Blockade of the integrin α L β 2 but not of integrins α 4 and/or β 7 significantly prolongs intestinal allograft survival in mice

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Abstract

Background—Small bowel transplantation remains a difficult therapeutic option endangered by a high rate of rejection and infectious complications. To improve these clinical results, it is mandatory to set up animal models to test alternative immunosuppressive regimens which may lead to immunotolerance.

Aims—To determine the value of blockade of α L β 2 (LFA-1) and α 4 and β 7 integrins (α 4 β 1, α 4 β 7, and α E β 7) in the prevention of rejection of fetal small bowel grafts in mice and the effect of the association of calcineurin dependent drugs in anti-LFA-1 treated mice.

Methods—Adult recipient mice engrafted with allogeneic fetal small bowel received a short course of anti- α 4 and/or anti-LFA-1 monoclonal antibodies (mAb) with or without FK506 or cyclosporin A. In addition, in a set of experiment, β 7 $^{-/-}$ mice were used as recipients. Graft biopsies were performed and processed for standard histology.

Results—Blockade of the pathways of the integrins α 4 and β 7 had a modest or no effect on intestinal graft survival. In contrast, transitory, short administration of anti-LFA-1 monoclonal antibody alone, when started before engraftment (day -1), allowed long term survival of intestinal grafts, even when associated with calcineurin dependent drugs. However, early withdrawal of FK506 reversed the immunosuppressive effect of anti-LFA-1 treatment.

Conclusion—These results suggest that firstly, anti-LFA-1, but not anti- α 4 mAb treatment, may be useful in improving the results of intestinal transplantation, and secondly, that this treatment is not incompatible with long term administration of tacrolimus currently used in the prevention of small bowel graft rejection in humans.

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Intestinal transplantation is a potential alternative to total parenteral nutrition for the treatment of chronic intestinal failure. The advent of cyclosporin A (CyA)¹ and particularly the powerful immunosuppressant tac-

rolimus (FK506 or Prograf) resulted in a significant increase in survival rates and thus allowed the development of this organ transplantation in several centres.^{2,3} However, rejection and infection remain two major contributors to morbidity and mortality in these patients. The international survey data reported by D Grant in 1995 involving 180 intestinal transplants in 170 patients showed three year graft survival rates with tacrolimus of 47% for the intestine alone, 40% for the liver-small bowel, and 43% for multivisceral grafts.⁴ The mortality rate was high (49%) as a result of infectious complications (42%) and/or multivisceral failure (30%). More recent data from the International Transplant Registry did not show further improvement.⁵ The high level of integration of the lymphoid system associated with the gut (GALT: gut associated lymphoid tissue) and the intrinsic septic nature of this organ may account for the difficulties encountered when transplanted. Thus the development of safe methods for achieving tolerance across major histocompatibility barriers remains a major goal of research in transplantation immunology and particularly in the case of intestinal transplantation.

Two members of the integrin family are potential targets for immunosuppression in intestinal transplantation. The first is LFA-1, a member of the β 2 integrin family expressed on all leucocytes. Via its interaction with intercellular cell adhesion molecule (ICAM) type 1 and 3, LFA-1 provides key accessory signals during T cell activation and thereby plays a critical role in a range of leucocyte functions, such as immunoglobulin and lymphokine production and induction of T cell mediated target lysis.^{6,7} Furthermore, via its interaction with the ligands ICAM-1 and 2 on endothelial cells, LFA-1 also plays a key role during leucocyte extravasation.⁸ In a previous study, we demonstrated that a non-depleting anti-LFA-1 antibody efficiently protected against rejection of major histocompatibility complex (MHC) incompatible heart and bone marrow in a mouse model.⁹ The importance of the LFA-1 molecule in the prevention of graft rejection was further emphasised in other experimental

Abbreviations used in this paper: CyA, cyclosporin A; ICAM, intercellular cell adhesion molecule; GALT, gut associated lymphoid tissue; LP, lamina propria; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; VCAM-1, vascular cell adhesion molecule 1; IL-2, interleukin 2; NFAT, nuclear factor of activated T cells; MHC, major histocompatibility complex.

studies as well as in clinical observations in bone marrow and renal transplantation.^{10, 11}

Another potential target for immunosuppression in small bowel transplantation is an integrin $\alpha 4 \beta 7$ chain which associates with either $\beta 1$ or $\beta 7$. $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ are expressed on almost all leucocytes and bind to fibronectin, a matrix protein, and to vascular cell adhesion molecule 1 (VCAM-1) on inflamed vessels. In addition, $\alpha 4 \beta 7$ binds to MadCAM-1, its main ligand expressed on intestinal vessels of Peyer's patches, mesenteric lymph nodes, and in lamina propria (LP).^{12, 13} Hence $\alpha 4 \beta 7$ plays a major role in the migration of lymphocytes into the GALT and LP. This was definitively demonstrated by generation of $\beta 7^{-/-}$ mice which exhibited an overall hypoplasia of the GALT.¹⁴

In the present work we have studied the effect of transitory administration of anti-LFA-1 monoclonal antibody (mAb), anti- $\alpha 4$ mAb, or both, on the prevention of rejection of fetal small bowel grafts in fully MHC incompatible mice. The value of disruption of $\beta 7$ mediated migration and activation pathways was also tested using $\beta 7^{-/-}$ recipient mice. Finally, the effect of CyA and FK506 on immunosuppression induced by anti-LFA-1 mAb treatment was evaluated.

