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ORIGINAL ARTICLE

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Allograft survival following immunization with membrane-bound or soluble peptide MHC class I donor antigens: factors relevant for the induction of rejection by indirect recognition

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Abstract T cells recognize foreign antigens in the form of peptide fragments resulting from antigen processing by antigen-presenting cells. In contrast to this indirect recognition, MHC molecules of foreign cells can be directly recognized by T cells. Direct recognition has for a long time been considered the only mechanism responsible for transplant rejection. Recent studies have provided evidence of a role of indirect recognition in rejection. In the current series of experiments, we studied the influence of indirect alloactivation, induced either by donor MHC class I peptides or by membrane-bound MHC I molecules, on heart allograft rejection in rats. Recipients were immunized before transplantation with synthetic donor MHC I peptides. The animals developed antibody and T-cell responses. Depending on the rat strain, peptide pretreatment either had no effect on graft survival (DA→PVG; untreated controls 8.5 ± 0.6 days, treated rats 9.5 ± 0.6 days) or led to accelerated rejection (DA→LEW; untreated controls 7.5 ± 0.3 days, treated rats 5.1 ± 0.2 days; $P < 0.0002$). Importantly, sensitization by indirect activation induced acute rejection in a donor-recipient combination (LEW.1A→LEW.1WR2) in which neither direct nor indirect recognition led to rejection (untreated controls > 400 days, pretreated rats 15 ± 4.2 days). Another group of animals was immunized with allogeneic or congenic erythrocytes carrying the MHC I antigen from which the peptides were derived. Although the immunization elicited a measurable immune response, it did not lead to accelerated rejection. We conclude that sensitization by indirect recognition is able to initiate an acute rejection even in recipients in which neither direct nor indirect recognition is effective, and that this effect is strain-dependent. The form in which the donor antigen is administered is decisive for the induction of rejection by indirect activation.

Key words Allorecognition, MHC antigens, rat · Rejection, MHC antigens, rat · MHC antigens, rejection, rat · Heart, rat, allorecognition

Introduction

In order to be recognized by T cells, foreign antigens must be taken up by antigen-presenting cells (APC) APC, digested, and re-expressed on the cell membrane in association with self-MHC molecules [2, 25]. There are only a few exceptions to this indirect pathway of antigen recognition. One concerns foreign MHC molecules. MHC antigens presented on donor cells are directly recognized by recipient T cells. There is a general consensus that activation by direct recognition plays a major role in allograft rejection [3, 13]. MHC alloanti-

gens can also be indirectly recognized. Fangmann and colleagues were the first to show that priming by indirect allorecognition influences skin graft rejection in rats [6]. Other studies have supported the concept that indirect recognition has a role in the allograft rejection response [1, 4, 7, 12, 16, 18, 22].

In the current series of experiments we analyzed the capacity of indirect priming induced by donor MHC I antigens, either in the form of soluble peptides or expressed on cell membranes, to activate the immune response and to promote acute graft rejection in various rat strains.

Materials and methods

Donor-recipient rat combinations

The following donor-recipient combinations were studied: DA→LEW (group 1), DA→PVG (group 2), and LEW.1A→LEW.1WR2 (group 3). In groups 1 and 2, the donor and the recipient were MHC class I/II and non-MHC mismatched (DA = RT1-$A^aB^aD^aC^{av1}$, LEW = RT1-$A^lB^lD^lC^l$, PVG = RT1-$A^cB^cD^cC^c$). In group 3 the donor and the recipient were derived from congenic strains in which the only mismatch was the RT1-A^a antigen (LEW.1A = RT1$A^aB^aD^aC^a$ and LEW.1WR2 = RT1$A^uB^aD^aC^a$). Groups of 6–14 animals were tested.

Immunization of recipients

Peptides

Two peptides corresponding to the hypervariable region of donor MHC class I (RT1-A^a) [6, 17] were synthesized. The peptides corresponded to the α helical regions of the α1 and α2 domain. Their sequence was α1: HN-Pro-Glu-Tyr-Trp-Glu-Gln-Gln-Thr-Arg-Ile-Ala-Lys-Glu-Trp-Glu-Gln-Ile-Tyr-Arg-Val-Asp-Leu-Arg-Thr-OH and α2: H2N-Thr-Arg-Asn-Lys-Trp-Glu-Arg-Ala-Arg-Tyr-Ala-Glu-Arg-Leu-Arg-Ala-Tyr-Leu-Glu-Gly-Thr-Cys-OH. The porcine neuropeptide Y served as a negative control. Its sequence was: HN-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile- Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-amide.

