

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

### II. Autoradiographic Estimates of the Frequency of Donor Lymphocytes Which Respond to each Ag-B-Determined Antigenic Complex\*

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Results presented in the accompanying paper (1) suggested that the frequency of nonimmune lymphocytes which can recognize the Ag-B-determined antigenic complex of the recipient in a graft-vs.-host (GVH)<sup>1</sup> system is about 12% of T lymphocytes. Antigenic recognition was assessed by the sequestration of antigen-sensitive cells from the traffic stream of lymphocytes recirculating through the spleen and lymph nodes of the F<sub>1</sub> hybrid recipient. The system can be regarded as analogous to an in vitro antigen-binding assay except that in this case the antigen was fixed in lymphoid tissue and the number of lymphocytes which were bound to antigen in vivo was measured. As is the case with counts of antigen-binding cells in vitro it was not known how many of the cells which were scored could respond to antigen by other criteria such as cell division or the production of effector cells.

The present experiments were also based on the injection of parental strain lymphocytes into irradiated F<sub>1</sub> hybrid recipients. They were designed to measure the proportion of donor lymphocytes which responded to transplantation antigens (a) by morphological transformation to large pyroninophilic cells or (b) by entering the S-phase of the cell cycle.

The data support other work (2-4) indicating that an exceptionally high proportion of T lymphocytes proliferate in response to antigens determined within a single complex locus, e.g. Ag-B. Although the basic significance of this is still unknown it is an empirical observation which must be considered in accounting for the diversity of T-cell recognition.

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<sup>1</sup> Abbreviations used in this paper: GVH, graft-vs.-host; LAD, lymphocyte-activating determinant; MLC, mixed lymphocyte culture; PALS, pararteriolar lymphoid sheath; TDL, thoracic duct lymphocytes.

## Materials and Methods

**Rats.** Donors of thoracic duct lymphocytes (TDL) were male AO rats and the recipients were either (AO x DA)<sub>F</sub><sub>1</sub> or (AO x HO)<sub>F</sub><sub>1</sub> hybrids. Some experiments were done in an Ag-B compatible combination by transferring HO lymphocytes into (HO x August)<sub>F</sub><sub>1</sub> hybrids. The hybrid recipients had received 400 rads of whole-body  $\gamma$ -irradiation from a <sup>60</sup>Co source 3 days before cell transfer and had been subjected to thoracic duct cannulation on the day before cell transfer. The object of this last procedure was to use precisely the same GVH system as in the previous series of experiments so that the results would be comparable (1).

**Radioactive Labeling of Donor Lymphocytes.** Parental strain lymphocytes were labeled in vitro with [5-<sup>3</sup>H]uridine at 5  $\mu$ Ci/ml. (5). They were reinjected i.v. into the donor and recovered from its lymph after 18–30 h. Three reasons for this autologous passage have already been stated (1). A fourth reason was that the labeled donor cells were spaced out by unlabeled donor cells in the recipient's spleen which greatly facilitated scoring in autoradiographs.

In other experiments donor lymphocytes were labeled by injecting the donor with [<sup>3</sup>H]thymidine. Two regimes were used, as follows: (a) daily i.p. injections for 3 wk followed by an interval of 2 wk to allow "short-lived" lymphocytes (6) to disappear, and (b) twice daily injections for 7 days followed by an interval of 2 days to label "young" or recently produced lymphocytes. The dose of [6-<sup>3</sup>H]thymidine (TRA.61, Radiochemical Centre, Amersham, England) was 1  $\mu$ Ci/g body wt/day.

**Radioactive Labeling of the Recipient.** In some experiments donor lymphocytes were not labeled before injection. The number of lymphocytes in the recipient which had entered S-phase was measured by giving the recipient a continuous i.v. infusion of [6-<sup>3</sup>H]thymidine (TRA.61), total dose of 1  $\mu$ Ci/g body wt, from 6 h after injection of the donor cells to 23 h. The recipients were killed 1 h later.

**Histology and Autoradiography.** Sections of spleen and lymph node were fixed in formol-alcohol or 2% glutaraldehyde and paraffin sections were cut at 3–5  $\mu$ . Triplicate sections were dipped in Ilford K5 emulsion and exposed for different intervals, e.g. 3 days, 7 days, and 14 days. Sections were stained through the emulsion with methyl green and pyronin.

**Prevention of Donor Cell Division.** The division of donor cells normally begins between 18 and 24 h after injection into irradiated F<sub>1</sub> hybrids (Results section 3). Division was blocked in metaphase by the i.v. injection of colcemid at a dose of 4 mg/kg body wt at 18 h after the donor cell injection. This dose is sufficient to block the cell division for a period of 6 h (C. E. Ford, personal communication).

**Enumeration of the Number of Donor Cells Responding to Antigen.** When labeled donor lymphocytes were injected the fraction which had transformed morphologically were counted. The numerator of this fraction was labeled cells which had (a) abundant, pyroninophilic cytoplasm, (b) an enlarged, open nucleus, and (c) a prominent pyroninophilic nucleolus. Two out of three of these criteria made a cell positive. (Fig. 1 and ref. 7). Pyroninophilic cells blocked in metaphase were also scored as positive. The denominator was all labeled cells in the spleen. A small number of cells were included in a "doubtful" category; some of these may have been in transition. The great majority of labeled lymphocytes were within the pariarteriolar lymphoid sheath (PALS). When unlabeled donor cells were injected into recipients which were infused with [<sup>3</sup>H]thymidine the fraction of cells which had responded was calculated by counting as the numerator labeled lymphocytes in the PALS and as the denominator all lymphocytes with distinct nuclei in the PALS (Figs. 3 and 4).

In all experiments similar counts of responding and nonresponding cells were performed in controls in which AO lymphocytes were injected into irradiated and cannulated recipients. The cells were scored using a X 40 oil immersion objective with the aid of a plain 20  $\times$  20 square grid as an eyepiece graticule. Sections of spleen were scanned systematically.

