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Dora I. Ninova
Deanna M. Ferguson
Peter J. Wettstein
Ruud A. F. Krom

Liver allograft rejection in rats depleted of CD8$^+$ cells

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D.I. Ninova · D.M. Ferguson · R.A.F. Krom (✉)
Division of Transplantation,
Liver Transplant Clinic, Ei-3G,
Mayo Clinic and Mayo Foundation,
200 First Street S.W.,
Rochester, MN 55905, USA
Fax: + 1 507 266 1856

P.J. Wettstein
Departments of Surgery and Immunology,
Mayo Clinic and Mayo Foundation,
200 First Street S.W.,
Rochester, MN 55905, USA

Abstract The mechanism(s) of rejection or tolerance induction is a competitive, complex process that presumably involves interactions between multiple subpopulations of T lymphocytes. We investigated the roles of CD8$^+$ cytolytic and CD4 + helper T cells in rat strains that tolerate liver allografts and that differ at both the major histocompatibility complex (MHC) (RT1) and minor histocompatibility genes. Orthotopic liver transplantation (OLT) with arterial reconstruction was performed with Brown Norway (BN) (RT1^n) donors and Lewis (RT1^l) recipients, some of which were untreated, others treated with anti-CD8 antibody, and still others treated with anti-CD4 antibody. Liver graft rejection was monitored for 28 days on the basis of two criteria: (1) serum levels of AST enzyme at 3-day intervals and (2) liver biopsies at weekly intervals and at the time of sacrifice at the end of the study period. In the untreated control group, an elevation of AST was found to peak at day 6 after grafting, and it remained elevated until day 28 (AST 542 ± 72 U/l). Histologically, signs of severe rejection were first observed on day 9; these changed to moderate rejection about day 21 and to mild rejection by day 28, when the animals were sacrificed. Recipients pre-treated with anti-CD8 demonstrated a significant elevation of AST within 6 days that, unlike in the control recipients, continued to rise sharply through the observation period (AST 1127 ± 181 U/l, $P = 0.009$ vs control group). Liver biopsies showed mild rejection at day 9 and moderate rejection at days 21 through 28. Recipients pretreated with anti-CD4 showed a time course of enzyme elevation and severity of rejection that was not significantly different from that observed in the control group. However, anti-CD4 treatment resulted in only 75 % depletion of CD4$^+$ cells in peripheral blood as compared to complete elimination of CD8$^+$ cells following anti-CD8 treatment. Functional studies of spleen and liver-infiltrating lymphocytes obtained after 28 days showed low proliferative response in mixed lymphocyte culture with both BN and PVG stimulator spleen and lymph node cells. These results suggest that in this donor/recipient combination, removal of CD8$^+$ cells increases the severity of rejection as demonstrated by a progressive rise in AST and histology. Moreover, OLT in this combination results in a profound, nonspecific inhibition of proliferative T-cell responses to MHC alloantigens.

Key words Liver transplantation, rat, CD8$^+$ cells · CD8$^+$ cells, liver transplantation, rat · Rejection, CD8$^+$ cells, liver transplantation

Introduction

Liver allografts between donors and recipients incompatible at the major histocompatibility complex (MHC) can be accepted permanently without immunosuppression in some species, especially in inbred rats [11, 14, 18]. The survival of liver grafts is strain-dependent, and different types of responses to liver transplants have been described in rats ranging from acute rejection to indefinite survival [39]. In the case of acute

Table 1 Histological findings graded to score the severity of rejection

Grade	I Overall liver architecture changes	II Portal tracts	III Lobules	IV Central vein
0	Normal liver	Normal	Normal	Normal
1	Mild	Slightly expanded by cellular infiltrate	Inflammatory cells scattered through the parenchyma, no necrosis	Occasional inflammatory cells
2	Moderate	Swelling with inflammatory infiltration	Inflammatory infiltration of the sinusoids, degeneration and necrosis of hepatocytes	Inflammatory infiltration
3	Severe	Intense infiltration and edema	Intense infiltration of sinusoids, separation of liver cell plates, widespread degeneration and necrosis of hepatocytes	Intense infiltration

rejection, highly elevated blood enzyme levels and histologically severe rejection persist until the death of the recipient. In nonrejecting rat strain combinations, an immune response is also observed; moderately elevated enzyme levels and histologically identified rejection appear by the 2nd week and then return to normal by the 4th week [18]. Moreover, the outcome of liver allografting reflects the level of responsiveness of different recipient strains to a defined combination of major and minor histocompatibility antigens.