Erythrocytes

Erythrocytes were purified from heparinized blood by two consecutive centrifugations on double (1.077 and 1.119 g/ml) and single (1.077 g/ml) sodium metrizoate + polysaccharide density gradients, respectively. FACS analysis showed no contamination (0 %) with lymphocytes. The following erythrocytes were used for immunization: in group 1, DA erythrocytes (RT1-A^a), in group 2, congenic PVG-R1 erythrocytes (RT1-A^a); and in group 3, congenic LEW.1A erythrocytes (RT1-A^a). In groups 2 and 3, the only incompatibility between the erythrocyte donor and recipient was the RT1-A^a antigen. The animals were otherwise MHC and non-MHC identical. We chose RT1-A^a-bearing erythrocytes for immunization because this antigen is highly expressed on the erythrocyte membrane [10].

Immunization schedule

The recipients were immunized s.c. with a mixture of α1 + α2 peptide (combined 100 μg + 100 μg) in complete or incomplete Freund's adjuvant (FA) 1 and 2 months before transplantation. Other recipients were immunized s.c. following the same schedule with FA only, control peptide in FA, or 8×10^9 erythrocytes in FA. Another group of recipients received the same number of erythrocytes by i.v. treatment.

Detection of antibodies

Antipeptide antibodies (ELISA)

Antibodies were measured in the sera of immunized animals. Microtiter plates were coated with 0.5 μg/well of α1- or α2-peptide and the remaining active groups were blocked with PBS + 1 % gelatin. The test sera were applied to the plates (dilution series 50 μl/well). Thereafter, mouse F(ab')2 anti-rat Ig-AP (50 μl/well, dilution 1:5000; Jackson Immunoresearch Lab, West Grove, Pa., USA) and substrate (250 μg p-nitrophenyl phosphate disodium/well; Sigma Chemical, St. Louis, Mo., USA) were added stepwise. Each step was followed by extensive washing with PBS + 0.05 % Tween. A reference serum with known antipeptide antibody activity was used as a positive control and sera without antibody as negative controls. Optical density (OD) was determined at 405 nm. The reaction was stopped at an extinction of 700 OD in the positive control.

Antidonor cell antibodies (FACS)

Donor peripheral blood mononuclear cells (10^5) were incubated with 25 μl recipient serum before peptide immunization, after immunization (containing antipeptide antibodies), and during rejection, as well as with serum obtained before and after immunization with erythrocytes. The T cells were labelled with 0.4 μg R73-phycoerythrin (mouse IgG1-anti-rat α/β T-cell receptor) and mouse F(ab')2 anti-rat-F(ab')2-FITC (Jackson Immunoresearch) was added. Antibodies directed against donor T cells were determined in the FACScan.

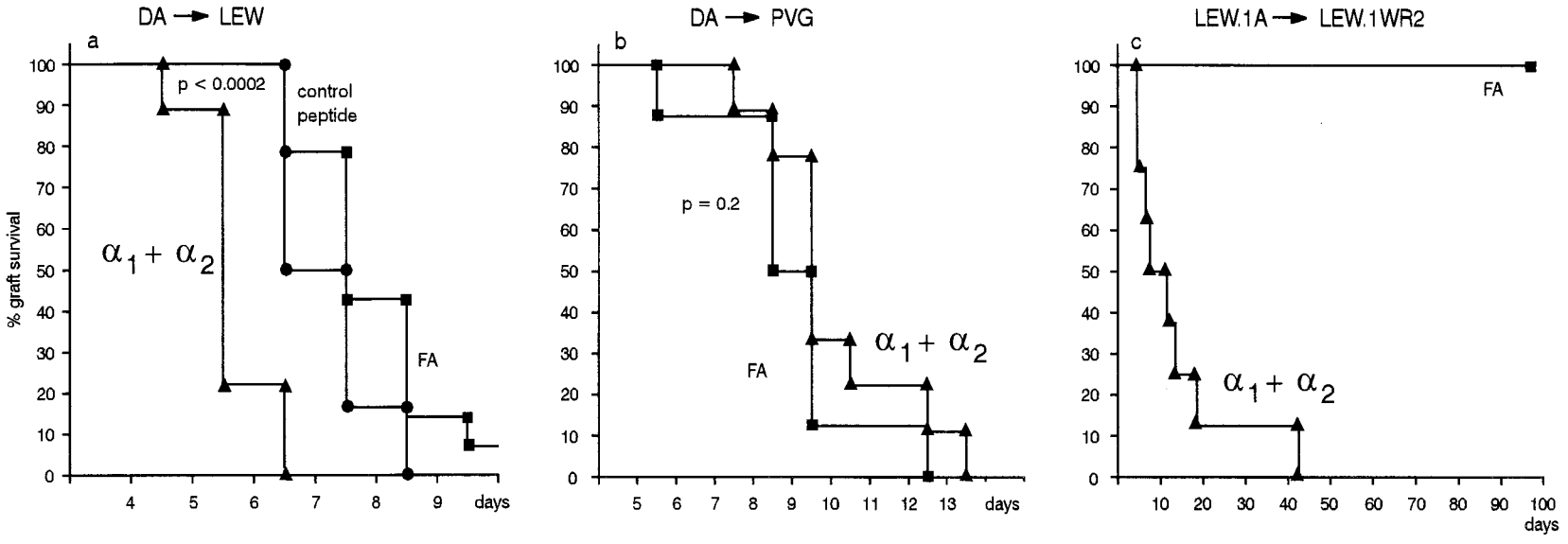
Detection of T-cell sensitization against peptides

