EARLY CELLULAR EVENTS IN A SYSTEMIC GRAFT-VS.-HOST REACTION

I. The Migration of Responding and Nonresponding Donor Lymphocytes

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In primary immune responses only a small minority of lymphocytes are responsive to each antigen, for example in the primary antibody responses to sheep erythrocytes and flagellin the proportion has been estimated as 1 in 10^5 and 1 in 10^6 respectively (1, 2). When a systemic graft-vs.-host (GVH)[1] reaction is initiated by the intravenous (i.v.) injection of lymphocytes into a susceptible recipient a minority of donor small lymphocytes transform into large, dividing cells while the majority remain unresponsive (3, 4). Several lines of investigation have shown that the frequency of responsive cells in a nonimmune population is much greater in the case of GVH reactions than in antibody responses. After injection of labeled lymphocytes into newborn allogeneic recipients 30% of the labeled cells in the spleen were noted to have undergone blastic transformation (4); by limiting dilution assay at least 1–2% of donor cells were estimated to be reactive after injection into allogeneic chicken embryos (5), and in one-way mixed leukocyte culture 1–3% of rat blood leukocytes were found to enter cell division in response to alloantigen (6). Considering the importance which has been ascribed to the high frequency of nonimmune cells reactive against alloantigens (7, 8) none of these estimates can be regarded as fully satisfactory.

The starting point of the present method of estimation is that after the i.v. injection of parental strain lymphocytes into an irradiated F_1 hybrid recipient (9) or an irradiated allogeneic recipient (10) the donor lymphocytes which are recovered from the recipient's thoracic duct lymph are specifically unresponsive against the alloantigens of the rat through which they have been passaged from blood to lymph. Histological observation has suggested that most reactive cells accumulate in the spleen and fewer are present in lymph nodes (3). There is thus a partial separation of reactive and nonreactive cells within the tissues of the recipient which can be ascribed to the recruitment and immobilization of reactive cells. This separation has been confirmed by labeling parental strain lymphocytes with [^{3}H]uridine and an unresponsive population with [^{14}C]uridine. It has been possible to gain more exact information on the organ distribution of

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[1] *Abbreviations used in this paper:* GVH, graft-vs.-host; PBS, phosphate-buffered saline; TDL, thoracic duct lymphocytes.

reactive and nonreactive cells after i.v. injection and to use this to make an estimate of the proportion of reactive cells in a nonimmune population. A few of the data have been published in preliminary form (11).

Materials and Methods

The plan of a typical experiment is illustrated in Fig. 1.

Rats and Transplantation Antigens. Male rats of the highly inbred strains AO (Ag-B 2) and HO (Ag-B 5) were used as donors of thoracic duct lymphocytes (TDL). The recipients were (AO x DA)F_1, (AO x HO)F_1, or (HO x August)F_1 hybrids weighing 180–220 g. The inbred DA and August strains are Ag-B 4 and Ag-B 5 respectively. Two Ag-B incompatible strain combinations were studied viz. AO → (AO x HO)F_1 and AO → (AO x DA)F_1 and one Ag-B identical combination viz. HO → (HO x August)F_1. Although they are Ag-B identical the first set skin allograft rejection time between the HO and August strains averages 10 days, which is only about 2 days longer than in most Ag-B different combinations (12).

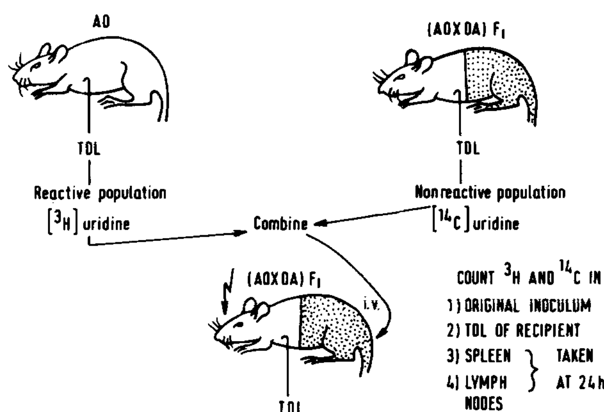


FIG. 1. A GVH reaction was initiated by the i.v. injection of TDL from a parental strain donor (top left) into an adult F_1 hybrid recipient which had been lightly irradiated 3 days previously and subjected to thoracic duct cannulation 1 day previously. Before injection the donor cells were labeled with either [^{3}H]uridine or [^{14}C]uridine. Their localization in the recipient was measured relative to that of a reference population of nonreactive TDL, e.g. from an F_1 hybrid (top right), which were labeled with the other isotope and combined with the reactive cells before injection. The distributions of the reactive and nonreactive populations were measured by liquid scintillation counting of both isotopes in the tissues of the recipient.

Irradiation. Hybrid recipients were subjected to 400 rads of whole-body γ-irradiation from a ^{60}Co source 3 days before injection of the donor cells. This was less than half the mid-lethal dose for these strains. Higher doses of radiation seriously interfere with lymphocyte recirculation as has been recently documented (13).

Thoracic Duct Cannulation. Thoracic duct cannulation was performed as previously described (14). Cannulated rats were restrained in a Bollman cage and given a continuous i.v. infusion of Dulbecco's phosphate-buffered saline (PBS) by means of a tail vein cannula. Donor cells were injected through this cannula over a few minutes.

