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Anti-integrin (LFA-1, VLA-4, and Mac-1) antibody treatment and acute cardiac graft rejection in the rat

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Abstract Cell adhesion molecules mediate interactions with other cells and extracellular matrix, control cell infiltration in sites of inflammation, and regulate cell activation. Previous studies have shown that treatment of rat cardiac transplant recipients with a combination of antibodies against the T-cell integrins LFA-1 and VLA-4 gave a modest prolongation of graft survival. Current experiments were designed to examine the effect of blocking Mac-1, an important monocyte adhesion receptor and mediator of monocyte migration, together with anti-LFA-1 and anti-VLA-4 antibodies on cardiac graft survival and on the graft rejection pattern. The anti-Mac-1, CD11b-specific antibody OX-42 did not affect graft survival time although it did decrease the graft infiltration by rounded, ED-2-positive macrophages.

Key words Anti-integrin, rejection, rat · Rat, rejection, anti-integrin · Antibodies, heart transplantation, rat · Heart transplantation, rat, rejection

Introduction

The adhesion of cells to other cells or to extra-cellular components of the tissue microenvironment is a basic function of cell recognition and migration and underlies many biological processes, including transplant rejection. Cell adhesion events are of critical importance in the T-cell recognition of alloantigens, the trafficking of recipient immune and inflammatory cells into the graft, and the execution of cell-mediated effector functions (reviewed in [12]). The graft endothelium of primary vascularized grafts is of particular importance as it constitutes the anatomic barrier between the blood and the tissue interstitium and may also serve as the primary target structure for the post-transplant immune response. In vitro adhesion studies of allogeneic T lymphocytes and cultured endothelial cells have shown the existence of several adhesion pathways [16, 22, 23], but there is less data on the importance of these pathways in graft rejection in vivo. The blockade of adhesion molecules with monoclonal antibodies has resulted in variable degrees of prolongation of graft survival, depending on the experimental model used as well as on the isotype and epitope specificity of the antibodies [5, 12, 15, 19]. We reported previously that anti-LFA-1 antibody treatment of rats with a cardiac allograft results in a modest prolongation of graft survival [18], as observed by other investigators [11]. Anti-VLA-4 antibodies gave only a marginal prolongation of graft survival, and the effect of both antibodies together was not more than that of anti-LFA-1 antibodies alone [18]. It is of interest that a combination of the same antibodies administered to recipients of pancreas islet grafts induced long-term graft survival [25].

Most of the work on anti-adhesion molecules in organ transplant models has focussed on lymphocyte-endothelial cell interactions, in line with the fundamental role of lymphocytes in acute rejection. However, the

number of monocytes and macrophages assessed by histopathological techniques in grafts with acute rejection tends to exceed the number of T lymphocytes [13, 17]. The contribution of monocyte-endothelial cell adhesion pathways in acute rejection models has not yet been adequately examined.

T cells express only the CD11a (LFA-1) form of the CD11/CD18 complex, but monocytes express three members of the CD18 family: CD11a, CD11b (Mac-1), and CD11c/CD18. In vitro studies using monoclonal antibodies to CD11a and CD11b have shown that both these integrins, as well as VLA-4, mediate monocyte transendothelial migration [1, 14]. Recently, VLA-4, CD11a/CD18, and CD11b/CD18 have also been shown to mediate monocyte migration to chronically inflamed joints [7]. Therefore, we investigated the effect of blocking Mac-1 (CD11b/CD18) together with LFA-1 and VLA-4 on the graft survival rate and the graft rejection pattern of vascularized cardiac allografts in the rat.

Materials and methods

Male Lewis (LEW) and LEW x Brown Norway (BN) [(LEW xBN)F_1] rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind., USA). The animals were housed in the University of Calgary Animal Resources Centre and maintained on standard rat chow and tap water. Animal care and experimentation was in accordance with the principles outlined in the most recent "Guide to the Care and Use of Experimental Animals" by the Canadian Council on Animal Care; the study was approved by the University of Calgary Review Committee.