PBMC (5×10^4) of nonimmunized or peptide-immunized LEW rats were incubated with 10 μg of α1, α2, or α1 + α2 peptide in RPMI + 5 % FCS. After 3 days, 20 μl ^{3}H-Thymidine (5 Ci/mmol) was added. Counts per minute were measured and the index of stimulation was calculated.

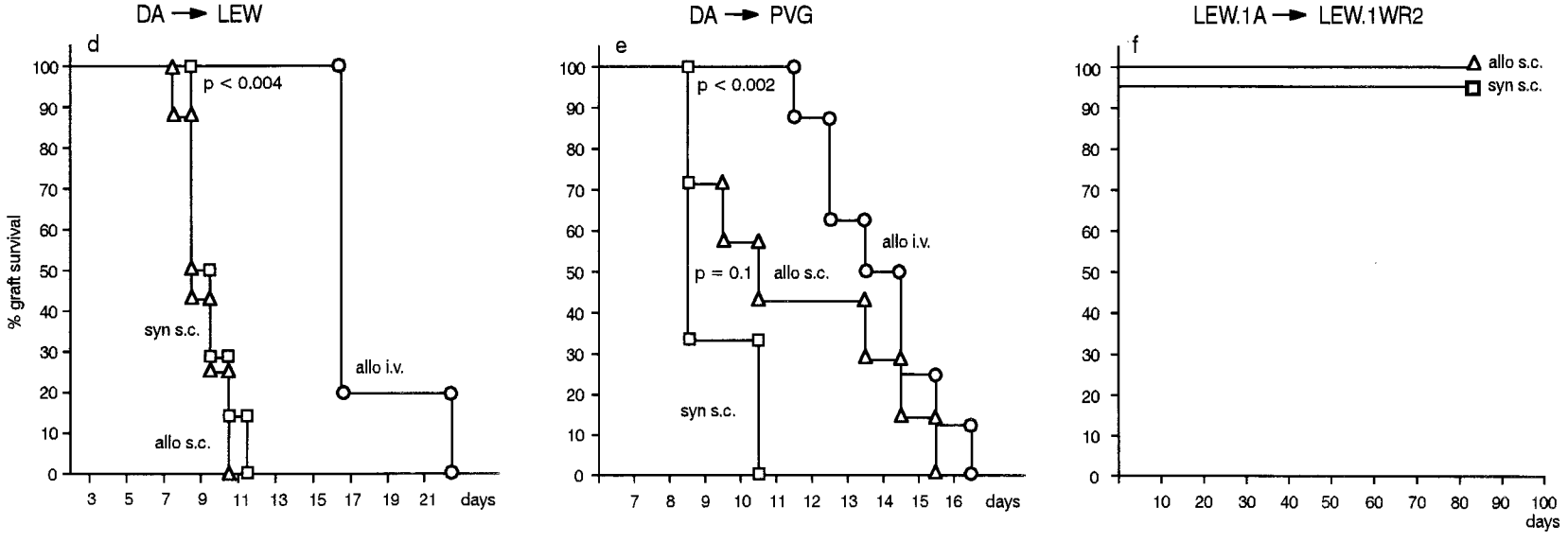
Heart transplantation

Transplantation was performed as previously described [15]. Briefly, the aorta and pulmonary artery of the donor heart were anastomosed to the aorta abdominalis and vena cava of the recipient. Graft survival was monitored by electrocardiogram. Rejection was defined as the moment when the transplanted heart stopped beating.