The basis of differences in competitive processes of rejection or tolerance induction still remains obscure. Even though the rejection is initiated successfully in some cases, the response does not lead to a complete rejection. Different mechanisms have been proposed to be involved in tolerance induction, among them: peripheral deletion of donor-specific T cells [20]; nondeletional mechanisms of functional inactivation or clonal anergy [2, 26, 31] involving altered regulation of costimulatory signaling pathways [13, 20, 27, 29] and cytokine expression [1, 9]; veto mechanism [34]; specific active suppression mediated by suppressor T cells [3, 4, 16] or suppressive cytokines [24, 33]; loss of antigenicity of the graft due to low expression of MHC antigens [28] or to the involvement of blocking anti-MHC antibodies, soluble MHC antigen-antibody complexes [32], and anti-idiotypic antibodies against MHC-specific alloantibodies [22]; and the establishment of long-term cellular microchimerism [30]. The mechanism of liver allograft rejection is a complex process that presumably involves interactions between multiple T-lymphocyte subpopulations. No information is available regarding the involvement of $CD4^+$ and $CD8^+$ T cells in the rejection process of liver allografts. The aim of this study was to investigate the roles of $CD4^+$ and $CD8^+$ T-cell subpopulations in the rejection of orthotopic liver grafts between inbred rat strains that tolerate liver allografts and that differ at both the major histocompatibility complex (RT1) and minor histocompatibility genes.

Materials and methods

Liver transplantation

Lewis (LEW), Brown Norway (BN), and PVG rats were purchased from Harlan Sprague Dawley (Indianapolis, Ind.) and were housed in a conventional facility under protection of isolator cages and laminar flow racks. Orthotopic liver transplantation (OLT) with arterial reconstruction was performed with LEW ($RT1^l$) rats as recipients and BN ($RT1^n$) as donors. The recipient animals were divided into three groups: (1) untreated control rats ($n = 10$), (2) rats treated with anti-CD8 antibody ($n = 6$), and (3) rats treated with anti-CD4 antibody ($n = 6$). For comparison of donor-specific alloreactivity in vitro, syngeneic LEW-to-LEW liver graft recipients and normal nontransplanted LEW rats were examined as responders and PVG spleen cells were used as third party stimulators.

Liver graft rejection monitoring

Recipient animals were monitored for rejection for 28 days on the basis of two criteria: (1) serum levels of aspartate aminotransferase (AST) at 3-day intervals and (2) liver biopsies at weekly intervals. Biopsy specimens from grafted livers were taken at laparotomy and formalin-fixed and paraffin-embedded. The severity of rejection was evaluated using histological criteria for rat liver allograft rejection [17] graded on a scale from 0 to 12. Attention was focused on four areas, each of which was graded from 0 to 3. A total of 12 points

Table 2 AST Levels (U/l) in control and depleted rat groups. Values represent mean ± SEM

	Day 6	Day 21	Day 28
Control group $n = 10$	790 ± 210	716 ± 150	542 ± 72
CD8-depleted group $n = 6$	596 ± 303	1164 ± 151	1127 ± 181[a]
CD4-depleted group $n = 6$	1080 ± 320	912 ± 88	890 ± 281

[a] $P = 0.009$ vs control group on day 28

(pts) was possible, which described the maximal lesions (Table 1). Rejection was evaluated as severe (10–12 pts), moderate (8–10 pts), or mild (< 8 pts). Histological analysis was performed blinded.