Radioactive Labeling of TDL. Lymphocytes were labeled in vitro with [5-^{3}H]uridine (Radiochemical Centre, Amersham, England, TRA. 178) at 5 μCi/ml or [^{14}C]uridine (U) (Radiochemical Centre, Amersham, England, CFB. 51) at 1.5 μCi/ml as has been described in detail (14). The cells were centrifuged, resuspended in PBS, and reinjected into the original donor. Labeled cells were recovered by collecting thoracic duct lymph from 18 to 30 h after injection. This stage of autologous blood to lymph passage can be regarded as part of the labeling procedure which ensures that (*a*) the labeled cells transferred to the final recipient consist entirely of accredited recirculating lymphocytes; damaged cells and most large lymphocytes are removed, (*b*) most of the radioactive uridine has

entered the acid insoluble fraction from which loss of label is slower and more regular than is the earlier loss from the acid soluble pool (15), and (*c*) before injection into the final recipient the labeled cells are subjected to the minimum of handling in vitro.

Measurement of Radioactivity. Samples of the mixture of lymphocytes injected into the final recipient, the thoracic duct output of the recipient collected in four 6-hourly fractions and the spleen and pooled mesenteric and cervical lymph nodes of the recipient removed when it was killed 24 h after donor cell injection were processed (14) for dual isotope counting on a Beckman LS-250 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) with external standardization. Absolute activities of ^{3}H and ^{14}C were computed with reference to separate calibration curves for spleen samples and other tissues. After lymphocytes have been labeled in vitro with [^{3}H]uridine or [^{14}C]uridine the isotopes are not lost at exactly the same rate (16). This differential loss of label was compensated by performing duplicate experiments simultaneously. Each experiment was identical except that the isotopes (^{3}H, ^{14}C) were swapped between the reactive and the control populations. This allowed the ratio of the two populations in each lymphoid compartment to be calculated by a method which eliminated distortion due to the differential elution of ^{3}H and ^{14}C. (See Appendix.)

Graft-vs.-Host. GVH activity was measured by the popliteal lymph node assay (17). Untreated parental strain TDL were injected into the opposite foot pad of F_1 hybrids for comparison with the cells being tested.

Immunological Tolerance. Immunological tolerance was produced by the i.v. injection of 50×10^6 F_1 hybrid bone marrow cells into AO rats on the day of birth. At 8–9 wk of age they were tested by making cell suspensions of cervical lymph nodes obtained by biopsy. Only those rats from which 30×10^6 TDL produced no reaction were accepted as tolerant. In these strain combinations 0.3×10^6 TDL from normal donors produce a significant reaction.

Results

In the Recipient do the ^{3}H and ^{14}C Labels Remain Associated with the Lymphocytes which were Originally Labeled? This essential question was attacked by injecting a labeled population of lymphocytes of (AO x DA)F_1 hybrid origin into a recipient in which the cells would not survive. The transfer of radioactive label released from the killed cells to intact lymphocytes was found to be extremely small; therefore in the definitive experiments the transfer of label from one population to another when lymphocytes were not killed en masse should be even less and is assumed to be negligible. TDL from an (AO x DA)F_1 hybrid were labeled in vitro with [^{14}C]uridine and as a control population TDL from an AO donor were labeled with [^{3}H]uridine. Each population was autologously passaged from blood to lymph; they were then combined, centrifuged, and resuspended in neat, inactivated anti-DA serum produced by skin grafting AO rats. The cytotoxic titer of the serum for lymphocytes was 1 in 32 by a method described previously (18). After incubation for 30 min at 37°C in the absence of complement the cells, which were > 95% viable at that stage, were injected i.v. into an unirradiated AO recipient which had been immunized by successive DA skin grafts. Thoracic duct lymph was collected for 24 h after injection and the washed lymphocytes were taken for scintillation counting. It was found that relative to the amounts of label injected the recovery of ^{14}C in TDL collected from the recipient was only $\frac{1}{400}$th of the recovery of ^{3}H. A second experiment was performed in which the AO cells were labeled with [^{14}C]uridine and the (AO x DA)F_1 cells with [^{3}H]uridine. The result was similar in that only $\frac{1}{600}$th of the ^{3}H label was recovered compared to the recovery of the ^{14}C label. Other published evidence supports the validity of labeling lymphocytes with radioactive uridine to follow their migration into lymphoid tissue (15, 19).

The Distribution of Parental Strain and F_1 Hybrid Lymphocytes in Irradiated F_1 Hybrid Recipients. Two populations of TDL from an AO strain donor and from an F_1 hybrid donor, were labeled with the alternative isotopes, autologously passaged from blood to lymph, combined, thoroughly mixed and injected into an F_1 hybrid recipient after a measured sample had been taken to count the $^3H/^{14}C$ ratio in the original inoculum (Fig. 1). The ratio of $^3H/^{14}C$ was measured by scintillation counting in the original inoculum, the thoracic duct cells collected from the recipient and the spleen and lymph nodes of the recipient which was killed 24 h after injection (Fig. 1). The ratio of the label associated with the reactive population to the ratio associated with the nonreactive, reference population was calculated in relation to the ratio in the original inoculum which was standardized to 1.00. Thus a ratio above 1.00 in any compartment indicated a surplus of reactive cells (Table I).

