# THE CELLULAR BASIS OF ALLOGRAFT REJECTION IN VIVO

## II. The Nature of Memory Cells Mediating Second Set Heart Graft Rejection*

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A striking feature of immune responses mounted against the major histocompatibility complex (MHC)[1] in vivo is the long duration of the specific memory of prior exposure to these antigens. Second-set skin graft rejection can be demonstrated several years after primary sensitization in mice and rats (1). Adoptive transfer of second-set rejection to naive animals with population of lymphocytes from immune donors has established that these cells carry specific memory (1, 2).

In most immune responses, including those to weak alloantigens, long term memory involves a large increase in immune responsiveness. This is not apparent in the intact animal sensitized to antigens of the MHC in which second-set skin graft rejection is usually only accelerated by 1–2 days over first-set rejection (3–5). Using an adoptive transfer system it is possible to demonstrate that there is in fact a large increase in potency in cells carrying memory for the MHC and that the reason this is not apparent in the intact animal is that the latter is 'saturated' with respect to the actual number of specific allograft reactive cells necessary to destroy the graft (6). Thus even large increases in potency are not reflected by a large increase in the speed of graft rejection. The adoptive allograft assay in irradiated rats described in the accompanying paper (7) has been used to investigate the nature of the changes in the lymphoid system which characterise the state of memory in rats immune to antigens of the MHC.

## Materials and Methods

*Rats.* In addition to the inbred strains described in the accompanying paper, the strains PVG-H-1$^w$ (AO)[2] congenic with PVG-H-1$^c$ but carrying the H-1$^w$ MHC, and AO-H-1$^w$ were obtained from Dr. Jonathan Howard, ARC Institute of Animal Physiology, Babraham, Cambridge.

*Sensitization.* Two sequential orthotopic-full thickness skin grafts were applied 14 days apart to the flank as previously described (8). Sensitized rats were used in experiments 3–12 mo after the second skin graft.

Irradiation, heart grafting, cell preparations, immunofluorescence, treatment with antimitotic agents and histology were as previously described (7).

* Supported by grants from the National Health and Medical Research Council, the N. S. W. State Cancer Council, and the University of Sydney Cancer fund.

[1] *Abbreviations used in this paper:* HU, hydroxyurea; Ig+, lymphocytes bearing surface immunoglobulin detectable with fluoresceinated rabbit antibodies to rat immunoglobulins; LNC, lymph node cells; MHC, major histocompatibility complex; MST, mean survival time; TDL, thoracic duct lymphocytes; VBL, vinblastine sulphate.

[2] H-1$^w$ is the current provisional nomenclature for the AO MHC. It will probably be altered in the near future to H-1$^v$ to permit H-1$^w$ to be reserved for wild-type alleles.


J. Exp. Med. © The Rockefeller University Press · 0022-1007/78/1001-0890$1.00
Volume 148 October 1978 890–902

## Results

*Potency of Cells from the Lymphoid Tissue of Sensitized Donors.* The relative potency of lymphoid cells of thymus, lymph node, thoracic duct lymph, spleen, and bone marrow of naive donors in the adoptive restoration of the allograft response has been described in the accompanying paper. The changes in potency of cell preparations from these tissues in PVG rats sensitized against DA alloantigens was measured. The results indicate that not only do all tissues show a dramatic and specific increase in potency (Table I), but a redistribution of allograft reactive cells has occurred. $10^6$ spleen cells from sensitized donors cause graft rejection with a more rapid tempo (mean survival time (MST) 16.5 ± 1.5 days) than $5 \times 10^8$ spleen cells from naive donors (MST 21 ± 1.1 days). Between $5 \times 10^6$ and $2 \times 10^7$ LNC or TDL from immune donors gave approximately the same restorative effect as $5 \times 10^8$ LNC or TDL from naive donors. The increase in potency is therefore greater than 500-fold with spleen cells and 25–100 fold with LNC and TDL. Even the thymus, which contains virtually no allograft reactive cells in naive donors, is a source of cells capable of causing graft rejection if the donor is sensitized. It is also noteworthy that, while a saturating dose of cells (>$5 \times 10^8$) from naive donors was incapable of causing graft rejection in less than 8 days, some animals reconstituted with cells from immune donors rejected their grafts with a second-set tempo of 6–7 days.

