SPLENOMEGALY AS A HOST RESPONSE IN GRAFT-VERSUS-HOST DISEASE

J. G. HOWARD, D. MICHIE AND M. SIMONSEN

From the Department of Surgical Science and Medical Research Council Research Group on Transplantation, University of Edinburgh; and University Institute of Pathological Anatomy, Copenhagen

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THE graft-versus-host reaction is provoked by introducing homologous lymphoid cells into an immunologically defenceless recipient, for example by injecting an F1 hybrid between two inbred strains with cells from one of the parent strains. It was shown by Simonsen (1957) that spleen enlargement is an early and reliable sign of this reaction. Since the splenomegaly represents a cellular increase, rather than an increase of stromal elements or of fluid content, it has seemed natural to regard the added splenic mass as being largely composed of donor cells which have settled and proliferated there. Direct proof that this may occur has emerged from the studies in chicken embryos by Biggs and Payne (1959) using the sex chromosomes as markers. On the other hand, Davies and Doak (1960) injected mouse spleen cells labelled with a cytological marker (of mutational origin) into homologous newborn recipients and found that the mitoses observed in the spleen tissue during the ensuing period were almost entirely confined to cells of host type.

Whereas Davies and Doak estimated the proportion of donor cells among all dividing cells, we have attempted to estimate the proportion of donor cells among all immunologically competent cells, using the spleen assay method developed by Simonsen and Jensen (1959). At the same time the donor cells were characterized as to whether they were still capable of reacting against a secondary host of the same genotype as the primary host or whether they were only capable of reacting against the antigens of some third strain. Simonsen (1960) has claimed that adult donor cells can under certain conditions acquire tolerance of the primary host while otherwise retaining the power of immune response.

MATERIALS AND METHODS

Plan of the assays

A cell suspension is prepared from the supposedly chimerical spleen of an animal undergoing a graft-versus-host reaction, and injected into infant mice of such a type that they may react with splenomegaly to the donor component, but not to the host component. This object is achieved in the following two circumstances:

Serial passage.—The chimerical suspension is tested in secondary hosts isologous with the primary hosts. A positive reaction indicates activity of the donor component against host strain antigens.

Discriminant assay.—The suspension is tested in F1 hybrids between the original donor strain and some third strain. The hybrids are chosen to be immunologically mature enough to eliminate the host component of the suspension and thus to respond only to the donor component. A positive reaction indicates that the donor component has retained potential activity against antigens of the third strain.

The principles involved are illustrated in Fig. 1. An F1 hybrid between two inbred strains, A and B, suffers a graft-versus-host reaction from injected A cells. Its spleen enlarges, and contains its own hybrid cells (shown as crosses) with an admixture of donor cells (shown as circles). A suspension from the spleen is assayed both against isologous (AB) and semi-isologous (AC) infant hybrids. Provided that the latter are sufficiently mature immunologically to reject AB cells as foreign (in practice, after the 4th day of life) they will react with splenomegaly only to the presence of A cells in the mixture. In a more obvious fashion, the isologous AB hosts will react only to the A cells, to the extent that these donor cells have not acquired any degree of tolerance of B antigens during their sojourn in the primary host.

