complications from the irreversible requirement of parenteral nutrition [1]. Hirschsprung's disease [2, 3] and Crohn's disease [4] patients are two examples. Recent effective immunosuppressive drugs, well-controlled postoperative care, and advances in diagnostic techniques have significantly improved the outcome of SBT [5]. Immunosuppressants such as mycophenolate mofetil, tacrolimus, and steroids, are routinely used for long-term management after transplantation [6, 7].

Acute cellular rejection (ACR) is a major cause of impaired colonization by the transplanted small intestine, and it frequently accompanies chronic and irreversible changes such as ulcers and lamina propria fibrosis. ACR has remained a risk factor that impedes functional recovery of the intestinal graft [1, 8, 9]. On the other hand, pathologists frequently encounter various pathologies of the intestinal allograft [10–12]. For example, mechanical failure of the graft due to operation during

surgery may occur during the early phase after transplantation. Cytomegalovirus (CMV) enteritis and Epstein–Barr virus -related enteritis are severe side effects and post-transplantation lymphoproliferative disorders/diseases [13–15]. It is often difficult to make a differential diagnosis of the ACR findings. However, histologic diagnosis is critical for the selection of immunosuppressants and their respective doses. Tacrolimus, cyclosporin, and steroids are commonly prescribed in the early stages of rejection [16]. If an excessive dose is administered, the occurrences of CMV enteritis and EBV enteritis become inevitable.

Among the various histological features, crypt epithelial cell apoptosis has been evaluated as a highly reproducible finding. However, other histological findings have been proposed at different institutions. We have also previously suggested other findings as indicators of ACR [17–19].

2. Diagnostic criteria for ACR

2.1 Crypt apoptosis

Crypt apoptosis is considered a unique feature of ACR in SBT. The crypt is an architectural element that is located at the base of the villous epithelium and serves as the source of mucosal cells. Paneth cells, stem cells, and reserve stem cells are included in the crypt. Enterocytes are differentiated from reserve stem cells in the crypt and migrate to the tips of villi through the transit amplifying zone [20]. The kinetics of differentiation and loss of enterocytes contribute to the maintenance of quick renewal for mucosal homeostasis. The supply of enterocytes becomes interrupted by apoptosis in the crypt, and the shortening of villi becomes unavoidable. When ulceration occurs, the lesion is susceptible to infectious enteritis such as CMV- and EBV-related enteritis, for a significant period of time [21, 22].

Pathologically, the diagnosis of small bowel transplant rejection is based on the appearance of 6 or more apoptotic lesions per ten crypts [3, 4] (**Table 1**). The detection of crypt apoptosis is commonly used because of its high reproducibility. Nevertheless, discussions about the number of lesions per crypt were held at the Banff Conference 2019. In the case we experienced, if more than six apoptotic cells were detected in the crypts, subsequent ulceration is inevitable, and infection from the ulcer site might occur. Therefore, we considered that immunosuppressants should be administered when apoptotic cells were observed in the crypt [18].

Previous apoptosis findings have shown that the cells are eosinophilic with an intensely stained nucleus [17, 18] (**Figure 1**). Cells with lobulated nuclei, such as neutrophils and apoptotic cells, can be confused morphologically; therefore, careful observation is necessary. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining is one method to avoid this confusion. This staining procedure involves an enzyme-mediated reaction. First, the fragmented DNA is labeled with biotin containing terminal deoxynucleotidyl transferase. The labeled DNA then reacts with streptavidin for staining. Both labels with 3,3′-diaminobenzidine (DAB) and fluorescein isothiocyanate (FITC) are available for visualization of the apoptotic body [18].

As apoptosis progresses, fragmented cell debris (apoptotic bodies) are observed in or around the crypt. Increasing the dose of the immunosuppressive drug suppresses the progression of apoptosis. Therefore, quick detection of apoptosis is critical for effective immunosuppression therapy [18, 19].