TABLE I

Distribution of Reactive and Nonreactive Lymphocytes in AgB-Incompatible F_1 Hybrid Recipients

Reactive population	Nonreactive population	Antigen*	Adjusted count ratios (reactive/nonreactive)			
			Original inoculum	Spleen‡	Lymph-nodes§	TDL‖
Normal AO	Normal F_1	HO	1.00	1.12	0.87	0.77
				1.21	1.01	0.90
		DA	1.00	1.28	0.96	0.87
				1.32	0.92	0.91
Normal AO	Filtered AO	HO	1.00	1.32	0.99	0.88
				1.22	1.00	0.92
		DA	1.00	1.10	0.92	0.84
				1.20	0.94	0.91
	Above combined	Mean ± SE		1.22 ± 0.03	0.95 ± 0.02	0.874 ± 0.018

Each figure is derived from a pair of experiments (^3H, ^{14}C swapped, see Appendix).
* The antigen expressed by the F_1 recipient against which AO cells react.
‡ Spleen from recipient killed 24 h after i.v. injection.
§ Pooled mesenteric and cervical lymph nodes taken after 24 h.
‖ Thoracic duct lymphocytes collected throughout the 24 h period.

In every experiment there was a surplus of the reactive cells in the spleen (mean = +22%) and a deficit in the lymph (mean = −12.6%) (Table I). In the lymph nodes the reactive/nonreactive ratio was always intermediate between the ratio in the spleen and that in TDL. There was no significant difference between the (AO x DA)F_1 and the (AO x HO)F_1 hybrid recipients, which was expected because the strength of the reactions are equal by the popliteal lymph node assay.

Exactly similar experiments were done in which parental strain (HO) and F_1 hybrid lymphocytes were injected into (HO x August)F_1 hybrid recipients, which

were Ag-B identical in contrast to the first two combinations. In this case no surplus or deficit of reactive cells was found in any compartment. The average of six experiments indicated an even distribution of the two populations (Table II). This was also expected since 30×10^6 HO cells are required to produce a standard GVH response (10 mg) in (HO x August)F_1 recipients which is about 100 times the number required in the above Ag-B different combinations.

The Distribution of Normal Parental Strain Lymphocytes and Specifically Unresponsive Parental Lymphocytes in Irradiated F_1 Hybrid Recipients. The marked difference found between the distribution of parental and F_1 hybrid lymphocytes may be attributable to the sequestration of a reactive minority in the spleen and, to a lesser extent, in the lymph nodes and to their failure to migrate into thoracic duct lymph. An alternative explanation is that parental and F_1 hybrid cells differ in their physiological migration pattern. This was tested by substituting for the labeled F_1 hybrid TDL parental strain TDL which

TABLE II

Distribution of Reactive and Nonreactive (HO) Lymphocytes in AgB Compatible, (HO x Aug)F_1 Hybrid Recipients

Reactive population	Nonreactive population	Adjusted count ratios (reactive/nonreactive)			
		Original inoculum	Spleen‡	Lymph nodes§	TDL‖
Normal HO	Filtered HO	1.00	0.99	0.98	1.00
			1.02	0.96	1.00
			0.99	1.06	1.03
		Mean ± SE	1.00 ± 0.01	1.00 ± 0.03	1.01 ± 0.01

Each figure is derived from a pair of experiments (^{3}H, ^{14}C swapped, see Appendix).
‡, §, ‖ See Table I.

had been shown to be inactive against the F_1 hybrid antigens by GVH assay (Fig. 2). Thoracic duct lymphocytes from four AO donors were pooled; half of the population were labeled in vitro with [^{3}H]uridine and the other half with [^{14}C]uridine. The ^{3}H-labeled AO cells were re-injected i.v. into AO donors as usual and the passaged cells recovered between 18 and 30 h after injection. The ^{14}C-labeled AO cells were similarly passaged from blood to lymph in irradiated (AO x DA)F_1 hybrid recipients in order to eliminate the responsive cells (9). The two populations were then combined and injected into an irradiated (AO x DA)F_1 hybrid recipient. As previously, samples were taken of the inoculum before injection into the final recipient and from three compartments of the recipient. Four such experiments were done and isotopes were again swapped between the reactive and nonreactive AO populations in alternate experiments.

The results (Table I) were very similar to those described in the previous section. There was an average surplus of 21% of the reactive label in the spleen and a deficit of 11% in the thoracic duct population with intermediate ratios in the lymph nodes.

