

## The influence of MHC class II antigen blockade by perfusion with a monoclonal antibody on rat renal graft survival

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**Abstract.** To decrease immunogenicity of the rat kidney, grafts were perfused with an anti-MHC class II monoclonal antibody (mAb). How effectively this procedure blocked class II-positive cells, which were mainly dendritic in appearance, was checked by immunostaining renal sections after perfusion and comparing them with *in vitro* stained sections. Optimum conditions were applied for graft pretreatment before transplantation. This procedure prolonged graft survival, though not satisfactorily from the biological point of view ( $9.6 \pm 0.8$  versus  $7.7 \pm 0.5$  days in the control group;  $P < 0.02$ ). The dendritic cells were not killed but blocked. Several hours after transplantation, the mAb dissociated from these class II-positive cells. It was also shown that donor cells migrate into the recipient's spleen early after transplantation. The number of these cells was smaller when the transplanted organ was perfused with the mAb. Further studies are suggested to deplete the graft of donor dendritic cells more adequately. They should also combine graft perfusion with anti-class II mAb and recipient immunosuppression at reduced doses.

**Key words:** Class II antigen blockade, rat, renal transplantation – Monoclonal antibody, class II antigen blockade – Renal transplantation, rat, class II antigen blockade

The immunogenicity of the graft and the immunological reactivity of the recipient are the main factors that determine allogeneic graft rejection. Immunogenicity depends on the presence of minor and major histocompatibility complex (MHC) class I and II gene products on the cell surface. It has been shown that class II antigen expression is not stable, and various protocols may change the extent to which these antigens are expressed [1, 2, 16, 19, 22, 24, 25]. It has also been demonstrated in studies on the heart and pancreas [3, 21] that rat strains may be divided into

two groups. One has a high density of interstitial dendritic cells (DC) that express class II antigens and the other a low density. For successful transplantation in different rat strain combinations, it may be advantageous to know the class II MHC antigen expression and to take it into account.

To decrease the immunogenicity of the graft, various procedures have been used [4, 9, 13, 14, 17, 18, 23]. Evidence is accumulating to support the hypothesis that graft immunogenicity may be altered by pretreatment with anti-class II monoclonal antibodies. However, evidence of the effectiveness of perfusion procedures, as presented in the literature, is quite fragmentary.

In the present study, MHC class II antigen expression was investigated in rat renal grafts to analyze the effectiveness of the perfusion procedure in pretreating the graft and to define the optimum conditions for blocking class II antigens expressed on DC. The efficacy of the method was assessed morphologically by the immunoperoxidase technique on renal tissue sections. Kidneys pretreated under optimum conditions were used for allogeneic rat transplantation.

### Materials and methods

#### Animals

Inbred male LEW (RT1 l), DA (RT1 a), and CAP (RT1 c) rats, bred in our department, were used in the study. All rats were 3–4 months old. Male DA and LEW rats were used as transplant donors and recipients.

#### Monoclonal antibodies (mAb)

Organ perfusions were done with an anti-class II mAb (29A1) of the IgG1 type that reacts with the I-E subregion. This mAb was produced at the Department of Immunology in Kiel. For immunoperoxidase staining, the following mAbs were also used: MRC Ox6, which reacts with monomorphic determinant of rat class II antigen (I-A region), and MRC Ox3, directed against a polymorphic determinant of class II antigen present on cells from animals of the LEW strain but not of the DA or CAP strains (Serotec, Kidlington, Oxford, UK).

### Organ perfusion

The left kidney was removed under ether anesthesia, perfused with 1.5 ml of cold phosphate-buffered saline (PBS) and then with mAb 29A1 (2.0 ml culture supernatant + 0.5 ml 20% glucose), and placed into an ice-cold bath for 20, 30, 60, or 120 min. The kidney fragments were snap-frozen and stored at  $-80^{\circ}\text{C}$ .

### Histology

Seven-micrometer cryostat sections were cut, air-dried, fixed in acetone, and stored at  $-20^{\circ}\text{C}$  until use. MHC class II antigen expression in organs perfused with cold PBS was demonstrated using a three-stage immunoperoxidase technique. The first step was to incubate the organ sections with the mAb. In two further steps, peroxidase-conjugated antiglobulin antibodies were used: rabbit anti-mouse Ig (Dianova, Hamburg, FRG) and goat anti-rabbit Ig (Daco, Copenhagen, Denmark). After staining with diaminobenzidine, the slides were colored with hematoxylin. Sections of organs perfused with 29A1 were analyzed for the effectiveness of mAb binding to class II antigen-positive cells with the two-stage immunoperoxidase technique, the second and the third stages of the three-stage method.