Material and methods

MICE

Pregnant female (gestational age 16–20 days) and adult male (8–10 weeks old) C57BL/6 (H-2^b) mice were purchased from CDTA (CNRS Orleans, La Source, France). Pregnant female and adult male (8–10 weeks old) C3H/He (H-2^k) mice were obtained from the Centre d'Élevage R Janvier (Le Genest Saint Isle, France) and IFFA-CREDO (L'Arbresle, France), respectively. Adult C57BL/6 (H-2^b) $\beta 7^{-/-}$ mice (8–12 weeks old) were obtained from Dr N Wagner. C3H/He(H-2^k) were used as donors for syngeneic controls and for $\beta 7^{-/-}$ recipient mice. In all other cases C57BL/6 (H-2^b) were used as intestine donors and C3H/He (H-2^k) as recipient mice. All mice were raised in our animal facility in conventional conditions in accordance with the guidelines of the National Institutes of Health's *Guide for the care and use of laboratory animals* (Bethesda, Maryland, USA).

IMMUNOSUPPRESSIVE DRUGS

CyA (kind gift from Sandoz, Bâle, Switzerland) was given for 14 days (5 and 20 mg/kg/day) via a subcutaneous Alzet osmotic pump (model No 2002, Alza Corporation, Charles River, France) which was implanted at the time of grafting and removed 21 days after transplantation. Serum levels of CyA were assessed in three animals from each group who received CyA alone from samples obtained by retro-ocular puncture (TDx, whole blood monoclonal cyclosporin, Abbott Laboratories, USA).

Tacrolimus (FK506) was kindly provided by Fujisawa GmbH (Dr Murato, Munich, Germany). Lyophilised tacrolimus was diluted in sterile saline serum and administered as 0.5 ml

intraperitoneally at a dose of 1 mg/kg/day on post-transplant days 0–7. Serum levels of tacrolimus were measured in three animals who received tacrolimus alone from samples obtained by retro-ocular puncture (INx, Tacrolimus II, Abbott Laboratories, USA).

MONOCLONAL ANTIBODIES

Production

Hybridomas for anti-CD11a mAb (clone M17/4.411.9, rat IgG2a κ anti-mouse CD11a mAb) and anti- $\alpha 4$ mAb (clone PS/2, rat IgG2b κ anti-mouse mAb) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) grown in RPMI 1640 (GIBCO, Grand Island, New York, USA) supplemented with 10% fetal calf serum and 30 μ g/ml of gentamycin, and injected into Pristane primed nu/nu six week old Swiss mice (IFFA-CREDO) to obtain ascites. Anti-CD11a and anti- $\alpha 4$ mAb were used as delipidated (Seroclear, Calbiochem, Meudon, France) ascites fluid. Protein concentration was estimated at 5 mg/ml and 2.5 mg/ml, respectively, using an enzyme linked immunosorbent assay (ELISA) according to standard procedures.

The anti-CD11a mAb was previously shown to inhibit LFA-1-mediated adhesion as it blocked the formation of conjugates in a cytotoxic cytolytic activity (CTL) mediated killing assay.¹⁵ In addition, similar to our previous study, a concentration of 1.25 μ g/ml of anti-LFA-1 mAb inhibited the in vitro mixed lymphocyte culture (MLC) by 100%. Finally, this antibody was not depleting as no reduction in blood leucocyte counts was observed after in vivo treatment of mice with a total dose of 0.5 mg (data not shown).⁹

The blocking effect of anti- $\alpha 4$ mAb was examined in an adhesion assay using the AKR/Cum TK1 cell line, selected for its property to strongly express $\alpha 4$.¹⁶ Briefly, 10⁷ TK1 cells labelled with 5 μ g/ml of BCECF-AM (Calbiochem) were incubated with different dilutions of either anti- $\alpha 4$ ascites or control decomplexed rat serum for 15 minutes at 37°C. After two washes, cells were added to microwells precoated (18 hours at 4°C) with fibronectin (CS-1, Sigma) or bovine albumin (Sigma) (50 μ g/ml) and stimulated with 100 ng/ml of PMA (Sigma). After 30 minutes at 37°C, microwells were washed five times by plate inversion and the number of cells remaining bound to the plate was quantified using a Cytofluor (Cytofluor 2350, Millipore, St Quentin-en-Yvelines, France). A concentration of 25 μ g/ml of anti- $\alpha 4$ mAb fully inhibited adhesion of TK1 cells to fibronectin by 100%.