*Significance of the MHC in Allograft Memory.* It is known that memory to weak alloantigens is characterized by a large, demonstrable increase in potency (3). It is therefore possible that the large increase in the potency of memory cells shown in PVG rats immunized with DA skin was due to the multiple minor alloantigenic loci at which these rat strains differ and not to differences at the H-1 locus. The irrelevance of these minor antigenic differences to the increase in potency was established using rats congenic with PVG except for the H-1 locus. PVG-H-$1^c$ were immunized with either AO-H-$1^w$ or PVG-H-$1^w$. The former differ from PVG at the H-1 and multiple minor loci, the latter only at H-1. The potency of spleen cells from naive and sensitized animals in each strain combination was compared. In both cases $5 \times 10^6$ cells from sensitized animals restored graft rejection with a more rapid tempo than $5 \times 10^8$ cells from naive animals (Table II) showing that the increase in potency, where immunization is restricted to the H-1 locus, is no less than where additional minor antigens

TABLE I

*Heart Graft Rejection* in Irradiated PVG Rats‡ Restored with Cells from Various Lymphoid Tissues of Immune Donors*

| | Restorative Inoculum | | | | |
|---|---|---|---|---|---|
| Source | $2.5 \times 10^8$ | $5 \times 10^7$ | $2 \times 10^7$ | $5 \times 10^6$ | $10^6$ |
| LNC | ND | 8.1 ± 0.2 (10) | 8 ± 0.4 (8)§ | 10 ± 0.9 (8) | 23 ± 0.2 (4) |
| TDL | ND | 6.8 ± 0.3 (4) | 9.2 ± 0.3 (10) | 10.8 ± 1.2 (6) | ND |
| Spleen | ND | ND | 7.4 ± 0.5 (7) | 9.5 ± 0.5 (8) | 16.5 ± 1.5 (4) |
| Thymus | 8.4 ± 0.5 (5) | ND | 21.2 ± 2.6 (6) | ND | ND |
| Blood | ND | ND | 11.2 ± 1.3 (5) | ND | ND |

* Graft rejection is expressed as the mean rejection time ± SEM (number of rats).

‡ MST grafts in irradiated rats not reconstituted with cells was 59 ± 5.3 days.

§ $2 \times 10^7$ cells from these donors restored 3rd party LEW or BN graft rejection to 29.4 ± 2.3 days which is equivalent to the restorative effect of this number of naive cells (thus demonstrating specificity).

TABLE II
*Role of MHC in Memory*

| Skin and* heart donor strain | Restorative inoculum | |
|---|---|---|
| | $5 \times 10^8$ Naive spleen cells | $5 \times 10^6$ Sensitized spleen cells |
| AO-H-$1^w$ | 18 ± 1 (5) | 10.8 ± 0.6 (5) |
| PVG-H-$1^w$ | 27.4 ± 4.6 (5) | 13.2 ± 0.4 (5) |

* PVG rats were sensitized by skin grafting from either AO-H-$1^w$ or PVG-H-$1^w$ and acted as donors, 3 mo later, of spleen cells to irradiated PVG rats bearing heart grafts from the original sensitizing strains.

are involved. However the additional minor antigens were not completely without effect as, with both naive and sensitized cells, grafts differing at H-1 only were rejected with a slower tempo than those differing for H-1 plus other minor antigens.

*Role of T and B Cells in Memory.* It has previously been established that T cells from naive animals are capable of causing graft rejection in the absence of B cells or B-cell products (7). The relative roles of T and B cells in the increased potency of cellular inocula from sensitized donors were examined in the following ways. The in vitro techniques already described (7) were used to remove Ig+ cells from LNC preparations obtained from PVG donors sensitised against DA. When used to restore irradiated PVG recipients grafted with DA hearts the separated Ig− cells were approximately equipotent with the inocula from which they were derived on a 'per T cell' basis (Table III). The activity of separated Ig− cells against 3rd party LEW grafts (MST 27.8 ± 1.2) was also approximately the same as that of the unfractionated inoculum (MST 23.2 ± 3.1). It was concluded that the specific increase in potency of inocula derived from sensitised hosts was a function of the Ig− cells within these inocula. To exclude the possibility that this memory effect was mediated by the minority population of B cells or their progeny, which were not removed by the anti-Ig adsorbents, the adoptive recipients of both the purified inocula and the unfractionated inocula from which they were derived, were screened for the presence of alloantibody. Antibody was only detected in the adoptive recipients which received the unfractionated inocula. No antibody was present in the serum of recipients of purified inocula of Ig− cells (Fig. 1). The presence or absence of serum antibody made no difference to graft rejection. It thus appeared that memory was not dependent on either B cells or alloantibody synthesis and was a property of Ig− lymphocytes.

