Changes of CD8+CD28- T regulatory cells in rat model of colitis induced by 2,4-dinitrofluorobenzene

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Abstract
AIM: To determine the changes of CD8+ T subsets especially CD8+CD28- T regulatory cells in rat model of experimental colitis induced by 2,4-dinitrofluorobenzene (DNFB).

METHODS: The rat model of experimental colitis was induced by enema with DNFB. Ten days later, colonic intraepithelial and splenic lymphocytes were isolated from colitis animals (n=16) and controls (n=8). The proportion of CD8+ T cells, CD8+CD28+ T cells and CD8+CD28- T regulatory cells were determined by flow cytometry.

RESULTS: The model of experimental colitis was successfully established by DNFB that was demonstrated by bloody diarrhea, weight loss and colonic histopathology. The proportion of CD8+ T cells in either splenic or colonic intraepithelial lymphocytes was not significantly different between colitis animals and controls (spleen: 34.6±7.24 % vs 33.5±9.41 %, colon: 14.0±8.93 % vs 18.0±4.06 %, P>0.05). But CD8+CD28- T regulatory cells from colitis animals were significantly more than those from controls (spleen: 11.3±2.26 % vs 5.6±1.01 %, colon: 6.50±5.37 % vs 1.07±0.65 %, P<0.05). In contrast, CD8+CD28+ T cells from colitis animals were less than those from controls (spleen: 23.3±6.14 % vs 27.8±9.70 %, P=0.06; colon: 7.52±4.18 % vs 16.9±4.07 %, P<0.05). The proportion of CD8+CD28- T regulatory cells in splenic and colon intraepithelial CD8+ T cells from colitis animals was higher than that from controls (spleen: 33.3±5.49 % vs 18.4±7.26 %, colon: 46.0±14.3 % vs 6.10±3.72 %, P<0.005).

CONCLUSION: Experimental colitis of rats can be induced by DNFB with simplicity and good reproducibility. The proportion of CD8+CD28- T regulatory cells in rats with experimental colitis is increased, which may be associated with the pathogenesis of colitis.

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INTRODUCTION
It is well known that immune system was involved in the pathogenesis of inflammatory bowel disease (IBD) [1-4]. Several extraintestinal autoimmune phenomena are accompanying IBD and immunosuppressive agents could alleviate the disease. Abnormalities in T-cell mediated immunity have also been noted in these patients [5]. But the exact mechanism remains to be clarified. The changes in T-cell subsets were controversial in the literature and should be deeply explored [6]. Although CD8+ T cells represent a major T-cell subset, there is little information available regarding the role of CD8+ T cells in the pathogenesis of colitis. CD8+ T cells specific for an epithelial cell-derived antigen have been shown to induce intestinal inflammation. It is possible that, like CD4+ T cells, functionally distinct CD8+ T-cell subsets may coexist. Thus we discussed the changes of CD8+ T-cell subsets in experimental colitis.

Another important part of ongoing studies of IBD was to use animal model to replicate some of the clinical features of this disease entity and to explore the mechanisms of IBD [7]. One of the important lessons learned from experimental models has been that recognition of the role of T regulatory cells plays in chronic intestinal inflammation. The ability of T cells to regulate colitis was first shown by studies of Powrie et al, who demonstrated that OX-22+ T cells could suppress the colitis induced by transfer of CD4+OX22 high T cells [8]. In the subsequent studies, several types of regulatory T cells have been identified. The T1r cells were initially reported to produce IL-10 and regulate the development of colitis in CD45RB model [9]. The effectiveness of immunosuppressive drugs in IBD may be partly related to the fact that T1r cells can be also generated in the presence of dexamethasone and vitamin D3. CD4+CD25+ T regulatory cells also have been demonstrated to prevent colitis in the CD45RB model [10]. A recent human study also showed that CD8+CD28- T cells activated by the interaction with intestinal epithelial cells through gp-180 possessed a regulatory activity [11], which perhaps play an important role in the pathogenesis of colitis. However, until now there is no study reported about the roles of CD8+CD28- T regulatory cells in colitis.

