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Clonal deletion as the mechansim of abrogation of immunological memory following liver grafting in rats

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Summary. In the rat strain combination of DA into PVG, an orthotopic liver graft has the ability to abrogate an existing state of sensitization against donor (DA) antigens. Fifty-four percent of PVG rats sensitized against DA by skin grafting accepted a DA liver graft permanently, and about half of these became systemically tolerant of DA MHC antigens, as demonstrated by permanent acceptance of a subsequent (second-set) DA skin or heart graft. The cellular basis of this tolerant state was studied *in vivo*. An adoptive transfer assay provided evidence for functional deletion of DA-reactive cells responsible for graft rejection from the recirculating lymphocyte pool. There was no evidence of a role for suppressor T cells in maintaining tolerance. However, a graft-versus-host assay showed normal reactivity in thoracic duct lymphocytes from tolerant animals. Hence, specific clonal deletion is apparently responsible for the abolition of immunological memory by liver grafting, but is selective in respect of the sets of alloreactive lymphocytes affected.

INTRODUCTION

Since the first reports of liver transplantation in the pig, it has been recognized that this organ does not obey the normal rules of graft rejection, often showing prolonged survival without the need for immunosuppression (Cordier *et al.*, 1965; Calne *et al.*, 1967). We have previously reported that in the fully allogeneic rat strain combination of DA (MHC haplotype $RT1^a$) grafted to PVG ($RT1^c$), liver transplants are never rejected, whereas rejection of other organs, such as kidney, heart or skin, occurs rapidly (Kamada, Brons & Davies, 1980); in fact, liver grafting induces a state of specific, systemic immunological tolerance to donor antigens, such that the recipient will also subsequently accept skin, heart or kidney grafts from the same strain (Houssin *et al.*, 1980; Kamada, Davies & Roser, 1981a; Kamada & Wight, 1984; Kamada, 1985). Investigations *in vivo* using an adoptive transfer technique demonstrated a specific functional deletion of alloreactive clones from the T-cell repertoire of PVG rats rendered tolerant by DA liver grafts (Davies, Kamada & Roser, 1983). *In vitro*, the lymphocytes of such DA-tolerant PVG rats failed to produce cytotoxic T cells against DA targets, though the mixed lymphocyte reaction against DA cells was undiminished (Kamada *et al.*, 1981a; Davies *et al.*, 1983).

More recently, we have shown that the tolerance-inducing capacity of allogeneic liver occurs even in immunized recipients, leading to the specific reversal of immunological memory. Thus, about 50% of PVG rats which had been presensitized to DA antigens by skin grafting survived indefinitely after orthotopic DA liver grafting, and accepted subsequent (second-set) grafts of skin from DA but not from third party strains

Abbreviations: GVH, graft-versus-host reaction; MST, mean survival time; TDL, thoracic duct lymphocytes.

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(Kamada, Davies & Roser, 1981b). Here, tolerance was induced even though sensitization increases the potency of alloreactive T cells at least 100-fold over that of normal PVG rats (Hall, Dorsch & Roser, 1978). This remarkable ability to abrogate an existing state of transplantation immunity against a full haplotype MHC difference is unique to liver grafts. It is also apparently restricted to rats; for example, preimmunized pigs severely rejected liver allografts from the donor against which they had been presensitized, such grafts being accepted by non-immunized pigs (Calne *et al.*, 1969; Galmarini *et al.*, 1971). The present experiments were designed to investigate the cellular basis of the tolerant state in PVG rats following the reversal of immunological memory by liver transplantation.

MATERIALS AND METHODS

Animals

PVG ($RT1^c$), DA ($RT1^a$), AO ($RT1^u$) and WAG ($RT1^u$) rats were obtained from OLAC (1976) Ltd., Bicester, Oxfordshire.

Transplantation

(a) Liver. Orthotopic liver transplantation (DA donors, PVG recipients) was performed by our published technique (Kamada & Calne, 1983). No immunosuppressive treatments were given.

(b) Skin. Full thickness orthotopic skin grafting (DA or AO donors, PVG recipients) was carried out according to the method of Roser & Ford (1972).

(c) Heart. Heterotopic auxiliary heart grafting (DA or WAG donors, PVG recipients) was performed in the neck using the method of Heron (1971). Rejection was defined by cessation of heart beat on neck palpation and confirmed by histological examination of the grafts.