*Role or Recirculating Cells in Memory*

RECIRCULATION IN IRRADIATED HOSTS. Although the distribution of potent memory cells in sensitized animals did not correspond with the distribution of recirculating cells, by analogy with other systems memory T cells might be expected to belong to the recirculating pool. To determine whether the cells responsible for the increased potency of TDL and LNC suspensions from sensitized donors were recirculating T cells, inocula of each of these preparations were injected intravenously into irradiated syngeneic recipients bearing a freely-draining thoracic duct cannula. The kinetics of lymphocyte recirculation and the yields of T and B cells from the thoracic duct lymph of these intermediate hosts were identical with the results obtained with naive cell populations as previously described (7). The potency of the T cells which recirculated

TABLE III

*Comparison of Restorative Potency of Ig-Cells Separated in Vitro and Unfractionated LNC*

| Method of separation | Restorative inoculum | |
|---|---|---|
| | Unfractionated LNC 2 × $10^7$ | Ig-cells* 1.2 × $10^7$ |
| Anti-Ig column‡ | 9.1 ± 0.7 (7)‖ | 11.2 ± 0.7 (9)‖ |
| Anti-Ig plate§ | 9.2 ± 0.6 (7)¶ | 9.4 ± 0.6 (5)¶ |

* This number is equivalent to the T cell content of unfractionated LNC which contained 34% Ig+ cells.
‡ Yield of Ig− cells was 90%, purity 96%.
§ Yield of Ig− cells was 60%, purity 99%.
‖ $0.1 > P > 0.05$.
¶ $0.9 > P > 0.8$.

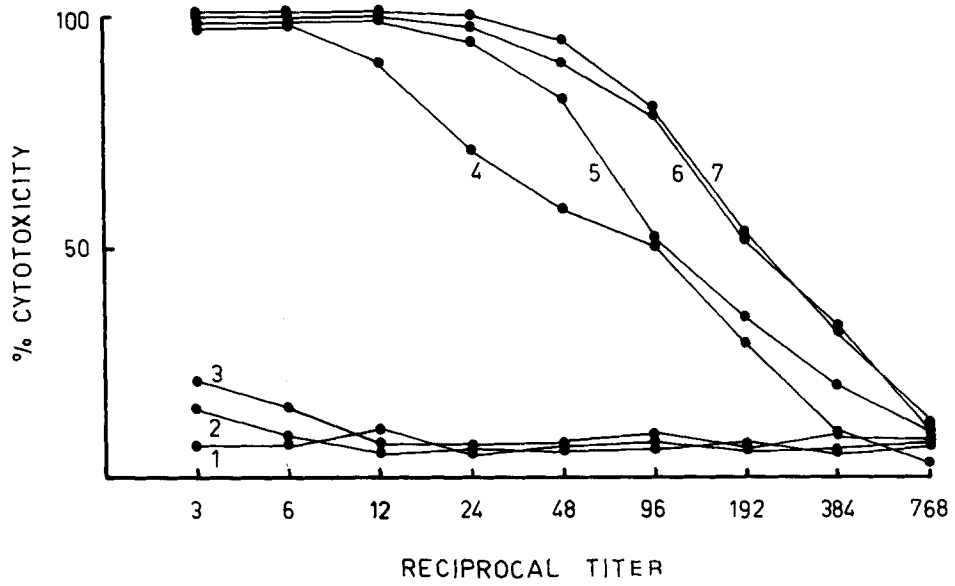


FIG. 1. Serum cytotoxic antibody titers in individual irradiated PVG rats which have rejected DA heart grafts. Serum was taken on the day of rejection. 1, 2, 3, restored with 1.2 × $10^7$ Ig- cells separated in vitro from sensitized LNC. 4, 5, 6, 7, restored with 2 × $10^7$ unfractionated, sensitized LNC. In both groups grafts were rejected with a similar rapid tempo (7–15 days).

within 24 h was compared with that of the unfractionated sensitized donor cell populations on a "per T cell" basis. It was evident that the cells responsible for the increased potency of sensitised populations were quantitatively excluded from the recirculating pool (Table IV). Collections of recirculating cells were continued for 48 h. Like the earlier collections, later collections did not show the increase in potency shown by unfractionated sensitised populations indicating that memory does not reside in a population which recirculates either with the same tempo or a little slower than the bulk of T cells.