The animals were monitored for vascular thrombosis or biliary stricture at the time of biopsy or sacrifice to exclude animals with these complications from the study.

Monoclonal antibodies

T-cell depletion in vivo was accomplished by i. v. administration of monoclonal antibodies (mAbs) specific for rat $CD8^+$ cells (mAb OX-8) [6] and rat $CD4^+$ cells (mAb W3/25) [38]. Hybridoma cells secreting the antibodies were grown in tissue culture and injected into pristine-primed BALB/c-nu/nu mice to produce ascites. The protein concentration of ascites fluid was quantitated and 3 mg was injected i. v. 1 day prior to transplantation, followed by weekly administration of 3 mg W3/25 and 1.5 mg OX-8. Depletion of specific T-cell populations was confirmed by flow cytometric analysis of peripheral blood lymphocytes 24 h after each antibody injection. FITC-labeled conjugates of W3/25 and OX-8 antibodies were purchased from Serotec (Cambridge, UK).

Mixed lymphocyte cultures (MLC)

The animals were sacrificed after 28 days, and lymphocytes from spleens and livers were isolated and examined for donor-specific alloreactivity. Livers were briefly perfused in situ with Ca^{++}- and Mg^{++}-free Krebs solution and then harvested. Pieces of these livers were pressed through 100-μm pore size nylon mesh into RPMI cell culture medium (GIBCO, Grand Island, N. Y.) containing 50 μg/ml gentamicin. The lymphocytes were purified on a Ficoll-Diatrizoate gradient, density 1.094 (Lympholyte-Rat, Cedarline, Ontario, Canada). Priming of recipient animals and normal, nontransplanted LEW rats was accomplished by intraperitoneal injection of 5×10^7 allogeneic rat spleen cells from the donor strain 1 week before sacrifice.

MLCs were performed according to the technique described previously with slight modifications [36]. Briefly, responder splenic or liver-infiltrating lymphocytes were incubated with irradiated allogeneic or syngeneic stimulator spleen cells. Responder and stimulator cells were incubated at 5×10^5 cells/well in microplates in RPMI 1640 medium supplemented with 5% fetal calf serum, 200 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 50 μg/ml gentamicin. The cells were pulsed after 4 days with 2 μCi/well of ^{3}H-thymidine and harvested onto glass fiber filter paper after an additional 24-h incubation. The mean (± SD) cpm of ^{3}H-thymidine uptake was calculated from quadruplicate wells for each combination. Control cultures contained responder cells alone or responder cells and syngeneic stimulator cells. MLC reactivity was expressed as Δ cpm = cpm allogeneic stimulation – cpm syngeneic stimulation.

Statistical methods

We compared the AST levels and the histology scores for rejection within each experimental group using a one-way analysis of variance with repeated measures in time with Bonferroni adjustment. To compare the measurements between groups at different time points, we used a two-sample t-test.

Results

Orthotopic liver transplantation was performed with BN rats as donors and LEW rats as recipients. The animals were divided into three groups: control recipients, recipients treated with anti-CD8 antibody, and recipients treated with anti-CD4 antibody. There were no biliary or vascular complications in any of the recipients.

In the untreated control group, an elevation of AST levels was found to peak at day 6 (AST 790 ± 210 U/l) and persisted until day 28, when the animals were sacrificed (AST 542 ± 72 U/l; Table 2). Individual values of AST levels in control OLT recipients are presented in Fig. 1. Histological signs of rejection were observed on day 9, showing severe rejection that changed to moderate rejection about day 21 and to mild rejection by the end of the observation period. Table 3 summarizes the severity score for rejection according to the scale presented in Table 1.