## Results

**The Proportion of Donor Cells Responding by Morphological Transformation.** The mean proportion of labeled donor cells in the spleen which had unequivocally transformed into large, pyroninophilic cells was 17.3% (Table I). Over 4,000 labeled cells were scored in six experiments; the individual scores were reasonably consistent (19.2; 16.3; 15.2; 17.1; 20.3; 12.2). There was no difference between (HO  $\times$  AO)<sub>F</sub><sub>1</sub> and (DA  $\times$  AO)<sub>F</sub><sub>1</sub> recipients which was expected because of the equal GVH activity of AO cells against these two recipients by the popliteal lymph node assay (8).

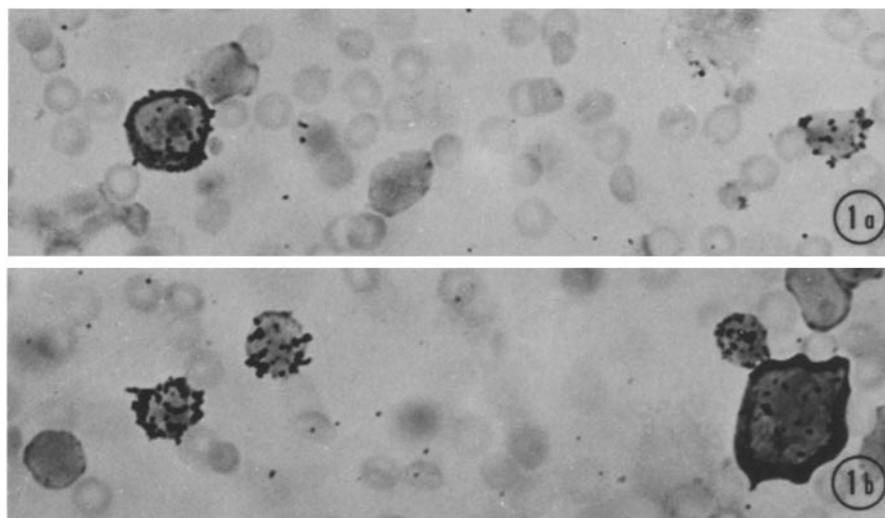


FIG. 1. Autoradiograph of a cell suspension made from the spleen of an  $F_1$  hybrid recipient which was killed 24 h after the i.v. injection of parental strain TDL. The donor cells had been labeled in vitro with  $[^3H]$ uridine. (a) Transformed cell with pyroninophilic cytoplasm and nucleolus. Labeled small lymphocyte on right. (b) Four labeled cells of which one has transformed. Methyl green-pyronin with green filter.  $\times 970$ . For appearance of transformed cells in tissue sections see ref. 7.

In control experiments in which labeled AO lymphocytes were injected into irradiated AO recipients only 1.0% of the labeled cells had transformed. Thus the excess which had transformed in the presence of an Ag-B antigen was 16.3% (Table I). In the non-Ag-B strain combination the proportion which had transformed was 1.0% (Table I), which is consistent with previous results (1)

TABLE I  
*Donor Lymphocytes Labeled with  $[^3H]$ Uridine In Vitro*

	Proportion of labeled lymphocytes in the recipient's spleen which had transformed into large pyroninophilic cells after 24 h			
	Definitely transformed	Definitely and possibly transformed	No. of cells counted	No. of exps.
	<i>mean % <math>\pm</math> SE</i>	<i>mean % <math>\pm</math> SE</i>		
AO $\rightarrow$ (AO $\times$ HO) $F_1$ and AO $\rightarrow$ (AO $\times$ DA) $F_1$	17.3 $\pm$ 1.2	20.6 $\pm$ 1.5	4,158	6
AO $\rightarrow$ AO (syngeneic background)	1.0 $\pm$ 0.3	2.1 $\pm$ 0.4	2,201	3
Specific for alloantigen	16.3 (5.7)*	18.5 (6.5)*		
HO $\rightarrow$ (HO $\times$ Aug) $F_1$ Non-Ag-B	1.0	1.3	674	2

\* Reduced by "selection factor" ( $\times 0.35$ ). See text.

suggesting that the proportion of donor lymphocytes which can recognize these weak transplantation antigens is too small to be detected.

*The Proportion of Donor Cells Responding by DNA Synthesis.* Approximately  $10^9$  parental strain lymphocytes were injected i.v. into irradiated, cannulated  $F_1$  hybrid recipients which then received a continuous i.v. infusion of [ $^3H$ ]thymidine. The proportion of labeled cells among all the lymphocytes in the PALS was counted (Fig. 3). The mean value obtained by counting a total of 5,000 cells in three experiments was 18.9% (Table II) and the proportion was again remarkably consistent (18.2; 19.2; 19.4).

The first group of control experiments consisted of injecting  $10^9$  AO lymphocytes into irradiated and cannulated AO recipients which were infused with [ $^3H$ ]thymidine. In this case the mean proportion of labeled cells in the PALS was 2.4% (Fig. 4). It is not known how many of the labeled cells were donor and how many were residual host cells but since the figure was used as a "background" to subtract from the figure in the spleen  $F_1$  hybrid recipients given parental strain TDL the relative donor/host contributions to this "background" is not of critical importance. Subtraction of the background (18.9% - 2.4%) suggest that 16.5% of the cells in the PALS have responded to transplantation antigen (Table II). This is remarkably similar to the 16.3% of labeled donor cells which were found to have transformed morphologically (Table I). The denominator of the fraction is all the lymphocytes in the PALS. They have been assumed to be of donor origin because in AO and  $F_1$  hybrid rats which were irradiated 4 days before killing and cannulated 2 days before killing the PALS were extremely deficient in lymphocytes (Fig. 2). The  $F_1$  hybrid recipients of  $10^9$  TDL showed spectacular replenishment of their PALS whether the cells were of parental strain or syngeneic (Figs. 3, 4, 6, 7). A large donor cell inoculum was used so that the very few residual host lymphocytes in the PALS could be regarded as negligible.

