Transpl Int (1995) 8: 13–19

ORIGINAL ARTICLE

Xian-Chang Li
Robert Zhong
Linfu Zhu
David Grant

Donor-specific cytotoxicity induced by allogeneic intestinal epithelial cells in a sponge matrix model

Received: 16 December 1993
Received after revision: 19 April 1994
Accepted: 21 April 1994

X.-C. Li · R. Zhong
L. Zhu · D. R. Grant (✉)
Department of Surgery,
University Hospital,
P. O. Box 5339, London, Ontario,
Canada N6A 5A5
Fax: +1 519 663 3858

X.-C. Li · R. Zhong · L. Zhu · D. R. Grant
The John P. Robarts Research Institute,
London, Ontario, Canada

Abstract Small intestinal epithelial cells (IEC) constitutively express MHC class II molecules. However, little is known about the role of IEC in intestinal allograft rejection. The present study examined whether IEC can induce the development of cytotoxic T cells in vivo using a sponge matrix model. IEC isolated from ACI ($RT1^a$) rats were injected into polyurethane sponges implanted i. p. in Lewis ($RT1^l$) rats. Sponge grafts with ACI splenocytes or Lewis IEC were used as controls. The sponge grafts were removed and sponge-infiltrating cells (SIC) were harvested on post-operative days (POD) 7, 10, and 14. The phenotype of SIC was determined by FACS analysis and the cell-mediated cytotoxicity was measured using a chromium release assay. Non-specific inflammatory cells accumulated in the IEC sponge allografts during the first 10 days. By POD 14, however, 61 % of SIC were T lymphocytes and 36 % expressed cytotoxic T cell marker (OX-8). The cytotoxicity in IEC sponge allografts was detectable on POD 7 and POD 10, and markedly elevated on POD 14. The cytotoxicity induced by allogeneic splenocytes appeared in the sponge grafts on POD 7, peaked on POD 10, and declined thereafter. The allospecific cytotoxicity induced by IEC was dependent on host macrophages as pretreatment of animals with gadolinium, a rare earth metal that inactivates macrophages, abrogated the induction of cytotoxicity. We conclude that: (1) the migration and maturation of cytotoxic T cells can be induced in vivo by IEC and (2) IEC may contribute to the increased severity of intestinal rejection through interaction with macrophages.

Key words Cytotoxicity, intestinal epithelial cells · Intestine

Introduction

Despite recent advances in immunosuppression, graft rejection continues to be a major barrier to successful intestinal transplantation [28]. The factors contributing to the increased frequency and severity of graft rejection are still poorly defined. Graft rejection has been attributed to the abundant lymphoid tissue in the small bowel, but depletion of lymphoid cells from the intestinal graft has failed to prevent graft rejection [12, 26]. We hypothesized that small intestinal epithelial cells (IEC) bearing MHC class II molecules [5] may contribute to graft rejection by acting as antigen-presenting cells. Previous studies have shown that human colonic epithelial cells can induce proliferation of allogeneic T lymphocytes in vitro, although the degree of stimulation was 5- to 20-fold lower than that achieved with peripheral blood adherent cells [18]. A recent study in our laboratory has shown that IEC can induce strong proliferation of allogeneic T cells when co-cultured with macrophages [17]. The present study was undertaken to examine the phenotype and the allospecific cytotoxicity of cells infiltrated in sponge matrix graft containing allogeneic IEC. This model permits an assessment of in vivo immune response directly at the site of allostimulation [21].

Materials and methods

Animals

Inbred male Lewis ($RT1^l$) and ACI ($RT1^a$) rats were purchased from Harlan-Sprague Dawley (Indianapolis, Ind., USA). PVG ($RT1^c$) rats were purchased from Bantin and Kingman (Fremount, Calif., USA). Animal use and care conformed to the guidelines established by the Canadian Council on Animal Care [10].

Antibodies

The following mouse anti-rat antibodies were obtained from Cedarlane (Hornby, Ontario, Canada): OX-4, which binds to rat MHC class II molecule; OX-41, which binds to rat macrophages and granulocytes; OX-33, which binds to B cells; W3/13, which binds to T cells; W3/25, which stains CD4+ T lymphocytes and some of the macrophages; OX-8, which stains CD8+ T lymphocytes and NK cells; and FITC-conjugated goat anti-mouse IgG. FITC-conjugated anti-rat pan-cytokeratin antibody was purchased from Sigma (St. Louis, Mo., USA).