Further experiments were performed using as the reference population TDL

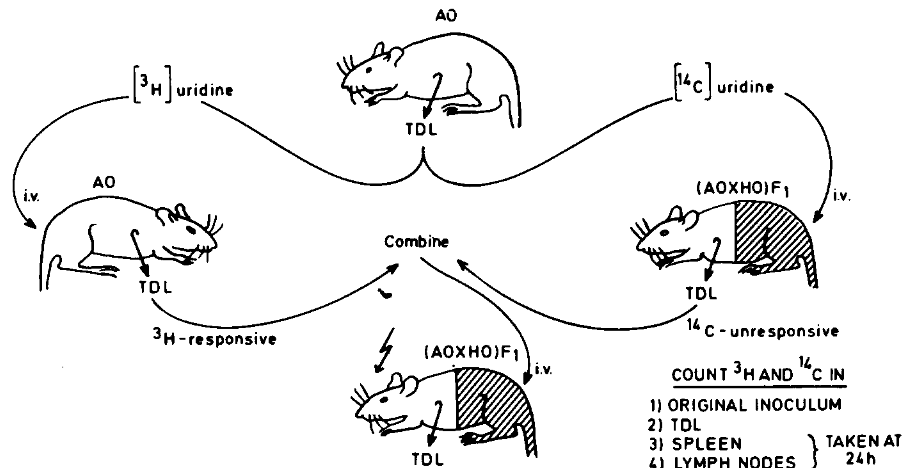


FIG. 2. The distribution of reactive and alternatively labeled nonreactive populations was again compared after i.v. injection into an irradiated, cannulated F_1 hybrid recipient (bottom). In this version AO TDL were made unresponsive after labeling in vitro by passage from blood to lymph in an F_1 hybrid recipient (right). The reactive population consisted of AO TDL which had been passaged in AO rats (left).

from AO donors which had been made tolerant to either the DA or HO antigen by the neonatal injection of F_1 hybrid marrow cells. In four experiments in which AO cells tolerant of the DA antigen were injected into the (AO x HO)F_1 hybrids, the same pattern of results was again found with a 32% surplus of the normal, reactive population in the spleen and a 16% deficit in thoracic duct lymph (Table III). However, in experiments in which AO cells tolerant of the HO antigen were injected into (AO x HO)F_1 recipients the results were inconsistent; in one pair of experiments the usual pattern was found but in the other two there was little difference between the distribution of the reactive and tolerant cells. Thus, the results, using cells from tolerant donors as a reference population broadly

TABLE III
Distribution of Normal and "Tolerant" Lymphocytes in AgB-Incompatible F_1 Hybrid Recipients

Reactive population	Nonreactive population	Antigen*	Adjusted count ratios reactive/nonreactive)			
			Original inoculum	Spleen‡	Lymph nodes§	TDL‖
Normal AO	AO from tolerant donor	DA	1.00	1.25	1.08	0.85
				1.39	1.08	0.83
		HO	1.00	1.08	1.10	0.97
				1.04	0.93	1.07
			Mean ± SE	1.18 ± 0.08	1.07 ± 0.05	0.92 ± 0.06

Each figure is derived from a pair of experiments (^3H, ^{14}C swapped, see Appendix).
*, ‡, §, ‖ See Table I.

confirmed the previous results but there was more variation between individual experiments.

The Distribution of Normal Parental Strain Lymphocytes and Parental Lymphocytes which were Unresponsive Against a Third Party Strain in Irradiated F_1 Hybrid Recipients. If the differential distribution of reactive and nonreactive populations depends on the recognition of transplantation antigen by some of the cells in the reactive population then normal (reactive) cells and cells which are unresponsive against a "third party" antigen should not show the usual differential distribution when injected together into an F_1 hybrid. This was tested in experiments which were of similar design to those depicted in Fig. 2 except that AO lymphocytes which were specifically unresponsive against HO antigens were combined with alternatively labeled normal AO cells and injected into a (DA x AO)F_1 hybrid. The results of eight experiments with the usual alternation of isotopes and F_1 hybrid types showed that the results were, in a sense, even better than predicted (Table IV). Instead of the usual surplus of normal AO cells in the spleen there was a barely significant deficit (3%) and instead of the usual deficit in the thoracic duct population there was a variable surplus averaging 11%. These results suggest that lymphocyte populations which are depleted of cells responsive to one transplantation antigen become slightly more responsive to a second antigen. This is consistent with GVH assays (9, 20) which tend to show slightly greater activity against third party hybrids. A firm conclusion is that the differential distribution recorded in sections (2) and (3) cannot be attributed to different physiological migration properties because it is clearly dependent on the antigenic constitution of the recipient as is shown by comparing the results shown in the lower half of Table I and Table IV.

The GVH Activity of the Donor Population Present in the Spleen 24 h after Injection with F_1 Hybrid Recipients. Parental strain lymphocytes which have migrated from blood to lymph in an irradiated F_1 hybrid recipient are deficient in GVH activity against the antigens of the F_1 hybrid (9) and are also numerically deficient compared to a reference, nonreactive population (Tables I and III). The

TABLE IV
Distribution of Normal and "Filtered" Lymphocytes in "Third Party" F_1 Hybrid Recipients

Reactive population A	Reactive population B	Recipient antigen*	Adjusted count ratios (reactive A/reactive B)			
			Original inoculum	Spleen‡	Lymph nodes§	TDL‖
Normal AO	Filtered AO	DA	1.00	0.96	1.02	1.16
				0.99	0.98	1.07
		HO	1.00	0.94	1.00	1.19
				1.00	1.00	1.04
			Mean ± SE	0.97 ± 0.01	1.00 ± 0.01	1.11 ± 0.04

Each figure derived from a pair of experiments (^3H, ^{14}C swapped, see Appendix).
*, ‡, §, ‖ See Table I.