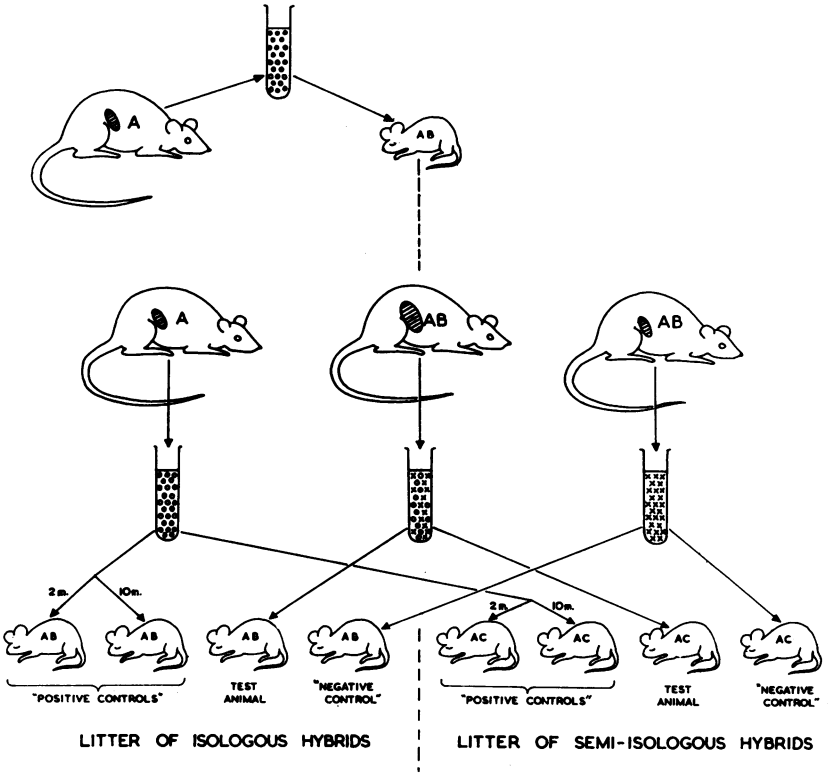


FIG. 1.—Diagrammatic representation of the discriminant spleen assay.

To control the assumptions stated above, litter-mates of the test animals must be injected with pure suspensions of AB cells. These serve as " negative controls " and provide the base-line against which to assess any splenomegaly observed in the test animals. " Positive controls " are also included. These consist of other litter-mates injected with pure suspensions of known numbers of A cells. If positive controls at more than one dose level are included, the degree of splenomegaly shown by the test animals can be interpreted in a quantitative fashion.

Mice

For the main series of assays, graft-versus-host disease was produced by injecting spleen cells from adult mice of the A inbred strain into F1 hybrids between A and C57BL. The semi-isologous hosts used in spleen assays were usually F1 hybrids between A and ASW, and sometimes between A and DBA/2. In a supplementary series C3H cells were injected

into (C3H × ST/A) F1 hybrids, and (C3H × DBA/2) F1 mice were used as semi-isologous hosts.

Cell suspensions

These were prepared by gentle trituration of the spleen in Hanks' solution (pH 7·4) with a glass piston blender, followed by passage through a fine-mesh stainless steel sieve. Cell counts were taken over a field of 1 sq. mm.; when the cells were very unevenly dispersed over the field, an additional three fields were counted and an average taken. The percentage of cells staining with 1/2000 Trypan Blue was frequently determined also, as an index of cell-damage, and this was found to vary between 10 and 30 per cent.

X-rays

These were delivered as whole-body irradiation at the rate of 150 r per min. given as a vertical beam from a Westinghouse machine under the following conditions: 230 kv. at 15 ma.; target-skin distance 50 cm.; 0·5 mm. Cu and 1·0 mm. Al filters.

Statistical analysis

The numerical treatment of the data followed the principles described by Michie, Woodruff and Zeiss (1961). All computation was performed on log-transformed data, which were finally reconverted to antilogarithms. Means therefore represent geometric and not arithmetic means.

RESULTS

Young (A × C57BL) F1 hybrids were injected intraperitoneally with adult A cells, and their spleens were weighed and assayed after varying intervals of time. Spleen weights, expressed as a ratio to body weights, reached levels 2–3 times control values at 8–10 days after injection, and then showed an irregular subsidence, the organ often becoming shrunken and relatively acellular. Cell density was not altered during the phase of splenic enlargement, so that an increase of spleen weight can be taken as a direct index of increased cell numbers.

It thus seemed that there should be no difficulty in detecting donor cells in assays of enlarged spleens, provided that the enlargement was chiefly due to proliferation of donor cells. Yet at the height of the splenomegaly our assay system was unable to detect even a trace of donor activity. A typical result is that shown in Table I. Two members of a litter of (A × C57BL) F1 hybrids were injected at the age of 8 days with 25 million adult A cells each. When killed 7 days later the relative spleen weights of the injected mice averaged 2·7 times those of their uninjected litter-mates. One of the enlarged spleens was used to provide a cell suspension for passage into young isologous and semi-isologous hybrids. No splenomegaly occurred in the secondary hosts. In the case of the semi-isologous hosts (A × ASW) we can utilize the positive control figures to estimate the admixture of immunologically competent donor cells in the inoculum as less than $\frac{2 \cdot 5}{3\,2 \cdot 0}$, *i.e.* less than 7·8 per cent of all competent cells. In the case of the isologous hosts the corresponding upper bound is 9·4 per cent.