In two of the three parental to  $F_1$  hybrid experiments the 24-h specimen of spleen was taken as a biopsy, a second dose of colcemid was injected and the i.v. infusion of [ $^3H$ ]thymidine was resumed for 5 h. The rats were killed after 1 more h, i.e., 30 h after the injection of the donor cells. In this case the proportion of

TABLE II  
*Recipient Infused Continuously with [ $^3H$ ]Thymidine*

	Proportion of labeled cells among the lymphocytes in the periarteriolar sheaths of the recipient's spleen	
	Recipient killed after 24 h	Recipient killed after 30 h
	<i>mean % ± SE</i>	<i>mean %</i>
AO → (AO x HO) $F_1$ and AO → (AO x DA) $F_1$	18.9 ± 0.4 (5,219/3)*	21.9 (2,140/2)*
AO → AO (syngeneic background)	2.4 (4,140/2)*	3.1 (2,130/2)*
Specific for alloantigen	16.5	18.8
Reduced by selection factor (x0.35)	5.8	6.6

\* No. of cells counted/no. of experiments.

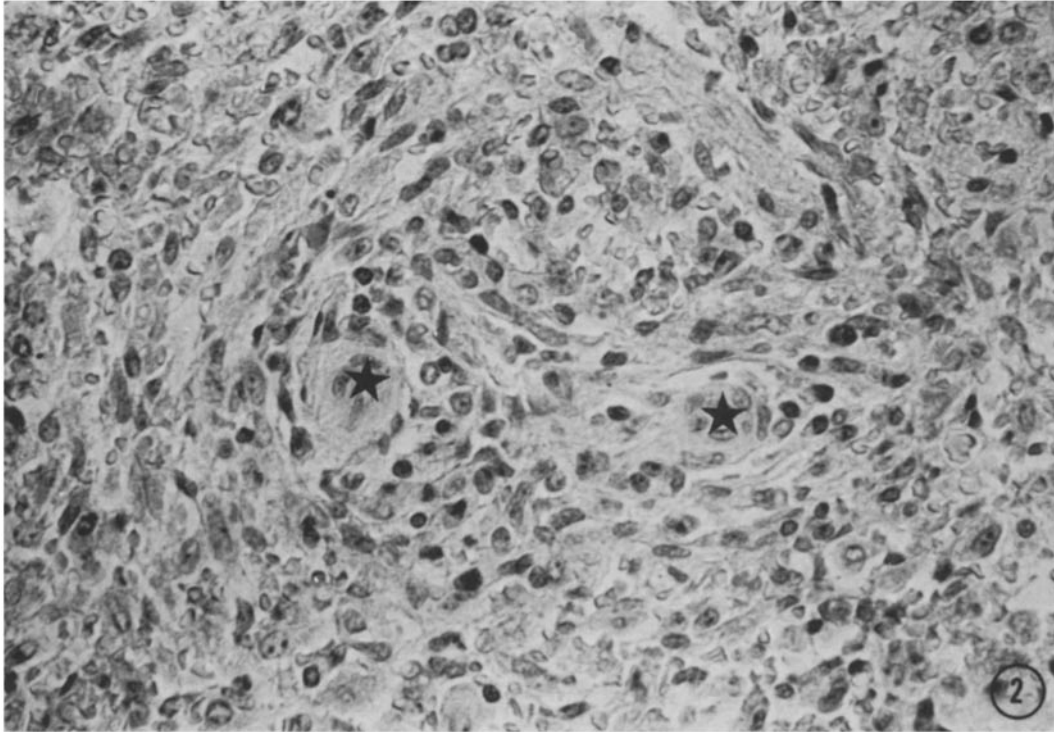


FIG. 2. Spleen of irradiated  $F_1$  hybrid which had not received an i.v. injection of lymphocytes. The PALS is very shrunken and few lymphocytes remain around the central arterioles (★). Compare with Figs. 3 and 4. Methyl green-pyronin with green filter.  $\times 600$ .

lymphocytes in the PALS which were labeled rose to 21.9% which was 18.8% above the background. This suggested that only a small number of parental strain cells (2-3%) first started to synthesize DNA between 24 and 30 h after transfer into  $F_1$  hybrid recipients.

*Donor Cell Division Does not Begin Until After 18 h Following i.v. Injection.* In the experiments already described cells in metaphase were numerous in the PALS of recipients killed at 24 or 30 h after donor cell injection. Irradiated, cannulated  $F_1$  hybrid recipients of  $500 \times 10^6$  donor cells were given colcemid (4 mg/kg) after 12 h and killed after a further 6 h. Only 0.4% of all the lymphocytes in the PALS had been arrested in metaphase (Fig. 6). The donor lymphocytes had been labeled in vitro with [ $^3$ H]uridine before injection and the percentage of labeled cells in mitosis were also counted on autoradiographs. The result, 0.46% in mitosis, was very similar to the proportion of mitoses in all the lymphocytes in the PALS. Thus there was no evidence of excessive donor cell division at that stage. By contrast the proportion of mitoses among labeled cells in spleens taken at 24 h and 30 h after i.v. injection with colcemid block from 18 h onwards was 4.1% and 6.5% respectively. (Fig. 5) (Table III). These data are consistent with previous evidence that donor cell mitosis does not begin until a lag of just less than 24 h. (7, 9). The administration of colcemid at 18 h in the previous experiments ensured that a negligible number of the cells which were