present experiments have confirmed that there is a surplus of the reactive population in the spleen. Thus the donor cells which are selected out into the spleen might be expected to show specifically increased GVH activity compared to the original donor population. An attempt was made to test this prediction by preparing cell suspensions of the recipients' spleen 24 h after the injection of parental strain cells. The number of viable donor cells was estimated by the use of an alloantiserum directed against the F_1 hybrid antigen as previously described (9). The donor cells in the spleen were assayed against specific and third-party F_1 hybrids by the popliteal lymph node assay. Paradoxically, the GVH activity of the donor cells in the spleen was slightly reduced against the specific antigens and was approximately equal to normal TDL against third-party antigens (Table V). This anomaly was first described by Simonsen (21) who found that the donor cells in the spleens of F_1 hybrid mice 24 h after injection were partially "tolerant". The poor functional activity of the donor cells in the spleen can be reconciled with the evidence that reactive cells are enriched in the spleen by postulating that the large pyroninophilic cells to which reactive donor lymphocytes have transformed after 24 h, are not transplantable and therefore do not count in GVH assays. Two similar experiments were performed in which the GVH activity of donor cells recovered from the F_1 hybrid spleen after 12 h was assayed. At this stage blastic transformation of donor cells can barely be detected histologically. Compared to normal parental TDL there was little or no change in the GVH activity of these donor cells recovered from the recipient's spleen (Table V).

What Happens to the Surplus of Reactive Cells in the Spleen when a Single Cell Suspension is Prepared From it? If the large pyroninophilic cell is not transplantable it may be because it is less well represented in the single cell suspension than are the nontransformed lymphocytes or because it fails to survive or migrate to appropriate sites in the recipients (22). The possibility that the surplus of reactive cells in the recipients' spleen at 24 h may be under-represented in the cell suspension used for GVH assay was tested by scintillation counting of a cell pellet taken from such a suspension. In five experiments the large surplus of the label associated with the reactive cells in the whole spleen had consistently fallen to a much smaller surplus in the cell suspension (Table

TABLE V

The GVH Activity of Donor (AO) Lymphocytes after Residence in an F_1 Hybrid Spleen

Hours between injection and removal of spleen	F_1 hybrid recipient	GVH activity of donor cells*	
		Against specific F_1	Against‡ 3rd party F_1
12	$(AO \times HO)F_1$	0.81	ND
	$(AO \times DA)F_1$	0.82	1.01
24	$(AO \times HO)F_1$	0.46	1.76
	$(AO \times DA)F_1$	0.76	0.56

* GVH activity compared to normal AO TDL, expressed as a potency ratio (20).

‡ Against $(AO \times DA)F_1$ if the donor cells were in an $(AO \times HO)F_1$ recipient and vice versa.

VI). This supported the notion that the lower-than-expected GVH activity of donor cells in the spleen was because the cells which had reacted did not go into suspension. Further support for this view was obtained from the autoradiographic experiments described in the accompanying paper (32). Repeated washing of the cell suspension did not reduce the surplus of reactive cells further and it is not known whether these reactive cells are more easily broken up when the cell suspensions were prepared or whether they are more adherent to the fibrous remnants of the spleen.

Is the Migration of the Nonreactive, Reference Population Altered by the Presence of a Reactive Population? Several workers have described an early inhibition of lymphocyte migration after antigen reaches a lymph node or the spleen (23, 24). The reaction of the parental strain lymphocytes against the F_1 hybrid antigen might have perturbed the distribution of the alternatively labeled F_1 hybrid lymphocytes which were used as a reference population. This was tested by injecting [^{3}H]uridine-labeled F_1 hybrid lymphocytes into two irradiated recipients which were matched for weight and sex. One recipient received the labeled F_1 hybrid cells combined with an excess of normal parental strain TDL, the other received an equal number of the labeled F_1 hybrid cells combined with unlabeled F_1 cells to make up equal numbers of total cells injected into each recipient. In four such experiments no difference was found in the distribution of the labeled F_1 hybrid cells between spleen, lymph nodes, and lymph whether or not a GVH reaction had been initiated by parental strain cells (Table VII). This supports the validity of using F_1 hybrid lymphocytes as a reference population.

TABLE VI

Effect of Making a Single Cell Suspension from the Spleen on the Ratio of Reactive Label/Nonreactive Label

Exp. no.*	Spleen 24h after injection		TDL collected throughout 24h
	Counted as intact organ	Counted as single cell suspension	
2	1.21	1.09	0.90
4	1.32	1.02	0.91
5	1.32	1.14	0.88
6	1.22	1.07	0.92
8	1.20	1.03	0.91
Geometric mean ± SE	1.25 ± 0.03	1.07 ± 0.02	0.90 ± 0.01

* Refers to experiments presented in Table I.