In previous studies, several models of experimental colitis have been described such as TNBS, DDS, DNCB and CD45RB T-cell infusion. 2,4-dinitrofluorobenzene (DNFB) - inducing experimental colitis in mice was demonstrated to be a suitable cell-immune model [12-14]. Thus we have established the same model in rats to explore the changes in CD8+ T cell subsets especially CD8+CD28- T regulatory cells and their possible roles in the pathogenesis of DNFB-induced experimental colitis.

MATERIALS AND METHODS

Animals
Specific pathogen-free Sprague-Dawley male rats weighing 250-300 g were kept in standard laboratory conditions (room temperature of 22 °C with a controlled 12-hour light-dark cycle and free access to animal chow and water). Animal care was in compliance with guidelines from the Peking University Health Science Center Policy on Animal Care and Use. The rats were allowed to adapt to our laboratory environment for
one week before the onset of the experiment.

Reagents
DNFB was purchased from Acros Organics (NJ, USA), olive oil and acetone from Sigma Chemical Co. (St. Louis, Missouri, USA). Mouse anti-rat PE-conjugated-CD8b mAb (clone 341, Mouse IgG1) and FITC-conjugated-CD28 mAb (clone JJ319, Mouse IgG1) were from Serotec (Oxford, UK). Isotype control PE-conjugated mouse IgG1 and FITC-conjugated mouse IgG1 were from BD Pharmingen (San Diego, CA). RPMI 1640 and FCS were obtained from Gibco BRL (Gaithersburg, MD).

Induction of colitis
The animals in the experimental group (n=16) were pretreated with two sensitizing doses of 1 ml of 0.5 % w/v DNFB solution by rubbing the substance on previously shaved abdominal skin, 96 and 72 h before challenge.[15] The DNFB solution was prepared by diluting the original DNFB preparation with 4:1 acetone and olive oil. Freshly prepared solutions were used for each application. All the animals in the experimental group received a rectal enema with 0.4 ml of 0.2 % w/v DNFB solution in acetone and olive oil into the lumen of the colon by means of an 8-cm plastic catheter with an outer diameter of 0.9 mm, attached to a 1-ml syringe. Rectal enemas were administered under light ether anesthesia. The animals in the control group (n=8) received a rectal enema with 0.9 % w/v saline only.

Histology
Animals were killed on day 10 after the challenge enema. The entire colon was dissected, opened longitudinally, washed in ice-cold PBS. The ratio of colon / body weight was calculated, which has been shown to be a marker of colonic inflammation.[15]

Macroscopic damage was assessed, according to a previously established score as follows[16]: 0: no ulcer and no inflammation, 1: no ulcer and local hyperemia, 2: ulceration without hyperemia, 3: ulceration and inflammation at one site only, 4: two or more sites of ulceration and inflammation, 5: ulceration extending more than 2 cm, 6-10: increment of 1 for each centimeter of ulceration greater than 2 cm.

Randomized tissue samples from the site of DNFB application were subsequently excised for histology. Paraffin sections were stained with haematoxylin and eosin. Microscopic damage was quantified by image analysis of stained sections in a blinded fashion as follows[17]: 0: No evidence of inflammation, 1: Low level of lymphocyte infiltration seen in ≤10 % high power field and no structural changes observed, 2: Moderate lymphocyte infiltration seen in 10-25 % high power field and crypt elongation, bowel wall thickening that did not extend beyond the mucosal layer and no evidence of ulceration, 3: High level of lymphocyte infiltration seen in 25-50 % high power field, high vascular density, and thickening of the bowel wall that extended beyond the mucosal layer, 4: Marked degree of lymphocyte infiltration seen in ≥50 % high power field, high vascular density, crypt elongation with distortion and transmural bowel wall thickening with ulceration.