MHC I Peptides



Erythrocytes



Statistics

Results are expressed as mean ± SEM. Data were compared using the Wilcoxon signed rank test and the Mann-Whitney U-test. Heart allograft survival rates were calculated according to the Kaplan-Meier method.

Results

Peptide immunization induces accelerated graft rejection, while erythrocyte immunization does not

Recipients were immunized with synthetic peptides corresponding to the hypervariable region of the RT1-A^a antigen. In the first group, the peptides induced accelerated graft rejection (Fig. 1 a). Interestingly, in the second group (Fig. 1 b), peptide pretreatment did not influence graft survival. A dramatic effect was noted in the third group (Fig. 1 c). Whereas untreated controls presented a graft survival of more than 400 days (all hearts are presently still beating!), peptide-pretreated animals rejected their grafts within 15 ± 4.2 days.

All erythrocytes used for immunization carried the RT1-A^a antigen. Pretreatment with allogeneic erythrocytes did not induce accelerated rejection in any group (Fig. 1d–f). Moreover, when the erythrocytes were injected i.v., a prolonged graft survival was induced in groups 1 and 2 (Fig. 1). In group 2 even s.c. immunization appeared to prolong graft survival, however, this was not statistically significant.

◀ **Fig. 1a–f** Heart-allograft survival after immunization with donor MHC class I peptides or donor MHC class I bearing erythrocytes. Recipients were immunized with either (**a–c**) α1 + α2 peptides (s.c.) or (**d–f**) RT1-A^a erythrocytes. Controls received Freund's adjuvant (*FA*) only, control peptide + FA, or syngenic erythrocytes + FA. The recipients were transplanted with a DA or LEW.1A heart. Peptides induced accelerated rejection (**a,c**) or had no effect (**b**). Erythrocytes had either no effect or prolonged graft survival

Peptide and erythrocyte immunization induces antibody formation

All peptide-immunized recipients developed antipeptide antibodies (Fig. 2). Allogeneic erythrocytes elicited antibodies in group 1 after s.c. injection (Fig. 3).

Antipeptide antibodies do not bind to donor cells

To complete this experimental study, the binding of antipeptide antibodies to donor cells and T-cell sensitization were analyzed, although similar data have already been published [6]. The findings show that the antipeptide antibodies do not bind to donor cells (Fig. 4).

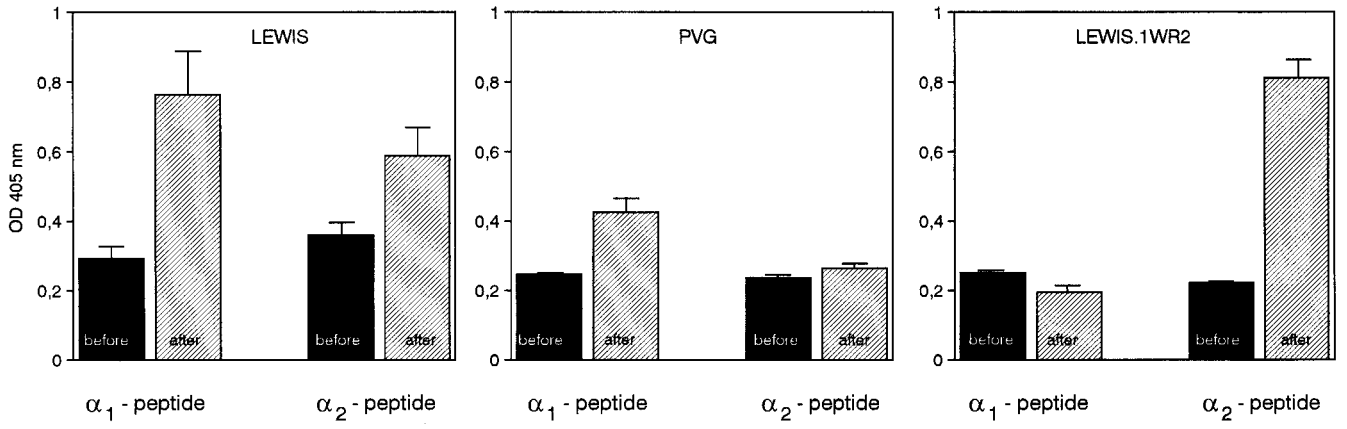
Peptide immunization induces T-cell sensitization

T-cell proliferation against peptides was studied in group 1. As shown in Fig. 5, T cells sensitized against both α1 and α2 peptides were detected in immunized but not in nonimmunized animals.