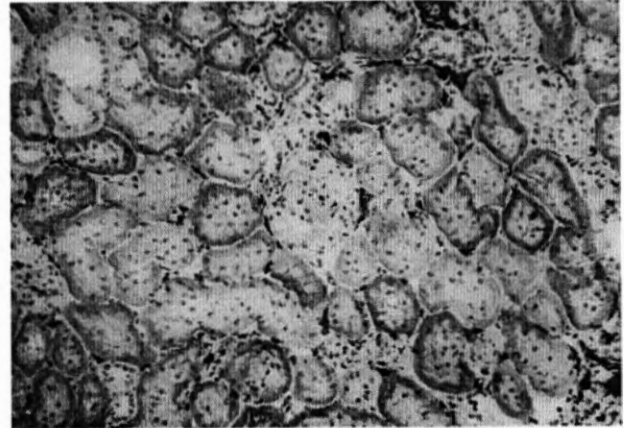
### Renal transplantation

Renal transplantations were performed according to the method employed by Lee [12] on rats weighing 250–300 g. In brief, the donor kidney was perfused with mAb 29A1 and incubated at  $4^{\circ}\text{C}$  for 60 min. Both of the recipient's own kidneys were removed. After incubation, the mAb was washed out of the donor kidney with PBS. The distal aorta and vena cava of the graft were connected to the re-

**Table 1.** Survey of MHC class II antigen expression on cells of the normal rat kidney in various inbred strains and an analysis of its ontogenesis

Rat strain	Age (weeks)	Class II-positive cells				Staining intensity of dendritic cells <sup>a</sup>
		Cells/glomerulus		Interstitial cells/mm <sup>2</sup>		
		n	$\bar{x} \pm \text{SD}$	n	$\bar{x} \pm \text{SD}$	
DA (RT1 a)	3	18	$0.1 \pm 0.3$	11	$103 \pm 30$	2+
	6	21	$0.8 \pm 0.7$	11	$96 \pm 25$	4+
	9	21	$1.6 \pm 1.4$	11	$113 \pm 19$	4+
	12	36	$0.9 \pm 1.0$	39	$78 \pm 24$	4+
	3–6		$P < 0.05$		NS	
	3–9		$P < 0.05$		NS	
	3–12		$P < 0.05$		NS	
LEW (RT1 l)	3	19	0	23	$19 \pm 11$	1+
	6	35	$0.6 \pm 0.7$	19	$23 \pm 11$	2+
	9	24	$1.5 \pm 1.2$	19	$37 \pm 16$	3+
	12	64	$1.2 \pm 1.4$	38	$38 \pm 21$	4+
	3–6		NS		NS	
	3–9		NS		$P < 0.01$	
	3–12		NS		$P < 0.01$	
CAP (RT1 c)	3	29	$0.1 \pm 0.3$	15	$78 \pm 30$	3+
	6	24	$2.2 \pm 1.5$	14	$80 \pm 16$	4+
	9	20	$3.4 \pm 1.6$	11	$127 \pm 26$	4+
	12	70	$2.2 \pm 1.5$	27	$78 \pm 23$	4+
	3–6		$P < 0.01$		NS	
	3–9		$P < 0.01$		$P < 0.05$	
	3–12		$P < 0.01$		NS	
DA 12–LEW 12		NS			$P < 0.01$	
CAP 12–LEW 12		$P < 0.05$			$P < 0.01$	

<sup>a</sup> Grading of staining intensity: 1+, weak; 2+, medium; 3+, strong; 4+, very strong



**Fig. 1.** Constitutive expression of MHC class II antigen in normal rat kidney. Sections of LEW rat kidney incubated with an anti-class II mAb (Ox6) and identified by the immunoperoxidase method. Interstitial dendritic cells and the cytoplasm of epithelial cells in proximal tubules are stained

spective structures of the recipient via end-to-side anastomoses. A ureter-ureter anastomosis was constructed over a fine polyethylene internal stent. The warm ischemia time did not exceed 20 min. If an obvious technical failure accounted for the death of the recipient, the animal was excluded from the study. Kidneys were transplanted in the following groups: (a) LEW to LEW for technical control after kidney perfusion; (b) group 1: DA to LEW, kidneys perfused with PBS; (c) group 2: DA to LEW, kidneys perfused with mAb 29A1; (d) group 3: LEW to DA, kidneys perfused with mAb 29A1; and (e) group 4: DA to LEW, kidneys perfused with mAb 29A1, anti-mouse IgG, and complement or mAb 29A1 and complement. Graft rejection was defined as death of the animal.