IEC isolation

A modification of the method described by Bjerknes et al. was used [4]. The thoracic cavity was opened and the animal was perfused through the left ventricle of the heart with 100 ml of calcium-magnesium-free Hanks' balanced salt solution containing 10 mM EDTA (CMF-HBSS-EDTA) for 15 min. The inferior vena cava was cut for subsequent drainage. The jejunum and ileum were removed, dissected free of fat, mesentery, and Peyer's patches, then opened lengthwise and cut into 1- to 2-cm pieces. The epithelium was released from the underlying tissue by gentle vortex and further digested with Dispase (Boehringer, Laval, Quebec, Canada) at 37 °C for 20 min. The supernatant was pooled and the epithelial cells were further purified using discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden). Cells from the interface of 10/30 % gradients were harvested and used in this study.

Sponge graft implantation

The procedure described by Robarts and Häyry [21] was used with slight modifications. Briefly, a polyurethane sponge ($10 \times 10 \times 15$ mm) was implanted intraperitoneally (i.p.) into each Lewis rat. Before closure of the abdominal wall, each sponge graft was injected with 2×10^6 ACI splenocytes or IEC in 0.5 ml phosphate-buffered saline. Lewis rats bearing sponge grafts injected with the same number of IEC from Lewis rats were used as a control. Each group had contained three animals. The sponge grafts that were well vascularized on post-operative day (POD) 3 were removed on POD 7, 10, and 14. The sponge-infiltrating cells (SIC) were harvested by repeated pressing of the sponge grafts in a tissue culture dish. The red blood cells were lysed using red blood cell lysing buffer and remaining cells were washed and used for phenotypic and cytotoxic analysis.

Gadolinium treatment

Animals were treated with two doses of gadolinium chloride (15 mg/kg; Aldrich Chemicals, Milwaukee, Wis., USA) dissolved in phosphate-buffered saline i.p. for 7 days before sponge implantation. This treatment decreased the number of macrophages and impaired macrophage function as previously described [9, 22].

Flow cytometry

Cells harvested from sponge grafts were stained with the following mouse anti-rat antibodies: OX-4, OX-8, OX-41, OX-33, W3/13, and W3/25 at 4 °C for 45 min. The cells were washed twice with phosphate-buffered saline and further stained with FITC-conjugated goat anti-mouse IgG. Control preparations were stained with FITC-conjugated goat anti-mouse IgG alone. The cells were washed and analyzed using FACS. Results were expressed as percentage of positive cells. For each sample, 2×10^4 viable cells were counted.

Cytotoxicity assay

The chromium release assay was used [1]. Target cells were prepared by culturing splenocytes (5×10^6 cells/ml) with Concanavalin A (Con A; 5 μg/ml, Sigma) for 48 h and were labeled with ^{51}Cr (250–500 mCi/mg; Amersham, UK). The ^{51}Cr-labeled target cells (4×10^4 cells/well) were placed into 96-well round-bottom culture plates (Linbro, McLean, Va., USA) along with effector cells to achieve the effector/target cell ratio of 100 : 1. Total culture volume was 0.2 ml in each well and all assays were performed in triplicate. After 4 h of incubation at 37 °C, the plates were centrifuged at 700 *g* for 10 min and 0.1 ml supernatant was removed from each well and counted in a gamma counter (Beckman, Fullerton, Calif., USA). Total counts were determined by lysing the target cells with 0.1 % Triton-100 (Sigma). The supernatant from target cells cultured with medium alone was used to determine the spontaneous release. Background ^{51}Cr release was 10 %–15 %. Cytotoxicity was calculated using the formula:

$$\frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}} \times 100$$

Cell proliferation assay

Mixed lymphocyte culture was performed in 96-well round-bottom tissue culture plates with RPMI 1640 medium containing 5 % FCS, 100 U/ml penicillin and streptomycin, 25 mM Hepes, and 2×10^{-5} M 2-ME. IEC from ACI rats irradiated with 2500 rads were used as stimulator cells. Varying numbers of stimulator cells were mixed in triplicate with T lymphocytes (2×10^5) purified from the spleen of Lewis rats using nylon wool columns. (FACS analysis showed 95 % of the responder cells expressed W3/13 T cell marker. The purity of T cells was further confirmed by lack of a proliferative response to the stimulation of Con A). The total culture volume was 0.2 ml in each well. In some experiments, peritoneal macrophages isolated from Lewis rats were added. The cells were cultured at 37 °C in a 5 % CO_2 humidified incubator for 5 days and 18 h before cell harvesting each well was pulsed with 1 μCi of ^3H-TdR (40–60 Ci/mmol; Amersham, UK). The cultures were harvested and counted using a scintillation counter (Beckman).

Statistical analysis

The data are expressed as mean ± SD of three animals in each group and compared using an analysis of variance with a P value below 0.05 as significant.

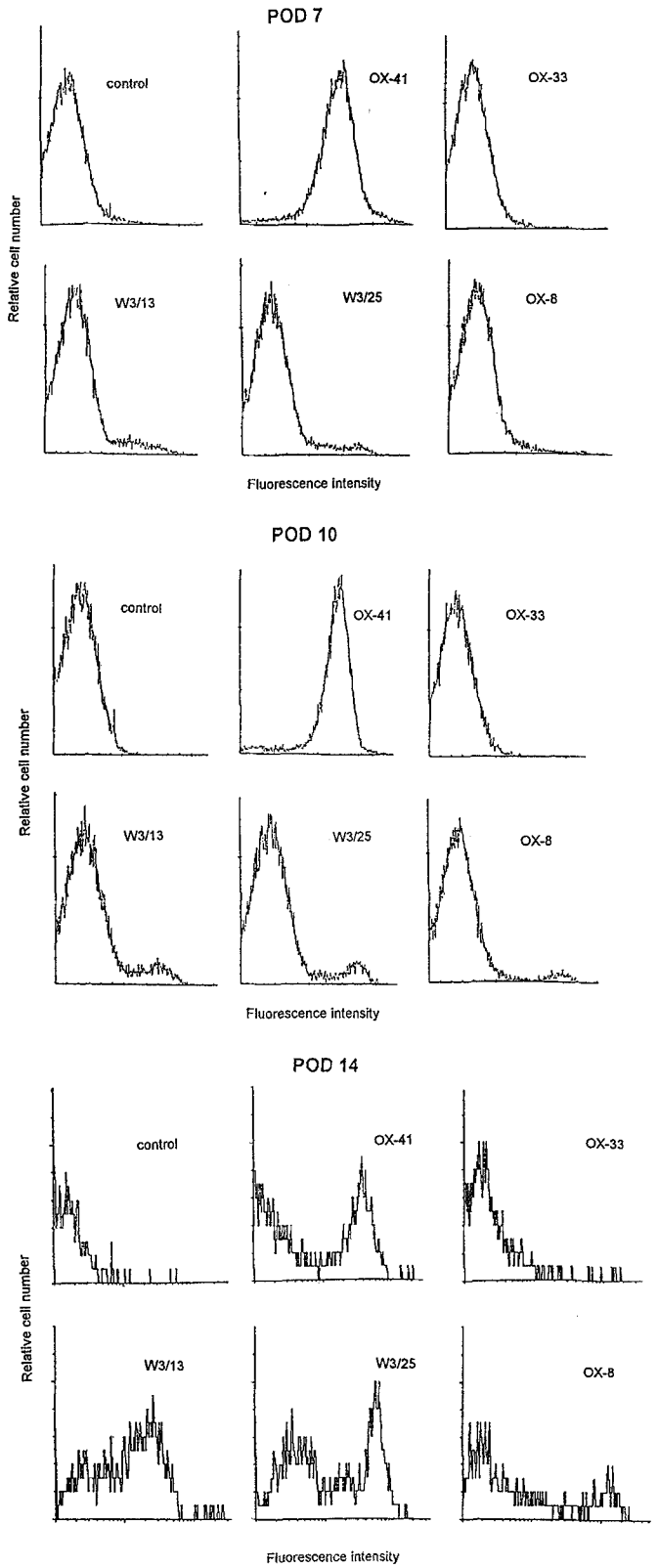

Fig. 1 The phenotye of cells infiltrating intestinal epithelial cells *(IEC)* sponge allografts analyzed by FACS