Donor hearts were transplanted heterotopically into the abdominal cavity using an end-to-side anastomosis of the graft ascending aorta to the recipient abdominal aorta; the graft pulmonary artery was anastomosed to the recipient inferior vena cava [17]. The reanastomosis time was usually between 20 and 30 min. Graft survival was monitored by daily palpation of the graft, and rejection was defined as cessation of a detectable heartbeat.

Monoclonal antibody treatment

MRC OX-42 is a mouse IgG_{2a} monoclonal antibody that reacts with rat CD11b of Mac-1 and was a gift from Dr. D.W.Mason (University of Oxford, Oxford, UK) [20]. It blocks Mac-1-mediated phagocytosis, neutrophil and monocyte adhesion, and in vivo neutrophil and monocyte migration [6, 9]. TA-2 is an $IgG_1\kappa$ mouse monoclonal antibody that reacts with the α_4 chain of rat VLA-4 and blocks VLA-4-mediated lymphocyte-endothelial cell adhesion in vitro and in vivo [8, 10]. TA-3 is also an $IgG_1\kappa$ mouse monoclonal antibody that reacts with the CD11a chain of rat LFA-1 and blocks lymphocyte adhesion, proliferation, homotypic aggregation, and in vivo lymphocyte migration [6]. TA-2 and TA-3 were administered in a dose of 0.75 mg each per day, while OX-42 was given at a dose of 0.62 mg per day for a period of 12 days. These doses were previously found to provide blood antibody concentrations that were 10–30 times those required to saturate the receptor on the leukocyte by immunofluorescent staining. B9 is a monoclonal antibody of the $IgG_1\kappa$ isotope that reacts with pertussis toxin and was used as a control antibody.

In addition to the studies of graft survival, three separate groups of five animals were treated with OX-42 alone, a combination of TA-2, TA-3, and OX-42 antibodies, or no treatment. These grafts were removed after 5 days for histological and immunohistological evaluations.

Immunohistology

Grafts removed on day 5 after transplantation were used for immunohistology to assess the phenotypic characteristics of graft-invading cells; recipients' own hearts were used as control tissues. Tissues were quick-frozen in compound in cryomolds and embedding rings (Tissue-Tek). Cryostat sections, 5 μ thick, were cut at −20 °C, thaw-mounted on glass slides, fixed in cold acetone, and incubated for 1.5 h with mouse anti-rat cell surface monoclonal antibodies diluted in phosphate-buffered saline (pH 7.4) in a humidified chamber; the binding of the mouse antibodies was visualized by staining with fluorescein-labelled goat anti-mouse IgG (Organon Teknika, Scarborough, Ontario) diluted in normal rat serum to absorb antibodies that cross-react with rat immunoglobulins. Control stainings were done with an isotype-matched irrelevant monoclonal antibody, followed by incubation with fluorescein-labelled anti-mouse IgG or by direct incubation with the fluorescein-labelled anti-mouse IgG without a primary antibody. Cellular infiltration was graded semiquantitatively. The shape of the cells was assessed as either round or dendritic-shaped, as in previous experiments [17]. Dendritic-shaped cells are present in normal, non-inflamed tissues and represent a population of bone marrow-derived "passenger" cells. The following antibodies were used for immunohistochemistry: CD45, an antibody against the leukocyte common antigen; ER 13, an antibody against a monomorphic class II MHC antigen; and ED-1, ED-2 [11], ED-7, ED-8, and ED-9 antibodies, a series of monoclonal antibodies against monocyte/macrophage antigens. Anti-ED-7, ED-8, and ED-9 antibodies recognize different epitopes of the CR3 molecule [2].

Statistics

Graft survival data were analyzed according to Cox's proportional hazards model and a one-way analysis of variance after logarithmic transformation of the data; differences between treatment groups were analyzed using Wilcoxon rank-sum tests with correction for the number of comparisons done.