This result was quite typical of what was obtained from the whole series of some 50 assays of the spleens of recipient mice, done at intervals ranging from 24 hr. to 54 days after the initial injection of adult A spleen cells. The early period was the most thoroughly investigated, and in the case of the 24-hr. tests an attempt was made to force the concentration of donor cells above the threshold of detectability by the injection of totals of 3–4 × 10^7 cells divided between the intraperitoneal and intravenous routes, instead of the customary 5–25 × 10^6 i.p.

TABLE I.—*Specimen Discriminant Spleen Assay*

Primary hosts : (A × C57BL) F1 mice 8 days old.

Identity No.	Injected with		After 7-day interval: Body wt. (g.)	Spleen wt. (mg.)	
1	$2·5 \times 10^7$	A cells	8·39	133	Presumed chimaera chosen for analysis
2	$2·5 \times 10^7$	A cells	8·32	141	
3	—		8·00	50	Pooled to provide negative controls
4	—		8·14	52	Pooled to provide negative controls
5	—		8·26	53	

Isologous test litter : (A × C57BL) F1 mice 7 days old.

Identity No.	Injected with	After 10-day interval, relative spleen weight (mg. spleen/10 g. body wt.)
1	$3·2 \times 10^7$ Chimaera cells	56
2	$3·2 \times 10^7$ Chimaera cells	54
3	$3·2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	54
4	$3·2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	51
5	1×10^7 A cells (positive controls)	161
6	3×10^6 A cells (positive controls)	80

Semi-isologous test litter : (A × ASW) 1 mice 5 days old.

Identity No.	Injected with	After 10-day interval, relative spleen weight (mg. spleen/10 g. body wt.)
1	$3·2 \times 10^7$ Chimaera cells	65
2	$3·2 \times 10^7$ Chimaera cells	64
3	$3·2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	71
4	$3·2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	55
5	1×10^7 A cells (positive controls)	134
6	5×10^6 A cells (positive controls)	136
7	$2·5 \times 10^6$ A cells (positive controls)	100

With one doubtful exception, no donor-cell activity was detected even following this intensive treatment.

The supplementary series of assays, in which the C3H→(C3H × ST/A) F1 combination was used, gave a rather different quantitative picture, although the qualitative conclusion, that the bulk of the enlarged spleen is of host origin, was the same. Estimates of the number of active donor cells, as a proportion of the total number of active cells present, are given in Table II. It appears not only

TABLE II.—*Proportion of C3H Donor Cells in 25 Chimaeric (C3H × ST/A) F1 Spleens, Estimated by Assay Against (C3H × DBA/2) F1 Mice*

Interval (days)	Estimated per cent anti-DBA activity	Average
1	0, 11, 12, 14, 21, 22, 35, 40	19·5
4	1, 10, 12, 15, 17, 39	15·7
11–28	0, 6, 9, 9, 13, 18	9·3
47–58	1, 4, 5, 7, 20	7·4

that these constitute a fairly small minority of the total, but also that they diminish in number relative to the host with the passage of time, even during the early period when the spleen is enlarging. Inocula ranging from 2×10^7 to

1×10^8 donor cells were used for establishing chimaerism, although 5×10^6 cells are sufficient in this combination to provoke a 2–3-fold enlargement of the spleen. The circumstances were thus more favourable for the detection of donor activity than in the main series described above.

Evidence from irradiated hosts

If, as suggested by the foregoing results, immunologically competent donor cells never constitute more than a small fraction of the spleen's competent cell population, even during the phase of enlargement, it seems likely that the greater part of the splenomegaly reaction is attributable to an increase of the host cell population. The alternative possibility is that donor cells have proliferated, but have become immunologically inert.