Results

Epithelial cell isolation and characterization

The in vivo perfusion and in vitro Dispase digestion method was adapted to purify IEC in single cell suspension with high viability, good yield, and optimal purity. Microscopic examination after CMF-HBSS-EDTA treatment showed depletion of the epithelium with excellent preservation of the lamina propria and the crypts. FACS analysis showed high purity of epithelial cells with 97 % positive for cytokeratin staining and less than 0.1 % OX-33+ cells (B cells), less than 0.1 % OX-41+ cells (macrophages and granulocytes), and 2.5 % W3/13+ cells (T cells). Thirty-six percent of cells were OX-4+ (MHC class II). The viability of IEC was greater than 70 % as determined by the trypan blue exclusion method.

Phenotype of cells infiltrating IEC sponge allografts

In sponge grafts injected with syngeneic IEC, the majority of SIC were macrophages and granulocytes at all time points. As shown in Fig. 1, macrophages and granulocytes predominated the cellular infiltrates in IEC sponge allografts on POD 7 (92 %). The phenotype of SIC maintained the same pattern on POD 10 with only a slight increase in the T-cell population. On POD 14, however, 61 % of SIC were T lymphocytes and 36 % expressed cytotoxic T cell marker (OX-8). Macrophages decreased to 40 % on POD 14. B lymphocytes were less than 6 % at all time points. After gadolinium treatment, there was an early influx of granulocytes in IEC sponge allografts and the macrophage population was less than 10 % at all time points. A high percentage of B cells (33 %) was present in IEC sponge grafts on POD 14. T lymphocytes showed almost the same kinetics of infiltration as in untreated animals.

Cytotoxicity induced by allogeneic IEC in vivo

As shown in Fig. 2, cytotoxicity in IEC sponge allografts was present at a detectable level on POD 7 and POD 10, and markedly elevated on POD 14. No cytotoxicity was detected in sponge grafts injected with syngeneic IEC at any time point. Cytotoxicity induced by allogeneic splenocytes appeared in sponge grafts on POD 7, peaked on POD 10, and then declined thereafter. SIC isolated from IEC sponge allografts showed a detectable level of cytotoxicity against donor (ACI) as well as third-party (PVG) target cells on both POD 7 and POD 10. The cytotoxic effect of SIC was donor-specific and no cytotoxicity was detected against third-party target cells on POD 14 ($P < 0.05$; Fig. 3). The allospecific cytotoxicity of

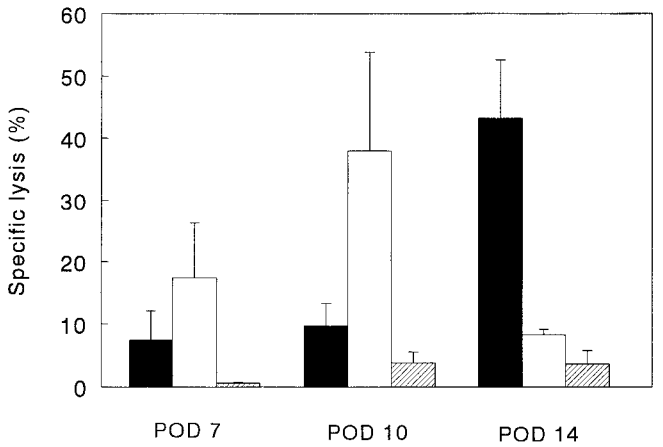

Fig. 2 Cytotoxicity of cells from sponge grafts implanted in Lewis rats. Cells harvested from sponge grafts injected with ACI splenocyte (□), ACI IEC (■), or Lewis IEC (▨) were used as effector cells. The ^{51}Cr-labeled T lymphoblasts of ACI rats were used as target cells. The effector/target cell ratio was 100 : 1. The specific killing was determined by chromium release. The data are presented as mean ± SD of three animals in each group