The factors that cause such apoptotic responses in the crypt and lamina propria are poorly understood. It is possible that cytotoxic T lymphocytes (CTLs) can directly

Histologic grade			
Indeterminate	Crypt apoptosis and related findings	Lymphocytic apoptosis in the lamina propria [18]	
	Up to 6 apoptotic bodies per 10 crypts	None	
Mild	>6 apoptotic bodies per 10 crypts Confluent apoptosis	Isloated apoptotic bodies in the lamina propria	Phagocytosis of apoptotic bodies by macrophages [18]
Moderate	Increased inflammation, epithelial injury	A few apoptotic body cluster in the lamina propria	Aggregation of macrophages [18]
Severe/exfoliative	Mucosal ulceration	Apoptotic bodies aggregate in the lamina propria	Granuloma consisting of macrophages

Table 1.
Histological criteria for ACR of the intestinal allograft [10]. The findings under the lymphocyte and macrophage categories refer to our previous study [18].

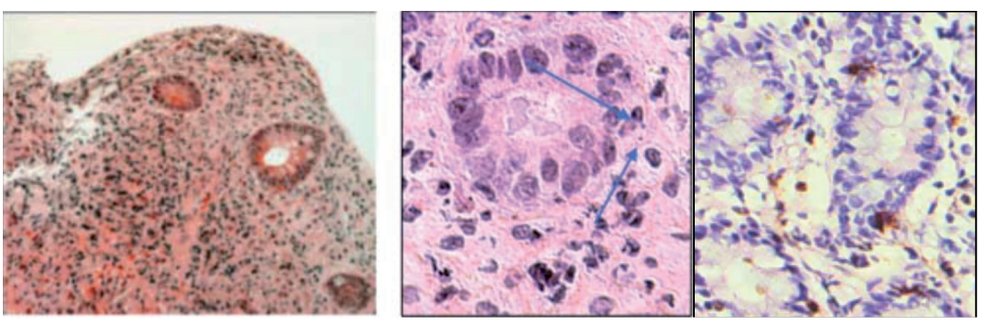

Figure 1.
Histology of ACR of the intestinal allograft. The onset of ACR. Eosinophil infiltrates are observed in the ulcerated mucosa (left, 100×). Apoptotic bodies are observed in the crypt (indicated by arrows, middle, 200×; right, 100×, TUNEL-stained with 3,3'-diaminobenzidine).

attack the crypt of the graft. However, it is not always histologically evident that CTLs directly infiltrate near the crypt and remain near this area. There is also a noteworthy research report suggesting that CD8-positive CTLs are not always involved in ACR [23]. At the basic research level, rejection of the apoptosis-inducing factors perforin and granzyme B released from CTLs has been reported [24]. Therefore, the destruction of the mucosal immune system by local increases in complement and inflammatory cytokines is thought to be the cause of apoptosis.

2.2 Immunohistochemical monitoring

In addition to the crypt apoptosis, apoptotic lymphocytes are identified by systematic immunostaining of lymphocyte surface antigens: T cell surface antigens CD3, CD4, and CD8; B cell surface antigens CD20 and CD79a; natural killer cell surface antigen CD56; and activated lymphocytes Fas and its ligand (FasL) [25]. FasL, also known as CD95L, is a surface antigen of activated cytotoxic T cells and NK cells are observed at the onset of rejection [18] (**Figure 2**, upper panels).

Apoptotic bodies are also been observed in the lamina propria and Peyer's patch (PP) distant from the crypt, and the macrophages that phagocytose them often aggregate to present granuloma-like findings. Notably, these bodies are stained with