In order to decide this question we investigated the effect of X-irradiation of the host animal immediately before injection of spleen cells, with the object of depressing the postulated proliferation of host cells. Owing to the short survival time of infant mice subjected to higher dosages of irradiation, adult hybrid recipients were used for this purpose. In Table III the results of this experiment are

TABLE III.—*The Effects of X-irradiation and Injection of Parental-strain Spleen Cells on the Spleen Weight of (A × C57BL) F1 Hybrid Adults*

No. of spleen cells injected	Interval between injection and autopsy	Irradiated hosts			Non-irradiated hosts	
		X-ray dose	Injected with A spleen cells	Uninjected	Injected with A spleen cells	Uninjected
$8{\cdot}4 \times 10^7$	6 days	800 r	82 (5)*	47 (3)	84 (3)	40 (3)
9×10^7	7 days	600 r	91 (2)	40 (3)	116 (3)	
$10{\cdot}7 \times 10^7$	9 days	375 r	124 (3)	40 (3)	93 (4)	

* Spleen weights are expressed as mg./10 g. body weight. Figures (in parenthesis) indicate the number of mice in each group.

set out, from which it can be seen that when the dosage of donor cells is increased in proportion to body weight the splenomegaly phenomenon can be as easily elicited in adult as in infant mice. Surprisingly the table shows no effect of prior irradiation, even at 800 r, on the spleen weight in (A × C57BL) F1 hybrids. (This unexpected finding is contrary to our experience with both A strain mice and (C57BL × CBA) hybrids, in which a considerable reduction in spleen weight has invariably followed irradiation at this dosage.) However, spleen weight turned out to be no guide to the nucleated spleen cell population of irradiated (A × C57BL) F1 hybrid mice, for the spleens (even when enlarged following injection of A cells) were packed with erythrocytes which were evidently contributing substantially to the mass of the organ. Furthermore, an analysis of the total number of nucleated cells revealed that irradiation, especially at 800 r, had indeed been effective in depressing cellular proliferation following injection of A spleen cells. Relevant data are shown in Table IV.

Histologically, mice which had received 800 r alone showed a gross reduction in the number of nucleated cells present in the spleen with an accompanying increase of red cells (Fig. 2). The Malpighian follicles were small and contained foci of mature plasma cells (Fig. 3), whilst the network of reticulo-endothelial

TABLE IV.—*The Effects of X-irradiation and Injection of Parent-strain Spleen Cells on the Numbers of Nucleated Cells in the Spleens of (A × C57BL) F1 Hybrid Adults*

No. of spleen cells injected	Interval between injection and autopsy	Irradiated hosts				Non-irradiated hosts
		X-ray dose	Injected with A spleen cells	Uninjected	Injected—uninjected difference	Injected—uninjected difference
$8{\cdot}4 \times 10^7$	6 days	800 r	30*	18	12	55
9×10^7	6 days	600 r	43	15	28	76
$10{\cdot}7 \times 10^7$	9 days	375 r	87	34	53	78

* Cell numbers are expressed as millions per 10 g. body weight, and represent the means of batches of not less than three mice.

cells throughout the pulp was clearly revealed by the reduction in cell density. Similarly irradiated mice injected with A spleen cells possessed spleens which, although enlarged, also showed a sub-normal nucleated cell density and congestion with red cells. In contrast with uninjected irradiated mice, however, numerous pyroninophilic cells (some large and of blast type) were scattered more or less evenly throughout the red pulp (Fig. 4). A few of these cells were observed to be in mitosis, but far fewer than in the proliferating spleen cell population of an unirradiated mouse undergoing graft-versus-host reaction.

In view of this evidence of a considerable host contribution towards the increase in spleen cell population during graft-versus-host reaction, host irradiation was used to increase the sensitivity of spleen assays in detecting donor cell activity. Infant (A × C57BL) F1 hybrids, in which no donor activity was previously detectable, were irradiated to 600–800 r and injected with A spleen cells. The results of spleen analyses performed 5 days later (Table V) show clear-cut evidence of donor cell activity in all cases, with no obvious difference between the isologous and semi-isologous tests.

TABLE V.—*Evidence of Donor Cell Activity in the Spleen 5 Days After the Injection of A Spleen Cells into Irradiated Infant (A × C57BL) F1 Mice*

Chimaera No.	X-ray dose	Anti-C57BL		Anti-ASW	
		Activity of donor component	Estimated equivalent donor cell (per cent)	Activity of donor component	Estimated equivalent donor cell (per cent)
219/1–5*	800 r	Highly significant	<30	Significant	>36
220/1–2 and 221/1–3	600 r	Highly significant	Approx. 50	Not significant	<53
235/1–6 and 236/1–4	600 r	Highly significant	>27	Significant	<27

* Numbers after oblique stroke indicate pooled litter-mate chimaeras.