As it has been shown that memory B cells enter the recirculating pool (9, 10) the capacity of the recirculating cells, obtained between 24 and 48 h after injection, to

TABLE IV

*Comparison of the Restorative Potency of Recirculating T Cells and the Unfractionated TDL from which they were Separated*

| Restorative inoculum | DA | LEW |
|---|---|---|
| $5 \times 10^7$ TDL | 6.8 ± 0.3 (4) | ND |
| $2.5 \times 10^7$ T Cells* | 12 ± 0.4 (5) | ND |
| $2 \times 10^7$ TDL | 9.2 ± 0.6 (10) | 24.5 ± 3.3 (4) |
| $1 \times 10^7$ T Cells* | 23.6 ± 1.0 (10) | 22.0 ± 1.1 (8) |
| $1 \times 10^7$ T Cells‡ | 20.5 ± 2.3 (7) | ND |
| $5 \times 10^6$ TDL | 10.8 ± 1.2 (6) | 39.0 ± 0.6 (3) |
| $2.5 \times 10^6$ T Cells* | 38.0 ± 0.7 (4) | 39.7 ± 1.5 (3) |

* Cells collected from the intermediate host 0–22 h after injection containing <2% Ig+ cells.

‡ Cells collected 22–48 h after injection containing 18% Ig+ cells.

adoptively restore a specific alloantibody response to the irradiated assay rats and the effect that this alloantibody had on graft rejection was examined. The results (Fig. 2) show that late collections of lymph from irradiated intermediate hosts contain sufficient memory B cells to adoptively restore synthesis in the recipient. Although antibody titers in some of these recipients reached ¼–½ of those of animals restored with unfractionated TDL or indeed of intact, sensitized animals, the presence of cytotoxic alloantibody did not accelerate graft rejection. The degree of restoration of the allograft response was in fact only equivalent to that in animals restored with an equal number of naive T cells (7).

*Recirculation in Normal Hosts.* The presence of memory cells in thoracic duct lymph of sensitised donors and their failure to recirculate through a secondary host are superficially contradictory observations. The irradiated rats used in these experiments supported the recirculation of B memory cells (Fig. 2) and irradiated intermediate hosts have previously been shown to support the recirculation of both the naive T cells necessary for graft rejection (7) and the suppressor T cells which mediate tolerance to MHC antigens (11). Nevertheless, it is possible that memory T cells failed to recirculate either due to an effect of irradiation or to exceptional fragility of memory cells. Because the potency of memory cells is so much higher than that of naive lymphocytes it was possible to directly examine whether memory cells recirculate after intravenous injection in unirradiated animals. In normal intermediate hosts the entry of memory cells into the thoracic duct is detected by a specific increase in the potency of the TDL on adoptive transfer. Normal PVG recipients with thoracic duct cannulae in situ were injected with $10^9$ TDL from PVG donors sensitized against DA antigen. Cellular inocula prepared from the TDL of these intermediate hosts did not show the specific increase in potency which characterises the presence of memory cells. They were equipotent against DA and third party (BN) grafts (Table V). TDL collected from a second group of intermediate hosts 15 days after the injection of specifically sensitized cells also failed to show the presence of memory cells; however memory cells were present in the spleen of this latter group of rats. Spleen cell preparations showed a specific increase in potency against DA grafts (Table V). The results show that although the injected memory cells had survived processing and injection, as was evident from their presence in the recipients spleens at 15 days, they did not appear in the thoracic duct lymph.

*Life Span of Memory Cells.* The memory effect in intact animals is known to last for