Recipient rats that were treated with the anti-CD8 mAb also demonstrated a significant elevation in AST within 6 days (AST 596 ± 303 U/l) which, unlike in the control recipients, continued to rise sharply throughout the 28-day observation period (AST 1127 ± 181 U/l, $P = 0.009$ vs control group on day 28; Table 2, Fig. 2). Liver biopsies showed mild rejection at day 9 and moderate rejection at day 21, which persisted through day 28. In the CD8-depleted recipient rats, rejection on day 9 was less severe than in the control group; however, the rejection did not show a declining course as it did in the control group on days 21–28 (Table 3). Distinct patterns of rejection, which may not be reflected in the rejection score, were not observed in any of the groups.

Recipient rats that were treated with the anti-CD4 mAb showed a time course of AST elevation not significantly different from that observed in the control group (Table 2, Fig. 3). Liver biopsy examination revealed that the onset and severity of rejection in CD4-depleted rats followed the same time course as that of the control group (Table 3). However, anti-CD4 treatment did not result in 100% depletion of $CD4^+$ cells in the peripheral blood as compared to complete elimination of $CD8^+$ cells following anti-CD8 treatment. As

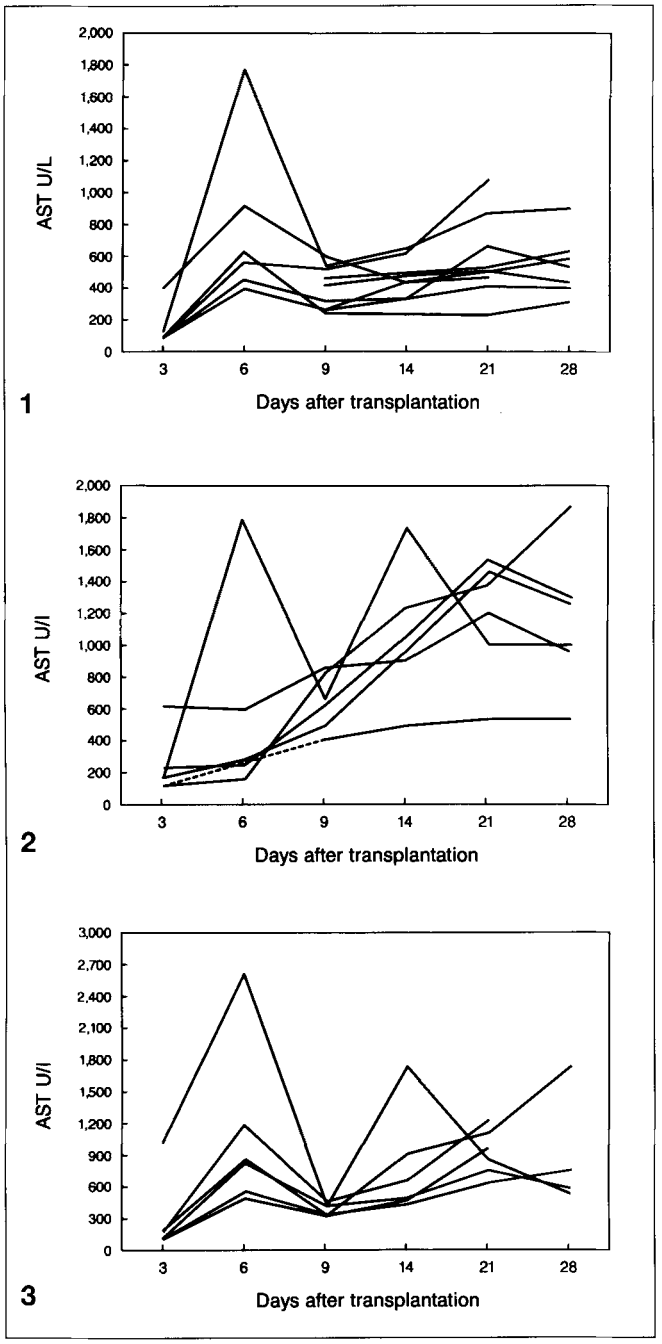

Fig. 1 AST levels in untreated LEW recipients of BN liver transplants

Fig. 2 AST levels in CD8-depleted LEW recipients of BN liver transplants

Fig. 3 AST levels in CD4-depleted LEW recipients of BN liver transplants

Table 3 Histological course of rejection in liver biopsies in control and depleted OLT rat groups