DISCUSSION

The conclusion of Davies and Doak (1960) that cellular proliferation in the spleen during graft-versus-host reaction in mice is largely of host origin has been confirmed by the present investigation, in which different strain combinations

and another experimental approach have been used. Our cell counts and histological observations on the effect of prior irradiation of the recipient on the cell population of the spleen supports this interpretation. So does the finding that immunologically competent donor cells only cross the threshold of detectability in one of our assay systems (A → A × C57BL) when the recipient has been irradiated, and that in the other (C3H → C3H × ST/A) they constituted on average only 14 per cent of the total number of competent cells present. A similar proportion of donor cells was found by Michie, Woodruff and Zeiss (1961) in non-irradiated A-strain mice which had been injected neonatally with CBA spleen cells.

Little can be said at this stage about either the nature or the significance of the host response. Clearly it is unlikely to be immunological, since there are no antigens present in the system which are foreign to the host. Gorer and Boyse (1959) interpreted the cellular proliferation following injection of A-strain spleen cells into (C57BL × A) F1 hybrids as a histiocytic response. On the other hand, using the C57BL → (C57BL × CBA) F1 system, Howard (1961) found a predominance of pyroninophilic cells in the spleen which were morphologically indistinguishable from plasma cell precursors. Furthermore, neither these nor the increased number of reticulum cells were demonstrably phagocytic *in vivo* or argyrophilic in tissue sections.

Death from irradiation set an upper limit of 5 days to the time interval during which we could seek evidence of donor cell activity in infant irradiated recipients in the A → A × C57BL system, but in the non-irradiated hosts of the C3H → C3H × ST/A series donor cells were found to persist for a much longer time. It is noteworthy that after 5 days' sojourn within irradiated (A × C57BL) F1 mice, A cells showed no evidence of tolerance of C57BL antigens—in fact they were as active against hybrids containing C57BL as they were against hybrids containing third-party ASW antigens. This contrasts strongly with the other strain combination used, C3H → (C3H × ST/A) F1, for Simonsen (1961) found that after only 24 hr. within (C3H × ST/A) F1 mice, donor cells had become tolerant of the host antigens. Presumably the discrepancy is attributable to differences in the donor-host combinations employed.