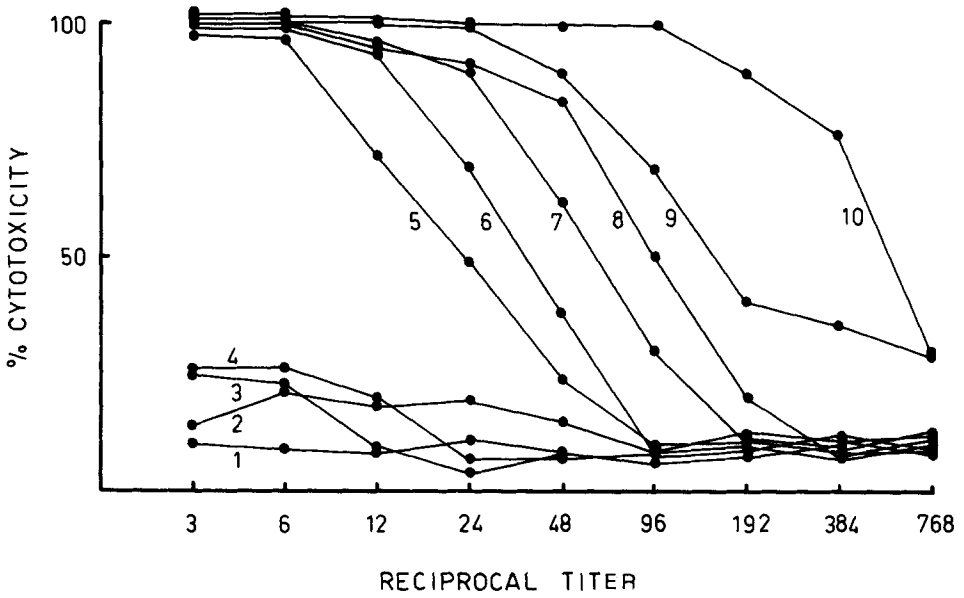


FIG. 2. Serum cytotoxic antibody titers in individual PVG rats which have rejected DA heart grafts. Serum was taken on the day of rejection. 1, 2 restored with $2 \times 10^7$ naive TDL (rejected 26, 30 days) 3, 4 restored with $10^7$ recirculating cells from sensitized donors (1–22 h collection <1% Ig+ cells) (rejected 26 days [2]) 5, 6, 7 restored with $10^7$ recirculating cells from sensitized donors (22–48 h collection, 18% Ig+ cells) (rejected 23 [2], 25 days) 8, 9, 10 restored with $5 \times 10^6$ unfractionated TDL from sensitized donors (43% Ig+ cells) (rejected 9 days [3]).

several years after sensitization (1). Because second-set graft rejection can be mediated by as few as $2 \times 10^7$ cells from such an animal, it contains more than 100 times the number of memory cells required for the memory effect to be observed. The potency of memory cells could therefore decrease by several orders of magnitude without the waning of memory becoming apparent on challenge of the immune animal with a second graft. Such a decrease in potency would indicate however, that memory cells have a measurably short life span. To establish whether potency wanes with time after immunization, inocula of cells were prepared from TDL, LNC, and spleen of immune donors at 3, 6, and 12 mo after sensitization and assayed in adoptive transfer. The results (Table VI) show that there is no decrease in the potency of LNC or spleen cells for at least 1 yr after sensitization. In TDL however the potency of memory cells did drop between 3 mo and 1 yr.

These data are consistent with either carriage of memory by extremely long lived cells or the continuous generation of relatively short lived cells for a prolonged period after sensitisation. The exclusion of memory cells from the rapidly recirculating pool indicates at least one qualitative difference between these cells and the recirculating T cell which mediates first set rejection. Experiments were therefore done to determine whether the memory cells belonged to the non recirculating short-lived category of lymphocytes.

Gowans and Uhr (12) described a simple method for removing short lived cells (predominantly large lymphocytes) from thoracic duct lymph. This method depends upon the greater vulnerability of dividing cells to the conditions of 24 hr incubation in tissue culture media. When TDL from rats bearing memory were incubated for 24

TABLE V

*Capacity of Memory Cells to Recirculate in Normal Intermediate Hosts*

| Restorative inoculum | Time after injection | DA | BN |
|---|---|---|---|
| $5 \times 10^7$ TDL* | 12–26 h | 11.2 ± 0.3 (7) | 11.2 ± 0.3 (6) |
| $5 \times 10^7$ TDL‡ | 15 days | 15.4 ± 0.5 (5) | 17 ± 1.2 (5) |
| $5 \times 10^7$ Spleen cells§ | 15 days | 17 ± 0.7 (4) | 28.4 ± 3 (5) |

The restorative effect on DA and BN graft rejection was not significantly different, $*P = 0.3$; $‡ P = 0.25$ indicating that memory cells are absent from the lymph.
The restorative effect on DA graft rejection is significantly greater than on BN graft rejection, § $0.02 > P > 0.01$ indicating that memory cells are present in the spleen.

h under these conditions approximately 20% of the cells failed to survive. This included the great majority of large and medium sized cells. Those cells which did survive were >99.5% typical small lymphocytes. $2 \times 10^7$ of these cells tested in the adoptive assay restored rejection time to 9.6 ± 0.5 days which did not differ significantly from the rejection time (10.2 ± 0.3) in animals restored with $2 \times 10^7$ of the pre-incubation population. Although this result indicates that memory is not carried by typical short-lived large lymphocytes it does not exclude the possibility that the memory cells in the thoracic duct might be small lymphocytes which have been recently derived from a dividing precursor. To answer this point, sensitized rats were treated with the inhibitors of cell division, vinblastine (VBL), or hydroxyurea (HU) and their lymphoid tissues then assayed for the presence of memory cells. VBL and HU were given to sensitized hosts by the regime previously described (7) which provides maximal antimitotic activity.