Discussion

When soluble MHC I peptides are injected into a recipient, they indirectly activate T cells [16]. In the current series of experiments, we sensitized rats of various strains with synthetic donor MHC I peptides and subsequently transplanted them with a heart allograft. Alternatively, the rats were immunized with MHC I in the form of intact molecules expressed on cell membranes. For this purpose, erythrocytes were ideal cells for two

Fig. 2 Antibody formation following immunization with donor MHC class I peptides. LEW, PVG, and LEW.1WR2 rats were immunized with α1 + α2 peptide. The anti-α1- or -α2-peptide antibody titer in serum (dilution 1:32) of four recipients in every group before and after immunization is shown



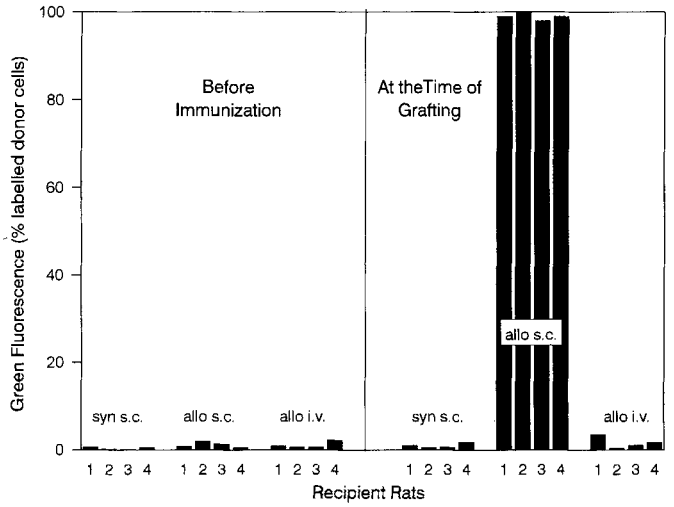


Fig. 3 Antibody formation following immunization with donor MHC class I bearing erythrocytes. LEW rats were immunized (s.c. or i.v.) with allogeneic (DA) or syngeneic erythrocytes and sera were collected before and after immunization. Serum antibodies against DA-derived T cells were measured by double fluorescence staining (anti-T-cell-PE, anti-rat-Ig-FITC) in a FACScan. The percentage of labelled donor T cells is shown. Subcutaneous immunization with erythrocytes induced antibodies

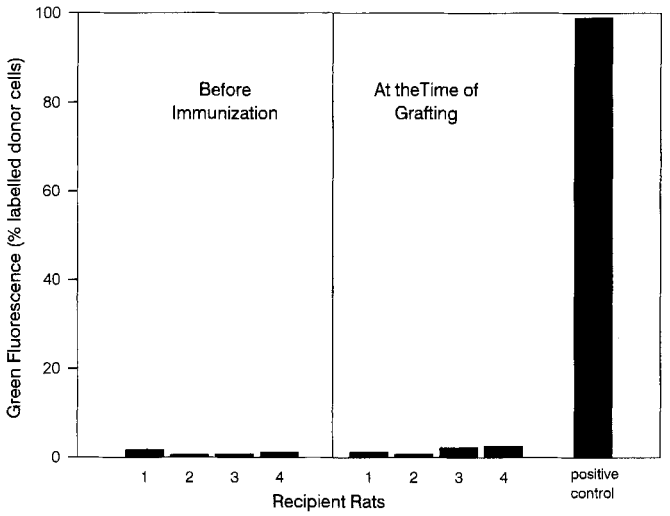


Fig. 4 Antipeptide antibodies do not bind to donor lymphocytes. DA lymphocytes were incubated with the serum of four LEW rats collected before immunization, after peptide immunization (containing antipeptide antibodies), and during rejection (containing anti-DA antibodies; positive control). Binding to donor T cells was measured by double fluorescence staining and the percentage of labelled cells is shown