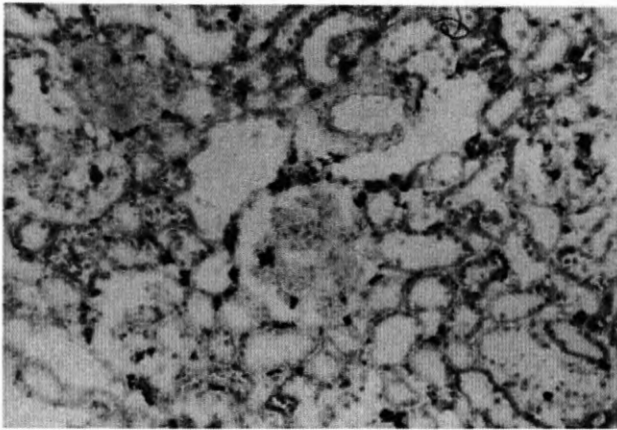
### Results

#### Constitutive expression of MHC class II antigens in the rat kidney

Our first study dealt with the constitutive expression of class II antigens on rat kidney cells because we wanted to know how many of these cells bind the mAb when the graft has been perfused with an anti-class II mAb. Table 1 presents the results of the *in vitro* immunohistological analysis of the labelling of class II-positive cells with mAb 29A1. The localization of these cells was predominantly interstitial, especially between renal tubules. The glomeruli contained some densely stained cells, localized within the mesangium. The mean number of class II-positive cells per square millimeter varied significantly between rat strains ( $P < 0.01$ ). Figure 1 shows the constitutive expression of class II antigen in the normal rat kidney.

#### Ontogenesis of class II expression in the rat kidney

Table 1 also presents results of the analysis of class II antigen expression by cells of the rat kidney in various age groups in three studied strains. In all of these strains, 3-week-old rats had a significantly lower number of posi-



**Fig. 2.** Labelling of class II-positive cells in LEW rat kidney after perfusion with mAb 29A1 and 60 min of incubation. Sections of kidney incubated with anti-immunoglobulin conjugated with peroxidase. Staining of interstitial dendritic cells between tubules

tive cells per glomerulus than 6- and 12-week-old rats ( $P < 0.05-0.01$ ). The number of class II-positive interstitial cells in 3-week-old DA and CAP rats was similar to that in 12-week-old rats. In kidneys from the LEW strain, however, the number of class II-positive interstitial cells was significantly smaller than in 9- and 12-week-old rats ( $P < 0.01$ ), and the staining intensity was weaker.

#### Labelling of class II-positive cells by monoclonal antibody

Table 2 presents the results of the binding of class II-positive cells by *ex vivo* perfusion with mAb 29A1, followed by incubation for varying periods of time. These studies showed that perfusion with the mAb and a 60-min incubation procedure are sufficient to label all cells that express class II antigen. Therefore, this procedure was accepted for pretreatment of grafts prior to transplantation. An example of class II antigen-positive cells labelled by the mAb is presented in Fig. 2.

#### Rat kidney transplantation

Table 3 presents the results of kidney transplantation with and without graft pretreatment with mAb 29A1. Blockade of class II-positive cells with the mAb in a perfusion procedure was able to prolong graft survival significantly. To investigate how mAb pretreatment of grafts with varying densities of interstitial DC affects graft survival, transplantation was done in two different systems. In the first - DA-to-LEW - the graft had a high density of DC, and in the second - LEW-to-DA - the density was low. Yet, no difference in graft survival was found between these groups. IgG1 mouse antibody is known not to activate complement. Therefore, to induce cytolysis of DC within grafts, perfusion was followed by a second perfusion with anti-mouse immunoglobulin and complement or

complement alone. These procedures did not, however, prolong graft survival either.