Flow cytometry

Saturation and modulation of receptor expression were analysed by flow cytometry on peripheral leucocytes of recipient mice during treatment with mAb. Non-treated control mice were analysed at the same time. Briefly, 50 μ l of blood harvested from the retro-orbital sinus were incubated with a 1:200 dilution of fluorescein conjugated mouse anti-rat IgG mAb (Jackson Laboratories, Baltimore, Penn-

sylvania, USA) (observed saturation) preceded or not by an incubation step with saturating concentrations of anti-LFA-1 (clone M17/4.411.9, PharMingen) or anti- $\alpha 4$ (used as ascites) mAb (maximal saturation). For negative controls, cells were incubated with isotype matched irrelevant mouse anti-human mAb. Saturation was defined as follows:

$$\frac{\text{mean fluorescence of observed saturation} - \text{autofluorescence}}{\text{mean fluorescence of maximal saturation} - \text{autofluorescence}}$$

Modulation was defined as follows:

$$\frac{(\text{mean fluorescence of maximal saturation} - \text{autofluorescence}) \text{ in non-treated mice}}{(\text{mean fluorescence of maximal saturation} - \text{autofluorescence}) \text{ in treated mice}}$$

All LFA-1 receptors were bound by the mAb during treatment whereas 60% were occupied 11 days after the last injection. Down modulation of LFA-1 receptors was calculated at 50% on day 3, 20% on day 13 (two days before the last injection), and 15% on day 26, 11 days after stopping the treatment. Study of the saturating conditions during treatment with anti- $\alpha 4$ showed that 100% of the receptors bound the anti- $\alpha 4$ mAb on day 3 and about 50% on day 13.

INTESTINAL TRANSPLANTATION AND HISTOLOGICAL EXAMINATION

Surgical procedures

Fetal small intestines were harvested, divided into a proximal and a distal segment, and transplanted into the space between the peritoneum and the right and left rectus abdominis, respectively, of adult recipient mice. Surgical procedures were performed aseptically after intraperitoneal anaesthesia with a mixture of ketamine (Imalgene, Rhône Mérieux, France), xylazine (Rompun, Bayer, Germany), and atropine (Meram, France). Animals that died within two days of transplantation were excluded from further analysis. Monoclonal antibodies were administered intraperitoneally to recipients at different doses according to different schedules.

Groups of animals

In the first large set of experiments displayed in fig 2, C3H/He (H-2^b) recipients were grafted with syngeneic fetal intestines (n=24) or with allogeneic C57BL/6 (H-2^b) fetal intestines left untreated (n=11), or were treated intraperitoneally with antibodies according to different regimens. Anti- $\alpha 4$ antibody was given at a dose of 70 μ g/day from day -1 to day 9 (n=5) or from day -1 to day 15 (n=5). Anti-LFA-1 antibody was given at a dose of 70 μ g/day from day -1 to day 9 (n=10), from day -1 to day 15 (n=5), and from day 0 to 9 (n=8). Both antibodies were also given together at the same respective doses from day -1 to day 15 (n=7). In the second set of experiments included in fig 2A, wild-type (n=8) or $\beta 7^{-/-}$ (n=19) C57BL/6

mice received C3H/He fetal intestinal grafts. In the third set of experiments (fig 3A), C3H/He recipients were grafted with allogeneic C57BL/6 fetal intestines and treated intraperitoneally with anti-LFA-1 antibody alone at a dose of 70 μ g/day from day -1 to day 15 (n=13), with CyA alone at 20 mg/kg/day via a subcutaneous osmotic pump (see above) (n=9), or with both anti-LFA-1 antibody and CyA (n=24). In the fourth set of experiments (fig 3B), C3H/He recipients were grafted with allogeneic C57BL/6 fetal intestines and treated intraperitoneally with anti-LFA-1 antibody alone at a dose of 70 μ g/day from day -1 to day 15 (n=8), with FK506 alone given intraperitoneally as 1 mg/kg/day from day 0 to 7 (n=21), or with both anti-LFA-1 antibody and FK506 from day 0 to 7 (n=21), or from day 0 to 30 (n=9).

Graft monitoring

Recipients were examined daily and biopsies of both proximal and distal grafts were performed either on days 5, 10, and 21 or on days 7, 14, and 30 in a random manner in each group. Additional biopsies were performed systematically on days 45, 60, and 90 in all recipients when the graft remained available at that time. Graft samples were fixed in 10% formalin and embedded in paraffin. Sections (4 μ m) were stained with haematoxylin and eosin for examination of graft morphological features and cellular infiltration.

Rejection was defined as follows: stage 1, absence of signs of rejection; stage 2, mononuclear cell infiltration of the LP without epithelial damage; stage 3, mononuclear cell infiltration of the LP with crypt epithelial damage but normal villous height; stage 4, LP polymorphic cell infiltration (mononuclear and polymorphonuclear cells) extended into the muscular layer, extensive gland destruction, and total villous atrophy. Histological findings were evaluated semiquantitatively in a blind manner by two independent observers (SS, FA). Grafts were considered as rejected when a histological score of rejection was 3 or more.

Statistical analysis

In each group, graft survival rate was calculated according to the Kaplan-Meier method. The log rank test was used to compare the graft survival period in each group. Differences were considered to be statistically significant at a confidence interval of 95% ($p < 0.05$).