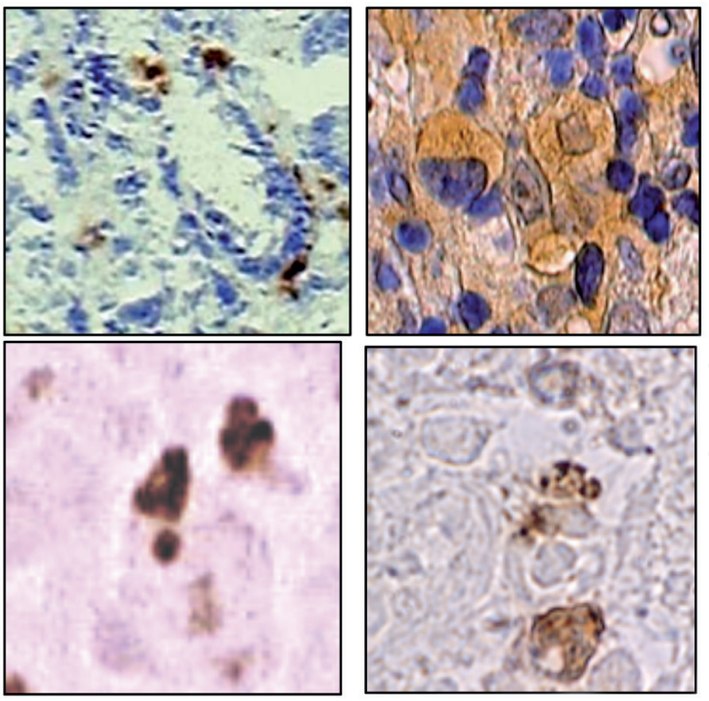

Figure 2.
FasL immunostaining of the intestinal allograft. FasL-positive lymphocytes in the lamina propria (upper left, 200×) and Peyer's patch (upper right, 400×) are shown. FasL-stained apoptotic bodies (lower left, 400×). Apoptotic TCRVα24 stained cells (lower right, 400×). TCRVα24 and FasL were visualized with DAB (3,3'-diaminobenzidine).

FasL and Fas, suggesting that the apoptosis relates to the FasL-Fas interactive reaction (**Figure 2**, lower panels). This result was first reported in our previous study [18].

3. Endoscopic examination and Peyer's patch response

Endoscopically, elevation of the small intestinal mucosa may be recognized and biopsied when clinical rejection is suspected. Since this elevation is observed in patients who are not receiving oral nutrition, the change may not be the result of irritation from the lumen of the small intestine and more likely due to the reaction of the Peyer's patches (PPs) to a load of patient cells on the graft mucosal immune system. In our cases, the biopsied Peyer's patches were injured at the onset of ACR (**Figures 3A** and **B**). Therefore, PP is one of the targets of ACR or other types of rejection (**Figure 3C**). Notably, B cells increased in number in the disintegrated PPs (**Figure 3**). As described later, IL-5 was increased in the intestinal allograft [17], which may promote the transient B cell growth in PP.

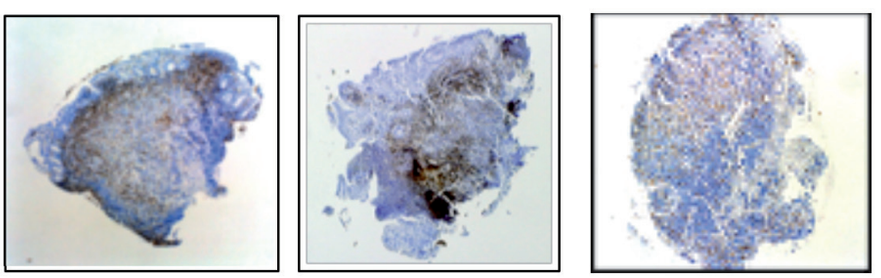

Figure 3.
Histology of a PP in an intestinal allograft. (A, B) A hyperplastic Peyer's patch stained with CD79a antibody before ACR (A) and at the onset of ACR (B). (C) CD8 staining of PP after 42 h at the onset of rejection. Many CD8+ CTLs infiltrate in PP. CD79 and CD8 were visualized by DAB. The photo magnitude is 100×.

4. Cases at Kyoto University Hospital

Here we review cases of SBT at Kyoto University Hospital [17, 18, 21, 22]. SBT was performed owing to intestinal malrotation and Hirschsprung's disease-related effects (**Figure 4**).