Results

Since T lymphocytes do not express the CD11b chain that is recognized by the OX-42 antibody, we did not assess the immunosuppressive efficacy of OX-42 alone but rather tested it in combination with an anti-VLA-4 and an anti-LFA-1 antibody. Treatment with the combination of OX-42, TA-2, and TA-3 antibodies for 12 days gave a median graft survival of 22.0 days (mean ± SEM 21.6 ± 3.5 days), which was significantly longer than that of untreated controls (median 7.0 days; mean 9.4 ± 1.5 days) or of animals treated with the negative control monoclonal antibody (median 10 days; mean 10.7 ± 0.6 days; $P < 0.05$). However, graft survival with the combination of OX-42, TA-2, and TA3 did not differ

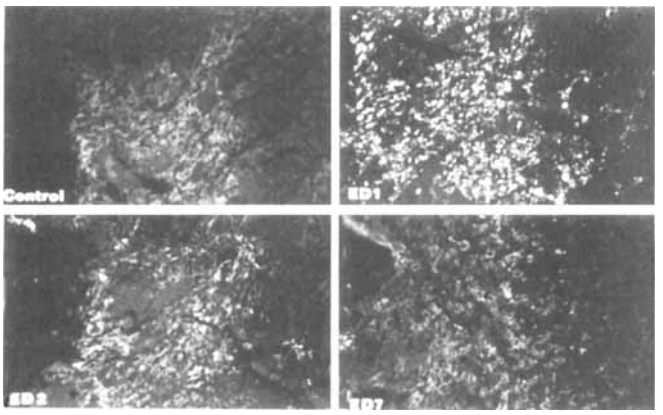

Fig. 1 Immunohistological staining of a (LEWxBN)F_1 cardiac graft removed from a LEW recipient after 5 days of treatment with OX-42, TA-2, and TA-3 antibodies. The *control panel* shows the background fluorescence of graft infiltrating cells; these cells were labelled in vivo with the mouse monoclonal antibodies and gave a dull green fluorescence. *Panel ED-1* shows the bright fluorescence of ED-1-positive cells (intra-cytoplasmic staining) that have invaded the graft; *panel ED-2* shows an occasional brightly positive cell (cell surface staining) in the graft, but most cells in the cellular infiltrate are ED-2-negative. The *ED-7 panel* shows the presence of many ED-7-positive cells (cell surface staining) in the graft interstitium

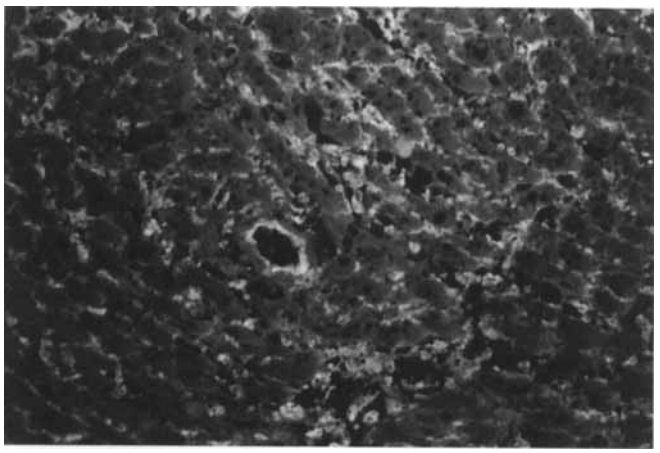

Fig. 2 Immunohistological staining of a (LEWxBN)F_1 cardiac graft removed from a LEW recipient after 5 days of treatment with OX-42; the section was incubated with ER-13, a monoclonal antibody against rat MHC class II antigen, and stained with fluorescein-labelled goat anti-mouse IgG. There is specific staining of the arterial endothelium and interstitial cells

from that of recipients treated with TA-2 only (median 14.5 days; mean 16.3 ± 1.5 days), TA-3 only (median 24.5 days), or the combination of TA-2 plus TA-3 antibodies (median 23.5 days), as established previously [18]. Histopathology of the grafts at asystole showed end-stage acute rejection, consisting of fibrinoid vascular necrosis, infiltration with mononuclear cells, interstitial hemorrhages, and myocyte necrosis.