Isolation of lymphocytes
Splenocytes were isolated by conventional methods. Red blood cells were removed from spleen cell suspensions by using a standard lymphocyte gradient (Chemical Co, Shanghai, China). Intestinal intraepithelial lymphocytes were isolated according to a previously published method[18]. An average of 2-4×10⁶ intraepithelial lymphocytes was yielded per colon.

Flow cytometry
A total of 1×10⁶ spleen or intraepithelial lymphocytes were prepared in 100 µl and anti-CD8b and anti-CD28 mAb were added to the cell suspension. The cells were incubated at 4 °C for 30 min, and then the cells were washed two times with PBS/10 % v/v FCS. The cells were finally resuspended in 250 µl PBS containing 2 % w/v paraformaldehyde and then analyzed by a FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA). 10 000 gated events were acquired for analysis. Isotype control was done according to the manufacturer.

Statistical analysis
Results were expressed as means ± standard deviation (SD). Comparison of results between groups was performed by unpaired t test. A probability (P) value <0.05 was considered as significant.

RESULTS

General findings
One day post-enema, diarrhea, hematochezia and reduced activity were observed in all experimental animals, but not in controls. Ten days later, the body weights of experimental animals were significantly lower than those of controls (P<0.05, as shown in Figure 1). The body weight of experimental animals decreased to the lowest on day 2 (92.8±5.0 % compared with pretreatment vs 106.4±1.4 % in controls, P<0.05). One rat died of colonic necrosis on the second day after DNFB enema.

Figure 1 Body weight of experimental animals was significantly lower than that of controls (P<0.01), ○ controls, ● colitis.

Colon inflammation
The ratio of colon/body weight in experimental animals was two times more than that in controls (0.015±0.004 vs 0.007±0.002, P<0.05). Oedema, hemorrhage, superficial ulcerations, and necrosis were observed in the colon of experimental animals. Moreover, macroscopical characteristics of chronic phase such as adhesions, thickening of the colonic segments with narrowing of the lumen and prestenotic dilatation, atrophy and even with megacolon formation, were obviously in experimental animals but not in controls. Microscopically, active inflammatory process was characterized by mononuclear infiltration, fibrosis, smooth-muscle hypertrophy and lymphoid hyperplasia (as shown in Figure 2 left). Both the macroscopical and microscopical damage scores in experimental animals were higher than those in controls (macroscopical: 5.2±1.4 vs 1.4±0.6, P<0.0001; microscopical: 3.3±0.5 vs 1.2±0.5, P<0.0001, Figure 2 right).

Splenic CD8+ T-cell subsets
There was no difference in the proportion of CD8+ T cells between experimental animals and controls. However, CD8+CD28- T regulatory cells of experimental animals were significantly more than controls (as shown in Figure 3). Even
though it was not significantly, CD8+CD28+ T cells of experimental animals were less than controls. In CD8+ T cells pool, the proportion of CD8+CD28- T regulatory cells of experimental animals was also significantly higher than that of controls (as shown in Table 1).

Table 1 Splenic CD8+ T-cell subsets of experimental animals and controls on day 10 after enema

|                           | Experimental colitis (%, n=16) | Control (%, n=8) | P value |
|---------------------------|--------------------------------|-----------------|---------|
| CD8+CD28-T                | 11.3±2.26                     | 5.64±1.01       | 0.0001  |
| CD8+CD28+T                | 23.3±6.14                     | 27.8±9.70       | 0.41    |
| CD8+T                     | 34.6±7.24                     | 33.5±9.41       | 0.83    |
| CD8+CD28-T/CD8+T          | 33.3±5.49                     | 18.4±7.26       | 0.003   |

Table 2 Intraepithelial CD8+ T-cell subsets of experimental animals and controls on day 10 after enema

|                           | Experimental colitis (%, n=16) | Control (%, n=8) | P value |
|---------------------------|--------------------------------|-----------------|---------|
| CD8+CD28-T                | 6.50±5.37                      | 1.07±0.65       | 0.049   |
| CD8+CD28+T                | 7.52±3.16                      | 16.9±4.07       | 0.004   |
| CD8+T                     | 14.0±6.83                      | 18.0±4.06       | 0.38    |
| CD8+CD28-T/CD8+T          | 46.0±14.3                      | 6.10±3.72       | 0.0001  |