Results

VALIDATION OF THE MODEL OF NON-VASCULARISED FETAL SMALL BOWEL TRANSPLANTATION

C3H/He animals grafted with allogeneic C57BL/6 fetal small bowel who received no treatment (n=11) showed consistent rejection of their grafts when biopsied on day 14. At this time, lesions were very severe with disappearance of epithelial cells and massive infiltration by mononuclear and polymorphonuclear cells (grade 4) (figs 1A, 2). In the reverse strain combination (C3H/He donor-C57BL/6 re-

Figure 1 Histology of intestinal grafts in mice treated with anti- $\alpha 4$ and anti-LFA-1 monoclonal antibodies (mAb). Paraffin embedded tissue sections stained with haematoxylin and eosin from: (A) grade 4 rejection of C57BL/6 fetal small bowel graft in untreated C3H/He mice on day 7; (B and C) syngeneic C57BL/6 fetal small bowel graft in C57BL/6 mice on day 14 (B) and day 60 (C); (D) grade 3 rejection of C57BL/6 fetal small bowel graft in C3H/He mice treated with anti- $\alpha 4$ mAb (0.7 mg of the total dose given from day -1 to +15) on day 7; (E and F) tolerated C57BL/6 fetal small bowel grafted in C3H/He mice receiving anti-LFA-1 mAb (0.5 mg of the total dose from days -1 to +9) on day 14 (E) and day 60 (F).

recipient, $n=8$), lesions of rejection were similar but all grafts were rejected after day 7 (fig 2A).

In recipients who received syngeneic C3H/He grafts ($n=24$), normal intestinal development with no sign of rejection was observed, irrespective of the day of biopsy. Seven animals were followed until postoperative day 60 and showed normal morphology of small bowel grafts. At this time, development of lymphoid follicles that were initially absent in the fetal graft were observed in the LP of some grafts (data not shown).

BLOCKADE OF THE INTEGRINS $\alpha L\beta 2$ (LFA-1), $\alpha 4$, AND $\beta 7$ (FIG 2)

Effects of $\alpha 4$ and $\beta 7$ blockade (fig 2A)

Only long term administration of anti- $\alpha 4$ (0.7 mg of the total dose given on days -1, 0, +1, +3, +5, +7, +9, +11, +13, +15; $n=5$) allowed a small but significant prolongation of graft survival compared with untreated recipients ($p=0.008$): 70% of animals rejected their graft on day 14 compared with 100% of untreated recipients. Furthermore, in biopsies performed on day 14, grading of rejection did not exceed 3 in $\alpha 4$ treated animals whereas it was 4 in all grafts performed in untreated recipients (figs 1D, 2A). Examination of saturating conditions during treatment with anti- $\alpha 4$ showed that 100% of the receptors bound the anti- $\alpha 4$ mAb on day 3 and about 50% on day 13 (data not shown). When given according to the short regimen (0.5 mg of the total dose given on days

-1, 0, +1, +3, +5, +7, +9; $n=5$), a transient protective effect was observed on day 7 but 70% of the grafts were rejected on day 14 (grade 4), a result not significantly different from that observed in untreated animals ($p=0.08$) (fig 2A).

Finally, survival of C3H/He (H-2^b) fetal small bowel allografts was studied in $\beta 7^{-/-}$ recipient mice (H-2^b) ($n=19$). Although rejection seemed to be slightly delayed, graft survival was not significantly different from that observed in wild-type C57BL/6 (H-2^b) recipient mice receiving C3H/He fetal small bowel allografts ($n=8$; $p=0.18$) (fig 2A).

Effect of $\alpha L\beta 2$ (LFA-1) blockade (fig 2B, C)

Animals treated with anti-LFA-1 mAb alone showed significant prolongation of allograft survival ($p=0.001$) (fig 1E, 1F, 2B). Results were similar irrespective of the dose or time of administration of the treatment (0.5 mg of the total dose given on days -1, 0, +1, +3, +5, +7, +9 ($n=10$) *v* 0.7 mg of the total dose given on days -1, 0, +1, +3, +5, +7, +9, +11, +13, +15 ($n=5$); $p=0.43$) and graft survival was more than 45 days for nine of 26 animals treated with anti-LFA-1 (see below). When animals received anti-LFA-1 mAb treatment according to the long protocol but with omission of the day -1 injection ($n=8$), results were dramatically different ($p=0.001$): a slight prolongation of graft survival was observed but all grafts were rejected by day 14 (fig 2B).

Combined treatment with anti- $\alpha 4$ mAb (0.7 mg of the total dose) and anti-LFA-1 mAb (0.7 mg of the total dose) from day -1 to day +15 ($n=7$) did not improve the survival of the grafts compared with anti-LFA-1 mAb treated mice ($p=0.11$) but was more efficient than anti- $\alpha 4$ mAb treatment alone ($p=0.019$) (fig 2C).

EFFECT OF CALCINEURIN DEPENDENT DRUGS ON THE IMMUNOSUPPRESSIVE EFFECT OF ANTI-LFA-1 mAb (FIG 3).

Previous work has shown that tolerance induced by CTLA-4-Ig and anti-CD40L antibody is abrogated by administration of CyA.¹⁷ This led us to test the effect of calcineurin dependent drugs on the immunosuppressive effect of anti-LFA-1.

CyA was delivered systematically at a total dose of 20 mg/kg/day ($n=9$) via an osmotic pump from day 0 to day 14, a dose which provided variable serum concentrations but always within the therapeutic range (2100 (400) ng/ml on day 7 and 1350 (895) ng/ml on day 14). With CyA treatment alone, 70% of grafts were rejected on day 14 and 100% on day 30 (fig 3A). Mice treated with anti-LFA-1 mAb (0.7 mg of the total dose from day -1 to day +15) and CyA (20 mg/kg/day from day 0 to day 14) ($n=24$) had identical rejection patterns to those treated with anti-LFA-1 mAb alone ($n=13$) ($p=0.36$). Both treatments were more efficient than CyA alone ($n=9$; $p<0.001$). However, 70% of the grafts were lost after day 30 (fig 3A).