Discussion

Lymphocytes which recognize and react to antigen are sequestrated from the traffic stream of migrating lymphocytes and in the particular case of a GVH reaction after the i.v. injection of lymphocytes the cells which have reacted are most conspicuous in the spleen (3). We have estimated the fraction of the whole lymphocyte population which leaves the recirculating pool in response to

TABLE VII
Lack of Effect of a GVH Reaction on the Distribution of a Labeled, Syngeneic Population

Unlabeled lymphocytes	Labeled F_1 hybrid lymphocytes	F_1 hybrid recipient	Exp. no.	Distribution of labeled lymphocytes % of injected dose: TDL of Recipient 0–6 h	TDL of Recipient 7–24 h	Recipient 24 h after injection: Spleen	Recipient 24 h after injection: Lymph nodes
Parental	(AO x DA)F_1	(AO x DA)F_1	1	0.066	5.9	18.6	51.5
(AO)			2	0.053	8.7	13.7	68.1
	(AO x HO)F_1	(AO x HO)F_1	3	0.046	ND	7.6	32.3
			4	0.129	ND	17.8	28.8
				0.074 ± 0.02	7.3	14.4 ± 2.5	45.2 ± 9.1
(AO x DA)F_1	(AO x DA)F_1	(AO x DA)F_1	1	0.084	10.1	13.3	53.2
			2	0.088	9.8	14.2	68.2
(AO x HO)F_1	(AO x HO)F_1	(AO x HO)F_1	3	0.079	ND	8.9	33.3
			4	0.079	ND	11.7	31.3
				0.083 ± 0.002	10.0	12.0 ± 1.2	46.5 ± 8.8

alloantigen by radioactively labeling lymphocytes before injection and precisely measuring their distribution in the tissues of the F_1 hybrid recipient. This approach is fraught with difficulties but these were largely overcome by rather elaborate measures which may appear to complicate the basically simple plan of the experiment.

In each experiment alternatively labeled reactive and nonreactive populations were injected simultaneously into F_1 hybrid recipients so that any consistent deviation of the ratio of the labels associated with each population would reflect an alteration of the migration of the reactive population accepting the evidence that the migration of the reference population was not perturbed by the GVH reaction (Table VII). Closely similar results were obtained using three types of reference population viz. F_1 hybrid lymphocytes, parental strain lymphocytes from tolerant donors and parental strain lymphocytes previously made specifically unresponsive by passage from blood to lymph in an F_1 hybrid rat.

The surplus of the reactive population found in the spleen and the deficit found in thoracic duct lymph was attributed to the sequestration of reactive cells consequent upon their recognition of antigen for three reasons. The first is that in a non-Ag-B strain combination, in which 100 times more cells were required to produce a standard GVH reaction compared with an Ag-B different combination, no differential distribution was detected which is to be expected if the label associated with the very small minority of reactive cells was 0.01 of the 22% surplus in the spleen found in Ag-B different combinations (i.e. about 0.2%). The second reason is that when normal (reactive) and specifically unresponsive populations were injected together with a "third-party" F_1 hybrid against which

they were both reactive the surplus of normal cells in the spleen and the deficit in thoracic duct lymph was not found. This showed that the differential distribution of these two parental strain populations depended on the antigenic constitution of the recipient. The third reason is that a balance sheet can be drawn up (Table VIII) from which can be calculated the total recovery of "reactive" and "nonreactive" labels relative to the amounts injected in the original inoculum. The same proportion of each label was accounted for within the limits of experimental error. This suggests that the cells which have reacted have no tendency to lose label either faster or more slowly than cells which have not reacted. The faster elution of label from activated cells if it had occurred (25) could not of course account for the deficit in label in lymph from which reactive cells are virtually absent (9).

TABLE VIII

Balance Sheet for Recoveries of "Reactive" and "Nonreactive" Labels

Compartment	(*a*) Distribution* of nonreactive label %	(*b*) Adjusted‡ count ratio reactive/ nonreactive	(*c*) = (*a*) x (*b*) Recovery of reactive label
Spleen	23.2	1.22	28.3
Lymph nodes	48.2	0.95	45.7
TDL	28.6	0.84	25.0
	100.0		99.0§

* Percent of radioactivity in spleen, lymph nodes, and TDL assuming total lymph node weight of 700 mg and uniform distribution between sampled lymph nodes and others.

‡ From Table I.

§ That is the recovery of the label associated with the reactive population is not significantly different from the recovery of label associated with the reference population.

From the balance sheet (Table VIII) and with reference to Figs. 3 and 4 equations can be written for x, the fraction of radioactivity associated with the reactive minority of cells in the whole reactive population, and a, the cells reacting in the spleen as a fraction of the cells reacting in spleen and aggregated lymph nodes taken together.

The simplest equation refers to the reactive/nonreactive ratio in lymph

$$(1 - x)/1 = 0.874. \quad (1)$$

This follows from the absence of reactive cells in lymph and gives $x = 0.126$. For the spleen

$$[ax + 0.232\,(1 - x)\,]\,/\,0.232 = 1.22 \quad (2)$$

and for lymph nodes

$$[(1 - a)x + 0.481\,(1 - x)\,]\,/\,0.481 = 0.95. \quad (3)$$

In each of Eq. 2 and 3 the first term in the numerator is the label associated with the reactive cells; the second term is the label associated with the

nonreactive majority in the reactive population; the denominator is the observed distribution of the nonreactive reference population and the right site is the observed reactive/nonreactive ratio.

With $x = 0.126$ equation (2) gives $a = 0.64$ and Eq. 3, $a = 0.70$. Thus we have a set of internally consistent data which indicate that approximately 12% of the radioactive label associated with the reactive population has been sequestered in the spleen and lymph nodes at the expense of the lymph; moreover approximately 67% of the sequestered radioactivity is present in the spleen and 33% in the lymph nodes.