To assess whether treatment with monoclonal antibodies had any effect on the histopathologic pattern of acute rejection, grafts were removed 5 days after transplantation and used for histopathologic and immunohistologic studies. Grafts removed from recipient animals treated with OX-42 showed a modest degree of vasculitis and extensive interstitial cellular infiltration, comparable to the lesions observed in untreated recipients. Grafts removed from recipients that had been treated with the combination of OX-42, TA-2, and TA-3 antibodies showed no vasculitis but only slightly less interstitial inflammation than control grafts.

Immunohistology studies of tissue sections incubated with goat anti-mouse IgG-FITC diluted in normal rat serum gave a dull background fluorescence staining of graft-invading cells, representing recipient blood cells that were labelled in vivo by the infused mouse IgG antibodies. This background fluorescent staining was strongest in grafts removed from animals that had been treated with three antibodies (Fig. 1, control). We also observed labelled cells in the vessel lumina and capillaries of the recipient's own heart in animals treated with the three-antibody regimen. White blood cell counts in these animals at sacrifice showed variable degrees of leukocytosis (up to 60×10^3 cells/mm^3) with a predominance of polymorphonuclear granulocytes. Animals treated only with OX-42 had a normal leukocyte count and a normal differential distribution ($6.9 \pm 1.2 \times 10^3$ leukocytes/mm^3; $68.2\% \pm 2.0\%$ lymphocytes, $23.6\% \pm 1.3\%$ polymorphonuclear granulocytes and $8.0\% \pm 1.6\%$ monocytes).

Immunohistological staining for class II MHC antigens showed expression on large vessel endothelium in control grafts and grafts removed from animals treated with OX-42 alone (Fig. 2). No class II antigens were found on the large vessel endothelium of grafts removed from recipients treated with the combination of OX-42, TA-2, and TA-3. Many of the interstitial graft-invading cells were class II-positive. The number of class II-positive cells was less in grafts removed from recipients treated with the combination of antibodies than in either control grafts or grafts removed from animals treated only with OX-42 antibodies.

Staining with anti-CD45, ED1, ED-7, ED-8, and ED-9 antibodies showed the presence of T cells and monocytes/macrophages in the grafts from recipients that had been treated with either OX-42 only, a combination of OX-42, TA-2, and TA-3 antibodies (Fig. 1, ED1), or no treatment. ED-7 (Fig. 1, ED-7), ED-8, and ED-9 gave virtually identical staining patterns, as expected.

Staining with ED-2, a monoclonal antibody specific for tissue macrophages [3], showed remarkable differences in the tissue infiltration patterns of ED-2-positive cells in animals treated with OX-42 antibody either