SUMMARY

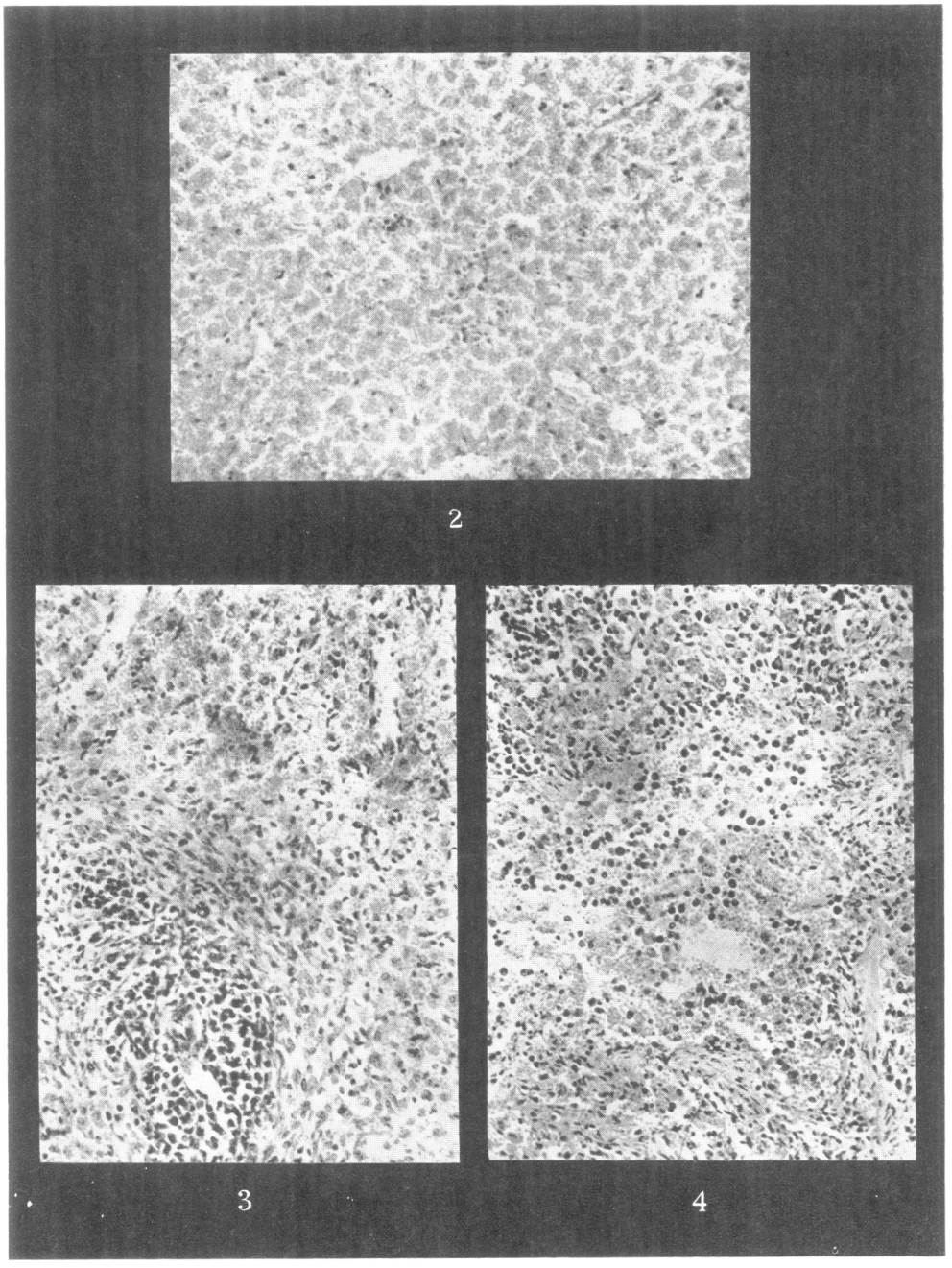
Graft-versus-host reaction was initiated in infant F1 hybrid mice by the injection of adult parental strain spleen cells. During the ensuing phase of spleen enlargement, the proportion of donor cells among all immunologically competent cells was estimated by means of the discriminant spleen assay of Simonsen and

EXPLANATION OF PLATE.

FIG. 2.—Red pulp of spleen of (A × C57BL) F1 mouse 6 days after whole-body irradiation (800 r). Note gross reduction in nucleated cell population and packed red cells. Pyronin-methyl green. ×177.

FIG. 3.—Malpighian follicle in spleen of mouse shown in Fig. 2. Note reduction in size, also focus of darkly staining mature plasma cells. Pyronin–methyl green. ×177.

FIG. 4.—Red pulp of spleen of (A × C57BL) F1 mouse 6 days after whole-body irradiation (800 r) followed by intravenous injection of $8{\cdot}4 \times 10^7$ A-strain spleen cells. Note pyroninophilic cells scattered throughout pulp (cf. Fig. 2). Pyronin–methyl green. ×177.



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Jensen (1959). Using the system A → (A × C57BL) F1, no donor cell activity could be detected during the period 1–54 days after injection of parental strain spleen cells. When the recipient had been previously irradiated (600–800 r), donor cells were readily detected at 5 days. Donor cells were detectable in the system C3H → (C3H × ST/A) F1 during the period 1–58 days after injection of C3H cells, without previous irradiation of the recipient, but they only constituted 14 per cent, on average, of immunological cells in the spleen.

The effect of previous irradiation of the recipient on the spleen enlargement which develops following injection of A spleen cells into adult (A × C57BL) F1 hybrids was examined histologically and by cell counting. Prior irradiation of the host was highly effective in depressing cellular proliferation in the spleen.

It is concluded that splenomegaly during graft-versus-host disease, at least in the two systems studied, is attributable in large degree to proliferation of host cells.

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