We then tested the effect of tacrolimus. The drug was first given intraperitoneally for seven days at 1 mg/kg/day ($n=21$), a dose that allowed

Figure 2 Effect of blockade of $\alpha L\beta 2$ (LFA-1), $\alpha 4$, and $\beta 7$ integrins on fetal intestinal allograft survival. (A) Only long term administration of anti- $\alpha 4$ mAb (0.7 mg of the total dose from day -1 to day +15) had a significant effect on allograft survival ($n=5$; $p=0.008$). The short regimen (0.5 mg of the total dose from day -1 to day +9) had no significant effect ($n=5$). In $\beta 7^{-/-}$ recipients, survival of the C3H/He fetal allografts ($n=19$) was slightly delayed but this was not significant compared with that in wild-type C57BL/6 recipient mice ($n=8$). (B) Anti-LFA-1 monoclonal antibody (mAb) treatment significantly prolonged graft survival, irrespective of the dose or time of administration (0.5 mg from day -1 to day +9 ($n=10$) or 0.7 mg from day -1 to day +15 ($n=5$); $p=0.001$ v untreated C3H/He recipient mice). Omission of the day -1 injection abrogated this immunosuppressive effect (0.43 mg of the total dose from day 0 to day +9 ($n=8$); $p=0.001$). (C) Combined treatment with anti- $\alpha 4$ mAb (0.7 mg of the total dose) and anti-LFA-1 mAb (0.7 mg of the total dose) did not improve the survival of the grafts compared with anti-LFA-1 mAb treated mice ($n=5$; $p=0.11$) but was more efficient than anti- $\alpha 4$ mAb treatment alone ($p=0.019$). The same group of animals treated with anti-LFA-1 antibody is shown in (B) and (C) which display experiments performed simultaneously. Results are expressed as cumulative per cent surviving, calculated according to the Kaplan-Meier method and compared using the log rank test.

serum concentrations within the therapeutic range (25 (5) ng/l). With this short treatment, 90% of recipients had rejected their grafts by day 14 and 100% by day 21 (fig 3B). In mice

Figure 3 Effect of calcineurin dependent immunosuppressive drugs on the tolerogenic effect of anti-LFA-1 monoclonal antibody (mAb). (A) Mice treated with anti-LFA-1 mAb (0.7 mg of the total dose from day -1 to day +15) and cyclosporin A (CyA) (20 mg/kg/day) via an osmotic pump for 14 days ($n=24$), and mice treated with anti-LFA-1 mAb alone ($n=13$; $p=0.36$) had identical rejection patterns. Both treatments were more efficient than CyA alone ($n=9$; $p<0.001$). (B) In mice treated with anti-LFA-1 mAb (0.7 mg of the total dose from day -1 to day +15) and tacrolimus (FK506 1 mg/kg/day) intraperitoneally from days 0 to 7 ($n=21$), graft survival was significantly increased compared with mice treated with tacrolimus alone ($n=21$; $p<0.001$). However, there was a significant decrease in graft survival a few days after withdrawal of tacrolimus ($p=0.004$). When tacrolimus (1 mg/kg/day) was given to anti-LFA-1 treated mice until day 30 ($n=9$), graft survival was not statistically different from that of mice treated with anti-LFA-1 alone ($n=8$; $p=0.21$) but all grafts were rejected a few days after withdrawal of the drug. Results are expressed as cumulative per cent surviving, calculated according to the Kaplan-Meier method and compared using the log rank test.

treated with anti-LFA-1 mAb (0.7 mg of the total dose from day -1 to day +15) and tacrolimus from days 0 to 7 ($n=21$), two observations were made. Firstly, graft survival was significantly increased compared with mice treated with tacrolimus alone ($p<0.001$). Secondly, there was a significant decrease in graft survival a few days after withdrawal of tacrolimus ($p=0.004$) (fig 3B). When tacrolimus (1 mg/kg/day) was given to anti-LFA-1 treated mice until day 30 ($n=9$), graft survival was not statistically different ($p=0.21$) from that of mice treated with anti-LFA-1 alone ($n=8$) but all grafts were rejected a few days after withdrawal of the drug (fig 3B).

Discussion

This study showed that blockade of the $\alpha 4\beta 1$ /VCAM and $\alpha 4\beta 7$ /MadCAM pathways by administration of anti- $\alpha 4$ mAb or disruption of $\alpha 4\beta 7$ and $\alpha E\beta 7$ receptors in $\beta 7^{-/-}$ recipient mice, respectively, had a modest or no effect on the survival of fetal allogeneic intestinal grafts.

In contrast, long term survival was obtained by blocking the LFA-1/ICAM-1 pathway by transitory, short term administration of anti-LFA-1 mAb alone. The immunosuppressive effect of the antibody when administered before engraftment (day -1) was not abolished by calcineurin dependent drugs. However, early withdrawal of FK506 reduced the effect of anti-LFA-1 treatment.