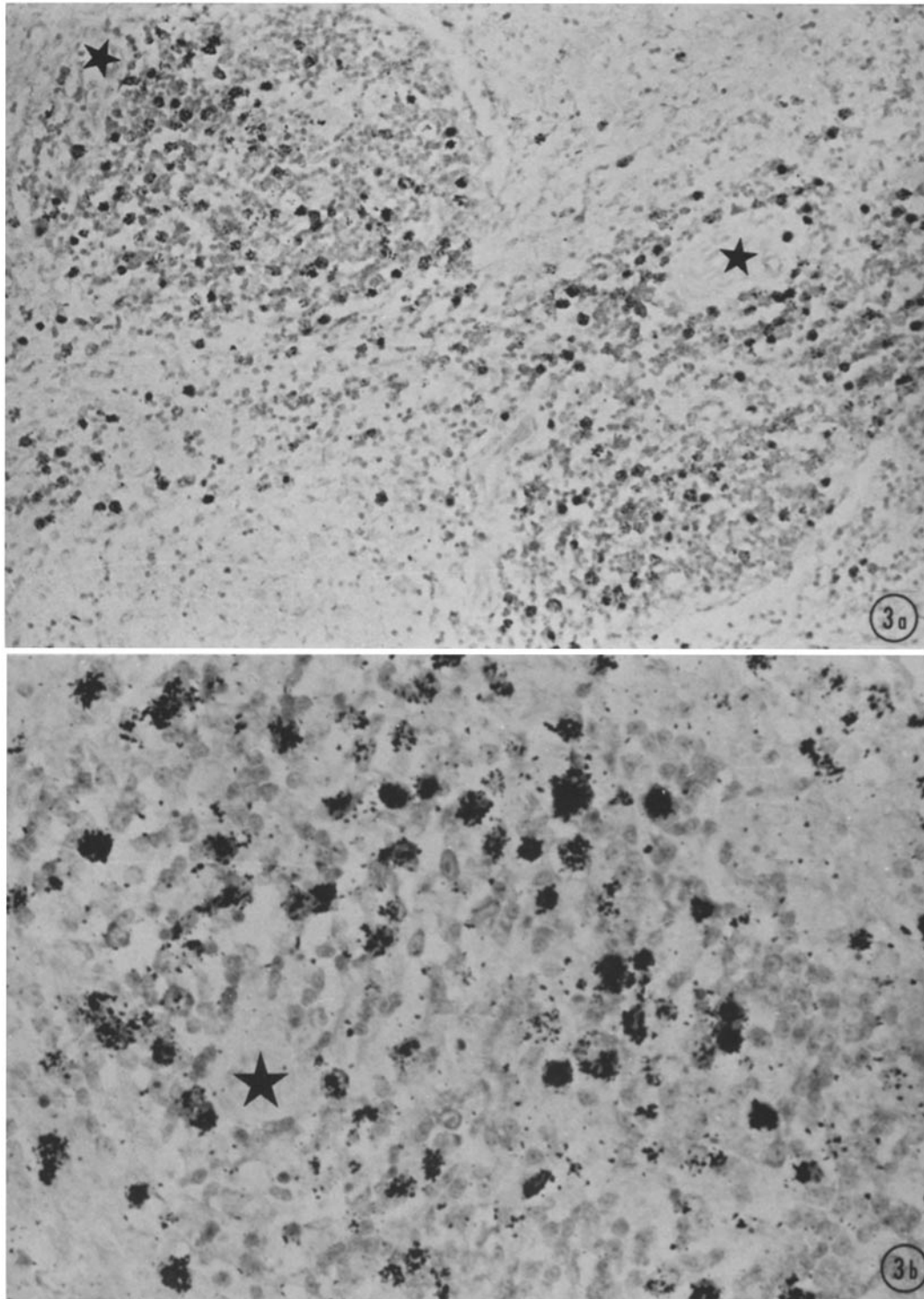


FIG. 3. Autoradiographs of the spleen of an irradiated F<sub>1</sub> hybrid which had been injected i.v. with parental strain lymphocytes and continuously infused with [ $^3\text{H}$ ]thymidine. A high proportion of the lymphocytes around the central arteriole (★) labeled. Methyl green-pyronin. (a)  $\times 245$ ; (b)  $\times 600$ .