Thoracic duct lymphocytes (TDL)

Lymph from different PVG rats (normal, DA-sensitized or DA-tolerant) was obtained by cannulation of the thoracic duct (Bollman, Cain & Grindlay, 1948). TDL were recovered by centrifugation at 4°, washed twice with Dulbecco's phosphate-buffered (A and B) saline containing 2% fetal calf serum (DAB/FCS), and counted and resuspended in DAB/FCS at a concentration of $2{\cdot}5 \times 10^7$ per ml.

Adoptive transfer assay

The method of adoptive transfer was as described by Dorsch & Roser (1974). Recipient PVG rats (groups of five or more) were irradiated with 850 rads, and one day later inoculated intravenously with 5×10^7 TDL; a heterotopic DA or WAG heart graft was transplanted into the neck of these animals on the same day and its survival time monitored.

Graft-versus-host assay

The popliteal lymph node weight assay was carried out using the method described by Ford, Burr & Simonsen (1970). Graded doses of TDL, from $1\text{–}9 \times 10^6$, were injected subcutaneously into the hind footpads of young $(DA \times PVG)F_1$ recipients (at least six per group). Seven days later, the recipients were killed and the popliteal lymph nodes removed, cleaned and weighed.

RESULTS

Induction of tolerance in sensitized rats by liver grafting

A group of 26 PVG rats were sensitized against DA antigens by grafting with DA skin; the grafts were all

Table 1. Survival of DA liver grafts in normal and DA-sensitized PVG recipients

PVG rat recipient	No. of animals	Days of DA liver graft survival	Mean survival time (days ± SD)
Normal	20	All > 100 days	> 100
DA-sensitized*	26	11,14,15,16,21,26,28,30,30,33,33,34 remainder all > 100 days (14)	24·3 ± 8·0 > 100†

* By grafting of DA skin 28 days before liver graft.
† MST of rejected grafts only.

Table 2. Survival of DA skin and heart grafts on normal, DA-sensitized, or DA-sensitized, DA liver grafted PVG recipients

PVG rat recipient	Survival times of subsequent grafts in individual rats (days)					
	DA skin	MST* ± SD	AO skin	MST ± SD	DA heart†	MST ± SD
Normal	7,8,8,8,8,9	8·0 ± 0·6	8,8,8,8,9,9	8·3 ± 0·5	7,7,8,8,8,8,9,9,9	8·1 ± 0·7
DA-sensitized‡	7,7,7,7,7,7	7·0 ± 0			4,4,5,5,5,6	4·8 ± 0·7
DA-sensitized and DA liver grafted§	32,48,52; > 100 (3)	44 ± 8·6; > 100	9,9,9,11 L	9·5 ± 0·9	> 100 (4)	> 100
DA-sensitized and PVG liver grafted¶ (controls)	7,7,7,7	7·0 ± 0				

* MST = mean survival time in days ± standard deviation.
† Heterotopic auxiliary heart graft in the neck.
‡ By DA skin graft.
§ Orthotopic DA liver grafting was 28 days after a DA skin graft.
¶ Orthotopic syngeneic liver grafting was 28 days after a DA skin graft.

rejected within 9 days. Twenty-eight days later, the liver of each of these rats was removed and replaced by an orthotopic graft of DA liver; a group of normal (non-sensitized) PVG rats was used as control recipients. The fate of the grafts is shown in Table 1. In contrast with the long-term survival of DA livers attained in all the normal PVG rats, 54% of the DA-sensitized recipients rejected their grafts within 34 days, leading to the death of these animals. Histological examination of the rejected livers showed the intense mononuclear cell infiltration and hepatocellular necrosis typical of acute rejection. However, the remaining DA-sensitized recipients survived indefinitely, and liver biopsy showed minimal signs of rejection.