	Days after OLT	Signs of rejection/Severity score (mean ± SEM, range 1–2 points)
Control	9	Severe (10.8 ± 0.2)
BN-to-LEW OLT	21	Moderate (8.2 ± 0.5)
	28	Mild (6.8 ± 0.4)
CD8-depleted	9	Mild (6.3 ± 0.3)[a]
BN-to-LEW OLT	21	Moderate (8.3 ± 0.3)
	28	Moderate (8.3 ± 0.3)[b]
CD4-depleted	9	Severe (10.3 ± 0.6)
BN-to-LEW OLT	21	Moderate (9.2 ± 0.7)
	28	Mild (7.0 ± 0.4)[a]

[a] $P < 0.001$ vs control group on day 9; $P = 0.02$ vs day 21 & 28
[b] $P = 0.033$ vs control group on day 28

shown in Table 4, the depletion of $CD4^+$ cells averaged 75 %, and the intensity of the fluorescence of the remaining $CD4^+$ peak on flow cytometry was lower than in non-W3/25-treated rats.

The liver graft recipients were examined at the end of the observation period for donor-specific reactivity against stimulator BN spleen cells in a primary MLC proliferation assay. The results of ^{3}H-thymidine incorporation of splenocytes of the responders and control animals are shown in Fig. 4. Spleen cells from normal LEW rats showed a strong proliferative response against BN stimulator cells only after i.p. priming with BN spleen cells. Nondepleted allogeneic OLT recipients did not show a proliferative response in MLC against BN stimulators, and the response was not increased after i.p. injection of BN spleen cells. The proliferative response of BN-to-LEW OLT recipients primed in vivo with PVG spleen cells was not higher against PVG stimulator cells in MLC than the response of BN-primed BN-to-LEW OLT recipients to BN stimulators. Syngeneic LEW-to-LEW OLT recipients demonstrated significantly higher proliferative activity than recipients of allografts only after priming ($P = 0.018$ vs untreated recipients; $P = 0.021$ vs CD8-depleted recipients). Surprisingly, they exhibited lower proliferative activity than primed, normal LEW rats (10 % of the response). Neither CD4- nor CD8-depleted rats responded at this point in time, even after spleen cell priming.

The proliferative response of lymphocytes isolated from transplanted livers was examined in order to identify possible differences in donor-specific responsiveness between spleen cells and liver-infiltrating cells. They showed a response not significantly different from spleen cells (Fig. 4).