DISCUSSION

DNFB, a highly reactive compound with hapten characteristics, could bind to and covalently modify cell surface proteins, changing their antigenic conformation and their immunogenicity. T lymphocytes could recognize and lyse hapten-modified autologous cells quite efficiently in presensitized animals [19]. Thus DNFB was capable of inducing a delay-type hypersensitivity reaction when applied to the skin or to the intestinal mucosa [20]. In addition, DNFB was more reactive in protein modification than DNCB [21,22]. This is a possible explanation for DNFB causing the lesions, more pronounced in both acute phase and chronic phase than in previously described models induced by DNCB. In 1992, Banic et al. demonstrated that experimental colitis of mice was induced by DNFB, mimicking inflammatory bowel disease by the intestinal histopathological and extraintestinal manifestations [12-14]. It also responded well to glucocorticosteroid and cyclosporine A, resembling TNBS-induced colitis [23,24]. Because of its simplicity and reproducibility this model represents a suitable preparation for investigating pathogenetic mechanisms of IBD especially that involves cellular immunity. We further explored this model in rats as the first time and the results were consistent with Banic et al. All morphologic changes in experimental group were similar to the changes characteristically observed in IBD in humans. Thus
experimental colitis model of rats induced by DNFB could be used to study the pathogenesis of IBD.

There is increasing evidence that IBD is a kind of autoimmune diseases. IBD could be envisioned as an imbalance between proinflammatory and anti-inflammatory cytokines. It is suspected that changes in certain cytokines could be a result of changes in proportion or function of certain T-cell subsets. In a series study of Jewell et al., however, no difference was found in CD4+ and CD8+ T cells of peripheral blood and colonic mucosa between IBD and healthy controls. Regrettably, subsets of CD4+ and CD8+ T cells were not further studied. Several studies demonstrated that T cells from IBD patients or experimental colitis model had diminished response to mitogen stimuli of T cells in IBD. Further study is urgently needed to explore the roles of T regulatory cells in pathogenesis of colitis. Our study showed the increase of CD8+CD28- T regulatory cells is a feedback to cell pools mentioned above can be explained. We suspect that changes in certain cytokines could be a result of functional features of suppression. Thus the changes of CD8+ T cells in experimental animals compared to controls, and decreasing population of CD8+CD28+ T cells at the same time. These results have not been reported before.

CD8+ T cells comprise cells that are in different states of differentiation and under the control of complex homeostatic processes. In a number of situations such as chronic inflammatory conditions, infectious diseases, ageing, immunodeficiency, iron overload, heavy alcohol intake and transplantation, major phenotypic changes, usually associated with an increase in CD8+CD28- T cells, took place. The CD8+CD28- T cells are characterized by functional features of suppression. CD8+CD28- T cells could suppress alloreactive immune responses in an antigen-specific, major histocompatibility complex (MHC)-restricted manner.

The emergence of expanded CD8+CD28- T-cell clones has been ascribed to extensively continuous or repeated antigenic stimulation leading to immune exhaustion, as suggested by the accumulation of such cells in patients with human immunodeficiency virus (HIV) infection and autoimmune disease. Although the nature of the signals that give origin to this T-cell subset is uncertain, growing evidence argues for the existence of an interplay between epithelial cells, molecules with the MHC-class I fold and CD8+ T cells. By stimulation of antigen especially intestinal epithelial cells, CD8+CD28+ T cells can proliferate of lymphocytes by binding with pectin in experimental colitis in the rat. Although the nature of the signals that give origin to this T-cell subset is uncertain, growing evidence argues for the existence of an interplay between epithelial cells, molecules with the MHC-class I fold and CD8+ T cells. By stimulation of antigen especially intestinal epithelial cells, CD8+CD28+ T cells can proliferate of lymphocytes by binding with pectin in experimental colitis in the rat.

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