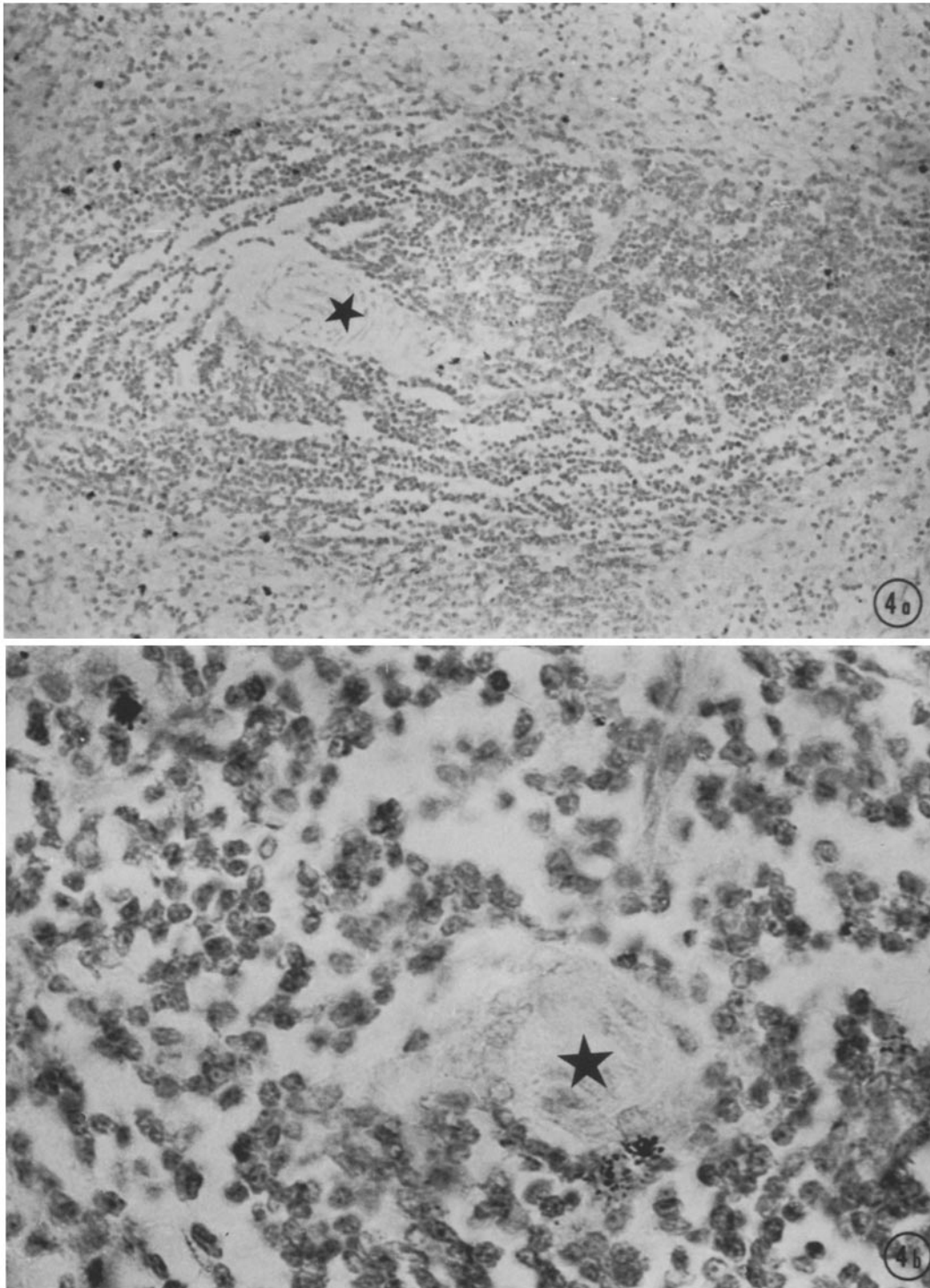


FIG. 4 Autoradiograph of the spleen of an irradiated F<sub>1</sub> hybrid which had been injected i.v. with syngeneic lymphocytes and continuously infused with [<sup>3</sup>H]thymidine. Only a small proportion of the lymphocytes around the central arteriole are labeled. Methyl green-pyronin. (a)  $\times 245$ ; (b)  $\times 970$ .

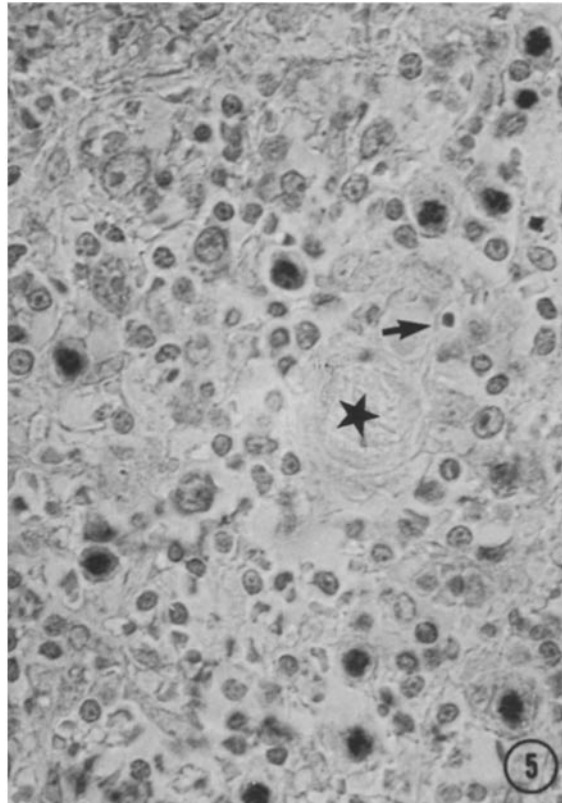


FIG. 5. Spleen from  $F_1$  hybrid recipient which was injected with parental strain lymphocytes 30 h previously and colcemid 18 and 24 h after injection. 10 or 11 mitoses are seen around the central arteriole (★). Some are not in the precise plane of focus but they are easily distinguishable from the pyknotic body (arrowed). Methyl green-pyronin.  $\times 700$ .

TABLE III  
*Proportion of Cells in Mitosis*

	Recipients killed after donor cell injection		
	18 h	24 h	30 h*
Donor cells labeled in vitro with [ $^3\text{H}$ ]juridine (% of labeled cells in mitosis)	0.46 (1,076)	4.1 (764)	6.5 (2,140)
Percent of all cells in periarteriolar lymphoid sheaths in mitosis	0.40 (1,772)	—	—

Colcemid injected 6 h before death.

\* Colcemid injected at 18 h and again at 24 h after donor cell injection.

scored as "responding" at 24 h were daughter cells derived from a smaller number of donor cells injected.

*The Early Recruitment of Lymphocytes Which are not Antigen-Sensitive.* The interaction of immune lymphocytes and antigen produces a factor



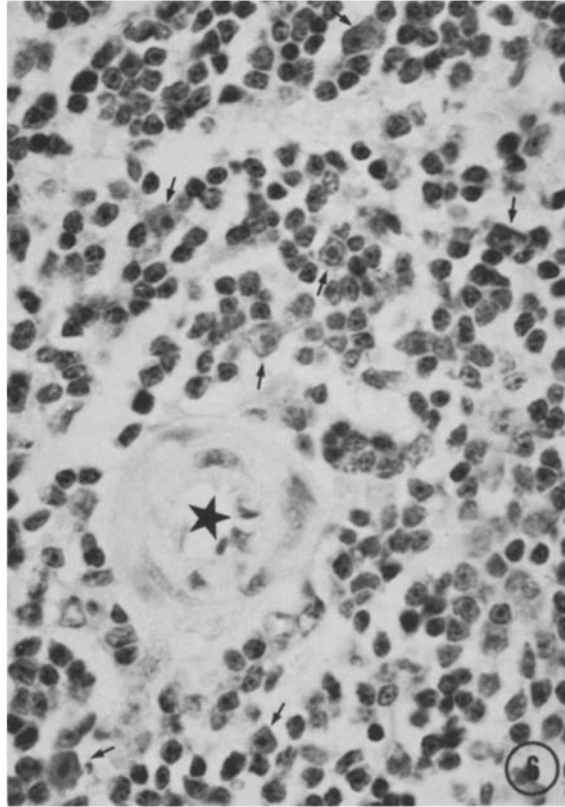


FIG. 6. Spleen from  $F_1$  hybrid recipient which was injected with parental strain lymphocytes 18 h previously and colcemid 12 h after injection. There are no mitoses around the central arteriole (★) although some cells (arrowed) show early blastic transformation. Methyl green-pyronin.  $\times 700$ .

which is mitogenic for lymphocytes (10). It could be argued that the number of antigen-sensitive cells is only a small minority of those scored; the majority may be recruited nonspecifically as a consequence of the response of the minority. However, the finding that early in a GVH reaction the mitotic response is almost exclusively of donor origin, even when the host lymphocytes are available, makes this unlikely (11).