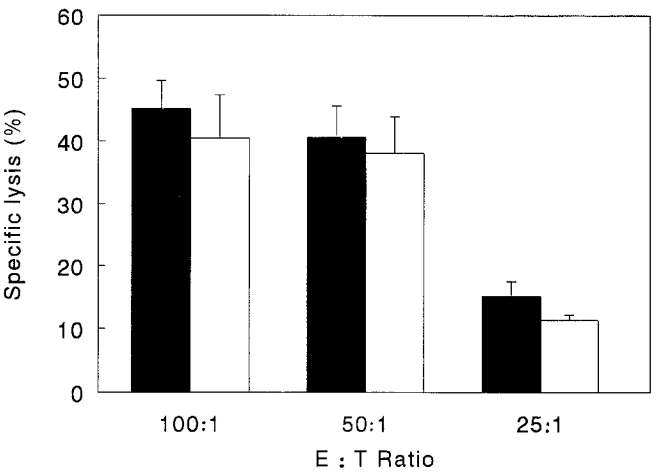

Fig. 4 Cytotoxicity of SIC (■) or nonadherent cells (□) isolated from IEC sponge allografts on POD 14. SIC were harvested from IEC sponge allografts. The nonadherent cells were prepared by adherent culture to deplete macrophages. The cytotoxicity of SIC or nonadherent cells against donor target cells *(ACI)* was assayed

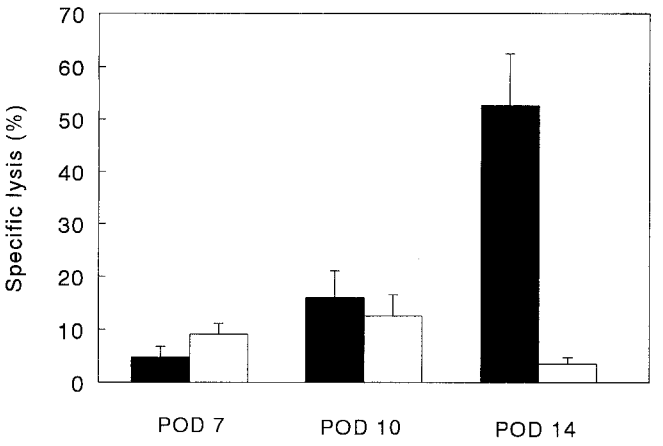

Fig. 3 Cytotoxicity of cells from IEC sponge allografts against donor (*ACI*, ■) or third-party (*PVG*, □) target cells. Sponge-infiltrating cells *(SIC)* were harvested from IEC sponge allografts implanted in Lewis rats and mixed with ^{51}Cr-labeled ACI or PVG target cells at a ratio of 100 : 1. The specific killing was determined by chromium release

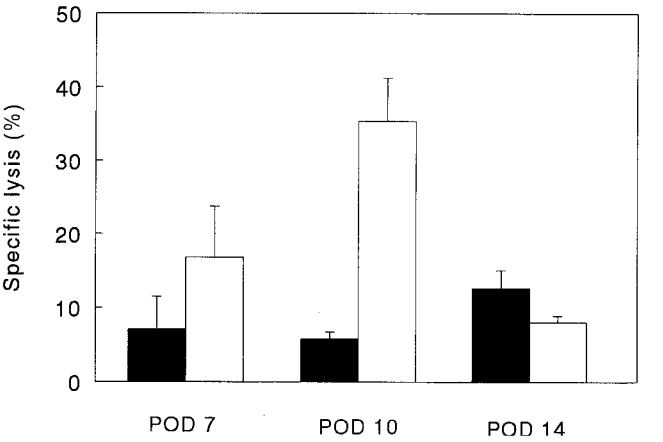

Fig. 5 Cytotoxicity of cells from sponge allografts after gadolinium treatment. The Lewis rats were treated with gadolinium for 7 days and then implanted with sponge grafts containing ACI splenocytes (□) or ACI IEC (■). SIC were harvested and assayed for cytotoxicity against ACI target cells

SIC harvested from IEC sponge allografts on POD 14 was not affected by depletion of adherent cells (Fig. 4), suggesting that the cytotoxic cells are T lymphocytes. In order to determine whether macrophages were involved in the induction of cytotoxicity by IEC, animals were pre-treated with gadolinium to block the macrophage function. As shown in Fig. 5, this treatment inhibited the development of cytotoxicity in the IEC sponge allografts. In contrast, cell-mediated cytotoxicity in splenocyte sponge allografts was not affected by this treatment ($P < 0.05$).