The $\alpha 4$ integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ are expressed on lymphocytes and bind to fibronectin and to VCAM-1, a receptor whose expression is upregulated during inflammation on endothelial vessels and antigen presenting cells. In addition, $\alpha 4\beta 7$ binds with high affinity to MadCAM-1, expressed mainly on high endothelium in Peyer's patches and mesenteric lymph nodes and on the flat endothelium of vessels in intestinal LP.¹⁸ A previous study in rats showed that anti- $\alpha 4$ antibody induced modest prolongation of survival of allogeneic islets.¹⁹ Furthermore, in mice, using the same anti- $\alpha 4$ antibody that we used, Isobe *et al* demonstrated significantly increased survival of cardiac allografts.²⁰ The recent demonstration in $\beta 7^{-/-}$ mice that the interactions between $\alpha 4\beta 7$ and MadCAM-1 play a major role in lymphocyte homing in the intestine¹⁴ suggests that blockade of these interactions may prevent adverse intestinal immune reactions. In agreement with this hypothesis, in vivo administration of anti- $\alpha 4$ antibodies could block the intestinal antihelminthic immune response in the intestinal mucosa of rats,²¹ and attenuate the colonic inflammation which develops spontaneously in the cotton-top tamarin^{22, 23} and the severity of an intestinal graft versus host reaction in mice.²⁴ Furthermore, in vivo use of an antibody directed against $\alpha E\beta 7$, the second $\beta 7$ integrin, significantly decreased mucosal inflammation in interleukin 2 (IL-2) $^{-/-}$ mice immunised with TNP-OVA.²⁵ In our model of intestinal transplantation, anti- $\alpha 4$ therapy allowed modest prolongation of graft survival. Furthermore, $\beta 7^{-/-}$ mice, which lack both $\alpha 4\beta 7$ and $\alpha E\beta 7$ integrins, rejected their intestinal graft with kinetics comparable with untreated wild-type mice. These negative results cannot be ascribed solely to the fetal origin of the grafts. Firstly, the MadCAM- $\alpha 4\beta 7$ pathway is likely functional in fetal intestine as strong expression of MadCAM-1 was observed in the fetal grafts before and during transplantation (data not shown). Furthermore, a preliminary report indicates that vascularised intestinal allografts are also rejected by $\beta 7^{-/-}$ mice (Kellersmann *et al*, unpublished data). The strong infiltrate observed in rejected grafts in $\beta 7^{-/-}$ and anti- $\alpha 4$ treated mice indicates that compensatory pathways, perhaps favoured by the ischaemia-reperfusion process, allowed lymphocytes to migrate into the allogeneic intestinal graft.

Blocking lymphocyte migration may also not be sufficient to prevent rejection. Notably, treatment with anti- $\alpha 4$ antibody, which interferes not only with migration into inflammatory sites but, to some extent, with interactions between T lymphocytes and antigen presenting cells, showed a modest but significant effect on

graft survival compared with $\beta 7^{-/-}$ recipient mice. Finally, the poor efficiency or lack of effect of blocking the $\alpha 4$ pathway contrasted with the striking effect of the anti-LFA-1 antibody.

The immunosuppressive effect induced by anti-LFA-1 mAb treatment has been reported by several groups, including ours, in experimental models of transplantation of bone marrow, heart, and pancreas islets. Recently, Kato and colleagues²⁶ reported long term survival of allogeneic fetal small bowel grafts in recipient mice receiving anti-LFA-1 treatment but this tolerogenic effect required simultaneous administration of anti-ICAM-1 mAb and at least four weeks of treatment. Here, we have demonstrated that a short course of anti-LFA-1 antibody alone is sufficient to induce significant prolongation of allogeneic intestinal grafts provided the treatment starts one day before engraftment. This latter result supports other *in vitro*⁷ and *in vivo*²⁷ studies where anti-LFA-1 mAb was unable to stop an ongoing allogeneic activation process.

The mechanisms underlying prevention of graft rejection by the anti-LFA-1 antibody are not yet elucidated. Its effect could not be ascribed either to cell depletion or to profound downregulation of LFA-1 surface expression.⁹ At the initial phase, anti-LFA-1 antibody may decrease the migration of neutrophils which occurs early during reperfusion after ischaemia²⁸ as well as migration of lymphocytes into the intestine.^{14, 29} Later on, when allograft rejection has induced an inflammatory reaction and thereby upregulation of its endothelial ligand ICAM-1,³⁰ the antibody may decrease recruitment of lymphocytes, monocytes, and neutrophils. However, the blocking effect of anti-LFA-1 antibody on leucocyte migration cannot easily explain the long lasting effect of the antibody several weeks or months after cessation of treatment.

Therefore, the beneficial effect of anti-LFA-1 antibody may more likely be ascribed to the well established function of LFA-1 in T lymphocyte activation. This function, first described *in vitro*,³¹ was more recently confirmed in LFA-1 defective mice.³² Interestingly, in these mice, the most affected immunological function was rejection of allogeneic tumours. LFA-1 favours the interactions between T lymphocytes and antigen presenting cells and thereby contributes to the initiation of the immune response. In addition, it promotes the effective phase by favouring contact between cytotoxic T cells and their targets.³³ Our observation that the antibody needs to be administered before initiation of antigen recognition and is no more efficient when given after the first day, strongly suggests that its main effect on the prevention of intestinal allograft rejection is at initiation of the allogeneic response. This result obtained *in vivo* is consistent with results obtained *in vitro*⁷ in a mixed lymphocyte reaction where the antibody blocked only when added before or immediately after initiation of the culture. Furthermore, recent *in vivo* studies using LFA-1 defective lym-

phocytes suggest that LFA-1 is critical for signalling via the T cell receptor.³²⁻³⁴