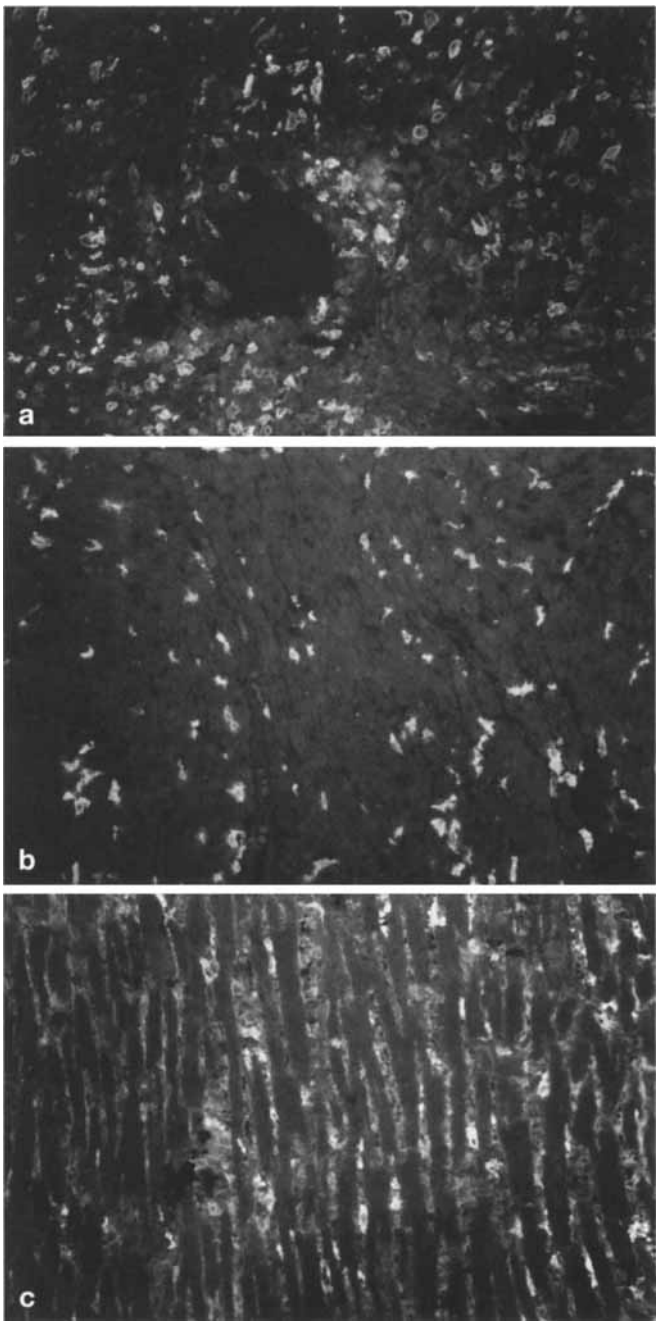

Fig. 3 **a** Immunohistological staining of a (LEWxBN)F_1 cardiac graft removed from an untreated LEW recipient after 5 days; the section was incubated with ED-2 antibody and stained with a fluorescein-labelled goat anti-mouse IgG. There is specific staining of rounded ED-2-positive cells in the interstitium. **b** Immunohistological staining of a LEW recipient native heart removed 5 days after transplantation of a (LEWxBN)F_1 cardiac graft in the abdominal cavity and treatment with OX-42 antibodies; the section was incubated with ED-2 antibody and stained with a fluorescein-labelled goat anti-mouse IgG. There is specific staining of dendritic-shaped ED-2-positive cells dispersed throughout the myocardium. **c** Immunohistological staining of a (LEWxBN)F_1 cardiac graft removed 5 days after transplantation in a LEW recipient that was treated with OX-42 antibodies; the section was incubated with ED-2 antibody and stained with a fluorescein-labelled goat anti-mouse IgG. There is specific staining of dendritic-shaped ED-2-positive cells dispersed throughout the myocardium

alone or in combination with TA-2 and TA-3. Previous experiments have shown that normal rat cardiac tissue contains a population of dendritic-shaped ED-2-positive cells dispersed throughout the myocardium [11]. Following cardiac transplantation, there is a rapid increase in the number of ED-2-positive cells in conjunction with acute rejection, but these ED-2-positive cells are round rather than dendritic-shaped (Fig. 3 A). A similar pattern of rounded ED-2-positive cell infiltration was also observed in cardiac grafts removed from animals treated with TA-2 and TA-3 antibodies. Cardiac grafts removed from animals treated with OX-42 antibodies, either alone or in combination with TA-2 and TA-3 antibodies, showed the baseline pattern of dendritic-shaped, ED-2-positive cells dispersed throughout the myocardium, as in non-transplanted hearts, but very few rounded ED-2-positive cells were found in such grafts; when present, they were mostly distributed in foci (Fig. 1, ED-2). Figure 3B shows the pattern of ED-2-positive dendritic cells in the recipient's own heart after treatment for 5 days with the combination of OX-42, TA-2, and TA-3 antibodies; there are ED-2-positive cells dispersed throughout the myocardium. Figure3C shows the ED-2-positive dendritic-shaped cells in the graft, in a pattern that resembles that of the recipient's own heart but not that of transplanted hearts in animals not receiving OX-42 antibodies (Fig. 3 A). Thus, OX-42 treatment of the recipient does not affect the baseline pattern of ED-2 expression in the heart, but it does inhibit the intragraft accumulation of rounded ED-2-positive cells.