In order to test whether they had been rendered systemically tolerant of DA antigens, all surviving rats received a further graft of DA skin, or a heterotopic DA heart graft, 60 days after the liver graft. Table 2 shows that, in contrast with the second-set rejection expected of sensitized animals, DA skin grafts on DA-sensitized, DA-liver-grafted PVG rats showed a very prolonged survival in some animals, and permanent survival in the remainder. The antigen-specificity of the tolerant state was demonstrated by normal rejection of third party (AO) skin grafts on the same animals. The tolerant state was further confirmed by the DA heart grafts, also performed 60 days after DA liver grafting; four out of four animals accepted the subsequent DA heart graft (Table 2). Thus, DA liver grafting had effectively reversed the pre-existing immunological memory to DA antigens and converted it into unresponsiveness.

Allograft reactivity of TDL from tolerant rats: adoptive transfer assay

TDL were collected from normal PVG rats, from PVG rats sensitized against DA antigens by skin grafting, and from DA-sensitized rats rendered tolerant of DA by liver grafting (above), and transferred to groups of sublethally irradiated syngeneic recipients. On the same day, the recipients were given a heterotopic auxiliary DA or WAG (third party) heart graft in the neck and its survival monitored. Control groups included irradiated PVG rats which were heart-grafted but received no cells, and non-irradiated normal or sensitized PVG similarly grafted with DA or WAG hearts.

The results are shown in Fig. 1. Panel I shows the rejection of DA heart grafts in normal, non-irradiated PVG rats (Group B; mean survival time (MST) 7·8 ± 0·7 days) and the more rapid rejection in PVG presensitized with a DA skin graft (Group A; MST 5·2 ± 0·8 days). The results of the adoptive transfer assay are given in Panels II and III. Panel II shows that DA heart grafts survived for a considerable period on irradiated PVG recipients before eventual rejection in 60% of animals, the remainder surviving indefinitely (Group E; MST at 75 days was 63·3 ± 11·5 days). In

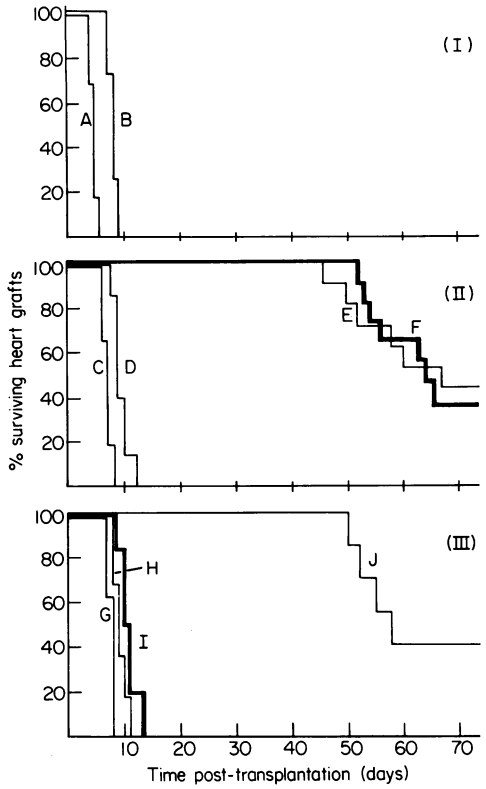

Figure 1. Adoptive transfer assay of allograft reactivity of TDL from normal, DA-sensitized or DA-sensitized, DA liver grafted PVG rats. The groups shown are as follows (mean survival time ± SD indicated). Panel I: (A) survival of DA heart grafts ($n=5$) implanted into non-irradiated PVG recipients sensitized against DA antigens by skin grafting 28 days prior to heart grafting (5·2 ± 0·8 days); (B) survival of DA heart grafts ($n=12$) in normal, non-irradiated PVG recipients (7·8 ± 0·7 days). Panel II: (C) survival of DA heart grafts ($n=6$) in irradiated PVG recipients injected with 5×10^7 TDL from PVG rats sensitized against DA by skin grafting (6·8 ± 0·7 days); (D) survival of DA heart grafts ($n=7$) in irradiated PVGs injected with TDL from normal PVG rats (9·5 ± 1·3 days); (E) survival of DA heart grafts ($n=10$) in irradiated PVGs without transfer of TDL (63·3 ± 11·5 days); (F) survival of DA heart grafts ($n=11$) in irradiated PVGs injected with TDL from DA-sensitized, DA liver-grafted PVG rats (64·5 ± 9·5 days). Panel III: (G) survival of WAG heart grafts ($n=8$) in normal (non-irradiated) PVG recipients (7·6 ± 0·5 days); (H) survival of WAG heart grafts ($n=6$) in irradiated PVGs injected with TDL from normal PVG rats (9·2 ± 1·2 days); (I) survival of WAG heart grafts ($n=6$) in irradiated PVGs injected with TDL as in Group F (9·8 ± 1·7 days); (J) survival of WAG heart grafts ($n=7$) in irradiated PVGs without transfer of TDL (65·0 ± 14·2 days).