This distribution of reactive cells between the spleen and lymph nodes is consistent with the simple notion that lymphocytes react in the tissue into which they first migrate from the blood as suggested by Gowans (3). In the rat the rate of lymphocyte traffic through the spleen has previously been estimated to be at least as great as through the aggregated lymph nodes (26). The size of the recirculating pool in the lymph nodes is larger than in the spleen because it takes lymphocytes on average three times as long to traverse the nodes and return to the blood compared with the spleen (16). There is a net redistribution from spleen to lymph nodes between 6 and 24 h after injection (16, 27) which accounts for the much higher surplus of reactive cells in the spleen than in the lymph nodes. Thus the reactive/nonreactive ratios in the lymph nodes were always intermediate between spleen and lymph (Table I) because the lymph nodes at 24 h after injection contain a population which has migrated there directly from the blood and a population which has been redistributed from the splenic pulp so that the reactive cells have already been "filtered out" (Figs. 3 and 4).

The slight reduction of GVH activity of donor cells in the spleen at 24 h after injection is anomalous since the scintillation counting data indicates that the reactive cells are enriched there. This can be satisfactorily explained on the basis that the large pyroninophilic cells to which reactive donor lymphocytes have transformed are poorly transplantable. When a single cell suspension as is used for a GVH assay was assessed by scintillation counting the surplus of the reactive label was greatly reduced compared to the whole spleen showing that these cells go badly into suspension. Other explanations for the poor transferability of activated T cells have been suggested by studies on the response to sheep erythrocytes in the mouse spleen (22). These are localization of the blast cells in inappropriate sites and a transient phase of tolerance. Either or both of these mechanisms may act in the present situation as well as the mechanical factor.

We conclude that the minority of cells which recognize each Ag-B antigenic complex carries $12 \pm 2\%$ of the radioactive label in the starting population (Fig. 3). Several points require to be emphasized. Firstly, not all of these cells necessarily proceed to blastic transformation, cell division, or a fortiori the production of specific cytotoxic cells. The method measures the removal of cells from the traffic stream which follows specific antigenic recognition and implies the presence of surface receptors with some affinity for the transplantation antigen.

Secondly, it cannot be immediately assumed that $12 \pm 2\%$ of all the donor cells are reactive to antigen since the label may not be evenly distributed between the

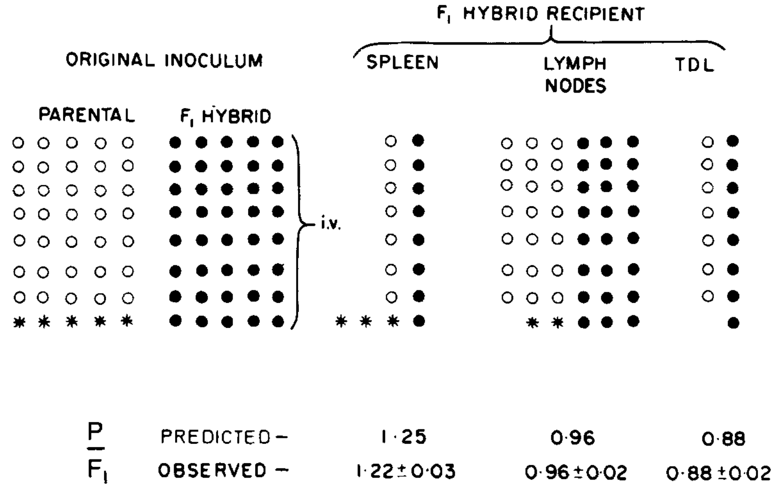
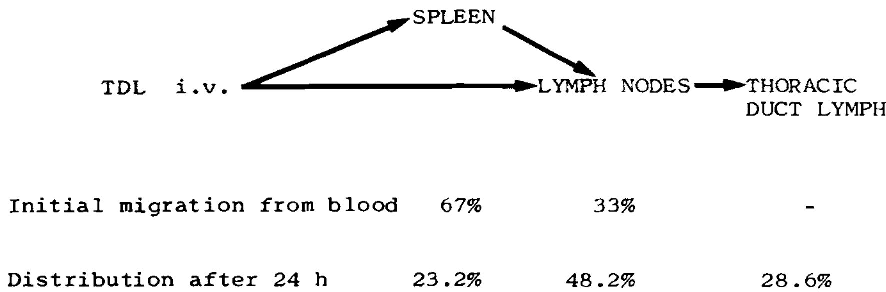


FIG. 3. 24-h distribution of TDL after i.v. injection into F_1 hybrid recipient. Interpretation of the differential distribution of responsive and nonresponsive populations in an F_1 hybrid recipient. *, reactive cells in the responsive population; O, nonreactive cells in the responsive population, ●, reference population e.g. F_1 hybrid cells. One in eight (12.5%) of the reactive population are reactive cells. The distribution of nonreactive cells after 24 h in the cannulated F_1 hybrid recipient is in the proportion of 1:3:1 between the spleen, lymph nodes and TDL. Three-fifths of the reactive cells lodge in the lymph nodes and two-fifths in the spleen because these are the sites of their initial migration from the blood. Two-thirds of the nonreactive cells which initially migrate to the spleen are later distributed to lymph nodes which thus have a small net deficit of reactive cells. The model has been designed to fit the observed values for the reactive/nonreactive ratios and the 24 h distribution of nonresponsive cells. The fit is not perfect only because, for the purpose of clarity, the number of "cells" has been limited.