Blockade of LFA-1 during triggering of the T cell receptor by alloantigens most likely results in induction of tolerance. This hypothesis is supported by the long lasting effect of the antibody as well as by in vitro studies demonstrating a reduced proliferative response in MLC with reduced IL-2 production of T cells harvested from mice rendered tolerant by in vivo administration of anti-LFA-1.³⁵ In addition, it has been shown that the state of hyporesponsiveness obtained by anti-LFA-1/ICAM-1 treatment can be restored in vitro³⁶ or in vivo³⁷ by administration of IL-2. A previous study demonstrated that CTLA-4-Ig and anti-CD40L antibody induced long term tolerance to cardiac grafts but that this effect was abolished by short administration of CyA.¹⁷ Considering the pivotal role of calcineurin dependent drugs in the current protocols used in humans to prevent intestinal graft rejection, we considered it necessary to test the effect of these drugs on the protective effect of anti-LFA-1 treatment. Administration of CyA for 14 days or tacrolimus for 30 days did not significantly affect the survival of the graft, suggesting that the drugs did not interfere with the active immunological process most likely involved in the immunosuppressive effect of anti-LFA-1 antibody. However, it is noticeable that the grafts were rapidly rejected following withdrawal of the drugs. Furthermore, early withdrawal of FK506 significantly reduced the protective effect of anti-LFA-1 treatment. It is possible that withdrawal of immunosuppressive calcineurin dependent drugs favours the reactivation of tolerant T cells. Thus in vitro studies have shown firstly, that CyA does not prevent accumulation of AP-1, a transcription factor for the IL-2 gene, and secondly, that following removal of CyA there is rapid nuclear translocation of preformed NFAT (nuclear factor of activated T cells) which associates with AP-1 in the nucleus to induce rapid accumulation of mRNA for IL-2.³⁸ These observations suggest that in our study, graft rejection which followed early withdrawal of tacrolimus may have been due to release of IL-2 which disrupts anti-LFA-1 induced immunosuppression.

In summary, in humans a beneficial effect of anti-LFA-1 treatment has been demonstrated previously in bone marrow and renal transplantation.¹⁰⁻¹¹ Our results suggest that anti-LFA-1 treatment may also be useful in improving intestinal transplantation and is not incompatible with the long term administration of tacrolimus currently used for the prevention of small bowel graft rejection in humans. The fact that antiviral responses are not affected in LFA-1 defective mice suggests that the beneficial effect on graft rejection may not be counterbalanced by severe depression of anti-infectious responses.¹⁴