The TDL output of VBL treated rats was only about half that of untreated animals. The TDL from these animals however showed a potent memory effect causing rejection of DA grafts with a more rapid tempo than third party grafts (Table VII). Similarly, after treatment with HU for 5 days (in doses sufficient to cause >95% depression of thymidine uptake by bone marrow cells) LNC and spleen cells from sensitized donors still showed the increase in potency characteristic of the presence of memory cells (Table VII).

These data confirm that memory cells are not rapidly proliferating and are not recently derived from a mitotically active precursor. To obtain an estimate of the longevity of memory cells the duration of survival of memory cells after adoptive transfer to irradiated rats was determined. Inocula of LNC from sensitised donors were injected i.v. into irradiated adoptive recipients. The homing pattern and survival of the cells carrying memory was determined by assaying the potency of various populations of lymphocytes obtained from the secondary recipients after 48 hr, 2, 10, and 56 days. The results show that memory cells are long-lived cells which home preferentially to the lymph nodes and spleen of secondary recipients and persist there for many weeks (Table VIII).

*Role of the Thymus in the Generation of Memory Cells.* We have previously shown that the T cells responsible for first set heart-graft rejection are long-lived cells which do not decrease significantly in numbers in the periphery after adult thymectomy (7). Taken with the profound deficit in these cells after neonatal thymectomy (13, 14) this seems to indicate that allograft reactive cells are generated soon after birth and

TABLE VI
*Duration of Memory*

| Restorative inoculum ($5 \times 10^6$ cells) | Time since donor sensitization | | |
|---|---|---|---|
| | 3 | 6 | 12 |
| | | *mo* | |
| LNC | 9.7 ± 0.5 (10)* | 8.8 ± 0.5 (4) | 9.8 ± 0.6 (4)* |
| Spleen cells | 10.4 ± 0.9 (7)‡ | 14.0 ± 1.3 (4) | 10.3 ± 1.2 (4)‡ |
| TDL | 10.8 ± 1.2 (6)§ | 16.0 ± 1.4 (5) | 17.0 ± 1.7 (5)§ |

*, ‡ There is no significant difference ($P > 0.45$) between either LNC or spleen cells taken at 3 and 12 mo after sensitization.

§ There is a significant difference between TDL taken at 3 mo and TDL taken at 12 mo after sensitization ($0.01 > P > 0.005$).

TABLE VII
*Effect of Mitotic Inhibitors on Memory Cells*

| Restorative inoculum | Mitotic inhibitor | Heart graft | |
|---|---|---|---|
| | | DA | LEW |
| $2 \times 10^7$ TDL | — | 9.7 ± 0.4 (4) | 25.5 ± 2.9 (4) |
| $2 \times 10^7$ TDL | VBL* | 10.2 ± 0.4 (5) | 18.0 ± 1.1 (4) |
| $2 \times 10^7$ LNC | — | 9.8 ± 0.8 (4) | 29.4 ± 2.3 (7) |
| $2 \times 10^7$ LNC | VBL* | 9.0 ± 0.4 (4) | ND |
| $2 \times 10^7$ LNC | HU‡ | 8.3 ± 0.5 (4) | 19.5 ± 1.8 (4) |
| $2 \times 10^7$ Spleen | HU‡ | 9.5 ± 0.6 (4) | ND |

* VBL was given intravenously to sensitized donors 36 and 12 h before beginning TDL collection which was continued for the next 12 h.

‡ HU was given intravenously twice daily for 5 days to sensitized donors. LNC and spleen cells were then assayed.

thereafter require no thymic influence for their continued function. Whether memory cells are generated from the thymus or require thymic influence for their generation or maintenance was similarly studied. Donor animals were thymectomised within several weeks before or after the application of sensitizing skin grafts. Assay of the potency of cellular inocula from these donors showed that neither procedure affected the expression of memory by their lymphoid tissues (Table IX). The potency of cellular inocula taken from animals thymectomized before sensitization was equivalent to that of nonthymectomized sensitized donors. This result indicated that memory cells are generated from a precursor pool present in the peripheral lymphoid tissue. However when irradiated cannulated intermediate hosts were injected with cells from a sensitized donor, although the memory cells were excluded from the recirculating pool, those cells which did recirculate exhibited the same potency as equal numbers of naive cells (Table IV) suggesting that the generation of memory cells in the sensitised cell donor has not concurrently diverted large numbers of the recirculating alloantigen reactive cells to the memory cell pool. Whether the precursor cells are the recirculating alloantigen reactive cells is not clear. The actual identity and location of memory cell precursors is being further investigated using the adoptive transfer systems described in these papers.