#### Analysis of dissociation of the mAb from cells after transplantation

To answer the question how long the mAb is bound to class II-positive cells after transplantation, three kidneys in each group were explanted after 4, 8, 24, and 48 h. Sections of all kidneys were stained with O<sub>x</sub>3, O<sub>x</sub>6, or anti-immunoglobulin conjugated with peroxidase only (to detect mAb 29A1 on the cell surface). Four hours after transplantation,  $37.5 \pm 2.4$  DC/mm<sup>2</sup> were labelled and

**Table 2.** Labelling of cells expressing class II MHC antigen by various perfusion-incubation procedures with the anti-class II monoclonal antibody 29A1

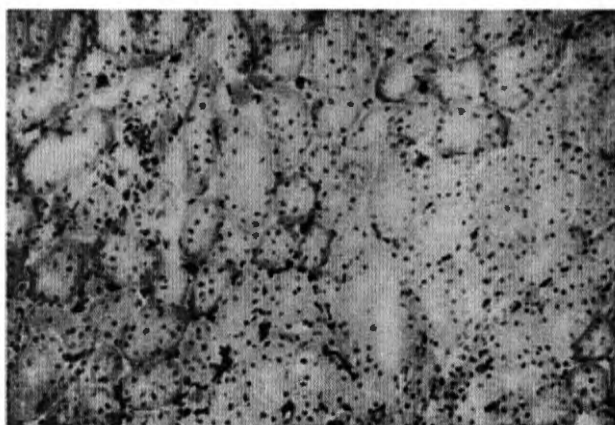
Rat strain	No. of organs	Incubation time (min)	Cells marked with mAb 29A1		Staining intensity <sup>a</sup>		
			Cells/glomerulus	Interstitial DC/mm <sup>2</sup>			
			<i>n</i>	$\bar{x} \pm SD$	<i>n</i>	$\bar{x} \pm SD$	
DA	2	30	41	1.8 ± 1.8	24	73 ± 19	2+
	2	60	21	1.7 ± 1.5	17	100 ± 32	3+
	2	120	40	1.5 ± 1.0	21	95 ± 23	3+
		30-Normal expression <sup>b</sup>		NS		NS	
		60-Normal expression <sup>b</sup>		NS		NS	
		120-Normal expression <sup>b</sup>		NS		NS	
	LEW	2	30	36	0.8 ± 0.8	11	50 ± 18
1		60	40	0.6 ± 0.5	22	50 ± 17	2+
2		120	35	1.5 ± 2.1	50	53 ± 17	3+
		30-Normal expression <sup>b</sup>		NS		NS	
		60-Normal expression <sup>b</sup>		NS		NS	
		120-Normal expression <sup>b</sup>		NS		$P < 0.05$	
CAP		1	20	38	1.1 ± 0.9	15	8 ± 10
	2	30	30	2.6 ± 1.4	20	89 ± 18	2+
	2	60	43	2.0 ± 1.2	25	91 ± 28	3+
	2	120	47	1.6 ± 1.2	20	86 ± 24	3+
		20-30		$P < 0.02$		$P < 0.001$	
		20-Normal expression <sup>b</sup>		$P < 0.02$		$P < 0.001$	
		30-Normal expression <sup>b</sup>		NS		NS	
		60-Normal expression <sup>b</sup>		NS		NS	
	120-Normal expression <sup>b</sup>		NS		NS		

<sup>a</sup> Grading of staining intensity: 1+, weak; 2+, medium; 3+, strong; 4+, very strong

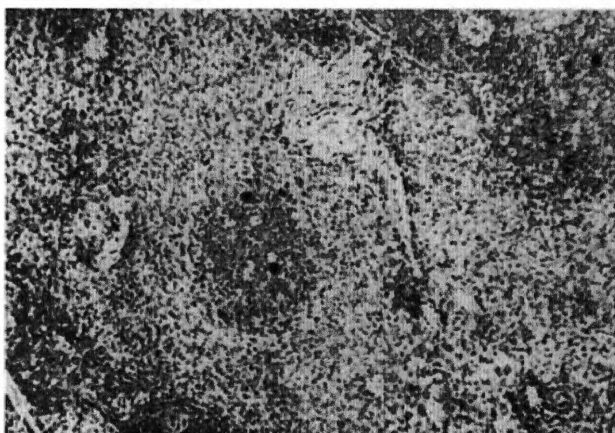
<sup>b</sup> Normal expression: number of class II-positive cells in *in vitro* staining of 12-week-old rat kidney