Jejunal or ileal grafts were monitored histologically. When fever, increased intestinal juice, abdominal pain, or C-reactive protein (CRP) elevation in peripheral blood (>0.5 mg/10^{-1} L) was observed, an endoscopic examination was performed. In particular, for the first 1 to 2 weeks after surgery, the examination was performed every other day, and a histological examination was also performed. Once the condition of the patient became stable, a histological examination was performed approximately once a week, and the state of the intestinal graft was monitored continuously for up to 2 months in the hospital. The patient received immunosuppressive therapy in combination with tacrolimus (trough concentration: 20 ng/mL) and methylprednisolone (30 mg/kg/day, 1 to 3 times). In the biopsy examination, diagnosis by hematoxylin and eosin staining and findings specific to rejection within 6 h were confirmed by immunostaining of frozen sections. For histological diagnosis, we stained the apoptosis-related proteins such as FasL and surface antigens of B cells, T cells, and NK cells in each case. Steroid pulse therapy was conducted following

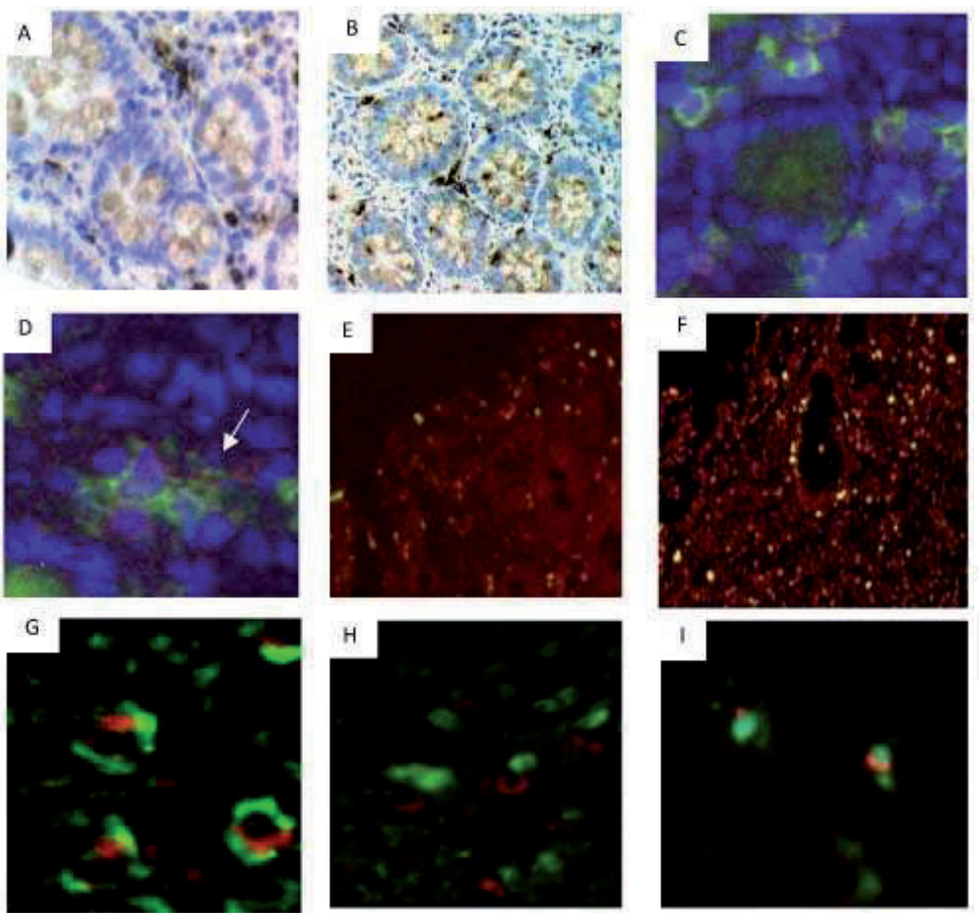

Figure 4.
Immunofluorescent staining of natural killer T cells in the intestinal allograft. Immunostaining of an intestinal allograft. Green signal, FITC and red signal, phycoerythrin [PE]. Nuclei are stained with DAPI (blue). Brown signal was visualized with DAB. (A) TCRVα24 (200×) and (B) TCRβ11 (200×). (C, D) TCRVα24 (green) and IL-4 (red) (IL-4 positive iNKT is indicated by an arrow). The observation magnification is 200× in both cases. (E, F) TCRVα24 (red) and TUNEL (green). (E) TUNEL+ (apoptotic) TCRVα24 + iNKT cells are observed at the onset of ACR (100×) and (F) 48 h after the onset of ACR (100×). Doubly stained cells were increased 48 h after the onset of ACR. (G) $CD1d^{+}$ dendritic cells. CD1d and CD11c were stained green and red, respectively. (H) TCRVα24 stained iNKT cells (red) and CD1d stained dendritic cells (green). (I) FasL+ (green) TCRVα24+ (red) iNKT cells. The observation magnification is 400 x in (G)-(I)."

detecting the immunological activation with the appearance of FasL-positive T/NKT cells and apoptotic bodies in the lamina propria. The treatment substantially prevented the progression of the crypt apoptosis [17, 18, 21, 22].

5. Cytokine production in the intestinal allograft

5.1 NKT cells and cytokines

NKT cells are resident in the large bowel and increase in number in the colorectal cancer tissue [26]. The NKT cells have a limited T cell repertoire, and the restricted types are called invariant types of NKT (iNKT) cells. During the onset of intestinal rejection, the α chain 24 (TCRVα24) and β chain 11 (TCRVβ11) on iNKT cells are positively stained (**Figure 4A** and **B**) [17]. iNKT cells are mainly involved in innate immunity against glycolipids with the assistance of CD1d + dendritic cells [27]. Since iNKT cells are not identified in the small intestine of healthy donors before transplantation, this finding to be an indicator of ACR [17, 28].