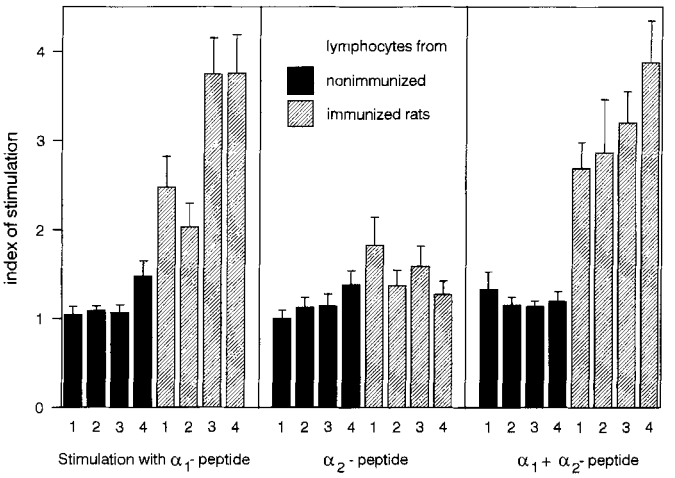


Fig. 5 T-cell proliferation against donor MHC class I peptides. Peripheral mononuclear blood cells of peptide-immunized and nonimmunized LEW rats were cultured with α1, α2, and α1 + α2 peptide. Cells without peptides served as control. ^{3}H-Thymidine incorporation (counts per minute) was measured and the stimulation index was calculated. Immunized animals presented a T-cell stimulation response against α1 ($P < 0.0001$), α2 ($P < 0.02$), and α1 + α2 peptide ($P < 0.0001$)

reasons. Firstly, they bear only MHC I locus A antigens [9]; thus, the antigenic material carried by erythrocytes was similar to that of the MHC peptides. Secondly, it is known that erythrocytes do not provide a costimulatory signal and, thus, are unable to directly activate recipient T cells. Therefore, if an immune response is obtained following erythrocyte injection, it must have been induced by indirect activation.

Indirect alloactivation by peptides induced accelerated rejection in LEW and LEW.1WR2 but not in PVG recipients. Donor erythrocytes, however, did not induce accelerated rejection in any strain. That rat erythrocytes are unable to sensitize transplant recipients even though they carry MHC I antigens has been reported previously by others [5, 24]. Erythrocytes might not promote rejection because indirect alloactivation does not occur, or indirect activation may take place but suppressive mechanisms are activated. In our first experimental group, there was antibody formation following immunization with erythrocytes, indicating that at least in these recipients indirect priming must have taken place. What, then, is the reason for the lack of accelerated rejection? In contrast to antipeptide antibodies, erythrocyte-induced antibodies bind to donor cells. It is known that antidonor cell antibodies are able to enhance graft survival in rats [8, 20, 21]. We considered the possibility that the graft-protective effect of erythrocyte-induced antibodies counteracted the accelerated rejection response. However, our results show that antibodies appear only after s.c. but not i.v. treatment. Interestingly, it was the group without antibodies that showed a significantly prolonged graft survival. This finding makes a graft-enhancing effect of antidonor cell antibodies highly improbable. Although erythrocytes are not able to directly activate lymphocytes, they can be directly recognized by T cells. T-cell receptor occupancy without acti-

vation is known to deliver an inactivating signal [14]. This could be the reason for the lack of accelerated rejection or even prolongation of graft survival in erythrocyte-transfused animals.

In group 3, donors and recipients differed only with respect to their MHC class I locus A antigens. As shown by the more than 400-day graft survival in untreated recipients, locus A antigens expressed on the transplanted heart were not able to induce rejection, either by direct or indirect recognition. Importantly, however, the activation of indirect recognition by immunization with locus A peptides induced rapid rejection. These results show that sensitization by indirect recognition is fully capable of initiating acute rejection, even in a weakly incompatible donor-recipient combination in which alloactivation is ineffective. Our results do not allow us to say conclusively whether indirectly sensitized T cells only amplify a latent rejection response caused by direct activation or whether they are capable of a genuine initiation of immunologic effector mechanisms. From a clinical point of view, these findings suggest that sensitization by indirect recognition as a result of pretransplant exposure to allogeneic MHC antigens (e.g., pregnancy, blood transfusion, previous graft) is capable of activating effector mechanisms that may destroy the allograft even in a weakly incompatible donor-recipient combination.

Although it was not the objective of this work to define the exact mechanism of graft destruction by indirect allorecognition, it is tempting to speculate about the possible mechanisms. In our experiments we obtained evidence that although recipients developed antibodies following peptide treatment, the antibodies did not bind to MHC molecules expressed on donor cells. Therefore, it is unlikely that the antibodies promoted rejection. We favor the hypothesis supported by previous experiments [4, 11, 19, 23] that sensitized T-helper cells in MHC peptide-immunized animals augment the destructive activity of cytotoxic T cells. For a direct graft-damaging effect of indirectly primed T helper cells, the only possible mechanism would be a delayed-type hypersensitivity reaction within the graft.

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