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- Revillon Y, Jan D, Sarnacki S, Goulet O, Brousse N, Ricour C. Intestinal transplantation in the child: experimental and clinical studies. *J Pediatr Surg* 1993;28:292-8.
- Abu-Elmag K, Todo S, Tzakis A, et al. Three years clinical experience with intestinal transplantation. *J Am Coll Surg* 1994;179:385-400.
- Langnas AN, Shaw BW Jr, Antonson DL, et al. Preliminary experience with intestinal transplantation in infants and children. *Pediatrics* 1996;97:443-8.
- Grant D. Current results of intestinal transplantation. *Lancet* 1996;347:1801-2.
- Grant D. Intestinal transplantation: 1997 report of the international registry. *Transplantation* 1999;67:1061-4.
- Davignon D, Martz E, Reynolds T, Kurzinger K, Springer TA. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effect on other T and B function. *J Immunol* 1981;127:590-5.
- Krensky AM, Sanchez-Madrid F, Robbins Z, Nagy JA, Springer TA, Burakoff SJ. The functional significance, distribution and structure of LFA-1, LFA-2 and LFA-3: cell surface antigens associated with CTL-target interactions. *J Immunol* 1983;131:611-16.
- Carlos TM, Harlan JM. Leucocyte-endothelial adhesion molecules. *Blood* 1994;84:2068-101.
- Cavazzana-Calvo M, Sarnacki S, Haddad E, et al. Prevention of bone marrow and cardiac graft rejection in an H-2 haplotype disparate mouse combination by an anti-LFA-1 antibody. *Transplantation* 1995;59:1576-82.
- Fischer A, Cavazzana-Calvo M, Jabado N, Sarnacki S. Monoclonal antibodies to adhesion molecules in bone marrow and organ transplantation. In: Chatenoud L, ed. *Monoclonal antibodies in transplantation*. Austin, TX, USA: RG Landes Company Publishers, 1995:137-45.
- Hourmant M, Bedrossian J, Durand D, et al. A randomized multicenter trial comparing leucocyte function-associated antigen-1 monoclonal antibody with rabbit antithymocyte globulin as induction treatment in first kidney transplantations. *Transplantation* 1996;62:1565-70.
- Berlin C, Bargatzke RF, Campbell JJ, et al. Alpha4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 1995;80:413-22.
- Bargatzke RF, Jutila MA, Butcher EC. Distinct roles of L-selectin and integrin alpha-4-beta-7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: The multistep model confirmed and refined. *Immunity* 1995;3:99-108.
- Wagner N, Löhler J, Kunkel EJ, et al. Critical role for $\beta 7$ integrins in formation of the gut-associated lymphoid tissue. *Nature* 1996;382:366-70.
- Springer TA, Davignon D, Ho MK, Kürzinger K, Martz E, Sanchez-Madrid F. LFA-1 and Lys-2,3, molecules associated with T-lymphocyte-mediated killing; and Mac-1, an LFA-1 homologue associated with complement receptor function. *Immunol Rev* 1982;68:171-95.
- Berlin C, Berg EL, Briskin MJ, et al. $\alpha 4 \beta 7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 1993;74:185-95.
- Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 1996;381:434-8.
- Lobb RR, Hemler ME. The pathophysiologic role of $\alpha 4$ integrins in vivo. *J Clin Invest* 1994;94:1722-8.
- Yang H, Issekutz TB, Wright JR Jr. Prolongation of rat islet allograft survival by treatment with monoclonal antibodies against VLA-4 and LFA-1. *Transplantation* 1995;60:71-6.
- Isobe M, Susuki JJ, Yagita H, et al. Immunosuppression to cardiac allografts and soluble antigens by anti-vascular cellular adhesion molecule-1 and very late antigen-4 monoclonal antibodies. *J Immunol* 1994;153:5811-18.
- Bell RG, Issekutz T. Expression of protective intestinal immune response can be inhibited at three distinct sites by treatment with anti $\alpha 4$ integrin. *J Immunol* 1993;151:4790-802.
- Podolsky DK, Lobb R, Benjalín CD, Pepinsky B, Sehgal P, deBeaumont M. Attenuation of colitis in the cotton-top tamarin by anti $\alpha 4$ integrin monoclonal antibody. *J Clin Invest* 1993;92:372-80.
- Hesterberg PE, Winsor-Hines D, Briskin MJ, et al. Rapid resolution of chronic colitis in the cotton-top tamarin with an antibody to a gut-homing integrin $\alpha 4 \beta 7$. *Gastroenterology* 1996;111:1373-80.
- Tanaka T, Ohtsuka Y, Yagita H, Shiratori Y, Omata M, Okumura K. Involvement of $\alpha 1$ and $\alpha 4$ integrins in gut mucosal injury of graft-versus-host disease. *Int Immunol* 1995;7:1183-9.
- Ludviksson BR, Strober W, Nishikomori R, Hasan SK, Ehrhardt RO. Administration of mAb against alphaEbeta7 prevents and ameliorates immunization-induced colitis in IL-2-/- mice. *J Immunol* 1999;162:4975-82.
- Kato Y, Yamataka A, Yagita H, Okumura K, Fujiwara T, Miyano T. Specific acceptance of fetal bowel allograft in mice after combined treatment with anti-intercellular adhesion molecule-1 and leukocyte function-associated antigen-1 antibodies. *Ann Surg* 1996;223:94-100.
- Le-Mauff B, Hourmant M, Rougier JP, et al. Effects of anti-LFA-1 (CD11a) monoclonal antibodies in acute rejection in human kidney transplantation. *Transplantation* 1991;52:291-6.
- Oubenaissa A, Mouas C, Boyrgeois F, et al. Evidence for an involvement of the neutrophil integrin lymphocyte function-associated antigen-1 in early failure of heart transplants. *Circulation* 1996;94:254-9.

- 29 Hamann A, Jablonski-Westrich D, Duijvestijn A, et al. Evidence for an accessory role of LFA-1 in lymphocyte-high endothelium interaction during homing. *J Immunol* 1988;140:693-9.
- 30 Fromont G, Cerf-Bensussan N, Patey N, et al. Small bowel transplantation in children: an immunohistochemical study of intestinal grafts. *Gut* 1995;37:783-90.
- 31 Van Seventer GA, Shimizu Y, Horgan KJ, et al. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T-cell receptor-mediated activation of resting cells. *J Immunol* 1990;144:4579-83.
- 32 Schmits R, Kündig TM, Baker DM, et al. LFA-1 deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. *J Exp Med* 1996;183:1415-26.
- 33 Figdor CG, Van Kooyk, Keizer GD. On the mode of action of LFA-1. *Immunol Today* 1990;11:277-9.
- 34 Bachmann MF, McKall-Faenza K, Schmits R, et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* 1997;4:549-57.
- 35 Chan BM, Zheng H, Wang H, et al. Treatment of cardiac allografts with established leukocyte infiltration by modulation of alpha4 (CD49d) and leukocyte function-associated antigen-1 (CD11a/CD18) integrin function. *Transplantation* 1998;66:277-83.
- 36 Bashuda H, Seino K, Ra C, Yagita H, Okumura K. Lack of cognate help by CD4+ T cells and anergy of CD8+ T cells are the principal mechanisms for anti-leukocyte function-associated antigen-1/intercellular adhesion molecule-1-induced cardiac allograft tolerance. *Transplantation* 1997;63:113-18.
- 37 Isobe M, Suzuki J, Yamazaki S, Sekiguchi M. Effects of exogenous IL-2 on tolerance induction by anti-ICAM-1 and anti-LFA-1 monoclonal antibodies. *Transplant Proc* 1996;28:1045-6.
- 38 Bemer V, Truffa-Bachi P. T cell activation by concanavalin A in the presence of cyclosporin A: immunosuppressor withdrawal induces NFATp translocation and interleukin-2 gene transcription. *Eur J Immunol* 1996;26:1481-8.