**Table 3.** Results of kidney transplantations in the rats

Donor strain	Recipient strain	No. of animals	Pretreatment of the graft	Graft survival (days)	$\bar{x} \pm SD$
LEW	LEW	2	Perfusion mAb 29A1	> 150, > 150	> 150
DA	LEW	10	Perfusion PBS	7, 8, 7, 8, 8, 7, 8, 8, 8, 8	7.7 ± 0.5
DA	LEW	5	Perfusion mAb 29A1	10, 9, 11, 9, 9	9.6 ± 0.8 $P < 0.02$
LEW	DA	3	Perfusion PBS	10, 8, 8	8.6 ± 1.1
LEW	DA	5	Perfusion mAb 29A1	9, 8, 9, 10, 9	9.0 ± 0.6
DA	LEW	5	Perfusion mAb 29A1 anti mouse IgG rat complement	9, 10, 8, 9	9.0 ± 0.8 $P < 0.05$
DA	LEW	2	Perfusion mAb 29A1 rabbit complement	8, 9	8.5 ± 0.5 NS



**Fig. 3.** Section of rat kidney perfused with mAb 29A1 and transplanted. Explantation done 4 h after grafting. Section incubated with anti-immunoglobulin conjugated with peroxidase. Interstitial dendritic cells are stained



**Fig. 4.** Demonstration of donor-specific class II-positive cells in recipient's peripheral white pulp of spleen. Spleen of DA rat transplanted with LEW kidney. Section stained with anti-donor-specific class II mAb (Ox3)

proximal tubular cells were weakly stained (Fig. 3). Sections stained with Ox3 and Ox6 showed  $42.9 \pm 5.8$  and  $83.8 \pm 1.5/\text{mm}^2$  class II-positive cells. Eight hours post-operatively, the number of labelled interstitial DC was  $29.3 \pm 4.2/\text{mm}^2$ . Staining of additional sections with mAb Ox3 showed  $33.8 \pm 9.0/\text{mm}^2$  donor-specific class II-positive cells. Twenty-four hours after transplantation, only sporadic cells were weakly stained ( $6.5 \pm 5.3/\text{mm}^2$ ). At the same time, staining of kidney sections with anti-class II mAb Ox6 demonstrated the presence of  $48.1 \pm 8.0/\text{mm}^2$  positive cells in interstitial tissue. Additional sections stained with anti-donor-specific class II antigen Ox3 (in the LEW-to-DA system) showed  $28.6 \pm 2.7$  cells/ $\text{mm}^2$  of donor origin. Thus, 24 h after transplantation, the number of donor DC was reduced and only 23% of them were labelled with mAb 29A1 given via perfusion. Forty-eight hours postoperatively, only a few cells were weakly stained, whereas  $28.8 \pm 7.1$  cells/ $\text{mm}^2$  reacted with Ox3. A similar number of Ox3-positive cells were observed on day 3 post-transplantation. These results showed that dissociation of the mAb begins earlier than emigration of DC and that

not all DC leave the graft during the first 3 days after transplantation.

#### *Migration of cells between the host and the graft*

On the basis of the observation that some regions of the kidney sections made 24–48 h after transplantation (LEW-DA) are depleted or even free of DC, recipient spleen and lymph nodes were analyzed for the presence of donor-specific class II cells. Donor-specific (LEW) class II-positive cells that reacted with mAb Ox3 were found in the peripheral white pulp of the recipient's (DA) spleen (Fig. 4). These cells were negative when stained with anti-mouse immunoglobulin to detect mAb 29A1 on their surfaces. There was a difference in the mean number of donor cells in the spleen between groups of rats pretreated with mAb 29A1 and those treated with PBS only ( $1.6 \pm 0.6$  versus  $3.3 \pm 1.7$  cells per periarteriolar lymphoid sheath). In mesenteric lymph nodes of rats transplanted with organs perfused with PBS only, sporadic cells of donor class II antigen were found, whereas in those perfused with mAb 29A1, none were found.

Immunomorphological studies performed with MRC Ox3 in the DA-to-LEW system demonstrated that infiltration of the graft by recipient-specific class II cells begins 2 days after transplantation.

#### **Discussion**

Class II-positive interstitial DC are able to present alloantigen as well as to act as accessory cells [11, 15], and they constitute a major component of the renal cells that may induce graft rejection. Therefore, studies on blockade or elimination of these cells from the graft have been proposed in the hope of prolonging the survival of the graft. Several studies have been done with organs perfused with anti-MHC class II mAbs.

In 1983, Otsubo et al. [17] demonstrated that after perfusion of canine kidneys with anti-mouse class II antigens crossreacting with canine class II antigen, it is possible to prolong graft survival by a mean value of 15 days (7 days in the control group). On the other side, Pollak et al. [18], who used the anti-rat class II mAb Ox4, showed that this perfusion did not alter the immunogenicity of the graft, as there was no prolongation of renal graft survival. Jablonski et al. [9] obtained similar results.