irradiated PVG rats given either normal (Group D) or DA-sensitized TDL (Group C), rapid rejection of the heart grafts was restored (MST 9·5 ± 1·3 and 6·8 ± 0·7 days, respectively), demonstrating the successful adoptive transfer of the allograft rejection response. In contrast, TDL from PVG rats in which immunological memory of DA priming had been abolished by DA liver grafting failed to increase the rate of rejection at all (Group F; MST 64·5 ± 9·5 days—cf. Group E), indicating the functional absence of DA-reactive cells from the TDL of these animals. The specificity of unresponsiveness is demonstrated in panel III, where it is seen that TDL from the same DA-tolerant donors caused efficient rejection of heart grafts from a third party strain (WAG, Group I; MST 9·8 ± 1·7 days).

Graft-versus-host reactivity of TDL from tolerant rats

As a further approach to defining the alloreactivity of lymphocytes from presensitized PVG rats tolerized by liver grafting, quantitative GVH assays were per-

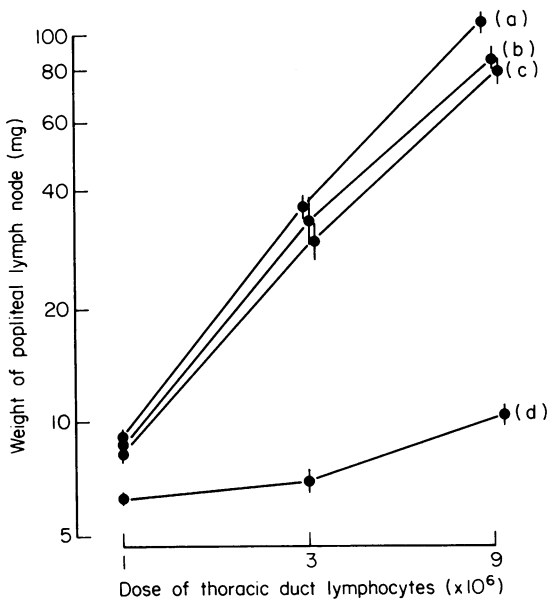

Figure 2. Graft-versus-host reactivity of TDL from normal, DA-sensitized or DA-sensitized, DA liver grafted PVG rats. The figure shows the mean weight of popliteal lymph nodes in (DA × PVG)F_1 rats receiving TDL from PVG rats sensitized against DA antigens by skin grafting (Group a), TDL from normal PVG rats (Group b), or TDL from PVG rats sensitized against DA and grafted with a DA liver (Group c). Group d is the control response with TDL from syngeneic (DA × PVG)F_1 rats. Vertical bars represent standard deviation.

formed in (DA × PVG)F_1 recipients. Figure 2 shows that the response, plotted as log popliteal lymph node weight, was linearly related to log cell dose, as described by Ford *et al.* (1970). Titrated TDL from DA-sensitized PVG rats (line a) produced a similar GVH response to those of unsensitized PVG rats (line b), the difference being significant only at 9×10^6 TDL. The GVH response of TDL from tolerized rats (line c) was identical with that of normal TDL, in spite of their failure to cause rejection in the adoptive transfer assay described above. The control response of syngeneic (DA × PVG)F_1 cells was negligible (line d).