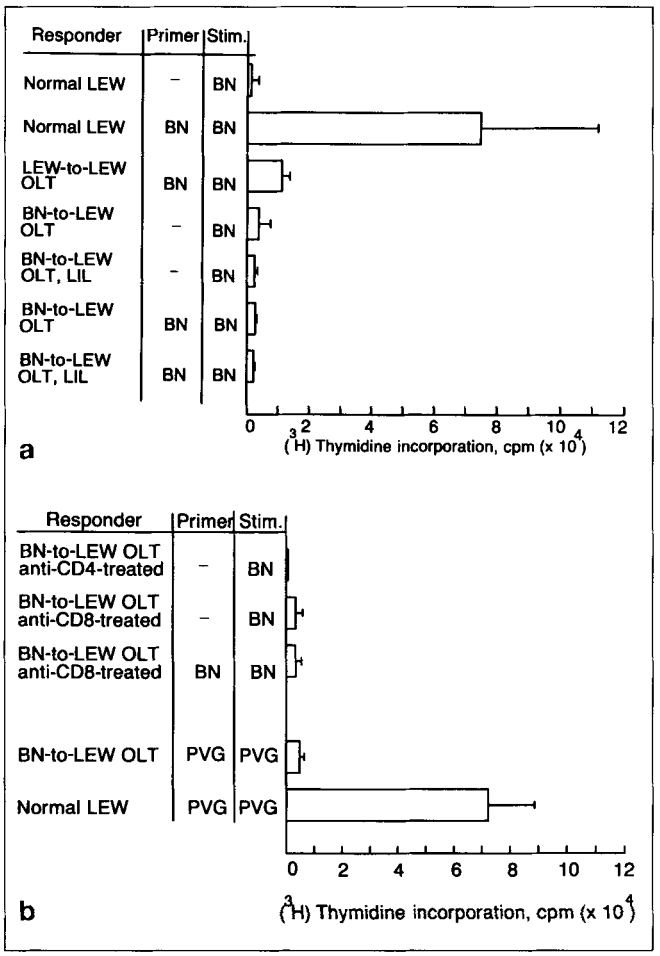

Fig. 4 **a** Reactivity of spleen and liver-infiltrating lymphocytes (LIL) from liver-grafted BN-to-LEW rats in MLC proliferation assay after stimulation with BN spleen cells. **b** Reactivity of spleen cells from liver-grafted BN-to-LEW rats in MLC proliferation assay after stimulations with BN and PVG spleen cells

Discussion

The role of T-cell subpopulations in rejection or nonrejection may be different in different RT1-incompatible strain combinations. Also, different requirements exist for T-cell subsets in the in vivo and in vitro responses to single and multiple minor histocompatibility antigens [37]. Numerous strategies for manipulation of T-cell responses with monoclonal antibodies have been developed with implications for experimental and clinical organ transplantation [35]. Anti-CD4 and, to a lesser degree, anti-CD8 therapy have been reported to induce tolerance in mice to MHC-mismatched heart allografts and xenografts [8]. The role of $CD8^+$ T cells in the rejection of class I MHC disparate allografts, in mediating signals in the generation of MHC-restricted cytolytic effector cells, and in generating suppression of $CD8^+$ T-cell responses is still controversial [3, 5, 21, 23].

Table 4 The effect of in vivo W3/25 and OX-8 treatment of OLT recipient rats on percentages of $CD4^+$ and $CD8^+$ T cells determined by flow cytometry

Recipient rats	Day	Test antibody	Percentage of specific fluorescence (peak fluorescence intensity)
CD4-treated	0	CD4	61.3 ± 2.8 (507 ± 42)
		CD8	20.5 ± 0.2 (623 ± 58)
	7	CD4	14.6 ± 2.5 (388 ± 12)
		CD8	43.7 ± 4.2 (607 ± 57)
	14	CD4	15.1 ± 0.9 (400 ± 12)
		CD8	51.8 ± 2.22 (614 ± 44)
	28	CD4	12.5 ± 2.2 (357 ± 27)[a]
		CD8	52.4 ± 2.3 (641 ± 39)
CD8-treated	0	CD4	57.9 ± 1.5 (488 ± 50)
		CD8	18.2 ± 0.4 (488 ± 50)
	7	CD4	77.2 ± 1.6 (488 ± 36)
		CD8	0.7 ± 0.4 (–)[b]
	14	CD4	80.1 ± 2.4 (484 ± 36)
		CD8	1.1 ± 0.6 (–)[b]
	28	CD4	73 ± 3.7 (597 ± 21)
		CD8	0.8 ± 0.3 (–)[b]
Untreated controls	28	CD4	61.8 ± 1.5 (588 ± 14)
		CD8	19.9 ± 1.1 (647 ± 53)

[a] $P = 0.002$ vs no treatment group and 0.04 vs day 0
[b] No discernable peak can be seen

The results of this study suggest that the course of rejection of BN liver grafts by LEW recipients is modulated by $CD8^+$ cells in a negative manner. As the liver grafts in this strain combination are generally accepted [11, 39], the initially strong immune response can be modulated and tolerance induction can occur.