Th1 cytokines, such as interferon-gamma (IFN-γ), generally act on the differentiation of CTLs, which promote rejection, while Th2 cytokines may suppress ACR of SBT. TCRVα24 (+) invariant NKT (iNKT) cells are positive for interleukin 4 (IL-4) in allografts of the intestine during rejection (**Figure 4C** and **D**) [8]. The apoptosis of iNKT cells are observed at the onset of rejection (**Figure 4E** and **F**), indicating that a part of apoptotic cells in the lamina propria are iNKT cells (**Figure 2**, lower right). CD1d+ dendritic cells are detected during the rejection process at the same time that the rejection progressed (**Figure 4G** and **H**). The involvement of iNKT cells in the rejection reaction has been discussed previously, and there is also an experimental report regarding their involvement in tolerance [29, 30]. However, the involvement of iNKT cells in rejection has not yet become apparent [31]. Furthermore, the mechanism by which the expression of IL-4 is directly involved in mucosal immune regulation remains unclear. However, IL-4 may suppress the action of CTLs that cause rejection. On the other hand, iNKT cells expressed FasL, indicating that they are activated in ACR (**Figure 4I**).

In addition, increased IL-5 production is also observed at the onset of rejection. IL-5 promotes eosinophil differentiation and chemotaxis [32]. This increase in production may explain the large number of eosinophils infiltrating the mucosa at the time of rejection [17]. Conventional T cells and iNKT cells may secrete IL-5 [17]. The role of eosinophils in rejection has often been debated [33] and there is a discussion on whether eosinophils may be the target of rejection therapy [34]. An increase in the rejection of eosinophils has also been reported in the transplanted liver [35]. In the small intestine, the presence of the mucosal immune system may further complicate the graft's immunological environment. Increased eosinophils, however, are histologically detectable and may provide useful information for the diagnosis of rejection, even in small bowel transplant grafts [5]. As a result of an imbalance in mucosal immunity, excess production of IL-4 and IL-5 may damage the mucosal epithelium. The administration of immunosuppressive drugs acts on iNKT cells in addition to cytotoxic T cells. Therefore, the distribution of immunocompetent lymphocytes in the mucosa is disturbed, and the treatment protocol should be developed further.