Irradiated  $F_1$  hybrid recipients were injected with unresponsive lymphocytes which had been labeled *in vitro* with [ $^3\text{H}$ ]juridine. They were unresponsive either because they were syngeneic  $F_1$  hybrid lymphocytes or because they were parental strain (AO) cells which had been passaged from blood to lymph in an irradiated  $F_1$  hybrid recipient (8). These labeled, unresponsive cells were injected together with a surplus of unlabeled, normal AO cells which initiated a GVH reaction. However, as shown in Table IV the number of labeled cells scored as responding by the same morphological criteria used previously was at the usual background level of about 1%. This finding excludes the possibility of nonspecific recruitment early in this GVH reaction and indicates that all the cells scored as "responding" were intrinsically antigen-sensitive. The related idea that more

TABLE IV  
*Unresponsive (labeled) Lymphocytes Injected with Excess of Responsive (unlabeled) Lymphocytes*

Source of unresponsive lymphocytes	Source of responsive lymphocytes	Mean % transformed at 24 h	
		Definitely transformed	Definitely and possibly transformed
Specifically unresponsive parental (AO)	Parental (AO)	1.1 (538)*	2.2
F <sub>1</sub> hybrid (syngeneic with recipient)	Parental (AO)	0.9 (676)*	1.3
F <sub>1</sub> hybrid (syngeneic with recipient)	None	1.2 (592)*	1.7

\* No. of cells counted.

cells respond to the background of nontransplantation antigens as a result of the allogeneic effect (12) is also excluded by this result.

*The Age of the Responding Cells.* Data on the response of blood leukocytes to Ag-B antigens suggested that young or short-lived lymphocytes (labeled by [<sup>3</sup>H]thymidine given for 10 days before isolation) are predominant among the lymphocytes which respond. The proportion of these recently formed cells was several times higher in the responding population than in the starting population of blood leukocytes (13). The intention of this experiment was to decide whether a similar predominance of young cells occurred in the population responding to Ag-B antigens in the GVH system or whether the responding population reflected the age spectrum of the starting population. Donor cells were labeled in vivo according to either a long-lived or a short-lived regime as described in the methods section and the percentage which had undergone morphological transformation was scored as in the experiments of group (1). There was no significant difference between the frequencies of transformed cells whether the labeled donor cells were "young" or "middle-aged" (Table V). Since these experiments were performed it has been found that the high proportion of labeled responding cells scored in mixed lymphocyte culture (MLC) after recent [<sup>3</sup>H]thymidine labeling of the donor can be attributed to reutilization of label released from dying granulocytes (D. B. Wilson, University of Pennsylvania School of Medicine, Philadelphia, personal communication).

With both labeling regimes the percentage of transformed cells in the spleen was just over half that found by the methods described in group (a) and (b). This discrepancy cannot be explained with confidence. It is possible that the intranuclear irradiation is sufficient either to delay the response of the cells or to kill some cells which have begun to respond to antigen.

*The Proportion of Transformed Lymphocytes in the Intact Spleen and in a Single Cell Suspension Produced from it.* The surplus of reactive parental strain lymphocytes in the spleen detected by scintillation counting was markedly and consistently reduced when a single cell suspension made from part of such a spleen was examined (1). To confirm the suggestion that this reduction was

TABLE V  
*AO Donor of Lymphocytes Labeled by Injections of [<sup>3</sup>H]Thymidine: Proportion Transformed in Spleen of F<sub>1</sub> Hybrid Recipient*

Labeling regime	Exp. no.	Recipient	Definitely trans-	Definitely and
			formed	possibly transformed
			%	%
Long-lived lymphocytes	1,004	(AO x DA)F <sub>1</sub>	11.5	12.7 (706)*
		(AO x HO)F <sub>1</sub>	9.7	12.1 (506)*
		AO (syngeneic)	1.4	2.4 (507)*
	1,614	(AO x DA)F <sub>1</sub>	9.9	11.1 (506)*
		(AO x HO)F <sub>1</sub>	8.9	10.0 (519)*
		AO (syngeneic)	0.5	0.9 (563)*
		Mean of parental to F <sub>1</sub>	10.0	11.4
Young lymphocytes	1,700	(AO x DA)F <sub>1</sub>	7.1	8.4 (547)*
		(AO x HO)F <sub>1</sub>	8.1	9.2 (557)*
		(AO (syngeneic)	3.6	4.6 (711)*
	28,113	(AO x DA)F <sub>1</sub>	12.9	14.3 (504)*
			Mean of parental to F <sub>1</sub>	9.1

\* No. of cells counted.

because cells which had responded went poorly into cell suspension the following experiment was performed. Two of the F<sub>1</sub> hybrid spleens in which the proportion of transformed [<sup>3</sup>H]thymidine-labeled cells were counted autoradiographically were also made into a single cell suspension which was washed once in PBS and the pellet was smeared after re-suspension in a few drops of neat serum. The smears were fixed in methanol and autoradiographs were prepared and stained with methyl green-pyronin. The proportion of cells which were scored as large pyroninophilic cells in smears by the same criteria as were used for section was reduced to about a quarter (Table VI) (Fig. 1).