*Histological Features of Graft Rejection in Adoptive Recipients.* A feature of rejecting grafts in normal animals is the number of mononuclear cells including T cells, B cells,

TABLE VIII

*Tissue Distribution in Irradiated Intermediate Hosts of Memory Cells*

| Restorative* inoculum | Time after injection of memory cells | Heart grafts | | *t* test |
|---|---|---|---|---|
| | | DA | BN | |
| $10^7$ TDL | 1–48 h | 23.6 ± 1.0 (10) | 22.0 ± 1.1 (9) | ($P = 0.3$) |
| $2 \times 10^7$ LNC | 2 days | 15.9 ± 0.9 (8) | 30.3 ± 0.9 (4) | ($P < 0.001$) |
| $2 \times 10^7$ LNC | 10 days | 18.8 ± 1.9 (6) | 27.0 ± 1.5 (4) | ($P < 0.005$) |
| $2 \times 10^7$ Spleen | 10 days | 10.3 ± 0.9 (4) | 27.3 ± 3.5 (4) | ($P < 0.01$) |
| $2 \times 10^7$ Spleen | 56 days | 15.3 ± 3.0 (4) | 30.0 ± 2.3 (3) | ($P < 0.02$) |

* These inocula were prepared from the tissues of irradiated recipients of $10^9$ LNC from sensitized donors at times indicated and were assayed in groups of irradiated secondary recipients bearing heart grafts which carried the sensitizing (DA) or 3rd party (BN) antigens.

TABLE IX

*The Effect of Thymectomy on Memory*

| Donor status | | Heart grafts | |
|---|---|---|---|
| Sensitized | ATx | DA | LEW |
| − | + | 20.2 ± 0.9 (5) | — |
| + | − | 8.4 ± 0.4 (9) | 31.5 ± 1.8 (4) |
| + | +* | 8.4 ± 1.1 (5) | 24.3 ± 1.5 (5) |
| + | +‡ | 8.0 ± 0.4 (5) | 31.0 ± 2.5 (4) |

All restorative inocula were $2 \times 10^7$ LNC.

* Donors ATx 28 days after last sensitizing skin graft rejected.

‡ Donors ATx 28 days before first sensitizing skin graft applied.

or plasma cells and macrophages seen in the grafted tissue at the time of rejection (15). It might be expected that the lymphoid tissues and heart grafts in animals reconstituted with very small numbers of memory T cells would show a much sparser cellular infiltrate. Haemotoxylin and eosin, or methyl green pyronin, stained sections of the recently rejected heart grafts and spleens of adoptive recipients of $5 \times 10^6$ sensitized Ig− cells showed that the cellular infiltrate in rejecting tissue was not significantly different from that in either normal animals or adoptive recipients of large numbers of unfractionated cells. The prolific cellular infiltrate seen in the rejected heart tissue argues for an intensive proliferation of memory cells in adoptive recipients. However, there was no evidence that this proliferation occurred in the lymphoid tissue outside the graft itself. The white pulp of the spleen in recipients of small numbers of memory cells showed a striking paucity of cells compared with the almost normal appearance of the white pulp when large numbers of reconstituting cells were used. The histologically simple background of the rejecting heart and lymphoid tissue in irradiated animals should provide an advantageous model for a more detailed analysis of the morphology of the cellular events relevant to the effector phase of graft rejection.

## Discussion

There have been repeated observations that long term memory of prior exposure to antigens of the MHC, in contrast to that for minor antigens, is not associated with a demonstrable increase in proliferative responses (16–19). It is however associated with a change in the effector arm of the allograft response which can be demonstrated in

vitro in CML assays (20–22). Recent sensitization results in the production of potent cytotoxic T cells. Although these rapidly diminish in numbers in animals sensitized with normal tissue they can be rapidly generated, apparently without proliferation, in lymphoid cell populations taken from animals bearing long-term memory if the cells are re-exposed to specific antigens. The results reported in this paper show that this change in the cell population in animals bearing long term memory to antigens of the MHC can be measured in vivo as a dramatic change in the capacity of cell populations to cause graft rejection. Allograft rejection can be procured by 500 times fewer cells if these are taken from a specifically sensitized animal and this increased potency endures for up to 12 mo after sensitization. This large increase in potency of cells bearing memory to MHC antigens is probably responsible for the enhanced efficiency of memory cells in terminating tolerance to transplantation antigens (2) and in rejecting long-standing grafts in immunologically privileged sites such as alymphatic skin pedicles (23, 24).