6. Histological tolerance of the intestinal allograft

Finally, we reviewed a case of histological tolerance reported [25]. This case involves a transplant in a 4-year-old male patient who had short bowel syndrome

and previously underwent a living small bowel transplant from his mother who was in her twenties. The patient underwent a small intestinal biopsy 2–3 times per week for one month. Immunological analysis was performed using CD3, CD4, CD8, CD20, CD56, CD79a, perforin, granzyme B, FasL, Fas, and TUNEL staining. No severe rejection with an increase in FasL-positive T cells was detected. The maximum level of CRP, an inflammation marker, was 1.0 ($mg/10^{-1}$ L) at POD67. In situ hybridization was performed using a Y-chromosome probe to evaluate rejection or tolerance for evaluation of the immunologic stability of the graft and chimerization [36], which comprises multiplex staining with a CD3 fluorescent substance, for monitoring allografts. **Figure 5** shows photographs of the graft 5 years after transplantation. A part of native T lymphocytes were replaced with Y-chromosome positive T lymphocytes from a male patient. This patient has been living for longer than ten years without any clinical symptoms, such as rejection, and is likely one of the first cases of operational tolerance.

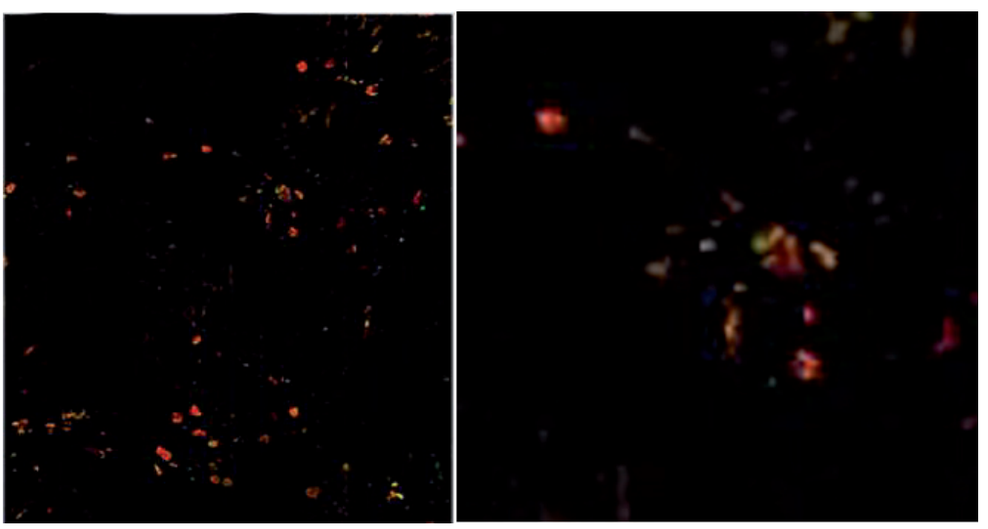

Figure 5.
Combined in situ hybridization of lymphocytes with the Y-chromosome probe (red: PE) and CD3-lymphocytic immunohistochemistry (green: FITC). The photos show the double-stained T cells carrying the Y-chromosomal investigation, indicating the male-donor derived lymphocytes in the female-derived intestinal allograft. Left (100×) and right (400×). The nuclei were stained red, indicating Y-chromosome positivity.

7. Conclusion

Early diagnosis of rejection of the transplanted small intestine is essential to facilitate the initiation of therapy that interferes with rejection progression. In addition to crypt apoptosis, apoptotic bodies in the lamina propria is considered useful for diagnosis. Furthermore, iNKT cell infiltration was another characteristic finding. Since histologic features of ACR have been studied extensively. Of note in future diagnoses are the issues of humoral and chronic rejection.

Appendices and nomenclature

ACR	acute cellular rejection
CMV	cytomegalovirus
CRP	C-reactive protein
CTL	cytotoxic T lymphocyte
EBV	Epstein–Barr virus

FasL	Fas ligand
FITC	fluorescein isothiocyanate
iNKT cells	invariant natural killer T cells
IL-4	interleukin 4
IL-5	interleukin 5
NKT cells	natural killer T cells
SBT	small bowel transplantation
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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