Macrophages in IEC-induced allogeneic T-cell activation in vitro

We further examined the interaction of IEC and macrophages in allogeneic T-cell activation in vitro. IEC were co-cultured with macrophages syngeneic to responder T cells. As shown in Fig. 6, both syngeneic macrophages and allogeneic IEC failed to induce significant T-cell proliferation. However, when IEC and macrophages were mixed and co-cultured with responder T cells, a strong T-cell proliferation was induced. Macrophages

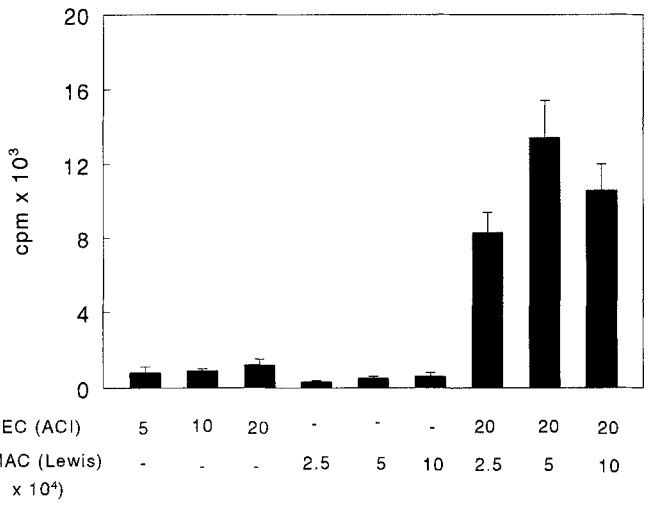

Fig. 6 The effect of macrophages *(MAC)* on IEC-induced T-cell proliferation. Responder T cells (2×10^5) from Lewis rats were mixed with equal numbers of IEC *(ACI)*, in which various numbers of MAC were added, as indicated. Responder T cells mixed with syngeneic MAC were included as a control

treated with gadolinium prior to cell culture failed to induce significant T-cell proliferation when stimulated by IEC (data not shown), suggesting that the activation of allogeneic T cells stimulated by IEC requires functional macrophages.

Discussion

The present study has demonstrated that IEC expressing MHC class II antigens can induce the migration and maturation of cytotoxic T cells in vivo and that this process requires the presence of functional macrophages, suggesting that IEC may contribute to the increased severity of intestinal rejection through interaction with macrophages.

The sponge matrix model has been extensively used to analyze the local cellular events involved in graft rejection. In the sponge grafts injected with allogeneic lymphoid cells, the host T-helper cells are activated in response to allogeneic MHC class II antigens and produce cytokines necessary for the local development of cytotoxic T cells [1]. Recent studies have demonstrated that specifically sensitized lymphocytes preferentially migrate from host lymphoid organs to the site of antigen deposition [2, 14, 20]. In the IEC sponge allograft model, we demonstrate the generation of allospecific cytotoxicity stimulated by parenchymal rather than lymphoid cells. Our data suggest that the local maturation of sensitized T lymphocytes appears to contribute to the allospecific cytotoxicity in IEC sponge allograft, since animals bearing sponge graft with allogeneic IEC have no detectable cytotoxicity outside of the sponge graft at any time point. In contrast, animals bearing sponge allograft with splenocytes develop significant cytotoxicity in the host spleen prior to the development of maximal cytotoxicity in the sponge graft. This difference is probably due to trafficking of cells out of the splenocyte sponge allograft into host central immune compartments where host T cells are sensitized.

The kinetics and phenotype of cellular infiltrate in IEC sponge allograft are similar to changes seen after intestinal grafting. The initial cells that accumulate in the sponge graft injected with allogeneic IEC are mainly non-specific inflammatory cells with a predominance of macrophages. The sensitized T cells are not present in IEC sponge allograft until 14 days after implantation. Early intestinal rejection is likewise characterized by a massive mononuclear cell infiltration and macrophages predominate the cellular infiltrate throughout the rejection process [15, 16]. At the onset of maximal clinical rejection, macrophages are observed in all graft compartments, particularly epithelium [16, 23]. The mucosal inflammation that occurs with graft rejection is accompanied by increased MHC class II expression on IEC [24, 25]. Therefore, the development of cytotoxic T cells, as observed in the sponge matrix grafts, may take place in intestinal epithelium during graft rejection.