*Dispersal of Labeled Cells in the Recipient's Spleen.* An unexpected observation was that in recipients of large numbers of parental strain cells, all the cells in the PALS were more widely separated than in recipients of equal numbers of syngeneic F<sub>1</sub> hybrid lymphocytes. (Figs. 3-6). When the injected lymphocytes had been labeled only about half the number of labeled cells were present per unit area of PALS when the GVH recipients were compared to controls given equal numbers of cells. There was apparently edema of the PALS which may have been a consequence of antigen recognition by the donor lymphocytes.

### Discussion

*The Frequency of Responsive Lymphocytes which is Indicated by the Data.* In the first 24 h of a systemic GVH reaction donor lymphocytes undergo

TABLE VI  
*Partial Removal of Large Pyroninophilic Cells by Making a Single Cell Suspension*

Exp. no.	Transformed cells counted in:	Parental → F <sub>1</sub>		Parental → parental	
		Definitely transformed	Definitely and possibly transformed	Definitely transformed	Definitely and possibly transformed
		%	%	%	%
1,300	Intact spleen	20.3 (777)*	22.9	0.9% (1,060)*	1.7
	Spleen cell suspension	4.7 (1,029)*	5.7	0.8 (528)*	1.3
1,301	Intact spleen	12.2 (764)*	13.9		
	Spleen cell suspension	3.7 (507)*	4.9		

\* No. of labeled cells counted.

blastic transformation followed by DNA synthesis followed by mitosis. These changes are most prominent in the thymus-dependent areas of recipients' spleen (4, 7). It is tedious but not difficult to count the proportion of donor cells in the spleen which have responded either by morphological change to a large pyroninophilic cell or by DNA synthesis. Only Porter and Cooper (4) have previously attempted to do so and they laid little emphasis on the figure of 30% of transformed cells which they observed. This is not by itself a meaningful figure because of three unknowns: (a) the proportion of donor cells which localized initially in the spleen and the proportion which remain at the time of scoring, i.e. 24 h, (b) the proportion of responding cells which have divided within 24 h, and (c) the extent of "nonspecific recruitment" of donor lymphocytes which are not intrinsically reactive to the F<sub>1</sub> hybrid antigens.

These gaps have been filled by the present experiments. The previous paper showed that 67% of injected donor lymphocytes migrate first from the blood into the spleen but only 23.2% of nonresponding cells are still there after 24 h. Therefore if  $x\%$  of donor lymphocytes in the spleen are scored as "transformed" the percentage of the injected lymphocytes which have responded can be shown to be  $23/67 x = 0.35x$ . This factor allows for cells which initially localized in the spleen but which, having failed to respond, moved on. Division of donor cells in response to F<sub>1</sub> hybrid antigens was found to begin between 18 and 24 h after donor cell injection. Colcemid was injected at 18 h to block cell mitosis up to the time of killing at 24 h. Therefore each cell which was scored as transformed at 24 h was derived from one of the donor cells injected.

Nonspecific recruitment of unresponsive lymphocytes was excluded by labeling either F<sub>1</sub> hybrid or specifically unresponsive donor strain lymphocytes. When injected with an excess of normal donor strain lymphocytes a vigorous blast cell reaction was seen but only the usual background of 1% of the labeled lymphocytes had transformed. This is not compatible with a major contribution of nonspecific recruitment to the 16-19% of cells which responded in previous experiments since both the specifically unresponsive parental lymphocytes and the F<sub>1</sub> hybrid lymphocytes were eligible for such recruitment.

Rat TDL consist of T and B cells in the approximate ratio of 2:1. (14). By both methods the cells which were scored either as transformed or nontransformed were mainly T cells for the following reasons. When TDL are labeled in vitro with [<sup>3</sup>H]uridine B cells label much more lightly than T cells (15); although all the cells in the recipient's spleen were scored, in fact almost all of the labeled cells were in the thymus-dependent PALS. Very few labeled cells were present in the B areas of the spleen. In the second method of estimation only the cells in the PALS were scored as labeled or unlabeled. By 24 h after the injection of TDL the segregation of T and B cells is complete (15, footnote 2). Although B cells enter the PALS a few hours after reaching the spleen they have moved on into the lymphocyte corona by 24 h.<sup>2</sup>

The final consideration is that enlargement of the transforming cells may theoretically produce an overestimate of their numbers because cells whose center lies at a greater depth within the section will be detected. If the mean diameter of a lymphocyte nucleus increases from 5 $\mu$  to 7 $\mu$  and the effective thickness of the section for autoradiography is 1 $\mu$  then because of the increase of cell diameter at right angles to the plane of section the proportion of transformed cells will be overestimated by a factor of  $(7 + 1)/(5 + 1) = 1.3$ . This factor reduces the estimate of responsive cells in the original inoculum from 6% to 4.5%. However the factor probably exaggerates the geometric effect since in the first method a large open nucleus was a criterion of transformation. Thus a fairly central cut of the transformed cell nucleus would be necessary for its recognition. Any other factors such as dilution of label would tend to minimize the proportion of transformed cells.

Our conclusion is that at least 4.5–6% of AO lymphocytes recognize and react to the unknown number of antigens determined within the Ag-B locus of either the DA or HO strains. A very unlikely alternative is that the number of cells responding to the antigenic differences at multiple weak (non-Ag-B) loci is greatly increased by the simultaneous presence of an Ag-B incompatibility. A congenic strain of HO rat which is homozygous for the Ag-B type 2 of the AO strain will soon be available and it is hoped to exclude this possibility by similar experiments to those described here between strains which differ only at the Ag-B locus.