Initial migration from blood	67%	33%	-
Distribution after 24 h	23.2%	48.2%	28.6%

FIG. 4. Distribution of syngentic TDL after i.v. injection. From the blood lymphocytes migrate into the spleen and lymph nodes in the ratio of 67:33. By 24 h many of the cells which were in the spleen have been redistributed to the lymph nodes and many of the cells which were in the lymph nodes have been released into the thoracic duct lymph.

cells which do react and those which do not. In fact T lymphocytes are labeled in vitro with radioactive uridine about 10–15 times more intensely than are B lymphocytes (28) and since about two-thirds of rat thoracic duct lymphocytes are T cells (29) the T-cell population will contain 20–30 times as much label as the B-cell component. Therefore it is mainly the T-cell population which has been followed and this study gives no information about B cells which anyhow are inert in GVH reactions (30, 31). Recently a case has been made that the frequency of B lymphocytes reacting to Ag-B antigens is not exceptionally high (18).

The possibility must be considered that it is only a very small but exceptionally heavily labeled population of T lymphocytes which can react to each of these Ag-B antigens although there are no a priori grounds for believing that this might be so. In one set of the autoradiographic experiments reported in the accompanying paper (32) the number of grains over cells which had been labeled with [^{3}H]uridine in vitro and which had undergone blastic transformation after 24 h in the recipient was on average less than over unchanged donor small lymphocytes. The enlargement of the reacting cells can no doubt account for the apparent dilution of label since relatively less cytoplasm will be present in the superficial layer in which tritium is detectable by autoradiography. However this geometric effect is quite small; it is proportional to the increased diameter of the cell which is from 7 μ to about 10 μ. If the reactive cells were more heavily labeled than the average T cell the difference must have been very small.

Two types of autoradiographic experiment have confirmed that at least 4–6% of donor cells react to each of these Ag-B antigens. This is a minimum estimate unlike the present estimate of 12%. The reconciliation of these two figures and the significance of such a high frequency of responding cells is discussed in the following paper (32).

Summary

A systemic graft-vs.-host (GVH) reaction was initiated by the intravenous injection of parental strain thoracic duct lymphocytes (TDL) into irradiated F_1 hybrid recipients with in-dwelling thoracic duct cannulae. The migration of the donor lymphocytes was followed by labeling them in vitro with either [^{3}H] or [^{14}C]uridine and measuring radioactivity by scintillation counting of the spleen and lymph nodes of the recipients removed 24 h after injection and in TDL collected throughout this period. The localization of labeled cells was always compared to that of a reference population of nonreactive lymphocytes, e.g. F_1 hybrid, labeled with the alternative isotope (Fig. 1). A consistent surplus of the reactive label was found in the spleen which was balanced by a deficit of the reactive label in TDL; lymph nodes gave intermediate values. The same distribution pattern was noted when the reference population was a specifically unresponsive population of the parental strain. This differential distribution depends on recognition of the recipient's Ag-B antigens because when normal lymphocytes were injected together with specifically unresponsive lymphocytes into a "third party" F_1 hybrid (against which both populations were reactive) there was no surplus of the normal cells in the spleen and no deficit in the lymph. Moreover in an Ag-B identical strain combination there was no detectable difference in the distribution of reactive and nonreactive populations.

The distribution of a labeled reaction population can be accounted for if a substantial minority of cells are immobilized in the spleen and lymph nodes as a consequence of antigen recognition (Fig. 3). When the donor cells in the spleen were assayed 24 h after injection there was paradoxically a slight reduction in their specific GVH activity, which is at least partly because they are under-represented in a single cell suspension. The size of the splenic surplus (23%) and the thoracic duct deficit (12%) suggested that the minority of nonimmune lymphocytes which recognize each Ag-B complex carry 12% of the radioactive label in

the original population. It is argued that this provides a near estimate of the frequency of T lymphocytes which can recognize each Ag-B antigenic complex.

Appendix

Lymphocytes labeled in vitro with [^{3}H]uridine lose ^{3}H faster than cells labeled with [^{14}C]uridine lose ^{14}C (16). The differential elution was fully compensated by performing simultaneous duplicate experiments with exchange of the ^{3}H and ^{14}C between reactive and nonreactive populations. Results were then calculated as follows:

Let the observed reactive/nonreactive ratio (^{3}H/^{14}C) in any compartment (spleen, lymph nodes, TDL) relative to the ratio in the original inoculum be a_1 and let the corresponding reactive/nonreactive ratio (^{14}C/^{3}H) in the duplicate experiment be a_2.

The ratios should be compensated by a factor m so that if there were no differential elution the ratio of ^{3}H/^{14}C would be a_1m. Then in the duplicate experiment the compensated ratio of ^{14}C/^{3}H is a_2/m.

The geometric mean ratio considering the two experiments together is given by

$$\sqrt{a_1m \times (a_2/m)} = \sqrt{a_1a_2}.$$

The ratios given in Tables I–IV and VI were all calculated as $\sqrt{a_1a_2}$. With respect to the key results given in Table I the same conclusions could have been drawn if the 16 experiments had been considered individually since the differential elution was small (about 5%) but the splenic surplus of reactive cells would have been less when the reactive/nonreactive labels were ^{3}H/^{14}C and greater when they were ^{14}C/^{3}H and so on.

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