The induction of cytotoxicity in the IEC sponge allograft involves a maturation period and functional macrophages are required for this process since pre-treatment of animals with gadolinium chloride, a rare earth metal that has been shown to decrease the number of macrophages and impair macrophage function [9, 22], prevents the generation of cytotoxic T cells. Several mechanisms may explain these findings. First, MHC class II antigens on the surface of IEC may be processed and presented by sponge-infiltrating macrophages to host T-helper cells in the context of self MHC class II molecules. The activated T-helper cells then produce cytokines that are required for the local development of cytotoxic T cells. This pathway has been described in vitro [11, 29, 31]. Second, IEC bearing MHC class II molecules may be unable to generate costimulatory signals required for T-helper cell activation because naive T-helper cell activation depends upon the recognition of MHC class II and antigen complex in the presence of costimulatory signals provided by antigen-presenting cells [19, 27, 30]. Thus, the activation of T-helper cells by IEC and subsequent generation of cytotoxic T cells may depend upon the costimulatory signals provided by sponge-infiltrating macrophages. Third, the induction of allospecific cytotoxicity by IEC may be MHC class I-dependent since IEC also express high-level MHC class I molecules. It has been shown that freshly isolated allogeneic hepatocytes that are MHC class I+ and class II– stimulate the development of cytotoxic T cells in the presence of macrophages [6–8].

The immunogenicity of solid organ transplants has been traditionally attributed to passenger leukocytes. There is increasing evidence that graft parenchymal cells also contribute to the rejection process, particularly when these cells express or can be induced to express MHC class II antigens [3, 8, 13]. The present study has shown that IEC can induce donor-specific cytotoxicity in vivo and that macrophages play a critical role in this process. Thus, techniques to prevent mucosal inflammation may reduce the risk of intestinal allograft rejection. Furthermore, strategies to inhibit macrophage migration and accumulation at the site of IEC deposition may also prevent epithelial cell damage in free cellular allotrasplantation.

Acknowledgements The authors thank Qiang Yu for her excellent assistance with the FACS analysis and Cate Abott for reviewing this manuscript. This work was supported by Medical Research Council of Canada grant MT-11457.