The scintillation counting method suggested that 12% of donor cells leave the recirculating pool as a consequence of recognizing the F<sub>1</sub> hybrid antigen (1). The estimates of 4.5–6% and 12% can be reconciled in three ways: (a) The 12% may be an overestimate because the responding cells may be more heavily labeled with [<sup>3</sup>H]uridine than the average of the starting population. This is unlikely to have been a large factor since there were fewer grains on average over cells which had transformed than over cells which had not. (b) The 4.5–6% may be an underestimate because more cells would have responded after the 24 h given the opportunity. This seems unlikely to be a major factor because by 30 h only a further 1% had responded. (c) Both estimates are substantially correct; the difference is because some cells recognize antigen and are immobilized but their

<sup>2</sup> Nieuwenhuis, P., and W. L. Ford. Comparative migration of B and T cells in the rat spleen and lymph nodes. Manuscript in preparation.

receptors do not bind antigen with sufficient affinity to produce blastic transformation. Our figures can also be reconciled with the estimate of 1-3% responding in MLC (2) because the starting population was blood leukocytes of which perhaps about half are T lymphocytes and also we may have studied stronger strain combinations. After we had chosen the AO to (AO × HO)F<sub>1</sub> and AO to (AO × DA)F<sub>1</sub> combinations for these experiments they were found to be the most powerful of nine Ag-B-incompatible combinations studied (16).

*The T-Cell Receptor—a Four Letter Word?* It remains to comment on the significance for antigenic recognition by T cells of results which indicate that 4-12% of T cells recognize each Ag-B complex and that different T cells respond to different Ag-B antigens (8, 17, 18). The idea that apparently nonimmune animals have experienced cross-reacting environmental antigens has not been supported by studies on the GVH and MLC activity of germ-free animals which is at least as great as that of normal animals (19, 20). The orthodox explanation is to assume that each Ag-B or *H-2* locus specifies a large number of lymphocyte-activating determinants (LAD), each of which is recognized by at most 1 in 10<sup>5</sup> of nonimmune lymphocytes. However our minimum estimate of 4% of responding cells would require 4,000 different antigens to be specified within each locus which seems more than is plausible. The model we favor envisages that the T-lymphocyte receptor is compounded of a small number of subunits, say four. (By analogy with antibody formation this seems plausible since immunoglobulin can of course be regarded as a composite recognition molecule consisting of two subunits, heavy and light chains, (21) each of which is controlled by a series of V-genes). It is proposed that the subunits of the T-lymphocyte receptor are determined by a closely linked series of nonallelic genes as others have also assumed (22, 23). We suggest that each individual has about 100 such genes of which a random selection of four is expressed by each cell. The four subunits, all of which are different, are combined into a functional receptor before the cell has had experience of antigen. The exclusion of the majority of genes is the generator of T-cell diversity because it produces subpopulations of cells which recognize ca.  $3 \times 10^6$  different antigenic patterns  $(100 \times 99 \times 98 \times 96)/(1 \times 2 \times 3 \times 4)$  through combinations of recognition subunits.

The recognition of antigens by virgin T cells leading to delayed hypersensitivity is by means of the composite receptor at least in the case of nontransplantation antigens. The large number of receptor subunit combinations permits the wide range and exquisite discrimination of delayed hypersensitivity. In transplantation immunity the recognition of target cell antigens by immune cells, in particular cytotoxic lymphocytes, is also by means of the composite receptor. However the initiation of responses to certain transplantation reactions, as exemplified by GVH and MLC reactions, is unique in the sense that LADs are recognized by all T cells which express the appropriate recognition subunit; the other subunits in the composite receptor are irrelevant in this situation. The explanation of this uniqueness may be connected with the peculiar presentation of alloantigens on the surface of the living cells in such a way that the intermeshing of complementary structures on the stimulating and responding cells vicariously activates the latter. This means that 4/100 T cells would recognize each alloantigen. In the present system 1-3 LADs within the Ag-B locus may be rec-

ognized by the responding population. This is based on the estimates of 4–12% for the frequency of responders.

This model has been influenced by the semi-instructive scheme of T-cell recognition advanced by Simonsen in 1970, although published later (23). We diverge from that model in that we envisage that T lymphocytes are fully precommitted during ontogeny before they encounter antigen. Although the evidence is completely inadequate at present a number of verifiable predictions stem from our model for example (a) T lymphocytes specific for a nontransplantation antigen, by virtue of the composite receptor, will also be activated by a number of LADs, by virtue of their receptor subunits; (b) only a small minority of the cells activated in GVH and MLC reactions will differentiate to cytotoxic effector cells, and (c) each T lymphocyte will proliferate in response to a small number of LADs, i.e., they will show a restricted pluripotentiality. This cognitive cross-reactivity of T-cell recognition will not necessarily be reflected in serological cross-reactions because whereas the B-cell dictionary consists of two letter words (heavy and light chains) each composed from different alphabets of about 5,000 words, the T-cell dictionary may consist of four letter words composed from a much shorter alphabet.

### Summary

A graft-vs.-host (GVH) reaction was initiated by the intravenous injection of parental strain (AO) lymphocytes into irradiated (AO × HO)F<sub>1</sub> or (AO × DA) F<sub>1</sub> hybrids. The proportion of donor T cells which had responded to the F<sub>1</sub> hybrid antigens within 24 h was estimated by two methods. (a) Donor lymphocytes were labeled with [<sup>3</sup>H]uridine in vitro before injection. The proportion of labeled cells which had morphologically transformed in the recipient's spleen was 17–19%. (b) A large number of unlabeled donor lymphocytes were injected into the irradiated recipient which was infused with [<sup>3</sup>H]thymidine until 1 h before killing. The proportion of labeled lymphocytes in the PALs was 19%. In both series of experiments syngeneic transfers were performed in which case the proportion of transformed cells was 1–2.4%. A similar low proportion was found after parental to F<sub>1</sub> transfer in a non-Ag-B strain combination.

These figures were used to calculate the frequency of responding cells in the injected population given three additional pieces of information: (a) the extent of selection in the spleen which transformed the estimate to 4.5%–6.0% responders; (b) division of donor cells was shown to be negligible under the conditions of the experiment; and (c) the nonspecific recruitment of lymphocytes was shown to be negligible. A speculative model of antigen recognition by T cells which accounts for the high proportion of responders is outlined.

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