Discussion

The current experiments show that the addition of OX-42 antibodies (anti-CD11b or anti-MAC-1) to a regimen of anti-LFA-1 and anti-VLA-4 antibodies does not prolong the graft survival time of cardiac transplants in rats beyond the level of graft survival obtained with anti-LFA-1 antibodies only. The addition of OX-42 antibodies also failed to decrease the extent of cellular infiltration in the graft interstitium, as assessed by standard light microscopy. The role of the CD11c chain cannot confidently be determined with the antibodies used although OX-42 may also react with CD11c, as suggested by several authors [6, 20, 24]. The rapid infiltration of the graft interstitium with recipient mononuclear cells in antibody-treated animals is unexpected vis-a-vis the in vitro and in vivo data that have shown complete inhibition of T-lymphocyte-dependent functions with TA-2 and TA-3 antibodies [8, 10, 25]. Our data suggest that cellular adhesion molecules other than the CD11/CD18 complex and VLA-4 are involved in acute rejection-dependent tissue infiltration by immune and inflammatory cells in cardiac allografts.

Very few studies have investigated the effect of monoclonal antibody treatment on the histopathological pattern of graft rejection. We reported recently that treatment of rat cardiac transplant recipients with anti-LFA-1 (DC11a) antibodies resulted in a slight reduction in the extent of interstitial cellular infiltration, assessed on day 5 after transplantation, compared with no treatment while anti-VLA-4 antibodies had no appreciable effect [18]. A study of similar design of rabbit cardiac transplants assessed on day 7 after transplantation found that administration of an antibody against the CD18 chain of the CD11/CD18 complex resulted in a significant reduction in the degree of interstitial cellular infiltration, whereas anti-VLA-4 antibody treatment enhanced the degree of interstitial cellular infiltration [21]. Of interest, however, is the observation that each antibody individually gave some reduction in the extent and degree of graft vasculitis [21]. We found that neither anti-LFA-1 nor anti-VLA-4 antibodies blocked the emergency of graft vasculitis, although each antibody was able to block the induction of class II MHC antigen expression on the graft arterial endothelium, a phenomenon that correlates with graft vasculitis [4]. We furthermore found that the combination of anti-LFA-1 plus anti-VLA-4 antibodies prevented early graft vasculitis completely, white little or no effect was found on the extent of interstitial cellular infiltration.

We demonstrate in the current experiments that treatment of graft recipients with OX-42 antibodies alone does not prevent induction of class II expression on graft arterial endothelium (Fig. 2), nor does this treatment inhibit graft vasculitis. It has been postulated that the endothelial induction of class II expression is primarily mediated by interferon-γ, a lymphokine generated by activated T lymphocytes and natural killer cells. Our finding that antibodies against T-cell integrins were effective in blocking induction of class II expression [18] while OX-42, an antibody against CD11b, had no effect on either class II expression or the histopathological evidence of graft vasculitis, is consistent with an important role of T lymphocytes in the pathophysiology of graft vasculitis.

We have hypothesized that long-term graft structure and function depend to a large extent on the characteristics of the graft-invading cells and the cytokines they produce [17]. The present experiments show that treatment of graft recipients with antibodies against the CD11b chain affects the phenotype of the macrophages that invade cardiac grafts or at least affects the expression of macrophage markers on graft-invading cells. Recipients treated with OX-42 antibodies showed only very few rounded ED-2-positive cells in the graft compared with grafts removed from untreated recipients or recipient animals that had been treated with anti-LFA-1 and/or anti-VLA-4 antibodies (Figs. 1, 3), although the pattern of dendritic-shaped intra-cardiac interstitial cells remained unaffected. As normal blood monocytes do not express that ED-2 antigen [3], the rounded ED-2-positive cells in grafts with unmodified rejection are either recently immigrated monocytes that have acquired the ED-2 marker or they are derived from the ED-2-positive population of cardiac interstitial cells, or both. In vivo treatment with OX-42 antibodies resulted in a change in either the composition or the phenotypic appearance of the graft-invading macrophages. Although such a modulation does not have an impact on the graft survival time, it remains to be established whether anti-Mac-1 antibodies will have an effect on the long-term graft function or structure in recipients in whom irreversible rejection is avoided using immunosuppressive drugs.

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