The cells bearing memory are long-lived, nonrecirculating, small Ig− lymphocytes, which are generated in the periphery by grafting with the sensitizing antigens. They thus appear to be a unique subpopulation of cells which correspond neither with T1 (recently thymus derived, spleen-seeking nonrecirculating cells) nor T2 (remotely derived from thymus, lymph node seeking, recirculating cells) (25). The progenitor cell from which they are generated remains unidentified but the methodology described in these papers should permit this aspect of their ontogeny to be studied.

The exclusion of these memory cells from the recirculating pool was a surprising findings. Cells bearing memory to a wide variety of other antigens have been shown to recirculate (9, 10, 26). Indeed Sprent and Miller (27, 28) have shown that the progeny of T cells activated in vivo against F1 hybrid alloantigens can be found in thoracic duct lymph some weeks after transfer to syngeneic recipients, albeit in small numbers. Our results do not exclude recirculation of a small minority of the cells bearing memory for alloantigen. The quantitative nature of our experiments shows that the great bulk of these cells are nonrecirculating. This would appear to be a unique feature of immune responsiveness to the MHC. The capacity of late-recirculating cells which contain small numbers of B cells to restore alloantibody synthesis (Fig. 2) argues that B-cell memory to MHC antigens does recirculate with a tempo akin to that of other B cells while only memory for the cell-mediated effector arm of the allograft response is nonrecirculating.

The presence of memory cells in the lymph of sensitized donors and their exclusion from the lymph of syngeneic recipients indicates an alteration in the physiology of memory cell recirculation in the donors as a consequence of their prior exposure to antigen. While experiments with mitotic inhibitors show that most memory cells in TDL and lymphoid tissue are not recently formed cells, it is possible that there is a constant small production of new cells as an enduring consequence of sensitization. The decay of memory in TDL populations over 12 mo is in accord with a gradual decrease in production of new cells with time after sensitization. Nevertheless the great bulk of memory cells are long-lived and can survive in vivo for at least 8 wk in the presumably antigen-free environment of the spleen of a naive intermediate host (Table VIII). The possible role of antigen in mobilizing these cells into the lymph is of obvious interest.

The role of the recirculating lymphocyte pool in immune responses to antigens of

the MHC is complex. Naive allograft reactive cells certainly inhabit this pool and indeed may be restricted to it (7). The suppressor T cells which mediate transplantation tolerance are also rapidly recirculating T cells (11). One would have predicted that allograft memory cells would likewise inhabit this pool. A major role of recirculation is thought to be the recruitment of specific antigen sensitive cells from a relatively small clone to localized depots of antigen (29). It may be significant that the failure of allograft memory cells to recirculate is probably compensated in vivo by their widespread distribution and greatly enhanced potency so that blood to lymph recirculation is not in fact required to guarantee that sufficient antigen sensitive cells will be available at a local lymphoid site of alloantigen deposition. It should be emphasized that while our results show that memory cells do not recirculate from blood to lymph through the lymph nodes they by no means suggest that memory cells are excluded from the circulation. The high concentration of memory cells in the spleen might indicate that they circulate in a pool of cells which both leave and re-enter the blood within the spleen.

The potency of memory cells in mediating graft rejection is not paralleled by their ability to reconstitute the architecture of lymphoid tissue of the recipient. The most striking appearances histologically were the dense cellular infiltrates in the rejected grafts of animals which showed an almost total lack of reconstitution of the organised lymphoid architecture of the spleen. It is apparent that most of the cellular activity in the organised lymphoid tissue during graft rejection in normal animals is not an essential prerequisite for tissue destruction. Whether it plays a role in the generation of memory cells will be further studied.

## Summary

An adoptive transfer system was used to study the cellular basis of memory in animals immunized by grafting with major histocompatibility complex incompatible tissue. Memory was characterised by a large (> 100 fold) increase in the potency of lymphocytes to precure graft rejection. This increase in potency endured for at least 1 yr after sensitization. The memory cells were shown to be Ig− small lymphocytes which were long lived and which did not recirculate from blood to lymph in normal recipients although they did home to lymphoid tissue from which they could be recovered several months later. The thymus was not required either for the generation of memory cells or their maintainance. Cells carrying memory for alloantibody synthesis did recirculate normally but alloantibody synthesis was shown not to be required for rejection.

We are grateful to Dr. Jonathan Howard for the generous gift of PVG-H-1$^{w}$(AO) and AO rats, to Dr. Adrienne Thompson for performing the in vitro T-cell separations and to Dr. I. Robinson, Department of Statistics, University of Sydney for statistical advice.

*Received for publication 17 April 1978.*

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