Studies on pancreas transplantation have shown that a blockade of class II-positive cells in the graft did not change graft survival [4, 13, 14, 23]. In these studies, the effectiveness of the DC blockade was analyzed. They indicated that nearly all cells were labelled.

To better define the effectiveness of the procedure used for kidney graft pretreatment, we analyzed MHC class II antigen expression in the normal rat kidney. The influence of age on class II antigen expression was also studied. The results confirmed and extended earlier studies on class II antigen expression in the kidney, which were, however, not quantitative [5, 6]. Our studies showed that all class II-positive DC were labelled after a procedure that included perfusion and 60 min of incubation.

Further studies have shown that this well-controlled procedure for graft pretreatment prolongs the graft's survival. The extent to which it does is not satisfactory, however, from the biological point of view. In light of these results, the question arose as to whether dissociation of an antibody is a factor responsible for this only short prolongation. Indeed, the studies done on sections of kidneys explanted at various time intervals after transplantation showed that the mAb became dissociated from the cell surface some hours after transplantation.

For several years the view has been accepted that sensitization of the host against kidney allograft occurs peripherally, within the graft. When we discovered in sections of our grafts explanted 24 and 48 h after transplantation that some regions of kidney tissue were free of DC, we considered this finding to be an effect of cytolysis induced by the mAb, allowing reduced graft immunogenicity. However, the brief survival of the perfused grafts led us to consider the alternative possibility that DC had migrated out of the graft and into the lymphatic tissue of the recipient and, thus, participated in the induction of graft rejection. For this reason we performed immunomorphological studies on the recipient's spleen and lymph nodes. As described above, it was found that as early as 1 day post-transplantation, donor-specific class II-positive DC begin to leave the graft and migrate into lymphatic tissues, preferentially the spleen. Similar studies on animals grafted with kidneys perfused with a mAb have shown that this procedure results in decreased migration of donor cells into the recipient's lymphatic tissues. Early migration of DC from a cardiac graft into the recipient's spleen and localization in peripheral white pulp was observed by Larsen et al. [10].

Taken together, this suggests that the reduction of DC within the graft explanted 24 and 48 h post-transplantation was the result of both depletion of their population and migration out of the graft.

We wish to point out that the number of class II-positive DC within the graft should be considered with caution, because some days after transplantation other cells, such as T lymphocytes or tubular epithelial cells, may be induced to express MHC class II antigen. In our perfusion experiments, however, induction of class II antigen on other cells may have been delayed, a suggestion that is supported by our earlier observation that only after mAb perfusion was class II antigen not expressed on distal tubular cells 4 days after transplantation.

Furthermore, considering that the mAb of IgG1 class, which we used in our study, does not activate complement, with the result that it becomes sufficiently cytolytic, the fact that some donor DC persisted within the graft is understandable. However, the combined perfusions with mAb, polyclonal anti-mouse immunoglobulin, and complement did not further prolong graft survival. Nor did destruction of DC within grafts by mAb targeted with A-chain ricin as immunotoxin [23] or by irradiation [9] inhibit graft rejection or prolong graft survival. Studies by Heart et al. [7] on retransplantation of rat kidney to another host showed only marginal prolongation of allogeneic graft survival. However, retransplantation of long-term surviving (DA x LEW) F1 grafts to fresh DA hosts,

resulted in very prolonged survival. The authors suggest that this result depends on the loss of donor DC from the graft.

On the second day after transplantation, we observed the beginning of infiltration of the graft by host cells. It may, therefore, be concluded that there is a bidirectional cell migration system between graft and host.

Our transplantation experiments showed that the survival of perfused grafts is only prolonged for about 3 days. This finding is in accordance with the observation that donor DC do not remain labelled with mAb for longer than 24 h. After that time, DC retrieved their alloantigen presentation ability and induced rejection.

Both migrating elements may participate in the induction of rejection by: (a) presentation of alloantigen by donor cells in the graft and later on in the recipient's spleen and (b) penetration of host cells into the graft and processing and presentation of alloantigen to T helper cells. The latter manner of alloantigen presentation has been suggested by Lechler and Batchelor [11], studied by Ishikura et al. [8], and confirmed by Sherwood et al. [20]. Participation of host cells in alloantigen presentation is good reason for combining mAb perfusion procedures with immunosuppression of the recipient. Thus, it may become possible (a) to apply appropriately reduced doses of immunosuppressive drugs and (b) to induce tolerance, allowing such treatment to be stopped several weeks or months after transplantation. Very promising studies along this line are in progress in our laboratory.

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