DISCUSSION

The unusual capacity of a liver graft to induce a state of specific unresponsiveness in normal recipients has been well documented in pigs (Calne *et al.*, 1969) and in certain inbred rat combinations, particularly that of DA grafted to PVG, as used here (Kamada *et al.*, 1980; Roser *et al.*, 1983). Even more remarkable is the ability of a liver graft to overcome the effects of antigen priming in this combination. Adoptive transfer assays have shown the TDL of PVG rats primed against DA to be at least 100 times more active in graft rejection than those of unprimed rats (Hall *et al.*, 1978), and of course the primed animals show the typical accelerated rejection of second-set DA skin grafts. Nevertheless, Table 1 demonstrates that such primed animals often accept grafts of DA liver permanently. Even in the 46% of primed PVG rats which eventually rejected their DA liver graft, the survival time (MST 24 days) was appreciably longer than in unprimed rats of several other strains, including BN, LEW and AUG (MST 11 days; Roser *et al.*, 1983). Permanent acceptance of the DA liver graft was accompanied by systemic tolerance in about half the animals, as evidenced by the survival of second-set DA skin or heart grafts for over 100 days (Table 2), while in the remainder skin graft rejection was considerably delayed.

We have examined the alloreactive potential of lymphocytes from these tolerant rats in two ways *in vivo*. In the first, the adoptive transfer assay, the capacity of recirculating lymphocytes (TDL) from DA-tolerant PVG rats to reject DA allografts was studied by transfer to irradiated syngeneic recipients. The usefulness of this assay is its ability to demonstrate whether a T-cell population is normally alloreactive, clonally deleted, or contains suppressor cells (Roser & Dorsch, 1979). Figure 1 shows that TDL from DA-sensitized PVG rats rendered tolerant by DA liver grafting displayed no graft-rejection activity in irradiated hosts, nor did they prolong the time taken for irradiated rats to recover their immunocompetence. These observations argue against major roles for either serum 'blocking factors' (free antigen or antigen–antibody complexes) or suppressor T cells in the maintenance of systemic tolerance after liver grafting. The former could be expected to be shed from the surface of T cells within a reasonably short time after transfer, permitting anti-DA cells to recover from a 'blocked' state, while suppressor T cells delay or prevent the return of heavily irradiated rats to immunocompetence (Roser & Dorsch, 1979). The results indicate the specific absence of DA-reactive lymphocytes from the recirculating pool (clonal deletion). The same conclusion was reached in our previous analysis of TDL from normal (unprimed) PVG rats following DA liver grafting (Davies *et al.*, 1983). It is possible that this functional deletion is the result of sequestration of DA-reactive clones within the liver, leading to their 'negative selection' out of the recirculating pool; alternatively, it may be due to death or inactivation of alloreactive T cells following contact with antigen in the liver or the circulation.

The other measure of *in vivo* alloreactivity studied here is the GVH response. In this assay, the activity of TDL from tolerant PVG was not significantly different from the response of normal PVG TDL, though slightly less than that of primed TDL. Thus, the tolerance induced by liver grafting in primed recipients is evidently selective, in that clones of DA-reactive cells responsible for graft rejection have been eliminated from TDL, while those proliferating in response to DA antigens in the GVH reaction are only marginally affected. Our result suggests that GVH-reactive cells are unlikely to be the prime effectors of graft rejection. A possible explanation for this dichotomy in the tolerant state ('split tolerance') may be suggested on the basis that the GVH response is primarily one of T cells directed against Class II MHC antigens, while the effector cells in graft rejection are principally the cytotoxic T cells responding to Class I alloantigens (Hall & Dorsch, 1984). While this is doubtless an oversimplification, it is the case that the liver is rich in Class I, but relatively poor in Class II, antigens (Hart & Fabre, 1979), and so might well trap or eliminate the cytotoxic subclass more readily than the GVH-reactive T cells.

These results are in agreement with our previous

observations on the nature of tolerance induced in normal (non-sensitized) PVG rats by DA liver grafting (Kamada *et al.*, 1980; Davies *et al.*, 1983). Thus, tolerance induced in MHC-primed or non-primed animals appears to have the same cellular basis. However, while clonal deletion is probably a sufficient explanation for the systemic tolerance which develops in PVG rats following a DA liver graft, it does not necessarily constitute the reason for non-rejection of the liver itself. Alloreactive T cells are certainly not eliminated immediately. In fact, a vigorous rejection response ensues at first in the grafted liver but, instead of progressing, seems to exhaust itself after 2 weeks (Kamada *et al.*, 1983). The mechanisms behind the protection of the DA liver in normal or primed recipients are not yet explained, and its long-term survival may involve the suppressive effect of soluble MHC antigen and anti-MHC antibodies (Roser *et al.*, 1983) in addition to the clonal deletion of reactive cells described here.

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