References

1. Ascher NL, Chen S, Hoffman RA, Stimmons RL (1983) Maturation of cytotoxic T cells within sponge matrix allografts. J Immunol 131: 617–621
2. Bishop DK, Jutila MA, Sedmak DD, Beattie MS, Orosz CG (1989) Lymphocyte entry into inflammatory tissues in vivo: qualitative differences of high endothelial venule-like vessels in sponge matrix allografts vs isografts. J Immunol 142: 4219–4225
3. Bishop GA, Waugh JA, Hall BM (1988) Expression of HLA antigens on renal tubular cells in culture. Transplantation 46: 303–310
4. Bjerknes M, Cheng H (1981) Methods for the isolation of intact epithelium from the mouse intestine. Anat Rec 199: 565–574
5. Bland P (1988) MHC class II expression by the gut epithelium. Immunol Today 9: 174–178
6. Bumgardner GL, Chen S, Hoffman R, Cahill DC, So SK, Platt J, Bach FH, Ascher NL (1989) Afferent and efferent pathways in T cells responses to MHC class I+, II-hepatocytes. Transplantation 47: 163–170
7. Bumgardner GL, Chen S, Almond SP, Ascher NL, Payne WD, Matas AJ (1990) Role of macrophages in the immune response to hepatocytes. J Surg Res 48: 568–572
8. Bumgardner GL, Matas AJ, Chen S, Cahill D, Cunningham TR, Payne WD, Bach FH, Ascher NL (1990) Comparison of in vivo and in vitro immune response to purified hepatocytes. Transplantation 49: 429–435
9. Callery MP, Kamei T, Flye MW (1989) Kupffer cell blockage inhibits induction of tolerance by the portal venous route. Transplantation 47: 1092–1093
10. Canadian Council on Animal Care (1984) Guide to the care and use of experimental animals, vol 1. Association of Universities and Colleges of Canada, Ottawa, Canada
11. Golding H, Singer A (1984) Role of accessory cell processing and presentation of shed H-2 alloantigens in allospecific cytotoxic T lymphocyte responses. J Immunol 133: 597–605
12. Gundlach M, Wasfie T, DÁgati V, Chabot J, Oluwole S, Hardy MA (1991) The role of passenger leukocytes in the immunogenicity of intestinal and cardiac allografts in the rat. Transplant Proc 23: 187–188
13. Hadley GA, Linders B, Mohanakumar T (1992) Immunogenicity of MHC class I alloantigens expressed on parenchymal cells in the human kidney. Transplantation 54: 537–542
14. Hanto DW, Hopt UT, Hoffman R, Simmons RL (1982) Recruitment of unsensitized circulating lymphocytes to sites of allogeneic cellular interactions. Transplantation 33: 541–546
15. Ingham Clark CL, Cunningham AJ, Crane PW, Wood RFM, Lear PA (1990) Lymphocyte infiltration patterns in rat small-bowel transplants. Transplant Proc 22: 2460
16. Kim PO, Levy GA, Craig M, Cullen J, Cohen Z (1990) Immune responses during small intestinal allograft rejection: correlation between procoagulant activity and histopathology. Transplant Proc 22: 2477–2479
17. Li XC, Quan D, Duff J, Grant D (1992) Rat intestinal epithelial cells as accessory cells in alloimmune responses. Transplant Proc 24: 1149
18. Mayer L (1987) The role of epithelial cells as accessory cells. Adv Exp Med Biol 216A: 209–218
19. Mueller DL, Jenkins MK, Schwartz RH (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annu Rev Immunol 7: 445–480
20. Orosz CG, Bishop DK, Ferguson RM (1989) In vivo mechanisms of alloreactivity. VI. Evidence that alloantigen deposition initiates both local and systemic mechanisms that influence CTL accumulation at a graft site. Transplantation 48: 818–824
21. Robarts PJ, Häyry P (1976) Sponge matrix allografts: a model for analysis of killer cells infiltrating mouse allografts. Transplantation 21: 437–445
22. Roland CR, Mangino MJ, Duffy BF, Flye MW (1993) Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. Transplantation 55: 1151–1158
23. Rosemurgy AS, Schraut WH (1986) Small bowel allografts: sequence of histologic changes in acute and chronic rejection. Am J Surg 151: 470–475
24. Schmid T, Oberhuber G, Korozsi G, Klima G, Margreiter R (1990) Altered distribution of MHC class II antigens on enterocytes during acute small bowel allograft rejection in rats. Transpl Int 3: 73–77
25. Schmid T, Oberhuber G, Koroszi G, Klima G, Margreiter R (1990) MHC class II antigens are expressed by enterocytes lining the crypts of Lieberkuhn during initial phases of rejection. Transplant Proc 22: 2064–2065
26. Stangl MJ, Lee KKW, Lee TK, Moynihan HL, Schraut WH (1990) Graft pretreatment with monoclonal antibodies prior to small-bowel transplantation. Transplant Proc 22: 2483–2484
27. Steinman RM, Young JW (1991) Signals arising from antigen-presenting cells. Curr Opinion Immunol 3: 361–372

28. Todo S, Tzakis AG, Abu-Elmagd K, Reyes J, Nakamura K, Casavilla A, Selby R, Nour BM, Wright H, Fung JJ, Demetris AJ, Van Thiel DH, Starzl TE (1992) Intestinal transplantation in composite visceral grafts or alone. Ann Surg 216: 223–234
29. Tsokos GC, Stocks NI, Clerici M, Shearer GM (1990) Human in vitro allogeneic responses. J Immunol 144: 2524–2528
30. Weaver CT, Unanue ER (1990) The costimulatory function of antigen-presenting cells. Immunol Today 11: 49–59
31. Weinberger O, Germain RN, Springer T, Burakoff SJ (1993) Role of syngeneic Ia+ accessory cells in the generation of allospecific CTL responses. J Immunol 129: 694–697