During the observation period of 4 weeks, LEW recipients of BN liver allografts demonstrated biochemical signs of persistent liver damage and morphological signs of declining rejection. However, CD8-treated recipients demonstrated different patterns of rejection. Initially, they had less severe rejection than control or CD4-treated recipients; at a later time, they showed increased rejection as defined by a progressive rise in liver enzyme levels and persisting liver damage as assessed by liver histology. The removal of $CD8^+$ cells seems to block the rejection in the early post-transplant period. This blockage is short-lived and can be explained by the predominant involvement of cytotoxic $CD8^+$ cells in the early post-transplant period. Later on, the removal of $CD8^+$ cells seems to increase the severity of rejection at the same time that it declines in the control group. This observation suggests that $CD8^+$ cell depletion may eliminate a specific subpopulation that modulates graft rejection.

The declining rejection in the control group may involve a peripheral deletion or anergy of donor-specific T cells provoked by a graft incapable of generating a second signal for activation once its passenger antigen-presenting cells have migrated out of the graft [20]. The

results of this study may imply that $CD8^+$ cells play an important role in tolerance induction during the initial immune response. Possible mechanisms may include the induction of anergic T cells or T cells on their way to apoptotic cell death, which have suppressive characteristics [19], and/or the induction of active, negative regulatory cells [25]. Further studies of $CD8^+$ cell-depleted recipient rats, using adoptive cell transfer techniques, are needed to substantiate the hypothesis that $CD8^+$ cells inhibit the immune response to orthotopic liver transplants between inbred rat strains.

The need for total elimination of $CD4^+$ T cells to achieve immunosuppression is still under investigation. Depleting and non-depleting CD4-specific mAb treatment regimens have been reported to produce a comparable state of tolerance [7, 10]. In our study, the remaining $CD4^+$ cells in CD4-treated animals did show decreased fluorescence intensity during the flow cytometry analysis using FITC-labeled W3/25 antibody, suggesting blocking of surface CD4 by the in vivo W3/25 antibody treatment. Despite this, our results demonstrate no effect of 75% CD4 depletion induced by W3/25 mAb treatment on the course of rejection. Taking into consideration the difficulties in totally depleting rat $CD4^+$ cells in vivo [5], we cannot, at this point, evaluate the requirement for $CD4^+$ cells for donor responsiveness in this rat strain combination.

It has been reported that in the BN-to-LEW combination, MLC proliferative responses of splenic lymphocytes from liver graft recipients were nonspecifically suppressed in the first 30 days after grafting and restored to normal at a later time [12]. MLC reactivity of Peripheral blood lymphocytes (PBL) from tolerant LEW or BN recipients of $(LEWxBN)F_1$ liver allografts has demonstrated a wide variation in individual rats and in time [15]. Our results show a lack of proliferative responses in MLC of splenic and liver lymphocytes of LEW recipients of BN liver grafts 4 weeks after transplantation, even after in vivo priming with allogeneic lymphocytes. Whether this response would be restored later is still to be determined. The lack of response of CD4-depleted rats is not surprising since the majority of proliferating cells in culture would be expected to be $CD4^+$. However, we were unable to demonstrate any difference in proliferative response of CD8-depleted rats at this point in time after transplantation. The immune response to certain RT1 alloantigens might be more suppressible than to others. Our in vitro proliferation assay results demonstrated strong nonspecific suppression of the immune response in the BN-to-LEW strain combination with modulation of the proliferative response to third party PVG cells. Despite biochemical and histological data of ongoing rejection in CD8-depleted rats 28 days after transplantation, no lymphocyte proliferation in vitro could be demonstrated. The assay for proliferation in vitro may not be a reliable estimate of in vivo reactivity at this time point. The reduced proliferative responses 28 days postgrafting most likely represent nonspecific suppression in that first, third party PVG cells do not stimulate higher proliferation than BN and, second syngeneic LEW-to-LEW responders exhibit lower proliferation with BN stimulator cells than normal LEW rats. In order to investigate the onset and the specificity of this phenomenon, it will be important to follow the proliferative response of CD8-depleted recipients at different time points after transplantation. These experiments are currently in progress.

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